Docosahexanoic acid signals through the Nrf2–Nqo1 pathway to maintain redox balance and promote neurite outgrowth

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ABSTRACT Evidence suggests that n-3 polyunsaturated fatty acids may act as activators of the Nrf2 antioxidant pathway. The antioxidant response, in turn, promotes neuronal differentiation and neurite outgrowth. Nrf2 has recently been suggested to be a cell intrinsic mediator of docosahexanoic acid (DHA) signaling. In the current study, we assessed whether DHA-mediated axodendritic development was dependent on activation of the Nrf2 pathway and whether Nrf2 protected from agrochemical-induced neuritic retraction. Expression profiling of the DHA-enriched Fat-1 mouse brain relative to wild type showed a significant enrichment of genes associated with neuronal development and neuronal projection and genes associated with the Nrf2-transcriptional pathway. Moreover, we found that primary cortical neurons treated with DHA showed a dose-dependent increase in Nrf2 transcriptional activity and Nrf2-target gene expression. DHA-mediated activation of Nrf2 promoted neurite outgrowth and inhibited oxidative stress-induced neuritic retraction evoked by exposure to agrochemicals. Finally, we provide evidence that this effect is largely dependent on induction of the Nrf2-target gene NAD(P)H: (quinone acceptor) oxidoreductase 1 (NQO1), and that silencing of either Nrf2 or NQO1 blocks the effects of DHA on the axodendritic compartment. Collectively, these data support a role for the Nrf2-NQO1 pathway in DHA-mediated axodendritic development and protection from agrochemical exposure.

INTRODUCTION
In a healthy cell, redox imbalance is controlled by enzymes that detoxify the cell of free radicals. These enzymes are part of the cell’s antioxidant response, a transcriptional pathway that activates ~800 antioxidant response genes. The master regulator of this pathway is a transcription factor called Nrf2. Our previous work has established that inhibition of the antioxidant response, in turn, leads to axonal pathology through loss of expression of microtubule stabilizing proteins, whereas reactivation of this pathway can rescue axonal neuropathology, and promote neurite outgrowth (Czaniecki et al., 2019). Agrochemical-evoked redox stress can alter transcription factor activity in neurons derived from patients with Parkinson’s disease (PD) as well as in agrochemically exposed animals relative to controls (Ryan et al., 2013) and we have further determined that the antioxidant response is among the transcriptional hubs altered in the brains of PD patients (Czaniecki et al., 2019). Indeed, exposure of neurons to agrochemicals triggers cellular stress events that mechanistically overlap with those evoked by mutations of the α-synuclein gene (SNCA) that result in familial PD, including neuritic retraction, axodendritic pathology, and oxidative stress (Ryan et al., 2013; Stykel et al., 2018; Czaniecki et al., 2019). Studies on the epidemiologic relationship between PD and exposure to

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Abbreviations used: ARE, antioxidant response elements; ChIP, chromatin immunoprecipitation; DEGs, differentially expressed genes; DHA, docosahexanoic acid; DMP, dimethyl fumarate; DPBS, Dulbecco’s phosphate-buffered saline; GO, gene ontology; HSE, heat shock element; LOEL, lowest observed effect level; MB, maneb; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NQO1, NAD(P)H:quinone acceptor oxidoreductase 1; PD, Parkinson’s disease; PQ, paraquat; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SA, steric acid.
agrochemicals determined that exposure to the herbicides paraquat (PQ) and maneb (MB) is associated with >200% increase in risk of PD (Pezzoli and Cereda, 2013). Moreover, in those individuals with causal familial mutations (e.g., SNCA-G209A, a mutation in the synuclein gene that causes the protein to misfold), agrochemical exposure correlates with disease onset at an earlier age (Gatto et al., 2010). Identifying ways to counteract the increase in disease risk associated with pesticide exposure would be of significant benefit.

Evidence suggests that n-3 PUFAs may act as activators of the Nrf2 antioxidant pathway (Zhang et al., 2014; Gruber et al., 2015; Zhu et al., 2018). In animal models of ischemic injury, docosohexaenoic acid (DHA) has been reported to activate the antioxidant response via an Nrf2-dependent mechanism, thereby reducing neuronal loss acutely induced by oxidative stress (Zhang et al., 2014). Mechanistically, n-3 PUFA-derived radicals have been reported to interact directly with Keap1, inhibiting Keap1-mediated degradation of Nrf2 and allowing for Nrf2 nuclear translocation and activation of antioxidant response elements (AREs; Gao et al., 2007; Mildenberger et al., 2017). In epidemiological studies, data from 7983 subjects who consumed a diet high in n-3 and n-6 PUFA, or more specifically DHA, have a significantly reduced risk of PD (de Lau et al., 2005). In experimental models of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent inhibitor of mitochondrial respiration and inducer of reactive oxygen species (ROS) accumulation, mice fed a diet high in n-3 PUFA were protected from loss of dopamine-producing neurons and displayed less axonal pathology relative to MPTP control animals (Bousquet et al., 2008). By contrast, dietary restriction of n-3 fatty acids has been shown to promote axodendritic pathology in dopaminergic and nondopaminergic neurons, including neuritic retraction and axonal varicosities (Cao et al., 2009; Cardoso et al., 2014). The role of Nrf2 in mediating the protective effects of DHA remains unclear, as it is difficult to distinguish the protective effects of the antioxidant response from the known anti-inflammatory effects of DHA.

In the current study, we therefore assessed whether DHA could counteract the neurobiological deficits evoked by PQ/MB exposure through activation of the Nrf2-dependent antioxidant response with a focus on axodendritic pathology and redox stress. To this end, we employed a series of gain of function, loss of function experiments to determine whether DHA stimulated axodendritic projections depend on Nrf2 transcriptional activity. Expression profiling of the DHA-enriched Fat-1 mouse brain relative to wild type (WT) showed a significant enrichment of genes associated with neuronal development and neuronal projection in Fat-1 mice with an equally strong enrichment of genes associated with transcriptional regulation. We find that DHA increases expression of Nrf2 target genes in both Fat-1 transgenic mouse brain relative to WT and in primary rat cortical neurons treated with DHA relative to steric acid (SA). Moreover, we find that DHA-mediated activation of Nrf2 inhibits oxidative stress and neuritic retraction evoked by exposure of neurons to PQ/MB. Finally, we provide evidence to suggest that this effect is largely dependent on induction and activation of the Nrf2-target gene NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), and that silencing of either Nrf2 or Nqo1 blocks the neuroprotective effects of DHA. Collectively, these data support a role for NQO1 in DHA-mediated protection from agrochemical exposure and in DHA-mediated neurite outgrowth.

RESULTS AND DISCUSSION

DHA induces expression of Nrf2-target genes

The Fat-1 mouse expresses an n-3 fatty acid desaturase that converts n-6 to n-3 fatty acids (Kang, 2007). As a result, these animals harbor an n-6 to n-3 fatty acid ratio of close to 1 compared with the 30:1 ratio of their WT counterparts (Hopperton et al., 2018, 2019). This model circumvents some of the confounding factors associated with traditional dietary supplementation of n-3 and n-6 fatty acids such as variation lipid source, purity, and the presence of secondary bioactives. Analysis of total cellular lipids composition of Fat-1 and WT brain tissue has shown that DHA is the most abundant PUFAs synthesized as a result of Fat-1 transgene expression (He et al., 2009), and yields highly consistent DHA levels in brain tissue between animals. Furthermore, expression profiling of brain tissue has revealed that expression of proinflammatory genes is attenuated in Fat-1 animals, consistent with animals fed diets enriched in fish oils (Hopperton et al., 2018). With a view of understanding whether attenuation of proinflammatory gene expression coincides with activation of genes-associated neuronal development and/or the Nrf2-transcriptional response, we contrasted the gene expression profiles of Fat-1 and WT animals. A volcano plot of differentially expressed genes (DEGs) with a false discovery rate of p < 0.05 yielded 1484 significantly altered genes (Figure 1A). Classification of these genes by gene ontology (GO) revealed that DEGs clustered to GO terms relating to neurite extension and transcription regulation of gene expression (Figure 1, B and C). Quantitative PCR (qPCR) analysis of brain tissue isolated from WT and Fat-1 mouse brain confirmed that expression of Nrf2-transcriptional target genes was enhanced in Fat-1 animals relative to control. A significant increase in expression of antioxidant genes including Nqo1, Gclc, Cat, and Gpx-3 was observed in Fat-1 brain compared with WT. Surprisingly, no difference in gene expression was observed for SOD1 or HO-1 (Figure 1D). Collectively, these data suggest heightened basal Nrf2 transcriptional activity in the Fat-1 brain is coincident with DEGs associated with neurite outgrowth and neuronal development, consistent with reports of enhanced neurogenesis in Fat-1 brain (He et al., 2009). That these differences were observed in otherwise healthy animals, suggests that marked differences in antioxidant systems may be present before brain insult or injury.

To establish whether this effect was DHA specific, or a general effect of increased metabolic flux, we turned to primary rat cortical neurons whose relative abundance better facilitates in vitro dose responses of increasing concentrations of fatty acids. When evaluating the antioxidant response in neurons, it is critical to ensure energy from lipids is balanced between treatment groups. Metabolic flux in neurons is primarily dependent on glycolysis as opposed to one synthesis via the pentose phosphate pathway offshoot. Promoters of HSE as opposed to ARE. It was also interesting that...
FIGURE 1: Fat-1 mouse brain shows increased expression of genes associated with neuronal development and transcriptional regulation of the antioxidant response. (A) Volcano plot of DEGs in Fat-1 mouse brain relative to WT, as assessed by microarray, identified 1484 significantly altered genes (dark gray). n = 3 independent animals, *p < 0.05, FDR. (B, C) Clustering of DEGs by GO term showed enrichment for terms associated with neuronal development (B) and regulation of transcription (C). (D) qPCR analysis of Fat-1 mouse brain relative to WT confirmed changes in expression of critical Nrf2-target genes. n = 3 independent animals; *, p < 0.05; **, p < 0.01, t test.

FIGURE 2: DHA treatment of neurons increases Nrf2-transcriptional activity and expression of Nrf2-target genes. (A–F) Primary rat cortical neurons were treated with increasing concentration of DHA or SA and expression of Nrf2-target genes was assessed by qPCR. n = 6 independent cultures; *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Dunnett’s test. (G) Luciferase reporter assay of ARE activation following Nrf2 overexpression in SH-SYSY neuroblastoma cells. n = 6 independent cultures; **, p < 0.01; t test. (H) Luciferase reporter assay of ARE activation following treatment with increasing concentration of DHA or SA in SH-SYSY neuroblastoma cells. n = 6 independent cultures; *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Dunnett’s test.
we saw no induction of SOD1, a canonical Nrf2 target, in either DHA-treated neurons or in Fat-1 animals. The kinetics and magnitude of the heat shock response varies in a stress- and cell type–dependent manner and has been shown to tightly regulate SOD1 expression in neurons (San Gil et al., 2017). Cell type–specific regulation may explain why SOD1 expression does not appear to be DHA dependent in primary neurons. To confirm that the effects of DHA on gene expression were specifically due to an increase in ARE transcriptional activation, we performed ARE-reporter assays following fatty acid treatment. Overexpression of Nrf2 showed that ARE-reporter activation is highly dependent on Nrf2 transcriptional activity (Figure 2G). Fatty acid treatment showed that DHA activates the ARE in a dose-dependent manner while SA does not, supporting the notion that SA does not signal through an ARE-dependent mechanism. Collectively, these data suggest that DHA induces expression of antioxidant enzymes both in vivo and in vitro through activation of AREs.

DHA-mediated ARE activation protects from ROS

To determine the functional consequences of DHA treatment with respect to ROS levels we used two contrasting methods to measure ROS production. CellROX is a generalized ROS reactive dye that measures ROS present throughout the cytosol while MitoSOX specifically measures levels of superoxide anion produced at the mitochondria. We first assessed the effect of fatty acid treatment on the baseline levels of ROS in neurons. We found that 100 μM DHA but not SA was able to lower the baseline levels ROS following 24-h treatment (Figure 3, A and B), consistent with the timeline of induction of Nrf2 target genes. We next assessed the effect of DHA in the context of mitochondrial stress. There is strong evidence linking agrochemical exposure with mitochondrial stress that culminates in neurodegeneration (Langston et al., 1983; McCormack et al., 2002; Gomez et al., 2007). Indeed, while the epidemiological association between PQ/MB exposure and PD onset has remained controversial in spite of an excess of epidemiological evidence, there is definitive causality with regard to PQ/MB and mitochondrial dysfunction (Gutman et al., 1970a,b; Gomez et al., 2007). We therefore exposed neurons to PQ/MB at levels below the EPA reported lowest observed effect level for oral exposure (http://www.epa.gov; Paraquat [CASRN 1910-42-5]/Maneb [CASRN 12427-38-2]), in order to assess the effect of DHA on ROS generation at the mitochondria. We

**FIGURE 3:** DHA treatment of neurons inhibits baseline redox stress and protects from mitochondrial stress evoked by agrochemical exposure. (A, B) Primary rat cortical neurons were treated with 100 μM DHA, SA, or vehicle for 24 h before staining with CellROX to measure cytosolic ROS levels. Representative micrographs are depicted in A and quantified in B. Scale bar = 50 μm; five independent cultures (150 neurons per culture); **, p < 0.01; two-way ANOVA; post hoc Dunnett’s test. (C, D) Primary rat cortical neurons were treated with 28 μM PQ and 10 μM MB for 15 min before the addition of 100 μM DHA, SA, or vehicle for 24 h and subsequently stained with MitoSOX to measure mitochondrial O$_2^-$ levels. Representative micrographs are depicted in C and quantified in D. Scale bar = 50 μm; six independent cultures (150 neurons per culture); **, p < 0.01; two-way ANOVA; post hoc Dunnett’s test.
found that in primary cortical neurons exposed to PQ/MB, DHA was not only able to reduce baseline levels of mitochondrial ROS but was also able to fully block ROS induced by PQ/MB exposure (Figure 3, C and D). Interestingly SA was also able to reduce ROS induced by PQ/MB albeit to a lesser extent. This is likely due to the effect of SA on expression of HO-1 through activation of an enhancer such as the HSE (Carratu et al., 1996; Okinaga et al., 1996; Alam and Cook, 2003), which would attenuate superoxide anion formation (Chao et al., 2013).

**DHA-mediated activation of Nrf2 rescues from agrochemical-evoked neuritic retraction**

Oxidative stress can trigger neuritic retraction that precedes cell death in multiple neurodegenerative disorders (Coyle and Puttfarcken, 1993; Adams et al., 1996; Giasson et al., 2000; Sykiotis and Bohmann, 2008; Yan et al., 2012). We find axo-dendritic neuropathology to be exacerbated by agrochemical exposure in the context of PD (Ryan et al., 2013; Stykel et al., 2018; Czaniecki et al., 2019). By contrast, DHA has been shown to stimulate neurite outgrowth and arborization in primary hippocampal neurons (Calderon and Kim, 2004). To assess whether DHA-mediated induction of Nrf2 could counteract the axodendritic deficits evoked by PQ/MB, we first determined the effect of PQ/MB exposure on neuritic retraction. PQ/MB exposure for 24 h resulted in a significant reduction in length of Tu1 labeled neurites. The effect of PQ/MB was inhibited by treatment with 100 μM DHA, whereas treatment with 100 μM SA was unable to inhibit neurite retraction (Figure 4, A and B). Moreover, DHA promoted baseline neurite outgrowth in the absence of PQ/MB two-way ANOVA; post hoc Tukey test.

(G) SH-SYSY were treated with 10 μM DMF before Nrf2 ChIP. Recruitment of Nrf2 to the Nqo1 promoter and subsequent induction of Nqo1 expression (inset) was assessed; n = 6 independent cultures; **, p < 0.01; ANOVA; post hoc Dunnet’s test. (H) Expression of Nqo1 following Nrf2 silencing assessed by qPCR. n = 6 independent cultures; **, p < 0.01; ANOVA; post hoc Dunnett’s test. (I) Lentiviral transformation of five unique Nqo1-targeted shRNAs coexpressing GFP were validated by Western blot analysis of Nqo1 expression and the most efficient vectors were carried forward for assessment of neurite length (E) following treatment with 100 μM DHA. n = 6 independent cultures (15 neurons per culture); *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Tukey test. (J) Neurite length of GFP fluorescent cells transduced with Nqo1-targeted shRNAs or control was assessed following treatment with 10 μM DHA in the presence and absence of 28 μM PQ/10 μM MB or vehicle. n = 6 independent cultures (15 neurons per culture); *, p < 0.05; **, p < 0.01; MANOVA; post hoc Tukey.

**FIGURE 4:** Nrf2 transcriptional activation is required for DHA-mediated neurite outgrowth. (A, B) SH-SYSY cells were treated with 28 μM PQ and 10 μM MB for 15 min before the addition of 100 μM DHA, SA, or vehicle for 24 h and subsequently labeled for Tuj1. Representative micrographs are depicted in A and quantified in B. Scale bar = 20 μm; n = 6 independent cultures (150 neurons per culture); *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Tukey test. (C) SH-SYSY overexpressing either Nrf2-GFP or GFP alone were treated with 28 μM PQ/10 μM MB and neurite length of fluorescent cells assessed. n = 6 independent cultures (15 neurons per culture); *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Tukey test. (D, E) Lentiviral transformation of five unique Nrf2-targeted shRNAs coexpressing GFP were validated by Western blot analysis of Nrf2 expression and the most efficient vectors were carried forward for assessment of neurite length (E) following treatment with 100 μM DHA. n = 6 independent cultures (45 neurons per culture); *, p < 0.05; **, p < 0.01; MANOVA; post hoc Tukey test.
whether DHA promoted recruitment of Nrf2 to the ARE sequence within the $Nqo1$ promoter, in a dose-dependent manner (Bang et al., 2013). We therefore performed chromatin immunoprecipitation (ChIP) of Nrf2 following DHA treatment and showed that DHA strongly promotes recruitment of Nrf2 to the ARE sequence within the $Nqo1$ promoter relative to a DNA region immediately upstream (Figure 4G), showing DMF to be a potent inducer of NQO1 protein expression (Figure 4G, inset). Moreover, silencing of Nrf2 corresponded to a loss of NQO1 expression (Figure 4H). To establish a causal link between Nqo1 induction and inhibition of PO/MB-evoked neuritic retraction, we silenced Nqo1 (Figure 4I) and assessed whether DMF could still be rescued from the effects of PO/MB. Loss of Nqo1 significantly reduced neurite length (Figure 4J). Analysis of neuritic retraction following PO/MB exposure in the context of Nqo1 knockdown showed that DMF no longer had any protective effect, suggesting that protection from PO/MB was dependent, in part, on Nqo1 expression. Collectively, these data demonstrate that DHA-mediated protection from PO/MB is dependent on Nrf2 activation and point to NQO1 as a key mediator of this protection.

**Nqo1 is a critical effector of DHA-mediated neurite outgrowth**

In our final set of experiments, we sought to confirm a role for Nqo1 in DHA-mediated signal transduction. We began by assessing whether DHA promoted recruitment of Nrf2 to the Nqo1 promoter. ChIP analysis of Nrf2 showed that DHA triggers significant recruitment of Nrf2 to the ARE within the Nqo1 promoter, relative to both vehicle-treated cells and a DNA region upstream of the Nqo1 promoter (Figure 5A). Interestingly, while 100 μM SA treatment did not yield significant induction of ARE in a luciferase-based reporter assay (Figure 2H), it did yield a moderate yet significant 0.2-fold increase in recruitment of Nrf2 (Figure 5A). This correlates with the modest increase in Nqo1 expression observed in primary neurons following 100 μM SA treatment by qPCR (Figure 2E). While this effect was not statistically significant ($p = 0.12$), together with the ChIP data suggests that SA is a very mild inducer of Nqo1 expression. To establish a causal link between Nqo1 induction and DHA signaling, we silenced Nqo1 and assessed whether DHA was able to 1) decrease baseline ROS levels as previously observed and 2) promote neurite outgrowth. Using lentiviral transduction, we expressed Nqo1-targeted shRNAs that coexpressed GFP in primary cortical neurons treated with DHA and analyzed ROS level using CellROX (Figure 5B). We found that Nqo1 knockdown significantly increased ROS production in primary neurons and that DHA was no longer able to inhibit the increase in ROS (Figure 5C). Finally, using the same knockdown system we assessed whether DHA could still promote neurite outgrowth following loss of Nqo1 expression. In addition to being unable to reduce ROS levels following silencing of Nqo1, DHA was unable to promote neuritic growth in the absence of Nqo1 (Figure 5, B and D). Collectively, these data show that DHA signaling of neurite elongation is mediated, in part, by Nrf2 induction of Nqo1.

NQO1 has been found to be enriched in astrocytes and dopaminergic neurons of the substantia nigra neurons. Expression is generally up-regulated in human disorders associated with enhanced oxidative stress (Floor and Wetzel, 1998; Murphy et al., 1998). Within the substantia nigra, NQO1 maintains dopamine metabolites in their reduced state, enabling their subsequent detoxification and clearance from the cell. Indeed, the relative increased protein oxidation in human substantia nigra of PD patients coupled with enriched NQO1 expression in this region, has led many to postulate that NQO1 may represent a therapeutic target in PD. Post-mortem analysis of tissue from PD patients reveals increased expression of NQO1 in both nigral astrocytes and dopaminergic neurons (van Muiswinkel et al., 2004), whereas pharmacological induction of NQO1 prevents the loss of dopaminergic neurons in the substantia nigra of MPTP-treated mice (Son et al., 2015). MPTP-treated Fat-1 animals as well as animals fed diets high in DHA are equally protected from loss of dopaminergic neurons (Bousquet et al., 2008; Bousquet et al., 2011). It is interesting to speculate as to whether NQO1 may represent a therapeutic target in multiple neurodegenerative diseases. For instance, evidence shows that NQO1 decreases baseline ROS in multiple cell types both in vivo and in vitro (Kim et al., 2013; Jo et al., 2016; Luo et al., 2018, 2019). Agrochemicals are just a few of an extensive list of toxins and toxicants from which NQO1 overexpression is protective by blocking induction of oxidative stress (Zafar et al., 2006; Jia et al., 2008; Kim et al., 2013; Son et al., 2015; Jo et al., 2016; Rasheed et al., 2020). We have shown previously that loss of microtubule stability is a critical event in axodontid retraction (Czaniecki et al., 2019). Multiple reports have shown that NQO1 ectopic expression stabilizes microtubules through direct binding (Wignall et al., 2004; Zhao et al., 2009; Dinkova-Kostova and Talalay, 2010), which may explain, in part, its ability to prevent axodontid retraction. While the agrochemicals studied herein are associated with increased risk of PD, oxidative stress and microtubule instability are associated with degenerative pathologies in multiple neurodegenerative disorders including Alzheimer’s disease, ALS,
and Huntington’s disease (McMurray, 2000; Itoh et al., 2013) highlighting the potential prophylactic benefit of nutraceuticals that promote NQO1 expression. Collectively, these data argue for a cell-autonomous protective signal elicited by DHA, in addition to the well characterized anti-inflammatory effects.

MATERIALS AND METHODS

Reagents

All reagents were sourced from Sigma-Aldrich unless otherwise stated. Fatty acids were supplied from Cayman Chemical Company in their nonesterified form. Upon arrival, they were resuspended in 95% ethanol (EtOH), aliquoted, layered with nitrogen gas, and stored in −80°C until required for experimental use.

Animals

The Fat-1 transgenic mouse was originally generated by Kang and colleagues (Kang et al., 2004). Housing and experiments abided by the regulations detailed by the Canadian Council on Animal Care for all animals used. At 2 mo of age, Fat-1 animals or their WT littermates, were anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS) before isolation of whole brain. Hemispheres were separated and flash-frozen in liquid nitrogen for downstream analysis.

Gene expression analysis

Microarray data from Fat-1 and WT animals (n = 3 experiments) were collected previously (Hopperton et al., 2018) and RAW data was downloaded and analyzed to clusters of DEGs associated with neuronal development and regulation of transcription. RAW data were analyzed with Transcriptome Analysis Console Software (Thermo-Fisher) to identify genes whose expression was statistically altered (p < 0.05) by at least onefold. Data were normalized by robust multiarray average. To group DEGs from Fat-1 animals into functional categories, the associated DEGs within both Fat-1 and WT animals were clustered for molecular function and biological process GO terms using the Database for Annotation, Visualization and Integrated Discovery v6.7 (Huang da et al., 2009a,b). Only level 4 and 5 terms were investigated, and the cutoff for the level of significance over background was p < 0.05 using Fisher’s exact test with correction for multiple hypothesis testing by the false discovery rate (FDR) algorithm. The background set of genes used was the entire human genome. After clustering, DEGs associated with the most significant GO terms were retained and fold changes visualized using GraphPad Prism 7.0.

Cell culture

Primary cortical cultures were from timed pregnant E18 Sprague Dawley rats (Charles River). Tissue was digested using filtered sterilized 17 U/mg Papain solution. Dissociated cortical neurons were seeded on plates previously coated overnight at 37°C with 0.15 mg/ml poly-d-lysine hydrobromide solution and washed twice with sterile water. A 50% medium change was performed every 3–4 d with Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM/F12) medium containing l-glutamine supplemented with hoc Dunnet’s test. (B–D) Lentiviral transformation of primary rat cortical neurons with two unique Nqo1-targeted shRNAs coexpressing GFP were used to simultaneously assess neurite length and ROS levels (CellROX) following 100 μM DHA treatment. Representative micrographs are depicted. Scale bar = 50 μm (B). CellROX is quantified in C, whereas neurite length is quantified in D. n = 6 independent cultures (15 neurons per culture); *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Tukey test.

FIGURE 5: NQO1 is a critical mediator of DHA signaling. (A) SH-SY5Y were treated with 100 μM DHA, SA, or vehicle for 24 h before Nrf2 ChIP and recruitment of Nrf2 to the Nqo1 promoter was assessed; n = 3 independent samples; *, p < 0.05; **, p < 0.01; ANOVA; post hoc Dunnet’s test. (B–D) Lentiviral transformation of primary rat cortical neurons with two unique Nqo1-targeted shRNAs coexpressing GFP were used to simultaneously assess neurite length and ROS levels (CellROX) following 100 μM DHA treatment. Representative micrographs are depicted. Scale bar = 50 μm (B). CellROX is quantified in C, whereas neurite length is quantified in D. n = 6 independent cultures (15 neurons per culture); *, p < 0.05; **, p < 0.01; two-way ANOVA; post hoc Tukey test.
0.1% β-mercaptoethanol, 1% antibiotic/antimycotic (HyClone), B27 (Life Technologies) and 0.7% bovine serum albumin (BSA) Fraction V (ThermoFisher).

SH-SY5Y neuroblastoma cells were maintained in a medium of DMEM/F12 containing 15% bovine calf serum (WWR), 1% MEM non-essential amino acids, 10 U/ml penicillin/streptomycin (GE Life Sciences), and 1% sodium pyruvate (Life Technologies). Cells were fed or passaged with trypsin every third day and maintained in a 37°C incubator with 5% CO₂. Cells were differentiated with retinoic acid (1 μM) for 7 d before fatty acid treatment. Cells were then treated overnight with fatty acids coupled to BSA at concentrations ranging from 1 μM to 100 μM. Knockdown of Nfe2l2 (Nr2) or Nqo1 was achieved by lentiviral expression of shRNA IRES GFP pGIPZ constructs (Dharmacon) targeted against Nfe2l2 gene or Nqo1 gene product or scrambled controls using sequences listed in Table S1.

**Reporter assays**

Cells were transfected using Lipofectamine 3000 (ThermoFisher) with ARE-Luciferase (Addgene) as well as a control Renilla-luciferase construct (Promega). Two days post transfection, the cells were harvested using the Firefly and Renilla Dual Luciferase Assay Kit (Bio- tium) according to the manufacturer’s protocol. Luminescence was assessed by a LUMistar Omega microplate reader (BMG Labtech).

Data were analyzed by calculating fold change after normalizing to Renilla-luciferase. Where Nr2 overexpression was employed, pcDNA3-Myc3-Nr2 (Addgene) or pcDNA3 vector control (Addgene) was cotransfected with the ARE constructs.

**qPCR**

RNA was extracted from samples using the RNaseasy Mini Kit (Qiagen). RNA (1 μg per sample) was reverse transcribed using the QuantiTech Reverse Transcription Kit (Qiagen). cDNA was diluted 1:4 in water. Two microliters of diluted cDNA were used for qPCR with PerFeCta SYBR Green FastMix ROX (Quanta Bioscience) in a StepOne Plus Real-Time PCR System (Applied Biosystems). Data was normalized to TUJ1 expression. The primer sequences used are listed in Supplemental Table S1.

**ChIP assay**

ChIP assays were performed using rabbit IgG or a rabbit anti-Nr2 antibody (Abcam) as previously described (Czaniecki et al., 2019). The AREs within the Nqo1 enhancer that were amplified for determination of Nr2 binding can be found in Supplemental Table S1. Levels of enrichment (n-fold) were calculated using the comparative cycle threshold method. For quantitative ChIP, the PCR was performed as described above.

**Western blot analysis**

Briefly, samples were separated on 4–12% gradient Bis-Tris SDS–PAGE gel and transferred onto 0.2 μm nitrocellulose. Membranes were probed with primary antibodies against Nqo1 (Santa Cruz; 1:200), Nr2 (Abcam; 1:1000), or GAPDH (Abcam; 1:10,000). Donkey anti-mouse (Bio-Rad, Mississauga, ON, Canada; 1:2000) and anti-rabbit (Bio-Rad, Mississauga, ON, Canada; 1:2000) horseradish peroxidase–conjugated secondary antibodies were used followed by clarity Western ECL blotting substrate (Bio-Rad, Mississauga, ON, Canada) to visualize bands on blots. Membranes were visualized with a LiCOR Odyssey Fc.

**Neurite analysis**

Neurite analysis was performed on TuJ1 stained primary cortical or neuroblastoma cells to identify neurites for analysis of length from single neurons. Where Nr2 or GFP expression was required, cells were transduced at least 3 d before analysis and the GFP signal was used to trace single neurons. Tracing was performed in Neurulucida 360 (MBF Bioscience) in a semiautomated manner. Experimenter input was primarily to resolve neurite intersections from two independent neurons that could not be distinguished in an automated manner; two to three single neurons per field of acquisition were chosen at random and experimenters were blind to the treatment conditions. The number of neurites extending from the soma of at least 45–150 neurons per sample was determined and total length was calculated by Neurulucida 360.

**Immunocytochemistry and fluorescence analysis**

For immunofluorescence, cells were fixed with 4% paraformaldehyde for 20 min, washed once with PBS, and blocked with 3% BSA and 0.3% Triton X-100 in PBS for 30 min. Cells were incubated with primary antibody overnight, and the appropriate Alexa Fluor (488, 594) conjugated secondary antibodies from Thermo Life Technologies were used at 1:1000. For MitoSOX and Cell ROX staining of ROS, cells were loaded with dye (Thermo Life Technologies, Mississauga, ON, Canada) in media for 15 min at room temperature. Excess dye was washed out with Dulbecco’s phosphate-buffered saline (DPBS). Cells were then processed as per the manufacturer’s protocol for visualization of differential fluorescence intensities. Imaging was performed on an Axio-Observer microscope with LED-based illumination and optical sectioning by structured illumination (Zeiss, North York, ON, Canada). Objectives used were Plan-APo 40×/1.4 oil DIC VIS-IR or Plan-APo 63×/1.4 oil DIC M27. Fluorescence intensity was measured using the Zen 2.3.

**Statistical analysis**

Data represent multiple replicate samples from minimum three independent differentiations, presented as mean ± SEM. Statistical significance was ascertained by Student’s t test or ANOVA (two-way or three-way) with appropriate post hoc testing (either Dunnet or Tukey); p < 0.05 was considered significant. All data were analyzed using either Prism7 (GraphPad Software) or in R (R-project version 3.5.3). Normality and variance of data sets were measured using Lilliefors test or an F test where appropriate. For data not fitting a normal distribution, nonparametric Mann-Whitney or Fisher Exact tests were employed.

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