BJP British Journal of Pharmacology

DOI:10.1111/bph.12030
www.brjpharmacol.org

Themed Section: Cannabinoids 2012, Part Two

REVIEW

N-acyl amines of docosahexaenoic acid and other *n*–3 polyunsatured fatty acids – from fishy endocannabinoids to potential leads

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Keywords
endocannabinoid system; n-3; PUFA, DHA, EPA; fish oil

Received
21 June 2012
Revised
15 September 2012
Accepted
15 October 2012

N–3 long-chain polyunsaturated fatty acids (*n*–3 LC-PUFAs), in particular α-linolenic acid (18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are receiving much attention because of their presumed beneficial health effects. To explain these, a variety of mechanisms have been proposed, but their interactions with the endocannabinoid system have received relatively little attention so far. However, it has already been shown some time ago that consumption of *n*–3 LC-PUFAs not only affects the synthesis of prototypic endocannabinoids like anandamide but also stimulates the formation of specific *n*–3 LC-PUFA-derived conjugates with ethanolamine, dopamine, serotonin or other amines. Some of these fatty amides show overlapping biological activities with those of typical endocannabinoids, whereas others possess distinct and sometimes largely unknown receptor affinities and other properties. The ethanolamine and dopamine conjugates of DHA have been the most investigated thus far. These mediators may provide promising new leads to the field of inflammatory and neurological disorders and for other pharmacological applications, including their use as carrier molecules for neurotransmitters to target the brain. Furthermore, combinations of *n*–3 LC-PUFA-derived fatty acid amides, their precursors and FAAH inhibitors offer possibilities to optimise their effects in health and disease.

LINKED ARTICLES
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Abbreviations
2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonoyl ethanolamine (anandamide); ALA, α-linolenic acid (18:3n-3); αLNEA, α-*N*-linolenoyl ethanolamine (conjugate of ALA); CHD, coronary heart disease; DEA, *N*-docosatetraenoyl ethanolamine; DHA, docosahexaenoic acid (22:6n-3); DHA-5HT, *N*-docosahexaenoyl serotonin; DHA-DA, *N*-docosahexaenoyl dopamine; DHEA, *N*-docosahexaenoyl ethanolamine; DPA, docosapentaenoic acid (22:5n-3); EPA, eicosapentaenoic acid (20:5n-3); *n*–3 LC-PUFA, (*n*–3) long-chain polyunsaturated fatty acid; NAEs, *N*-acyl ethanolamines; OEA, *N*-oleoyl ethanolamine; PEA, *N*-palmitoyl ethanolamine; SEA, *N*-stearoyl ethanolamine; TRVP1, transient receptor potential channel type V1

Introduction

Conjugates of fatty acids with ethanolamine, amino acids or monoamine neurotransmitters occur widely in nature (Di Marzo *et al.*, 2007; Farrell and Merkler, 2008; Connor *et al.*, 2010; Ezzili *et al.*, 2010). Chemically, they are categorized as fatty acid amides and further divided into subclasses, including the *N*-acyl ethanolamines (NAEs) and *N*-acyl amines

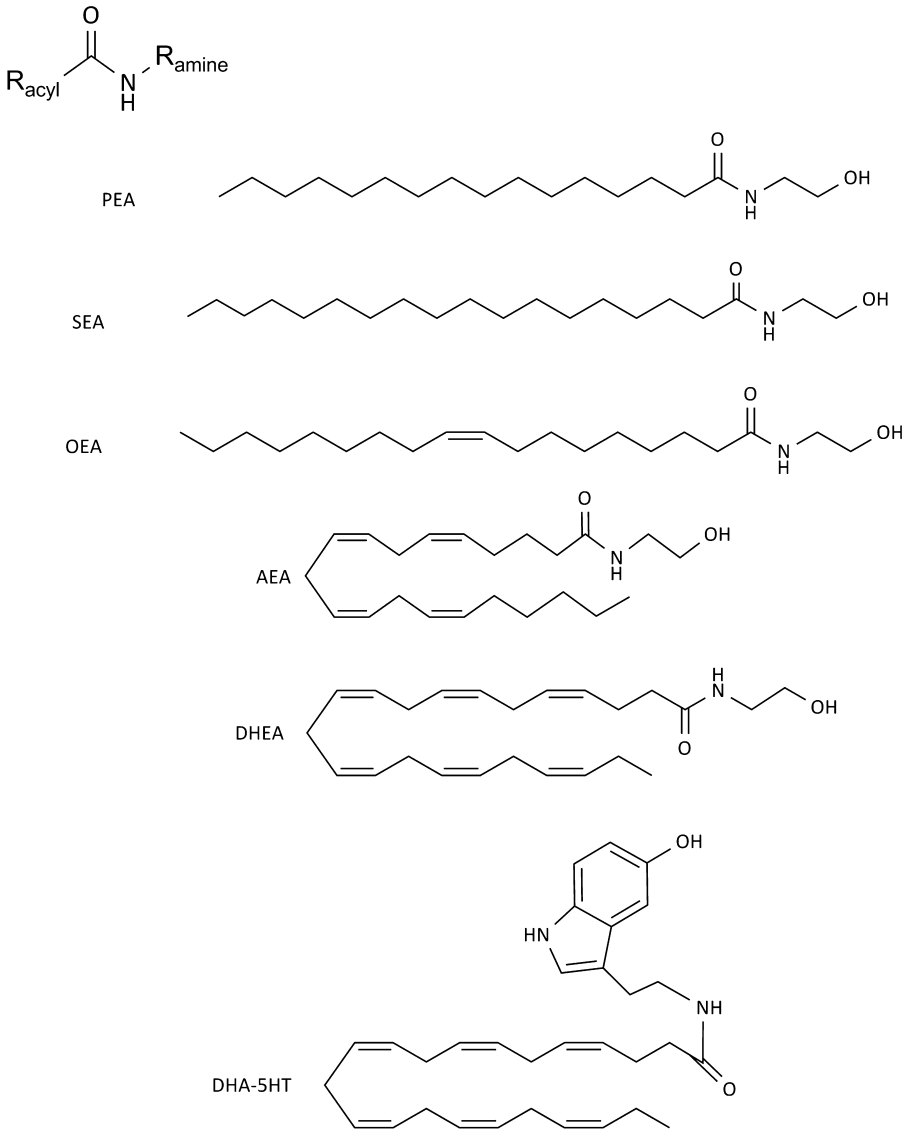


Figure 1

General chemical structure of fatty acid amides and some examples of conjugates of dietary relevant fatty acids with ethanolamine or serotonin.

(Lipid Maps class FA08; http://www.lipidmaps.org). The best studied representative to date is anandamide (*N*-arachidonoyl ethanolamine, AEA), a prototypic endocannabinoid well-known for its pleiotropic effects ranging from energy homeostasis to immune functioning (Di Marzo *et al.*, 2007). The biological and pharmacological properties of fatty (acid) amides do not follow their chemical classification and have shown to be very diverse. Anandamide (Figure 1) is a known ligand for both the cannabinoid type-1 (CB_1) and CB_2 receptors (receptor nomenclature follows Alexander *et al.*, 2011) and belongs to the NAE subclass. However, several other NAEs, such as *N*-palmitoyl ethanolamine (PEA), *N*-oleoyl ethanolamine (OEA) and *N*-stearoyl ethanolamine (SEA), show different receptor preferences, including affinity for GPR55, GPR18, GPR119, TRPV1 (transient receptor potential channel type V1) or PPARα, while often showing less or no affinity for CB_1 or CB_2 receptors (Alexander and Kendall, 2007; Di Marzo *et al.*, 2007; Farrell and Merkler, 2008; Hansen and Diep, 2009). The *N*-acyl amine subclass contains more than 80 different conjugates of long-chain fatty acids with amino acids (lipoamino acids; elmiric acids) or neurotransmitters (Burstein and Zurier, 2009; Connor *et al.*, 2010; Tan *et al.*, 2010).

For many of these molecules, relatively little is known so far about their biological significance or pharmacological potential. In many cases, only *in vitro* data are available, often obtained from testing single compounds. However, *in vivo*, fatty acid amides are known to occur in fluctuating mixtures of structurally related molecules with pleiotropic and tissue specific activities. With regard to the fatty acid moiety, the majority of studies so far have focused on conjugates of the most abundant fatty acids in higher animals, in particular those of arachidonic acid (20:4n-6), palmitic acid (16:0), oleic acid (18:1n-9) and stearic acid (18:0). Compared with these,

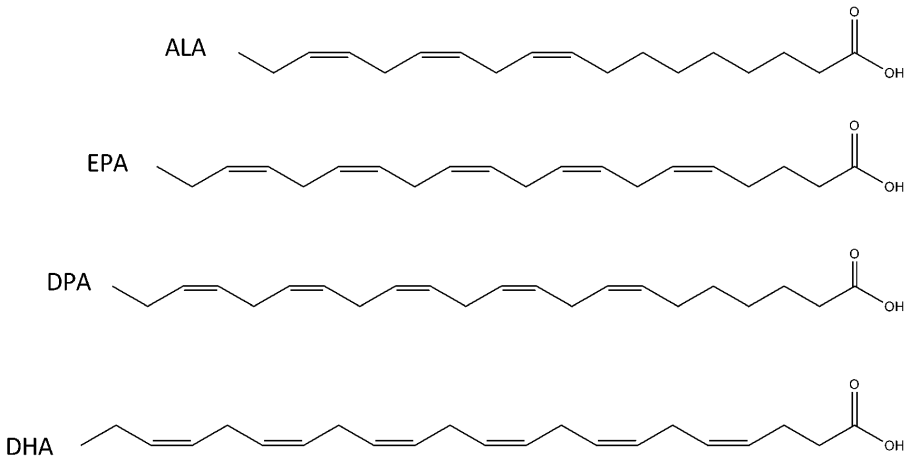


Figure 2
Chemical structure of four major natural *n-3* LC-PUFAs: ALA (18:3n-3), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3).

much less is known on the biology and pharmacology of fatty amides of long-chain polyunsaturated (*n*–3) fatty acids (*n*–3 LC-PUFAs), including those of the dietary most relevant α-linolenic acid (18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (see Figure 2).

Because of their (alleged) positive effects in health and disease, *n*–3 LC-PUFAs are of much interest both from a nutritional and a pharmacological perspective. As will be described below, several mechanisms have been proposed to explain these effects, but a possible involvement of fatty acid amides has not yet received much attention. During the last few years, new data have become available, suggesting that the formation and effects of fatty acid amides derived from *n*–3 LC-PUFAs may be more important than previously assumed. For example, although the existence of *N*-docosahexaenoyl ethanolamine (DHEA) in bovine brain was already reported in 1997 by the group of Raphael Mechoulam (Sheskin *et al.*, 1997), only few studies have further investigated its physiological role or pharmacological effects. However, evidence is increasing that several amine conjugates of *n*–3 acids possess an interesting spectrum of activities. Furthermore, at least some of them, including DHEA, are present in brain and gut tissue in concentrations similar to or even higher than those of AEA (Berger *et al.*, 2001; Wood *et al.*, 2010; Balvers *et al.*, 2012a).

The aim of the present review is to summarize and discuss these findings from a physiological and pharmacological perspective. The focus is primarily on DHEA, the most studied representative of this group, but other conjugates of DHA, EPA and α-linolenic acid with ethanolamine, dopamine, serotonin or other amines will also be given attention.

Long-chain *n*–3 PUFAs – presence, health effects and metabolic pathways

Polyunsaturated fatty acids contain more than one double bond in the aliphatic chain. The natural PUFAs are often categorized into two groups: the *n–6* (or ω-6) and the *n–3* (or ω-3) fatty acids, based on the position of the first double bond starting from the methyl (omega, ω) position. Mammals do not have enzymes to insert a double bond in the *n*–6 or *n*–3 position, and a lack of linoleic acid (18:2n-6) or α-linolenic acid (ALA, 18:3n-3) in the diet can give rise to symptoms of deficiency in humans (Hansen and Artmann, 2008; De Caterina, 2011). Significant amounts of ALA are found in a number of green plants, nuts, flaxseed (linseed) and some vegetable oils, including soybean and rapeseed oils (Calder, 2011). Via elongation of the acyl chain and insertion of extra double bonds ALA can be converted to EPA (20:5n-3) via the intermediate stearidonic acid (18:4n-3). EPA can be further metabolized to docosapentaenoic acid (22:5n-3; DPA) and finally to DHA (22:6n-3). However, endogenous conversion of ALA to EPA and DHA is very limited in humans, in particular in adults (Brenna *et al.*, 2009). Details on these pathways are described in a number of recent reviews (Russo, 2009; Calder, 2011; De Caterina, 2011).

EPA, DPA and DHA are particularly found in 'oily' fish (herring, salmon, mackerel), in certain algae and in 'krill oil'. One oily fish meal can provide between 1.5 and 3.5 g of these *n*–3 LC-PUFAs (Russo, 2009; Calder, 2011). Consumption of *n*–3 LC-PUFAs has been associated with a variety of positive health effects (Parker *et al.*, 2006; Carlson, 2009; Bazan *et al.*, 2011; Calder, 2011). However, for most of these presumed effects, the evidence in humans is far from conclusive, in particular when considering the DHA/EPA intake obtained from the commonly recommended one to two servings of oily fish per week. Benefits in humans seem to be most consistent for mortality from coronary heart disease and sudden cardiac death (Riediger *et al.*, 2009; de Roos *et al.*, 2009; De Caterina, 2011; Mozaffarian and Wu, 2011). At the same time, recent meta-analyses on the relation between consumption of fish and/or *n*–3 LC-PUFAs and the incidence of diabetes type 2, for example, did not provide clear evidence for such associations (Wallin *et al.*, 2012; Xun and He, 2012). In rodent studies, *n*–3 LC-PUFAs exhibit immune-modulating, anti-inflammatory and cellular protective properties (Calder, 2009; 2011). However, doses used in these

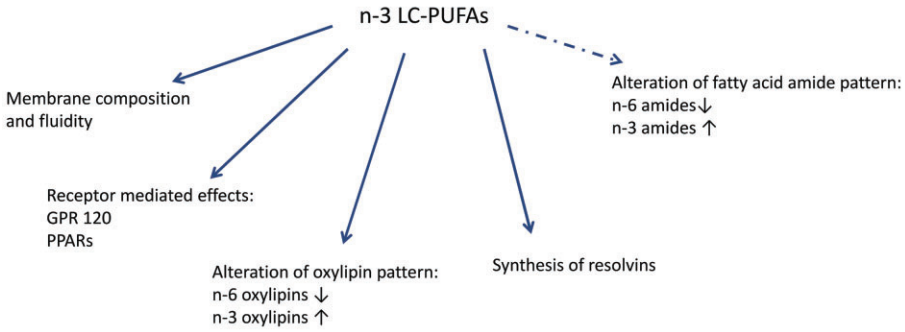


Figure 3

Schematic representation of different mechanisms via which *n–3* LC-PUFAs can influence biological processes. Dotted arrows depict the modulation of the endocannabinoid system as described in this review.

studies are often rather high, which complicates extrapolation to humans. Effects on inflammatory markers have been reported from human studies as well, although doses are sometimes high compared with those commonly obtained from the diet (see Tur *et al.*, 2012). The immune-modulating and/or anti-inflammatory effects have been explained from different mechanisms (see Figure 3), and it seems conceivable that these will at least partly be acting in parallel (Calder, 2011).

Dietary *n*–3 LC-PUFAs induce shifts in endocannabinoid patterns

The endocannabinoid system controls food intake and energy balance through a number of central and peripheral mechanisms (see Maccarrone *et al.*, 2010b). *Vice versa*, both the absolute and relative endocannabinoid tissue concentrations are determined by feeding status and dietary intake patterns. A number of studies in rodents and humans have shown that increasing the relative proportion of *n*–3 LC-PUFAs in the diet can lead to a decrease in the formation of the 'prototypic' endocannabinoids anandamide (AEA) and 2-AG (Batetta *et al.*, 2009; Banni and Di Marzo, 2010; Maccarrone *et al.*, 2010b). In the past, this was sometimes interpreted as an overall reduction of activity of the endocannabinoid system. For example, in the study of Watanabe *et al.* (2003), the conclusion was drawn that modulation of dietary *n*–3 PUFA status might provide a way to modify physiological and pathological events mediated by 2-AG through cannabinoid receptors in the CNS. However, although lower anandamide and 2-AG levels after fish oil diets have indeed been shown in many studies, these changes are a direct consequence from a shift in *n*–3/*n*–6 balance of membrane lipids. This results in compensatory increases in *n*–3 LC-PUFA-derived acyl conjugates. As will be discussed below, some of these molecules also show affinity for CB_1 or CB_2 receptors or share other activities with, for example, anandamide. Therefore, it seems important to pay attention to a broader spectrum of fatty amides and if possible include other classes of fatty acid metabolites, such as the oxygenated lipid species formed by COXs or lipoxygenases (Balvers *et al.*, 2012a).

Formation and turnover of *n*–3 LC-PUFA-derived fatty amides

Given the high proportion of its precursor DHA in phospholipids of brain synapses and retina, it is not surprising that DHEA was first discovered in brain tissue and retina (Sugiura *et al.*, 1996; Sheskin *et al.*, 1997; Bisogno *et al.*, 1999).

In 2001, Berger *et al.* demonstrated that brain levels of the ethanolamine conjugates of DHA and EPA, DHEA and EPEA (*N*-eicosapentaenoyl ethanolamine) in piglets were modulated by the amount of *n*–3 LC-PUFAs in the feed (Berger *et al.*, 2001). Since then several other studies in different species have confirmed an increased formation of DHEA and EPEA in various tissues after administering fish oil or individual *n–3* LC-PUFAs (see Maccarrone *et al.*, 2010b). A more recent example is the study of Artmann *et al.* (2008) who showed that a fish-oil rich diet given to rats increased jenunal levels of DHEA and EPEA, while at the same time decreasing levels of AEA, OEA and PEA. Specifically for brain, Wood *et al.* (2010) showed that 2 weeks of fish oil supplementation in mice caused a shift in NAE (and also glycerol-ester) patterns in favour of DHEA and EPEA at the expense of their arachidonoyl and oleoyl homologues. Remarkably, these studies also showed that even with control diets not enriched with *n–3* LC-PUFAs, brain concentrations of DHEA were higher than those of AEA and of the same order of magnitude as AEA in other tissues like ileum and liver. Tissue levels of EPEA appear to be low compared with those of DHEA but increase with fish oil diets and after LPS treatment, in particular in the gut (Artmann *et al.*, 2008; Balvers *et al.*, 2012a). Using deuterated (d5) DHA and EPA, we showed that differentiated 3T3-L1 adipocytes are able to synthesize DHEA and EPEA from their precursors (Balvers *et al.*, 2010). Recently, human breast and prostate cancer cell lines as well as hippocampal neuron cultures were also shown to perform these conversions (Brown *et al.*, 2011; Kim *et al.*, 2011a,b).

Pilot studies with human volunteers in our own lab showed that daily intake of fish oil food supplements (480 mg EPA plus 360 mg DHA per day) doubled plasma DHEA levels in 3 weeks. All these findings are consistent with the concept that the local relative availability of fatty acid precursors, which in turn is modulated by dietary intake of

lipids, determines the pattern of amide conjugates formed. The same holds true for the local availability of amines. For example, we showed that serotonin conjugates with fatty acids, including those of DHA and EPA, are formed by gut tissue, where most of the body's serotonin resides (Verhoeckx *et al.*, 2011). As expected, intestinal levels of DHA-serotonin and EPA-serotonin were higher in mice fed a fish oil rich diet. In addition to the ethanolamines EPEA and DHEA, several *n–3* LC-PUFA-derived fatty amides have so far been identified with different amines, in organisms ranging from *Homo sapiens* to *Hydra*. Examples taken from different studies are given in Table 1.

It is conceivable that *n*–3 LC-PUFA derived fatty amides will be formed and broken down via pathways similar to those described for other amides. Depending on the structure, synthesis can take place via different routes. An extensive review of these falls outside the scope of this paper, and readers are referred to several excellent recent reviews on this topic (Di Marzo *et al.*, 2007; Bisogno, 2008; Muccioli, 2010; Ueda *et al.*, 2010a). Briefly, according to the most studied transacylation–PDE pathway, NAEs are formed from glycerophospholipids via *N*-acylphosphatidyl ethanolamine (NAPE), by sequential catalysis involving Ca^{2+}-dependent *N*-acyltransferase and NAPE-hydrolyzing PLD. The biosynthetic NAPE precursor of DHEA has indeed been found in bovine retina (Bisogno *et al.*, 1999) and in rat brain (Sugiura *et al.*, 1996). Other pathways involve different enzymes including PLA2. The biosynthesis of conjugates with simple amino acids does not follow the phospholipid pathways (Bradshaw *et al.*, 2009). The breakdown of *n*–3 derived fatty acid conjugates is likely to follow routes similar to those described for other fatty acid amides. The primary NAE-degrading enzyme is fatty acid amide hydrolase (FAAH, now also known as FAAH-1), localized on the endoplasmatic reticulum (Bisogno, 2008). A second FAAH enzyme, now called FAAH-2, was found in humans, located on cytoplasmic lipid droplets (Wei *et al.*, 2006; Bisogno, 2008). Apparently, rodents do not possess FAAH-2. Both enzymes show distinct but overlapping substrate specificity and tissue distribution. To reach their sites of catabolism within the cell, NEAs are bound to different proteins including fatty acid binding proteins 5 and 7, heat shock protein 70, albumin and the FAAH-like AEA transporter protein (Kaczocha *et al.*, 2009; Fowler, 2012). Intracellular trafficking of NAEs is also important to reach the intracellular receptors (Maccarrone *et al.*, 2010a; Kaczocha *et al.*, 2012). DHEA inhibited the hydrolysis of [^{14}C]AEA although to a lesser extent than AEA itself (Bisogno *et al.*, 1999). This suggests that FAAH recognizes DHEA, but that it is a worse substrate than AEA. From their studies in the (human) LNCaP prostate cancer cell line, Brown *et al.* (2010) also obtained further evidence that FAAH metabolizes both EPEA and DHEA. Recently, a third NAE hydrolyzing enzyme, NAE-hydrolyzing acid amidase (NAAA), has been identified (Ueda *et al.*, 2010b). Next to hydrolysis, NAEs are substrates for oxidative enzymes including COXs, lipoxygenases (LOXs) and cytochrome P450 enzymes, yielding a range of prostamides (prostaglandin-amides) and hydroperoxy derivatives (Vandevoorde and Lambert, 2007; Woodward *et al.*, 2008: Dainese *et al.*, 2012 #3478; Rouzer and Marnett, 2011). A number of COX metabolites and LOX metabolites of NAEs have shown to possess biological activities (Vandevoorde and Lambert, 2007; Rouzer and Marnett, 2011). DHEA was oxidised by LOX in human PBMCs and mouse brain homogenates leading to the formation of several oxygenated molecules including, including 17-hydroxy-DHEA, 10,17-dihydroxy-DHEA and 15-hydroxy-16(17)-epoxy-DHEA (15-HEDPEA) (Yang *et al.* (2011). These authors also showed that some of these oxygenated metabolites possess biological activity, including effects on inflammatory processes, and may play important organ protecting roles.

Activities of DHEA and EPEA

Anti-inflammatory properties

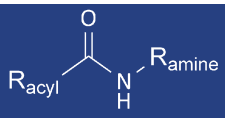
Several fatty amides, including (but not limited to) those binding to cannabinoid receptors, show anti-inflammatory or general immune modulating properties (Burstein and Zurier, 2009; Pandey *et al.*, 2009). Comparing a number of NAEs for their ability to inhibit NO release from stimulated RAW264.7 macrophages, we showed that DHEA was the most potent of the compound series tested, inducing a dose-dependent inhibition of NO release (Meijerink *et al.*, 2011). EPEA and DEA were also able to inhibit NO release, whereas anandamide and LNEA were inactive in this assay. Interestingly, the precursor of DHEA, DHA showed a much smaller effect. In the same cell line, DHEA significantly suppressed the production of the chemokine CCL2 (MCP-1) and in LPS-stimulated mouse peritoneal macrophages it reduced CCL2, IL-6 and NO production. The inhibition took place at a transcriptional level, as gene expression of CCL2 and inducible NOS was inhibited by DHEA. In differentiated 3T3-L1 adipocytes DHEA and EPEA reduced LPS-induced production of CCL2 and IL-6 (Balvers *et al.*, 2010). Both compounds were already effective at concentrations as low as 1 nM. Interestingly and similar to what has been shown for anandamide during inflammation, DHEA and EPEA tissue levels were found to increase after an inflammatory stimulus in mice fed fish oil (Balvers *et al.*, 2012b). This could indicate that these compounds have a role as endogenous anti-inflammatory mediators.

Other biological effects of DHEA and EPEA

Brown *et al.* (2010) suggested that DHEA and EPEA may possess potential anti-carcinogenic properties as the compounds displayed anti-proliferative and cell growth inhibitory effects in LNCaP and PC3 prostate cancer cells. NAEs had greater anti-proliferative potency than their parent compounds DHA and EPA. The inhibition resulted from an increased apoptosis and changes in cell cycle arrest. However, no consistent pattern was observed as specific effects exerted by the compounds differed between both *n*–3 NAEs and both cell lines. Hence, the mechanisms behind the anti-carcinogenic effects are still unclear. Increasing evidence suggests that DHEA and other DHA conjugates are important for brain development and the maintenance of brain functioning, and that they play roles in neuroprotection and the control of inflammation during disease or resulting from tissue damage. Like its parent compound DHA, DHEA and other conjugates, including that of dopamine are found in relatively high concentrations in brain (Sheskin *et al.*, 1997;

Table 1

General literature overview of *n*–3 LC-PUFA-derived fatty amides identified so far in different organisms and tissues and brief indication of their bioactivity and receptor affinity (if known)



R_{acyl}	R_{amine}	Selected references	**Presence shown in species (P)** **Receptor affinity studies (R)** **Bioactivity data available (B)**
DHA (22:6n-3)	Ethanolamine	(Sheskin *et al.*, 1997; Berger *et al.*, 2001; Artmann *et al.*, 2008; Balvers *et al.*, 2010; Wood *et al.*, 2010; Brown *et al.*, 2011; Meijerink *et al.*, 2011; Rapoport *et al.*, 2011; Tsuboi *et al.*, 2011)	**P**, **R**, **B** (see text)
	Dopamine	(Shashoua and Hesse, 1996; Bisogno *et al.*, 2000; Bezuglov *et al.*, 2001; Bobrov *et al.*, 2006; Ostroumova *et al.*, 2010; Dang *et al.*, 2011; Sakharova *et al.*, 2012)	**P**: rodent brain Fresh water hydra; see further text **B**: Hydra tissue development, mouse embryo development; uptake in mouse brain; antipyretic, analgesic, cataleptic in rats; FAAH inhibition; AEA uptake; inhibition of NO and cytokines; antioxidant and neuroprotective in rats, anti-Parkinson (see also text)
	Serotonin	(Verhoeckx *et al.*, 2011)	**P**: pig, mouse **B**: FAAH inhibition; GLP-1 release
	Glutamic acid and glutamine	(Tan *et al.*, 2010)	**P**: bovine brain
	GABA	(Tan *et al.*, 2010)	**P**: bovine brain
	Phenylalanine	(Tan *et al.*, 2010)	**P**: bovine brain
	Histidine	(Tan *et al.*, 2010)	**P**: bovine brain
EPA (20:5n-3)	Ethanolamine	(Berger *et al.*, 2001; Artmann *et al.*, 2008; Balvers *et al.*, 2010; 2012a,b; Wood *et al.*, 2010)	**P**,**R**,**B**, (see text)
	Dopamine	(Bisogno *et al.*, 2000; Bezuglov *et al.*, 2001)	**R**: CB_1 receptors **B**: antipyretic, analgesic, cataleptic in rats; FAAH inhibition; AEA uptake (see also text)
	Serotonin	(Verhoeckx *et al.*, 2011)	**P**: pig, mouse **B**: FAAH inhibition; GLP-1 release (see also text)
DPA (22:5n-3)	Ethanolamine	(Berger *et al.*, 2001)	**P**: pig brain
	Dopamine	(Bisogno *et al.*, 2000; Bezuglov *et al.*, 2001)	**R**: CB_1 receptors **B**: antipyretic, analgesic, cataleptic in rats
ALA (18:3n-3)	Ethanolamine	(Sheskin *et al.*, 1997; Movahed *et al.*, 2005; Meijerink *et al.*, 2011)	**R**: CB_1 receptors; no affinity; activates TRVP1 receptors **P**: rat mesenteric arteries **B**: No effect on NO and CCL2 release in RAW264.7 cells
	Serotonin	(Ortar *et al.*, 2007)	**R**: TRVP1 receptors **B**: FAAH inhibition
	Dopamine	(Bisogno *et al.*, 2000)	**R**: CB_1, CB_2 receptors **B**: FAAH inhibition; AEA uptake (see text)

Berger *et al.*, 2001; Tan *et al.*, 2010; Wood *et al.*, 2010). Although other several pathways are being proposed to explain the effects of DHA on brain (Bazan *et al.*, 2011), it is conceivable that mechanisms taking place via their amine conjugates will be involved here as well. For example, the presence of DHEA (called by the authors 'synaptamide') was demonstrated in mouse hippocampus and shown to be a potent stimulator of neurite growth and synaptogenesis in hippocampal neurons (Kim *et al.*, 2011a,b). Furthermore, it enhanced glutamatergic synaptic activity. Again, the bioactivities of DHEA were higher than those of the parent compound DHA. Yang *et al.* (2011) recently identified a series

of oxygenated metabolites from DHEA in mice brain that regulated leukocyte motility. The authors conclude that these metabolites might serve as anti-inflammatory and organ-protective mediators in brain.

A remarkable activity of EPEA with possible links to endocannabinoid function was reported for *Caenorhabditis elegans* (Lucanic *et al.*, 2011). This conjugate was not only present in the nematode but was also found to inhibit the typical dietary restriction induced lifespan extension (Lucanic *et al.*, 2011). The authors concluded that EPEA might have a role in ageing and represents a signal that coordinates nutrient status.

Are DHEA and EPEA members of the endocannabinoid family?

From their structural analogy to corresponding arachidonic acid *N*-conjugates, it is likely that there will be a number of candidate receptors to which different *n*–3 derived fatty amides may show affinity, including CB_1 and CB_2, GPR18, 55, 92, 119, TRVP1 and PPARs (Alexander and Kendall, 2007; Di Marzo *et al.*, 2007; de Novellis *et al.*, 2008). However, data on this are scattered, and the overall picture is not complete. Published data consistently suggest that DHEA and EPEA are relatively weak ligands for cannabinoid receptors. Binding affinity of DHEA to CB_1 receptors has been compared to anandamide in a number of studies (see Felder *et al.*, 1993; Sheskin *et al.*, 1997 for binding data). Low-affinity (compared with anandamide) binding of EPEA to CB_1 receptors has been shown (Adams *et al.*, 1995). More recently, Brown *et al.* (2010) reported values of 633 nM and 124 nM for binding of DHEA to mouse brain CB_1 receptors in the absence and presence of the FAAH inhibitor PMSF, respectively. For binding of EPEA to CB_1 receptors (in the presence of PMSF), slightly lower K_i values were found. The same authors also showed that DHEA and EPEA can bind to CB_2 receptors, although with a slightly lower affinities compared with those for CB_1 receptors. DHEA and EPEA behaved as CB_1 and CB_2 receptor agonists as indicated by their ability to produce a concentration related stimulation of [^{35}S]GTPγS binding to mouse brain and CHO-hCB_2 cell membranes. In both membrane preparations, DHEA displayed higher potency than EPEA. Using a commercially available human CB_2 receptor preparation (membranes from Sf9 cells; PerkinElmer, the Netherlands), we also found that DHEA binds to these receptors in the nanomolar range (K_i estimated 5.7 nM), with an approximately eight-fold lower affinity compared to Win55,212-2 (unpublished data).

Role of cannabinoid and PPAR receptors in biological effects of DHEA and EPEA

Although DHEA and EPEA have been shown to bind and activate CB_1 and CB_2 receptors, this has not been, so far, linked to their immune-modulating activities. In LPS-stimulated peritoneal macrophages collected from $CB_2^{-/-}$ mice, DHEA still produced a reduction of NO release, which would conflict with any involvement of the CB_2 receptor (Meijerink *et al.*, unpublished data). Similar studies in our lab with CB_1 receptor antagonists indicated that these receptors did not play a role either in this effect (unpublished data). In LPS-stimulated 3T3-L1 adipocytes, inhibition of IL-6 release by DHEA or EPEA could be blocked by a combination of a PPAR-γ (GW9662) and a CB_2 receptor antagonist (SR144528), while the individual antagonists showed much smaller effects (Balvers *et al.*, 2010). However, neither the combination nor the individual antagonists reversed the inhibitory effects of DHEA or EPEA on CCL2 release. These observations are in line with the studies of Brown *et al.* (2010), showing that the DHEA-mediated decrease in proliferation of their prostate cancer cell lines could not be blocked by CB_1 or CB_2 receptor antagonists. By contrast, the anti-proliferative potency of EPEA was reduced by AM281 and AM630, selective antagonists for CB_1 and CB_2 receptors respectively (Brown *et al.*, 2010). Finally, recent data on the effects of DHEA on neurite outgrowth and synaptogenesis in mice also show that these effects are apparently independent of interaction with CB receptors (Kim and Spector, 2012). Taken together, connections between biological effects of DHEA and EPEA found so far and receptor-specific interactions need to be analysed further.

Effects on COX-2

To further elucidate the underlying mechanism(s) of DHEA-exerted immune-modulatory activity, we studied its effects on different key inflammatory mediators. DHEA dose-dependently reduced levels of prostaglandins and thromboxane B_2 generated by COX-2 in LPS stimulated RAW264.7 macrophages. At low concentrations, DHEA caused a less pro-inflammatory secretory oxylipin profile in the activated macrophages, whereas its parent compound DHA did not change levels of metabolites formed by COX-2 in that concentration range. The activity of NF-κB and IFN-β, both important players of the MyD88-dependent and the MyD88-independent pathway, respectively, were not affected by DHEA (Meijerink *et al.*, unpublished data). As COX-2 protein expression was not altered these effects could be due to competition of DHEA or its oxygenated metabolites with arachidonic acid. Whether DHEA indeed acts as a substrate for COX-2, thereby generating active or non-active metabolites or whether it mainly exerts its effects by inducing a shift in pro-inflammatory mediators, remains to be investigated. Figure 4 shows a summarizing overview of the activities and putative mechanisms of DHEA and EPEA found so far.

Activities and possible molecular targets of *n*–3 LC-PUFA-derived fatty amides other than DHEA and EPEA

Dopamine- and other N-acyl-conjugates of DHA

Apart from DHEA, conjugates of DHA have been found with serotonin, dopamine, glycine, alanine, glutamine and glutamic acid, GABA, histidine and phenylalanine (Table 1). However, in most cases, information is limited to a demonstration of their existence, and even basic molecular properties have often not yet been established. As mentioned above, we found DHA-serotonin in the gut of mice fed a fish oil-rich diet. Studies on its biological effects and role are ongoing. Unlike its EPA analogue, DHA-serotonin did not inhibit

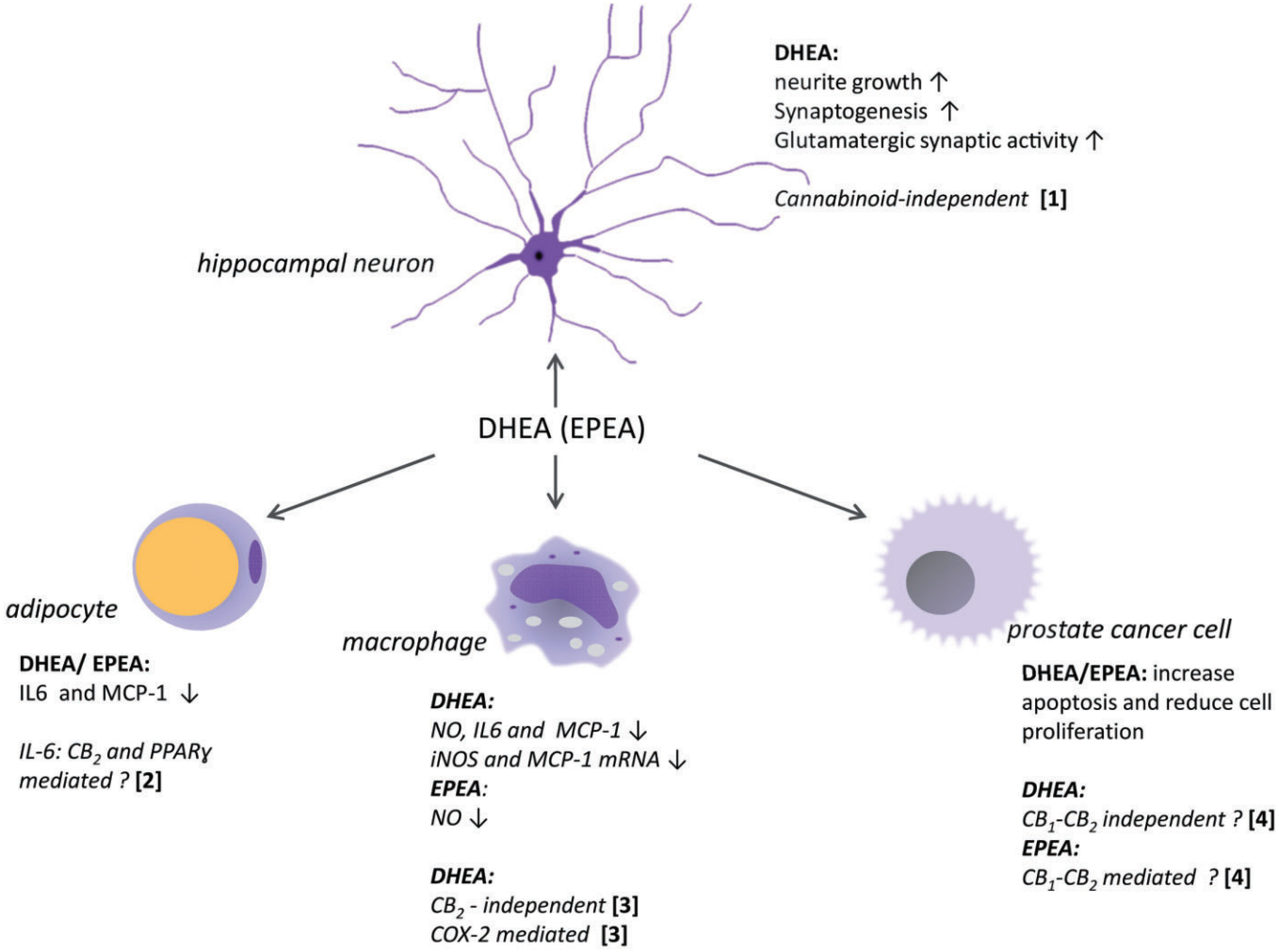


Figure 4

Summary of DHEA and EPEA effects on different cell types. (1): Kim and Spector (2012); (2): Balvers *et al.* (2010); (3): Meijerink *et al.* unpublished data; (4): Brown *et al.* (2010).

FAAH, and although it stimulated GLP-1 release *in vitro*, its potency was similar to that of its parent compound DHA (Verhoeckx *et al.*, 2011). More is known about the dopamine-conjugate of DHA. Like its analogues *N*-oleoyl dopamine (OLDA) and *N*-arachidonoyl dopamine (NADA) and also the ethanolamine conjugate DHEA (described above), this compound may be of interest because of its potential properties in relation to brain function and neuroprotection, including positive effects on hypoxic–ischaemic injury or brain inflammatory processes. This is in line with the observation that the parent DHA shows marked accumulation in the CNS, where it is a major component of brain synapses and retina and known to play important developmental roles (Rapoport *et al.*, 2011). Furthermore, DHA by itself is of great interest for its role in neuroprotection after brain hypoxia and ischaemia (Mayurasakorn *et al.*, 2011). *Vice versa*, high levels of dietary *n*–6 fatty acids contribute to reduced levels of DHA in the developing brain and inhibit secondary neurite growth (Novak *et al.*, 2008).

Nutritional *n*-3 (omega-3) deficiency also abolishes endocannabinoid-mediated neuronal functions (Lafourcade *et al.*, 2011). Testing a series of dopamine fatty acid conjugates, Bisogno *et al.* (2000) reported that DHA-dopamine is a better CB_1 receptor ligand than AEA tested under the same conditions. Synthesis of DHA-dopamine (and EPA-dopamine) and its further testing have also been reported by Bezuglov *et al.* (2001). The compound produced hypothermic, cataleptic and (some) analgesic effects as well as hypo-activity. An interesting application of DHA-dopamine was described by Shashoua and Hesse (1996) who studied the ability of different dopamine conjugates to act as carrier to increase brain dopamine content. Remarkably, and apparently in line with the tendency of DHA to accumulate in brain tissue, the DHA conjugate was the most active in increasing dopamine uptake by the brain. In addition, the conjugate depressed general locomotor activity of mice in a dose-dependent manner and suppressed the appetite of mice and rats. A similar concept was described by Yehuda (2002) who suggested that

N-(α-linolenoyl) tyrosine could potentially be used as an anti-Parkinson agent. Finally, Bobrov *et al*. (2006) showed that DHA-dopamine exhibited antioxidant activity and produced a dose-dependent protective effect on cultured granular cells from rat cerebellum under conditions of oxidative stress. It also decelerated the development of Parkinson's disease-like symptoms in a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model. At present, it is unclear whether this interesting application has been taken further since there appear to be no reports published since 2006.

Dopamine- and serotonin conjugates of EPA

Synthesis of EPA-dopamine has been reported by Bezuglov *et al*. (2001). The compound produced displacement of radioactive ligand from a CB_1 receptor preparation with a K_i of the same order of magnitude as that of NADA. As observed with DHA-dopamine (described above), the EPA analogue showed hypothermic, cataleptic and analgesic effects and also produced hypoactivity in rats. The analgesic effect, as measured with the hot plate test, was greater than that of DHA-dopamine and comparable with NADA. As *N*-arachidonoyl serotonin is a FAAH inhibitor (Maione *et al*., 2007; de Novellis *et al*., 2008), we investigated a series of other amides, including DHA-serotonin and EPA-serotonin for this effect (Verhoeckx *et al*., 2011)and found that EPA-serotonin, but not DHA-serotonin, was able to inhibit FAAH. However, we also found that some of the parent fatty acids, including AA and EPA had the same effect.

N-acyl conjugates of ALA

Data on the activity of amine conjugates from α-linolenic acid (18:3n-3) in animals appear to be scarce, and in some reports, the structure seems to be confused with that of its γ-isomer, which is an *n*–6 fatty acid. The ethanolamine conjugate (αLNEA) was found in rat mesenteric arteries and dorsal root ganglia, and shown to activate TRVP1 receptors (Movahed *et al*., 2005). In our laboratory, the compound was not active in inhibiting NO and CCL2 release from LPS-activated RAW264.7 macrophages (Meijerink *et al*., 2011). The serotonin conjugate of ALA was synthesized and further tested by Ortar *et al*. (2007). This compound also showed TRVP1 activity and furthermore inhibition of FAAH. Finally, and as described in a previous section, *N*-(α-linolenoyl) tyrosine may have potential in Parkinson's disease (Yehuda, 2002). Although it is possible that this molecule can be formed endogenously, to our knowledge this has not been demonstrated so far.

Conclusions and future perspectives

N-acylamines of DHA and other *n*–3 polyunsatured fatty acids are members of a large group of endogenous mediators of which the full biological significance remains to be established. Their formation is time- and tissue-specific and modulated by various endogenous (such as energy status or inflammation) and environmental factors, including diet. From a physiological perspective, it is important to realise that these molecules occur in fluctuating mixtures of structurally related molecules with pleiotropic and tissue-specific activities. To make it even more complicated, there is a constant interplay with other biochemical routes, including the formation of eicosanoids and different intermediates (LOX, COX, CYP450 products) (Balvers *et al*., 2012a; 2012b). Therefore, especially when studying their physiological roles, lipidomic and multi-target approaches are needed to fully comprehend their pathways and effects.

Given these complicating factors, evidence is accumulating that DHEA, DHA-dopamine and other *n–3* LC-PUFA-derived fatty amides possess several interesting properties that merit further studies in relation to for example inflammatory and neural disorders. In the brain, DHEA is present at levels comparable with those of AEA. Although its affinity for CB_1 receptors is lower than that of AEA, recent studies suggest that the compound or its metabolites do play important roles in normal brain functioning and modulation of inflammatory processes. Notwithstanding the association between dietary intake of *n*–3 LC-PUFAs and the formation of their respective fatty acid amides, including DHEA, their role and significance in mediating the alleged health effects of fish oil remains speculative. In this respect, fatty acid amides represent a group of molecules of interest to both the pharmacological and nutritional research fields. In addition to directly administering the compounds, the ability to use DHA conjugates as carriers of neurotransmitters through the blood–brain barrier also merits further investigation. Not only for dopamine as suggested before (Shashoua and Hesse, 1996) but perhaps also for serotonin. The fact that fatty acid amides are a part of endogenous pathways could be advantageous for pharmacological applications. At the same time, this may have consequences for their metabolic stability. Therefore, in future pharmacological studies, it is recommended to pay particular attention to their pharmacokinetic properties. In addition to their administration as single compounds, combinations with FAAH inhibitors (Pillarisetti *et al*., 2009) and (or) their fatty acid precursors could be of interest to further increase (local) concentrations.

Conflict of interest

The authors declare that they have no conflicts of interest.

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