Title: Distinctive effects of eicosapentaenoic and docosahexaenoic acids in regulating neural stem cell fate are mediated via endocannabinoid signalling pathways

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Running title: EPA, but not DHA induces proliferation in NSCs

Abbreviations: AA, Arachidonic acid, AEA, anandamide, DHA. Docosahexaenoic acid; EPA, eicosapentaenoic acid, 2-AG, 2-arachidonylglycerol

Title: Distinctive effects of eicosapentaenoic and docosahexaenoic acids in regulating neural stem cell fate are mediated via endocannabinoid signalling pathways

Emerging evidence suggests a complex interplay between the endocannabinoid system, omega-3 fatty acids and the immune system in the promotion of brain self-repair. However, it is unknown if all omega-3 fatty acids elicit similar effects on adult neurogenesis and if such effects are mediated or regulated by interactions with the endocannabinoid system. This study investigated the effects of DHA and EPA on neural stem cell (NSC) fate and the role of the endocannabinoid signalling pathways in these effects.

EPA, but not DHA, significantly increased proliferation of NSCs compared to controls, an effect associated with enhanced levels of the endocannabinoid 2-arachidonylglycerol (2-AG) and p-p38 MAPK, effects attenuated by pre-treatment with CB1 (AM251) or CB2 (AM630) receptor antagonists. Furthermore, in NSCs derived from IL-1β deficient mice, EPA significantly decreased proliferation and p-p38 MAPK levels compared to controls, suggesting a key role for IL-1β signalling in the effects observed. Although DHA similarly increased 2-AG levels in wild-type NSCs, there was no concomitant increase in proliferation or p-p38 MAPK activity. In addition, in NSCs from IL-1β deficient mice, DHA significantly increased proliferation without effects on p-P38 MAPK, suggesting effects of DHA are mediated via alternative signalling pathways. These results provide crucial new insights into the divergent effects of EPA and DHA in regulating NSC proliferation and the pathways involved, and highlight the therapeutic potential of their interplay with endocannabinoid signalling in brain repair.

Keywords: CB1 cannabinoid receptor, CB2 cannabinoid receptor, docosahexaenoic acid, endocannabinoids, eicosapentaenoic acid, neurogenesis

1 Introduction

Neurogenesis has consistently been shown in two regions of the adult brain, the subventricular zone (SVZ) of the olfactory bulb and the subgranular layer of the hippocampal dentate gyrus, where it occurs in all mammals studied to date, including man (Ehninger and Kempermann, 2008). Ageing is the greatest negative regulator of hippocampal neurogenesis (Kuhn et al., 1996), and adult neurogenesis may be required for hippocampal-dependent learning and memory (Zhao et al., 2008). Increased neurogenesis in rodents is seen following ischaemia (Takagi et al., 1999), stroke (Darsalia et al., 2005) and after seizures (Parent et al., 1997). These increases may be an attempt at brain self-repair and treatments that enhance neurogenesis and survival of new neurons in adults may provide a novel therapeutic approach to the treatment of neurodegenerative diseases.

Omega-3 polyunsaturated fatty acids (PUFAs) are essential for brain development and function and an elevated intake of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) may provide benefit in a range of neurological and neurodegenerative conditions (Dyall and Michael-Titus, 2008). EPA and DHA enhance adult hippocampal neurogenesis in a variety of animal models (Dyall, 2011b) and DHA has consistently been shown to promote differentiation of neural stem cells (NSCs) (Katakura et al., 2009; Kawakita et al., 2006; Ma et al., 2010; Rashid et al., 2013). Elucidating the mechanisms behind these effects has been complicated by the convergent pathways involved in regulating neurogenesis and the pleiotropic effects of omega-3 PUFAs. However, a number of potential targets have been identified including enhancing neurotrophin levels and regulating transcription factors (Dyall, 2011b).

The endocannabinoid system plays a key regulatory role in adult hippocampal neurogenesis (Jin et al., 2004; Rueda et al., 2002). Moreover, the endocannabinoid system directs cell fate specification of NSCs in the CNS (central nervous system) via interleukin-1β associated signalling pathways (Molina-Holgado and Molina-Holgado, 2010). The two main endocannabinoids, N-arachidonylethanolamide (AEA, anandamide), and 2-arachidonylglycerol (2-AG), which are derivatives of the omega-6 PUFA, arachidonic acid (AA) exert most of their biological effects by binding to the CB1 and CB2 cannabinoid receptors. The CB1 receptor is widely expressed in the brain, with high levels in the hippocampus, cerebellum and basal ganglia (Herkenham, 1991; Herkenham et al., 1991; Moldrich and Wenger, 2000). CB2 receptors were initially described in the immune system,

Abbreviations: AA, Arachidonic acid, AEA, anandamide, DHA. Docosahexaenoic acid; EPA, eicosapentaenoic acid, 2-AG, 2-arachidonylglycerol

but have more recently been identified in glia and subsets of neurons the CNS (Onaivi et al., 2012). Activation of cannabinoid receptors stimulates neurogenesis through a mechanism dependent on diacylglycerol lipase (DAGL: the enzyme primarily responsible for the synthesis of 2-AG) and the phosphoinositide-3 kinase／Akt pathway, an effect more pronounced in older animals (Goncalves et al., 2008; Molina-Holgado et al., 2007).

Studies into the pro-neurogeneic effects of endocannabinoids in the dentate gyrus have produced conflicting results. Adult rats treated with an AEA analogue show significantly decreased neurogenesis in dentate gyrus, and this is increased by a CB1 antagonist (Rueda et al., 2002). However, other evidence indicates that chronic treatment with an endocannabinoid agonist increases hippocampal neurogenesis in adult rats (Jiang et al., 2005), and CB1 receptor-knockout mice show significant reductions in neurogenesis (Jin et al., 2004). Pharmacological blockade CB2 inhibits the proliferation of NSC, and the proliferation of progenitor cells in young animals (Goncalves et al., 2008). A similar response is seen with a fatty acid amide hydrolase (FAAH) inhibitor that limits degradation of endocannabinoids (Aguado et al., 2005). Overall, the effects of the endocannabinoid system on neurogenesis appear a fine balance of receptor activation.

A number of independent lines of evidence suggest a complex crosstalk between omega-3 PUFAs and the endocannabiniod system. For example, an analogous series of ethanolamide endocannabinoids derived from omega-3 PUFAs has been identified (Berger et al., 2001; Wood et al., 2010), and omega-3 PUFA deficiency abolishes endocannabinoid-mediated neuronal functions in mice (Lafourcade et al., 2011). It may therefore be that some of the effects of omega-3 PUFAs on hippocampal neurogenesis are mediated via interaction with endocannabinoid signaling pathways. Furthermore, recent evidence suggests EPA and DHA have independent and divergent effects in the brain in both normal aging and neurodegenerative conditions (Dyall, 2015). Therefore, the aim of this study was to evaluate the effects of DHA and EPA on NSC fate and the potential role of endocannabinoid signalling pathways in these effects.

2 Material and methods

2.1 Neural stem cell (NSC) cultures

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and with international guidelines on the ethical use of animals. Cultures of NSCs

were prepared from the cortex of day 16 (ED16) mice C57BL6 mice Wild Type (WT, Charles River, UK), or interleukin-1β (IL-1β) deficient (KO) C57BL/6 mice (obtained from Prof. Yoichiro Iwakura, University of Tokyo, Tokyo, Japan), as described previously (Reynolds and Weiss, 1992; Rubio-Araiz et al., 2008). Briefly, the NSCs were manually dissociated and the cell suspension centrifuged at 500xg for 5 min. The cells were then cultured as free-floating neurospheres at a density of 20 cells/μL in Dulbecco's Modified Eagles Medium (DMEM) / F12 plus B27 and 20 ng/ml fibroblast growth factor (FGF) and epidermal growth factor (EGF) (Pepro-Tech, UK). Primary neurospheres were cultured for 7-10 days before secondary cultures were established with mechanical dissociated cells. Cell cultures were maintained by adding 6 μL of FGF and EGF every three days. Thereafter, the neurospheres were passaged every 5-7 days and experiments were performed after three to seven passages.

2.2 Fatty acid analysis

The incorporation of DHA and EPA into the membrane phospholipids of the NSCs from wild type and IL-1β knock-out mice was assessed as described previously (Dyall et al., 2007). After 3 days in culture, DHA or EPA (10 nM) was added to the cells and incubated for 24 hr at 37^{o}C. DHA and EPA stock solutions (1 mg/mL as free fatty acids, Sigma-Aldrich, U.K.) were prepared in ethanol and stored at -20°C under nitrogen until dilution to final concentrations in ethanol. The cellular pellet was collected by centrifugation at 500 x g for 3 min. Four determinations were made for AA, DHA and EPA for each cell type. The lipids from the pellets were extracted using the method of Folch and co-workers (Folch et al., 1957). The pellets were weighed and heneicosanoic acid (C21:0, 50 μg) was added as an internal standard. Fatty acid composition was measured after transesterification with 14% boron trifluoride in methanol (v/v) at 100°C (Morrison and Smith, 1964). Individual fatty acids were identified by gas chromatography with flame ionization detector (GC-FID) (7820A Agilent Technologies, USA) using a Omegawax capillary column (30 m × 0.25 μm × 0.25 mm i.d., Supelco, U.S.A.). Fatty acid methyl ester identity was confirmed by retention times compared to known standards. Quantification of peak area was by ChemStation software (Agilent Technologies, U.S.A.). The fatty acid concentrations were quantified against the C21:0 internal standard and corrections were made for variations in the detector response, determined previously.

2.3 Neural stem cell proliferation assays

NSCs from wild type mice were treated with DHA or EPA at final concentrations of 0.01, 0.1, and 1 μM as free fatty acids (Sigma-Aldrich, U.K.) in order to identify the optimum dose. DHA and EPA were diluted to final concentrations in ethanol from the stock solutions. The number of spheres formed was counted after 7 days in culture. From these experiments it was determined that 10 nM DHA and EPA produced the optimum effects and this dose was used in all subsequent analyses.

In the dilution assays, dissociated NSCs were treated with 10 nM DHA or EPA and the cells progressively diluted from 4000 to 500 cells in 96-well plates. The number of spheres formed was counted after 7 days in culture. In experiments employing the selective cannabinoid antagonists AM251 (0.5 μM) and AM630 (0.5 μM), dissociated neurospheres were treated with either antagonists and then diluted, as above, and number of spheres counted after 7 days in culture. In this experiment, the number of new neurospheres was plotted against the initial number of cells plated, and the slope of the lines obtained after the linear regression was used to compare the experimental conditions, from which the behaviour of the NSCs was evaluated (Campos et al., 2004; Tropepe et al., 1999).

2.4 5-Bromo-2'-deoxyuridine (BrdU) incorporation assays

The effects of DHA and EPA on cell proliferation were assessed using a colorimetric assay, based on the measurement of BrdU incorporation during DNA synthesis following the manufacturer instructions (Cell Proliferation ELISA, BrdU (colorimetric), Roche, Germany). Prior to experimentation, the NSCs were passaged creating a single cell suspension. A 96 well plate was then pre-treated with poly-ornithine for 4 hr. The NSCs were plated at a density of approximately 20,000 cells per well in 100 μL of medium. The plate was then incubated for three days at 37^{o}C. Following this, the cells were exposed to the selective cannabinoids antagonists AM251 or AM360 (both at 1 μM) for approximately 15 min prior to the addition of DHA or EPA (10 nM) and the plate was incubated for 24 hr at 37^{o}C. The BrdU labelling solution was diluted to a final concentration of 100 μM. 10 μL per well BrdU labelling solution was added to the cell culture and the plate was re-incubated for 6 hr. After this time, the plate was removed from the incubator and the labelling solution was removed from each well. 200 μl/well of FixDenat was then added to the cells which were then incubated for 30 min at 15°C. The FixDenat solution was then removed and 100 μl/well of anti-BrdU-POD working solution added and the plate incubated for a further 90 min at 15°C.

The antibody conjugate was removed and the plate washed three times and 100 µl/well of substrate solution added. The plate was left for approximately 30 min and then measured at 405 nm. This method was repeated for NSCs from IL-1β knock-out mice.

2.5 Quantitation of endocannabinoid concentrations in NSCs using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids was essentially as described previously (Henry et al., 2014; Kerr et al., 2012; Kerr et al., 2013a; Kerr et al., 2013b). In brief, cell pellets (1.5×10^6) were homogenised for 2 sec in 400 µL 100% acetonitrile containing deuterated internal standards (0.014 nmol anandamide-d8 and 0.48 nmol 2-AG-d8). Homogenates were centrifuged at 14,000 x g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness. Lyophilised samples were re-suspended in 40 µl 65% acetonitrile and separated on a Zorbax® C18 column (150 × 0.5 mm internal diameter) by reversed-phase gradient elution initially with a mobile phase of 65% acetonitrile and 0.1% formic acid which was ramped linearly up to 100% acetonitrile and 0.1% formic acid over 10 min and held at this for a further 20min. Analyte detection was carried out in electrospray-positive ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantification of each analyte was performed by ratiometric analysis using Masshunter Quantitative Analysis Software (Aligent Technologies, UK). The amount of each analyte in unknown samples was calculated from a standard curve of Relative response vs. Relative concentration for each analyte i.e. (Peak Area analyte$_{(undeuterated)}$/Peak area analyte$_{(deuterated)}$ vs (Concentration analyte $_{(undeuterated)}$/concentration analyte$_{(deuterated)}$ and expressed as nmol or pmols per 1.5×10^6 cells.

2.6 MAP Kinases cell signalling ELISA

The effects of DHA and EPA on mitogen-activated protein (MAP) kinases cell signalling pathways were investigated in the NSCs (PathScan® MAP Kinase multi-target sandwich ELISA, Cell Signaling Technology, USA). The levels of phospho-p44/42 MAPK (Thr202/Tyr204) (phosph-ERK1/2), phospho-p38 MAPK (Thr180/Tyr182), MEK1/2, phospho-MEK1/2 (Ser217/221), SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185) were analysed. The NSCs were treated with cell culturing media, excluding EGF and FGF, as they may influence the activation of cell signalling pathways. After 3 days, some NSCs were treated with selective cannabinoid antagonists AM251 or AM360 (both 1 µM) for

approximately 15 min at 37^{o}C. After the incubation period EPA or DHA was added at a final concentration of 10 nM for a 5 min incubation period at 37^{o}C. The controls for EPA and DHA were not treated. The NSC cultures from IL-1β knock-out mice were prepared as above, but without the addition of AM251 or AM630. After incubation the samples were centrifuged for 3 min at 500 × g. The MAP kinase activation was analysed following the manufacturer's instructions and the absorbance was read at 450 nm within 30 min.

2.7 Statistical Analysis

Results are presented as means (SD) of 3-6 independent experiments, each carried out in triplicate or quadruplicate. Statistical analyses was carried out using one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc multiple comparison test, or unpaired two-tailed Student's t-test. Statistical significance was set at $P<0.05$. Statistical analyses were performed using IBM SPSS statistics version 21 (Chicago, USA). The tables and graphs of the original data were produced using GraphPad Prism software version 6.00 for Windows (GraphPad, USA).

3 Results

3.1 The effects of DHA and EPA on NSC cell viability

To examine the effects of DHA and EPA on the NSC viability neurospheres were cultured with various concentrations DHA and EPA (Fig. 1). The only significant increases in the mean number of neurospheres were seen with 10 nM EPA (25.2% vs. control, $P<0.05$). However, both DHA and EPA significantly decreased their number at higher concentrations (all at $P<0.05$ vs. control). These findings suggest that both DHA and EPA promote the maintenance NSCs at nM concentrations, whereas these effects are not seen at higher concentrations. Therefore, all subsequent experiments were performed with DHA and EPA at concentrations of 10 nM.

3.2 DHA and EPA incorporation into NSCs from wild type and IL-1β knock out mice.

The AA, DHA and EPA contents of the NSCs from the wild type and IL-1β knock-out mice and media were analysed from cultures after 24 hours incubation with 10 nM DHA or EPA (Supplementary Table 1). The addition of DHA significantly increased the levels of DHA in both wild type and IL-1β knock out cells, by 22.0% and 20.3% respectively (both $P<0.05$ vs. controls). Similarly, the addition of EPA significantly increased the levels of EPA in both

wild type and IL-1β knock out cells (84.3% and 85.5%, respectively with both P<0.05 vs. control). There were no significant differences in phospholipid incorporation of either DHA or EPA between the NSCs from wild-type and IL-1β knock-out mice. Although there were decreases in the AA content following both DHA and EPA treatment these did not reach statistical significance for either cell type.

3.3 EPA, but not DHA increases proliferation of NSCs from wild-type mice

NSCs were treated with DHA or EPA and were progressively diluted from 4000 to 500 cells. The number of neurospheres was counted after 7 days in culture and was plotted against the initial number of cells plated. The slope of linear regression line was used to compare the proliferation in different experimental conditions (Fig. 2). With the DHA experiment, the regression slope for the control cells was slope 0.189 ± 0.013 (R^2=0.98) (Fig. 2A), which was not significantly different from the slope of the DHA treated cells, 0.173 ± 0.007 (R^2=0.99), nor, the slope for DHA treated cultures pre-treated with the selective CB1 receptor antagonist AM251 at 0.161 ± 0.024 (R^2=0.92). However, there was a significant decrease in proliferation with the prior addition of the CB2 receptor antagonist AM630 (0.118 ± 0.021, R^2=0.89, P<0.05 vs. all other groups). With the NSCs treated with EPA, the regression slope for the control cells was 0.162 ± 0.025 (R^2=0.91) (Fig. 2B), which was not significantly different from the slope of the EPA treated cells, 0.190 ± 0.021 (R^2=0.95), whereas the slope for cultures pre-treated with the selective CB1 (AM251) and CB2 (AM630) antagonists were 0.144 ± 0.016 (R^2=0.95) (P<0.05 vs. EPA group) and 0.087 ± 0.001 (R^2=0.94) (P<0.01 vs. control and EPA groups), respectively.

The effects of DHA and EPA on neurosphere proliferation were also analysed using BrdU ELISA. DHA treatment did not significantly alter the levels of BrdU absorbance compared with the Controls (Fig. 2 C). Prior administration with AM251 or AM630 decreased proliferation of DHA treated NSCs (both P<0.05 vs. control and DHA groups). In comparison, EPA treatment significantly increased BrdU absorbance compared to the Control group (P<0.05), and this effect was prevented by pre-treatment with AM251 (P<0.05 vs control and P<0.001 vs. EPA) and AM630 (P<0.05 vs. control and P<0.001 vs. EPA).

3.4 Effect of EPA and DHA on endocannabinoid levels in NSCs

The effect of EPA and DHA treatment on endocannabinoid levels in NSCs was assessed using LC-MS/MS (Fig 3). Treatment of the NSC with DHA or EPA resulted in significant increases in the levels of 2-AG by 112.6% ($P<0.01$) and 101.4% ($P<0.05$), respectively. There were no significant effects of treatment with either DHA or EPA on levels of AEA.

3.5 DHA and EPA induce different effects on MAPK cell signalling in NSCs from wild type mice

The effects of DHA and EPA on MAPK cell signalling in NSCs from wild-type mice are summarised in Figs. 4 and 5, respectively. There were no significant effects of DHA treatment, or pre-administration with AM251 or AM630, on the levels of phospho-ERK1/2, phospho-p38 MAPK or MEK1/2. Pre-treatment with AM251 followed by the DHA treatment significantly decreased the levels of phospho-MEK1/2 (-22.2% vs. control and -13.4% vs. DHA, both at $P<0.05$). Similar effects were seen with the pre-treatment by AM630 (-22.6% vs. control and -13.9% vs. DHA, both at $P<0.05$).

Phospho-p38 MAPK levels were significantly increased following EPA treatment (+23.8%, $P<0.05$ vs. control). This increase was inhibited by pre-treatment with both AM251 and AM630 (both $P<0.05$ vs. EPA). Neither EPA treatment, nor pre-treatment with AM251 or AM630 significantly altered the levels of phospho-ERK1/2 or MEK1/2. Consistent with the DHA treatment, the levels of phospho-MEK1/2 were not significantly altered following the addition of EPA compared to the controls, whereas pre-administration with both AM251 and AM630 prior to EPA treatment significantly decreased levels (-22.5% and -15.4% vs. control, and -15.5% and -8.3% vs. EPA, respectively, all at $P<0.05$).

3.6 Endogenous IL-1β is essential for the effects of EPA on NSC proliferation

NSCs from IL-1β knock-out mice were treated with either DHA or EPA, and the slope of the linear regression line was used to compare the proliferation in the different experimental conditions (Fig. 6). With the DHA experiment, the regression slope for the control cells was slope 0.155 ± 0.006 ($R^2=0.99$) (Fig. 6A), which was not significantly different from the slope of the DHA treated cells, 0.151 ± 0.015 ($R^2=0.96$). Similarly, there were no significant differences between the slopes of the control (0.129 ± 0.018, $R^2=0.93$) and EPA (0.102 ± 0.018, $R^2=0.89$) treated cells (Fig. 6B). The effects on neurosphere proliferation in IL-1β knock-out NSCs were also analysed using BrdU ELISA, shown in Fig. 6 (C). BrdU

absorbance was significantly increased in the DHA treated cells compared to the control ($P<0.05$ vs. control). Whereas there was a significant decrease in the absorbance of BrdU in the EPA treated NSCs compared to the control and DHA treated cells (both at $P<0.05$).

3.7 IL-1β deficiency modifies the effects of DHA and EPA on MAPK cell signalling in NSCs

The addition of DHA or EPA to NSCs from IL-1β knock-out mice produced significantly different effects to those observed in NSCs from wild type mice, Fig. 7. Following DHA treatment the levels of phospho-ERK1/2 were significantly decreased compared to the controls (-8.8%, $P<0.05$ vs. control). The levels of phospho-p38 MAPK were not significantly altered by the addition of DHA. There were significant decreases in the levels of phospho-MEK1/2 following DHA treatment (-23.0%, $P<0.05$ vs. control), whereas there were no significant changes in the levels of unphosphorylated-MEK1/2, nor the activation the MEK pathway. There were also no statistically significant effects of DHA treatment on the levels of phosphorylated or unphosphorylated-SAPK/JNK.

Phospho-p38 MAPK was significantly decreased following EPA treatment (-9.1%, $P<0.05$ vs. control). Phospho-MEK1/2 was significantly decreased following EPA treatment (-18.8%, vs. control, $P<0.05$), whereas unphosphorylated MEK1 was significantly increased (+7.1% vs. control, $P<0.05$). The activation of the MEK pathway, calculated from the ratio of phosphorylated to unphosphorylated-MEK1, was significantly decreased following EPA treatment (-41.0%, $P<0.001$ vs. control). There were no statistically significant effects of EPA treatment on the levels of phospho-ERK1/2 or phosphorylated and unphosphorylated-SAPK/JNK

4 Discussion

Overall the results indicate that the addition of DHA or EPA to NSCs has differing effects on NSC proliferation and that these effects are mediated via different cell signalling pathways. The data demonstrate that EPA increases cell proliferation, an effect accompanied by an increase in 2-AG formation, which acts via CB1 or CB2 receptors, to increase activation of the p38 MAPK signalling pathway. In comparison, although DHA also results in increased 2-AG levels in NSCs, there was no concomitant change in NSC proliferation or activation of p38 MAPK. Furthermore, the regulatory effects of DHA and EPA were reversed in NSCs

from IL-1β knockout mice, suggesting an important interaction between these omega-3 PUFAs and the neuro-immune system. CB1 and CB2 receptor agonists increase IL-1β levels in NSCs (Garcia-Ovejero et al., 2013), and it may be that the effects of EPA on 2-AG and p38 MAPK induces IL-1β thereby increasing proliferation, whereas pharmacological blockade of CB1 or CB2 or knockout of IL-1β attenuates this response, potentially via preventing the activation of other MAPKs, such as MEK1/2. These results provide important new insights into the interaction between omega-3 PUFAs and endocannabinoid signalling and the subsequent cellular mechanisms by which omega-3 PUFAs exert their effects on neurogenesis.

The initial cell viability assays showed the greatest numbers of neurospheres were induced by DHA and EPA when added at nM levels, with toxic effects above 1 µM, and therefore 10 nM was used in all the subsequent experiments. This concentration is lower than typically applied to NSCs, where 1 µM DHA has been shown to induce differentiation (Katakura et al., 2009; Kawakita et al., 2006). However, our analysis showed that even at these low concentrations there was significant enrichment of the membrane phospholipids, consistent with NSCs incorporating and metabolizing PUFAs readily (Langelier et al., 2010). Furthermore, the initial reports of cell viability assays showed cells were most viable when DHA was added at the lowest dose (0.1 µM) (Katakura et al., 2009). There are also differences in methodologies used, as in these other studies the fatty acids were dissolved in media containing 1.0% fatty-acid free bovine serum albumin (Katakura et al., 2009; Kawakita et al., 2006), whereas in the present study DHA was dissolved in ethanol and then diluted into the media..

DHA inhibited NSC proliferation consistent with previous studies (Katakura et al., 2013; Katakura et al., 2009); however, others have reported DHA enhances NSC proliferation (Sakayori et al., 2011). Sakoyori and co-workers suggested that the difference in effects on proliferation was the due to the lower DHA concentration promoting the maintenance of gliogenic NSCs. Since this was the same dose as was used in our study it is more likely that this discrepancy is due to differences in the experimental conditions. In our study we collected NSC from ED16 fetal mice cortices, whereas Sakayori and co-workers used ED16.5 rats and Katakura and co-workers used ED 14.5 rats (Sakayori et al., 2011). There were also differences in the concentrations of heparin, FGF and EGF between studies, all of which are crucial determinants of NSC fate (Kelly et al., 2005).

EPA treatment significantly increased NSC proliferation. The role of EPA in regulating NSC fate has only recently begun to be explored. In a recent paper EPA was shown to enhance neuronal differentiation of NSCs, but the effects on proliferation were not explored (Katakura et al., 2013). Furthermore, although EPA was shown to enhance differentiation, this group reported that DHA and EPA induced different effects on the transcription factors involved in regulating the cell cycle. EPA significantly increased the levels of Hes1, whereas with DHA treatment it was significantly decreased. Hes1 is a repressor type transcription factor, which inhibits neuronal differentiation and enhances proliferation in NSCs (Ishibashi et al., 1994; Ohtsuka et al., 2001). EPA, but not DHA, also significantly increased Hes6, and Hes6 acts in a positive-feedback loop with Hes1 to promote neuronal differentiation (Bae et al., 2000; Koyano-Nakagawa et al., 2000). It may therefore be that the effects of EPA on proliferation are based on a fine balance between these basic helix-loop-helix transcription factors and may also depend on experimental conditions employed.

The effects of EPA on proliferation were abolished by prior exposure of the NSCs to pharmacological blockade of either CB1 or CB2 receptors, suggesting the effects were mediated via endocannabinoid signalling pathways. Previous studies have shown cross-talk between omega-3 PUFAs and the endocannabinoid system in the brain (Lafourcade et al., 2011). Importantly, our results show for the first time that a single administration of DHA or EPA to NSC significantly increases the levels of the AA-derived endocannabinoid, 2-AG. Previous studies have reported omega-3 PUFA supplementation reduces levels of 2-AG (and AEA) across a range of tissues, including the brain (Artmann et al., 2008; Batetta et al., 2009; Matias et al., 2008; Watanabe et al., 2003; Wood et al., 2010); however, these studies are based on long term administration protocols and the effects appear driven by displacement of AA from phospholipids and subsequent competition for biosynthetic enzymes. This displacement was not seen in the present study as neither treatment altered AA levels likely due to the low doses of DHA and EPA applied, and therefore, this observation of an acute effect of DHA and EPA on 2-AG levels must be considered novel.

The results show both EPA and DHA similarly increase 2-AG levels, whereas only EPA induces proliferation. Differences between the effects of AA and DHA on NSC fate have been observed previously (Sakayori et al., 2011). Thus it is possible that EPA is acting via endocannabinoid signalling pathways to promote proliferation, whereas DHA may

additionally be acting via alternative pathways, such as through conversion to the endocannabinoid-like metabolite, synaptamide, which has been shown to induce neuronal differentiation of NSCs via activation of protein kinase A (PKA)/cAMP response element binding protein (CREB) (Rashid et al., 2013).

In the present study the EPA induced increases in NSC proliferation were accompanied by regulatory effects on MAPK signalling pathways with a significant increase in phospho-p38 MAPK. The p38 MAPKs have important roles in cytokine production and belong to the stress-activated MAPK family (Mittelstadt et al., 2005). However, additional functions for p38 MAPKs in regulating stem cell fate have begun to be elucidated, where for example, the adipokine adiponectin increases NSC proliferation via p38 MAPK signalling pathways (Zhang et al., 2011). These effects appear mediated via transient activation of p38 MAPK, as in both our study and the study by Zhang and co-workers phosphorylation of p38 MAPK declined by 30 min (data not shown). Pharmacological blockade of CB1 and CB2 inhibited both the proliferative effects of EPA and the accompanying increases in phospho-p38 MAPK, suggesting p38 MAPK activation was occurring downstream of CB1 or CB2. Consistent with these observations, cannabinoids have been shown to activate p38 MAPK, but not JNK via CB1 receptors in hippocampal slices (Derkinderen et al., 2001).

The cytokine IL-1β has a complex interaction with the p38 MAPK pathway and endocannabinoid signalling in regulating NSC fate (Garcia-Ovejero et al., 2013; Molina-Holgado and Molina-Holgado, 2010; Vela et al., 2002). Furthermore, we have previously shown an intricate balance exists between DHA and EPA and the inflammasome (Mandhair et al., 2012). Therefore the role of IL-1β in mediating the effects of DHA and EPA were investigated in NSCs derived from IL-1β deficient mice. In these NSCs from ILβ knock-out mice both DHA and EPA induced effects opposite to those observed in the cells from wild type mice, such that DHA significantly increased proliferation, whereas EPA significantly reduced it. The effects of DHA were seen without increases in the levels of phospho-p38 MAPK, indicating the involvement of different signalling pathways. Whereas in the EPA treated IL-1β knock-out NSCs there was also a decrease in the levels of phospho-p38 MAPK, consistent with a role in the proliferative effects of EPA. These results provide the first evidence that IL-1β has an essential role in the effects of DHA and EPA in regulating NSC fate.

The present results indicate that IL-1β is needed for the proneurogenic effect of EPA. However the effects of IL-1β in the control of neural precursors cells fate decisions remains controversial. Several studies indicate that IL-1β is antineurogenic (Koo and Duman, 2008; Ryan et al., 2013) by activation of the tumor suppressor p53 (Guadagno et al., 2015) or via adrenocortical activation (Goshen et al., 2008). Other findings suggest a proneurogenic effect of IL-1β (de la Mano et al., 2007; Xue et al., 2015). In previous studies from our group, we observed that genetic deletion of IL-1β increases basal proliferation of NSC (Garcia-Ovejero et al., 2013).

The results highlight the important differences in the effects of DHA and EPA in regulating NSC fate. DHA is quantitatively the most important omega-3 PUFA in the brain, and brain DHA levels are typically 250-300 times higher than EPA (Chen et al., 2009). The low levels of EPA are maintained by multiple mechanisms including β-oxidation, decreased incorporation, elongation to docosapentaenic acid (DPA, 22:5n-3) and lower phospholipid recycling (Chen et al., 2013). However, in spite of these quantitative differences the doses used in the present study are of physiological relevance (Contreras et al., 2000) and produce significant enrichment of the membrane phospholipids. Furthermore, evidence is now accumulating that many effects are specific and unique to the individual omega-3 PUFAs and it is essential to identify these fatty acid specific effects, as there has traditionally been a lack of discrimination between the different omega-3 PUFAs with results broadly attributed to the omega-3 PUFA series as a whole (Dyall, 2011a).

In conclusion, this study provides the first evidence that EPA induces NSC proliferation via direct effects on endocannabinoid and p38 MAPK signalling pathways, potentially mediated by IL-1β. However, further work is required to identify the downstream targets, which may include cell cycle regulatory transcription factors (Katakura et al., 2013). Furthermore, our work does not preclude synergistic/parallel effects on other signalling pathways. For example, the positive effect of omega-3 PUFA supplementation on neurogenesis in aging occur in parallel with restorative effects on the retinoic acid receptors, RARalpha and RXRbeta in CA1 and dentate gyrus regions of the hippocampus, suggesting some of the effects may be mediated by activity at these transcription factors (Dyall et al., 2010), or interaction between p38 MAPK and retinoid signalling (Piskunov and Rochette-Egly, 2012).

Further studies will address these down-stream effects. However, these results highlight important differences between the neurogenic effects of EPA and DHA and suggest that through modulation of the endocannabinoid system brain repair may be supported.

5 Acknowledgements

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Fig. 1. DHA and EPA show dose-specific effects on NSC proliferation. After seven days in culture the effects of different doses of DHA or EPA were quantified by counting the number of neurosphere formed. The results are expressed as a percentage of the control values as means (SD) and the groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test (N = 6 per group). Only EPA at 10 nM significantly increased proliferation, whereas at 100 nM and 1 µM both DHA and EPA significantly decreased proliferation. *P<0.05 vs. control.

Fig. 2. Divergent effects of DHA and EPA and selective endocannabinoid antagonists on NSC proliferation. Serial dilution assays of dissociated neurosphere cultured with 10 nM DHA or EPA in the absence or presence of selective CB1 (AM251) and CB2 (AM630) receptor antagonists (both 0.5 µM). The number of neurospheres was estimated 1 week later and plotted against the initial number of cells plated. The slopes of the lines obtained after liner regression were used to compare the experimental conditions as assessed by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test (N = 4 per group). (A) DHA and DHA with prior administration with AM251 did not significantly alter proliferation compared to controls, whereas AM630 significantly decreased the number of newly formed neurospheres (P<0.05 vs. all other groups). (B) EPA did not significantly alter proliferation, whereas prior administration of AM251 and AM630 significantly decreased proliferation (P<0.05 vs. EPA group and P<0.05 vs. control and EPA groups, respectively). The effects of DHA and EPA on cell proliferation were also assessed using a colorimetric assay, based on the measurement of BrdU incorporation during DNA synthesis. DHA did not enhance cell proliferation (Fig. 2 C), whereas EPA significantly increased proliferation (Fig. 2 D). Prior administration of AM251 or AM630 (both at 1 µM) significantly decreased proliferation following the addition of both DHA and EPA. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test and *P<0.05 vs. control, $^{\#}$P<0.05 vs. DHA treated and $^{\#\#}$P<0.001 vs. EPA treated (N = 6 per group).

Fig 3. EPA and DHA treatment increased levels of 2-AG but not anandamide (AEA) in NSCs. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test with * representing P<0.05 and ** P<0.01 vs. control (N = 3-4 per group).

Fig 4. Treatment of NSCs with 10 nM DHA in the absence or presence of AM251 and AM630 induces differential phosphorylation of MAP Kinases. The addition of 10 nM DHA did not significantly alter the levels of any of the MAP kinases measured; however, administration of AM251 and AM630 (both 1 µM) prior to DHA treatment significantly decreased phospo-MEK1/2 compared to control and DHA treated groups. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test with * representing $P<0.05$ vs. control and $^{\#}$ $P<0.05$ vs. DHA treated, (N = 6 per group).

Fig. 5. EPA induced significant increases in phospho-p38 MAPK, which are abolished by inhibition of cannabinoid receptors. EPA (10 nM) significantly increased the levels of phospo-p38 MAPK compared to the controls, which was abolished by pre-treatment with both AM251 and AM630. Consistent with the DHA treated cells, pre-treatment with both AM251 and AM630 (both 1 µM) prior to the addition of EPA significantly decreased phospo-MEK1/2 compared to control and EPA treated groups. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test, with * representing $P<0.05$ vs. control and $^{\#}$ $P<0.05$ vs. EPA treated, (N = 6 per group).

Fig. 6. IL-1β has an essential role on the proliferative effects of DHA and EPA in NSCs Serial dilution assays of dissociated neurosphere cultured with 10 nM (A) DHA or (B) EPA. The number of neurospheres was estimated 1 week later and plotted against the initial number of cells plated. There were no significant differences in the slopes of the lines obtained after liner regression under any of the experimental conditions as assessed by Student's t-test (N =4 per group). The effects of DHA and EPA on cell proliferation were also assessed using a colorimetric assay, based on the measurement of BrdU incorporation during DNA synthesis (Fig. 6 C). In these cells DHA significantly enhanced proliferation, whereas EPA significantly decreased proliferation. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test, with * representing $P<0.05$ vs. control and $^{\#}$ $P<0.05$ vs. DHA treated, (N = 4 per group).

Fig. 7. IL-1β is essential for the effects of DHA and EPA on MAPK cell signalling in NSCs. The effects of DHA and EPA on MAPK cell signalling in IL-1β deficient mice was assessed using a multi-target sandwich ELISA. In these cells DHA significantly decreased phospho-ERK1/2 and phospho-MEK1/2 levels, effects which were not seen in the cells from wild type

mice. Following EPA treatment the opposite effect on phospho-p38 MAPK was seen compared to the NSCs from wild type mice, such that there was a significant decrease in the levels. EPA also significantly decreased the levels of phospho-MEK1/2 and increased MEK1/2, leading to an overall significant increase in MEK1/2 activation. Groups were compared by one-way ANOVA followed by the Bonferroni post hoc multiple comparison test, with * representing $P<0.05$ vs. control (N = 4 per group).