

Effect of Zinc and DHA on Expression Levels and Post-Translational Modifications of Histones H3 and H4 in Human Neuronal Cells

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1. Introduction

Docosahexaenoic acid (DHA) is an important omega-3 fatty acid required for the development of the human central nervous system and the continuous maintenance of neuronal cell function. The DHA composition of the brain decreases with age possibly as a result of increased oxidative damage to the lipid membranes (Schaefer, Bongard et al. 2006). Epidemiological studies have shown that patients with Alzheimer's disease (AD) have significantly lower levels of omega-3 fatty acids in their plasma phospholipids (Moriguchi, Greiner et al. 2000; Friedland 2003).

There is an association between DHA levels in the brain and zinc homeostasis, which is particularly interesting as both are involved in neuroprotection. A reduction of DHA levels in the brain causes over expression of ZnT3, a transmembrane proteins that is associated with sequestration of cytoplasmic Zn²⁺ into synaptic vesicles (Jayasooriya, Ackland et al. 2005), resulting in zinc toxicity and neuronal cell death in cultured neuronal cells (Suphioglu, De Mel et al. 2010a; Naganska and Matyja 2006). Mice that lacked a zinc-transporting gene ZnT3 were shown to develop fewer and smaller plaques than Alzheimer's-prone mice with the gene (Lee, Cole et al. 2002), suggesting that altered zinc homeostasis may contribute to the plaque formation in AD. DHA, on the other hand, has neuroprotective properties against neurodegenerative diseases. Dietary supplement of omega-3 fatty acid may protect against Alzheimer's disease, through inhibiting amyloid plaque formation (Calon, Lim et al. 2004; Oksman, Iivonen et al. 2006; Florent-Bechard, Malaplate-Armand et al. 2007). DHA was also observed to significantly increase neuronal survival by preventing cytoskeleton perturbations, caspase activation and apoptosis (Florent-Bechard, Malaplate-Armand et al. 2007).

Our recent data has shown that histone gene and protein expression were affected by both zinc and DHA. The expression levels of histones H3 and H4, in human neuronal cells, were down-regulated by zinc and up-regulated by DHA (Suphioglu, Sadli et al. 2010b), suggesting a possible interaction between the two nutrients. Further investigations into the

effects of zinc and DHA on histone post-translational modifications (PTMs) were carried out. Zinc was found to reduce acetylation, while increasing histone deacetylase (HDACs) expression levels. This is consistent with dysfunctional acetylation-deacetylation apparatus seen in neurodegenerative diseases (Saha and Pahan 2006). DHA, however, showed an increase in acetylation of histones while it reduced the HDACs to the basal level, indicating that zinc and DHA have distinct epigenetic patterns. Both may be involved in neurodegenerative process possibly mediated by histone post-translational modification. Epigenetic mechanisms, including DNA methylation and histone modifications are critically important in mediating precise neural gene regulation. Studies reported that abnormal epigenetic regulation was associated with mental retardation and neurodegenerative symptoms (Al-Gazali, Padmanabhan et al. 2001).

Although it is still unclear that the change in DHA levels, which result in an alteration in zinc homeostasis, could contribute to the beta amyloid formation (Koh 2001) and neuronal cell death, a growing number of studies do in fact indicate this altered molecular interaction between zinc and DHA as a trigger of the pathology (Jomova, Vondrakova et al., 2010; Tougu, Tiiman et al., 2011).

In this chapter, we will summarize the current findings regarding molecular interactions between zinc and DHA that may provide a potential molecular mechanism to explain the beneficial effects of dietary DHA in neuroprotection.

1.1 Neurodegenerative diseases

As life expectancies are increasing and populations are ageing, neurodegenerative diseases have become a global issue (Nepal, Brown et al. 2008; Nepal, Ranmuthugala et al. 2008). Neurodegenerative diseases such as Alzheimer's disease is the leading cause of dementia in the elderly, which is characterized by molecular changes in nerve cells that result in nerve cell degeneration and ultimately nerve dysfunction and cell death (Dong, Wang et al. 2009). In 1995, Australia had a population of 18 million and 13,000 people were estimated to have dementia. It is predicted that Australia will have 25 million people in 2041, and 460,000 of these will have dementia (Jorm 2001). In other words, while our total population will increase by 40%, our population with dementia will increase by more than three-fold (Jorm 2001).

The expected human lifespan is now longer than ever due to improved hygiene, the discovery of medicines such as antibiotics, and economic welfare. The consequences for this aging population are the increased incidence of age-related diseases. Therefore, treatments to prevent age related neurodegeneration will have economic benefits as well as major impact on the quality of life of the patients (Karasek 2004). A great deal is already known about the pathology of neuronal diseases, but the molecular mechanisms underlying many of these diseases remain unknown. Thus, more research is needed to find the cause and to improve the treatment methods for these significant mental health problems.

1.1.1 Risk factors associated with neurodegeneration

The most consistent risk factor for developing neurodegenerative disease is aging (Pardon and Rattray 2008; Yankner, Lu et al. 2008; Fratiglioni and Qiu 2009). While it is possible to develop dementia early in life, the chances of developing it increases dramatically as people get older (Rocca, van Duijn et al. 1991). Although AD can strike people in their 30s, 40s, or 50s, the vast majority of cases of AD are diagnosed in people older than 65

(Breteler, van den Ouweland et al. 1992; Launer, Brayne et al. 1992; McDowell 2001). A family history of dementia, gender (women are more likely to develop dementia than men), a head injury in the past (Plassman, Havlik et al. 2000), atherosclerosis, high cholesterol, hypertension, diabetes and high homocysteine levels, excessive alcohol and tobacco consumption, exposure to environmental substances and non-healthy diets are some of the factors likely to increase risk of dementia (Larrieu, Letenneur et al. 2004; Letenneur 2004).

While there are some risk factors that cannot be controlled, such as genetics or age, many risk factors can be managed through lifestyle changes or appropriate dietary intakes. These dietary and lifestyle interventions cannot stop people from developing dementia but they may reduce the risk (Simopoulos 1999; Simopoulos, Leaf et al. 1999; Crawford, Bazinet et al. 2009). The adequate omega-3 fatty acid and zinc intake are examples of dietary factors associated with a substantially reduced risk of neurodegenerative diseases (Simopoulos 1991; Crawford, Bazinet et al. 2009; Devore, Grodstein et al. 2009).

1.2 The importance of zinc and DHA in neuronal cells

1.2.1 Zinc in the brain

Zinc is the second most prevalent trace element in the body and is present in particularly high concentrations in the mammalian brain (Weiss, Sensi et al. 2000), including synaptic vesicles where it is tightly bound to intracellular proteins and zinc finger-containing transcription factors (Frederickson, Hernandez et al. 1989). The concentration of intracellular free zinc in the brain is thought to be very low under physiological conditions (Frederickson, Hernandez et al. 1989; Outten and O'Halloran 2001). However, it can rise to >300 nM in response to injurious stimuli (Canzoniero, Turetsky et al. 1999).

Zinc plays an important role in growth and development, the immune response, neurological function and reproduction (Stefanidou, Maravelias et al. 2006). Zinc is also a constituent of many enzymes and is essential for the proper function of various enzymes including carbonic anhydrase (Lukaski 2005), RNA polymerase, and superoxide dismutase (Paik, Joung et al. 1999).

The role of zinc in cognitive function has been studied extensively in both children (Sandstead, Penland et al. 1998) and the elderly (Bertoni-Freddari, Mocchegiani et al. 2006). Zinc deficiency during fetal life is associated with developmental delays and low serum zinc levels in elderly is linked with poor global cognitive function (Golub, Keen et al. 1995), particularly verbal function, and also increases stress (Mocchegiani, Bertoni-Freddari et al. 2005; Mocchegiani, Malavolta et al. 2006). Zinc deficiency most often occurs when zinc intake is inadequate or poorly absorbed (Hambidge, Goodall et al. 1989; Golub, Keen et al. 1995; Paik, Joung et al. 1999), when there are increased losses of zinc from the body or when the body's requirement for zinc increases (Hambidge, Goodall et al. 1989). Nonetheless, despite its importance, recent studies have revealed that excess zinc release in the pathological condition is toxic to the central nervous system. Moreover, disruption of zinc homeostasis has been implicated in several neurodegenerative diseases, such as AD (Huang, Cuajungco et al. 2000; Watt and Hooper 2003) where excess extracellular synaptic zinc was found to induce the formation of amyloid plaques, the characteristic feature of AD brains (Linkous, Adlard et al. 2009; Zatta, Drago et al. 2009). These studies suggest the link between an altered neuronal zinc homeostasis and neurodegenerative disease progression.

1.2.2 Omega-3 fatty acids in the brain

Docosahexaenoic acid (DHA) is the predominant omega-3 fatty acid in the brain of mammals which comprises up to 15% of the concentration of fatty acids in the nervous system (Calderon and Kim 2004). It is found in the neuronal phospholipids in high concentrations in synapses (Bazan 2003). Epidemiological studies suggest that dietary DHA, which is commonly found in fish (Kalmijn, Launer et al. 1997), may modify the risk for certain neurodegenerative disorders (Hibbeln and Salem 1995). As evidence, decreased blood levels of omega-3 fatty acids have been associated with several neurodegenerative conditions, including Alzheimer's disease, schizophrenia and depression (Fenton, Hibbeln et al. 2000; Young and Conquer 2005). Communities with regular consumption of fish have shown to possess reduced prevalence of neurodegenerative disease and cognitive decline in general (Fenton, Hibbeln et al. 2000; He, Song et al. 2004; van Gelder, Tijhuis et al. 2007).

DHA can be linked with many aspects of neural function, including neurotransmission, membrane fluidity (Lauritzen, Hansen et al. 2001), ion channel (Lai, Wang et al. 2009), enzyme regulation (Strokin, Sergeeva et al. 2003) and gene expression (Qi, Seo et al. 2006). DHA is found in breast milk, and may be required for early visual (Bazan 2009) and brain development in children (Willatts 2002; Simmer and Patole 2004). Furthermore, studies in animal models have provided support for the protective role of omega-3 fatty acid. For example, mice fed on diets high in omega-3 fatty acids were shown to improve in neurological function, such as better regulation of nerve cell membrane excitability (Xiao and Li 1999), increased levels of neurotransmitters and higher density of neurotransmitter membrane receptors (Innis 2000). Hossain et al (1998) found that administration of DHA led to improvement in memory function and reduction in free radical levels while maintaining high level of antioxidant enzyme, suggesting a role of DHA in antioxidant defense (Hossain, Hashimoto et al. 1998). Study by Calon et al (2004) has reported that dietary DHA protects the cells against apoptosis by decreasing caspase activity (Calon, Lim et al. 2004). While others have supported this finding by showing that the enrichment of dietary DHA prevents apoptosis under damaging conditions (Gomez de Segura, Valderrabano et al. 2004). DHA also increases phosphatidylserine levels (PS) in neuronal membrane, which result in Akt translocation (Akbar, Calderon et al. 2005) and contributes to survival signaling by suppression of caspase-3 (Akbar, Baick et al. 2006).

1.2.3 Molecular link between DHA and zinc in neuronal cells: DHA decreases neuronal cell death in association with altered zinc transport

The alteration in both DHA and zinc homeostasis are key features of neurodegenerative disorders (Lukiw, Cui et al. 2005) (Cuajungco and Lees 1997). A study by Jayasooriya et al (2005) has demonstrated the link between an altered zinc homeostasis in the brain of rats fed on an omega 3-deficient diet (Jayasooriya, Ackland et al. 2005); this diet lead to a significant decrease in brain DHA levels. Though these data have shown a relationship between DHA and zinc homeostasis, the basis of a molecular mechanism has not been elucidated. We therefore used this fundamental idea to investigate the molecular mechanisms underlying the zinc and DHA interaction. With the use of human neuroblastoma cell line M17, we have shown that DHA reduces cellular zinc uptake, possibly mediated by the zinc transporter ZnT3 followed by a significant reduction in pro-apoptotic marker, caspase-3 (Suphioglu, De Mel et al. 2010a). This indicates the effect of DHA deficiency in the progression of neurodegenerative disease, which is partly mediated by altered zinc fluxes.

Zinc homeostasis in the brain is regulated and tightly controlled by Zn transporters, which are divided into two gene families; the ZnT proteins [solute-linked carrier 30 (SLC30)] and the Zip family [solute-linked carrier 39 (SLC39)] (Overbeck, Uciechowski et al. 2008; Lichten and Cousins 2009). ZnT and Zip proteins appear to have opposite roles in cellular zinc homeostasis, where ZnT transporters reduce intracellular cytoplasmic zinc by promoting zinc efflux from cells or into intracellular vesicles, while Zip transporters increase intracellular cytoplasmic zinc by promoting extracellular and, perhaps, vesicular zinc transport into cytoplasm (Murakami and Hirano 2008). In M17 cells, we detected the expression of these two zinc transporter families, including Zip1, Zip2, Zip3, Zip4, Znt1, ZnT2, ZnT3, ZnT4, ZnT5, ZnT6 and ZnT7 (Suphioglu, De Mel et al. 2010a). ZnT3 has been the focus of our studies, as it is associated with brain zinc accumulation, as well as Alzheimer's disease, the condition where the expression was found to be up-regulated (Zhang, Wang et al. 2008).

Progressive neuronal cell loss is a pathological hallmark of neurodegenerative diseases. Apoptosis or alternative pathways of neuronal death have been discussed in Alzheimer's disease and other disorders (Culmsee and Landshamer 2006). We propose that the alteration of zinc metabolism may play a significant role in cellular apoptosis, which is a key feature in the pathology of neurodegenerative disorders such as Alzheimer's disease (Mattson and Duan 1999). Using western blot analysis of human neuroblastoma M17 cell line, we observed a link between DHA treatment and inhibition of apoptosis, where more than 66% reduction in active caspase-3 protein levels was detected in cells treated with 20 μ g/ml DHA, compared with the untreated control (Suphioglu, De Mel et al. 2010a). The suppression of activated caspase-3 might be mediated by phosphatidylinositol 3-kinase-dependent pathway resulting in the phosphorylation of Akt and DHA may act through this pathway. Akbar et al. (2005) reported the beneficial effect of DHA in neurosurvival through an increase in phosphatidylserine concentration, which resulted in translocation and phosphorylation of Akt suppressing the activation of caspase-3 (Akbar, Calderon et al. 2005). Zinc on the other hand directly activates Akt by phosphorylation at Ser-473/Thr-308 in H1907 embryonic hippocampal cells, leading to activation of GSK-3 β and cell death (Min, Lee et al. 2007). Therefore, we hypothesize that DHA inhibits apoptosis through decreasing intracellular zinc ion concentration, which leads to an increase in Akt activity and neuronal survival.

In summary, dietary DHA deficiency is associated with neurodegenerative condition, which has shown to be a factor in zinc toxicity. DHA also inhibits cellular apoptosis in M17 cells through decreasing cellular zinc uptake and reduction of ZnT3 mRNA and protein levels. Therefore, zinc homeostasis plays an important role in neuronal cell survival and altered zinc homeostasis may contribute to the development of neurodegenerative diseases such as Alzheimer's disease.

1.3 Zinc and DHA affect neuronal histone levels

The connection between zinc homeostasis and DHA metabolism contributes significantly towards neuronal survival and neurodegenerative diseases. A greater understanding of the fundamental basis by which dietary DHA plays an important role in regulating zinc homeostasis, may lead to the development of effective strategies for the prevention and treatment of neurodegenerative diseases. In recent years, our research has been focusing on the key proteins that are regulated by both zinc and DHA and we have also studied how

zinc and DHA, alone and in combination might affect the expression levels of these novel proteins.

Two-dimensional gel electrophoresis and mass spectrometry were applied to identify the major protein changes in the protein lysates of M17 human neuronal cells that had been grown in the presence and absence of zinc and DHA. Four protein spots were selected for mass spectrometry analysis to reveal their identity as human histone variants H3 and H4. In order to investigate the change in the expression levels of the histones, proteomic findings were further investigated using western blot and real-time PCR analyses. Our results have revealed the differential expression of histones, particularly histone H3 and H4 in response to DHA and zinc supplementation (Suphioglu, Sadli et al. 2010b). In this study, we reported for the first time that both H3 and H4 were significantly down-regulated by zinc in the absence of DHA (zinc effect) and up-regulated following DHA treatment at the physiological zinc level (DHA effect), suggesting the interrelationship between zinc and DHA in neuroprotection, which is mediated by histones (Suphioglu, Sadli et al. 2010b).

1.3.1 Histones

Histones are a group of conserved, highly basic proteins that are rich in lysine (K) and arginine (R) (Kinkade and Cole 1966; DeLange and Smith 1971; Elgin and Weintraub 1975; Munishkina, Fink et al. 2004) (Table 1). Histones are the nuclear protein that are involved in the assembly of chromatin through electrostatic interaction between the highly negatively charged polymeric DNA and the positively charged histones, which play a determining role in stabilizing the nucleosomes at physiological conditions (Korolev, Lyubartsev et al. 2004). About 85% of the DNA in chromatin is represented by uniform units, the nucleosomes, which are the complexes of DNA double helix with five histone proteins (H2A, H2B, H3, H4, and H1) (Luger, Rechsteiner et al. 1997). The central part of the nucleosome is called the nucleosome core particle and consists of 147 bp DNA wrapped around the histone octamer, which is formed from one (H3/H4)² tetramer and two H2A/H2B dimers (Luger, Rechsteiner et al. 1997; Woodcock and Dimitrov 2001). The four core histones have similar isoelectric points (pI) and share a common structural motif called the histone fold, which facilitates the interactions between the individual core histones (Arents and Moudrianakis 1995). Flanking the core domains are the relatively unstructured *N*-terminal tail domains. The histone tails extend out from the face of the nucleosome and through the gyres of DNA

Histone type	Class (amino acid distribution)	M.W. (Da)	Sequence length	Isoelectric point (pI)
H1	Very lysine rich	~ 21,500	~215	
H2A	Lysine rich	14,004	129	10.9
H2B	Lysine rich	13,774	125	10.3
H3	Arginine rich	15,324	135	11.1
H4	Arginine rich	11,282	102	11.4

Table 1. Characteristics of histones. Molecular weight (MW) is given in Daltons (Da) and isoelectric points (pI) are shown

superhelix into the area surrounding the nucleosome (Luger, Rechsteiner et al. 1997). In contrast to the structural core histone proteins, histone H1 is associated into linker DNA, which connects the nucleosomes together, resulting in the formation of “beads-on-a-string” chromatin structure (Davie and Chadee 1998).

1.3.2 Possible mechanism of the effect of zinc and DHA on H3 and H4 expression

We observed a significant reduction in both mRNA and protein levels of histones H3 and H4 following zinc treatment suggesting that zinc may inhibit the transcription of histones H3 and H4 in M17 human neuronal cells (Suphioglu, Sadli et al. 2010b). Histones H3 and H4 possess multiple metal response elements upstream of their start codon, which indicates the possible involvement of zinc in their transcription. Previous studies showed that inhibition of DNA synthesis triggers a concerted repression of histone synthesis, indicating that sustained histone synthesis depends on continued DNA synthesis. We proposed that the termination of H3 and H4 synthesis may possibly be caused by the effect of zinc in inhibiting DNA synthesis (Suphioglu, Sadli et al. 2010b). Conversely, DHA was found to up-regulate H3 and H4 expression levels and abolished the effect of zinc, suggesting the potential contribution of DHA in increasing DNA synthesis, which result in the increase of histone protein levels. Our results are supported by previous studies, by which zinc regulates a variety of transcription and translation related factors, including the H3 histone family 3A protein (Barcelo-Coblijn, Hogyes et al. 2003). Since there’s association between alteration in histone subunit expression and DNA replication, the condition may then alter the expression of many other genes.

From this study, we propose that DHA may contribute positively to minimizing the onset of neurodegenerative disease through maintaining the integrity of DNA and histones H3 and H4 synthesis. The inhibition of DNA synthesis, which subsequently lead to the loss of neurons, however, is a pathological process of neurodegenerative disorders and potentially cause the death of the cells.

1.4 Histone post-translational modifications (PTMs) and gene activities

In addition to nucleosome assembly, studies have found that histones are potentially important carriers of epigenetic information. They, therefore, play significant role in regulating gene activities, such as DNA damage repair, replication and transcription through post-translational modifications (PTMs) (Hasan and Hottiger 2002). Core histones are characterized by the presence of fold domain (Alva, Ammelburg et al. 2007) and *N*-terminal tails which are exposed to nucleosomal surface (Ausio, Dong et al. 1989). These *N*-terminal tails of core histones are subjected to extensive post-translational modifications in many cellular processes (Ausio, Dong et al. 1989), which play pivotal roles in the epigenetic control of chromatin structure necessary for DNA accessibility during gene expression (Iizuka and Smith 2003). Some PTMs, including acetylation and phosphorylation, are reversible and are often associated with increase in gene expression. Other PTMs, such as lysine methylation, are often found to be more stable and participate in long term epigenetic maintenance (Bernstein and Allis 2005).

One of the best-studied post-translational modifications is the acetylation of lysine residues, which is a reversible process that is catalyzed by either histone acetyltransferases (HATs) or histone deacetylases (HDACs). The main acetylation sites in histone H3 of most species are at lysine 9, -14, -18 and -23 (Thorne, Kmiecik et al. 1990). Histone acetylation is a hallmark of

transcriptionally active regions, whereas hypoacetylated histones are associated with tightly compacted nucleosomes, resulting in transcriptional repression due to restricted access of transcriptional factors to their targeted DNA (Oliva, Bazett-Jones et al. 1990). The addition of an acetyl group by a member of HAT family create appropriate 'histone code' for chromatin modification and decrease the interaction between the negatively charged DNA backbone and the positively charged histone tail enhancing DNA accessibility to transcription factors (TFs), which therefore increase gene transcription. Conversely, HDAC removes the acetyl group and potentially leads to general repression of gene transcription (Dangond, Henriksson et al. 2001).

So far, in humans, 18 HDACs enzymes have been identified on the basis of similarity to yeast counterparts and classified based on sequence identity and domain organization as well as cofactor dependency (Heltweg, Dequiedt et al. 2003). The classic HDACs (Class I, II and IV) require Zn^{2+} for their activity, whereas the sir2-related HDACs (sirtuins) require (nicotinamide adenine dinucleotide) NAD^+ as cofactor (Koyama, Adachi et al. 2000). Class I HDACs (HDAC1, 2, 3 and 8), which are homologs of the yeast histone deacetylase RPD3, are found primarily in the nucleus of most cell lines and tissue types (Fischle, Emiliani et al. 1999; Fischle, Kiermer et al. 2001), whereas Class II HDACs (HDAC 4, 5, 6, 7 9 and 10) share a significant degree of homology with the yeast Hda1 and are able to shuttle in and out of the nucleus depending on different signals (Fischle, Emiliani et al. 1999; Fischle, Kiermer et al. 2001), suggesting a potential extranuclear functions by regulating the acetylation status of nonhistone substrates (Grozingler, Hassig et al. 1999; Heltweg, Dequiedt et al. 2003). Class III HDACs are composed of the Sirtuins (SIRT) proteins 1-7 and require NAD^+ for deacetylase activity in contrast to the zinc-catalyzed mechanism used by class I and II HDACs (Koyama, Adachi et al. 2000; Lo, Trievel et al. 2000; Blander and Guarente 2004). The most recently described HDACs are Class IV, which is represented by HDAC11. This enzyme is phylogenetically different from class I and II HDACs and is therefore classified separately (Gao, Cueto et al. 2002). So far, very little is known about its function and regulation (Yang and Seto 2008).

In addition to acetylation, important progress has also been made in the studies of other types of covalent modifications including methylation and phosphorylation of histones H3. It has long been known that histone H3 is methylated at a number of lysine (Lys) and arginine (Arg) residues. The major sites of Lys-methylation on histones identified so far are: Lys4, Lys9, Lys27, Lys36, Lys79 and arginine methylation takes place at R2, R17 and R26 (Sims, Nishioka et al. 2003; Lee, Teyssier et al. 2005). The addition of methyl-group on histone tail residues is catalyzed by histone methyltransferases (HMTs). These enzymes can catalyze mono-, di-, or trimethylation on lysine residues and this differential methylation provides further functional diversity to each site of lysine methylation. Similar to histone acetylation, histone methylation can also modulate histone interaction with DNA, which result in an alteration of nucleosome structures and functions and therefore contribute to different cellular process (Rice and Allis 2001). The specific methylation of histone tails such as H3(K4), H3(K36), and H3(K79) have been associated with active transcriptional activity (Strahl, Ohba et al. 1999; Berger 2007), whereas methylation of H3(K9), H3(K27) and H4(K20) have been correlated with gene silencing (Lee, Teyssier et al. 2005).

Histone phosphorylation have also been shown to occur on all histones, and are located within the highly conserved amino acid residues alanine, arginine, lysine and serine (Clayton and Mahadevan 2003). For histone H3, phosphorylation takes place specifically at Thr3, -11 and at Ser10, -28 (Hendzel, Wei et al. 1997; Hsu, Sun et al. 2000; Dai, Sultan et al.

2005). Studies have reported the involvement of H3 phosphorylation in transcriptional activation (Mizzen, Kuo et al. 1998; Clayton, Rose et al. 2000; Nowak and Corces 2000), chromatin fiber decondensation, and chromosomes compaction during cell division (Hendzel, Wei et al. 1997; Hsu, Sun et al. 2000). Histone H3 is phosphorylated during both mitosis and meiosis and initiated at different phase of the cell division in different organisms (Hans and Dimitrov 2001). The phosphorylation of Thr(T)3 of histone H3, which is catalyzed by kinase haspin occurs during mitosis and it plays an essential role in facilitating condensation and resolution of sister chromatids in the late G2 and prophase (Dai, Sultan et al. 2005). To ensure this orderly cell cycle progression, the regulation of chromatin structure and spindle activity must be precisely integrated. The inappropriate H3(T3) phosphorylation causes defects in chromatin structure which might hinder chromosome alignment in mitosis (Enomoto, Koyamazaki et al. 2001), leading to genomic instability (Dai, Sultan et al. 2005). Threonine-3 residue is found in histone H3 of all eukaryotes, suggesting a highly conserved and critical function for this residue (Dai, Sultan et al. 2005).

1.4.1 Histone post-translational modifications (PTMs) and neurodegenerative disease

As previously discussed, histone post-translational modifications (PTMs) play significant role in regulating gene activities. Therefore, aberrant pattern of epigenetic regulation has been linked to the development of neurodegenerative diseases such as Alzheimer's disease.

During normal neuronal condition, HATs and HDACs remain in a state of balance, which they counteract each other to ensure neurophysiological homeostasis. Such equilibrium (Figure 1A) is responsible for regulating gene expression leading to normal function of neuronal cell activity and memory formation (Saha and Pahan 2006). During neurodegenerative diseases, the acetylation homeostasis is altered when histone acetylation significantly decreases (Rouaux, Jokic et al. 2003), reflecting dysfunctional acetylation-deacetylation apparatus (Figure 1B). General loss of acetylating agent would cause excessive increase in HDAC activity, which is then associated with transcriptional repression (Figure 1B). Studies have reported that reduction in histone acetylation followed by the increase in HDAC activity or DNA methylation is common in many neurodegenerative and neuropsychiatric disorders (Faraco, Pancani et al. 2006; Fischer, Sananbenesi et al. 2007).

In recent years, the increasing numbers of structurally diverse HDAC inhibitors have been identified with the potential to target specific brain regions and in cell-specific manner to reverse disorder-specific epigenetic dysregulation (Abel and Zukin 2008). The HDAC inhibitors include: short-chain fatty acid (i.e. valproic acid) (Kothari, Joshi et al. 2009), hydroxamic acid (i.e. SAHA, TSA, oxamflatin) (Archin, Espeseth et al. 2009), cyclic tetrapeptides (i.e. trapoxin, apicidin) (Masuoka, Shindoh et al. 2008) and benzamide (i.e. MS-275) (Gahr, Peter et al. 2008). These HDACs inhibitors are aimed to inhibit its enzymatic activity and to remove the repressive blocks from promoters of essential genes and therefore induce active gene transcription (Saha and Pahan 2006). The X-ray crystallographic studies showed that this type of HDAC inhibitor act as a chelator of zinc ion in the catalytic site of HDACs which therefore block the substrate access to the active zinc ion and subsequently inhibit the deacetylation activity (Ficner 2009). However, it is still uncertain whether certain neurodegenerative disorders are mediated by a specific HDAC.

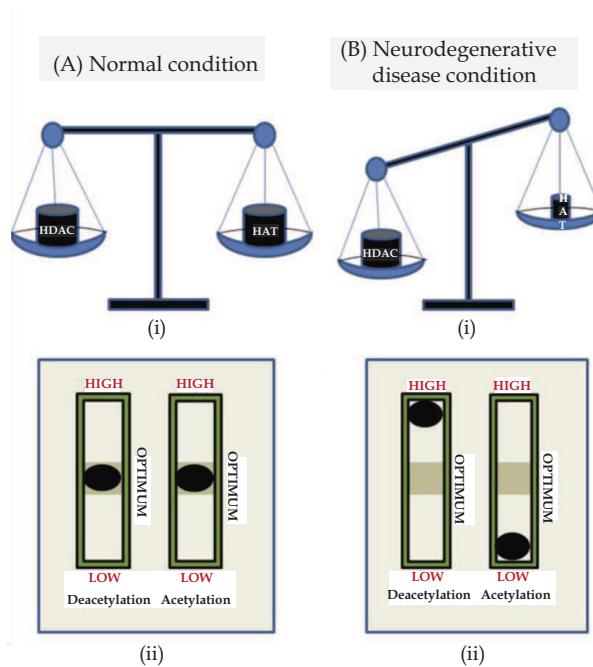


Fig. 1. Neuronal acetylation homeostasis. (i) Weights on the balance represent the protein level of HATs and HDACs. (ii) Enzymatic activity scale represents the activity and dark grey areas are physiologically optimal. (A) Under normal neuronal conditions, the level and activity of both HATs and HDACs are within their point of balance where they counteract each other to maintain internal equilibrium (homeostasis). (B) During neurodegenerative disease condition, acetylation homeostasis is altered resulting in the loss of HATs level and activity which balance towards an excessive production of HDACs and subsequent increase in deacetylation

Aging is also considered as the greatest risk factor for the development of the neurodegenerative diseases, such as Alzheimer's disease where neuronal function declination and gene expression alternation could be detected in the aging human brain (Giovacchini, Chang et al. 2002). Studies have found the altered pattern of histone modification in aging cells, such as, trimethylation of histone H4 at lysine 20, which was increased in kidneys and liver of the old-aged rat (Sarg, Koutzamani et al. 2002), and the level of H4 acetylation, which was decreased in the rat brain cortical neurons with age (Pina, Martinez et al. 1988). Several new methylated sites, such as H3(K24), H3(K128) and H2A(R89) were also detected in the study of aged mouse brain, however, no functional studies on these three sites had been reported (Wang, Tsai et al. 2009). It has been reported that in aging brains, most PTMs sites were found on histone H3 which has the longest N-terminal tails amongst other core histones (Wang, Tsai et al. 2009). These studies suggest the importance of proper epigenetic modification in biological activities and neuronal cell development, while the altered epigenetic regulation leads to neurodegenerative diseases.

1.5 Importance of zinc and DHA in epigenetics of human neuronal cells

1.5.1 Effect of zinc and DHA on acetylation levels of Histone H3(K9)

Proper regulation of gene expression in the nervous system is not only controlled by the transcriptional machinery but is also subject to modulation by epigenetic mechanisms such as histone modifications. Following our histone expression study, for the first time, we have also investigated the effect of zinc and DHA on post-translational modifications of histones, in particular histone H3 in human neuronal cells.

One-dimensional electrophoresis and western immunoblot analysis were used to investigate the change in post-translational modified histones of human neuronal cells that had been grown in the presence and absence of zinc and DHA. Our results showed that zinc decreased acetylation of H3(K9), whereas DHA increase H3(K9) acetylation. This suggests the potential involvement of zinc in the progress of neurodegenerative disease through an altered acetylation homeostasis in neuronal cells. During the acetylation dyshomeostasis, transcriptional regulation may be affected which has been reported to be one of the prime causes of neurodegenerative diseases (Saha and Pahan 2006). This altered gene transcription then caused opposite effects from normal gene regulation pattern in neuronal cells. This attributed to degenerative fate of neurons that subsequently reduced the expression of survival-associated genes by altered acetylation and at the same time, stimulated the expression of death-inducing genes (Saha and Pahan 2006).

The increase in Histone H3(K9) acetylation in response to DHA, however, indicates the ability of DHA to normalize the histone H3(K9) acetylation to the basal level (control) and abolishes the effect of zinc (Sadli et al., 2011, *unpublished results*). Therefore, DHA may contribute to neuroprotection through reinstating the altered acetylation dyshomeostasis caused by zinc toxicity, which would possibly up-regulate the expression of neuroprotective genes (Saha and Pahan 2006).

1.5.2 Effect of zinc and DHA on histone deacetylases (HDACs) 1, 2, 3

We performed western immunoblotting to investigate the change in the expression levels of histone deacetylase (HDACs) 1, 2, 3 using anti-HDAC1, 2 and 3 antibodies, where we found that zinc significantly up-regulated HDAC1, 2 and 3 expression levels compared with the control, while DHA significantly down-regulated HDAC1, 2 and 3 (Sadli et al., 2011, *unpublished results*). It's been reported that the activity of HDACs were increased in dying neurons, due to the loss of counterbalancing effect of HATs activity (Saha and Pahan 2006). From our results, we propose that the increase in zinc can also contribute to the neurodegenerative process through up-regulating HDACs enzyme expression levels, and therefore increasing the activity of histone deacetylation.

The HDACs catalytic domain contains a Zn^{2+} ion, in the active site, which contributes significantly to its catalytic activity (Vannini, Volpari et al. 2004; Ficner 2009). *In vitro*, the deacetylase activity of the purified HDAC homologue was observed only after incubation with zinc chloride (Finnin, Donigian et al. 1999), which suggests that HDAC activity requires a metal cofactor (Hassig, Tong et al. 1998). X-ray crystallographic studies have shown that HDAC inhibitors could chelate zinc ions in the catalytic sites of HDACs and therefore block substrate access to the active zinc ions and inhibit the deacetylation reaction (Marks, Richon et al. 2000; Ficner 2009).

It has been established in the scientific literature that the isotopic selective inhibition of HDAC enzyme may be the potential treatment for neurodegenerative diseases. It has also been demonstrated that the transcriptional dysregulation by HDACs may play significant

role in causing neurodegenerative disease and that HDACs therapy may prevent or slow down the neurodegenerative disease process. So far, the HDAC inhibitors investigated in treating neurodegenerative diseases are very limited and mainly focused on the well-established experimental drug trichostatin A (member of hydroxamic acid group) and the clinically used HDAC inhibitors sodium butyrate, valproic acid, phenylbutyrate and vorinostat, which belong to short chain fatty acid group that are known to be able to penetrate the blood-brain barrier (Butler and Bates 2006). From our observation, DHA, being a long chain n-3 fatty acid that is selectively allowed to cross the blood-brain barrier, is likely to have neuroprotective characteristic that mimic the behavior of HDACs inhibitors. This significantly down-regulates the HDACs expression levels and therefore induces histone acetylation, which then allow the transcription and expression of genes, in what had been a too tightly packaged chromatin structure in which certain genes do not get transcribed. Generally, increase in HDACs during neurodegenerative disease is associated with increase in gene repression and transcriptional dysfunction of certain transcription factors (TFs) such as CREB, which is important in regulating the expression of pro-survival elements such as Bcl-2 (Freeland, Boxer et al. 2001; Saha and Pahan 2006). In our study, we show how zinc contributes to dysfunctional acetylation homeostasis in M17 cells by up-regulating HDACs, which influence the reduction of HATs and consequently histone acetylation levels. DHA, however, is shown to reestablish the imbalance of acetylation homeostasis and therefore capable of correcting the down-regulation of specific genes caused by reduction in histone acetylation (Sadli et al., 2011, *unpublished results*). The mechanism by which DHA inhibits the HDACs expression is unclear, whether DHA directly chelates zinc ion from the catalytic sites of HDACs or hinder the zinc ion binding to the enzymes.

1.6 Link between cellular apoptosis and neurodegenerative diseases

A characteristic of many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and stroke - is neuronal cell death (Cavallucci and D'Amelio, 2011; Calissano, Matrone et al. 2009). Central nervous system tissue has very limited regenerative capacity and therefore it is important to limit the damage caused by neuronal cell death (Rossi and Cattaneo 2002) (Kuhn, Palmer et al. 2001). In recent years, the investigation regarding the contribution of caspases and neuronal apoptosis to neurodegenerative diseases has gained increasing attention. This evidence has been generated by using a variety of complementary approaches, including evaluating human tissue and using transgenic mouse and *in vitro* models (Kuhn, Palmer et al. 2001).

Studies have shown the imbalance level of pro-apoptotic (Bax, Bak and Bad) and anti-apoptotic Bcl-2 protein (Su, Deng et al. 1997; Kitamura, Shimohama et al. 1998), as well as caspase-3 and -6 in post-mortem brains of AD patients (Stadelmann, Deckwerth et al. 1999). In addition, immunohistochemical and biochemical studies reported the presence of active caspases and caspase-cleaved substrates around senile plaque and neurofibrillary tangles in neuron (Gastard, Troncoso et al. 2003). There's also a marked co-localization of hyperphosphorylated tau, caspase-3 and caspase-6 in TUNEL-positive neurons in the brainstem of AD patients (Wai, Liang et al. 2009), suggesting the potential involvement of apoptotic death in the etiology of AD.

Caspase-mediated apoptotic pathways have specifically been linked to the progression of AD, especially toward the formation of amyloid precursor protein (APP) and A β peptide production. Caspase-3-mediated APP stabilizes BACE1 (the β -secretase enzyme that is responsible for the cleavage of APP and the associated creation of beta-amyloid), which lead

to an increase in A β production (Tesco, Koh et al. 2007). Studies also indicate that caspases have been implicated in the mechanism of tau-mediated neurodegeneration of AD (Garcia-Sierra, Mondragon-Rodriguez et al. 2008) (Dickson 2004). According to this hypothesis, A β peptide was reported to promote neuronal pathological tau filament assembly by triggering caspase activation leading to tau cleavage, which in turn generate more tau pathological filaments (Tesco, Koh et al. 2007) that further contribute to increase of cellular dysfunction in AD (Fasulo, Ugolini et al. 2000).

1.6.1 Effect of zinc and DHA on Bcl-2 (anti-apoptotic marker) and caspase-3 (pro-apoptotic marker) expression levels

As mentioned previously, zinc toxicity is one of the important causes of cell death in neurodegenerative disease, including Alzheimer's disease (Naganska and Matyja 2006). It has been reported that intracellular zinc release, as a result of altered zinc metabolism, leads to the activation of caspase-3, which then subsequently trigger neuronal cell apoptosis (Zhang, Wang et al. 2004). In our study, we aimed to determine whether anti-apoptotic Bcl-2 and pro-apoptotic caspase-3 were involved in the cellular pathway affected by zinc and DHA interactions in M17 cells by investigating their expression levels using western blot analysis.

Both zinc and DHA have been shown to opposingly modulate the levels of Bcl-2 and caspase-3 in M17 cells (Sadli et al., 2011, *unpublished results*). An increase in zinc levels causes up-regulation of caspase-3 and down-regulation of Bcl-2 expression, suggesting the potential occurrence of apoptosis of zinc-induced M17 cells, which is representative of neurodegenerative conditions such as AD where intracellular zinc ion is elevated while DHA level is reduced. Conversely, DHA treatment of M17 cells increased expression levels of Bcl-2 and reduced caspase-3, which suggest that DHA exclusively activates the extracellular signal regulated kinase/mitogen-activated protein kinases (ERK/MPK) pathway to promote cell survival that lead to the up-regulation of Bcl-2 and inhibition of caspase-3 activation (German, Insua et al. 2006). Our observation was supported by Akbar et al. (2006), which showed the involvement of DHA in neuronal cell survival by driving Akt translocation resulting in activation of Bcl-2 and subsequent suppression of caspase-3 activity leading to inhibition of apoptosis in neuronal cells (Akbar, Baick et al. 2006).

Our findings with Bcl-2 and caspase-3 highlight the importance of DHA in neuroprotection and zinc toxicity in apoptosis. The blockage of caspase-3 activity by DHA might protect against the apoptotic cell death following zinc toxicity, which may offer a useful and alternative therapeutic strategy to delay neuronal loss associated with neurodegenerative diseases. Further investigations on the role of DHA in neuroprotection through inhibition of caspase needs to be done, which will provide additional insights into this cascade activity pathway. This in return will establish the idea whether cascade-induced zinc toxicity is a direct cause of apoptosis or a downstream consequence, which will eventually lead to cell death in neurodegenerative diseases.

1.7 M17 cell line as a model

Neurodegeneration is very difficult to study *in vivo*. Neuronal cells do not regenerate and cannot be observed or manipulated without removing them from the patients. For these reasons, *in vitro* models are very important options. An ideal cell line would possess similar characteristic as the *in vivo* neurons, while having the advantage of immortalization to

ensure continuous supply of cells. Immortalized cells are also convenient to handle and experiments can be performed during continuous conditions in which biochemical process can easily be studied.

Throughout our studies, M17, a neuronal-derived cell line was used. M17 cells are originally isolated from the bone marrow of a two year old male suffering from disseminated neuroblastoma (Global Bio-resource Center, 2007). Microscopic analysis shows that the cell type indicates a neuronal characteristic; being morphologically small in size and dense with triangular-shaped cell bodies. The advantage of this cell line is that it is of human origin, and by now, M17 cells constitute a well studied and defined cellular system. Our in-house results suggest that M17 cell line is a suitable model for studying the effects of DHA and zinc supplementation on the gene and protein expression profiles of neuronal cells throughout this study.

1.8 Application of proteomic and molecular analysis in neurodegenerative disease research

The need for protein-level analysis arises because the phenotype of human neuronal cells in relation to neurodegeneration corresponds to the functions of expressed and modified protein networks. Unlike the genome that is relatively static, the proteome is extremely dynamic and constantly adjusted in response to changing internal (e.g aging) and external events (e.g toxic exposure, drugs).

Proteins are composed of a variety of combinations of amino acids, and are subject to co- as well as post-translational modification, such as deletion of amino acid sequences and chemical modification of specific amino acids (e.g oxidation and phosphorylation) (Anderson, Matheson et al. 2001). These modifications will influence the activity state, function and interactions of proteins.

The word “*proteome*” is derived from proteins expressed by a genome, and it refers to all the proteins produced by an organism, first coined by Wilkins et al. in 1996 (Wilkins, Sanchez et al. 1996). In its wider sense, proteomic research assesses protein expression, modification, interaction and localization. By studying global patterns of proteins and their changes dynamically, proteomic research can improve our understanding of system-level cellular behavior. Although proteomics as an entity is relatively new, the methodological and theoretical foundations have been under development for more than three decades (Campostrini, Pascali et al. 2004). Two-dimensional protein electrophoresis, coupled with peptide mass fingerprinting analysis by mass spectrometry (MS), has become the most powerful techniques for proteome analysis (Binz, Muller et al. 1999).

In the future, downstream steps after genomics and proteomics will aim at understanding functional consequences of biomolecule interactions in different biological pathways in a system. Comparative studies to quantitate, identify and characterize the proteins expressed in normal neuronal cells and diseased cells will give insight into the mechanisms of neurodegenerative disease. This will allow the identification of novel diagnostic and treatment reagents for Alzheimer’s disease.

Proteomic analysis data has become an important resource in the investigation of neurodegenerative diseases. Proteomic profiling, in particular, has enabled researchers to investigate a vast number of proteins at once. Such principles have been utilized in order to detect specific alterations in the protein expression levels in various regions of the neurodegenerated brain compared to control brain. This may, in turn, facilitate the construction of hypotheses on the mechanisms by which the disease progresses.

When considering neurological disorders, one good example for the usability of two-dimensional gel electrophoresis (2-DE) in exploring new biomarkers, was the discovery of two unknown protein isoforms p130 and p131 that were suggested to be able to discriminate Creutzfeldt-Jakob disease from other type of dementia (Harrington, Merrill et al. 1986). Most efforts in understanding the pathogenesis using 2-DE-based expression proteomics have been made by comparing brain proteomes of AD patients and controls. Some of the first 2-DE studies examined the levels of AD brain proteins where they revealed alterations in the levels of a number of proteins, such as GFAP, tubulin, and creatine kinase (Smirnov, Shevtsov et al. 1991; Burbaeva 1992). Subsequently, the number of 2-DE studies has multiplied and at present, changes in the levels of more than 100 brain protein isoforms have been identified in neurodegenerative disorders (Fountoulakis, Juranville et al. 2002; Butterfield and Castegna 2003; Vlahou and Fountoulakis 2005). Despite the multiplicity of isoform specific protein changes, the findings still remain rather fragmented and novel hypothesis related to the pathogenesis of AD still remains to be revealed.

Proteomic methods were also successfully applied in the study of tau protein phosphorylation in AD where tau become phosphorylated and accumulated to form neurofibrillary tangles (Ksiezak-Reding, Binder et al. 1990). The increased phosphorylation of elongation factor II has also been demonstrated in AD brain by the 2-D approach (Johnson, Gotlib et al. 1992). As a consequence of rapid demographic aging, AD has become one of the most devastating socioeconomical challenges of the present-day. Now, the new hope of unraveling the secrets of AD is done by the so-called "new technologies" (i.e. proteomics) which have been suggested to represent a breakthrough in improving our understanding, diagnosis and treatment of AD.

1.9 Conclusions and future perspectives

We characterized the effect of zinc and DHA in modulating gene and protein expression in M17 human neuronal cells. This idea was based on the fact that both zinc and DHA play significant roles in neuroprotection and are known to interact biochemically. DHA treatment of M7 cells results in lower zinc transporter ZnT3 protein levels and reduction in pro-apoptotic marker caspase-3 indicates the involvement of zinc in pathways that regulate brain cell survival and that alteration in zinc homeostasis may contribute to the development of neurodegenerative diseases.

Both zinc and DHA may possibly be involved in the signaling mechanism that regulates histone expression levels in M17 human neuronal cells. Here we hypothesize that DHA may play a protective role by up-regulating histones H3 and H4, which accounts for the positive effect of DHA in minimizing the onset of neurodegenerative disease through facilitating DNA synthesis and therefore increasing histone protein levels.

Following our previous study, we also investigated the effect of zinc and DHA in regulating gene expression through histones post-translational modifications. Zinc was found to reduce histone acetylation and increase HDACs, which represent a critical step commonly underlying catastrophic neuronal dysfunction, whereas, DHA reinstated the imbalance of acetylation homeostasis indicating its potential neuroprotective ability to ameliorate neurodegenerative diseases. Reduction in acetylation along with parallel gain of HDAC levels represents the crux of the altered situation and we propose that DHA could possibly mimic the action of a HDAC inhibitors and therefore, reverses the zinc-mediated altered acetylation homeostasis.

Currently, the acetylation homeostasis system in neuronal cells is still in its infancy, so more research needs to be done in this field, especially in relation to neurodegenerative diseases. However, evidence provides us with some insights into the distinct epigenetic pattern and activity between zinc and DHA, which suggest their opposing role in the progression of neurodegenerative diseases. Our study highlights the functional mechanism in relation to beneficial effect of DHA in a number of ways and the involvement of zinc toxicity in cellular apoptosis (Figure 2).

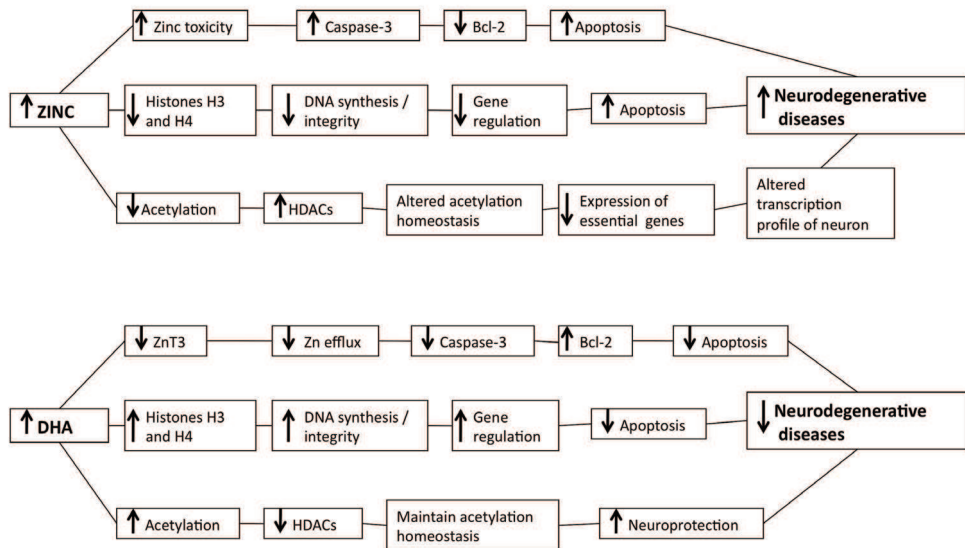


Fig. 2. Proposed model depicting the role of zinc and DHA in progression of neurodegenerative diseases. Various models representing neurodegenerative diseases are marked by irregular gene expression, altered epigenetic patterns as well as apoptosis. Cellular zinc toxicity contributes to neurodegenerative condition through a number of different pathways, which seem to be reversed by the presence of DHA. Zinc and DHA may share common pathways in the progression of neurodegenerative disease where DHA play a significant role in restoring the condition caused by altered zinc homeostasis

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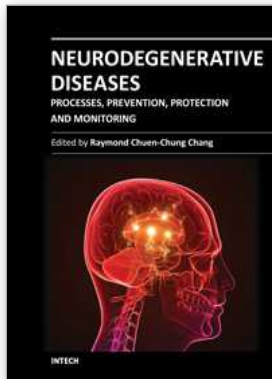
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Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring

Edited by Dr Raymond Chuen-Chung Chang

ISBN 978-953-307-485-6

Hard cover, 558 pages

Publisher InTech

Published online 09, December, 2011

Published in print edition December, 2011

Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring focuses on biological mechanisms, prevention, neuroprotection and even monitoring of disease progression. This book emphasizes the general biological processes of neurodegeneration in different neurodegenerative diseases. Although the primary etiology for different neurodegenerative diseases is different, there is a high level of similarity in the disease processes. The first three sections introduce how toxic proteins, intracellular calcium and oxidative stress affect different biological signaling pathways or molecular machineries to inform neurons to undergo degeneration. A section discusses how neighboring glial cells modulate or promote neurodegeneration. In the next section an evaluation is given of how hormonal and metabolic control modulate disease progression, which is followed by a section exploring some preventive methods using natural products and new pharmacological targets. We also explore how medical devices facilitate patient monitoring. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

How to reference

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Nadia Sadli, Nayyar Ahmed, M. Leigh Ackland, Andrew Sinclair, Colin J. Barrow and Cenk Suphioglu (2011). Effect of Zinc and DHA on Expression Levels and Post-Translational Modifications of Histones H3 and H4 in Human Neuronal Cells, *Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring*, Dr Raymond Chuen-Chung Chang (Ed.), ISBN: 978-953-307-485-6, InTech, Available from: <http://www.intechopen.com/books/neurodegenerative-diseases-processes-prevention-protection-and-monitoring/effect-of-zinc-and-dha-on-expression-levels-and-post-translational-modifications-of-histones-h3-and->

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