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Activation of WNT and CREB Signaling Pathways in Human Neuronal Cells in Response to the Omega-3 Fatty Acid Docosahexaenoic Acid (DHA)

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Abstract

A subset of individuals with major depressive disorder (MDD) elect treatment with complementary and alternative medicines (CAMs), including the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Previous studies in rodents suggests that DHA modulates neurodevelopmental processes, including adult neurogenesis and neuroplasticity, but the molecular and cellular mechanisms of DHA's potential therapeutic effect in the context of human neurobiology have not been well established. Here we sought to address this knowledge gap by investigating the effects of DHA using human iPSC-derived neural progenitor cells (NPCs) and post-mitotic neurons using pathway-selective reporter genes,

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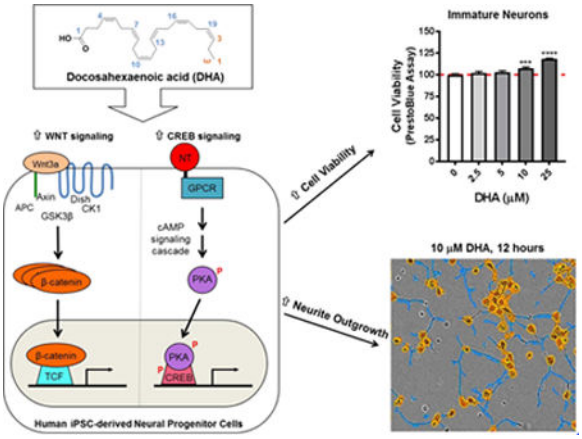
Disclosures

S.J.H. is a member of the scientific advisory board and has financial interest in Rodin Therapeutics and is an inventor on IP licensed to this entity that is unrelated to this study. S.J.H. is also a member of the scientific advisory board of Psy Therapeutics, Frequency Therapeutics, and Souvien Therapeutics, none of which were involved in the present study. S.J.H. has also received speaking fees from Amgen and Merck. R.H.P. is a member of the scientific advisory board of Psy Therapeutics. R.M. has financial interests in SHAPE Pharmaceuticals, Acetylon Pharmaceuticals and Regenacy Pharmaceuticals. He is also the inventor on IP licensed to these two entities that is unrelated to this study. W.-N.Z., N.K.H., J.W., P.S.C., B.A., I. K., A.S. reported no biomedical financial interests or potential conflicts of interest.

multiplexed mRNA expression profiling, and a panel of metabolism-based viability assays. Finally, real-time, live-cell imaging was employed to monitor neurite outgrowth upon DHA treatment. Overall, these studies showed that DHA treatment (0 – 50 μM) significantly upregulated both WNT and CREB signaling pathways in human neuronal cells in a dose-dependent manner with 2 to 3-fold increases in pathway activation. Additionally, we observed that DHA treatment enhanced survival of iPSC-derived NPCs and differentiation of post-mitotic neurons with live-cell imaging revealing increased neurite outgrowth with DHA treatment within 24 hours. Taken together, this study provides evidence that DHA treatment activates critical pathways regulating neuroplasticity, which may contribute to enhanced neuronal cell viability and neuronal connectivity. The extent to which these pathways represent molecular mechanisms underlying the potential beneficial effects of omega-3 fatty acids in MDD and other brain disorders merits further investigation.

Graphical abstract



Keywords

omega-3 fatty acid; docosahexanoic acid; neuroplasticity; depression; iPSC-derived human neurons; complementary and alternative medicine

Introduction

Major depressive disorder (MDD) is a common, and debilitating mental disorder that affects 10–15% of the population worldwide [1]. According to the DSM-V (Diagnostic and Statistical Manual of Mental Disorders), MDD is marked by a period of at least two weeks of depressed mood, irritability, fatigue, significant weight change, feelings of worthlessness, and possibly suicidal thoughts. With more than 300 million people suffering from depression globally [2], the burden of depressive disorders has increasingly become a public health concern [3, 4]. While selective serotonin reuptake inhibitors (SSRIs) are widely recognized as the first-line pharmacologic treatment of depression [5], not all patients reach remission and some experience adverse side effects [6–11].

In addition to possible alterations in serotonergic neurotransmission in depression, accumulating evidence for stress-related abnormalities in MDD patients has led to a ‘stress hypothesis’ for depression [12–14]. The associations between depression, oxidative stress and antioxidant status have been identified in post-mortem samples, together with elevated peripheral markers of oxidative damage in patients’ blood samples [15]. Since neurons are especially vulnerable to stress, dysregulation of stress pathways results in decreased neurogenesis, synaptogenesis and dendritic spine formation, and increased apoptosis of neurons, the structural abnormalities further leading to deficits of neuronal functions [16–18]. Indeed, multiple reports note decreased hippocampal volume among patients with depression, regardless of age of onset [19–25]. These findings indicate the potential for a pharmacological intervention that specifically targets stress-neuroplasticity.

As an alternative or addition to FDA-approved pharmacologic treatment, complementary and alternative medicines, the omega-3 fatty acids, such as docosahexaenoic acid (DHA; 22:6(n-3)) and eicosapentaenoic acid (EPA; 20:5(n-3)), and S-adenosyl methionine (SAMe), a major methyl donor in the brain, have emerged as promising candidates for controlling depressive symptoms [26]. Several clinical trials of DHA/EPA report their ability to alleviate MDD symptoms and increase remission rates [27–30], though the effectiveness of DHA as compared to EPA may require more confirmative trials [31–34]. In a combination therapy study, Gertsik et al. [35] found citalopram plus EPA/DHA/other PUFA (poly-unsaturated fatty acids) co-treatment significantly decreased the symptoms of MDD in comparison to monotherapy. A recent meta-analysis concluded that supplementation with omega-3 PUFAs provides benefits for reducing MDD symptoms, especially when taken as an adjunct treatment with antidepressants [36], although effect sizes may be modest [37]. Outside of controlled clinical trials in MDD, omega-3 PUFAs remain popular as dietary supplements and have become of growing interest in the potential treatment of age-related cognitive decline, cardiovascular disease, and cancer [38, 39]. In all of these contexts, the relative contribution of DHA versus EPA remains important to understand given growing recognition that different omega-3 PUFAs may function separately with varying potencies as well as function synergistically. This issue has further relevance for efforts to develop omega-3 PUFA-based pharmaceutical products that contain highly purified preparations, such as the ethyl ester derivative of EPA, icosapent ethyl, that is being explored for reduction of cardiovascular risk [40].

Of the omega-3 PUFAs, DHA is the most abundant in the brain and is essential for fetal brain development [41]. Literature on DHA reports a diverse role for the compound, including antioxidant and anti-inflammatory properties [42], as well as modulation of cell membrane fluidity [43, 44]. DHA further influences multiple brain development processes, such as neurotransmitter release, gene expression, myelination, neuroinflammation, and neuronal differentiation [45–47]. While the mechanisms of DHA action remain unclear, growing evidence indicates that it plays a role in regulating neurogenesis and cell survivability. DHA has been shown to increase neurite outgrowth and dendritic branching in rat embryonic hippocampal primary cultures [48], as well as to promote axon outgrowth in rat cortical neurons [49, 50]. To date, there has been little effort to characterize the mechanism of action of DHA in human rather than rodent cells. Understanding its effects

could not only inform us of the compound's potential benefits, but also facilitate in the identification of more targeted treatments.

In the present study, we employ human induced pluripotent stem cell (iPSC)-derived neuronal cells to investigate the impact of DHA at a molecular and cellular level. Our study provides potential insight into the molecular and cellular mechanisms of DHA's antidepressant effect, and provides evidence supporting the stress-neuroplasticity hypothesis of MDD. In particular, our studies demonstrate DHA's effect on the canonical WNT/β-catenin pathway, an essential pathway involved in cell fate, cell proliferation, and cell migration that has been implicated in diverse aspects of the pathophysiology of many neuropsychiatric disorders [51–54]. Additionally, we demonstrate that DHA modulates CREB (cAMP response element-binding protein)-mediated transcription, which also plays an essential roles in numerous neuronal biological processes, including cell survival, synaptic structure, and synaptic plasticity [55]. The crosstalk between these two signaling pathways has been reported [56], but is not well understood in the context of human neuroplasticity. By demonstrating that DHA exposure regulates these pathways and by elucidating functional changes at the level of the transcriptome and neural development, we provide new insight into how DHA may affect the neural substrates critical for neuroplasticity.

Methods and Materials

Culturing and Neural Differentiation of Human iPSC-derived Neural Progenitor Cells (NPCs)

All tissue culture ware used for iPSC-derived NPCs and neurons were prepared by a double-coating (poly-ornithine/laminin) procedure to ensure cell adherence [57]. NPC8330–8 line [58] was used throughout the work unless noted, and cultured as previously described [57, 58]. WNT signaling and CREB signaling reporter NPC lines were generated from NPC8330–8 and described in detail in our previous publications [57, 59]. Four additional NPC lines used in the L1000 gene expression assay were generated from iPSC lines derived from four human subjects. To obtain post-mitotic neurons, NPC differentiation was initiated by removal of growth mitogens (EGF, bFGF) from the complete NPC media, and cultures maintained with medium replacement twice per week.

Compounds

The main source of docosahexaenoic acid (DHA) used in the study was acquired from Sigma Aldrich, St. Louis, MO (D2534–100MG). Three additional sources of DHA were tested in the WNT reporter assays (D2534–1G, Sigma Aldrich; U-84-A, Nu-Chek Prep Inc., Elysian, MN; Cayman chemical, Ann Arbor, MI, 90310). Other compounds used in the study include: Oleic acid (Nu-Chek Prep Inc.; U-46-A), crebinostat (custom synthesized compound), CHIR-99021 (custom synthesis), forskolin (Sigma Aldrich; F6886), S-(5'-Adenosyl)-L-methionine chloride dihydrochloride (SAMe; Sigma Aldrich; A7007).

Analytical LC/MS was performed on a Waters 2545 HPLC equipped with a 2998 diode array detector, a 2424 evaporative light scattering detector, a 2475 multichannel fluorescence

detector, and a Waters 3100 ESI-MS module, using a XTerraMS C18 5 μm, 4.6 × 50 mm column at a flow rate of 5 mL/min with a linear gradient (95% A: 5% B to 100% B 570 sec and 30 sec hold at 100% B, solvent A = water + 0.1% formic acid, solvent B = acetonitrile + 0.1% formic acid). LC/MS analyses were performed on DHA sources 1–4 (Supplemental Figure S7). For each DHA source, in addition to the dominant peak for DHA (*m/z* $(M-H)^-$ 327.3, [calculated $C_{22}H_{32}O_2$: 328.24]), several minor components (less than 1%) conforming to various oxidized forms of DHA were detected.

WNT and CREB signaling reporter assays

The reporter assays were performed as previously described [57, 59]. WNT reporter assays were performed in the absence or presence of 10% Wnt3a-conditioned media (Wnt3a-CM) with a 24-hour treatment. Wnt3a is a WNT signaling ligand, a low level of which was used to activate the WNT signaling pathway. CHIR-99021, a potent GSK3α/β inhibitor and an activator of WNT signaling, was used in the WNT reporter assay as a positive control. CREB reporter assays were performed in the presence of 10 μM forskolin, an adenylate cyclase activator, with a 6-hour treatment. Forskolin and crebinostat are known to activate the CREB signaling pathways based upon our previous studies [60], and were therefore used as positive controls. Treatment with DHA was used in doses between 0 and 50 μM. At the end of treatment, cells were lysed with SteadyGlo reagent (Promega) and read for luminescence. Activities of WNT or CREB signaling stimulation are expressed as fold change over DMSO-treated samples.

L1000 mRNA Profiling Assay

NPCs in 384-well plates at 10,000 cells per well were treated with compounds, and at the end of treatment, lysed in lysis buffer and stored at −80°C. Plates were then processed for L1000 mRNA profiling at the Broad Institute LINCS (Library of Integrated Cellular Signatures) Center. The L1000 assay directly measures the expression of 978 landmark genes that can be extended to measure of the whole genome with a computational inference model [61, 62]. Gene expression levels are expressed as z-scores, calculated from the entire 384-well plate. zi = (xi–median(X)) / (MAD(X)×1.4826), where X is the vector of normalized gene expression of gene x across all samples on the plate. The “median” and “MAD” represent median and median absolute deviation [MAD = median(IXi-median(X)I)], respectively.

Quantitative RT-PCR (qRT-PCR) analysis

qRT-PCR was performed as previously described [63]. NPCs or differentiated post-mitotic neurons in 6-well plates were treated with DHA (0 – 50 μM) or vehicle for a period of time specified in experiments (4 or 18 hours). Cells were then collected in 0.75 mL of TRIzol Reagent (Zymo Research Corp., Irvine, CA) at the end of the treatment period. RNA was extracted using Direct-zol RNA MiniPrep Kit (Zymo Research Corp., Irvine, CA), and RNA concentrations were measured using NanoDrop (Thermo Fisher). LifeTech high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used for cDNA synthesis with 1 μg of RNA, followed by TaqMan qRT-PCR reaction. qRT-PCR was run on Roche 480 Light Cycler and Ct/Cp values, normalized to internal control *GAPDH*, were used to calculate fold changes to vehicle control DMSO for mRNA expression levels.

Cell viability assays

NPCs or differentiated neuronal cultures were seeded to pre-coated 96-well plates (Corning 3903) at a density of 25,000 cells per well, followed by compound treatments (0 – 100 μM DHA; 0 – 5 μM CHIR) for 48 hours. Unpaired t test analysis was used to determine statistical significance ($p<0.05$, $n=10$).

PrestoBlue Cell Viability Assay.—The PrestoBlue Cell Viability Assay (Molecular Probes, Invitrogen) evaluates the redox state of cells by measuring cellular levels of the reduced form of nicotinamide adenine dinucleotide (NADH). PrestoBlue Reagent was diluted 10x in culture media of compound-treated cells, and plates were read after one-hour incubation at 37°C. Cell viability of DMSO-treated was set to 100%.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay.—The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780) quantifies the amount of cytosolic LDH released from damaged cells into the cell culture media. Following compound treatment in cells, 50 μL of media from all wells was transferred into a flat, clear bottom 96-well plate (CLS3789, Corning), to which an equal volume of CytoTox 96 Reagent was added and incubated at RT for 30 min in dark. Cell death of DMSO-treated was set to 1, and fold change was calculated by normalization to DMSO-treated.

BrdU Cell Proliferation Assay.—The bromodeoxyuridine (BrdU) Cell Proliferation ELISA Kit (ab126572; Abcam, Cambridge, MA) allows for the quantification of DNA synthesis and cell proliferation. BrdU was applied to compound-treated and control wells to allow for its incorporation into the DNA of proliferating cells. Once cell treatment (48 hours with BrdU added for the last 24 hours) completed, cells were fixed and washed, and processed following the manufacturer's ELISA protocol. BrdU incorporation of DMSO-treated cells was set to 100%.

Live cell imaging and analysis

NPCs grown in T75 flasks were differentiated for five days, then dissociated and seeded to 96-well plates (Corning 3904) at a density of 17,000 cells per well with 150 μL of media. Immediately after compound addition (0 – 20 μM DHA; 0 – 20 μM CHIR), plates were placed on IncuCyte ZOOM live-cell imaging platform (Essen BioScience, Ann Arbor, MI) in an incubator of 37°C with 5% CO_2. Nine images (20x) were captured from each well, which were repeated every hour for 24 hours. Phase contrast images were acquired at a resolution of 0.61 μm/pixel and segmented using IncuCyte ZOOM software (2016A). NeuroTrack Analysis package was used to segment unlabeled images and extract the following cellular growth metrics: Cell-Body Cluster Area and Neurite Length per Cell-Body Cluster Area. The metric data were exported and analyzed for dose response using R Studio and MatLab. Time course data for Neurite Length was aggregated by computing area under the curve (AUC) using trapezoidal integration of the baseline subtracted growth curve over the 24-hr time window. The values extracted from the initial image were used as baseline. 95% confidence intervals were calculated using the standard error multiplied by a t-statistic with N-1 degrees of freedom, where N=number of images segmented (63 for each dose of the compound treatments; 450 for DMSO treatment).

Statistical analysis

Data are expressed as Mean ± SEM. Unpaired t tests were used to determine treatment significance when comparing two samples. Stars of significance indicate a significant effect for a treatment dose compared to control (* $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$, **** $p < 0.0001$). When more than three doses of a compound were tested, a one-way ANOVA was used to determine statistical significance of any treatment effect relative to the control as well as to determine if the response was dose dependent. Tukey's honest significant difference (HSD) test was used to determine averages that were significantly different from one another as an ANOVA post-hoc test. P value comparing treatment to control: * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$, **** $p < 0.0001$. ‡ denotes a treatment that was significantly different from all other treatments tested. † denotes a treatment that was significantly different from control and some, but not all, other treatments tested.

Results

DHA upregulates WNT signaling in human NPCs

We previously reported on the development of a high-throughput WNT/β-catenin signaling reporter system generated in iPSC-derived NPCs [57]. In a screen of a compound library assembled with multiple commercially available FDA-approved, bioactive drug libraries and custom bioactive compounds, we observed that DHA in two pathway-biased libraries (Nuclear Receptor Ligand Library and WNT Pathway Library) showed a Wnt3a-dependent increase of WNT reporter activity (Supplemental Figure S1). To confirm this finding, we acquired an independent source of DHA and measured the WNT reporter response to 6 doses of DHA in the absence and presence of Wnt3a-conditioned media (Wnt3a-CM). DHA demonstrated a dose-dependent increase of WNT signaling in NPCs at concentrations of 12.5 and 25 μM with a maximum induction of about 3-fold in the presence of 10% Wnt3a-CM (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$), whereas no induction was detected in the absence of Wnt3a-CM (Figure 1).

To examine the specificity of DHA's effect, we tested oleic acid (OA), a structurally distinct, omega-9, monounsaturated fatty acid, in the same WNT reporter system. Unlike DHA, OA showed no increase of WNT reporter activity in the presence of 10% Wnt3a-CM (Figure 1D). Furthermore, to confirm our findings from our primary DHA source, and to determine if batch-to-batch variability of DHA impacted our findings, we tested three additional sources of DHA in either media alone or 10% Wnt3a-CM (Figure 1E). Results from DHA source 3 appeared comparable to those from our primary source, showing Wnt3a-dependent WNT reporter activity at 12.5, 25, and 50 μM concentrations (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$). Source 2 (different batch of source 1) and source 4 demonstrated weaker WNT reporter activity, with an approximately 1.5-fold increase at the highest concentration of 50 μM, suggesting a potential batch effect. No activity was detected in the media alone condition (data not shown). Collectively, across all four sources of DHA, we found DHA treatment significantly increased WNT reporter activity in Wnt3a-dependent and dose-dependent manners, suggesting WNT pathway activation upon exposure of human NPCs to DHA.

DHA upregulates endogenous WNT-regulated genes in NPCs

To investigate the molecular underpinnings for the observed WNT signaling enhancement by DHA in the engineered reporter system, we proceeded to measure endogenous WNT signaling-regulated genes in NPCs using the L1000 assay. We noted that several known WNT-regulated genes are amongst the set of measured landmark genes, which we have shown by L1000 profiling in NPCs to respond to Wnt3a and CHIR-99021 treatment (Supplemental Figure S2). Wnt3a-CM produced a significant increase in expression of several WNT-related genes, including *CCND1* (Cyclin D1), *CXCR4* (C-X-C Motif Chemokine Receptor 4), *HES1* (Hes Family bHLH Transcription Factor 1), *ID2* (Inhibitor of DNA binding 2), and *JUN* (Jun Proto-oncogene, AP-1 Transcription Factor Subunit), and a decrease in expression of *SOX2* (SRY-box 2). Dose-dependent changes in expression levels of the six WNT genes were detected for CHIR-99021, and the data revealed that CHIR-99021 regulates the expression of these genes in a similar fashion to Wnt3a with the exception of *JUN*, which was up-regulated by Wnt3a but down-regulated by CHIR-99021, indicating a unique signature of CHIR-99021 in WNT signaling regulation. The time-course measurement shows that the expression of these WNT genes peak at different time points, suggesting the dynamic expression of different genes in human NPCs in response to WNT pathway activation.

To determine if DHA similarly regulates endogenous WNT-regulated genes, we examined an additional L1000 data set collected with multiple dose treatments of DHA for 24 hrs. The expression of the six WNT genes described above was extracted from a larger dataset for CHIR-99021, DHA, and for comparison of a different type of neutriceutical SAMe (S-adenosyl methionine). However, we found that SAMe did not activate WNT signaling in the WNT reporter assays in NPCs (data not shown). Shown in Figure 2, the data for CHIR-99021 was consistent with previous findings. DHA, in a dose-dependent manner, increased the gene expression of *CXCR4*, *HES1*, *ID2*, and *JUN*, and decreased that of *CCND1* and *SOX2*, whereas SAMe showed no regulation of these WNT genes, consistent with its lack of activity in the WNT reporter assay. Our findings of the six WNT genes in response to DHA treatment measured by L1000 profiling was replicated in four additional iPSC-derived NPC lines (Supplemental Figure S3). Measured by the L1000 assay, 5 out of 6 WNT genes showed the same regulation pattern for DHA, compared to Wnt3a, except *CCND1*, which was down-regulated by DHA but up-regulated by Wnt3a, suggesting that DHA has a unique signature in regulating WNT signaling.

DHA enhances expression of the adult neurogenesis marker PROX1

WNT signaling is a significant regulator of adult neurogenesis in the hippocampus (Figure 3A) [52], and the WNT-regulated gene, *PROX1* (Prospero Homeobox 1) is a marker gene for hippocampal dentate gyrus (DG) granule cell maturation and intermediate progenitor cell maintenance [64–66]. Suggested by our finding of DHA-induced up-regulation of WNT signaling, we proceeded to test if DHA promotes adult neurogenesis by monitoring the expression of *PROX1*. We treated NPCs at the initiation of differentiation with DHA in the absence or presence of 10% Wnt3a-CM and measured mRNA expression of *PROX1* (Figure 3B). Notably, *PROX1* expression was significantly increased by DHA treatment in combination with Wnt3a at both 4 hours and 18 hours (Figure 3C). One-way ANOVA with

Tukey's HSD post-hoc testing revealed that this effect was significant at both time points ($P<0.001$ at 4 hours; $P<0.0001$ at 18 hours), but only dose-dependent at 18 hours with a 50 μM treatment. This response was absent in cells without Wnt3a-CM addition, demonstrating a WNT-dependent increase in expression.

DHA upregulates CREB signaling in NPCs

Examination of L1000 mRNA expression data revealed that the expression of *EGR1*, an L1000 landmark gene, was induced up to 10-fold by 24-hour DHA treatment in NPCs in multiple independent measurements (Supplemental Figure S4). The *EGR1* L1000 data prompted us to investigate whether DHA enhances CREB signaling, using a CREB signaling reporter system developed in the lab [59]. We treated the reporter NPCs for 6 hours with DHA at 8 concentrations in 2-fold dilution, as well as two known activators in CREB signaling: forskolin [67] and crebinostat [60]. As expected, forskolin and crebinostat significantly increased CREB reporter activity (Figure 4A). DHA showed a 2.5-fold increase of CREB signaling at the concentration of 50 μM. This response was dose-dependent at values of 25 μM and 50 μM DHA (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$). Oleic acid, used here as a negative control, did not stimulate CREB activity at any of the tested concentrations (Figure 4A).

Having detected the induction of CREB signaling in the reporter system, we went on to determine if DHA treatment leads to the regulation of CREB signaling target genes in human iPSC-derived NPCs and neurons by qRT-PCR analysis. In NPCs, 6-hour DHA treatment significantly increased mRNA expression of *EGR1* in a dose-dependent manner (Figure 4B), consistent with the data from L1000 analysis (Supplemental Figure S4). This increase in expression followed a dose-dependent pattern (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$), with a 3-fold increase at a treatment concentration of 50 μM. In two-week differentiated neurons, in addition to *EGR1*, we also probed *BDNF* (Brain Derived Neurotrophic Factor), which plays an important role in the survival of neurons. We detected small, but significant, increases in *BDNF* expression at 50 and 100 μM, and about 3-fold increase in *EGR1* expression at 100 μM (Figure 4C). These data indicate that DHA up-regulates the transcription of CREB signaling target genes in both NPCs and post-mitotic neurons.

DHA enhances human iPSC-derived neuronal cell viability

Having shown the activation of WNT and CREB signaling pathways by DHA, we proceeded to examine potential phenotypic effects of pathway activation in human neuronal cell cultures. We conducted a series of cell viability assays to assess DHA's impact on the viability and survival of human NPCs and immature post-mitotic neurons. Three cell states were used to determine the effect of DHA: i) NPCs (Figure 5A, D), ii) NPCs at the initiation of differentiation (Figure 5B, E), and iii) neuronal cultures differentiated for one week (Figure 5C, F). Cells were seeded in 96-well plates and treated with DHA at the concentrations of 2.5, 5, 10 and 25 μM, as well as CHIR-99021 (5 μM) and vehicle control DMSO. After two days, cell viability was measured via the PrestoBlue assay, which measures the metabolic state of viable cells through NADH redox potential (Figure 5A, B, C). For all three cell states, we detected a statistically significant increase of cell viability

with CHIR-99021 (5 μM) treatment. Similarly, DHA treatment at three concentrations (5, 10 and 25 μM) showed a statistically significant increase and dose-dependent response in cell viability for almost all cell states (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$).

To determine if reduced cell death contributed to the observed increased cell viability measured by PrestoBlue for both DHA and CHIR-99021, we turned to a lactate dehydrogenase (LDH) assay, which quantifies the LDH released from dead cells with permeable membranes (Figure 5D, E, F). For all three cell states described above, DHA treatment at both 5 and10 μM, lowered LDH release levels, with a significant dose-dependent response for these treatments (one-way ANOVA with Tukey's HSD post-hoc, $P < 0.0001$). These data suggest that reduced cell death contributed to the apparent increased cell viability measured by PrestoBlue at these doses, but not above 25 μM where reduced LDH levels was not observed. Similarly, reduced LDH levels was also detected in CHIR-99021-treated NPCs and NPCs released into differentiation, whereas a modest increase was observed in 1-week differentiated neuronal cultures, also suggesting that CHIR-99021 treatment reduces cell death across cell states.

To more directly determine whether cell proliferation contributes to the increased cell viability measured by PrestoBlue assay in NPCs and NPCs after the initiation of differentiation, we conducted a BrdU incorporation assay. In this assay, BrdU incorporates into newly synthesized DNA only in actively replicating cells. Proliferating NPCs or NPCs induced to differentiate were seeded and treated with DHA at varying concentrations (2.5–25 μM) for a total of 48 hours, with the last 24 hours in the presence of BrdU. Cells were then fixed, and an ELISA performed to quantify the amount of BrdU incorporation. As shown in Supplemental Figure S5, apart from the significant increase of BrdU incorporation detected for CHIR-99021 treatment in both cell states, a statistically significant increase in BrdU incorporation was only detected for DHA at 5 μM in NPCs, a condition in which the PrestoBlue assay of NADH levels did not detect any increase (Figure 5A). No increase was detected for other concentrations of DHA tested other than the decrease detected for 5 μM in NPCs at the initiation of differentiation and 25 μM in both cell states. Therefore, our results suggest that cell proliferation is likely not a contributing factor to the increased cell viability by DHA thereby distinguishing DHA mechanistically from CHIR99021 treatment.

To resolve the seemingly conflicting observation that CHIR-99021, depending on the assay, apparently both increased cell death and increased cell viability on 1-week differentiated neuronal cultures, we performed an experiment in which 1-week differentiated neuronal cultures were dissociated, seeded and treated as described above. After 2 days, the culture was fixed and immunostained for neuronal dendritic marker MAP2 and nuclei, followed by image acquisition and quantification of nuclei (Supplemental Figure S6). Fewer neuronal processes and more NPC-like cells were evident in the CHIR-99021-treated cultures in comparison to the DMSO- or DHA-treated. Quantification of cell nuclei number showed that CHIR-99021 significantly increased the number of cells, whereas no difference between DHA and DMSO was detected. These results indicate that CHIR-99021 promotes NPC proliferation and may block or delay neuronal differentiation from the resulting cell population thereby accounting for both increased cell viability and increased cell death

simultaneously detected for CHIR-99021 and further distinguishing its mechanism of action from that of DHA.

DHA promotes neurite outgrowth of human neuronal cells

Previous studies in rodents suggest that DHA increases neurite length [49, 50]. Thus, in addition to DHA's impact on the viability of NPCs and neuronal cultures, we next sought to determine whether DHA treatment affects neurite outgrowth on human neuronal cultures. Given our data supporting a possible role for DHA in pathways related to neuronal plasticity, stress, and cell fate, we implemented real-time, live-cell imaging of human neuronal cultures to measure neurite outgrowth over 24 hours of DHA treatment. For these assays we seeded 5-day differentiated neuronal cultures In 96-well plates and applied treatments of DMSO (0.02%), DHA (0.63 – 20 μM), and CHIR-99021 (0.63 – 20 μM). Using the IncuCyte Zoom live-cell imaging and analysis platform, images taken from wells were first segmented for cell bodies and neurites prior to analysis of neurite parameters (Figure 6A). Total neurite length was normalized by cell body area on a per image basis. DHA treatment resulted in a dose-dependent increase in the average neurite length in comparison to DMSO-treated cells (Figure 6B, left), whereas CHIR-99021 treatment reduced neurite outgrowth (Figure 6B, right). These analyses were combined from two biological replicate experiments (in total, n=7 wells, 9 images/well for drug treatments; n=50 wells, 9 images/well for DMSO treatment). The area under the curve (AUC) was computed across the 24-hour window to provide an aggregate measure of growth (Figure 6C). DHA treatment at 10 μM and 20 μM significantly increased neurite outgrowth. In contrast, CHIR-99021 treatment at 5, 10, and 20 μM significantly decreased average neurite length, consistent with the data presented above (Supplemental Figure S6), indicating that CHIR-99021 attenuates, while DHA enhances, neuronal differentiation.

Discussion

Our current study provides pre-clinical evidence for the beneficial effects of DHA, a popular complementary medicine for MDD, in multiple pathways that function as critical mediators of neuroplasticity.

Using a WNT reporter assay, we found that DHA enhances WNT activity in a Wnt3a-dependent manner. The WNT pathway plays an essential role in processes such as cell migration, cell fate determination, and neurogenesis [51, 52, 68]. The promotion of WNT signaling may lead to improved cell survival, particularly throughout differentiation. DHA's enhancement of WNT signaling and WNT gene expression indicates the promotion of factors to protect, support, and promote differentiation of neurons. Notably, DHA treatment significantly increased the mRNA expression of the key WNT signaling pathway regulated gene *PROX1* (Prospero Homeobox 1) [64]. PROX1, a transcription factor, acts as a critical regulator of neurogenesis in both the embryonic central nervous system and in the adult DG of the hippocampus [69, 70]. Prior studies in rodent cells have shown that PROX1 expression in the hippocampal dentate gyrus (DG) plays a key instructive role in post-mitotic granule cell fate with over expression of PROX1 in immature hippocampal CA3 pyramidal neurons being sufficient to drive cells toward a granule cell fate [65]. Conversely

the removal of PROX1 from immature DG neurons has been shown to cause a switch to CA3 pyramidal neuron properties [66]. While the length of our DHA treatments were of a rather short-term nature, future studies with prolonged DHA exposure should be performed to ultimately determine if *PROX1* induction is sustained enough to alter hippocampal dentate granule cell maturation as well as proliferation of intermediate progenitor cells.

Beyond WNT signaling, motivated by evidence to support DHA's potential role in memory [71, 72], we found that DHA also enhanced the CREB pathway and CREB-regulated genes. CREB activity has been linked to neuroprotective pathways and neuronal plasticity [73], and is critical in neuronal development. For example, Mantamadiotis et al. [74] found postnatal deletion of CREB in mice led to hippocampal neurodegeneration in the CA1 pyramidal cell layer and DG. CREB target genes, such as *BDNF* and *EGR1*, are critical for neuronal survival and cell fate [75]. BDNF is a key regulator of neuronal plasticity in the hippocampus [76] and decreased gene expression has been linked to depression [77] and Alzheimer's disease [78]. EGR1 is a transcriptional regulator of the incorporation of immature neurons into hippocampal circuitry in the adult mammalian brain, with its expression supporting the recruitment of new neurons [79].

In our present study, we showed that DHA activates the WNT signaling pathway, an activity shared with CHIR-99021, which in our previous work has been demonstrated repeatedly to increase WNT signaling [57]. When NPCs and NPCs at the initiation of differentiation were analyzed, both compounds behaved similarly in viability assays, i.e., increased cell viability measured by PrestoBlue detection of NADH levels and decreased cell death by an LDH assay. However, our study also revealed differences in the cellular effects of these two compounds. Using immature neurons differentiated for only 1 week, we found that CHIR-99021 increased cell viability as well as increased cell death (Figure 5), which prompted us to perform immunocytochemistry experiments that showed that CHIR-99021 enhanced cell proliferation (BrdU incorporation) at the same time that other cells in the culture are *en route* to becoming post-mitotic neurons (Supplemental Figure S6). In our live-cell imaging assay monitoring neurite outgrowth, we detected DHA promoting neurite outgrowth, whereas CHIR-99021 reduced neurite outgrowth and increased cell body cluster area (Figure 6). Since two components – neuronal regeneration and differentiation – exist at the time of cell seeding and compound treatment, the reduced neurite outgrowth detected for CHIR-99021 could result from CHIR-99021 either blocking neuronal differentiation or promoting regeneration, therefore, delaying neuronal differentiation, which may or may not catch up at a later time. In addition to the cellular phenotypic differences between CHIR-99021 and DHA, they do differ in modulation of CREB signaling: DHA activates CREB signaling, but CHIR-99021 does not (unpublished data), which underscores a possible mechanistic basis for their different phenotypic outcomes.

In various assays, the effective concentrations of DHA vary. Activation of WNT and CREB signaling reporter activities was generally detected with concentrations above 12.5 μM, but a drop could appear at high concentrations, such as 50 and 100 μM, signaling either a cytotoxic or competing biological effect. However, regulation of WNT signaling-regulated genes measured by L1000 or regulation of CREB signaling-regulated genes measured by qRT-PCR was mostly detected at high concentrations of DHA (50 and 100 μM).

Furthermore, DHA at 5, 10, and 25 μM were detected to increase cell viability measured by PrestoBlue, whereas only 5 and 10 μM, but not 25 μM, decreased cell death. Different effective concentrations of DHA have also been reported in literature. A concentration of 1.5 μM DHA/albumin was shown to increase neurite outgrowth from cultured rat primary hippocampal neurons after six days of treatment [48], while 25 μM DHA enhanced cell survival in mouse neuroblastoma Neuro2A cells after 48 hours [80]. These differences may be attributed to the nature of different types of measurement or to the state of cells at the time of assays. In addition to varying concentrations, varying time frames were used for distinct experiments. Each was optimized according to the assay, and the differences in timing to note an effect of DHA may again be related to the differences what was being measured or differences in cell states across experiments. More investigation is necessary in order to understand the difference of efficacious concentrations of DHA in various assays.

In terms of potential molecular mechanisms that link DHA treatment to the activation of WNT and CREB signaling, one potential effect is through modifying lipid rafts [43, 44]. Lipid rafts are specialized plasma membrane domains and one of their major functions is to modulate signaling originating from the plasma membrane. It has been shown that addition of DHA to cells changes lipid raft stability [43] as well as modifies G-protein signaling [44]. It would be interesting in the future to investigate whether the addition of DHA to NPCs or neurons modifies the properties of lipid rafts on membranes to alter WNT and CREB signaling.

A limitation of our study is the fact that our cellular phenotypic experiments did not address how DHA impacts synaptic signaling or circuits. Neurite outgrowth is not indicative of what happens at the synapse; in fact, the impact is on developing neurons. It is critical in the future to further explore whether DHA impacts synaptic structure using immunostaining or if it affects synaptic activities by multi-electrode arrays, or patch-clamp electrophysiology. A second limitation of the present study is that total fatty acid levels in cells were not determined for each experiment. In retinoblastoma and neuroblastoma cells, DHA has been shown to elongate to tetracosahexaenoic acid and retroconvert DHA to EPA [81]. No study to date has examined this phenomenon in neural progenitor cells and neurons, yet this remains a possible underline mechanism for our results.

Conclusions

Our use of an *ex vivo* human iPSC-derived neuronal cell culture system to probe the effects of DHA at the molecular and cellular levels shows that DHA activates WNT and CREB signaling pathways, which may contribute to increased neuronal cell viability, and neuronal connectivity. Thus, our study provides insight into the molecular and cellular mechanisms of DHA's effect on neuroplasticity, which may in turn be leveraged in the future to develop more effective therapeutics and preventative treatments for MDD and related neuropsychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Human iPSC-derived neuronal cell culture system was used to investigate the molecular and cellular mechanisms of DHA's effect on neuroplasticity
- DHA activated WNT and CREB signaling pathways in human neural progenitor cells
- DHA enhanced the survival of iPSC-derived NPCs and differentiating post-mitotic neurons
- DHA increased neurite outgrowth monitored by real-time live-cell imaging

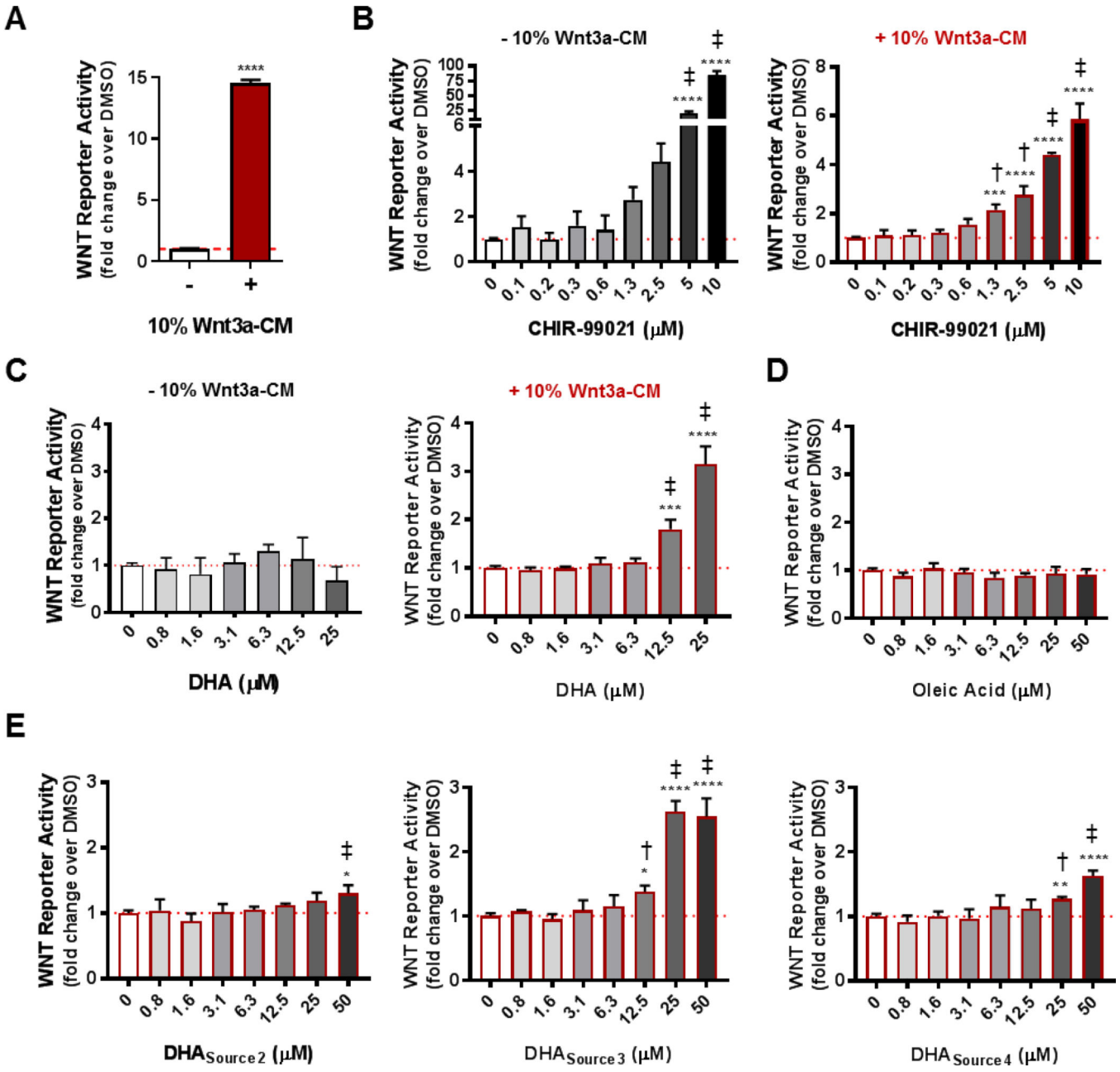

Figure 1. Wnt3a-dependent increase of WNT signaling in human NPCs by DHA.

(**A**) The Wingless/Integrated (WNT) reporter system in human iPSC-derived NPCs is responsive to WNT signaling ligand Wnt3a. About 15-fold induction of the reporter activity was detected from 10% Wnt3a-conditioned media (Wnt3a-CM). (**B**) CHIR-99021, a potent GSK3α/β inhibitor and an activator of WNT signaling, demonstrated a dose-dependent increase in WNT reporter activity in either the absence or the presence of 10% Wnt3a-CM. (**C**) DHA increased WNT reporter activity in the presence of 10% Wnt3a-CM but not in the absence, indicating the Wnt3a-dependent induction of WNT signaling. Dose-dependent effect was detected for 12.5 and 25 μM by Tukey post-hoc. (**D**) Oleic acid (OA) showed no WNT reporter activity. (**E**) Multiple additional sources of DHA were shown to increase

WNT reporter activity in the presence of 10% Wnt3a-CM. Bars with red outline denote the test was done in the presence of 10% Wnt3a-CM. Dashed red line drawn at 1 indicates fold changes are calculated by normalization to DMSO-treated (0 μM). Error bars display standard error of the mean (SEM) of quadruplicate measurements in one of 3 biological replicates. One-way ANOVA with Tukey's HSD post-hoc testing confirmed dose-dependent effects. P value comparing treatment to control: * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$, **** $p < 0.0001$. ‡: Denotes a treatment that was significantly different from all other treatments tested. †: Treatment that was significantly different from control and some, but not all, other treatments tested.

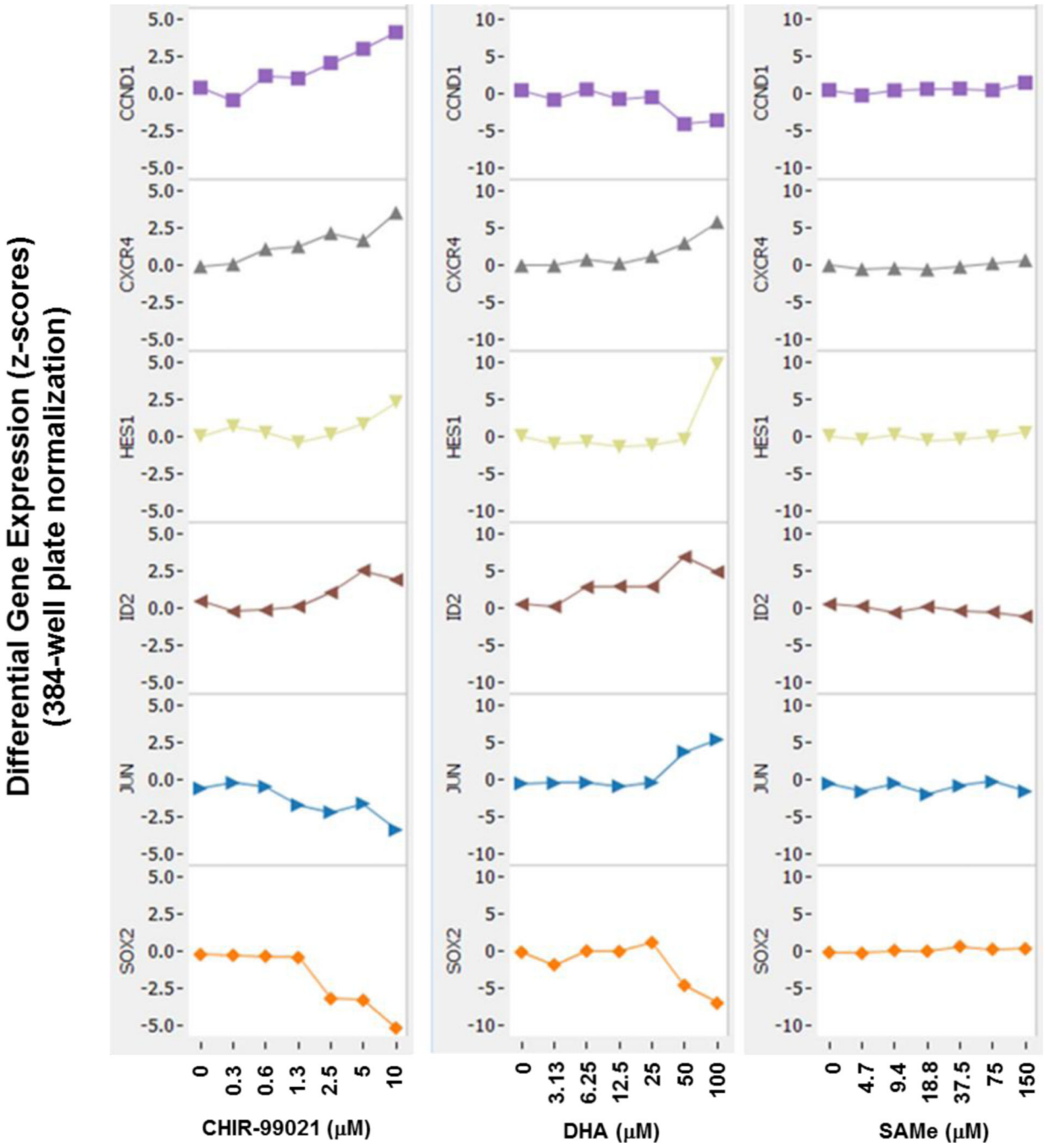

Figure 2. DHA regulates expression of WNT signaling-regulated genes.

Selected mRNA expression profiles of 6 WNT signaling-regulated genes generated by L1000 mRNA expression profiling assay for DHA, as well as CHIR-99021 and S-adenosyl methionine (SAMe). Each mRNA expression level is expressed as z-scores, which were calculated by normalizing across the 384-well microplate. Data points represent the mean of 3 biological replicates, presented by the BREW level data from L1000 pipeline. Graphs were generated with the built-in utility in the custom data-mining tool GENE-E, developed by the LINCS Center at the Broad Institute.

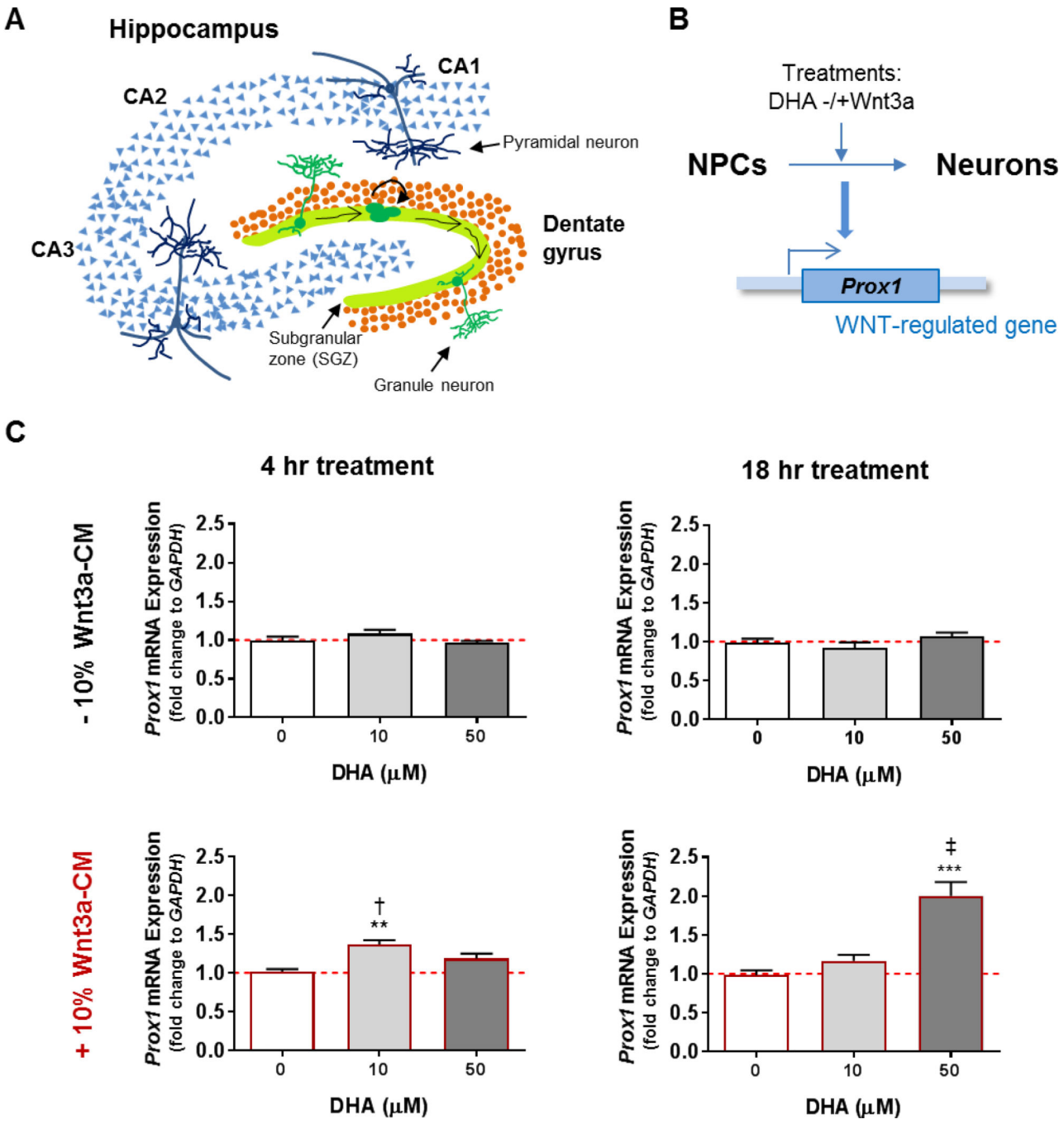

Figure 3. DHA promotes expression of hippocampal adult neurogenesis marker *PROX1*.

(**A**) Schematic representation of hippocampal regions and dentate gyrus granule neurons. Neural stem cells give rise to granule neurons that integrate into existing circuitry in the dentate gyrus. (**B**) Overview of the treatment protocol for measuring mRNA expression levels. NPCs were treated with DHA while simultaneously initiating differentiation through growth factor withdrawal. After incubation, WNT-regulated Prospero Homeobox 1 (*PROX1)* expression was probed using qRT-PCR. (**C**) mRNA expression of *PROX1* gene after 4 and 18 hours of treatment with DHA in either media alone or Wnt3a-CM. All gene expression

values were analyzed with $2^{-\Delta\Delta Ct}$ method, and first normalized to internal control *GAPDH*, then to DMSO-treated (0 μM), indicated by the dashed red line at 1. Error bars display standard error of the mean (SEM) of 3 biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's HSD post-hoc test (* $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$). ‡: Denotes a treatment that was significantly different from all other treatments tested. †: Treatment that was significantly different from control and some, but not all, other treatments tested.

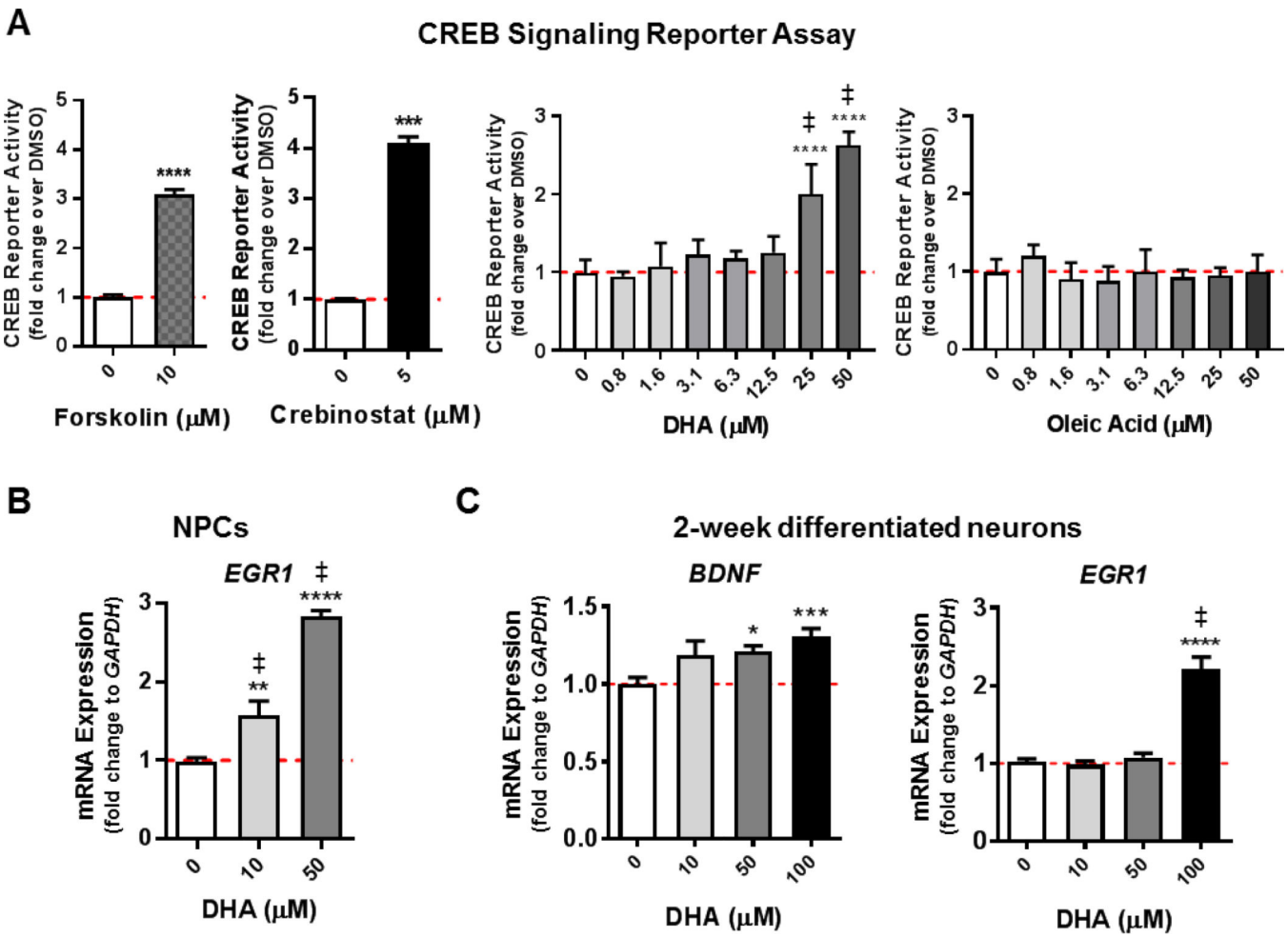

Figure 4. DHA up-regulates CREB signaling and target gene expression.

(**A**) cAMP response element binding (CREB) signaling reporter assays in human NPCs showing 6-hr DHA treatment increased CREB reporter activity at 25 and 50 μM, as well as two positive controls forskolin and crebinostat. No induction of CREB reporter activity was detected from oleic acid. Dashed red line at 1 indicates fold changes are calculated by normalization to DMSO-treated (0 μM). Error bars display standard error of the mean (SEM) of quadruplicate measurements. (**B**) Increase of Early Growth Response 1 (*EGR1*) mRNA expression was detected by qRT-PCR after 6-hour DHA treatment in NPCs. (**C**) Increased Brain Derived Neurotrophic Factor 1 (*BDNF*) and *EGR1* expression in 2-week differentiated neurons was detected by qRT-PCR following 6-hour DHA treatment. All bar graphs normalized to DMSO-treated (0 μM), indicated by the dashed red line at 1. Error bars display standard error of the mean (SEM) of 3 biological replicates. Statistical significance determined by unpaired t-test and one-way ANOVA with Tukey's HSD post-hoc to determine dose-dependency (* $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$, **** $p < 0.0001$). ‡: Denotes a treatment that was significantly different from all other treatments tested. †: Treatment that was significantly different from control and some, but not all, other treatments tested.

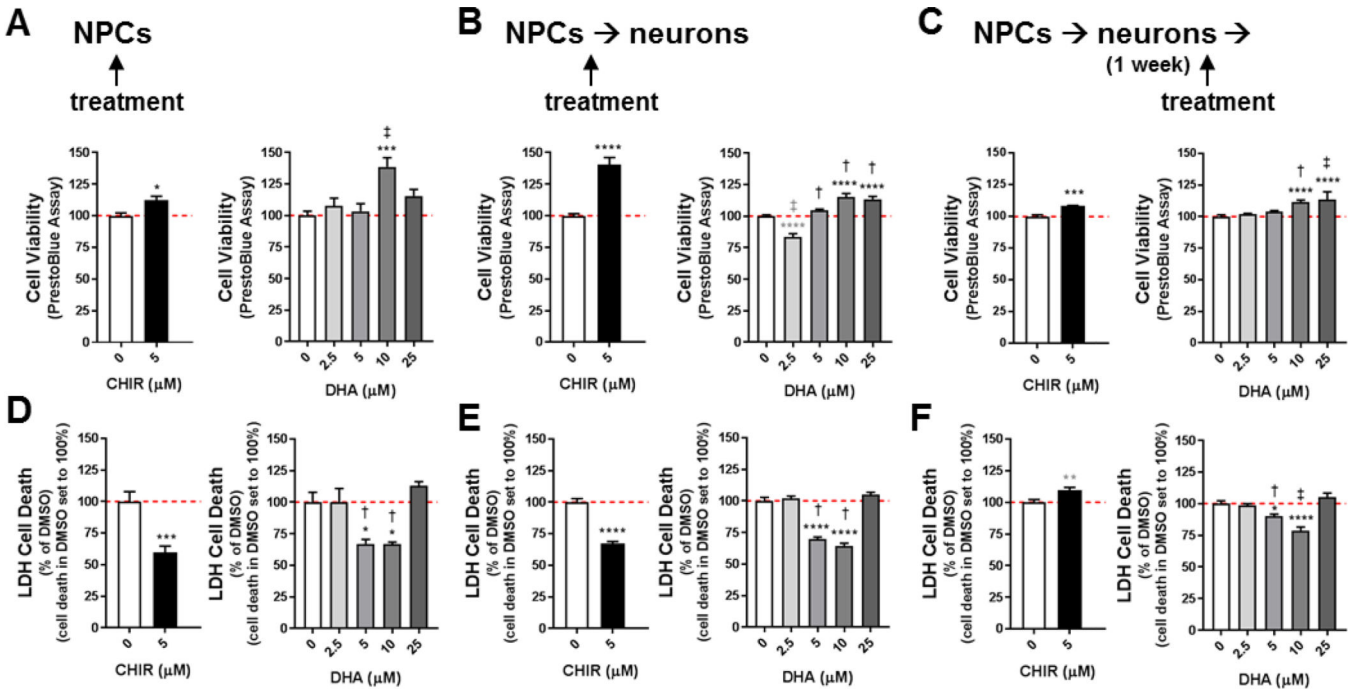

Figure 5. DHA enhances neuronal cell viability.

A PrestoBlue assay was used to measure the redox state from live cells via levels of reduced NADH, and an LDH assay was used to monitor cell death after 1–2 days of DHA (2.5, 5, 10, 25 μM) or CHIR-99021 (5 μM) treatment. For PrestoBlue assay, cell viability of DMSO-treated (0 μM) was set to 100%, indicated by dashed red lines. For LDH assay, cell death of DMSO-treated (0 μM) was set to 1, indicated by dashed red lines, and fold change was calculated by normalization to DMSO-treated. Assays were performed on (**A,D**) NPCs, (**B,E**) NPCs at the initiation of differentiation, and (**C,F**) one-week differentiated neurons. (**A,D**) DHA at 10 and 25 μM increased NPC viability, and at 5 and 10 μM reduced cell death. (**B,E**) For NPCs released into differentiation, and (**C,F**) on one-week differentiated neurons, DHA increased cell viability at 5, 10, and 25 μM, while reduced cell death at 5 and 10 μM. 5 μM CHIR-99021 was used as a positive control, which increased cell viability for all three cell states, reduced cell death for NPCs and NPCs released into differentiation, but increased cell death for one-week differentiated neurons. N=10. Error bars display SEM. Statistical significance determined by unpaired t-test and one-way ANOVA with Tukey's HSD post-hoc to determine dose-dependency. * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $0.0001 \leq p < 0.001$, **** $p < 0.0001$. ‡: Denotes a treatment that was significantly different from all other treatments tested. †: Treatment that was significantly different from control and some, but not all, other treatments tested.

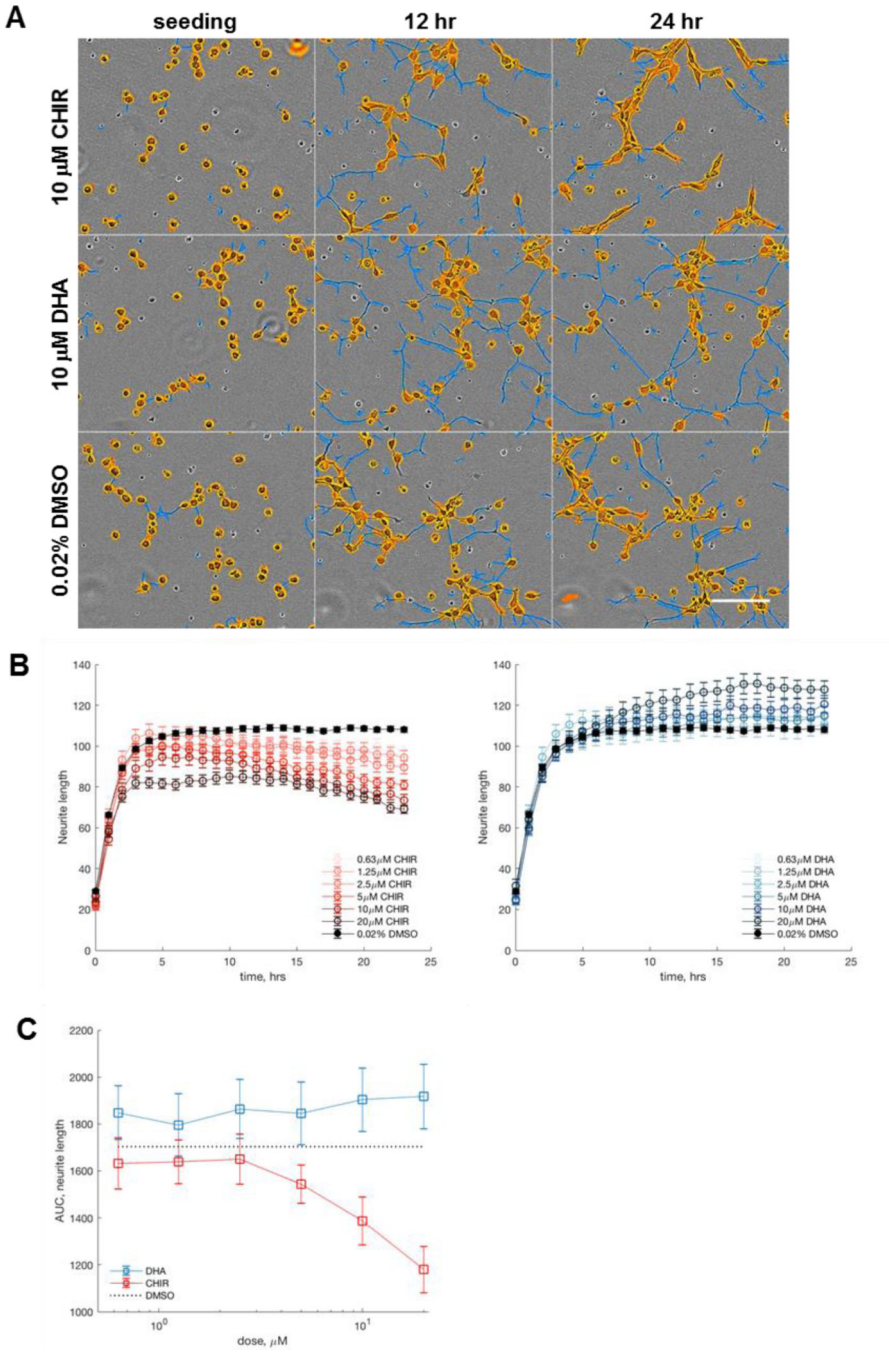

Figure 6. DHA promotes neurite outgrowth.

NPC cultures differentiated for 5 days and subsequently dissociated and seeded in 96-well plates were treated with DHA and CHIR-99021 (0.63 – 20 μM), as well as vehicle control DMSO. Plates were immediately placed on IncuCyte ZOOM live-cell imaging platform. 9 images (20x) were captured from each well, which was repeated every hour for 24 hours. Images were processed with NeuroTrack Analysis for cell body cluster area and neurite length. (**A**) Representative images of segmented neurites (blue) and somas (yellow) for 10 μM CHIR-99021 (CHIR), 10 μM DHA, and 0.02% DMSO (rows) for 3 timepoints

(columns). Scale bar: 100 μm. (**B**) Treatment with DHA (left) increased neurite outgrowth in a dose dependent manner over the 24-hour time course in comparison to DMSO-treated cells. CHIR-99021 treatment (right) resulted in decreased neurite outgrowth. Neurite length measurements were normalized to cell body cluster area. (N=63 images for each compound dose; N=450 images for DMSO treatment; error bars indicate SE). (**C**) Aggregate measure of neurite outgrowth (Area Under the Curve, AUC) showed significant increase for 10 and 20 μM DHA, and significant decrease for 5, 10, and 20 μM CHIR-99021 treatment (N=63 for each compound dose; N=450 for DMSO treatment; ANOVA, $p<0.01$; error bars indicate 95% CI).