n-3 polyunsaturated *N*-acylethanolamines are CB_2 cannabinoid receptor-preferring endocannabinoids

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Abstract

Anandamide, the first identified endogenous cannabinoid and TRPV1 agonist, is one of a series of endogenous *N*-acylethanolamines, NAEs. We have generated novel assays to quantify the levels of multiple NAEs in biological tissues and their rates of hydrolysis through fatty acid amide hydrolase. This range of NAEs was also tested in rapid response assays of CB_1, CB_2 cannabinoid and TRPV1 receptors. The data indicate that PEA, SEA and OEA are not endocannabinoids or endovanilloids, and that the higher endogenous levels of these metabolites compared to polyunsaturated analogues are a correlate of their slow rates of hydrolysis. The n-6 NAEs (AEA, docosatetraenoyl and docosapentaenoyl derivatives) activated both CB_1 and CB_2 receptors, as well as TRPV1 channels, suggesting them to be 'genuine' endocannabinoids and 'endovanilloids'. The n-3 NAEs (eicosapentaenoyl, docosapentaenoyl and docosahexaenoyl derivatives) activated CB_2 receptors and some n-3 NAEs (docosapentaenoyl and docosahexaenoyl derivatives) also activated TRPV1 channels, but failed to activate the CB_1 receptor. We hypothesise that the preferential activation of CB_2 receptors by n-3 PUFA NAEs contributes, at least in some part, to their broad anti-inflammatory profile.

Abbreviations

AEA, *N*-arachidoylethanolamine, C20:4, n-6

ALEA, α-linolenoylethanolamine, C18:3

DHEA, *N*-docosahexaenoylethanolamine, C22:6

DPEA, *N*-docosapentaenoylethanolamine, C22:5

EPEA, *N*-eicosapentaenoylethanolamine, C20:5

FAAH, Fatty acid amide hydrolase

LEA, *N*-linoleoylethanolamine, C18:2, n-6

LNEA, *N*-linolenoylethanolamine, C18:3, n-3

NAE, *N*-acylethanolamine

NAPE, *N*-acylphosphatidylethanolamine

NEA, nervonoylethanolamine, *N*-tetracosaenoylethanolamine, C24:1

OEA, *N*-oleoylethanolamine, C18:1, n-9

PEA, *N*-palmitoylethanolamine, C16:0

SEA, *N*-stearoylethanolamine, C18:0

Author contributions

Initial design and planning of the study was conducted by DAB, AJB, VC and SPHA; execution of the experimental was conducted by NA, PC, WH and CO; all the authors contributed to analysis of the data; SPHA wrote the initial draft and all the authors commented and approved the manuscript.

Acknowledgements

We thank Prince Sattam bin Abdulaziz University, the Kingdom of Saudi Arabia; the Biotechnology and Biological Sciences Research Council and Philadelphia University, Jordan for providing funding for Nahed Alharthi, Pete Christensen and Wafa Hourani, respectively, to complete their PhD studies. We are grateful to Dr Michael Garle for assistance with the enzyme assays.

1. Introduction

Anandamide (*N*-arachidonoylethanolamine; AEA; C20:4, n-6) is a polyunsaturated fatty acid *N*-acylethanolamine (PUFA-NAE), which was identified as the first endogenous cannabinoid following isolation from pig brain in 1992 [1]. Two additional n-6 PUFA-NAEs were isolated from pig brain and found to bind to CB_1 cannabinoid receptors: *N*-dihomo-γ-linolenoylethanolamine (C20:3, n-6) and *N*-docosatetraenoylethanolamine (C22:4, n-6) [2]. Since then, two further PUFA-NAEs with n-3 fatty acid sidechains, *N*-eicosapentaenoylethanolamine (EPEA, C22:5, n-3) and *N*-docosahexaenoylethanolamine (DHEA, C22:6, n-3) were also identified in pig brain [3] and found to be influenced by dietary fatty acid composition. These NAEs are members of an extended family of endogenous lipid derivatives, which also include saturated (such as *N*-palmitoylethanolamine, PEA, C16:0), monounsaturated (such as *N*-oleoylethanolamine, OEA, C18:1, n-9) and diunsaturated (such as *N*-linoleoylethanolamide, LEA, C18:2, n-6) analogues. While the PUFA-NAEs, including particular n-3 analogues (e.g. DHEA and EPEA) and n-6 analogues (AEA, C20:3, n-6 and C22:4, n-6), have been shown to bind to CB_1 cannabinoid receptors [1, 2, 4], PEA, OEA and LEA have been reported only to bind at concentrations higher than 10^{-4} M [4, 5]. The saturated, monounsaturated and diunsaturated NAEs are also poor binding ligands at CB_2 cannabinoid receptors [4, 5], but with the exception of AEA, PUFA-NAEs have not been investigated.

AEA is not only an endogenous ligand at GPCR cannabinoid receptors, but is also able to activate the TRPV1 receptor [6]. The TRPV1 receptor is sensitive to noxious heat, protons and dietary components, such as capsaicin, resulting in the gating of cations, particularly calcium, at the cell surface (for review, see [7, 8]). Responses of the TRPV1 receptor to NAEs other than AEA have not been investigated in depth. While PEA appears inactive at concentrations up to 30 μM [6], OEA was able to activate the rat recombinant TRPV1 receptor, but only in the presence of protons or phorbol esters [9]. SEA (and other shorter chain saturated NAEs) were unable to activate the TRPV1 receptor directly, but appeared to enhance the effects of AEA through an unclear mechanism [10]. In an investigation of human recombinant TRPV1, calcium ion elevation was enhanced by C18 NAEs, where a modest structure-activity relationship was identified [11]. While the unsaturated analogue, SEA was considered inactive, OEA (C18:1, n-9), LEA (C18:2, n-6) and LNEA (*N*-linolenoylethanolamine, C18:3, n-3) produced responses similar in profile to AEA, although OEA appeared slightly less effective [11]. In addition, a further n-6 PUFA NAE was tested, *N*-docosatetraenoylethanolamine (DTEA, C22:4, n-6), which was slightly less effective than AEA [11]. In an independent study using human recombinant TRPV1 receptors, SEA, OEA, LEA and PEA at 10 μM were either ineffective or evoked responses of ~10 % of those evoked by AEA and DHEA, which were equieffective [12].

The NAEs appear to be synthesised from membrane phospholipid-associated precursors termed NAPEs (*N*-acylphosphatidylethanolamines), predominantly by a form of phospholipase D termed NAPE-PLD (for review, see [13]). The NAEs are hydrolysed primarily by an enzyme termed fatty acid amide hydrolase (FAAH), but may also be transformed through other routes *in vitro*, although the relevance of these latter pathways is unclear (for review, see [14]). One of the potential influences on the spectrum of NAEs in biological samples appears to be the precursor fatty acids. Thus, it has been observed that changing the diet can lead to changes in the levels of NAEs in CNS and other tissues [15-17]. Given the reported beneficial effects of dietary supplementation with n-3 PUFAs (for reviews, see [18, 19]), we were prompted to identify whether we could find evidence for distinct roles of n-3

and n-6 PUFA NAEs, as well as saturated and monounsaturated NAEs, at their most well-established molecular targets, CB_1, CB_2 and TRPV1 receptors.

In this study, therefore, we have developed a mass spectrometry survey method to investigate the levels of NAEs in liver and midbrain. We have examined this spectrum of NAEs as agonists at CB_1, CB_2 and TRPV1 receptors, and their metabolism through FAAH. The differential activities at the receptors suggests a potential differential contribution of n-3 and n-6 PUFA NAEs to physiological and pathological signalling. Our results suggest that n-3 fatty acid derivatives may provide benefit by activating CB_2 receptors with limited activity at CB_1 and TRPV1 receptors. Furthermore, given the influence that dietary lipid composition is reported to have on PUFA-NAE levels [15, 16], our results imply that altering PUFA intake can change the balance of endocannabinoid tone to emphasise CB_2 receptor activation *in vivo*.

2. Methods

2.1 N-Acylethanolamine analysis by LC-MS/MS

Tissues and organs were collected from Sprague-Dawley rats (Charles River, UK). The animals were stunned by a blow to the head followed rapidly by decapitation, typically within 3–4 sec. Tissues were rapidly dissected, collected and frozen immediately on dry ice. All samples were subsequently stored at -80 ^{o}C until required. Tissues (100-150 mg for midbrain and 750-800 mg for liver) were extracted by a previously published method [20] with the exception that the solid phase extraction stage was not used. Briefly, tissue was homogenized in ethyl acetate:hexane (9:1, v/v) followed by centrifugation, removal and evaporation of supernatant. The final dried extract was redissolved in mobile phase A before analysis by LC-MS-MS.

All survey scans and MS/MS experiments were conducted on a 4000 QTRAP hybrid triple-quadrupole–linear ion trap mass spectrometer (Applied Biosystems, Manchester, UK) equipped with a TurboIon source used in positive ion electrospray mode. A Windows XP (Microsoft, Redmond, WA, USA) workstation running Analyst (version 1.4.1) was used for data acquisition and processing. Accurate mass measurements were conducted on a Waters QToF Premier (Waters, UK) and controlled by Windows XP workstation running MassLynx (version 1.4).

Chromatography was carried out on a Shimadzu series 10AD VP liquid chromatography system equipped with binary pumps, a vacuum degasser and an SILHTc autosampler and column oven (Shimadzu, Columbia, MD, USA). The LC column was a Thermo Hypersil–Keystone HyPurity Advance column (100 x 2.1 mm i.d., 3 µm particle size), with a mobile phase flow rate of 0.3 ml/min. Gradient elution mobile phases consisted of A (water, 1 g/L ammonium acetate, and 0.1 % formic acid) and B (acetonitrile, 1 g/L ammonium acetate, and 0.1% formic acid) at pH 3.6. The gradient started at 45 % B, increasing to 55 % after 2 min and then increasing again to 65 % at 6.5 min; this was maintained until 9 min, with subsequent re-equilibration at 45 % B for 6 min. Column temperature was maintained at 40 °C and sample temperature was maintained at 4 °C in the autosampler during analysis.

An internal standard (AEA-d8) was used with calibration standards of AEA, OEA, PEA, docosatetraenoyl (C22:4), dihomo-γ-linolenoyl (C20:3) ethanolamines to aid with identification and relative quantitation of NAEs. Precursor ion (*m/z* 62) and neutral loss (*m/z* 90) scans were used to identify NAEs, in conjunction with accurate mass determinations, analysis of authentic reference standards and database searches (Lipid Maps, www.lipidmaps.org).

2.2 Hydrolysis of NAEs by rat liver membranes: a novel ethanolamine detection method

Rat liver particulate fractions were prepared essentially as previously described [21]. Fresh liver was dissected from Wistar rats (6-9 weeks old and 150-250 g weight) killed by cervical dislocation and exsanguination. After washing in 0.05 M potassium phosphate buffer, pH 7.4, tissue was roughly chopped with scissors in homogenising buffer and then homogenized (1:10 w/v) using a Polytron homogeniser. Following centrifugation at 10 000 g for 20 min at 4 °C, the pellet containing the nucleus and un-homogenised material was discarded and the supernatant layer was re-centrifuged at 30 000 g for 60 min at 4 °C. The membrane pellet was resuspended in 2 volumes of original wet weight using the homogenising buffer and stored until required at -80 °C. The protein concentration was determined by the Lowry protein assay [22].

A two-step analysis of rat liver membrane NAE hydrolysis was conducted separated into a conventional hydrolysis step and a detection step. For NAE hydrolysis, rat liver membranes (10 µg/mL) were incubated in the presence of 1 mg/mL BSA in 0.1 M phosphate buffer, pH 7.4. After 30 min incubation at 37 °C in the presence of each of the 14 individual NAEs, reactions were terminated with the addition of trichloroacetic acid to a final concentration of 3 % (w/v) before centrifugation to clear the precipitated protein at 13 000 g for 10 min. An aliquot of the supernatant layer was diluted 1:3 with a solution of naphthalene-2,3-dicarbaldehyde (dissolved in acetonitrile to 1 mg/mL and then diluted 1:5 in 0.2 M borate buffer, pH 11.0). The fluorescent product was quantified at ex 420 nm/em 480 nm using a standard curve of ethanolamine.

PEA, SEA and OEA were synthesized in the School of Chemistry (by SPHA), while AEA was obtained from Tocris Cookson (Bristol, UK). All other NAEs were from Cayman Chemicals (Cambridge Bioscience, Cambridge, UK).

2.3 Action of NAEs at CB_1 and CB_2 receptors: ERK and $[Ca^{2+}]i$ elevations

Human recombinant CB_1 and CB_2 cannabinoid receptors expressed stably in Chinese hamster ovary cells were grown in DMEM/F12 supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 0.4 or 0.5 (CB_1 or CB_2 cultures, respectively) mg/mL G418. ERK phosphorylation was quantified using an “in-cell Western” protocol previously described [23]. In brief, cells were seeded at a density of 40 000 cells/well and assessed 24 h later, including a period of 6 h of serum starvation.

Intracellular calcium ion levels were quantified using a FlexStation [24]. Positive controls for ERK activation and intracellular calcium elevation were 10 % FCS and 10 µM ATP, respectively.

AM251 and JWH015 were from Ascent (Cambridge, UK) and Tocris (Avonmouth, UK), respectively, while HU210 and ATP were from Sigma (Poole, UK).

2.4 Action of NAEs at TRPV1 receptors: $[Ca^{2+}]i$ elevations

Imaging of intracellular calcium ion levels was conducted in adult rat acutely dissociated dorsal root ganglion cells [25]. Cells on coverslips were exposed to agonists for a two min period with a minimal time interval between agonist exposures of 45 min. A typical protocol involving sequential exposure to an NAE at 10 µM, followed by the vehicle (0.1 % ethanol or DMSO) and then 10 µM capsaicin to focus on TRPV1-expressing cells.

Capsaicin and capsazepine were from Sigma (Poole, UK).

2.5 Data and statistical analysis

ERK phosphorylation responses in transfected Chinese hamster ovary cells in response to administration of the 14 NAEs individually were assessed initially as ratios of pERK and ERK immunoreactivity, using the dual

channel scanning facility of the Licor Odyssey system, having subtracted background levels (wells with cells processed identically except for the omission of incubation with primary antibodies). Calcium responses in transfected Chinese hamster ovary cells were assessed using a FlexStation as the peak response following agonist exposure, having subtracted the mean baseline response prior to agonist addition. Responses to the 14 individual NAE ligands for both ERK activation and calcium elevation were compared to their vehicle controls using repeated measures ANOVA and Dunnett's multiple comparison test.

In the rat DRG neurones, responses to NAEs were normalised to the responses evoked by 10 µM capsaicin in that individual cell (cell-matched controls) for graphical representation (Figure 4). Untransformed responses to NAEs and capsaicin from multiple experiments were compared to vehicle controls in the same cells by 2-way ANOVA with post-hoc Dunnett's multiple comparison.

3. Results

3.1 N-Acylethanolamine analysis by LC-MS/MS

Using the survey scanning methodology (Figure 1), a differential pattern of expression was observed when comparing rat liver and midbrain. Levels of PEA (C16:0), *N*-octadecadienoylethanolamine (C18:2) and EPEA (C22:5) appeared similar in the two tissues, while midbrain levels of *N*-eicosatetraenoylethanolamine (C20:4) appeared modestly increased relative to liver. All the other NAEs that exceeded the lower limit of detection were much higher in the midbrain compared to liver: SEA (C18:0), *N*-octadecenoylethanolamine (C18:1), *N*-eicosanoylethanolamine (C20:0), *N*-eicosaenoylethanolamine (C20:1), *N*-docosaenoylethanolamine (C22:1), *N*-docosatetraenoylethanolamine (C22:4) and *N*-tetracosaenoylethanolamine (24:1).

<< Figure 1 here >>

3.2 Hydrolysis of NAEs by rat liver membranes

Although a number of NAEs have been examined for their ability to interfere with the FAAH-mediated hydrolysis of [^{3}H]-AEA [26], this approach fails to differentiate whether the NAEs are acting as inhibitors of [^{3}H]-AEA hydrolysis or substrates for the same enzyme. Therefore, to identify whether any of the NAEs identified in the survey scanning LC-MS/MS were substrates for FAAH activity, we generated a novel assay (Alharthi et al., in preparation). This assay allows quantification of ethanolamine derived from hydrolysis of any NAE, thereby permitting estimating substrate affinity and maximal velocity.

A further consideration for analysis of the NAEs identified in the biological samples was that the survey scanning methodology allows identification of the mass of the fatty acyl sidechain, but fails to identify the potential location of unsaturations in the fatty acyl sidechain. Commercial sources allowed the testing of 14 different NAEs (Table 1). Of the NAEs identified in the survey scan, only *N*-eicosaenoylethanolamine (C20:1) was not readily commercially available. For *N*-docosapentaenoylethanolamine (C22:5), both n-3 and n-6 analogues were available.

In the novel assay, hydrolysis of all 14 NAEs could be detected using rat liver particulate preparations as a source of FAAH activity. Using a range of substrate concentrations allowed analysis of Michaelis-Menten kinetics. Substrate affinities divided into high affinity (K_m values of 2-7 µM) and low affinity (K_m values of 12-18 µM) clusters (Table 1). The high affinity cluster included all the NAEs with two or more unsaturations, while the low

affinity cluster included all the saturated or monounsaturated NAEs. One exception to this was *N*-docosaenoylethanolamine (C22:1, n-9), which exhibited high affinity despite being a monounsaturate. Similarly, two clusters of NAEs could be identified based on the maximal velocities of hydrolysis (V_{max} values). However, while the low affinity cluster also exhibited low maximal rates of hydrolysis, not all the high affinity substrates showed high maximal rates of hydrolysis. Notably, the n-6 version of *N*-docosapentaenoylethanolamine (C22:5, n-6) and *N*-docosahexaenoylethanolamine (C22:6, n-3).

<< Table 1 here >>

The involvement of FAAH as the hydrolytic enzyme responsible for the generation of ethanolamine was confirmed through the use of URB597. This FAAH-selective inhibitor [27] showed high potency and complete inhibition of the hydrolysis of multiple NAEs (Table 2).

<< Table 2 here >>

3.3 Action of NAEs at CB_1 and CB_2 receptors: ERK and $[Ca^{2+}]i$ elevations

The potential for NAE activation of cannabinoid receptors was assessed using recombinant CB_1 and CB_2 cannabinoid receptors expressed in Chinese hamster ovary cells. In order to reduce any influence of ligand metabolism (see above, Section 3.2), two rapid assay styles were chosen with a timeframe of $\leq$5 min. Thus, elevation of intracellular calcium was compared to responses to 10 µM ATP as a positive control (Figure 2).

In CHO cells expressing the human recombinant CB_1 cannabinoid receptor, the synthetic cannabinoid HU210 produced a response which was approximately 10 % of the peak response to 10 µM ATP (Figure 2A). The majority of NAEs failed to evoke a significant elevation of intracellular calcium compared to the vehicle controls (Figure 2A). Notably, the NAEs which were able to evoke a significant calcium elevation in CB_1-expressing CHO cells were all n-6 PUFA derivatives (C20:4, n-6; C22:4, n-6 and C22:5, n-6). These responses were slightly higher than peak calcium responses to HU210 (Figure 2A). The responses to PUFA-NAEs were blocked in the presence of the CB_1 receptor antagonist AM251 or following pre-incubation with pertussis toxin (data not shown).

Similarly, using CHO cells expressing human recombinant CB_2 cannabinoid receptors, elevation of intracellular calcium was compared to responses to 10 µM ATP (Figure 2B). The CB_2 receptor-selective agonist JWH015 was able to evoke a relatively large peak of calcium levels, to about 60 % of the ATP peak response. A distinct pattern of activation in response to the range of NAEs was observed when compared to the results using CB_1 receptors (Figure 2A). Thus, although monounsaturated and saturated NAEs were also ineffective as CB_2 receptor agonists, all the n-3 and n-6 PUFA NAEs evoked significant calcium ion elevations in these cells (Figure 2B). The magnitude of these responses were slightly less than the peak calcium responses to JWH015 (Figure 2B). The responses to PUFA-NAEs were blocked in the presence of the CB_2 receptor antagonist AM630 or following pre-incubation with pertussis toxin (data not shown).

<< Figure 2 here >>

A second assay style was investigated, which allowed rapid measurement of agonist-evoked responses and which should minimise the influence of ligand metabolism (see Section 3.2). Using a whole cell ERK activation assay and a 5 min timepoint (the peak of ERK activation, data not shown), the profile of activation of cannabinoid receptors by NAEs was investigated (Figure 3). HU210 evoked a large increase in ERK phosphorylation in CHO cells expressing CB_1 receptors to approximately four-fold vehicle levels (Figure 3A). In these cells, saturated and monounsaturated NAEs were ineffective in eliciting ERK phosphorylation. As with the calcium elevation assays,

the n-6 PUFA NAEs all evoked significant increases in ERK phosphorylation, while the n-3 analogues failed to increase ERK activation significantly (Figure 3A). ERK phosphorylation in the presence of the n-6 PUFA NAEs was slightly less than that evoked by HU210 – about three-fold (Figure 3A). These responses were blocked in the presence of the CB_1-selective antagonist AM251 or following preincubation with pertussis toxin (data not shown).

Examining ERK activation in CB_2 receptor-expressing CHO cells identified that JWH015 was able to produce a significant response, to about 350 % of basal levels (Figure 3B). As with the intracellular calcium assay, saturated and monounsaturated NAEs were ineffective, while the n-3 and n-6 PUFA NAEs were able to enhance ERK activation (Figure 3B).

<< Figure 3 here >>

3.4 Action of NAEs at TRPV1 receptors: $[Ca^{2+}]i$ elevations

Using acutely dissociated cells from adult rat dorsal root ganglia, the 14 NAEs were investigated as stimuli of intracellular calcium ion levels. The protocol allowed matching of responses to NAEs to the archetypal TRPV1 agonist capsaicin in the same cell (Figure 4). Saturated or mono-unsaturated NAEs failed to elicit significant calcium elevations in capsaicin-responsive DRG neurones. However, the n-3 and n-6 PUFA NAEs all evoked significant calcium responses in these cells, where the responses to the n-6 analogues were similar to the size of responses evoked by capsaicin (Figure 4). Responses to EPEA (C22:5, n-3) were also similar to the capsaicin responses, while the other n-3 analogues appeared to generate smaller responses. The elevation of intracellular calcium ion levels by AEA, DTEA, and DPEA was completely blocked by the TRPV1 receptor antagonist capsazepine (10 µM, data not shown).

<< Figure 4 here >>

4. Discussion

In this report, we have examined the levels of multiple NAE species and their actions at CB_1, CB_2 and TRPV1 receptors. It is apparent that n-3 PUFA NAEs are less effective at CB_1 cannabinoid and TRPV1 receptors than n-6 PUFA NAEs, while they appear equally effective as agonists at CB_2 cannabinoid receptors. It is possible that this differential influence at these three molecular targets of PUFA NAEs contributes to the long-term beneficial effects of dietary n-3 PUFAs.

4.1 Saturated and monounsaturated NAEs

Using assays of rapid receptor activation ($\leq$5 min), the saturated NAEs (PEA, SEA and *N*-arachidylethanolamine) failed to activate CB_1, CB_2 or TRPV1 receptors (Figures 2, 3 and 4). This lack of activity of PEA and SEA is consistent with the literature. PEA was reported not to bind to CB_1 receptors [28], and although PEA was initially suggested to have functional activity at CB_2 receptors [29], a number of publications since have identified that PEA fails to bind or activate CB_2 receptors [30-32]. SEA failed to bind to rat CB_1 or CB_2 receptors [33], and it has also been reported to be inactive at rat and human TRPV1 channels [11]. We have been unable to find information on the pharmacological properties of *N*-arachidylethanolamine (C20:0) beyond a lack of effect *in vivo* on catalepsy, hypothermia or sleeping time in mice at doses where AEA altered all three [34].

Of the monounsaturated NAEs tested in the current study, OEA (C18:1, n-9), C22:1, n-9 and C24:1, n-9, all three were without effect at CB_1 or CB_2 cannabinoid receptors and failed to activate the TRPV1 receptor (Figure2,

3 and 4). There is an abundance of literature on OEA, which has actions at an orphan receptor GPR119 [35] and the nuclear hormone receptor PPARα [36]. OEA was reported to have low binding affinity (2-3 μM) at rat CB_1 or mouse CB_2 receptors [5] and human CB_1 or CB_2 receptors [37]. It has been reported to function as a TRPV1 agonist, although the human receptor appears more responsive than the rat version [9, 11]. The docosa and tetracosa monounsaturated analogues, the ethanolamides of erucic (C22:1, n-9) and nervonic acid (24:1, n-9), have previously been described in biological samples [38, 39] but otherwise have received very little pharmacological attention.

Although it appears that these saturated and monounsaturated NAEs exhibit modest or no activity at CB_1, CB_2 or TRPV1 receptors, their levels are relatively high in both liver and midbrain (Figure 1). These levels are a consequence of flux through formation and transformation pathways of the distinct NAEs. While the synthetic pathway/s are more difficult to assay, the predominant hydrolytic enzyme, FAAH, has been investigated through a variety of different assays (for examples, see [21, 40, 41]). Some of these assays rely on synthetic substrates which generate spectrophotometrically-measurable products [41], while others use radiolabelled versions of a natural substrate [40]. While this latter assay has become a 'Gold Standard' for measurement of *ex vivo* enzyme activities, it lacks versatility in identifying kinetic characteristics of alternative endogenous substrates; that is, an alternative substrate cannot be distinguished from an inhibitor. For this reason, we developed a more flexible assay based on determination of the ethanolamine product of NAE hydrolysis (Alharthi, in preparation, describes the assay characteristics in more detail). Application of this assay to the saturated and monounsaturated NAEs, with rat liver particulate preparations as a source of FAAH activity, identified that these were all substrates for the enzyme with measurable kinetic parameters (Table 1). However, comparison of these saturated and monounsaturated NAE substrates with the PUFA NAEs shows that they are relatively poor FAAH substrates with higher K_m values and lower V_{max} values (Table 1). The enzymatic synthesis and hydrolysis of the precursors of the NAEs, *N*-acylphosphatidylethanolamines, has been suggested to have little selectivity for acyl groups (for review, see [42]). An implication of the distinct rates of FAAH-mediated catabolism (Table 1) is that, assuming the synthesis of all these NAEs have similar rates, the slow rates of hydrolysis by FAAH mean that these saturated/monounsaturated NAEs will accumulate to greater levels than the PUFA NAEs, which is indeed the case (Figure 1).

4.2 n-3 vs n-6 PUFA NAEs

We chose to focus on two rapid responses to CB and TRPV1 receptors as this is likely to reduce the potentially confounding influence of metabolism of the NAEs. Using the two different downstream signalling pathways of CB_1 and CB_2 receptor activation (Figures 1 and 2), there are clear distinctions between the n-3 and the n-6 PUFA NAEs. AEA, C22:4, n-6 and C22:5, n-6 NAEs elicit responses in both ERK and calcium ion assays of CB_1 and CB_2 receptor function (Figures 1 and 2) indicating that all three compounds are 'genuine' endocannabinoids. They are also effective as agonists in the TRPV1 assay and so might also be termed 'endovanilloids' (Figure 4).

However, the profile of the n-3 PUFA NAEs is more complex. EPEA, C22:5, n-3 and DHEA all activate CB_2 receptors in both ERK and calcium ion assays (Figures 1 and 2). In contrast, the activity of these three n-3 PUFA NAEs at CB_1 receptors was not different from the background levels (Figures 1 and 2). These data suggest that these ligands are endocannabinoids with a CB_2 preference. As potential 'endovanilloids', EPEA, C22:5, n-3 and

DHEA elicited activation of the rat TRPV1 receptor, although C22:5, n-3 appeared more effective than EPEA and DHEA (Figure 4).

Relatively few studies have examined PUFA-NAE stimulation of rat TRPV1. In comparing AEA responses in rat DRG neurones and rat recombinant TRPV1 channels expressed in HEK293 cells, responses with the recombinant system were more potent [43]. The rat DRG neurones were near confluent populations assessed for calcium responses using a FLIPR, where the response to 30 µM AEA was reported to be 63 % of the responses to capsaicin [43]. Using a $^{45}Ca^{2+}$ uptake assay, which was conducted over 20 min (compared to the calcium assays of ~2 min), 10 µM AEA responses were less than 20 % of the magnitude of the capsaicin response, but could be amplified to equivalent levels to the capsaicin response in the presence of PMSF, an inhibitor of FAAH activity [44]. Responses to C22:4, n-6 and C18:2, n-6 NAEs were only measured in the presence of PMSF and were ~95 % and ~80 % of the capsaicin response, respectively. It is likely that FAAH activity is more of an influence in the more dense cellular preparations and so the apparently equivalent response of AEA compared to capsaicin observed in the present report may reflect a reduced metabolism of NAEs. If responses to capsaicin across all the preparations (0.476 ± 0.044 ΔRFU) are compared to responses to AEA (0.298 ± 0.026 ΔRFU) and expressed as a percentage, the numerical value obtained (63 %) is identical to the estimate identified using the human TRPV1 in recombinant expression and measuring population calcium levels [43].

Anandamide has been identified to act at multiple TRP channels in recombinant expression [45], which would also be expected to be present in the rat DRG neurones investigated in the current study, albeit at levels lower than the TRPV1 channels. AEA has been reported to inhibit TRPM8 channel function and to activate TRPA1 channels but with 3-fold lower potency than at TRPV1 channels [45]. Thus, it is possible that responses to PUFA NAEs observed in the rat DRG neurones are not limited to TRPV1 channels, although the effects of capsazepine point to a predominant role for TRPV1 channels.

In the assay of rat FAAH-mediated hydrolysis, all the PUFA NAEs exhibited higher affinity compared to the saturated/monounsaturated NAEs (Table 1). The majority of the PUFA NAEs showed high maximal rates of hydrolysis similar to AEA, with the exception of DHEA and C22:5, n-6 (V_{max} values approximately one third of that to AEA, Table 1). An implication of this is that (assuming similar rates of production) steady state levels of PUFA NAEs will be low. In the main, this is borne out by the spectrum scanning method (Figure 1). Levels of C20:4 (presumably AEA), C22:4 and C22:5 were all below 5 pmol/g in both liver and midbrain, while C20:5 and C22:6 in these samples were below the limits of detection. In contrast, PEA was measured at 70-85 pmol/g in these tissues (Figure 1); more than ten times the levels of the PUFA NAEs. One exception to the prediction is LEA, which showed high affinity and maximal rates of hydrolysis by FAAH (Table 1), but was present at approximately 15 pmol/g levels in the two tissues, more than five-fold the AEA levels (Figure 1). One possible explanation is that FAAH-mediated hydrolysis, while important in determining the levels of NAEs, may not be the only route of metabolism of PUFA NAEs. Oxidative metabolism of PUFA NAEs through cyclooxygenase-2, lipoxygenases, or epoxygenases [14] may show some preference for the tetraenoyl and pentaenoyl NAEs compared to LEA as a dienoyl NAE derivative.

4.3 Implications of supplementing levels of n-3 NAEs

CB_1 cannabinoid receptors have been implicated in adverse effects of diet, such that the CB_1 antagonist rimonabant was licensed in Europe (albeit for less than two years) for the indication of metabolic disorder and

type 2 diabetes (for reviews, see [46, 47]). CB_1 receptor activation is associated with increased feeding behaviour, particularly of palatable food (for review, see [48]). An increased level of n-3 compared to n-6 PUFA NAEs would, therefore, provide a reduced tone through CB_1 receptors, which would be expected to result in anorexia and a reduced potential for metabolic disorder. At the same time, an increased CB_2 receptor activation would provide a continuous anti-inflammatory tone. Given the potential to change the spectrum of plasma and tissue NAEs through dietary supplementation with fish oil [17], this seems a realistic option.

At first sight, the activity of n-3 PUFA NAEs at the TRPV1 receptor might be considered as a less positive aspect of attempting to increase n-3 PUFA NAEs, since the TRPV1 receptor is acutely associated with sensory neurones and pain sensation (for reviews, see [8, 49, 50]). Some of the n-3 PUFA NAEs appeared less effective than the n-6 analogues, notably *N*-eicosapentaenoylethanolamine, but nevertheless evoked significant elevations of intracellular calcium ion levels (Figure 3). However, dietary TRPV1 activation has been reported to reduce insulin resistance and liver steatosis in high fat diet-fed obese mice [51] and to alter brown adipose tissue metabolism [52], as well as improving exercise endurance [53]. It is possible that promoting EPEA generation at the expense of the n-3 docosapenta- and docosahexa- NAEs, which were more effective as TRPV1 agonists (Figure 1) may be a more successful strategy to accentuate an anti-inflammatory and anti-obesity tone.

Much of the emphasis of studies of risk and benefit of dietary variation of n-3 PUFAs intake (for reviews, see [18, 19]) has been on oxidative metabolites of PUFAs through cyclooxygenase and lipoxygenase pathways, which clearly play a substantive role in long term inflammatory/anti-inflammatory effects and gradations in risk. Our results identify that metabolism of dietary n-3 PUFAs to n-3 PUFA-NAEs *in vivo* may provide a parallel, independent pathway to push the balance towards reduced risk and increased benefit.

In summary, we have identified a selective action on n-3 PUFA NAEs as endogenous CB_2 cannabinoid receptors, which we hypothesise may contribute to the long-term beneficial actions of n-3 PUFA metabolites in the diet.

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6. Legends to Figures

Figure 1: LC-MS/MS quantification of NAEs in rat liver and midbrain tissues. The insert highlights the PUFA NAE data on an amplified scale. Data are mean ± SEM of NAE levels expressed as pmol/g wet tissue weight from five (midbrain) or six (liver) animals. Although the survey scanning method is able to determine numerous other NAEs (notably EPEA, C20:5 and DHEA, C22:6), these were below limits of detection in these samples.

Figure 2: Elevation of intracellular calcium ion levels measured using a FlexStation, with Chinese hamster ovary cells expressing human recombinant CB_1 (A) or CB_2 receptors (B). Data are means ± SEM from six (A) or five (B) different experiments conducted in triplicate. *P<0.05, ***P<0.001 vs vehicle controls using repeated measures ANOVA with Dunnett's multiple comparison.

Figure 3: Phosphorylation of ERK measured using an in-cell immunostaining assay, with Chinese hamster ovary cells expressing human recombinant CB_1 (A) or CB_2 receptors (B). Data are means ± SEM from four (A) or five (B) different experiments conducted in triplicate. **P<0.01, ***P<0.001 vs vehicle controls using repeated measures ANOVA with Dunnett's multiple comparison.

Figure 4: Elevation of intracellular calcium ion levels measured using a calcium imaging apparatus, with rat dorsal root ganglion neurones *in vitro*. Data are means ± range from two (C20:0; C22-1, n-9), or means ± SEM from three (C16:0; C18:0; C18:3, n-3; C18:2, n-6), four (C18:1, n-9; C24:1, n-9), five (C22:5, n-3), six (C22:6, n-3; C20:4, n-6; C22:4, n-6; C22:5, n-6) or eight (C20:5, n-3) separate preparations. Within each preparation, 5-171 cells were assessed (mean ± SEM of 51 ± 6; median 33 cells). ***P<0.001 vs vehicle controls using two way ANOVA with Dunnett's multiple comparison.