The Effects of Choline Availability From Gestation to Early Development on Brain and Retina Function and Phospholipid in a Mice Model

by

SHOUG ALASHMALI

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Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

R3T 2N2

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Abstract

Choline is known to be essential for brain development and neural function, but its impact on the retina, as a type of neural tissue, is unknown. This study examined the effects of choline during fetal development on membrane phospholipid (PL) compositions and functions in neural tissues, brain and retina. Pregnant C57 BL/6 mice were fed one of the 4 choline modified diets from gestation to early development: i) deficient (Def, 0g/kg), ii) control (Cont, 2.5g/kg), iii) supplemented with choline chloride (Cho, 10g/kg), iv) supplemented with egg phosphatidylcholine (PC) (PC, 10g/kg). On postnatal day (PD) 7, pups were culled to 4 from each dam, and kept on the same respective diets until 45 PD. On PD 35, memory function was measured by Morris water maze and on PD 45, retina function by an electroretinogram. Brain and retina were obtained for PL analysis by ^{31}P NMR. Animals on the Def and PC diets were lower in body weights on PD 7, in comparison to the other two groups. While the Def group caught up in weights to its Cont counterparts, the PC group's weight stayed consistently low until PD 45 ($P<0.03$). As for brain function, Cho and PC supplemented groups showed enhanced cued learning task, and spatial memory abilities, respectively, whereas the Def group showed the poorest memory recollection ($P<0.05$). The ERG amplitudes of rod driven photoreceptors and inner neural cell functions were significant ($P<0.05$) in the following order: Cont > Def > PC > Cho, at all light intensities, without reaching statistical significances in cone-driven responses. There were no differences in major PL compositions in the brain and retina. PC enriched group had increased subclasses of ether PL, PEaa and PCaa in the brain. These results indicate that while the addition of choline supplementation is beneficial for fetal brain development and function during early developmental stages, its contributions in the retina were minor. The effect of choline to

the membrane PL structure was negligible for the stage of development in the given experimental design.

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List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
BACE1	β -site amyloid precursor protein cleaving enzyme-1
CNS	central nervous system
CREB	cAMP-response element binding protein
ChAT	choline acetyltransferase
CK	choline kinase
CoA	coenzyme A
CDP-choline	cytidine diphosphate choline
CMP	cytidylyl monophosphate
CT	cytidylyl transferase
CPT	1, 2-diacylglycerol choline phosphotransferase
DG	diacylglycerol
ECF	extracellular fluid
GABA	glutamate; gamma aminobutyric acid
GPC	glycerophosphocholine
G-protein	guanine nucleotide-binding proteins
HC3	hemicholinium-3
CHT	high-affinity choline uptake transporter
LTP	long term potentiation
Lyso-PC	lysophosphatidylcholine
MRI	magnetic resonance image

MCI	mild cognitive impairment
MAPK	mitogen-activated protein kinase
NMDA	N-methyl D-aspartate
NF-κB	nuclear factor kappaB
OCT2	organic cation transporter 2
PNS	peripheral nervous system
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT-I	phosphatidylethanolamine-N-methyletransferase
PEMT-II	phosphatidyl-N -methylethanolamine-N-methyltransferase
PI	phosphatidylinositol
PS	phosphatidylserine
P-choline	phosphocholine
CTP	phosphocholine cytidylyl transferase
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PLs	phospholipids
PKC	protein kinase C
SM	sphingomyelin
SMase	sphingomylenase
SGZ	subgranular zone
SVZ	subventricular zone
WHO	world health organization

1. INTRODUCTION

The nervous system is very complex, and it is divided into two main parts; the central (CNS) and peripheral nervous system (PNS). When the nervous system malfunctions, due to genetics, diseases, and/or nutrition, many neurological problems may occur, which can lead to epilepsy, Alzheimer disease and other dementias, cerebrovascular diseases, Multiple Sclerosis, Parkinson's disease, and/or brain tumours. The World Health Organization (WHO) estimated in 2006 that neurological disorders and their consequences affect as many as one billion people worldwide. Approximately 6.8 million people, irrespective of age, sex, education or income, die every year as a result of neurological disorders (WHO, 2007). WHO also identified no clear treatment strategies without side effects, leaving those who were affected with neurological diseases disabled and helpless even after diagnoses.

There are regions of the brain that have important sites of neurogenesis, which controls behavioural development, cognitive ability, and other neural cell functions. It is made up of different structures and regions, each associated with a unique task. The brain needs many nutrients, ranging from macro- to micronutrients, which regulate its development and maintenance from early on in the embryonic stage. Although we do not know the region-specific nutrients required in the brain, numerous investigations over the past 40 years have shown that the chemistry and function of both developing and mature brain are influenced by diet (Fernstrom, 2000).

Recent understandings of this organ has been extended with the finding that essential nutrients are involved in its formation and cognitive function, such as iron, zinc, omega-3 fatty acids, folate, several B vitamins, and choline (McCann et al., 2006). Some of these nutrients affect mostly the biochemistry in the brain, whereas others affect behaviour and function (Fernstrom, 2000). Among them, choline is the most commonly

studied nutrient for neuronal function. In particular, pre and/or early postnatal choline supplementation has been shown to enhance hippocampal and prefrontal cortical performance on attention and spatial learning (Zeisel, 2006). Since choline serves as an essential precursor for the neurotransmitter acetylcholine (ACh) (Blusztajn and Wurtman, 1983; Sahley et al., 1986; Blusztajn et al., 1986), and for major neural cell membrane structural components phosphatidylcholine (PC) and sphingomyelin (SM) (Tayebati et al., 2011), the increase or decrease of choline intake may be tightly related to the changes in membrane lipid composition during neuronal development in the brain and other neural cells in the retina.

A review of the background related to brain, memory and neurological functions will focus on choline supplementation, as choline chloride and PC (lecithin), influencing PL in neural cell in the brain and retina during developmental stages.

1.1. Brain and Neuronal Health during Development

The spectrum of brain disorders is large and includes hundreds of diseases that are either psychological, neurological or both. The total cost related to treating brain disorders around the world was estimated at 798 billion euros in 2010 (Gustavsson et al., 2011). In 1998, the mortality rate of children (0 to 1 year) in the US, attributable to strokes or "cerebrovascular disorder[s]", was 7.8/100, 000 (Murphy, 2000). It has been shown that cerebral infarctions are considered as the underlying cause of neonatal seizures by 12% in infants greater than 31 weeks of gestation (Lynch et al., 2002). Other studies have shown that babies born prematurely or of diseased mothers are at increased risk of adverse neurodevelopmental and behavioral outcomes (Ramenghi et al., 2007). Examples include fetal alcohol spectrum disorders (Thomas et al., 2010). Previous

studies draw attention to the critical period of development known as the perinatal stage, where the brain is at its most vulnerable time to comorbid conditions.

Prenatal and/or early postnatal (perinatal) periods are essential for proper brain formation and organ function later in life (Zeisel, 2006). During this time, the brain grows rapidly by synthesizing millions of cells and producing billions and trillions of connections, or synapses, between each one (neurogenesis). Neurogenesis processes during the developmental stage can be examined by magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI), which shows the changes in signal intensities of gray and white matter as well as the rapid advancement of myelination during this period (Almli et al., 2007). Any insult during this period can result in adverse neurogenesis.

Postnatal neurogenesis is the continuous production and addition of neurons to the brain after birth and throughout life; this can be observed in both invertebrate and vertebreates (Belvindrah et al., 2009). Neurogenesis has been observed in two brain regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Yoshizaki and Osumi, 2010). Since postnatal reduction in hippocampal neurogenesis may contribute to mental illnesses such as schizophrenia and major depression during adult stages (Yoshizaki and Osumi, 2010), healthy brain development of neonates is essential. Knowledge of the effects of environmental factors such as nutrition is important for the identification and understanding of brain biochemistry. Moreover, understanding the brain structure and function can be helpful for improving therapeutic strategies of special clinical populations, such as children with motor dysfunction, visual processing deficits, learning disabilities, or attention problems (Zeisel, 2006).

1.2. The Nervous System; Important Structures in Neuronal Signalling

1.2.1. The Brain

During embryonic development, the brain first forms as a tube, the anterior end of which enlarges into three hollow swellings that form the brain, and the posterior of which develops into the spinal cord. The human brain can be divided into three parts: forebrain, midbrain and hindbrain. The largest part of it is the cerebral cortex, which can be broken down into many functional regions called "lobes": frontal lobe, parietal lobe, occipital lobe, and temporal lobe, each associate with unique functions (Carlson, 2010; Farabee, 2010). The different lobes together create and maintain cognitive function, which may be affected by certain nutrients.

One major role of the brain is to transfer information from the PNS to the CNS, process the information in the CNS, and send back the information to the PNS. This transfer of information is known as *neuronal signalling.* Neuronal signaling occurs via many structures such as synapses, which are important for the physical (i.e. photoreceptors in retina) and chemical (i.e. neurotransmitters) changes in a variety of organs (Carlson, 2010). The chemical changes in neural function are very important, and typically work constantly and efficiently in order to maintain normal behavioural and memory functions.

1.2.1.1. Memory

Based on both brain region and the type of learning involved such as perceptual, general information, or motor, there are different types of memory (Carlson, 2010) that regulate function. Memories can be classified into groups: declarative and non-declarative. Declarative memory is usually dependant on hippocampal formation. It is associated with facts and general information (semantic memory) and perceptions of events

organized in time, which are identified by a particular context (episodic memory). However, non-declarative memory (procedural memory) does not depend on the hippocampal formation; it is a collective term for perceptual, stimulus-response and motor memory (Carlson, 2010). A third type of memory, which also does not depend on hippocampal formation, is working memory. The term is often used interchangeably with short-term memory, which is defined as the memory of a stimulus or an event that lasted for a short while – usually on the order of a few seconds (Carlson, 2010). Many studies have investigated the dietary effects of a variety of nutrients such as choline on long and short-term memory (working memory) among different age groups of animals and human models (Zeisel, 2006; Buchman et al., 2001; Tees and Mohammadi, 1999; Pyapali et al., 1998; Williams et al., 1998; Meck and Williams, 1997b; Meck et al., 1988; Li et al., 2004).

1.2.2. The Retina

Like the brain, the retina is also a type of neural tissue specialized in the conduction of electrical impulses that convey information. During embryonic development, the retina and the optic nerve originate as outgrowths of the developing brain, so the retina is considered part of the central nervous system (CNS) (Sensory Reception, 1987). The retina constitutes the interior lining of the eye and contains rod and cone cells, collectively known as photoreceptors. The human retina contains approximately 120 million rod and 6 million cone cells. Indeed, the retina consists of several layers of neuronal cell bodies: their axons and dendrites, and the photoreceptors. The primate retina is divided into three main layers: the photoreceptive layer, the bipolar cell layer and the ganglion cell layer (Dowling, 1966). In addition, the retina contains horizontal cells and amacrine cells, both of which transmit information in a direction parallel to the surface of the retina and thus combine messages from adjacent photoreceptors (Carlson, 2010).

1.2.3. Neurons and Synapses

The neuron is the functional unit of the nervous system. Based on function, there are different types of neurons such as catecholaminergic, serotonergic, noradrenergic, cholinergic, etc. The primate retina contains approximately 55 different types of neurons: one type of rod, three types of cones, two types of horizontal cells, ten types of bipolar cells, 24-29 types of amacrine cells, and 10-15 types of ganglion cells (Masland, 2001).

Moreover, a neural plasma membrane is composed of a lipid bilayer which has many phosphatide subunits (e.g., PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI)) (Cansev et al., 2008) are incorporated and aggregated in cells to form the major constituents of their membranes. Protein molecules are also embedded within the bi-lipid membranes, which are called signal proteins. Signal proteins such as protein kinase C (PKC), respond to the binding of specific substances, such as hormones (Carlson, 2010) and different concentrations of ceramide (Dutta et al., 2011), by sending messages into the cell interior and activating certain mechanisms. Therefore, signal proteins control interior cellular functions and contribute to membrane permeability by acting as transporters (Carlson, 2010).

Another component of the nervous system are the glial cells which account for nervous tissue such as the Schwann cells that cover neurons with a myelin sheath (Carlson, 2010). SM, as PLs and SLs components, are important in maintaining nervous tissue by supporting, protecting and nourishing the neurons as a major part of the myelin sheath and neural cell membrane.

When an action potential reaches the synapse on the axon terminal of a neuron, it causes presynaptic terminals to secrete chemical messengers called neurotransmitters. Neurotransmitters are transmitted across the synaptic gap of a sending neuron to the

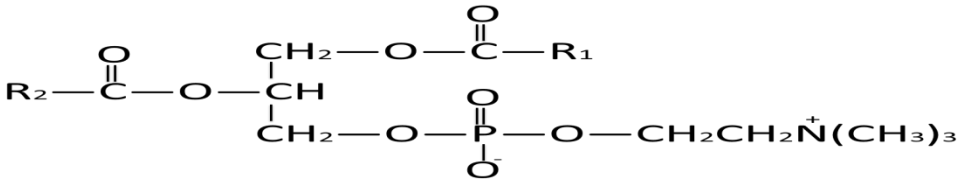
receiving one. They bind to post-synaptic receptors on the dendrite in order to send unique signals. Based on the variety of neural cells there are also many neurotransmitters. Each have a unique structure, postsynaptic effect, location and purpose, yet they are all able to function in concert; e.g., dopamine, serotonin, noradrenaline, glutamate (GABA) and ACh (A Review of the Universe, 2013). Since all of these neurotransmitters and their neurons are connected, the effect of environmental factors, e.g. nutrition, on one of these aspects may affect others. Farabee (2010) stated that nutritional supplementation, which could result in morphological changes including larger cell body and increased numbers of dendritic branches of one neuron, may have either direct or indirect effects on others. The reduced number of brain synapses is a major factor causing patients to develop cognitive dysfunction. The provision of nutrients such as choline may contribute in part to increase the number of brain synapses, which in turn might improve the behavioral function in normal and diseases conditions.

1.3. Choline

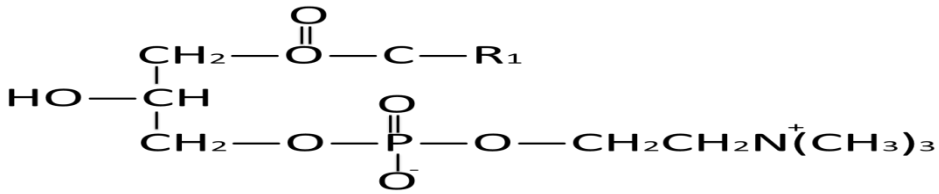
Choline (2-hydroxy-N, N, N-trimethylethanaminium) has been recognized as an important nutrient for many brain and nerve functions. This nutrient exists in the brain as a free base (e.g. water-soluble phosphocholine (P-choline) and glycerophophocholine (GPC)), and as constituents of membrane phospholipids (e.g. PC, SM and lyso-phosphatidycholine (lyso-PC)) (**Figure 1-1**)**.** Free choline levels in the brains of humans and rats reportedly vary between 36–44 μM (Ross et al., 1997) and 30–60 μM (Klein et al., 1993) respectively, and are much lower than choline metabolites, such as PC, P-choline, GPC and lyso-PC. Choline-containing phospholipids, PC and SM, are precursors for intracellular messenger molecules, diacylglycerol (DG), Cer and sphingosine-1-phosphate (Sph-1-P). Choline is also a precursor for ACh, an important neurotransmitter

involved in muscle control, memory, and many other functions. Thus, the altered choline concentration may disturb membrane integrity and cell signalling, thereby influencing overall function of the brain.

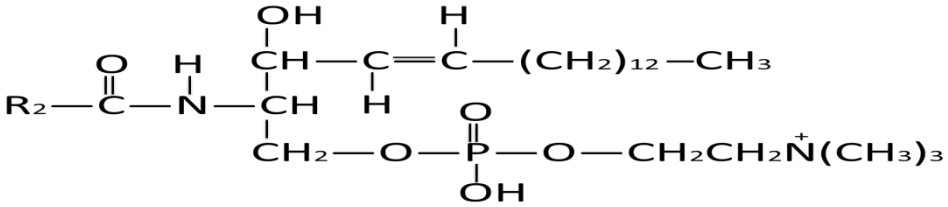
Choline



Phosphatidylcholine



Lysophosphatidylcholine



Sphingomyelin

Figure 1-1: Structures of choline and major choline-containing phospholipids.

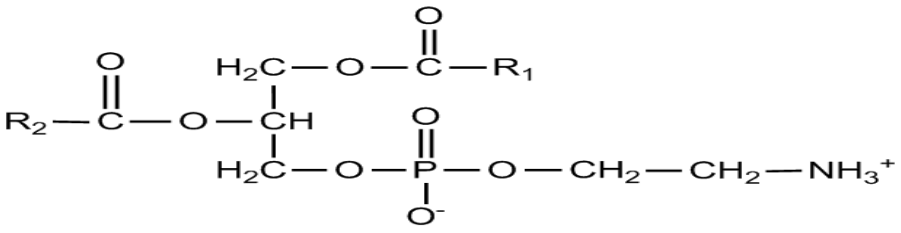
1.3.1. Choline as a Source of Membrane Phospholipids

1.3.1.1. Endogenous Synthesis

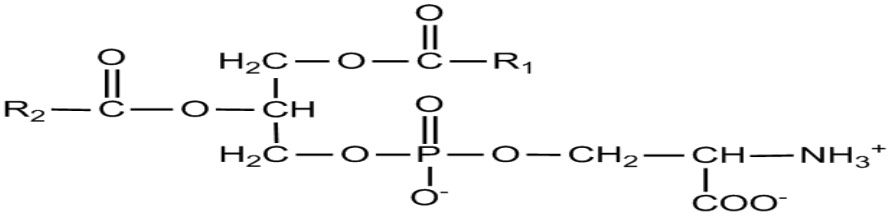
Endogenous choline is produced principally in the liver (Bremer And Greenberg, 1960), but also to a small extent in the brain (Crews et al., 1980). Brain cells – including nerve terminals (Holbrook and Wurtman, 1988) contain all the enzymes needed to synthesize choline via both methylation reactions and from pre-existing synthesized PC (Kennedy Cycle) in the cell membrane. Therefore, membrane phospholipids; PC and SM, can also serve as reservoirs for choline (Lajtha, 2009).

1.3.1.1.1. Methylation Reactions

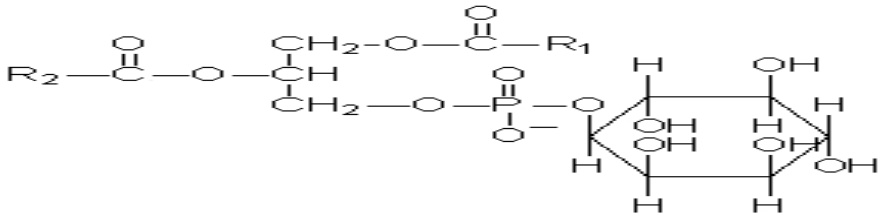
Choline *de novo* synthesis starts by the addition of three methyl groups to the amine nitrogen of phosphatidylethanolamine (PE) **Figure 1-2**. These methylation reactions are catalyzed by two enzymes, phosphatidylethanolamine-N-methyletransferase (PEMTI), which converts PE to its monomethyl derivative, and phosphatidyl-N-methylethanolamine-N- methyltransferase (PEMTII), which adds the second and third methyl groups (Bremer and Greenberg, 1960) in order to form PC. PC can then be broken down by a family of phospholipase enzymes; phospholipase D (PLD), phospholipase A2 (PLA2) and phospholipase C (PLC) (Lajtha, 2009), which act either directly or indirectly on the different bonds of PC to yield free choline as the product. As a result, free choline is liberated by methylation reactions, and can be further used to synthesize PC. Hence, the activation of each of these enzymes is tightly regulated and, in general, initiated by the interaction of a neurotransmitter or other biologic signal with a receptor coupled to a G-protein (Sandmann and Wurtman, 1991; Sandmann and Wurtman, 1990).



Phosphatidylethanolamine



Phosphatidylserine



Phosphatidylinositol

Figure 1-2: Structures of non-choline containing phospholipids. Adapted from (Lajtha, 2009).

1.3.1.1.2. Pre-existing Synthesized PC (Kennedy Cycle)

Choline can also be liberated from PC molecules, which are already formed from choline through the cytidine diphosphate choline cycle (CDP-choline cycle), also known as the Kennedy cycle. The Kennedy cycle involves three sequential enzymatic reactions to form PC as shown in **Figure 1-3**. In the first reaction, choline is catalyzed by choline kinase (CK), yielding P-choline. In the second reaction, the molecule is catalyzed by CTP: phosphocholine cytidylyl transferase (CT) to yield cytidylyldiphosphocholine (also known as CDP-choline or as citicoline) (Lajtha, 2009). Much of the CTP that the human brain uses for this reaction is derived from circulating uridine, whereas in rats it is derived from cytidine (Wurtman et al., 2000). The last reaction is catalyzed by CDP-choline: 1,2-diacylglycerol choline phosphotransferase (CPT), and yields PC. This PC is again broken down by phospholipase enzymes into choline.

Since the PC-synthesizing enzymes that act on all choline precursors have high affinities for their substrates, choline and its precursors in the blood can affect the overall rate of PC synthesis (Cansev et al., 2008). Indeed, because choline kinase's (CK) Michaelis-Menten constant (Km) for choline (2.6 mmol/L) (Spanner and Ansell, 1979) is much higher than the usual brain choline levels (30 to 60 μmol/L) (Ross et al., 1997), local choline administration increases brain phosphocholine levels in rats (Millington and Wurtman, 1982) and humans (Babb et al., 2004) (**Table 1-1**). The proportion of any membrane phospholipids represented by PC can vary depending on the species and age of animal, the particular brain region or cell type being studied, as well as the membrane function within the cell (e.g., nuclear membrane and plasma membrane) (Suzuki, 1981).

PE is synthesized via the Kennedy cycle, where ethanolamine is substituted for choline. PS, the third major structural phospholipid is produced by exchanging a serine molecule for choline in PC or ethanolamine in PE (Sastry, 1985). The PI synthesis process

is different as a result of reacting CDP with inositol; this reaction is catalyzed by CDP-diacylglycerol inositol phosphatidyltransferase (Kuksis, 2003) (**Figure 1-2**).

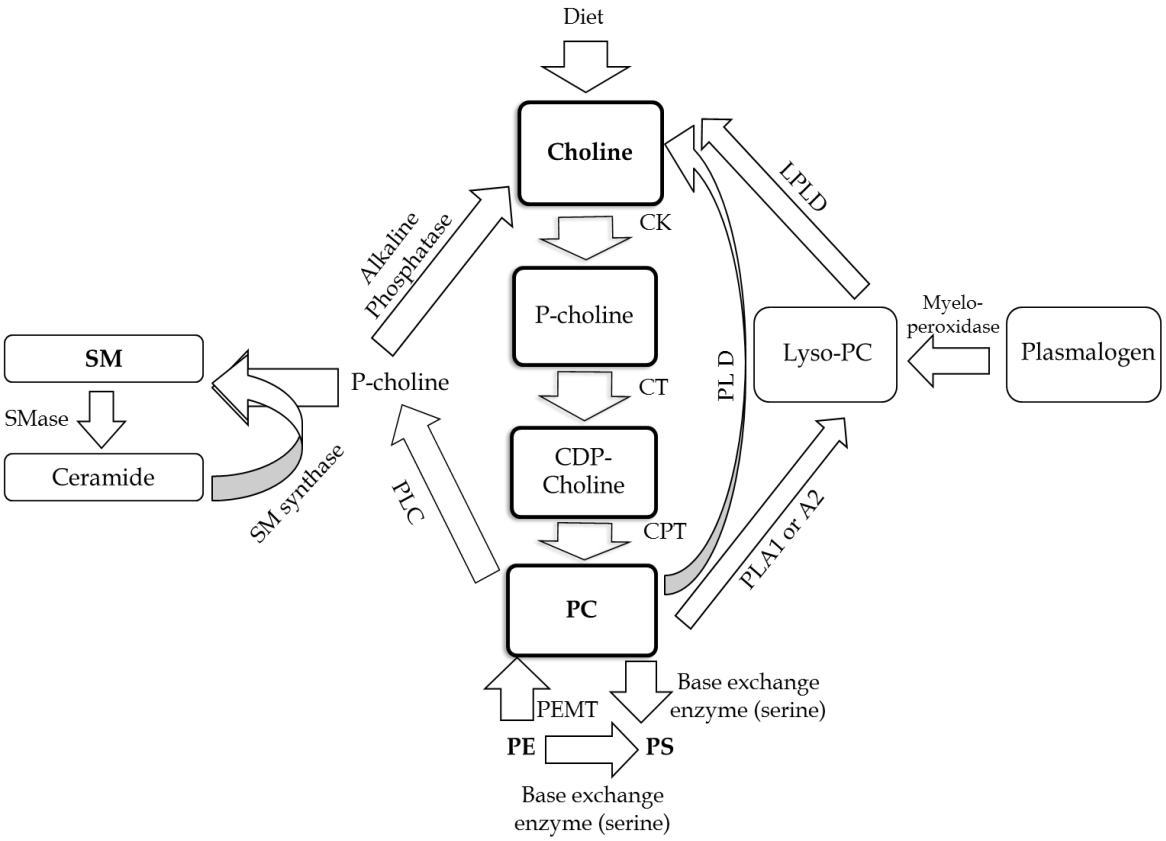


Figure 1-3: Intermetabolism of PC synthesis via (CDP)-choline cycle or "Kennedy cycle" and SM biosynthesis.

1.3.1.2. Uptake of Circulating Choline into the Brain and Retina

Choline in the brain can also be obtained entirely, or in part, from the circulatory system (Cansev et al., 2005; Wurtman et al., 2006). The brain can obtain circulating choline and various other nutrients (e.g., neutral and basic amino acids, glucose, adenine, or adenosine) (Pardridge, 1986; Pardridge and Oldendorf, 1977) via two routes, transporter and facilitated diffusion. Photoreceptor cells of the retina (cone and rod cells) are also considered as important cells of the body that use a high affinity uptake system of choline (Farabee, 2010; Masland, 1980). Small amounts can pass from the blood to the cerebrospinal fluid through the action of a specific transport protein; organic cation transporter 2 (OCT2) (Sweet et al., 2001); however, orders of magnitude more pass bidirectionally between the blood and the brain extracellular fluid (ECF) by facilitated diffusion. This process is catalyzed by a different transport protein, such as hexose, monocarboxylic acid, neutral and basic amino acid and amine, which are localized within the endothelial cells that line the brain's capillaries (Pardridge and Oldendorf, 1977). Hence, choline that is obtained through circulation via these routes can be used for both phosphorylation and acetylation.

1.3.1.3. Choline and PC Functions in the Brain and Retina

Choline, and/or its metabolites, assures the structural integrity and signaling functions of cell membranes, especially during neurogenesis and synaptogenesis (Zeisel, 2000). As a constituent of PC, choline is critical for the synthesis of neural membrane PLs, such as in the brain and retina, and is important for cell formation as well as tissue repair.

PC is highly heterogeneous, actually representing a family of compounds with differing fatty acid compositions (Lee and Hajra, 1991) and consequently, differing

chemical and physical properties. The major fatty acid compositions observed in brain PC are palmitic acid, stearic acid, oleic acid, archidonic acid (AA) and docosahexaenoic acid (DHA) (Lim and Suzuki, 2000). AA and DHA fatty acids under brain PC are more affected with dietary lipid supplementation, in particular a DHA diet (Lim and Suzuki, 2000). Thus, neural cell membrane PLs can be improved with exogenous sources of fatty acids or nutrients in order to maintain their optimal functions.

Retina membranes are also highly enriched in choline-containing PL. PC comprises about 45% of the total PLs found in the photoreceptor layer of the retina (Masland and Mills, 1980). Photoreceptors are uniquely high in DHA, which also constitutes approximately 50% of PLs in rod photoreceptor (Tuo et al., 2009). Retina PC is the only PL that contains very- long-chain (C24–C36) polyunsaturated fatty acids (VLC-PUFA) (Aveldano and Sprecher, 1987; Poulos, 1995), which are localised on the outer segments of the photoreceptor (Rotstein and Aveldano, 1988). Exogenous choline has been shown to be highly incorporated into retina PL, specifically into PC. Choline functions to maintain the steady state of newly formed photoreceptor membranes, which is significantly considering retina cells have one of the highest rates of membrane synthesis in the body (Masland and Mills, 1980).

PC also plays a critical role in generating second messenger molecules for membrane signal transduction; the process by which hormones and other substances transmit messages from the cell's surface to its interior. Therefore a broad spectrum of cell activities such as growth, gene expression, ion transport, and energy utilization are regulated (Canty and Zeisel, 1994). In addition, PC activates phospholipases inside the cell membrane, which degrades phospholipids (as mentioned above). The degradation products, free fatty acid and DG, can act upon a key regulatory enzyme, such as PKC, which in turn promotes cell growth (Canty and Zeisel, 1994). PC is also important for

SM synthesis by providing the P-choline moiety (Cansev et al., 2008; Kennedy and Weiss, 1956).

As free choline and PC are metabolically inter-changeable, they may have similar effects on the brain and retina as behavioral and neuroprotective nutrients. In multiple studies of preterm infant feeding, it was recognized that specific nutrient intake as choline and PC, which is involved in forming cell membrane and myelinisation, affected several neural functions such as visual and mental functions later on in childhood (Crawford, 1993; Carlson et al., 1994).

1.3.2. Choline as a Source of Acetylcholine

1.3.2.1. Endogenous Synthesis

Choline is considered a basic component of the neurotransmitter ACh. ACh is synthesized in cholinergic neurons, principally their terminals, by the ChAT (choline acetyltransferase) -mediated acetylation of free choline; ChAT catalyzes the reaction: choline + acetyl-CoA $\rightarrow$ ACh + CoA (Blusztajn and Wurtman, 1983). However, choline is unable to permeate the cell membrane, and requires a transporter to enter the cell. High affinity choline uptake transporter (CHT), which imports choline from the extracellular space to presynaptic terminals, plays a critical role in the regulation of acetylcholine synthesis in cholinergic neurons (Okuda et al., 2002), thus CHT is considered as the rate-limiting step of ACh synthesis. ACh levels in different brain regions are presented in **Table 1-1**.

Recent evidence links changes in CHT capacity with the ability to perform tasks that are related to attention processes and capacities (Sarter and Parikh, 2005). There are many other important components in the regulation of presynaptic cholinergic transmission beside ChAT, ACh neurotransmitter and CHT, such as acetyl coenzyme A,

acetylcholinesterase (AChE), muscarinic receptors and choline (Sarter and Parikh, 2005). By the hydrolysis of the ACh released from a cholinergic terminal by AChE, free choline molecules can be produced and can again be used in ACh synthesis. This process terminates the neurotransmitter's physiologic actions, i.e., its ability to combine with and activate its pre- or postsynaptic muscarinic or nicotinic receptors (Lajtha, 2009).

1.3.2.2. Acetylcholine Function

ACh is an important neurotransmitter because of its role in the brain's memory center, the hippocampus (Biasi, 2011). Its synthesis rate can be affected under specific conditions such as during neuronal firing (Blusztajn and Wurtman, 1983; Sahley et al., 1986), and also in postsynaptic ACh-dependent functions like the control of the rat striatal (Cohen and Wurtman, 1976). Apparently, many factors influencing the ACh synthesis rate are involved under the previous and following conditions; for example, the treatments which increase brain choline (e.g., administering choline (Tayebati et al., 2011; Cansev et al., 2008; Cohen and Wurtman, 1975), PC (Tayebati et al., 2011; Magil et al., 1981), or consuming either supplements or choline-rich foods (Tees and Mohammadi, 1999; Cohen and Wurtman, 1976; Magil et al., 1981). Limited information is available on whether different forms of choline, PC (lecithin) or choline chloride, increase ACh concentration differently in brain.

Table 1-1. Choline, PC and ACh concentrations in plasma and different regions in mice brain.

Choline and PC	Plasma	Brain regions			White matter %	Gray matter %	Reference
		Cortex	Hippocampus mol x 10^{-10}/mg	Cerebellum			
PC (22-26 mo)	2.11	1.84	1.52	0.37	33% (Suzuki, 1981)	42% (Suzuki, 1981)	(Muma and Rowell, 1986)
Choline (22-26 mo)	5.18	5.44	5.11	4.96	-	-	(Muma and Rowell, 1986)
ACh (12wk)	-	0.016	0.023	-	-	-	(Chung et al., 1995)

1.4. Essentiality of Choline during Development

Choline is the most studied nutrient related to brain development and memory function. Although choline deprivation is not lethal in rodents and humans, since it can be synthesized endogenously, increasing evidence show the necessity of choline, especially during embryogenesis and neonatal development. In 1998, the Institute of Medicine and the National Academy of Sciences in the USA classified choline as an essential nutrient. During development, there is a progressive decline in blood choline concentration of the fetus and neonate, which has been detected by a radio-enzymatic assay using choline kinase and radioactive ATP. Contrarily, blood choline concentrations are seven times higher than in adults (Zeisel et al., 1980a; Zeisel and Wurtman, 1981). This decline in blood choline concentration occurs during the first weeks after birth in rodents and humans, indicating the critical time to provide choline (Zeisel, 2006; Zeisel, 2000). Therefore, sufficient amounts of choline are necessary during development due to rapid organ growth and membrane biosynthesis. In the last decade, many studies have accumulated concerning effects of choline and PC supplementation or deficiency during

development on neurological function in rodent offspring (McCann et al., 2006). However, limited information is available regarding the effect of dietary choline and PC on the lipid profile (PLs) during development stages, which may fortify the evidence of choline essentiality during growth.

1.4.1. Supplementation of Choline and PC on Neuronal Disorders

Many studies have shown the relationship between neurodegenerative and mental diseases with plasma and tissues choline concentrations. For example, rats with glaucoma, a neurodegenerative disease of the visual system, have 40% lower choline levels in the glaucomatous visual cortex than the control group (Chan et al., 2009). In humans, lower plasma choline concentrations have been associated with higher anxiety levels compared to the high plasma choline groups in the adult and elderly (Bjelland et al., 2009). These studies imply the importance of choline provision to prevent and/or delay the progression of neurodegenerative and mental diseases, although the robust data are still needed in future studies.

Since choline and PC administration significantly increase choline levels in the blood 200-256% (Hirsch and Wurtman, 1978; Jope, 1982), brain 49% (Hirsch and Wurtman, 1978) and to some extent the level of ACh neurotransmitter 19% (Hirsch and Wurtman, 1978; Babb et al., 2004; Cohen and Wurtman, 1975; Jope, 1982; Domino et al., 1983), choline and/or PC intake through either pharmacological or dietary supplementation has been studied as a neuroprotective tool for different neuronal diseases.

1.4.1.1. Pharmacological Administration

Studies in epilepsy and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (AD) in humans, have shown that pharmacological choline

supplementation can improve memory deficit problems, attention deficit disorders, hyperactivity disorders, neuromuscular disorders and general manic disorders in adults and aging (Chan et al., 2009; Bjelland et al., 2009; Zeisel and da Costa, 2009). In Dull mice, an animal model of dementia, 100 mg of egg PC consumed daily by oral gavage for 41- 46 days improved memory function in passive avoidance performances compared to the control group. The authors concluded that this was due to many factors including increase in serum choline concentration, brain choline content (cortex and hippocampus), ChAT enzyme activity and ACh amounts compared to levels of control group (Chung et al., 1995).

On the other hand, prenatal choline supplementation as choline chloride, enhanced N-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission in adult rat model (Montoya and Swartzwelder, 2000). Since 60% of NMDA receptors are located in the lipid rafts of neural cells (Besshoh et al., 2005; Fullekrug and Simons, 2004), choline may influence lipid raft structures and further cell membranes and by extension signal transduction, synaptic plasticity and cell survival (Delint-Ramirez et al., 2010). Prenatal choline administration also reportedly enhanced long-term potentiation (LTP) (Pyapali et al., 1998), PLD activity (Holler et al., 1996), and dendritic spine formation (Williams et al., 1998) in the rat hippocampus during adult stages.

Thomas et al (2010) studied the effects of prenatal choline supplementation on behavioural alterations associated with prenatal alcohol exposure in a rat model. They have found that choline supplementation (250 mg/kg/day, oral gavage) of pregnant dams during a gestational period of 5 to 20 days reduced the severity of fetal alcohol effects. Rat offspring showed improvement particularly on tasks that required behavioral and memory flexibility, measured by the T-maze and the Morris water maze (Thomas et al., 2010). Limited information is available regarding which form of dietary choline (PC or

choline chloride) may have the highest potential for improving neurological and memory functions during development stages.

1.4.1.2. Dietary Supplementation

1.4.1.2.1. Pre/Post natal

Many studies have investigated the neuroprotective effects of pre and/or early postnatal choline intake as a dietary supplement. A study has shown that pregnant dams that were supplemented with choline chloride (4.6 mmol/kg) for 6 days during the gestational days 12-17 protected both adolescent and adult offspring against neurotoxicity induced by NMDA receptor antagonists and subsequent changes in brain function (Guo-Ross et al., 2002). Another study reported that postnatal female rats (30 days old) fed a low level (0.8g/kg of diet) of choline chloride diet for 60 days had suppressed levels of neurotoxicity induced by MK-108, which causes neuronal degeneration in the retrosplenial cortex region of the brain (Biasi, 2010).

In Rett syndrome, a neurodevelopment disorder of grey matter in the brain which mainly affects motor function, perinatal choline chloride supplementation in water improved motor performance in a mouse model through striatal nerve growth factor. This suggests that neuronal proliferation and survival induced by choline may contribute to improvement of the syndrome (Nag et al., 2008). All of these studies are presenting the neuroprotective effects of choline during the perinatal period. PC as a choline source may have similar effects on a variety of neurological diseases during growth. However, no current information is available during developmental stages.

1.4.1.2.2. Adult

Dietary choline has also improved neurological diseases in adults. In AD patients, the responders showed moderate improvements in factor score 1 (FAC1), which

includes orientation, learning and memory (psychological tests and scores) after being on a treatment of 20-25 g/day of PC (soybean lecithin) administered as a milk shake for 6 months (Little et al., 1985). This improvement may depend on the timing of choline supplementation; that is, before or after the neurological disease onsets.

PC has also been investigated as a dietary supplement during gestational and adult periods in Dull mice. Moriyama et al. (1996) fed animals from early gestation until 10 weeks, or postnatally from 4 to 10 weeks. During these periods, mice were supplemented with either 2% or 8% egg PC of diet. They found that both 2% and 8% PC diets were more effective on memory acquisition and retention during step-down type passive avoidance measurements in group 1(prenatally) than control Dull mice. In postnatally-fed Dull mice, 2% PC diet also showed memory improvement, however, it was limited to memory retention function ($P<0.05$) (Moriyama et al., 1996). On the 8% PC diet, there was no effect observed in dull mice, but marginal improvement (non-significant) in memory function. This study shows the importance of providing PC as a choline supplement during gestational and developmental stages, a critical period of brain development, which will decrease the likelihood of having neurological diseases in later life.

1.4.2. Supplementation of Choline and PC on Memory Function

The hippocampus is the brain's memory center, and it is sensitive to pre- and postnatal insults. Therefore, long-lasting changes in its activity brought on by early malnutrition can be harmful to many neuronal and behavioral processes (Walker, 2005). As stated earlier, choline is known as an important component of the brain for ACh synthesis in cholinergic neurons, which has a role in memory function by high-affinity reuptake of choline (Zeisel, 2006; Tayebati et al., 2011). Thus, the alterations in choline levels may affect ACh synthesis and also the cellular membrane composition in terms of PLs, which

also influence overall brain function. Hippocampal development has been demonstrated, after pre and/or postnatal choline supplementation, as facilitating neuroplasticity and improving cognitive functions such as learning and memory in offspring (Zeisel, 2006; Zeisel, 2000; Shaw et al., 2004). Choline supplementation, either pharmacologically or through dietary supplementation, on memory function has been studied among different models.

1.4.2.1. Pharmacological Administration

Choline and/or PC supplementation during the developmental stage has shown improvement on memory function via pharmacological dosage. Supplementation of pregnant or lactating rats with choline chloride during perinatal period (embryonic days 12–17 and/or postnatally 16–30 days) resulted in long-lasting improvements in spatial memory of offspring (adult and aged groups), which was measured by a radial-arm maze tests (Williams et al., 1998; Meck et al., 1988; Meck and Williams, 1997c) and the Morris water maze test (Tees and Mohammadi, 1999). Moreover, rats received choline perinatally at 25 mM of choline chloride supplemented in water during the gestational period of 12-17d, followed by injection of their pups (postnatally; 1-30 day) with 25 mM choline chloride solution. Their offspring (3-6 months old) exhibited increased memory capacity and precision on a peak-interval timing task performance (Meck and Williams, 1997a).

These beneficial effects could be the results of the morphological alterations that occur in the brain after choline supplementation during fetal life. Those alterations include larger soma and increased numbers of primary and secondary basal dendritic branches (Williams et al., 1998; Li et al., 2004). Indeed, choline supplementation has been shown to yield morphological changes of neurons and brain regions after feeding dams a 5 ml/L solution of 70% choline chloride, followed by a subcutaneous injection of

pups as dose of 250 mg/kg choline chloride. The supplemented group performed more accurately on tests of spatial memory and radial arm-maze, and increased the neuronal size and circularity of neuronal immunoreactivity in some regions of the basal forebrain compared to control groups (Williams et al., 1998).

The effect of intraperitoneal choline chloride injection (6–60 mg/kg) in combination with glucose has also resulted in improved passive avoidance behavior in 3-month-old mice (Kopf et al., 2001). Consequently, perinatal supplementation of choline enhanced memory and learning functions, changes that endure across a lifespan. Since most of these studies supplemented pharmacological doses of choline chloride via the intraperitoneal or intravenous route, it is of interest to see if similar effects on neurons and memory function can be achieved via dietary intake levels of choline and/or PC via the normal digestive system during the developmental stage.

1.4.2.2. Dietary Supplementation

1.4.2.2.1. Pre/Post natal

The dietary intake of choline during the pre/postnatal period has presented a variety of effects on memory and brain function. Indeed, choline supplementation between 11-18 days of gestation, provided in water as 25mM choline chloride, has increased memory performance and behavioural capacity in the neonate (18-19 days) rat model (Mellott et al., 2004). These effects have been explained by an increase in processing speeds as a result of accelerated hippocampal maturation after choline supplementation, which further leads to an increase in the probability of attention performance. Another reason for increasing memory capacity after choline supplementation is the activation of hippocampal mitogen-activated protein kinase (MAPK) and cAMP-response element binding protein (CREB) in response to the stimulation of glutamate, NMDA or

depolarizing concentrations of K+ controlled by choline supplementation (Mellott et al., 2004). These developmental effects are also associated with a reduction in the age-related decline of attention processes (Meck and Williams, 1997c). As a protective agent, perinatal choline supplementation in water (25 mM choline chloride) starting on the 11th day of gestation and continuing until postnatal 7 has protected rats from memory deficits induced by epileptic seizures (Holmes et al., 2002). The dietary intake of PC on neuronal functions and as a protective nutrient has not yet been studied under normal conditions during the development stage.

Studies have also determined that prenatal choline provided in water as 3.5 g/l choline chloride during 12-18 days of gestation, decreased the rate of apoptosis of neuronal cells in the hippocampus and basal forebrain of 18-day-old fetuses (Holmes-McNary et al., 1997), and increased brain cell division (Albright et al., 1999a; Albright et al., 1999b). On the other hand, choline deficiency during critical periods of brain formation resulted in memory and cognitive deficits that persisted until the late stages of adulthood (Zeisel, 2006). Moreover, this deficiency leads to a decrease in processing speed and forces rats to selectively respond to stimuli rather than process them in parallel by dividing attention among relevant events (Meck and Williams, 1997c; Meck and Williams, 1997a).

Recent study has investigated the effects of PC supplementation in pregnant women (from 18 wk gestation through 90 d postpartum) on the cognitive abilities of their offspring. Pregnant women (n=140) were randomly assigned supplementation with either PC (750 mg, 65% and 36% higher than AI of pregnant and lactating women, respectively) or placebo, along with moderate-choline diet intake (~360 mg) (Cheatham et al., 2012). This study concluded that PC supplementation of pregnant mothers eating moderate amounts of choline did not enhance infants' brain functions (short-term

visuospatial memory, long-term episodic memory, language development and global development) at 10 and 12 months of age. This could be due to the limited PC supplementation period, which did not include the first trimester of pregnancy (one of the critical periods for brain development). Another possible reason is that PC supplementation was stopped in mothers after the first 3 months of lactation, while the behavioral functions of infants were not measured until 10 and 12 months of age. These studies present controversial results about the essentiality of choline during perinatal periods including whole gestation and lactation periods for behavioural developments.

1.4.2.2.2. Adult

Acute administration of choline, either choline chloride or PC, to rodents (Tees and Mohammadi, 1999) and humans (Ladd et al., 1993) improved short-term memory in adults. Choline supplementation between 11-18 days of gestation, provided in water as 25mM choline chloride, increased memory performance and behavioural capacity in mature (2-4 months) and aged (24-26 months) rat models (Meck and Williams, 1997c). In humans, eighty college students were provided with PC (soybean lecithin) either 10g or 25g. Regardless of the dose, PC improved explicit memory function at 90 min post-ingestion on a serial learning task (Ladd et al., 1993). Chronic consumption of a choline-rich diet as PC (8%) by SEC/1ReJ aged mice (17 months) reportedly counteracted the age-associated decline in learning and memory by increasing avoidance performance by nearly 30% than in control group (Leathwood et al., 1982). Later studies found that the dietary intake of 5% egg-PC in 3-week-old mice for 7 months had resulted in a decreased amount of time to reach the maze exit, and diminished straying into blind alleys than in control group (Lim and Suzuki, 2000).

A summary of studies focusing on the relationship between PC supplementation and behavioural functions is presented in **Table 1-2.** These findings indicate that memory

improvement is enhanced by a dietary intake of PC, which leads to an increase in choline concentration, the precursor to ACh in brain. Considering that PC is a major membrane constituent, its supplementation may alter membrane composition and neural cell function, thereby affecting memory function. However, this hypothesis has not yet been tested in the above studies.

Table 1-2. Summary of studies focusing on the relationship between PC supplementation and behavioural functions

PC study	Source	Dose	Supplemented Period	Behavioural test	Effect
(Cheatham et al., 2012)	Dietary PC capsules (egg source)	6 gel caps per day of pregnant women (750 mg choline/day)	From 4 ½ mo of gestation to 3 mo of lactation (~8 mo)	• Short term memory • Long term episodic memory • Language development • Global development	• No enhancement of the cognitive abilities of infants at 10 and 12 months of age
(Lim and Suzuki, 2000)	Dietary PC (egg source)	5% of diet of 3 wks CD 1 male mice model	7 mo	• Learning ability by using maze with blind alleys	• Less time to reach maze exit • Enhanced Maze-learning ability
(Moriyama et al., 1996)	Dietary PC (egg source)	2 % and 8% PC of diet for each group	Two groups: • First group fed from early gestation until 10 weeks. • Second group during 4 to 10 weeks (postnatally)	• Memory acquisition and retention of step-down type passive avoidance test	• 2% and 8% PC diet were more effective on memory acquisition and retention of Dull mice but not in normal mice in group 1 • Postnatally fed Dull mice, 2% PC diet improved only memory retention function • No effect observed in postnatally fed Dull mice (group 2)
(Chung et al., 1995)	PC (egg source)	100 mg daily by oral gavage of 10 wk old Dull mice	41- 46 day (fed for 6 weeks)	• Passive avoidance performance	• Improved memory function in Dull mice compared with control

(Ladd et al., 1993)	PC (soybean source)	Either 10g or 25g to eighty college students	At 60 and 90 min	• Serial learning task	• Improved explicit memory at 90 min postingestion • Slight improvement after 60 min of PC ingestion
(Little et al., 1985)	PC (soybean source) as milk shake	20-25 g/day to fifty one of AD patients	6 months	Psychometric tests and factor scores: • FAC 1 (verbal learning and memory) • FAC 2 (visuo-spatial and constructional) • FAC 3 (self-care)	• An improvement in a subgroup of relatively poor compliers and intermediate levels of plasma choline were shown
(Leathwood et al., 1982)	Dietary PC (soybean source)	0, 2, 4, and 8% PC of diet for either 6 or 17 months of SEC/1ReJ mice model	4 days	• Shuttle-box (avoidance performance)	• PC did not affect the younger mice performance • Older mice at highest dose of PC increased avoidance performance by nearly 30%

1.5. Dietary Sources of Choline

1.5.1. Choline Sources

Choline was considered a non-essential nutrient until 1998. Afterwards, the Food and Nutrition Board of the Institute of Medicine (USA) considered choline an essential water-soluble nutrient in animal and human diet (Institute Of Medicine, Dietary Reference Intakes, 1998). Choline is classified as a member of the vitamin B complex (Biasi, 2011), because it is a water-soluble compound that performs some functions similar to those by vitamins. Choline can be provided in food forms as a free molecule or as phosphatides, such as P-choline, PC (lecithin), GPC or SM, as well as many other supplemental forms including choline chloride, choline bitartrate, citicoline, and isolated PC from soy or egg yolk.

The US Department of Agriculture (USDA) Nutrients Databases gives examples of foods that contain choline. This includes vegetables and fruits (spinach, potato, cauliflower, tomato, banana, orange, lentil, bean, pea, almond, nut, peanut), cereals (brown rice, soybean products, bean curd, oats, sesame seed, barley, and wheat germ), and meats (liver, muscle meat, fish, chicken, milk and egg yolk) (Zeisel et al., 2003). Among these foods, eggs are the 2nd highest choline and PC (lecithin) enriched source (Zeisel, 2000) (**Table 1-3**).

Lecithin is a common name for PC that has been used to designate its commercial preparation from different food sources. The lecithin preparation contains other phospholipids such as PE, cholesterol and TG, however, the most abundant phospholipid is PC, which contains about 13% choline by weight. Despite the fact that most studies are using choline chloride as a choline supplement, it has been shown that lecithin or PC raises blood choline concentrations more effectively and persists for up to 8 hours (Zeisel et al.,

1980). Most commercial lecithin is prepared from soybeans (soy lecithin) or eggs (egg lecithin) which have significantly different fatty acid compositions (Magil et al., 1981). Egg lecithin contains more saturated fat (41.8%); whereas soy lecithin includes primarily unsaturated fats such as linoleic acid and α-linolenic acid (65.9-83.4%) (Zeisel et al., 1980). Although egg lecithin contains fatty acid chains that are longer than soy lecithin, their effects on serum choline, brain choline and ACh levels were similar (Magil et al., 1981).

Table 1-3. Choline and choline phospholipid content of some common foods

Food	Choline (mg/serving)	Lecithin (PC) (mg/serving)	Sphingomyelin (mg/serving)	Total choline
Apple (1 medium)	0.39	29.87	1.51	4.62
Banana (1 medium)	2.85	3.26	1.66	3.52
Beef liver (3.5 oz)	60.64	3362.55	134.68	532.28
Corn oil	0.004	0.13	0.05	0.03
Coffee (6 oz)	18.59	2.05	2.96	19.29
Egg (1 large)	0.22	2009.80	81.90	282.32
Grape juice (6 oz)	8.99	2.11	0.66	9.37
Human milk (1 cup)	2.10	27.08	31.83	10.29
Infant formula (1 oz)	0.818	2.97	1.10	1.38
Milk (whole, 1 cup)	3.81	27.91	14.57	9.64
Orange (1 medium)	2.91	53.03	2.45	10.40
Peanut butter (2 tbsp)	12.96	97.39	0.21	26.09
Whole wheat bread (1 slice)	2.52	6.57	0.20	3.43

Adapted from (Canty and Zeisel, 1994).

1.5.2. Choline Intake

Zeisel et al. estimated that healthy adults in the United States consume about 6 g per day of lecithin and 0.6-1.0 g per day of choline based on the dietary intake data from the 1970s (Zeisel et al., 1980). Since many Americans currently have reduced their

consumption of eggs, meats, and dairy products due to their fear of high-cholesterol containing fatty foods, this may be an overestimating regarding current intakes (Canty and Zeisel, 1994). Based on another study, an average American diet which high in fatty foods cause only small elevations in plasma choline. However, purified lecithin supplements compared to normal diets are likely to have a greater effect on choline plasma after ingestion (Zeisel et al., 1980b).

Choline plasma levels can rapidly increase several folds after ingestion of choline-rich foods. For instance, the consumption of a 5-egg omelet (containing about 1.4 g of total choline) increased the level from 9.8 μM to 36.6 μM within 4 h (Hirsch and Wurtman, 1978). Prolonged fasting reduced human plasma choline levels from 9.5 μM to 7.8 μM after 7 days (Savendahl et al., 1997). Similarly, removal of all choline-containing foods from the diet for 17–19 days gradually lowered plasma choline, from 10.6 μM to 8.4 μM in humans (Zeisel, 2000; Zeisel et al., 1991) and from 12.1 μM to 6.3 μM in rats (Klein et al., 1998). These data indicate that plasma choline cannot be fully sustained by endogenous stores and must be constantly supplied by an exogenous source through diet.

A daily intake of choline among males and females on an ad libitum diet was 8.4 and 6.7 mg/kg, respectively. Those amounts were almost similar to the current recommended adequate intakes for males and females by the Institute of Medicine: 7 mg/kg (Fischer et al., 2005) (**Table 1-4**). This implies that choline levels can be achieved by consuming a normal diet during adulthood. However, since the developmental stage is more critical and fragile, it is recommended that infant formulas contain at least 7 mg of choline per 100 kcal (16.7 μg/kJ), based on the choline content in human milk (U.S.Congress.Infant formula, 1980). The choline moiety of human milk is approximately 1400 μmol/L as 500 μmol/L free choline, 400 μmol/L choline in phospholipids (Zeisel et al., 1986) and 400-500 μmol/L choline as GPC and P-choline (Rohlfs et al., 1993).

Table 1-4. Recommended Adequate Intakes (AI) and Upper Limit (UL) for choline

Population	Age	AI (mg/day)	UL (g/day)
Infants	0-6 months	125 mg/day, 18	N/A
	6-12 months	mg/kg	N/A
Children	1-3 years	150	1
	4-8 years	200	1
	9-13 years	250	2
Males	14-18 years	375	3
	19 years and older	550	3.5
Females	14-18 years	550	3
	19 years and older	400	3.5
Pregnancy	All ages	425	3-3.5
Lactation	All ages	450	3-3.5
		550	

Adapted from the (Institute of Medicine, Dietary Reference Intakes, 1998).

Choline is safe when used appropriately. However, there may be side effects in higher concentrations such as sweating, increased salivation, gastrointestinal pain, diarrhea, nausea, vomiting, faint or dizziness, hypotention, depression, and/or a fishy body odor (Biasi, 2011). To avoid any risks, a daily consumption of choline should not exceed the tolerable uptake level for choline as shown in **Table 1-4**.

2. RESEARCH PLAN

Rationale

Perinatal nutrition is critical for fetal brain development. Any disruptions during this period can adversely affect neurogenesis, which may subsequently lead to abnormal neurodevelopment as well as neurodegenerative disorders in later life. Postnatal reduction in hippocampal neurogenesis also contributes to a part of the symptoms observed in mental illnesses such as schizophrenia and major depression during adulthood (Yoshizaki and Osumi, 2010). Thus, healthy brain development of the fetus and neonates is essential for a healthy adult life, which implicates the importance of maternal and infant nutrition.

Choline (2-hydroxy-N, N, N-trimethylethanaminium) is the most studied nutrient related to brain development and memory function. In particular, pre and/or early postnatal choline supplementation enhances hippocampal and prefrontal cortical functionality and performance on attention and spatial learning (Pyapali et al., 1998; Meck and Williams, 1997b; Montoya and Swartzwelder, 2000; Meck and Williams, 1997c; Meck and Williams, 1997a; Meck and Williams, 1999). Deficiencies of choline during pregnancy lead to altered memory function (Meck and Williams, 1997b; Meck et al., 1988; Meck and Williams, 1997c; Meck and Williams, 1997a; Mellott et al., 2004; Meck and Williams, 1999; Meck et al., 1989) and increased birth defects (Fisher et al., 2001; Fisher et al., 2002). Although humans are capable of synthesizing this compound and surviving without additional consumption, choline has been classified as an essential nutrient by the Institute of Medicine and National Academy of Sciences in USA (1998). However, whether similar positive effects are also found in other neural tissues remains unknown.

Like the brain, the retina is also a type of neural tissue specialized for the conduction of electrical impulses that convey information. During embryonic

development, the retina and the optic nerve originate as outgrowths of the developing brain, so the retina is considered part of the central nervous system (CNS) (Sensory Reception, 1987). The retina is highly enriched in the choline containing phospholipid, PC, which comprises about 45% of the total PL in photoreceptors (Masland and Mills, 1980). Moreover, retina PC is the only PL that contains novel retina specific very- long-chain (C24–C36) polyunsaturated fatty acids (VLC-PUFA) (Aveldano and Sprecher, 1987; Poulos, 1995; Suh et al., 2009). This indicates that the status of choline may influence the level of PC, and ultimately alter the visual processing of the retina. An earlier study showed that retina incubated in a choline medium had primarily more PC synthesis than control retina (Masland and Mills, 1980). However, there is little information available whether choline is indeed essential for retinal function.

Optimum PL composition is important for maintaining membrane physicochemical status and cell-to cell interactions. As a component of the polar head of PC and SM, choline contributes to membrane integrity, thereby positively influencing brain function as mentioned above. This could be one of the mechanisms of choline-induced improved memory and spatial learning. However, earlier studies have shown conflicting data for the metabolism of choline into brain PL. In a short term supplementation, choline had no effect on brain PL concentrations (Foot et al., 1982; Jope et al., 1984), whereas in a long term administration, PC was increased in different brain regions (Muma and Rowell, 1986). The majority of these studies used either pharmacological doses of choline via intragastric injections, or tested its contribution to PL compositions without testing brain function. By providing choline modified diets (deficient and supplemented) from gestation to early developmental stages, this study will investigate the essentiality of choline on neural tissues, retina and brain by measuring their functions as well as PL compositions as a mechanism.

Objectives

The main goal of the present study is to determine the essentiality of choline during development on neural tissues of the brain and retina. By providing choline deficient or supplemented diets from the gestation to early developmental stage, this study will specifically:

1. Examine the influence of choline modified diets on both spatial memory in brain and cone and rod function in retina
2. Examine the influence of choline modified diets on membrane PL compositions in the brain and retina

Hypothesis

Choline-modified diets, provided from the gestation to early developmental stage, will influence both brain and retina function and induce changes in their PL profiles. It is specifically hypothesized that:

Compared to normal choline containing diet:

1. Choline supplementation will improve both retina and brain functions
2. Choline supplementation will increase choline containing PL classes
3. Choline deficient diet will decrease both retina and brain function by decreasing choline containing PL classes

3. EXPERIMENTAL DESIGN AND METHODS

1. Animals and diets

Male and female C57BL/6 mice (about 10-11 wk old, 8 males and 16 females) were purchased from Charles River Laboratories (St. Constant, PQ). After acclimatization for approximately 1 week, 2 females were placed with one male (two : one mating) until pregnancy occurred (approximately 1-2 wk and confirmed by palpitation test, then males were removed) and were randomly assigned to one of the 4 choline modified diets (n=4 dams per diet group): i) choline deficient diet (Def, 0g/kg diet), ii) normal choline (Cont, choline chloride (Choline Cl), 2.5g/kg diet), iii) choline supplemented diet (Cho, Choline Cl, 10 g/kg), iv) egg phosphatidylcholine supplemented diet (PC, egg yolk lecithin, 80g/kg, 80g PC x 0.137 choline=10.96g/kg, Leathwood *et al*., 1982) (**Table 3-1**). Mineral mix was reduced in the diet composition of Cho group, however, it was increased in Def and PC diets depending on the choline content of the diet. This adjustment was due as the mineral mix contained compounds which helped in the production of choline in the body. Egg yolk PC (lecithin) was purchased from Q.P.Co (Tokyo, Japan, PL-100LE, 80% pure PC). Since lecithin (PC) is a lipid, the difference in fat content was adjusted with canola oil for the diet mixtures; fatty acid analysis of Egg Yolk PC and canola oil are shown in **Table A-1**. The calorie density of each diet group was 4.38 Kcal/g diet by providing approximately 35% of calories as fat, 45% carbohydrate and 20% protein, reflecting the macronutrient distribution recommendation by the Dietary Reference Intakes for pregnant and lactating women (Institute of Medicine, Dietary Reference Intake, 2005).

Table 3-1. Composition of experimental diets (g/kg)

	Cont	Def	Cho	PC
Casein	218.5	218.5	218.5	218.5
Corn Starch	294.0	294.0	294.0	294.0
Glucose (Dextrose)	200.0	200.0	200.0	200.0
Non-Nutritive cellulose	50.0	50.0	50.0	50.0
Vitamin Mix†	10.0	10.0	10.0	10.0
Mineral Mix‡	50.0	52.5	42.50	52.5
L-Cysteine	2.5	2.5	2.5	2.5
Choline Chloride	2.5	-	10.0	-
Inositol*	2.49	2.49	2.49	2.49
Tert-butylhydroquinone	0.01	0.01	0.01	0.01
Canola Oil**	170.0	170.0	170.0	90.0
Egg Yolk Lecithin***	-	-	-	80.0

Diet ingredients were purchased from Dyets Inc. (Bethlehem, PA)
except canola oil, Inositol and Egg Yolk Lecithin
† Vitamin Mix; Thiamin HCl, Riboflavin, Pyridoxine HCl, Niacin, Calcium Pantothenate, Folic Acid, Biotin, Cyanocobalamin, Vitamin A Palmitate, Vitamin E Acetate, Vitamin D3, Vitamin K1.
‡ Mineral Mix; Calcium, Phosphorus, Potassium, Sodium, Chloride, Selenium, Magnesium, Ferrous, Manganese, Zinc, Chromium, Iodine, Fluorine, Boron, Silicon, Nickel, Sulfur, Lanthanum, Vanadium, Molybdenum.
*Inositol (Bio-Serv, Frenchtown, NJ)
**Canola Oil (Loblaws Inc., Toronto, Canada)
***Egg Yolk Lecithin (PL-100LE, Q.P.Co, Tokyo, Japan)
Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

After parturition, pups from each litter were culled to 4 at postnatal day 7 (PD 7) to receive equal amounts of milk. After weaning at PD 21, dams were removed and pups followed the same designated diet for a further 3.5 weeks (PD 45). This feeding period covers two sensitive times in rodent brain development in relation to choline associated long-lasting enhancement of spatial memory (the first occurs during embryonic days 12 to

17, and the second during PD 16 to PD 30) (Zeisel, 2006). Body weight and dietary intake were monitored weekly. Animals had access to diets and water ad libitum and were raised under controlled environment conditions (18-22 C°, 30-60% relative humidity, 0700-1900 h lighting period). The study protocol was approved by the University of Manitoba, Office of Research Ethics & Compliance and Animal Care Committee. Memory function and retinal function were measured followed by tissue collection.

2. Memory function measurement by Morris Water Maze (MWM)

On PD 35, mice were tested for 8 consecutive days on a standard Morris water-maze (MWM) behavioral test, consisting of 2 days of cued learning and 6 days of spatial learning using an established method in Dr. Albensi's laboratory (Department of Pharmacology and Therapeutics) (Glazner et al., 2010; Oikawa et al., 2012). The maze consisted of an 81 cm circular pool, filled with water (26-28°C) and made opaque white with powdered milk. A hidden platform, around 7 cm in diameter, was submerged approximately 0.5 cm below the water surface in the center of the selected target quadrant. This maze was surrounded by a white curtain, which had four visual cues (star, triangle, circle, and arrow) that were positioned in front of each other above water level; this curtain also blocked the availability of distal cues in the room. Several parameters were measured in order to evaluate memory and visual functions (e.g., path length, escape latency, swim speed, time in target quadrant and number of passes over the missing platform).

2.1. Cued learning

Cued learning task of MWM is considered a control procedure to 1) test the animal's ability to swim to a cued goal, 2) test the visual function and 3) eliminate the problems of animals not acquiring appropriate skills during swimming before presenting them with the spatial version of MWM (Vorhees and Williams, 2006). In this task, animals

were tested for 4 trials/day (4 trials =1 block = 1 day) with intertrial interval (ITI) of 30-35 min over 2 consecutive days. The platform was hidden, only marked by a mounted flag (approx. 12 cm) that extended above the water surface. However, the location of the goal and the starting point were both moved randomly –by using a random generator program- to new positions during each trial to prevent the learning of spatial task. Mice were allowed to swim for 90 sec to find the hidden platform and were required to remain on the platform for 15 sec after which the mice returned into their cage.

2.2. Spatial memory

2.2.1. Acquisition phase

After the last block of cued learning trials, mice underwent one block over 5 days of acquisition as a first phase of spatial task. Similar to cued learning version, the platform was submerged under the water's surface, however, it was not marked by any flag. Moreover, the platform's position was randomly selected from one of four possible positions and remained stationary for the all trials during the acquisition phase, whereas the starting position was differ allowing the animals to use distal cues to navigate a direct path to the hidden platform.

2.2.2. Retention phase

The retention phase of MWM began a day after the last block of the acquisition phase. During the retention phase (also known as probe trial), the platform was removed from the pool and each mouse was given 90 sec to search for the position of the missing platform. For the retention phase, each animal was also tested for 1 block for 1 day. Other parameters measured during this phase included the number of passes over the targeted quadrant (real passes) and the number of passes over the other three quadrants (fake passes). In addition, the amount of time the mouse spent in the targeted quadrant was

measured. This information allowed us to calculate the annulus crossing index (ACI), which was measured by subtracting the mean number of fake passes from the number of real passes. ACI can be interpreted based on its value; if it is positive that is indication of a selective search of the missing platform position, an index around zero is a reflective of a random search (non-specific), and a negative index means a selective search in quadrants other than the target quadrant (Oikawa et al., 2012). All mice were videotaped and the videos were analyzed for previous parameters with Matlab (Natick, MA, USA). Schematic of MWM is shown in **Figure A- 1**.

3. Retina function by electroretinography (ERG)

Retina function was determined using a full field electroretinogram (ERG) at PD 45, with the UTAS-4000 data system (LKC Technologies Inc., Gaithersburg, MD). ERG is a more objective measurement of the neuronal function since the forebrain develops into the retina during embryogenesis. To ensure maximum sensitivity, animals were dark adapted over night before recording. To assess rod and cone function, mixed scotopic responses and photopic responses were used on the retina as previously published by our laboratory (Suh *et al*., 2009). One eye per animal was analyzed, based on the criteria of the highest maximal scotopic a-wave amplitude. A schematic of full-field ERG apparatus is in **Figure A- 2**.

ERG responses were recorded bilaterally under dim red light. Mice were under anesthesia with an intraperitoneal injection of a mixture of ketamine (62.5 mg/kg) and xylazine (12.5 mg/kg), and body temperature was maintained at 38°C with a heating pad. Pupils were dilated with 1% tropicamide placed on the cornea to prevent corneal dehydration and allowed electrical contact with the recording electrodes (gold loop). Platinum needles (25-gauge) implanted subcutaneously behind each eye served as

reference electrodes. The ground electrode consisted of a platinum needle inserted behind the neck.

3.1. Dark adapted ERG

A series of flash stimuli with increasing intensity was applied to generate dark-adapted, rod-driven responses (Suh et al., 2009). Stimuli consisted of single white (6500 K, xenon bulb) flashes repeated 3 to 5 times to verify the responsiveness reliability and obtain an average. For intensity responses, stimuli were presented at 15 increasing steps of intensity varying from –3.7 to 2.39 log sc cd·s/m^2 in luminance. Interstimulus intervals were increased from 10 sec up to 2 min at the highest intensity to allow maximum rod recovery between consecutive flashes. The amplitude of the a-wave, which represents the activity of the photoreceptor, was measured as the difference between base line at 0 sec and the lowest point of the negative a-wave trough. B-wave amplitude, which partially represents the activation of the post-synaptic ON-bipolar cells in the inner retina, was measured from the a-wave negative peak relative to the b-wave positive apex. The oscillatory potentials (OP) amplitudes, that represent the activation of amacrine and ganglion cells of inner retinal neurons, were calculated using EMWIN software (LKC Technologies Inc., Gaithersburg, MD).

3.2. Light-adapted ERG

After scotopic recordings, animals were light adapted at 30 cd/m^2 background light for the photopic intensity responses (cone-driven intensity), which were recorded with stimulus intensities at 11 increasing steps ranging from -1.22 to 2.86 log cd·s/m^2. A total of 3 to 5 light-adapted responses were flashed for each light intensity. B-wave and OP amplitudes were measured as described above for dark-adapted responses.

4. Lipid Analysis

4.1. Chemicals, materials and preparation of standard stock solutions

Phospholipid standards; phosphatidylcholine (PC) (from egg yolk), L-α-phosphatidylethanolamine (PE) (chicken egg), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) (from egg yolk) were purchased from Sigma-Aldrich (Oakville, ON). Assay properties for all compounds were ≥ 95 % (HPLC). Chemical structures are shown in **Figure 1-1** and **Figure 1-2**. Deuterated chloroform ($CDCL_3$) (99.8%) for the nuclear magnetic resonance machine (NMR) was purchased from Sigma (MO, USA). K_4EDTA (EDTA tetra potassium) salt was obtained from Pfaltz & Bauer Inc. (CT, USA). To minimize risk of compound degradation, standards and working solutions were prepared as extraction were initiated by an appropriate dilution of the concentrated standard stock solutions with additional chloroform ($CHCl_3$): methanol (CH_3OH) (1:1) (Fisher scientific, Ontario, Canada).

4.2. Brain and retina lipids

Total lipid extraction from brain and retina was based on a modified method of Folch et al. (Folch et al., 1957). Approximately 100 mg of brain and 6-7 retinas (pooled) were homogenized in 0.025% $CaCl_2$ for approximately 1-2 min and then 10 mL of $CHCl_3$: CH_3OH (2:1, vol/vol) was added, vortexed, and centrifuged at 2400 and 360x g, respectively, for 15 min at 4°C. The lower layer was transferred to a GC vial and evaporated to dryness under nitrogen gas with three rinses using $CHCl_3$:CH_3OH (1:1, vol/vol), then reconstituted with 1 mL of $CHCl_3$:CH_3OH (1:1). The samples were stored at -80°C until analysis took place.

4.2.1. Phospholipid analysis with ^{31}P NMR

Brain and retina (6-7 retina pooled) samples, which contained approximately 3-4 mg and 0.5-1 mg of lipid, were prepared for NMR analysis in the $CDCL_3$:CH_3OH: H_2O (10:4:2) solvent system as described by Pearce (Pearce et al., 2009). The lipid extracts were redissolved in 0.94 mL of $CDCL_3$:CH_3OH (10:4). After a brief vortex, 134 µL of deionized water containing 0.2 M K_4EDTA (pH 6.0) was added (Edzes et al., 1992). The mixture was vortexed again and the two phases were allowed to settle around 20 min before transferring lower phase to a 5-mm NMR tube, which was allowed to settle for at least an hour and equilibrated to 25°C in the NMR probe.

4.2.1.1. NMR conditions and analysis

Phosphorus-^{31}NMR was performed at 202.46 MHz on a Bruker Avance 500 spectrometer (Fallanden, Switzerland) using methods similar to those described previously (Pearce et al., 2009). The ^{31} P pulse width (reference pulse width) of 10 µs was equivalent to a 73° pulse angle. Other typical conditions were: repetition time TR, 4.38 s; spectral width, 1 kHz in 8K points; typical number of transients, 512. ^{31}P chemical-shift scales were referenced to 85% H_3PO_4 at 0 ppm by setting the chemical shift of PC peak to - 0.51 ppm (Pearce et al., 2009). This solvent system of PLs had ^{31}P spin-lattice relaxation times ranged from 1.2 to 2 sec. Therefore, the typical conditions in particular TR and tip angle used here provided good signal-to-noise ratio.

The resonances were quantified relative to the summed areas of all PL class resonances using the program SpinWorks 1D (NMR lab, Dep. of Chemistry, University of Manitoba). NMR free induction decays (FIDs) were zero-filled once, multiplied by an exponential function (1 Hz line narrowing for resolution enhancement), and followed by Fourier transformation. The resulting spectra phased and fit with Lorentzian line shapes

using a direct parameter organization. Individual PL class is presented as the relative concentration of total PL.

5. Statistical analysis

The effect of diet was analyzed by nested one-way analysis of variance (ANOVA) using SAS 9.3 (SAS Institute Inc., Toronto, ON). The significance effect of the diet treatment was defined by Least Squares (LS) means. For MWM experiments, nested one-way ANOVA was used with repeated measures (Mixed Model). ERG data were analyzed using quadratic growth model with PROC MIXED provided by SAS. All data expressed as LS mean ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

4. RESULTS

Choline-modified diets on body and tissue weights

Body weight & food intake

Average litter numbers from each dam in diet group were similar (**Table 4-1**), although the PC group had the lowest dam size. Only 3 out of 4 females fed the PC diet had a successful pregnancy. The dams from this diet group had lower body weights over pregnancy and lactation periods compared with other experimental groups (P=0.05, **Table A-2**). In terms of litters weight, at PD 7, litters from dams fed the PC or Def diets had significantly lower body weights compared to other groups (P<0.03) (**Figure 4-1**). By the end of experimental day of PD 45, the animals from the Def diet group caught up in body weight to its control counterparts, while the weight of PC fed animals remained consistently low (Cont, 22.68 ± 0.73g; Def, 21.86 ± 0.55g; Cho, 23.91 ± 0.76g, PC, 19.31 ± 0.73g; P<0.03). Dietary intake of pups after weaning was similar in Cont, Def and Cho supplemented groups, but significantly lower in the PC dietary group (Cont 6.57 ± 0.24, Def 6.11 ± 0.18, Choline Cl 6.32 ± 0.20, Egg PC 5.40 ± 0.11g per day; P=0.001) (**Figure 4-2**), which was strong in odor. This low intake of PC diet may have contributed to the lower body weights. The effects of dietary treatment on body weight are presented as weekly in **Table A-2** (Appendix). There were no choline treatment effects on the sex ratio of pups or the length for opening eyes (eye growth).

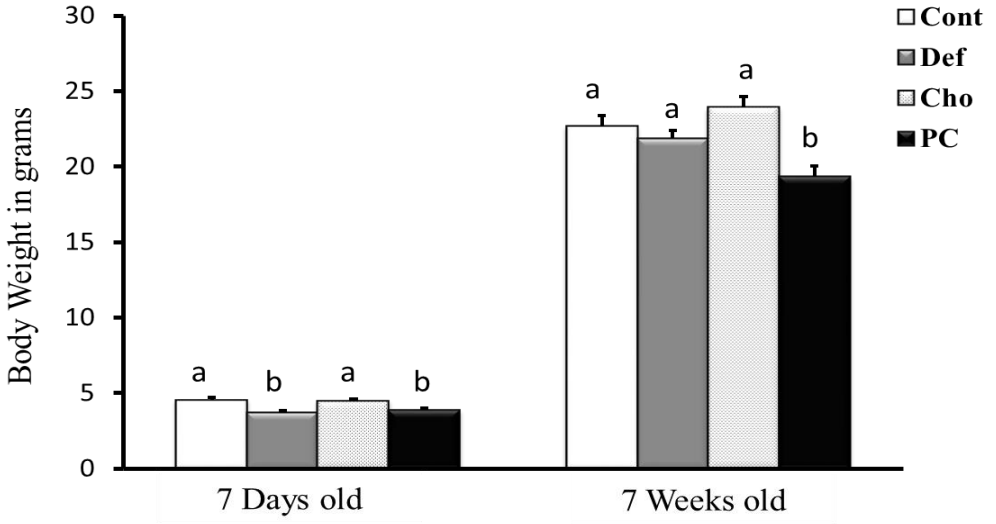


Figure 4-1: Effect of choline-modified diets on body weights at postnatal day 7 and week 7. Data expressed as least square (LS) mean ± SEM (n=9-15 mice/group). Significant effects of diet during lactation (P<0.03) and post-weanling period (P<0.03) on pups weights were identified by nested one-way analysis of variance. Different superscripts within a parameter indicate statistical differences. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

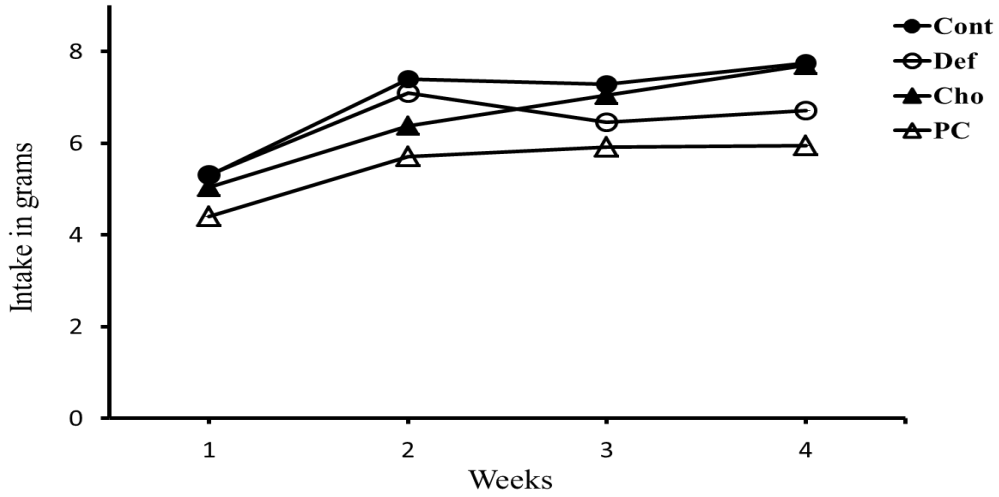


Figure 4-2: Effect of choline-modified diets on feed intake from weanling to postnatal week 7. Data expressed as LS mean ± SEM (n=9-15 mice/group). Significant effect of diet (P=0.001) was identified by nested repeated one-way analysis of variance. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC. (See **Table A-** for tabulated weekly value).

Organ weights

Brain and liver weights (g/100g body weight) were measured to determine the effects of choline diet on organ mass (**Figure 4-3**). Weights for the brain were not affected by Cho or PC diet. Mice fed the Cho enriched and Def diet had significantly larger liver weights (P<0.05) (**Table 4-1**).

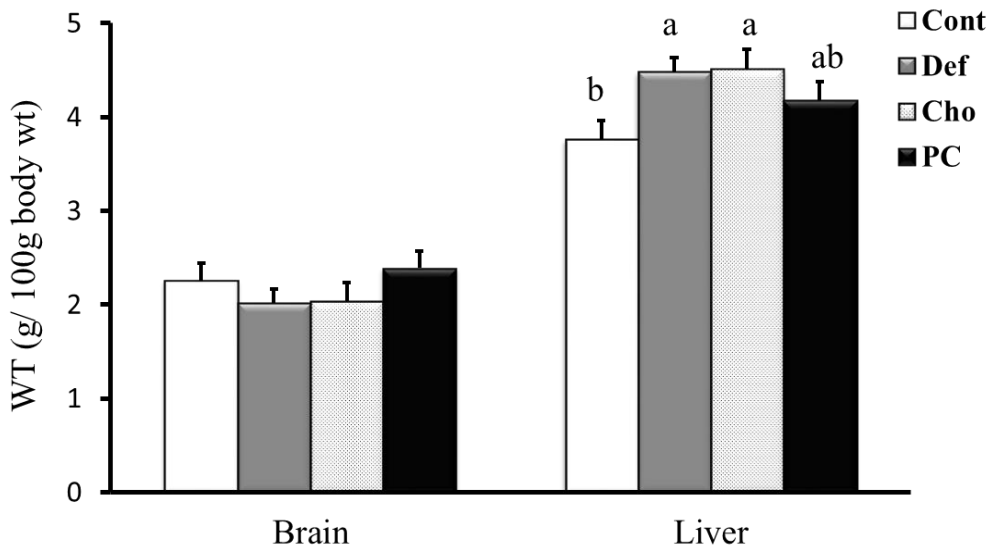


Figure 4-3: Effects of choline-modified diets on brain and liver weights. Data expressed as LS mean ± SEM (n=9-15 mice/group). Significant effects of diet (P<0.05) at end points were identified by nested one-way analysis of variance. Different superscripts within a parameter indicate statistical differences. Cont, control with normal amount of choline; Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Table 4-1. Effect of choline-modified diets on litter numbers and tissue weights

	Cont	Def	Cho	PC
Litters (n)	8.50 ± 0.65	8.75 ± 0.48	7.25 ± 0.75	6.75 ± 1.55
Brain (g)	0.49 ± 0.04	0.44 ± 0.03	0.49 ± 0.04	0.46 ± 0.04
Liver (g)	0.85 ± 0.05	0.98 ± 0.04	1.07 ± 0.06	0.81 ± 0.05

All data expressed as LS mean ± SEM (n= 9-15 mice/group). Significant effects of diet were identified by nested one-way analysis of variance. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Choline-modified diets on memory function

To test the effect of dietary choline on spatial memory and cued learning (visual function), the MWM behavioral paradigm was used.

Cued learning

During 2 days of training, the effects of choline were tested on path length (cm), escape latency (sec) and swim speed (sec/cm). On trial day 1, animals fed Cho supplemented diet had the shortest swimming length ($P<0.05$), took the least amount of time ($P<0.05$) and had the fastest swim speed ($P<0.01$) in reaching the cued platform compared to the other groups (**Figure 4-4 A, B and C**). However, on the second trial day, there were no differences in cued learning parameters among diet groups except for animals fed-Cho, who had higher swim speeds ($P<0.05$) in comparison to the Def group. Increased swim speed parameters in the MWM is related to both improved memory and absence of deficits in motor functions.

Spatial memory

Acquisition phase

The effect of choline on spatial memory task was tested by measuring path length (cm), escape latency (sec), and swim speed during a 5 day acquisition phase. During this period, path length (**Figure 4-5 A**) was getting shorter as the days progressed among all diet treatments groups ($P<0.05$). However, no diet effect was identified in this parameter. During the acquisition phase, the escape latency time was also significantly shorter in days with training ($P<0.0001$) (**Figure 4-5 B**). Among diet groups, animals fed PC diet had ($P\leq 0.05$) shorter escape latency time compared to the animals fed other diets.

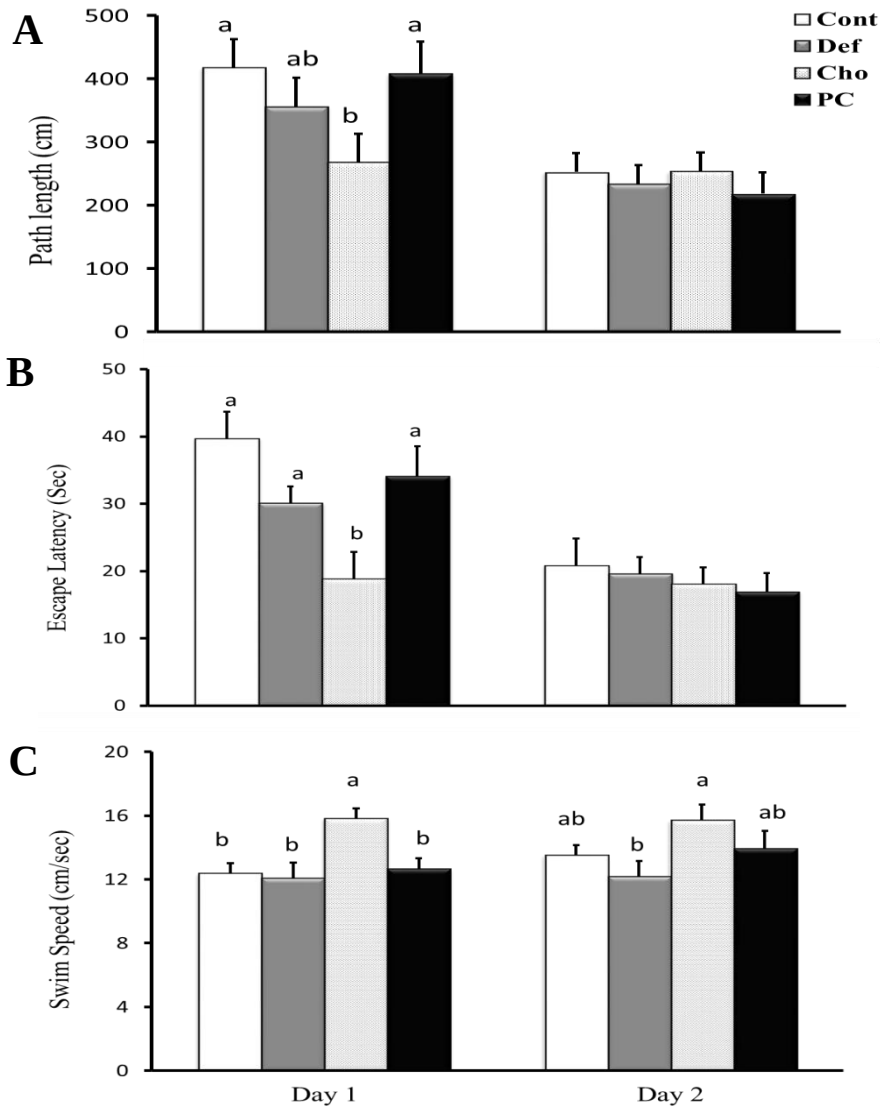


Figure 4-4: Effect of choline-modified diets on cued learning task. Data expressed as LS mean ± SEM (n=10 mice/group). Significant effects of diet were identified by mixed model analysis of variance with repeated measurements over 2 days of training. Different superscripts within a parameter indicate statistical differences. Cued learning parameters: (A) path length (cm), (B) escape latency time (sec), (C) swim speed (cm/sec). Significant diet effects: A, B and C (NS). Significant day effects: A and B ($P<0.0001$), C (NS). Significant interaction (diet and day) effects: B ($P<0.05$). Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

In terms of swim speed, as the days progressed, the swim speed rate was generally higher ($P<0.0001$), suggesting no overt motor deficits in animals, such as an impaired ability to swim. Animals fed PC diet trended toward ($P=0.07$) a higher speed rate from the 3rd day of training compared to the rest of the animal groups (**Figure 4-5 C**). This was qualified by a significant interaction between diet and day, indicating that swim speed varied as a function of the day and diet treatment ($P<0.0001$).

Retention Phase

Time in target quadrant (sec), passover frequency (missing target), and annulus cross index (ACI) were measured as part of memory retention, which took place on day 8. Animals fed choline deficient and supplemented diet spent more time in the missing target quadrant (where the platform was) ($P<0.05$) compared to animals fed Cont and PC diets. However, animals fed the deficient diet had the least number of passes over the missing platform (passover frequency) compared to the rest of the groups (**Figure 4-6 B**), which resulted in the lowest ACI ($P<0.05$). Among diet groups, Cho and PC supplemented groups had higher ACIs in spatial bias for the platform position in the target quadrant, indicating that Cho and PC supplementation may improve memory retention by accurately recalling the position of the platform compared to the Def group.

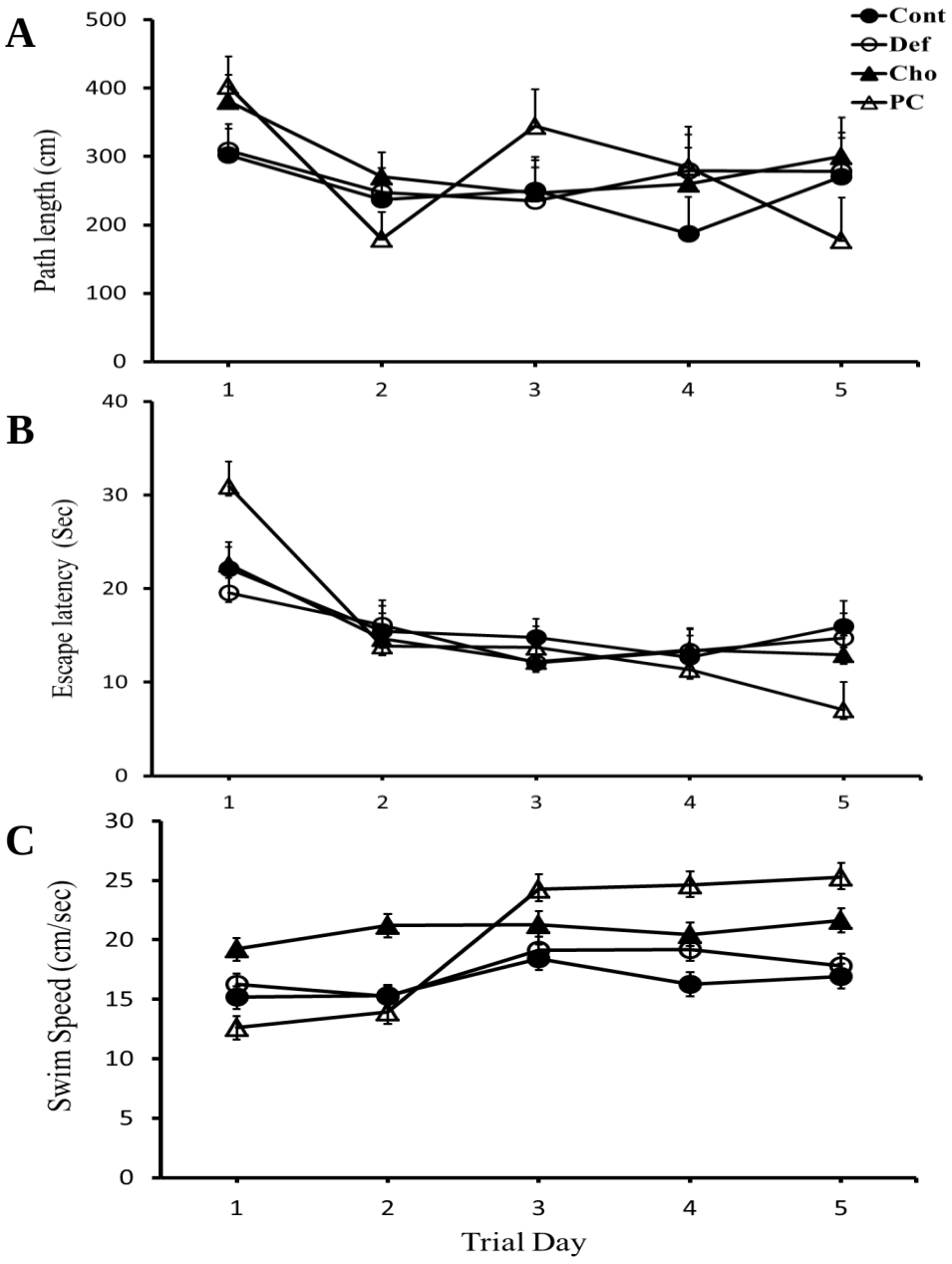


Figure 4-5: Effect of choline-modified diets on acquisition phase of spatial memory task. Data expressed as LS mean ± SEM (n=10 mice/group). Significant effects of diet were identified by mixed model analysis of variance with repeated measurements over 5 days of training. Spatial memory parameters: (A) path length (cm), (B) escape latency time (sec), (C) swim speed (cm/sec). Significant diet effects: A (NS), B (NS), C (P=0.07). Significant day effects: A (P<0.05), B and C (P<0.0001). Significant interaction (diet and day): A (NS), B (P≤0.05), C (P<0.0001). Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

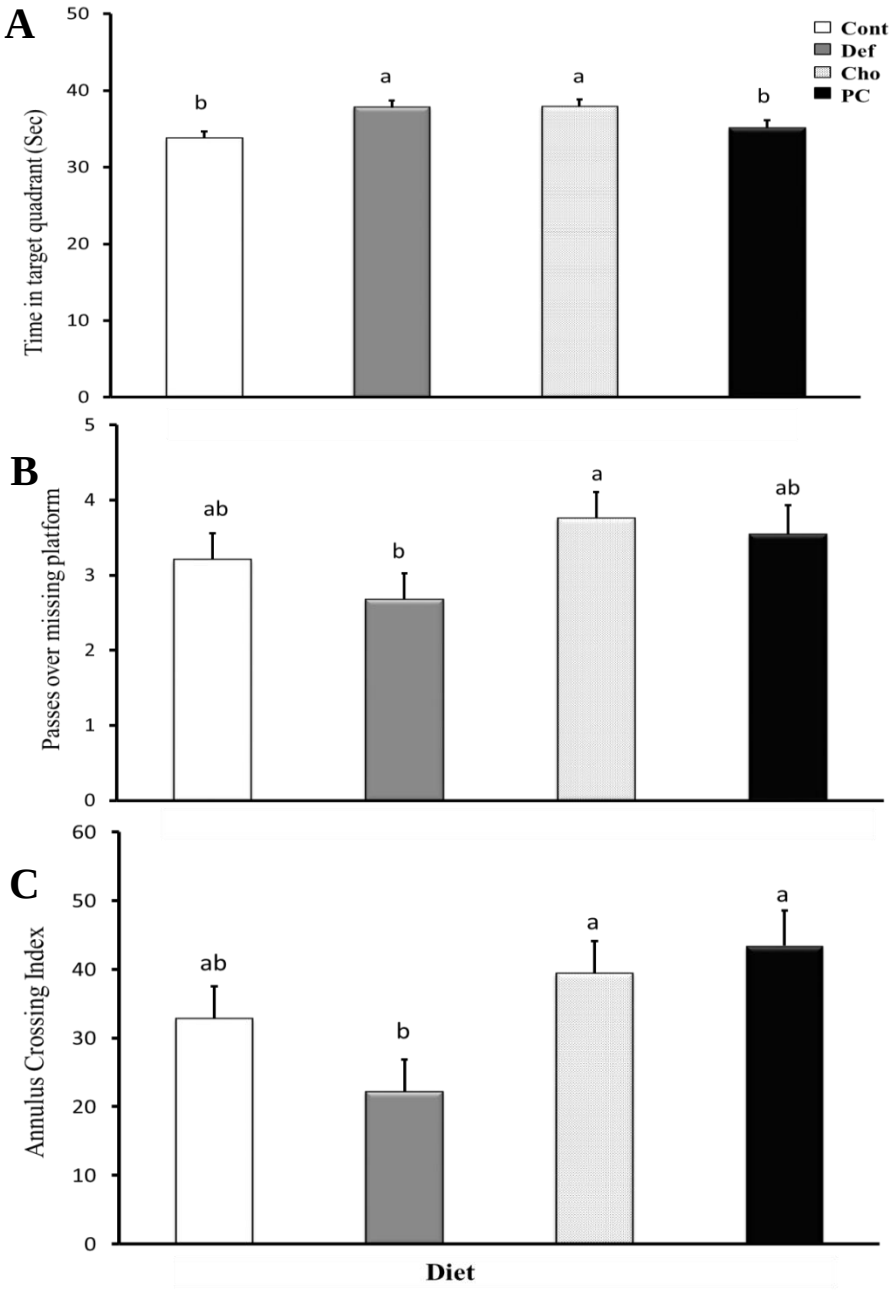


Figure 4-6: Effect of choline-modified diets on retention phase (probe trial) of spatial memory task. Data expressed as LS mean ± SEM (n=10 mice/group). Significant effects of diet were identified by nested one way analysis of variance of 1 day of training. Different superscripts within a parameter indicate statistical differences. Retention phase parameters: (A) time in target quadrant (sec), (B) passes over the missing platform, (C) Annulus crossing index (ACI, $P<0.05$). Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Choline-modified diets on retina function

Retina (rod and cone) functions were measured using ERG recordings to determine the effects of choline.

Choline on dark-adapted responses

Under dark-adapted conditions, typical a-wave (photoreceptor) traces of choline-modified diet treatments are shown in **Figure 4-7 A**. During ERG recordings, Cho and PC supplemented animals had significant lower rod-driven photoreceptors (a-wave, **Figure 4-7 B;** $P<0.05$) and a trend of lowering bipolar cell amplitudes among Cho-fed animals only (b-wave, **Figure 4-7 C**; $P=0.053$) at all light intensities compared to other experimental groups (Cont and Def). This effect was most apparent at the highest stimulus strengths (elicited by 2.4 log cd·s/m^2). The implicit time of rod driven a- and b-waves were tend to be delayed in Cho and PC enrichment compared to other groups. This suggests delayed outer retina photoreceptor response in the dark in choline supplementation groups (**Figure A- 4 A and B**). Rod driven amplitudes of oscillatory potentials were also reduced at increasing flash intensities in Cho and PC supplemented animals compared to the other groups, although it did not reach statistical significances (**Figure 4-7 D**).

Choline on light-adapted responses

The typical b-wave traces of different choline-modified supplemented animals under light adapted conditions are shown in **Figure 4-8 A.** Animals on Cho and PC enrichment diet had lower b-wave amplitudes, indicating an lower processing of retina function, not reaching statistical significance (**Figure 4-8 B**). Cone driven oscillatory potentials (**Figure 4-8 C**) and implicit time (**Figure 4-8D**) were also shown no statistically different among diet groups.

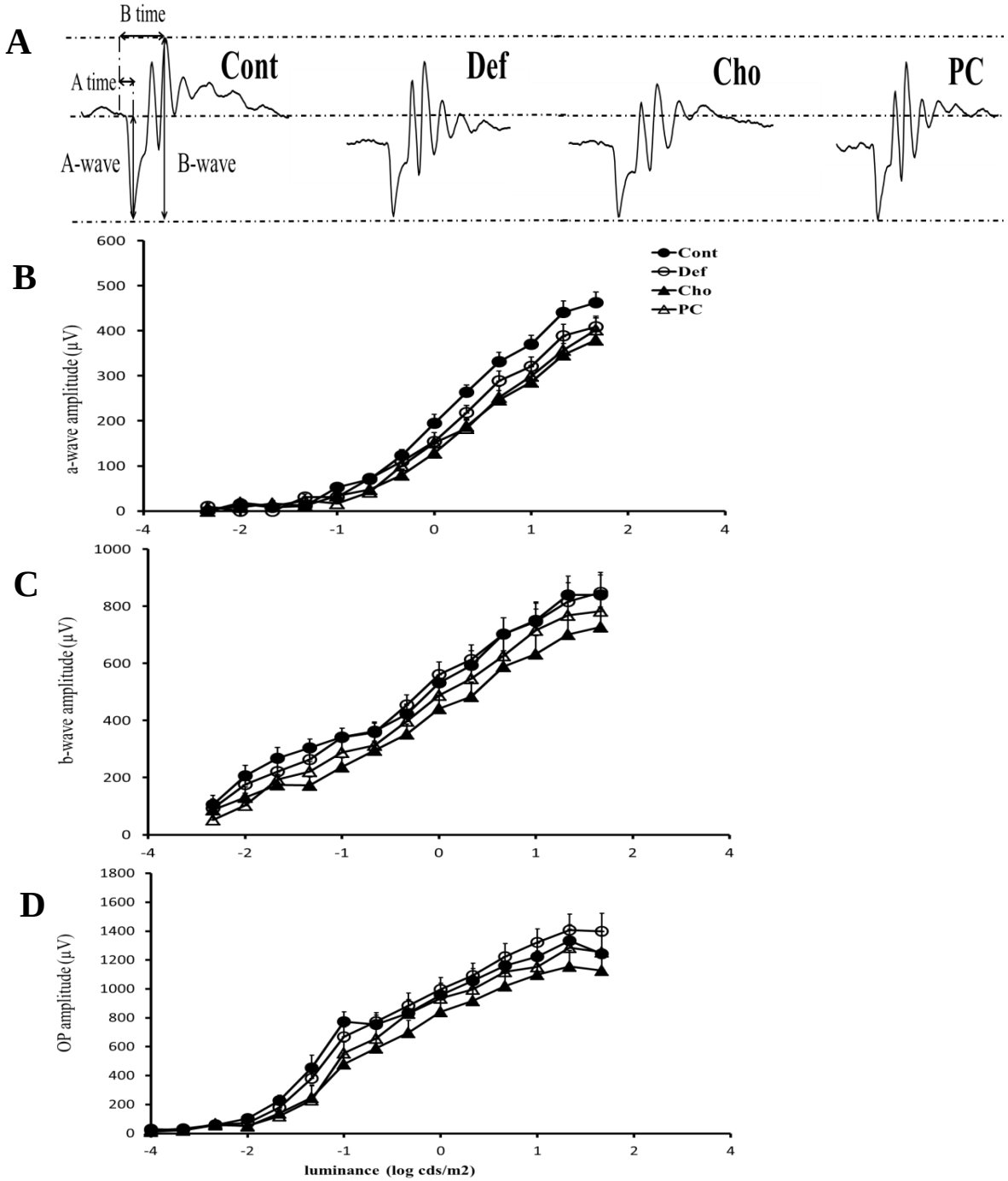


Figure 4-7: Effect of choline-modified diets on ERG dark adapted responses of the retina. Data expressed as LS mean ± SEM (n=6-8 mice/group). Significant effects of diet were identified by using quadratic growth model with PROC MIXED provided by SAS. Representative ERG traces: (A) Typical traces of maximal ERG recording. Scotopic responses: (B) A-wave amplitude; (C) B-wave amplitude; (D) Sum oscillatory potential (OP) amplitude. Significant light effects: B, C and D ($P<0.0001$). Significant diet effects: B, C and D (NS). Significant interaction effects: B ($P<0.05$), C ($P=0.05$), D (NS). Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

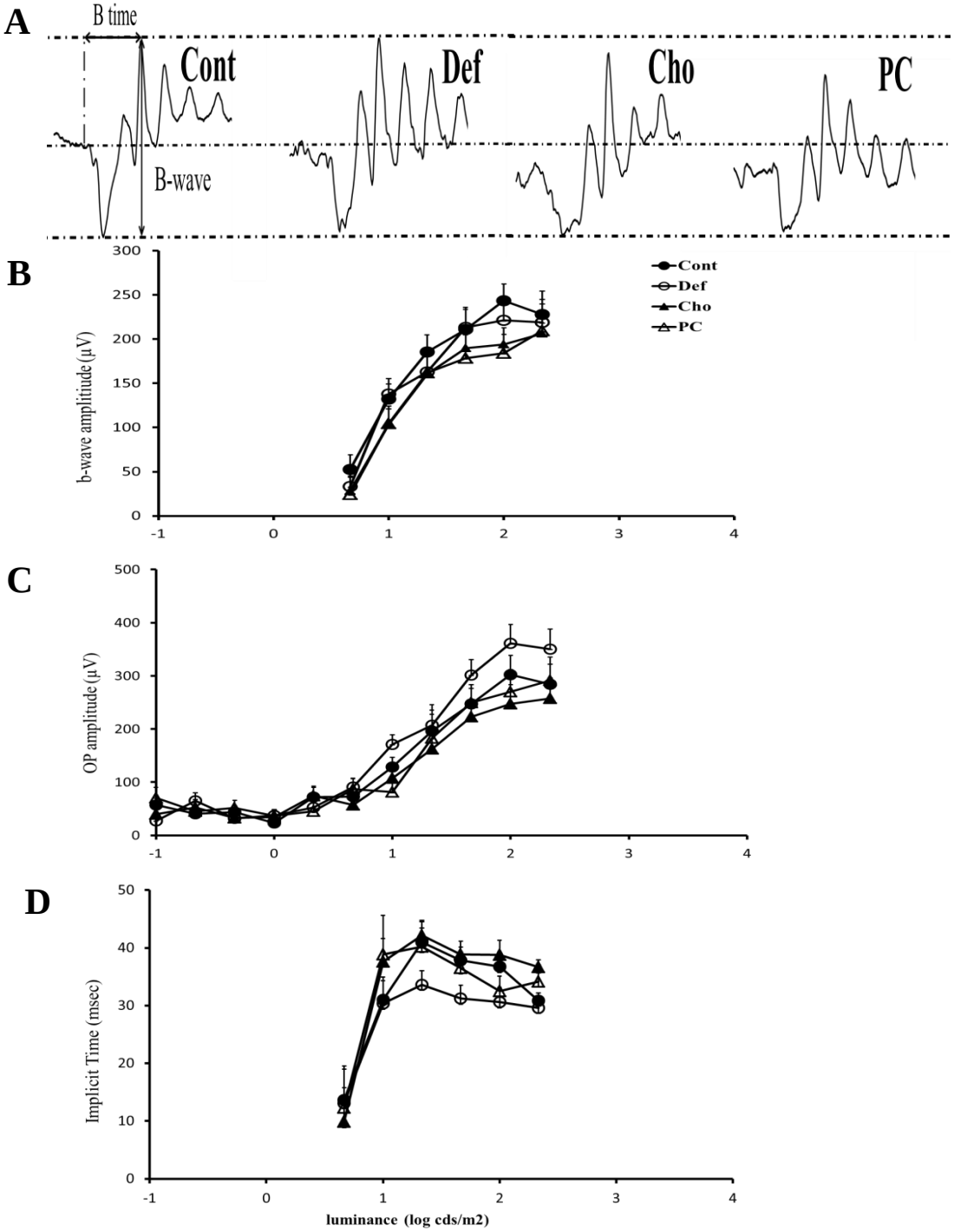


Figure 4-8: Effect of choline-modified diets on ERG light adapted responses of the retina. Data expressed as LS mean ± SEM (n=6-8 mice/group). Significant effects of diet were identified by using quadratic growth model with PROC MIXED provided by SAS. Representative ERG traces: (A) Typical traces of maximal ERG recording. Photopic responses: (B) B-wave amplitude; (C) Sum oscillatory potential (OP) amplitude; (D) B-wave implicit time. Significant light effects: B, C and D ($P<0.0001$). Significant diet effects: B, C and D (NS). Significant interaction effects: B, C and D (NS). Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Choline-modified diets on phospholipids in brain and retina

NMR analysis

The effect of choline-modified diets on phospholipid composition in the brain and retina were determined using NMR. The typical spectrum from PL classes in the brain and retina are shown in **Figure 4-9** and **Figure 4-10**, respectively.

In the brain

Under the present experimental conditions, the major PL classes (PC, PI, SM, PS, and PE) and subclasses of PL (1-O-alkyl-2-acyl-sn-GPE (PEaa), plasmenylethanolamine (PEp) and 1-O-alkyl-2-acyl-sn-GPC (PCaa)) were identified (**Table 4-2**). PL components were as follows: PC > PE > PEp > PS > LysoPE > PI~SM > PEaa > PCaa. The amount of PEp was 16-19% of the total brain PL and 81-99% of the total brain PE which shows the brain is a PEp enriched tissue. Choline-modified diets did not significantly affect the major PL composition regardless of choline deficiency and supplementation. Animals supplemented with Cho and PC had a trend of elevated total PC ($P=0.06$) than other two diet groups. Choline containing SM was not affected by dietary intervention. Both PCaa and PEaa were found highest in animals fed the PC diet and lowest in animals on Def diet ($P<0.05$).

In the retina

The major PL classes were only detected in the retina in order of amounts: PC> PE > PEp > PS > PI > LPE > SM. Unlike the brain, the amount of PEp was 8-10% of total retinal PL and 34-41% of retinal PE. The subclasses of ether lipids (PE_{aa} and PC_{aa}) were not detected, perhaps due to the small sample size. The average, relative total PL levels (**Table 4-3**) were not affected by choline-modified diets.

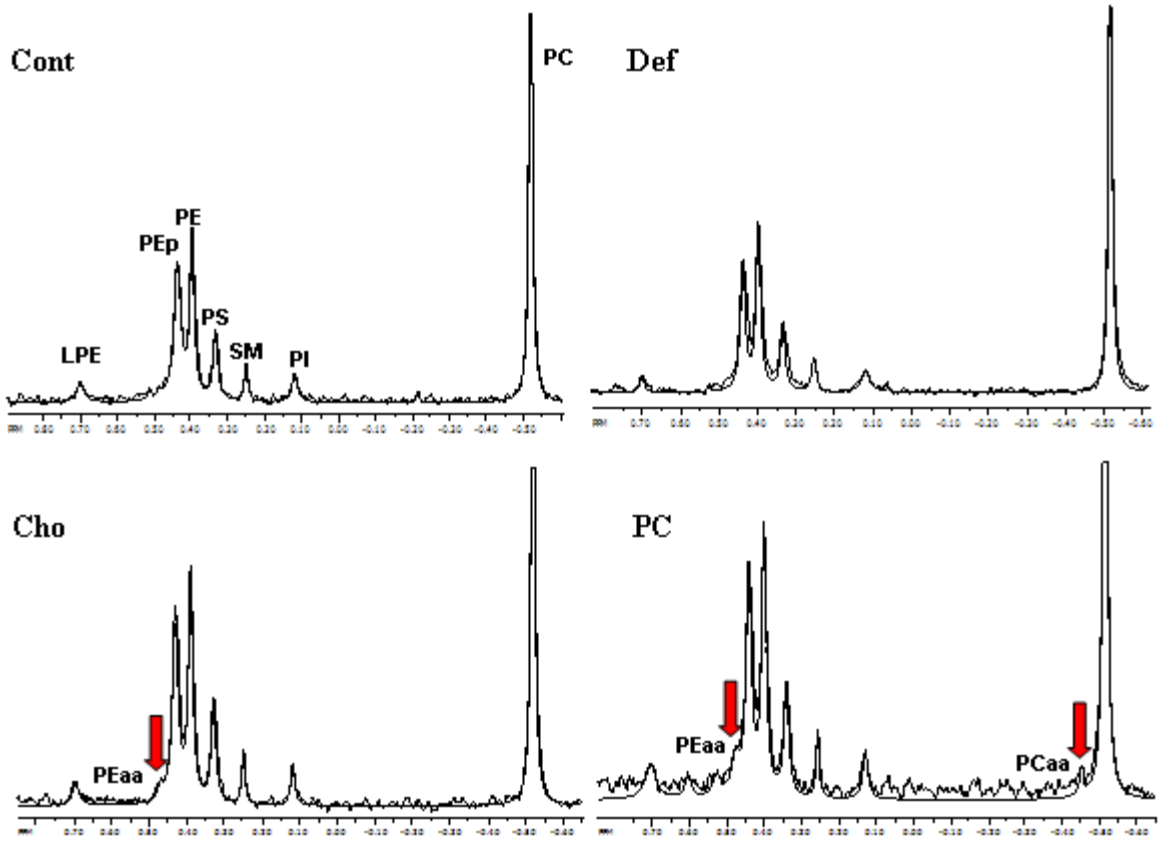


Figure 4-9: Effect of choline-modified diets on phospholipid composition in the brain. Data expressed as LS mean ± SEM (n=7-8 mice/group). The PC peak in ^{31}P NMR spectrum is off-scale. Chemical shifts are referenced to 85% H_3PO_4 at 0.00 ppm by setting the chemical shift of PC to -0.51ppm. Also shown are the individual lines used to fit the various PL class and subclass resonances. The number of lines was used: one for each PL metabolite. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Table 4-2. Effect of choline-modified diets on phospholipid compositions in the brain

PL class (%, total PL)	Cont	Def	Cho	PC
LPE	4.21 ± 0.23	4.24 ± 0.23	4.35 ± 0.26	4.54 ± 0.23
PEaa	1.60 ± 0.45	0.57 ± 0.45	1.69 ± 0.51	2.51 ± 0.46
PEp	18.88 ± 0.82	17.93 ± 0.82	16.27 ± 0.92	16.06 ± 00.83
PE	19.00 ± 0.62	20.33 ± 0.62	20.02 ± 0.70	19.86 ± 0.63
SM	4.08 ± 0.23	4.32 ± 0.23	4.08 ± 0.26	3.98 ± 0.24
PS	11.00 ± 0.44	11.05 ± 0.44	9.98 ± 0.50	9.63 ± 0.45
PI	3.76 ± 0.32	4.60 ± 0.32	4.13 ± 0.35	3.97 ± 0.32
PCaa	0.33 ± 0.27	0 ± 0	0.49 ± 0.30	0.86 ± 0.27
PC	36.32 ± 0.93	36.96 ± 0.93	38.79 ± 1.04	38.59 ± 0.95

All data expressed as LS mean ± SEM (n=7-8 mice/group). Significant effects of diet were identified by nested one-way analysis of variance. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

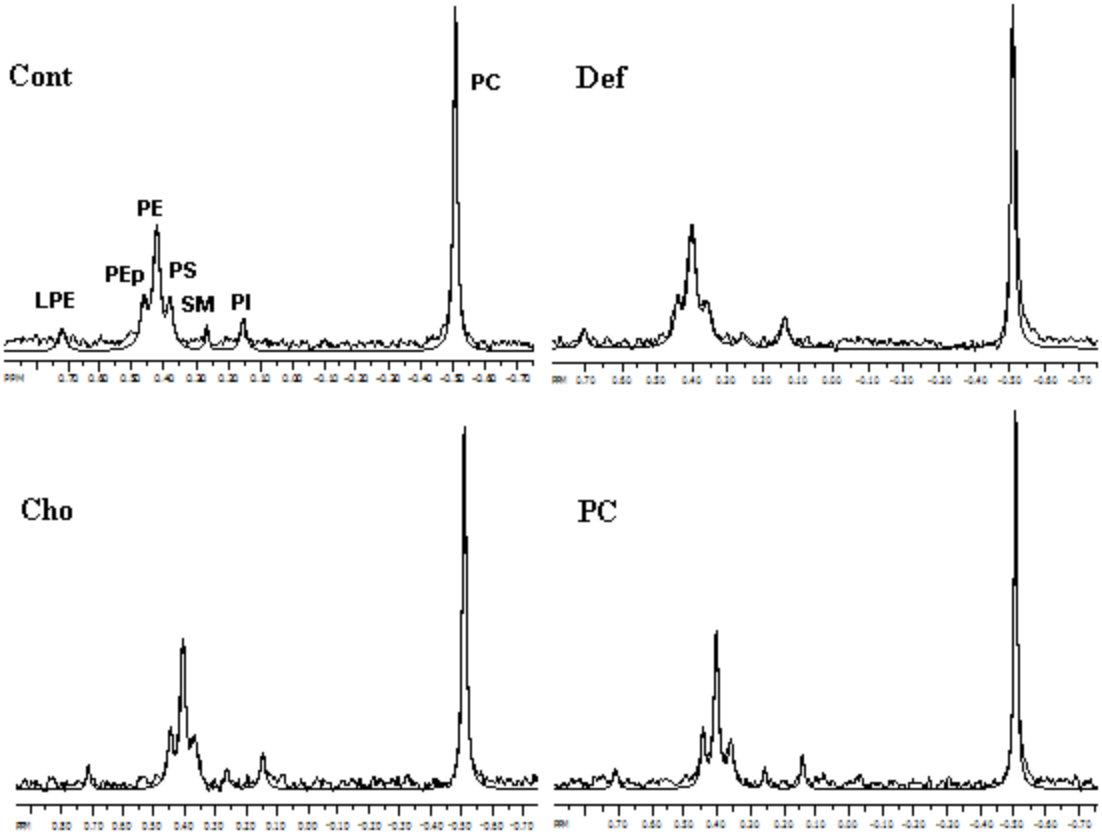


Figure 4-10: Effects of choline-modified diets on phospholipid compositions in the retina. Data obtained from n=3-4 mice/group. Chemical shifts are referenced to 85% H_3PO_4 at 0.00 ppm by setting the chemical shift of PC to -0.51ppm. Also shown are the individual lines used to fit the various PLs class and subclass resonances. The number of lines was used: one for each PL metabolite. Cont, control; Def, choline deficient diet; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

Table 4-3. Effect of choline-modified diets on phospholipid compositions in the retina

PL class (%, total PL)	Cont	Def	Cho	PC
LPE	3.79 ± 0.41	4.17 ± 0.30	3.98 ± 0.65	4.57 ± 0.74
PEp	9.47 ± 1.09	9.64 ± 0.97	8.90 ± 1.81	8.23 ± 0.30
PE	23.39 ± 1.02	23.89 ± 1.73	24.35 ± 1.39	24.15 ± 1.99
SM	3.55 ± 0.47	3.09 ± 0.35	3.25 ± 0.30	3.60 ± 0.35
PS	8.18 ± 0.43	8.58 ± 0.93	8.92 ± 0.09	8.50 ± 0.58
PI	5.12 ± 0.32	5.38 ± 0.41	5.20 ± 0.34	4.44 ± 0.35
PC	46.49 ± 1.72	45.24 ± 1.57	45.40 ± 0.36	46.52 ± 2.70

All data expressed as mean ± SEM (n=3-4 mice/group, 6-7 retinas pooled). Significant effects of diet were identified by nested one-way analysis of variance. Cont, control; Def, choline deficient; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

5. DISCUSSION

Although the essentiality of choline has been known for improving brain and neural cell functions, no studies have been performed regarding its impact on retinal function as a part of the CNS. This is the first study to examine the impact of choline on both the retina, as well as the brain in one study. By providing mice with choline modified diets (supplemented and deficient) during a whole gestational period to the early developmental stages (PD 45), the present study examined behavioral (memory) and electrophysiological (visual) functions and tissue PL compositions. The present study found that choline supplementation improved MWM performance, improved cued learning task by Cho (choline, 1g/100g diet) and spatial memory task by PC (choline, 1g/100g diet). Choline deficiency (choline, 0g/100g diet) contributed the least amount of aid on memory function. The impact of any of these diets on retina function was significant by lowered amplitudes of rod driven responses in the choline supplemented group (choline chloride and egg PC). Cone-driven amplitudes were also lower in Cho and PC groups, although it did not reach statistical significance. The present study also found that choline modified diets trended toward altering PL compositions in the brain, but not in the retina. The two choline supplemented diets increased total PC concentrations, and specifically PCaa and PEaa in the brain. Our results indicate that while additional choline supplementation is beneficial for brain development during pregnancy and early developmental stages, it is not essential for the retina. The contribution of choline to the membrane structure was minor at this stage of growth.

5.1. Influence of choline-modified diets on food intake and body weight

Food consumption for pups on choline-modified diets was similar except for the PC group. Unlike other diet mixtures, the PC diet had a strong odor, which may have contributed to the lower intake in dams and pups resulting in lower body weights throughout the experimental period. Similar observations were made in an earlier study (Suh, not published) when weanling rats were fed DHA containing egg PC. Whether soy PC would elicit the same result remains unknown. Other choline enriched studies used different amounts of PC (Lim and Suzuki, 2000; Chung et al., 1995; Moriyama et al., 1996; Leathwood et al., 1982), however, there was no report regarding animal food consumption or changed in body weight. One double-blind randomized controlled trial of pregnant women consumed egg PC supplementation for eight months and showed no change in infants' birth weights (Cheatham et al., 2012). On the other hand, choline supplementation (Choline Cl) during the gestational period had a better weight-protective effect under diseases conditions; such as fetal alcohol spectrum disorder (Thomas et al., 2010). Interestingly, this present study showed that pups on the choline deficient diet had the lowest body weights during the lactating period, measured at PD 7, PD 14 and PD 21, but observed a sudden increase in weight allowing them to catch-up starting from PD 28 to the Cont and Cho groups' weight. It would have been interesting if the stomach content of the weanling pups were measured to reflect the milk composition of lactating dams.

5.2. Influence of choline-modified diets on memory and visual memory functions

In this present study, MWM, an assessment for hippocampal dependent spatial memory, was used for studying the spatial and visuo-spatial memory function in mice fed

choline modified diets, from gestation throughout subsequent developmental stages. Regarding cued learning for visual memory, animals fed a Cho supplemented diet took less time, shorter swimming length and had a higher swim rate when finding the flagged platform, indicative of better visual memory function and no overt motor deficits (ability of animals to swim) among tested groups. During the spatial memory test in the acquisition phase, mice fed supplemented PC took less time and had a higher swim speed rate to find the hidden platform, suggesting that the intake of choline in the form of egg PC, improved memory function. Mice fed both these supplemented diets (Cho and PC) also had a stronger recall memory based on the annulus crossing index: subtracting the mean number of fake passes from the number of real passes, during retention phase. Mice fed the deficient diet showed the worst recollection abilities. Our findings agree with previous studies that reported improved spatial and visuo-spatial memory functions with Cho during the gestational stage (Tees and Mohammadi, 1999; Williams et al., 1998; Meck and Williams, 1997b; Meck et al., 1988; Moriyama et al., 1996; Meck and Williams, 1997c; Mellott et al., 2004). Similarly, diets provided with 5% and 8% egg PC (w/w diet) allowed adult mice to show improved maze-learning abilities (Lim and Suzuki, 2000; Moriyama et al., 1996). Along with other studies that show the long-lasting effects of prenatally provided choline on brain structure and function (Napoli et al., 2008; Glenn et al., 2008), our data confirms the importance of choline provision throughout growth stages.

PC is absorbed as lipid (50%) and enters the lymphatic system by passing the liver, whereas the aqueous soluble part enters the portal vein and presented to the liver. On the other hand, choline, as a water soluble component, is directly absorbed and enters the portal vein, and then taken up by the liver (Cheng et al., 1996), where 50% of the circulating choline is removed (Haubrich et al., 1975). The liver converts the remaining

choline to betaine and the rest is exported as PC (Zeisel et al., 1980). Therefore, different biological and functional effects can be seen from supplementing choline in the form of PC rather than choline. However, the difference between Cho and PC supplementation on the memory test was non-significant in the present study.

Choline availability during the prenatal period has been shown to influence neurochemical, electrophysiological, and functional activities of different brain regions including the hippocampus and prefrontal cortex in developing rodents (Tees and Mohammadi, 1999; Williams et al., 1998; Meck and Williams, 1997b; Meck et al., 1988; Meck and Williams, 1997c; Mellott et al., 2004; Albright et al., 1999a; Albright et al., 1999b; Meck et al., 2007). Indeed, it has been concluded that prenatal choline improves cognitive functions such as learning and memory in offsprings throughout a variety of mechanisms. For example, prenatal choline activates the hippocampal mitogen-activated protein kinase (MAPK) and cAMP-response element binding protein (CREB) in response to the stimulation of glutamate, NMDA or depolarizing concentration of K+ (Mellott et al., 2004); reduces the apoptotic rate of neuronal cells in the hippocampus and basal forebrain (Holmes-McNary et al., 1997); increases cell division within the neuroepithelial layer of the hippocampus and septum (Albright et al., 1999a; Albright et al., 1999b); and alters cell differentiation in developing animals (Zeisel, 2006). Another important mechanism is choline's long-lasting effect on brain structure and function. For instance, prenatal choline supplementation leads to an increase of growth factors (insulin-like growth factor II mRNA and its receptors) in the hippocampus and frontal cortex (Napoli et al., 2008), and a higher rate of hippocampal neurogenesis that persists until the late stages of adulthood (Glenn et al., 2008). Furthermore, prenatal choline administration increases the size of neural cells (Meck et al., 2007), the number of dendritic spine formations (Williams et al., 1998; Li et al., 2004), and the release of acetylcholine

(Chung et al., 1995). Thus, cholinergic neurotransmission systems are highly efficient for months even after completing a choline supplementation treatment. Finally, the hippocampal long-term potentiation (LTP), a putative mechanism for learning and memory processing, was reportedly enhanced after prenatal choline supplementation (Pyapali et al., 1998; Li et al., 2004), which agreed with our findings. In contrast, prenatal choline deficiency reduces hippocampal neurogenesis, resulting in memory and cognitive deficits that persist until late stages of adulthood (Zeisel, 2006), and induces cell apoptosis (Holmes-McNary et al., 1997). Moreover, it leads to a decrease in processing speed and forces rats to selectively react to stimuli rather than process them in parallel by dividing attention among relevant events (Meck and Williams, 1997c; Meck and Williams, 1997a). Thus, any of these potential effects could be influencing the outcome of following prenatal choline deficiencies. Although our present study did not measure similar parameters, choline deficiency negatively impacted memory retention.

Another possible mechanism for improving brain structure and function is the effect of choline (Cho and PC) intake on the neural cell membrane. Choline is also considered an important component of the phospholipids (PC, PE, PI, PS, SM) in cell membranes, methyl group metabolism, and cholinergic neurotransmission (Zeisel and Blusztajn, 1994). Indeed, PC is one of the major components of the neural cell membrane which occupies about 37% of neuronal membrane lipids (Ansell et al., 1973). Further work should assess choline uptake transporters, fatty acids and enzyme levels, and establish a dose-response between different forms of choline supplementation and brain function indices.

5.3. Influence of choline-modified diets on retinal function

To date, this is the first study examining the effects of choline on retinal function measured directly by ERG responses. The major finding is that animals provided Cho and PC (10g/kg of diet) significantly lowered rod-driven retinal function (a-wave) in comparison to other diet groups. Moreover, Cho-fed animals only trended toward lowering rod-driven (b-wave) as well. Lowered cone-driven retinal function was also observed among Cho and PC, although it did not reach statistical significance. Previous studies have shown choline accumulation in photoreceptor cells from the extracellular environment by a high uptake of choline in a cyclic daily rhythm (Masland and Mills, 1980). Since blood choline concentrations of fetuses and neonates are seven times higher than adults (Zeisel et al., 1980a; Zeisel and Wurtman, 1981), additional provision of choline may have led to a lower retina response. In regards to choline deficiency, the retinal function of male C57BL/6 mice measured at PD 45 displayed similar electrophysiological rod driven functions to the control group. Even cone-driven responses to the light were faster than other groups, indicating choline may not be essential for retinal function with the present experimental design. Since mice fed the deficient diet had caught up in growth after lactation, whether the retina also grew normal needs to be confirmed.

As a general comparison, there was a greater reduction in cone b-wave amplitudes than rod b-wave amplitudes. This might be due to a reduced sensitivity of mice cone pathways, rather than dietary treatment differences, as mice are rod dominant, comprising ~97% of retinal photoreceptors with cones accounting for the remainder (Carter-Dawson and LaVail, 1979). Overall, provision of choline supplementation from gestation to early developmental age (PD 45) significantly alters performance of spatial memory tasks in

MWM, but not retinal function. Choline may be processed differently in different types of neural cells. Further investigation of neural molecular changes would elucidate how choline may differentially affect brain and retina physiological pathways in normal developing animals and animals that experience adverse prenatal nutrition.

5.4. Influence of choline-modified diets on phospholipid profiles in the brain and retina

5.4.1. In brain

This present study tested the enhanced effects of choline supplemented diets on MWM learning ability, via modulated PL compositions of the brain as one of the mechanisms. Unlike other studies (Muma and Rowell, 1986; Foot et al., 1982; Jope et al., 1984; Nardella et al., 1991; Jope and Jenden, 1979), nested one-way ANOVA revealed no overall differences in individual PL classes among diet groups, although both supplemented groups tended to have a higher trend in total PC. Some studies found changing in PL compositions including decreased PC and PE levels while increasing PS and SM in AD and Schizophrenic brains. These changes can be related to the impairment in memory and learning abilities among these diseases. Perhaps, in a normal healthy condition, the influence of choline in PL might be minor. ^{31}P NMR, most useful in the detection of the structural organization of biological membrane systems (Sotirhos et al., 1986), also resolved phospholipid subclasses such as PEp, PE_{aa} and $PC_{aa.}$ Both cho and PC supplementation tended to decrease the PEp level in the brain, yet PC increased the PEaa and PCaa. Since these ether lipids are involved in myelin and synaptic membranes (Igarashi et al., 2011), these minor increases in membrane lipids may have improved memory function. Since no other studies measured these PL subclasses with choline treatment, future confirmation studies are needed. Subsequently, future research should

examine the enzyme activity involved in de novo PL synthesis and the possibility that they are induced by choline enrichment.

5.4.2. In retina

Regardless of choline deficiency or supplementation, the retina PL composition was not affected in the present study. Therefore, the lower amplitudes of ERG under Cho and PC-fed groups seem not related to PL levels. This result does not agree with the previous findings that exogenous choline is mainly incorporated into PC in the retina by high affinity uptake, in order to maintain the steady state of renewing photoreceptor membranes (Masland and Mills, 1980), perhaps due to the in vitro conditions. A more likely explanation of similar PL levels among all groups would seem to be a change in enzymes involved in de novo PL synthesis and catabolism during the growth stage, which was not measured in the present study. In the brain, choline uptake transporters are unoccupied at regular plasma choline concentrations (Pardridge and Oldendorf, 1977), however, it was inhibited when choline reached higher concentrations (Wecker and Trommer, 1984). Because the retina is a part of the CNS, high choline availability in the plasma may limit the retina choline uptake system. Future studies should determine choline levels in the plasma, retina and brain and correlate them to PL metabolism in the retina.

Rhodopsin is a visual protein in the retina, which is positively affected by choline (Kimura and Hosoya, 1952), and also highly correlated to the PL in retina neural membranes (Roy et al., 2011). Thereby, the lower ERG responses seen in Cho supplementation groups could be a result of changing rhodopsin levels, which should have been tested. Another possible explanation is docosahexaenoic acid (DHA) levels in the retina of the four choline-modified diet groups. Retina photoreceptors are mostly

composed of lipids containing DHA, which constitutes approximately 50% of the rod photoreceptor PL (Tuo et al., 2009). DHA plays an important role in the vital functions of retinal photoreceptors including development, survival, and the inhibition of apoptosis (Organisciak et al., 1996; Rotstein et al., 1996). It would have been of interest if DHA was measured in the retina.

Although they are similar neural tissues, the PL distribution was distinctively different between the retina and the brain. Compared to the brain, the retina is highly enriched in diacyl (1-acyl-2-acylglycero-), a subclass of PC (36.3-38.8% vs 45.2-46.5%), PE (19.0-20.3% vs 23.4-24.4%), and lower in PS (9.6-11.1% vs 8.2%-8.9%). The concentration of PEp (1-O-alkenyl-2-acylglycero-PE) was almost two times higher in the brain than in the retina (16.1-18.9% vs 8.2-9.6%), indicating that PL distribution is tissue specific. Although it is not significant, the PEp in brain and retina tended to be lower in both Cho and PC supplemented groups. Whether these minor changes of PEp contribute to the neural cell function is unknown.

Significance of Research

This is the first study to explore the relationship between the effects of choline on both brain development and the retina in one study. It serves as a pilot study to explore the effect of two sources of choline enrichment on neural cell function, examining PL compositions in conjunction with MWM and ERG outcomes. While choline supplementation, especially in the form of PC, improved memory function, it did not have improving effects on retina function in a mouse model. Additionally, it appears that choline enrichment is paired with increasing total PC and ether lipids (PE_{aa} and PC_{aa}) in the brain, with no changes in retina PL, which has never been assessed. The link between brain and retina function, fatty acids compositions and choline uptake receptors /enzymes

should be explored and confirmed in the next stages of the project as the data and results are novel findings. Additionally, markers of lower visual function should be examined with a range of choline enrichment doses within the retina, to determine whether there is damaging or accumulating effects.

Strengths & limitations

This is the first known study to assess brain and retina function in response to two different sources of choline enrichment, as well as its impact on the brain and retina's major classes and subclasses of PL. We established a diet capable of providing choline in its choline-rich food form (as salt or in ester form) in lieu of a purified form, with the intention of having a more representative model of dietary metabolism. Additionally, compared to the other studies that supplemented egg PC for longer period with no impact on memory function (Lim and Suzuki, 2000; Chung et al., 1995; Moriyama et al., 1996; Leathwood et al., 1982), this study found improving effects on memory tasks, even though choline was administered only during the perinatal stages of development. This is drawing our attention to a critical period of brain formation and development; first during prenatal stage from 12-17, and second during postnatal stage from 16-30 (Zeisel, 2006).

There are several limitations to our exploratory study. First, it is assumed that the observed effects of choline as either Cho or PC were due to the choline composition in the diet. Other biologically active components or changes in fatty acid compositions may have been present in all four choline-modified diets, but were not investigated. The energetic content of the four choline-modified diets might also be different because of the fat calorie adjustment by the use of two different forms of fat; canola oil and egg PC. They may have different densities of energy, which were not analyzed. The purity of the egg PC compound, as 80% PC with 18% PE, may have also resulted in our findings (no significant changes) in neural cell membrane compositions of brain and retina due to the

competitive relationship that may produce between PC and PE when they are incorporated into neural cells.

Although we were able to assess visual function outcomes and its related PL compositions, we did not have a sufficient sample size to test fatty acids, specifically enzymes and transporters known to be involved in choline metabolism in the retina. Therefore, we were unable to draw conclusions regarding the mechanism of action involved in lower ERG responses in choline enrichment groups imparted by the results. Moreover, our feeding treatment was designed to ensure there would be a perceived effect in memory and visual function and to establish evidence of functional similarities in neural cells of the brain and retina. Consequently, the diet provided a large dose of choline (Cho vs PC w/w; 10g/kg) for the pregnancy, lactation and, early growth periods, which would most accurately be equivalent to a population of pregnant mothers during the three trimesters of pregnancy and lactation when they would be consuming large amounts of choline. Our dose is not traditionally achievable in humans by diet alone, and is a higher dietary dose (4x higher choline than the recommended intake for rodents). In this study, the effect of choline supplementation on pregnancy versus lactation periods cannot be distinguished.

Furthermore, our model used only male animals, as such, the data may not accurately portray findings in a female population since the sex hormone estrogen has been known to impact gene expression and increase PC synthesis (Noga and Vance, 2003). This may lead to a further reduced sensitivity to choline deficiency in females (Tessitore et al., 1995).

Lastly, supplementing different choline doses for a longer term would be more suitable in future studies of choline and visual function, as they may yield more pronounced results. In our study, only one dose of two different sources of choline was

provided for a short-term, with improved effects on brain function only, not retina. A variety of choline doses provided may present the optimal dose for both brain and retina functions during development stages.

Recommendations for future research

There are several options for follow-ups to this study to further enhance the understanding of the effects of choline supplementation on neural cell metabolism and function, in particular the retina function. The first recommendation is to assess dose responses relative to choline supplementation, memory, and visual function by MWM and ERG outcomes, respectively. Our feeding design was adopted to ensure maximum tissue effects and to establish evidence of functional similarities in neural cells of the brain and retina. Consequently, we provided a larger dose of choline than the recommended intake in mice. We used a level of choline supplementation previously determined in a study improving memory function for Dull mice (mice with dementia), which was 4x higher than the recommended intake, and would qualify an uncommon level in humans (Moriyama et al., 1996). Although the previous study authors reported no detrimental effects on brain function in the control group, there may be differing thresholds in retina function, which may have contributed to the loss of ERG amplitudes in choline enriched groups. As this dose of choline has never shown any detrimental effect on other body organs, an assessment of a variety of doses, starting at 5% (w/w) is required for future consideration, and for a better understanding of lipid metabolism and influence on retina.

Due to the small sample size and time limitation, we were unable to assess different enzyme and transporter functions within the brain and retina; any of which may have contributed to the improving effects on brain function and to the loss of ERG amplitudes of choline enriched groups in the retina. Future research should consider

correlating MWM and ERG results with these measurements, since little is known about choline transportation and utilization within the brain and retina during development. Choline and its metabolites may have accumulated in the ocular tissue and caused a slight visual dysfunction, which suggests further examination is required.

Conclusion

Choline supplementation, especially in the form of egg PC, is beneficial to the brain but not retina. In terms of PLs compositions, choline enrichment as Cho did not change PL levels, compared to PC supplementation in the brain. Thus, this draws our attention to another unexplored mechanism that may have improved memory function apparently with PC than Cho supplementation, which requires further investigation. In the retina, it remains to be confirmed as to whether the decrease in ERG amplitudes and not changing PL components is reflective of a higher availability of choline or inhibition of choline uptake for PL components involved in neural cell membrane structures. At present, the underlying mechanism of improving memory function with choline enrichment is well understood, however, the mechanism of deteriorating visual function with choline supplementation remains unknown. Although previous studies have suggested a need for choline supplementation on brain and neural cell functions, especially during developmental stages (Pyapali et al., 1998; Meck and Williams, 1997b; Montoya and Swartzwelder, 2000; Meck and Williams, 1997c; Meck and Williams, 1997a; Meck and Williams, 1999), there is little evidence to confirm that choline supplementation has any benefit within the retina, as a part of neural cells. Considering the dynamic effect on neural cell, and the enhancing effect of ether lipids and PC in the brain but not the retina, supplementation of choline at high concentrations used in this study needs to be confirmed before recommending doses at present time.

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Appendix A

1. Morris Water Maze (MWM)

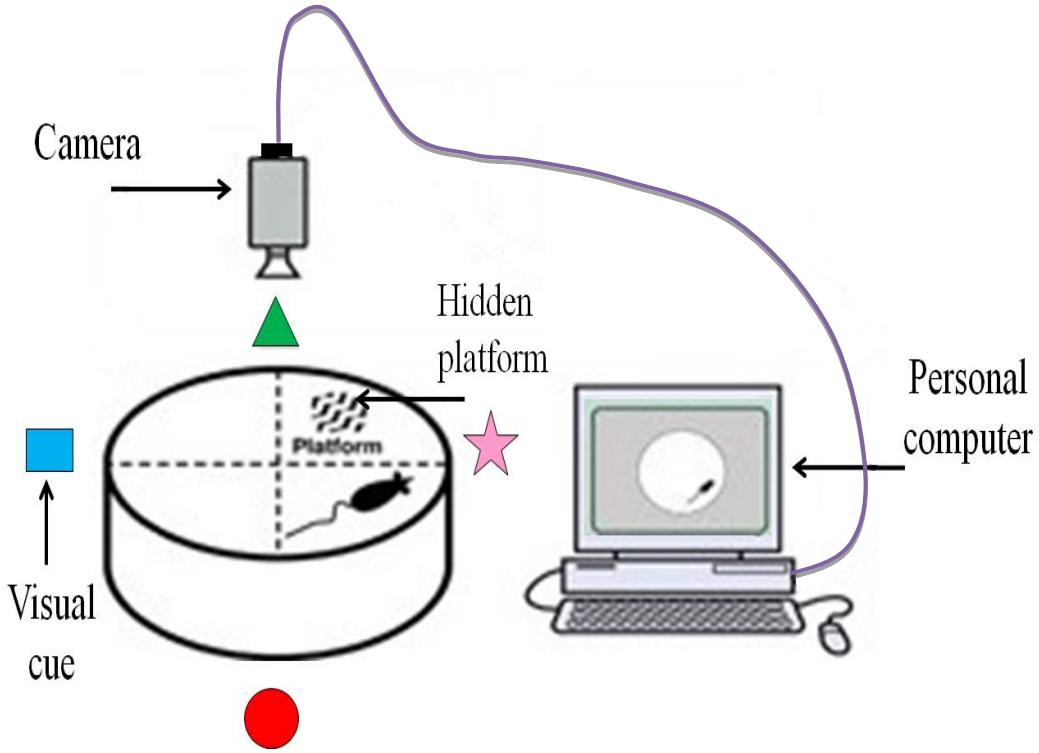

Figure A- 1: Schematic of a full field Morris water maze.

2. Electroretinogram

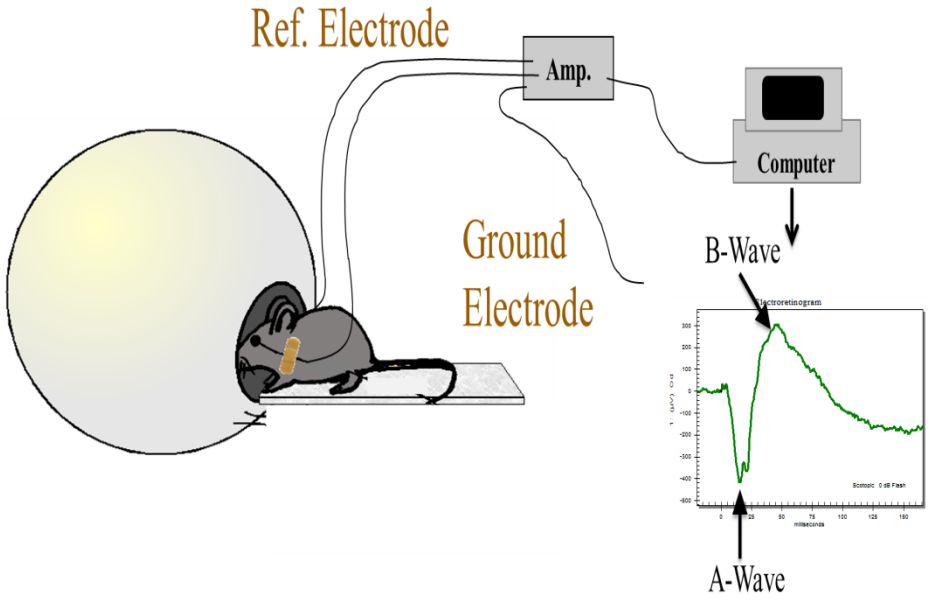

Figure A- 2: Schematic of a full field electroretinogram apparatus.

3. Lipid analysis (H-plate)

PLs bands in brain tissue were separated on silica gel H-plate. A representative chromatogram is shown in **Figure A- 3.** The lipids migrated in this system in the order neutral lipid (NL) > sulfatide > PE > PI > PS > PC > SM. However, our main focus is on the main cellular membrane PLs (PE, PI, PS, PC and SM). PC and PE fractions are more expressed than other PLs bands in all diet treatment groups, followed by PS component. The corresponding quantitative amounts of PLs bands displaying on H-plate were analyzed by using NMR technique.

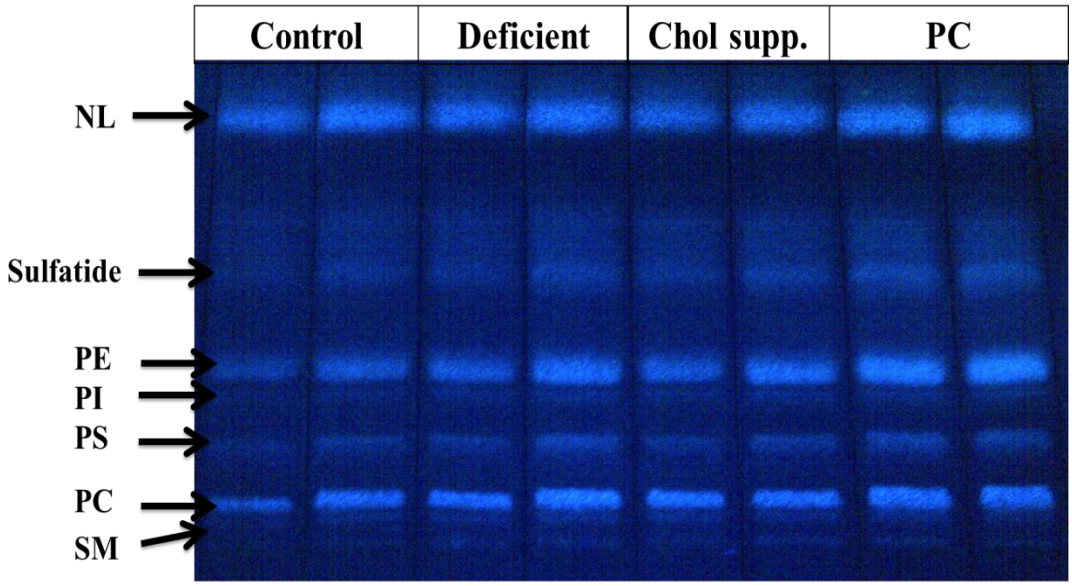

Figure A- 3: Effects of choline-modified diets on phospholipids components of the brain. Data extracted from n= 8 mice/group. PC and PE are abundant in the brain compared to the other phospholipids. Cont, control; Def, choline deficient diet; Cho, supplemented with Choline Cl; PC, supplemented with egg PC.

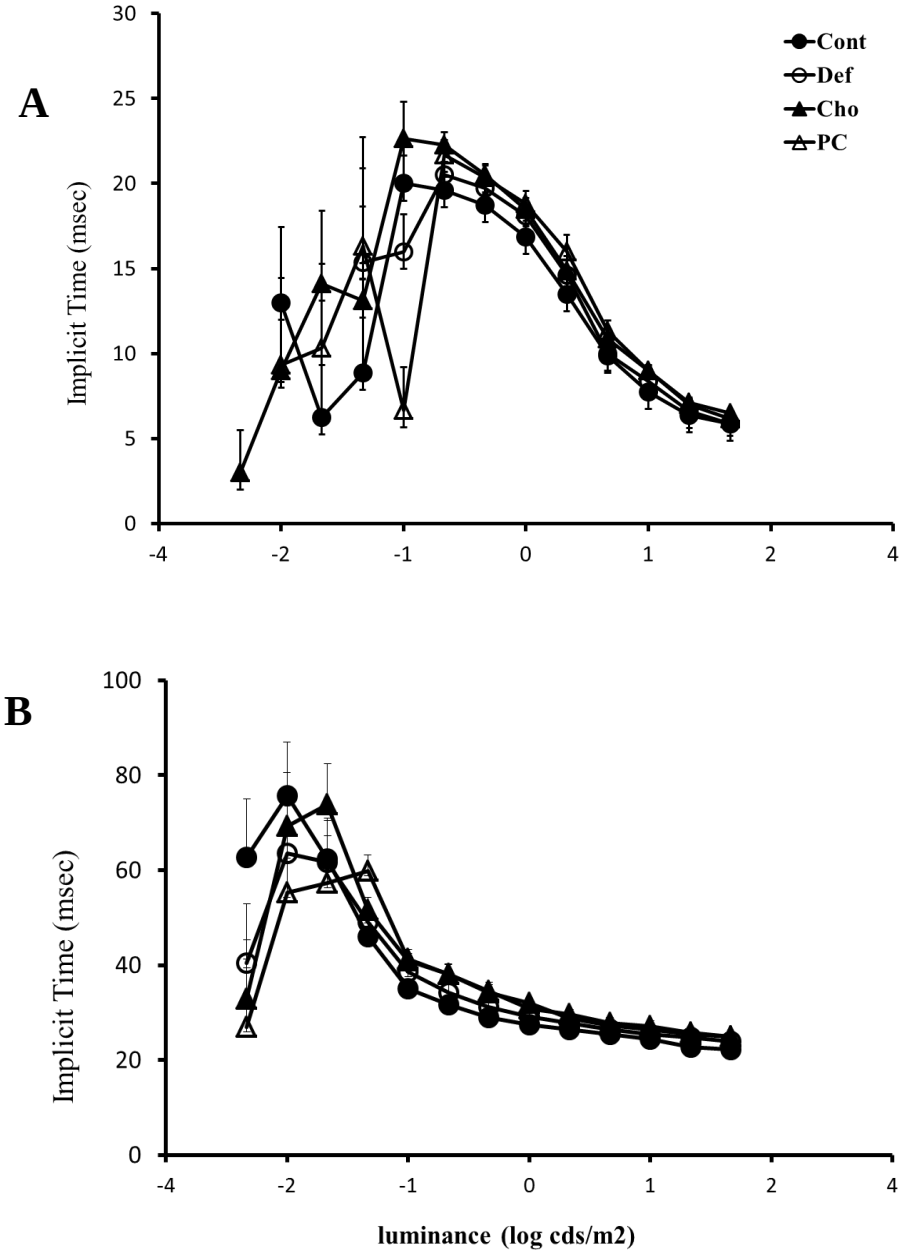

Figure A- 4: Effects of choline-modified diets on dark adapted implicit times in mice. Data expressed as LSMEAN ± SEM (n=8 mice/group). Significant effects of diet were identified by nested one-way analysis of variance. Scotopic response: (A) dark-adapted intensity response of a-wave implicit time; (B) dark-adapted intensity response of b-wave implicit time. Significant diet effects: A and B (NS). Cont, control diet; Def, choline deficient diet; Cho, choline chloride supplemented; PC, egg phosphatidylcholine supplemented diet.

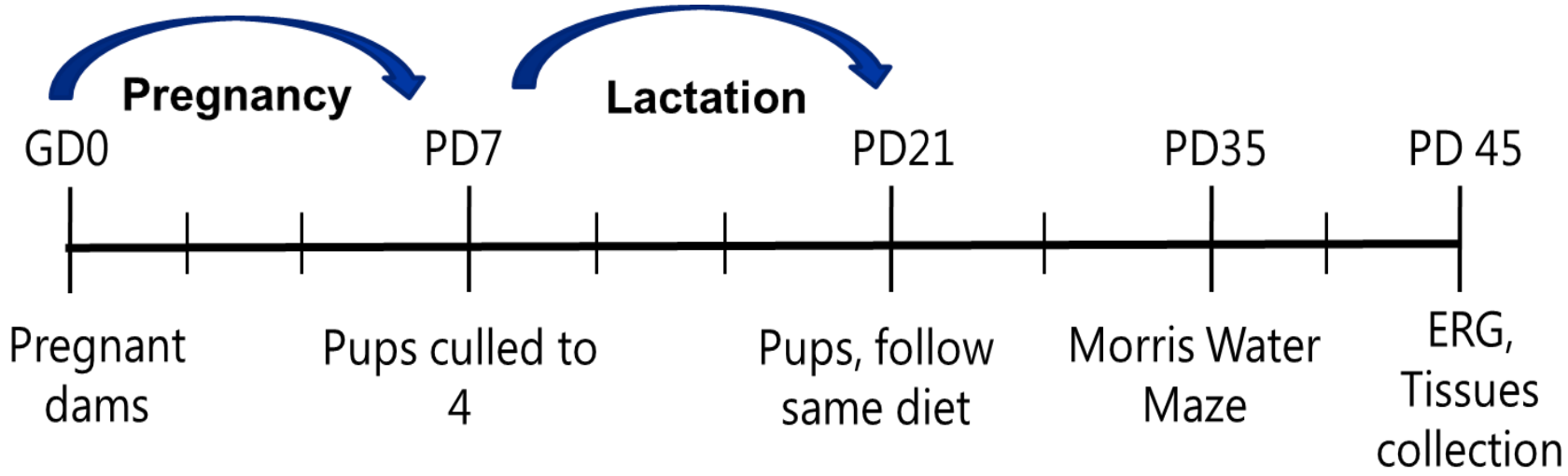

Figure A- 5: Experimental Design of the study.

Table A-1. Fatty acids analysis of canola oil and egg PC.

Fatty Acids	Canola Oil (%)	Egg Yolk Lecithin (egg PC) (%)
Palmitic acid (C16:0)	4.11	29.33
Palmitoleic (C16:1)	-	1.15
Margaric acid (C17:0)	-	0.21
Stearic acid (C18:0)	1.85	14.45
Oleic acid (C18:1)	58.23	22.01
Linoleic acid (C18:2)	18.19	14.26
γ-Linolenic acid (C18:3) (n-6)	0.46	0.19
α- Linolenic acid (C18:3) (n-3)	7.29	0.10
Arachidic acid (C20:0)	0.60	0.11
Gondoic acid (C20:1)	0.94	0.20
Eicosadienoic acid (C20:2)	-	0.36

Dihomo-γ-Linolenic acid (C20:3)	-	0.40
Arachidonic acid (C20:4)	-	5.53
Behenic acid (C22:0)	-	0.10
Adrenic acid (C22:4)	-	0.32
Lignoceric acid (C24:0)	-	1.76
Nervonic acid (C24:1)	-	0.12
Docosapentaenoic acid (C22:5)	-	0.13
Docosahexaenoic acid (C22:6)	-	1.60

Table A-2. Weekly body weights of dams and pups

Group	Week	Cont	Def	Cho	PC	Diet (P<)
Dams	1	19.55 ± 0.32	20.15 ± 0.41	18.58 ± 0.08	18.18 ± 0.28	0.002
	2	20.95 ± 0.31	22.40 ± 0.71	20.85 ± 0.88	20.03 ± 0.20	0.08
	3	25.73 ± 0.75	26.88± 0.84	23.30 ± 1.68	23.45 ± 1.40	NS
	4	36.43 ± 3.86	36.65 ± 5.35	31.43 ± 4.40	29.30 ± 7.25	NS
	5	25.73 ± 1.42	27.23 ± 0.99	26.20 ± 0.61	25.38 ± 1.34	NS
	6	27.90 ± 0.86	31.70 ± 1.15	30.05 ± 1.63	31.10 ± 6.18	NS
	7	29.43 ± 0.73	32.45 ± 4.25	25.80 ± 1.01	25.78 ± 0.98	0.06
Pups	1	4.57 ± 0.12	3.73 ± 0.09	4.52 ± 0.12	3.87 ± 0.12	0.03
	2	7.86 ± 0.21	6.69 ± 0.16	8.75 ± 0.22	6.84 ± 0.21	NS
	3	11.56 ± 0.22	9.41 ± 0.17	11.82 ± 0.23	9.44 ± 0.22	0.06
	4	18.48 ± 0.33	14.74 ± 0.25	17.68 ± 0.34	13.53 ± 0.33	0.02
	5	21.51 ± 0.56	19.11 ± 0.42	21.99 ± 0.58	17.21 ± 0.56	0.01
	6	22.40 ± 0.68	21.51 ± 0.51	23.66 ± 0.71	18.89 ± 0.68	0.02
	7	22.68 ± 0.73	21.86 ± 0.55	23.91 ± 0.76	19.31 ± 0.73	0.02

Data expressed as LS mean ± SEM (n=9-11 mice/group) in grams for pups. For dams, data expressed as mean ± SEM in grams (n=4 mice/group). Week 7 weights measured prior to fasting and termination.

Table A-3. Weekly feed intake of pups after weanling

Week	Cont	Def	Cho	PC	Diet (P<)
1	5.44 ± 0.23	5.32 ± 0.06	4.95 ± 0.07	4.43 ± 0.14	NS
2	7.05 ± 0.66	7.22 ± 0.51	6.06 ± 0.24	5.68 ± 0.11	NS
3	6.72 ± 0.37	6.36 ± 0.21	6.83 ± 0.36	5.90 ± 0.08	NS
4	7.08 ± 0.47	5.72 ± 0.09	7.47 ± 0.35	5.45 ± 0.13	0.04

All data expressed as LS mean ± SEM (n=9-11 mice/group) in grams. Week 4 intakes measured before fasting