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Neuroprotectin D1 Induces Neuronal Survival and Downregulation of Amyloidogenic Processing in Alzheimer's Disease Cellular Models

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Abstract The mediator neuroprotectin D1 (NPD1) is an enzymatic derivative of the omega-3 essential fatty acid docosahexaenoic acid. NPD1 stereoselectively and specifically binds to human retinal pigment epithelium (RPE) cells and neutrophils. In turn, this lipid mediator induces dephosphorylation of Bcl-x_L in a PP2A-dependent manner and induces PI3K/Akt and mTOR/p70S6K pathways leading to RPE cell survival during oxidative stress-induced apoptosis. As a proof of principle of its systemic in vivo bioactivity, NPD1 attenuates laser-induced choroidal neovascularization in mice. Using human neural cells transfected with amyloid precursor protein (APP)sw (Swedish double mutation APP695sw, K595N, M596L), NPD1 was shown to regulate secretase-mediated production of Aβ peptide, downregulates pro-inflammatory gene expression, and promotes cell survival. In human neural cells overexpressing beta-amyloid precursor protein (βAPP), the lipid mediator suppressed Aβ42 shedding by downregulating β-secretase (BACE1) while activating the α-secretase (ADAM10), thus shifting the βAPP cleavage from the noxious amyloidogenic pathway into a non-amyloidogenic, neurotrophic pathway. Furthermore, downregulation of Aβ42 peptide release by NPD1 may be dependent upon PPARγ activation. In conclusion, NPD1 exhibits anti-inflammatory, anti-amyloidogenic, and anti-apoptotic bioactivities in human neural cells in part via PPARγ signaling and through the targeting of α- and β-secretase systems.

Keywords Omega-3 fatty acids · Docosahexaenoic acid · Cyclooxygenase-2 · 15-Lipoxygenase-1 · Age-related macular degeneration

Abbreviations

15-LOX-1	15-Lipoxygenase-1
ARCD	Age-related cognitive decline
AMD	Age-related macular degeneration
ADAM10	Alpha-secretase distintegrin and metalloproteinase 10
AD	Alzheimer's disease
APP	Amyloid precursor protein
AA	Arachidonic acid
βAPP	Beta-amyloid precursor protein
CNS	Central nervous system
CA1	Cornu ammonis 1
COX-2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
ERK1/2	Extracellular signal-regulated kinases
FA	Fatty acid
HN	Human neural
IL	Interleukin
MAP	Mitogen-activated protein
NPD1	Neuroprotectin D1
PLA_2	Phospholipase A_2
RPE	Retinal pigment epithelium
TNF-α	Tumor necrosis factor alpha

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Docosahexaenoic Acid-Derived Neuroprotectin D1

Docosahexaenoic acid (DHA, 22:6; n-3) is enriched in the central nervous system (CNS); it plays a key role in brain function and has been implicated as a positive effector in slowing down neurodegenerations and eliciting neuroprotec-

tion [1–6]. Neuroprotectin D1 (NPD1) is derived from the stereoselective oxygenation of DHA by 15-lipoxygenase-1 (15-LOX-1) [7]. The quantity of unesterified (free) DHA (and arachidonic acid (AA)) in photoreceptors, retinal pigment epithelium (RPE) cells, and the brain is tightly regulated by phospholipase A_2 (PLA_2), reacylation, and peroxidation [8–10]. Free DHA is released from membrane phospholipids in a hydrolysis reaction catalyzed by PLA_2 as a response to numerous stimulations including ischemia, seizure, and neurotransmitter receptor occupancy. The free fatty acid (FA) may be reincorporated into membrane phospholipids, first by becoming the substrate of docosahexaenoyl-coenzyme A synthesis for channeling through acyltransferases, which incorporate this FA into phospholipids. For example, RPE cells modulate the uptake, conservation, and delivery of DHA to photoreceptors through these pathways [11]. Alternatively, oxidases such as 15-LOX-1 may initiate conversion to bioactive mediators. RPE cells utilize a specific DHA-phospholipid pool as a precursor for NPD1 synthesis.

NPD1 Induces Homeostatic/Pro-Survival Signaling

NPD1 in response to cellular and systemic insults induces a homeostatic response (Fig. 1) [12–14]. Specifically, NPD1 upregulates anti-apoptotic proteins (Bcl-2 and Bcl-xL) and downregulates pro-apoptotic proteins (Bax and Bad) in response to cellular oxidative stress and cytokine activation leading to an overall pro-survival transcriptome [12–15]. The stereoselective mediator, NPD1, provides a specific mechanism to understand DHA-mediated modulation of neuroinflammation and neuroprotection. NPD1 elicits neuroprotection in brain ischemia–reperfusion and in oxidative-stressed retinal cells [14–16]. DNA microarray profiling suggests a downregulation of pro-inflammatory genes as well as of pro-apoptotic genes of the Bcl-2 gene family to be involved [13]. Thus NPD1 is a mediator that executes protective bioactivity of DHA in the CNS. Deficiency of NPD1 and of the enzyme involved in its formation, 15-LOX-1, has been observed in Alzheimer's disease (AD) brain. Also, NPD1 further influences beta-amyloid precursor protein (βAPP) processing and decreases Aβ42 release [13], and its precursor, DHA, elicits an Aβ42-lowering effect both in vitro and in vivo [17–19]. In addition, free radical-mediated DHA peroxidation products accumulate during ischemia and neurodegeneration. These oxidation products in turn may form protein adducts and other cytotoxic molecules that promote further free radical injury [20–22].

Decreased DHA is Associated with Cognitive Decline

Learning during aging is enhanced in a senescence-accelerated mouse by dietary omega-3 FAs that results in higher DHA content in hippocampal and amygdala phospholipids. DHA is incorporated into brain phospholipids, and dietary omega-3 PUFAs are associated with a delay in cognitive decline [23]. Also, omega-3 deficiency results in impaired performance in spatial learning [24]. It is remarkable that the CNS displays a high degree of molecular structural recognition throughout DHA signalolipidomics since omega-6 20:5 fails to substitute DHA, which provides evidence for loss of spatial task performance [25]. The molecular characterization of the transporters/receptors engaged in these events remains to be ascertained. DHA deficiency is associated with normal aging, AD, hyperactivity, schizophrenia, and peroxisomal disorders. Higher dietary DHA intake is inversely correlated with risk of AD. DHA dietary supplementation is associated with improved immediate and delayed Verbal Recognition Memory scores, but not working memory or executive function tests. For the CANTAB Paired Associate Learning (PAL), a visuospatial learning and episodic memory test, members of the DHA group scored higher on the test than controls. DHA was well tolerated, with no reported treatment-related adverse events. A current study during a 24-week supplementation with 900 mg/day DHA shows that improved learning and memory function occurred in patients with age-related cognitive decline, supporting the notion that DHA supplementation has benefits for supporting cognitive health during aging. The studies listed here complement several others described in other parts of this review. Overall, we aim to convey the clear need for further mechanistic studies to precisely define how DHA participates in successful aging.

Neuroprotectin D1 in Cellular Models of Alzheimer's Disease

Alzheimer's disease is a complex, multi-factorial neurodegeneration of the elderly characterized by progressive cognitive impairment and, at the cellular level, by synaptic damage, intracellular neurofibrillary tangles and βAPP processing dysfunction that leads to overabundance of the 42 amino acid amyloid-beta (Aβ) 42 peptide. In turn, the Aβ42 oligomer triggers neuroinflammation, synaptic toxicity, and apoptosis. The oligomer accumulates as an aggregate and becomes a major component of senile plaques [26–31]. Aβ42 peptides are generated from βAPP via sequential cleavage by beta- and gamma- (β- and γ-) secretases, or a second pathway may be activated through alpha-secretase distintegrin and metalloproteinase 10 (ADAM10) that cleaves βAPP to yield sAPPα (non-amyloidogenic or neurotrophic pathway).

The CNS response to injury and to the onset (and progression) of neurodegeneration includes the release of free DHA and AA along with the synthesis of stereospecific

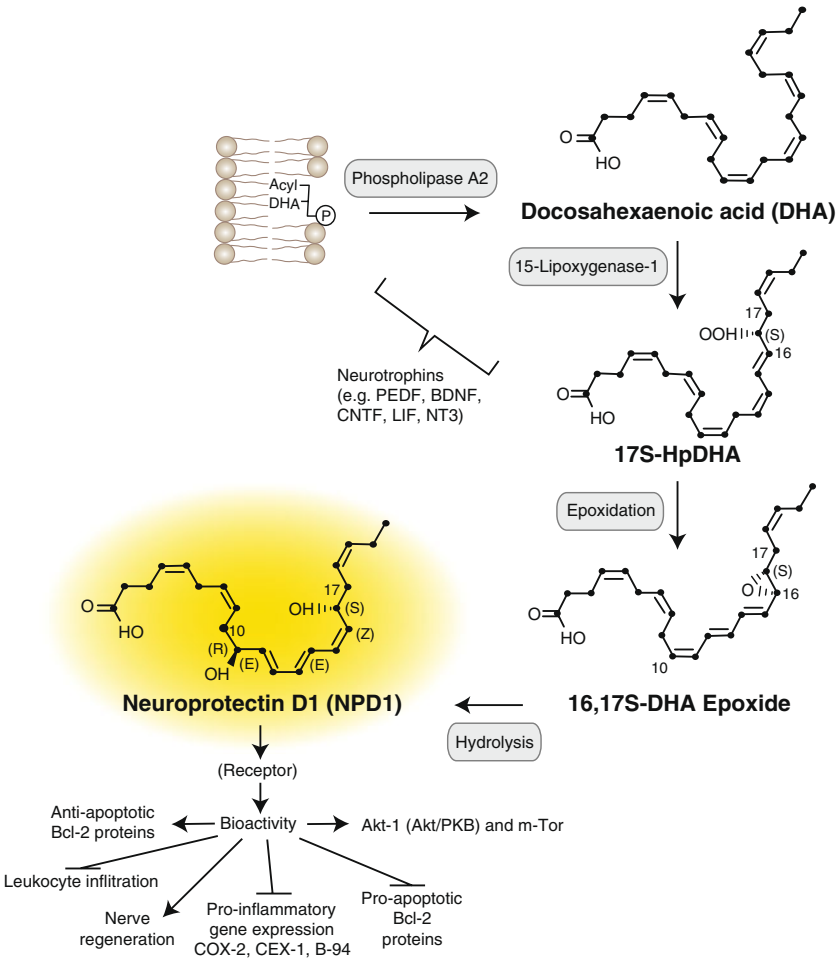

Fig. 1 Biosynthesis of neuroprotectin D1 (*NPD1*). A membrane phospholipid containing a docosahexaenoyl chain at sn-2 is hydrolyzed by phospholipase A2, generating free (unesterified) DHA (22:6). Lipoxygenation is then followed by epoxidation and hydrolysis, to generate NPD1. Thus far, a binding site for NPD1 has been identified in retinal pigment epithelium cells and polymorphonuclear cells

docosanoid derivatives. Human neural (HN) progenitor cells in primary culture during 8 weeks display an eightfold enhanced synthesis and release of Aβ40 and Aβ42 peptides that resembles Aβ deposition during brain aging and in AD. In HN cells, Aβ42 triggers apoptosis and changes in gene expression that emulate neurodegenerative events characteristic of AD. DHA partially counteracts cognitive decline in the elderly [32]. Moreover, omega-3 essential FA-rich diets are associated with a trend in reduced risk for mild cognitive impairment (MCI) and with MCI conversion to AD, whereas DHA has been shown to be beneficial in transgenic AD models [17, 32–35]. The DHA-derived NPD1 displays neuroprotective bioactivity in brain and retinal cells against various insults, including oxidative injury, ischemia–reperfusion, and inflammation [13–15, 36, 37]. Both AD brain [13] and the 3xTg-AD mouse exhibit reductions in DHA and NPD1. In our laboratory, we further characterized the anti-inflammatory and anti-apoptotic activity of NPD1 in cultures of HN cells stressed with the Aβ42 oligomer, and studied the NPD1-mediated modulation of α- and β-secretase activity that resulted in reduced shedding of Aβ42 [38].

Neuroinflammatory neurodegeneration associated with Aβ42 is an important contributory event to AD neuropathology [39, 40]. In our laboratory, primary HN cells were used, since human primary neurons do not survive well in the absence of glial cells [13, 41]. While we cannot exclude the possibility that glial cells provide some neuroprotective "shielding", both neuronal and glial cells release cytokines when exposed to Aβ42 that, in turn, activate more microglia and astrocytes that reinforce pathogenic signaling. NPD1 is anti-inflammatory and promotes inflammatory resolution [14, 15, 37, 42]. In HN cell models of Aβ42 toxicity, microarray analysis and Western blot analysis revealed downregulation of pro-inflammatory genes (cyclooxygenase-2, tumor necrosis factor alpha, and B94), suggesting NPD1's anti-inflammatory bioactivity targets this gene family, at least in part [13]. These effects are persistent, as shown by time-course Western blot analysis in which protein expression was examined up to 12 h after treatment by Aβ42 and NPD1 [38].

Although counteracting Aβ42-induced neurotoxicity is a promising strategy for AD treatment, curbing excessive Aβ42 release during neurodegeneration is also desirable.

DHA could lower the Aβ42 load in the CNS by stimulating non-amyloidogenic βAPP processing, reducing PS1 expression, or by increasing the expression of the sortilin receptor, SorLA/LR11 [17, 19, 43, 44].

In contrast to a previous report by Green et al. [34] that suggested that Aβ peptide reductions in whole brain homogenates of 3xTg AD after dietary supplementation of DHA were the result of decreases in the steady state levels of PS1, our lab showed that NPD1 had no effect on PS1 levels in primary HN cells, but that a significant increase in ADAM10 occurred in conjunction with a decrease in BACE1. These later observations were further confirmed by both activity assays and siRNA knockdown. NPD1 reduced Aβ42 levels released from HN cells overexpressing APPsw in a dose-dependent manner. Our examination of other βAPP fragments revealed that after NPD1 addition a reduction in the β-secretase products sAPPβsw and CTFβ occurred, along with an increase in α-secretase products sAPPα and CTFα, while levels of βAPP expression remained unchanged in response to NPD1. Hence these abundance- and activity-based assays indicate a shift by NPD1 in βAPP processing from the amyloidogenic to non-amyloidogenic pathway. Previously, sAPPα was found to promote NPD1 biosynthesis from DHA [13], while studies in our lab showed that NPD1 works to stimulate sAPPα secretion, creating positive feedback and neurotrophic reinforcement. Secreted sAPPα's beneficial effects include enhanced learning, memory, and neurotrophic properties [31]. NPD1 further downregulated the β-secretase BACE1 and activated ADAM10, a putative α-secretase. Our ADAM10 siRNA knockdown and BACE1 overexpression-activity experiments confirmed that ADAM10 and BACE1 are required in NPD1's regulation of βAPP. Therefore, NPD1 appears to function favorably in both of these competing βAPP processing events.

PPARγ activation leads to anti-inflammatory, anti-amyloidogenic actions, and anti-apoptotic bioactivity, as does NPD1. Some FAs are natural ligands for PPARγ, which have a predilection for binding polyunsaturated fatty acids [45–47]. NPD1 is a PPARγ activator, as shown by using both human adipogenesis and cell-based-transactivation assay [38]. NPD1 may activate PPARγ via direct binding or other interactive mechanisms [48, 49]. Analysis of βAPP-derived fragments revealed that PPARγ does play a role in the NPD1-mediated suppression of Aβ production. Overexpressing PPARγ or incubation with a PPARγ agonist leads to reductions in Aβ, sAPPβ, and CTFβ similar to that with NPD1 treatment, while a PPARγ antagonist abrogates these reductions. Activation of PPARγ signaling is further confirmed by the observation that PPARγ activity decreases BACE1 levels, and a PPARγ antagonist blocks this decrease. Thus, the anti-amyloidogenic bioactivity of NPD1 is associated with activation of the PPARγ and the subsequent BACE1 downregulation (Fig. 2). The difference between the bioactivity of NPD1 concentrations for anti-apoptotic and anti-amyloidogenic activities ([50 nM] vs. [500 nM]) may be due to the different cell models used (i.e., Aβ-peptide stressed vs. βAPPsw-overexpressing HN cells) and/or related mechanisms. Although Aβ-lowering effects of PPARγ have been reported, the molecular mechanism of this action remains unclear. Induction of βAPP ubiquitination, which leads to enhanced βAPP degradation and reduced Aβ peptide secretion, has been suggested [46]. Alternatively, Aβ clearance might be involved, or regulation by PPARγ may be due to enhancement of insulin sensitivity and increases in brain insulin-degrading enzyme [45]. The decreases in BACE1 may be the cause for Aβ reduction [41, 50]. A reason for these conflicting reports may be that cell models and culture conditions used vary (HN cells transiently overexpressing βAPPsw or cell lines using stable βAPP expression). Similar to the model of Sastre et al. [50], our cells underwent increases in Aβ production. Excessive Aβ causes inflammatory responses in both neuronal and glial cells [41]. Since inflammatory signaling plays a role in AD pathogenesis, we believe HN cell cultures are a valuable model for Aβ42-mediated cellular actions. The fact that comparable results of our study were obtained at a much lower drug concentration ([0.5 μM] of rosiglitazone vs. [10–30 μM] in previous reports) [38] underscores the highly sensitive nature of HN cells after βAPP transfection. It is still possible that PPARγ may repress BACE1 by antagonizing activities of other transcription factors that promote BACE1 expression, such as STAT1, nuclear factor kappa-B (NF-κB), and AP1 [51]. It is noteworthy that BACE1 expression in HN cells was increased after βAPP overexpression. The fact that PPARγ did not affect the levels of sAPPα and CTFα, aside from the PPARγ antagonist being unable to reverse NPD1-elicited increase in these fragments, clearly shows that PPARγ is not essential for NPD1's regulation of the non-amyloidogenic pathway. Further analysis of ADAM10 showed no change occurring in ADAM10 following PPARγ activation, nor did PPARγ antagonists affect NPD1-enhanced expression of mature ADAM10. Therefore, modulation by NPD1 of α-secretase and βAPP processing is independent of PPARγ. ADAM10 is synthesized as an inactive zymogene and is processed to its mature form by cleavage of the pro-domain by pro-protein convertases (PPCs), such as furin and PC7 [52]. Other evidence also demonstrates that protein kinase C (PKC) and mitogen-activated protein (MAP) kinase, particularly extracellular signal-regulated kinases (ERK1/2), are involved in regulation of α-secretase activity [23, 48, 53]. No cross-talk between the PPCs and PKC or MAP kinases has been reported. Since only the mature ADAM10 was increased in the studies performed in our lab, it is likely that the PPCs are implicated in NPD1 actions.

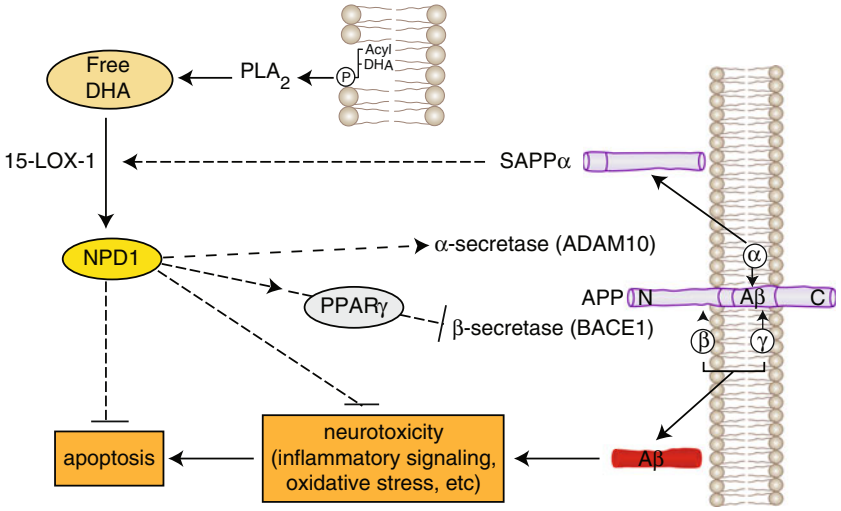

Fig. 2 Mechanism for neuroprotectin D1 (NPD1) induction of non-amyloidogenic and neurotrophic bioactivity. DHA (22:6) is excised by phospholipase A2 (*PLA$_2$*) to yield *free DHA*; in turn, *free DHA* is 15-lipoxygenated to generate *NPD1* which then activates a neuroprotective signaling. These events are mediated, in part, by shifting βAPP processing from an amyloidogenic into a neurotrophic, non-amyloidogenic pathway and by inhibiting apoptosis, blocking inflammatory signaling, promoting cell survival. *BACE1* activity is suppressed and α-secretase (*ADAM10*) activity is stimulated, thus downregulating Aβ42 peptide release . *NPD1* signaling to *BACE1* and *ADAM10* may be mediated via other neuromolecular factors. The *ADAM10* cleavage product *sAPPα* further induces the conversion of *free DHA* into NPD1, thus constituting a positive, neurotrophic feedback loop

PPARγ antagonist GW9662 also failed to reverse the anti-apoptotic effect of NPD1, indicating that PPARγ is not implicated in NPD1 anti-apoptotic bioactivity. NPD1 attained this neuroprotection at a concentration of [50 nM], at which its PPARγ activity is far from physiologically relevant in the in vitro system. There is compelling evidence that NPD1 is endowed with strong anti-inflammatory, anti-amyloidogenic, and anti-apoptotic bioactivities in HN cells upon exposure to Aβ42 oligomers, or in HN cells overexpressing βAPP_{sw}. These results suggest that NPD1's anti-amyloidogenic effects are mediated in part through activation of the PPARγ receptor, while NPD1's stimulation of non-amyloidogenic pathways is PPARγ-independent. NPD1 stimulation of ADAM10, coupled to suppression of BACE1-mediated Aβ42 secretion, clearly warrants further study since these dual secretase-mediated pathways may provide effective combinatorial or multi-target approaches in the clinical management of the AD process.

The hippocampal cornu ammonis 1 (CA1) region, the area of cortex most heavily damaged by AD, displays 1/20 of the NPD1 of age-matched controls, even though the difference in free DHA was only twofold lower; these changes were not present in other brain regions [13]. Potent protective bioactivity of NPD1 was shown in various models of neuroinflammatory pathology, including age-related macular degeneration (AMD) [12, 15, 54, 55], stroke [14, 56], AD [13, 38, 44, 56], and oxidative stress [15, 16, 57]. These observations implicate NPD1 as an integral homeostatic modulator of long-term function and highlight the needs of DHA accretion in the CNS.

Neuroprotectin D1 Targets Neuroinflammatory Signaling

The pro-inflammatory cytokine, interleukin (IL)-1β, stimulates Aβ40 and Aβ42 secretion as a function of HN cell aging. Conversely, DHA suppresses both Aβ40 and Aβ42 peptide release with concomitant NPD1 synthesis. Also, NPD1 inhibits Aβ42-induced apoptosis in HN cells. Pro- and anti-apoptotic proteins are modulators proximal to mitochondrial and cellular damage. Pro-apoptotic Bik and Bax were enhanced by Aβ42, but not by NPD1, whereas Bcl-2, Bcl-xL, and Bfl-1(A1) were increased in the presence of NPD1. Bfl-1(A1) increased almost sixfold. NPD1 also induces the anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL in oxidatively challenged human RPE cells and promotes cytoprotection. Since photoreceptor survival is dependent on RPE cell integrity, the protection of RPE cells has important implications in vision and AMD. A further suggestion for the significance of NPD1 in AD is the finding that hippocampal CA1 regions from AD patients show a dramatic reduction in NPD1. Thus the interplay of DHA-derived neuroprotective signaling aims to counteract pro-inflammatory, cell-damaging events triggered by multiple, converging cytokine and amyloid peptide factors in AD. Amyloid peptide mediates oxidative stress and microglial-derived cytokines, such as IL-1β and tumor necrosis factor alpha, supporting progressive inflammatory episodes in AD. These noxious stimuli further orchestrate pathogenic gene expression programs in stressed brain cells, thereby linking a cascade of caspase-mediated cell death

pathways with apoptosis and neuronal demise. Neural mechanisms leading toward NPD1 generation from DHA thereby appear to redirect cellular fate toward successful brain cell aging. The Bcl-2 pro- and anti-apoptotic gene families, neurotrophins, sAPP alpha, and NPD1 lie along a cell fate–regulatory pathway whose component members are highly interactive and have potential to function cooperatively in brain cell survival. Agonists of NPD1 biosynthesis, NPD1 analogs or dietary regimens may be useful for exploring new preventive/therapeutic strategies for stroke, AMD, AD, and other neurodegenerative diseases. Other mechanisms have been proposed to explain DHA's anti-apoptotic and anti-inflammatory effects, including maintenance of plasma membrane integrity, activation of Akt signaling [58], and conversion into other derivatives [21, 33]. These findings also provide clues for NPD1's potential targets. NPD1 inhibits NF-κB activation and cyclooxygenase-2 (COX-2) expression in brain ischemia–reperfusion [14], while Aβ peptide-induced apoptosis is associated with ERK and p38 MAPK-NF-κB-mediated COX-2 up-regulation [59]. Neuroprotection mediated by NPD1 may further involve components of signaling pathways upstream of NF-κB activation and DNA-binding [13].

The importance of DHA in maintaining cellular integrity and homeostasis has been underscored in many studies linking decreased levels of brain DHA to cognitive decline, specifically AD (Fig. 2) [13, 60]. When rats are fed a diet depriving them of both DHA and ALA precursors, specific physioneurological changes take place. Rats show decreased performance in water-maze challenges, decreased visual acuity, and increased tendencies towards aggression and depression [61, 62]. Multiple studies have shown decreased levels of DHA in both the esterified phospholipid and free form in brains from Alzheimer's patients compared to controls. One of the more recent publications also measured the levels of omega-3 precursors in liver and brain samples of Alzheimer's patients. Omega-3 precursors were similar in brain samples and increased in liver samples, but the amount of DHA (free and esterified) was decreased in both tissues. This same study also compared the gene expression profiles of hepatic enzymes involved in the conversion of omega-3 precursors to DHA from the same donor pool of Alzheimer's patients and controls. Hepatic tissue samples from the Alzheimer's patients showed reduced expression of peroxisomal bi-directional protein (an enzyme involved in the final step of DHA synthesis from DPA, an ALA elongation product) and increased metabolic precursors of DHA [60]. Various forms of retinitis pigmentosa and Usher syndrome type 1, two retinal pathologies, have been linked to decreased plasma DHA levels [12, 63]. These data imply a link between impaired conversion of omega-3 precursors in the liver to decreased DHA bioavailability in the brain and retina, putting these vital systems at risk for disease. Moreover, in experimental stroke, systemically administered DHA protects the penumbra in a remarkable fashion: the time window extends up to 5 h after 2 h of middle cerebral artery occlusion, with concomitant NPD1 synthesis [64].

The results summarized in this review highlight the specificity and potency of NPD1 as a homeostatic mediator of CNS function and integrity, particularly when confronted with injury or neurodegeneration.

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