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## **DOCOSAHEXAENOIC ACID PROTECTS FROM AMYLOID AND DENDRITIC PATHOLOGY IN AN ALZHEIMER'S DISEASE MOUSE MODEL**

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### **ABSTRACT**

Genetic data argues that Alzheimer's disease (AD) can be initiated by aggregates of a 42 amino acid beta amyloid peptide (A $\beta$ 42). The A $\beta$  aggregates, notably small oligomer species, cause a cascade of events including oxidative damage, inflammation, synaptic toxicity and accumulation of intraneuronal inclusions; notably neurofibrillary tangles. Cognitive deficits are likely to begin with a failure of synaptogenesis and synaptic plasticity with dendritic spine loss and dying back of dendritic arbor. This is followed by neuron loss in key areas involved in learning and memory. Significant prevention or delay of clinical onset may be achievable by modifying environmental risk factors that impact the underlying pathogenic pathways. Because low fish intake and low blood levels of the marine lipid, docosahexaenoic acid (DHA) have been associated with increased AD risk we have tested the impact of depleting or supplementing with dietary DHA on AD pathogenesis in transgenic mice bearing a mutant human gene known to cause AD in people. We reported that even with intervention late in life dietary DHA depletion dramatically enhanced oxidative damage and the loss of dendritic markers, while DHA supplementation markedly reduced A $\beta$ 42 accumulation and oxidative damage, corrected many synaptic deficits and improved cognitive function. Loss of brain DHA was exacerbated in mice expressing the mutant human AD transgene, this is consistent with evidence for increased oxidative attack on DHA oxidation in AD. Treatment with the curry spice extract curcumin, a polyphenolic antioxidant that inhibits AP aggregation, has been strongly protective in the same mouse model. Many Western diets are typically deficient in DHA and low in polyphenolic antioxidant intake. These and other data argue that increasing dietary intake of both DHA and polyphenolic antioxidants may be useful for AD prevention.

*Key words:* Alzheimer's disease, DHA, antioxidant, brain.

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## INTRODUCTION

### A $\beta$ 42 causes AD

Alzheimer's Disease (AD) is a progressive degenerative disease that attacks vulnerable brain regions resulting in impaired memory, thinking and behavior. It affects about 4 million Americans today. Numbers are predicted to rapidly increase with the aging of the population in the US and other industrialized countries and to reach an estimated 14 million in the US and 50 million worldwide. It is characterized by the accumulation of two major lesions; neuritic plaques with an insoluble core comprised largely of filamentous aggregates of beta amyloid peptide (A $\beta$ ) and neurofibrillary tangles of hyperphosphorylated tau protein forming paired helical filaments (PHF).

Neurodegeneration leading to synapse and neuron loss in selected pyramidal cell layers of cortex and hippocampus as well as subcortical cholinergic and other large projection neurons are widely believed to drive cognitive decline. The A $\beta$  peptide is a 40–42 amino acid fragment derived by proteolytic cleavage near and within the single transmembrane domain of a larger beta amyloid precursor protein (APP). The longer 42 amino acid form of A $\beta$  aggregates much more rapidly leading to neurotoxic soluble and fibrillar or protofibrillar aggregates that are commonly hypothesized to initiate the disease. Strong support for this view came from the identification of mutations in APP at or near both cleavage sites and in the "presenilin" component of the " $\beta$ -secretase" protease complex that cuts out A $\beta$ 40 or A $\beta$ 42. These have been identified as causes of increased A $\beta$ 42 production and familial autosomal dominant early onset AD (Selkoe, 1997).

### How does A $\beta$ 42 cause cognitive deficits?

While APP and presenilin mutations and other genetic evidence collectively implicate increased A $\beta$ 42 production from birth as sufficient to cause disease three or four decades later, it remains unclear what A $\beta$ 42-related mechanisms are taking decades to develop and are directly responsible for the memory and cognitive decline in AD. The assumption initially was that neurotoxicity and neuron loss from amyloid fibrils was responsible for causing AD. This was supported by observations of amyloid toxicity in vitro and the early and fairly extensive (~50%) neuron loss in vulnerable regions in AD. However, while neuron loss likely contributes to dementia, at early stages one might predict that 50% neuron loss would be adequately compensated by compensatory sprouting from remaining neurons, as is the case with Parkinson's disease and in many examples of head injury and stroke. In fact, normal cognitive function has been found in many individuals with less than half of normal brain weight and presumably proportionately lower neuron numbers (Jackson and Lorber, 1984). This suggests that in the absence of disease low neuron

numbers can be compensated for. However, in AD it is unclear that the extensive aberrant axonal sprouting is functional and not simply misdirected to plaques (Phinney *et al.*, 1999). Available data also indicates a failure of compensatory dendritic sprouting in many regions (Coleman, 1987; McKee *et al.*, 1989). Instead most evidence is for a major loss of arbor and spines on remaining pyramidal neurons (Moolman *et al.*, 2004). Dendritic spine loss is likely to matter because it has been closely associated with cognitive deficits in people. Thus, dendritic spine loss or dysgenesis has long been known as a common morphological feature of mental retardation syndromes (Purpura, 1974). Spine formation and morphology are regulated by local actin dynamics and the identification of genetic mutations, causing a growing list of these mental retardation syndromes, supports a role for defects in the postsynaptic machinery regulating spine actin assembly and disassembly with these known causes of cognitive deficits (Ramakers, 2002).

Furthermore, *in vitro* evidence for amyloid induced neurotoxicity and cell death has not received strong support from amyloid laden transgenic mice which typically lack widespread neuron and synapse loss. The limited amyloid toxicity in these *in vivo* models requires explanation if A $\beta$ 2 is central in causing AD. In recent years an increasing number of investigators have come to de-emphasize amyloid plaques which correlate relatively poorly with dementia in favor of a more important role for smaller, more soluble oligomeric A $\beta$  species. Thus, while fibril and amyloid toxicity and cell death at 20–100  $\mu$ M concentrations has previously been extensively studied, soluble A $\beta$ 42 oligomer species with subtler toxic effects at much lower nM doses have recently been shown to induce LTP and memory deficits (Klein *et al.*, 2001; Walsh *et al.*, 2002). In particular, very low levels of 12-mer A $\beta$  oligomer occurs at the onset of memory deficits in APP<sup>sw</sup> transgenic mice and this form of oligomer is sufficient to cause memory deficits when purified from APP<sup>sw</sup> mouse brains and injected into healthy young animals (Lesne *et al.*, 2006). Additional support for a role for these species came from observations that cognitive deficits are very rapidly reversible by anti-A $\beta$  antibodies without reducing total insoluble A $\beta$  in APP transgenic animal models arguing for the significance of toxic A $\beta$  effects beyond insoluble fibrils and from factors other than irreversible neuron loss.

Another open question is the impact of modifiable environmental risk factors, both those already implicated by epidemiology and factors as yet undiscovered. Environmental risk factors are clearly central to reducing the majority of late onset cases which are not obviously caused by genetic factors. Thus, our group and others have attempted to screen environmental risk factors in APP transgenic models and produced evidence that non-steroidal anti-inflammatory drugs like ibuprofen (Lim *et al.*, 2000), antioxidants (Lim *et al.*, 2001), cholesterol (Refolo *et al.*, 2000) and exercise (Lazarov *et al.*, 2005) can modify A $\beta$  accumulation and plaque pathogenesis.

Brain structural material is 60% lipid. Specific omega-3 and omega-6 fatty acids have been known for some time to be required by mammals for the

brain cell membrane structures and function (Crawford *et al* 1999, Crawford & Sinclair, 1972). Reduced consumption of the omega-3 fatty acids found in fish, notably docosahexaenoic acid (DHA), has also been associated with increased risk for AD (MacLean, 2005). DHA is the only omega-3 fatty acid of significance in the brain (Crawford *et al* 1976). It is enriched in neurons and synapses where it has been implicated in multiple useful functions while chronic DHA deficiency from dietary depletion appears to cause cognitive deficits (Lim and Suzuki, 2000; Salem *et al.*, 2001; Catalan *et al.*, 2002). With 6 double bonds DHA can be rapidly oxidized by oxygen radical attack, oxidized forms of DHA are enriched in an AD brain (Nourooz Zadeh *et al.*, 1999; Reich *et al.*, 2001). Oxygen radical attack, not only on lipids but also DNA and protein, occurs early in AD and may play a significant role in AD pathogenesis (Castellani *et al.*, 2006). Because DHA competes with the n-6 series arachidonic acid for esterification into phospholipids diets are typically judged sufficient in the context of both absolute levels and n-6/n-3 ratios.

## METHOD

### DHA depletion or supplementation in an Alzheimer transgenic mouse model

Since common laboratory mouse chows are typically based on NIH 31 and have been optimized by nutritionists they are low in saturated fat, enriched for omega 3 fatty acids with soy and/or fish meal and typically have ratios of n-6/n-3 on the order of 4:1, similar to a “traditional Japanese diet”. Therefore, in order to study the impact of added DHA, we studied the effects of a DHA-depleting safflower oil-based mouse chow plus or minus DHA supplementation on synaptic and amyloid endpoints in Tg2576 APP transgene positive and negative control mice. The experimental diets began at 17 months of age when amyloid pathology is well-established and burdens are already comparable to those found in AD.

## RESULTS

Results (Table 1) showed transgene-dependent loss of DHA, drebrin and PSD-95 (but not synaptophysin) on the DHA-depleting diet (Caton *et al.*, 2004).

Table 1 demonstrates that except for synaptophysin and GFAP, changes reported are all statistically significant (DHA- vs DHA+). Docosapentanoic acid (DPA), a lipid marker for DHA deficiency, was significantly elevated by DHA depleting diet and reduced by DHA supplements, consistent with reciprocal change in DHA levels. For Westerns for synaptic molecules (PSD-95, synaptophysin, CamKIIalpha), caspase-cleaved actin (fractin),

TABLE 1

Effects of Omega-3 (DHA) depletion (-) and replacement (DHA+) in Tg2576. Tg+ vs Tg- (transgene negative) mice raised on PMI 5015 breeder chow to 17 months and on indicated DHA-depleting safflower oil alone or DHA+ supplemented diets from 17–22 months of age

Diet: n-6/n-3 ratio	Breeder chow 7/1	DHA- 85/1	depleted DHA+ 5/1	supplemented
Parameter				
DPA (cortex)	0.54	1.668	0.198	
DHA (cortex)	17.91	15.14	19.63	%total FA
Abeta-42	106.1 ± 11.3	96.8 ± 7	49.2 ± 8.7	ng/mg tissue
Abeta-40	17.81 ± 19.7	294.1 ± 36.2	154.4 ± 23.2	ng/mg tissue
Carbonyls	8.9 ± 3.8	32.4 ± 5.4	14.0 ± 3.3	O.D.
Fractin	159 ± 23	668 ± 153	235 ± 41	% of Tg-std Diet
Drebrin	38 ± 5	6 ± 2	40 ± 6	% of Tg-std Diet
PSD-95	125 ± 8	23 ± 5	84 ± 11	% of Tg-std Diet
CamKII	145 ± 32	15 ± 8	101 ± 16	% of Tg-std Diet
Synaptophysin	100 ± 5	104 ± 4	115 ± 3	% of Tg-std Diet
n				
GFAP	158 ± 5	158 ± 36	161 ± 17	% of Tg-std Diet

the dendritic spine marker, drebrin and the astrogliosis marker GFAP, all the samples were assayed for protein in the same assay and equal protein loading was confirmed by densitometry of Coomassie stained gels. Consistent with selective postsynaptic (PSD-95, drebrin) rather than protein loading differences, GFAP and synaptophysin on the same blots did not change. Except for A $\beta$  values (Sandwich ELISA) all analysis is by Westerns and reflects either relative O.D. or % of Tg- on standard diet values. Protein carbonyls were negatively correlated with drebrin ( $r^2=0.47$ ), PSD 95 ( $r^2=0.60$ ) and fractin/ actin ( $r^2=0.44$ ), while drebrin and PSD-95 were also negatively correlated with fractin/actin ( $r^2=0.59$  and  $0.64$ , respectively) this is consistent with a close association between oxidative damage, caspase activation and postsynaptic marker loss. Real time PCR showed that PSD-95 mRNA was 48% reduced ( $p=0.003$ ) in Tg+ positive animals by BAD diet. The mRNA changes confirmed a transgene-dependent excitatory postsynaptic phenotype that could not be derived from generalized proteolysis. Except for synaptophysin and GFAP all the changes in Table 1 are significant DHA-dependent changes.

Although the study was not powered adequately to study behavior at 22 months cognitive function measured in the Morris Water maze also appeared to decline with DHA depletion (Caton *et al.*, 2004). We attributed the transgene dependent loss of DHA from mouse brain to increased oxidative damage which we measured as protein carbonyls. A follow up study showed

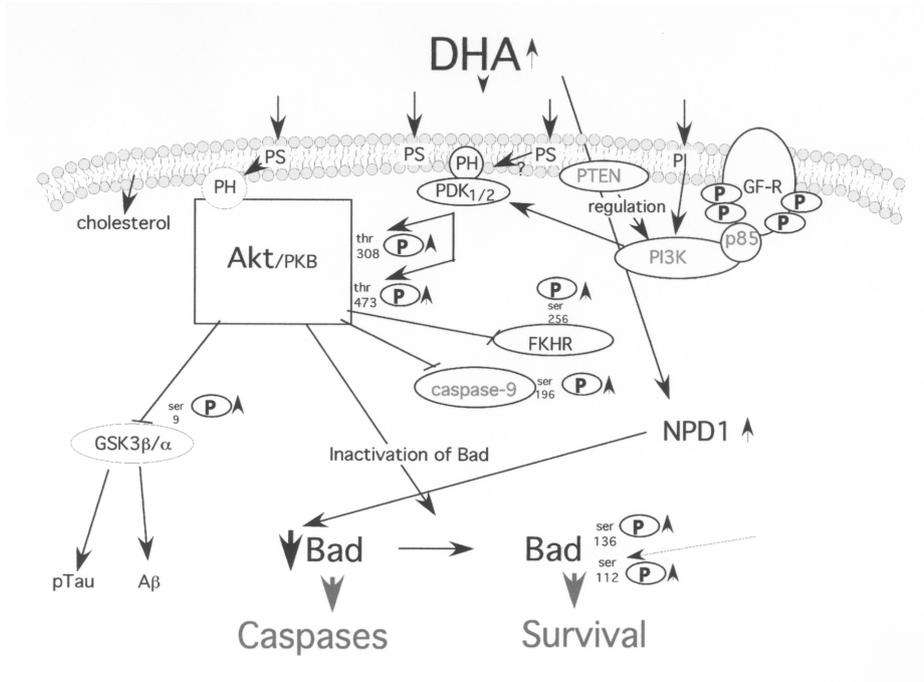


Figure. 1 Schematic of DHA actions. Increasing DHA increases membrane phospholipids including phosphatidylinositol (PI) and phosphatidylserine (PS). PS on the inner membrane promotes docking of proteins with plectstrin homology domains (PH), including components of the P13 kinase pathway, PDK and Akt, a major “survival signaling kinase. Increased Akt activity from increased D14A suppresses active glycogen synthase kinases (GSK3 $\alpha$  and  $\beta$ ) through inhibitory phosphorylation. Inhibiting GSK3 $\alpha$  limits gamma secretase and A $\beta$  production while inhibiting GSK3 $\beta$  suppresses tau phosphorylation and A $\beta$  toxicity. A $\beta$  production may also be reduced by lowering lipid raft cholesterol. Akt also promotes survival by phosphorylating forkhead (FKHR) and the pro-apoptotic caspase regulator, Bad. Caspase activation may be further limited by the DHA metabolite, NPD1, which controls expression of multiple caspase regulatory proteins, including Bad. Although not shown here, both Akt and the P13-K p85 subunit are both substrates for caspases. Our data in DHA-depleted APP mice showed reduced p85 P13-K protein as well as reductions in mRNA for pathway components, p85 P13-K, the P13-K regulator, PTEN and caspase 9 (shown in grey).

that a major player in memory, CaM kinase II alpha, was also markedly reduced by DHA depletion. Other significant factors in memory were altered by APPsw transgene on the safflower oil diet. For example, NMDA receptor subunits NR2B and NR2A were similarly reduced as a function of diet and transgene but poorly protected by DHA supplements (Calon et al 2005).

## DISCUSSION

### Mechanism of DHA protection

We interpreted the protective effects of DHA in terms of increased P13-K> Akt signaling leading to increased neuroprotection including increases in anti-apoptotic phosphoBAD and decreases in caspase cleaved actin (Calon *et al.*, 2004). Subsequent research shows that dietary lipid and the AD transgene can act on PAK kinases. In particular, A $\beta$  oligomers appear to dysregulate signaling from rac>PAK kinases that control actin dynamics in dendritic spines of excitatory neurons resulting in major losses in active soluble PAK and drebrin (Zhao *et al.*, 2006). Major drebrin loss occurs in AD and in the DHA-depleted Alzheimer model mice.

An alternative mechanism of DHA protection may be through enzymatic oxidation by lipoxygenases to a neuroprotective lipid, neuroprotectin D1 (NPD1), that can upregulate anti-apoptotic and downregulate pro-apoptotic regulatory proteins, for example BAD (Bazan, 2005). Consistent with this possibility we also found increased BAD protein with DHA depletion (Calon *et al.*, 2005). Another example of potent neuroprotective activity that may be related to generation of NPD1 (Bazan 2005) comes from observations that DHA supplementation can limit neurodegeneration associated with stroke in vivo (Belayev *et al.*, 2005). This data is consistent with earlier observations of the protective effects of DHA in other situations involving excitotoxicity (Saugstad, 2004).

AD patients have been reported to have dramatic 20-fold losses in NPD1 in the vulnerable CA1 region (Lukiw *et al.*, 2005). The magnitude of the lipoxygenase-derived NPD1 deficits clearly exceeded losses in DHA and expected neuron and synapse loss. Finally, insoluble beta amyloid and plaque counts were reduced by DHA supplementation of the DHA depleting diet (Lim *et al.*, 2005). Unpublished data from our lab confirms in another set of aged Tg2576 mice that DHA supplementation can markedly reduce A $\beta$  accumulation, but the mechanisms involved remain unclear. APP processing involves APP finding cholesterol modulated secretases in lipid rafts. The DHA mediated action could involve effects on membrane protein mobility, APP processing (possibly by DHA), cholesterol compartmentalization and lipid raft structure (Stillwell *et al.*, 2005). Alternatively, DHA stimulation of Akt activation (Akbar *et al.*, 2005) and downstream inhibition of GSK3 $\beta$  could be limiting gamma-secretase and A $\beta$  production (Phiel *et al.*, 2003). In addition, amyloid reduction may be mediated in part by an induction of the amyloid carrier transthyretin. Transthyretin has been shown to be induced in the CNS by dietary fish oil (Puskas *et al.*, 2003) and implicated in A $\beta$  clearance (Schwarzman *et al.*, 1994; Stein *et al.*, 2004). Finally, both DHA and NPD1 were reported to reduce A $\beta$  production by cultured human neurons suggesting that NPD1 signaling may influence secretase levels or activity (Lukiw *et al.*, 2005). Whatever the mechanism or mechanisms involved,

there is mounting *in vitro* and *in vivo* evidence from multiple groups that DHA treatment can reduce A $\beta$  levels.

Our data is consistent with an A $\beta$  oligomer and omega-3 interaction effects on excitatory neurons because these are enriched in PSD 95 and drebrin (Aoki *et al.*, 2005) as well as CaM kinase II $\alpha$ . Soluble oligomeric abeta species have been reported to bind focally to post synaptic PSD-95 positive sites on CamKII $\beta$  positive excitatory hippocampal neurons in culture (Lacor *et al.*, 2004) and lead to NMDA receptor subunit endocytosis (Snyder *et al.*, 2005). These direct soluble A $\beta$  oligomer effects on excitatory synapses, involved in learning and memory, provide a basis for a synaptic plasticity defect related to spine dysfunction and loss in excitatory pyramidal neurons as a significant contributing factor in the cognitive deficits in AD.

Given that part of the transgene-dependent loss of DHA appeared related to oxidative damage, we hypothesized that antioxidant treatment might protect against the impact of DHA-depletion. This appeared to be true of the potent antioxidant and NSAID curcumin which significantly limited oxidative damage, amyloid accumulation (Lim *et al.*, 2001) and postsynaptic marker loss in excitatory neurons (PSD-95, drebrin, CaM kinase II alpha (unpublished results)). Because curcumin has multiple activities, including direct inhibition of A $\beta$  oligomer formation and oligomer-mediated toxicity (Yang *et al.*, 2005), curcumin inhibition of synaptic damage cannot be interpreted solely in terms of antioxidant activity but may involve multiple actions.

Nevertheless, our collective data suggests a role for oxidative damage in synaptic damage. For example, we have found that curcumin or antioxidant cocktails can limit synaptic and cognitive deficits occurring in rat intracerebroventricular A $\beta$  infusion models where exogenous A $\beta$  drives deficits (Frautschy *et al.*, 2001). Others have found that antioxidant cocktails can limit cognitive deficits in aging dogs (Milgram *et al.*, 2005). Further, more recent, reports from many groups are finding that natural products enriched in other polyphenolic or flavonoid antioxidants including green tea (Rezai-Zadeh *et al.*, 2005), red wine (Dickstein *et al.*, 2005) and pomegranate juice (Hartman *et al.*, 2005) can protect against amyloid pathology in transgenic models. Overall, these results argue that increased consumption of diets or supplements enriched in both the omega-3 fatty acid DHA and polyphenolic or other antioxidants should be tested for their ability to chronically limit pathogenesis and help prevent or treat Alzheimer's disease.

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