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

Jennifer Drolet[a], Brodie Buchner-Duby[a], Morgan G. Stykel[a], Carla Coackley[a], Jing X. Kang[b], David W. L. Ma[c], and Scott D. Ryan[a,d,*]

[a]Department of Molecular and Cellular Biology and [c]Department of Human Health and Nutritional Sciences, The University of Guelph, Guelph, ON N1G 2W1, Canada; [b]Laboratory for Lipid Medicine and Technology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129; [d]Neurodegenerative Disease Center, Scintillon Institute, San Diego, CA 92121

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 docosohexanoic 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.**

Monitoring Editor
Coleen Murphy
Princeton University

Received: Sep 21, 2020
Revised: Dec 15, 2020
Accepted: Jan 22, 2021

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 agrochemical 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

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E20-09-0599) on January 27, 2021.

*Address correspondence to: Scott Ryan (sryan03@uoguelph.ca).

Abbreviations used: ARE, antioxidant response elements; ChIP, chromatin immunoprecipitation; DEGs, differentially expressed genes; DHA, docosohexanoic acid; DMF, 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; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SA, steric acid.

© 2021 Drolet *et al*. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

"ASCB®," "The American Society for Cell Biology®," and "Molecular Biology of the Cell®" are registered trademarks of The American Society for Cell Biology.

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, docosohexanoic 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.

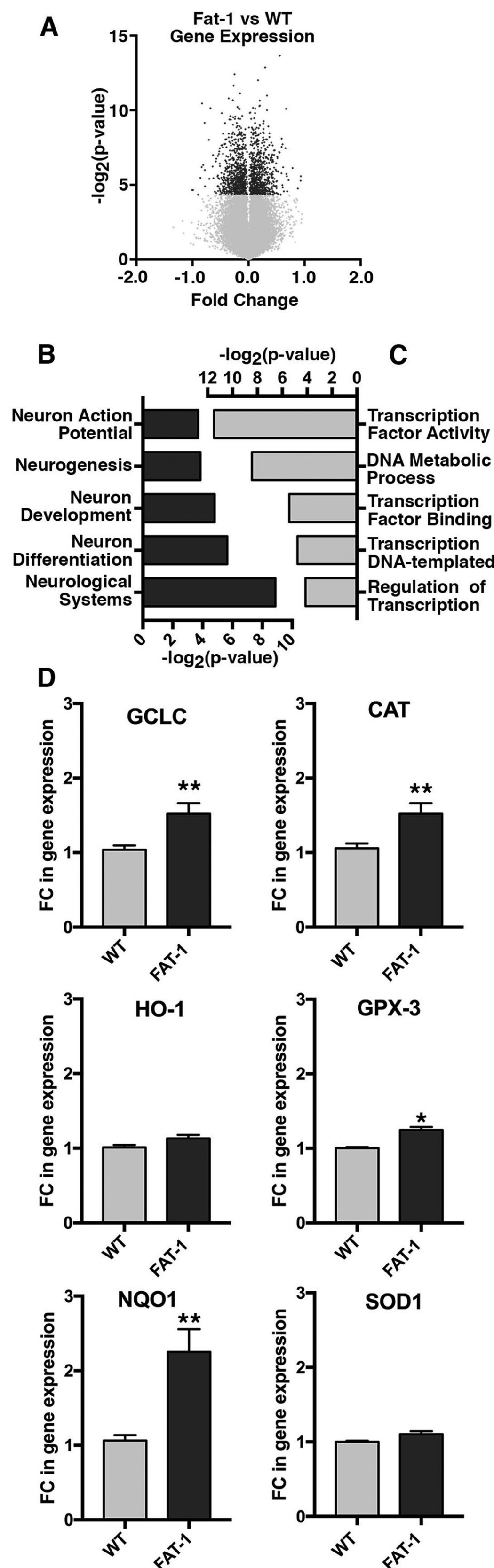
In the current study, we therefore assessed whether DHA could counteract the neurobiological deficits evoked by PQ/MB exposure through activation of the Nrf2-dependent antioxidant response with a focus on axodendritic pathology and redox stress. To this end, we employed a series of gain of function, 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 PUFA 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 neuritogenesis 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 β-oxidation, which plays a role in redox homeostasis through glutathione synthesis via the pentose phosphate pathway offshoot. Promoting excess β-oxidation could, as a result, alter redox balance. Thus, to control for the general effects of fatty acid addition and ensure specificity of DHA-mediated signal transduction, we choose SA as a saturated fatty acid control. DHA treatment (24 h) resulted in a specific increase in expression of CAT, GPX-3, and NQO1 relative to SA, with NQO1 displaying the strongest dose-dependent effect (Figure 2, A–F). Interestingly, while HO-1 was not induced in neurons following treatment with DHA or in *Fat-1* animals, it was induced by SA treatment suggesting that DHA triggers a gene expression profile distinct from that of SA (Figure 2A). Indeed, *HO-1* is regulated by multiple conserved enhancers that are induced by multiple stimuli (Alam and Cook, 2007; Milani *et al.*, 2011). These are not limited to AREs but also include heat shock elements (HSEs; Okinaga *et al.*, 1996; Yoo *et al.*, 1999) and an intronic SP1 enhancer (Deshane *et al.*, 2010; Milani *et al.*, 2011). Saturated fatty acids such as SA and palmitic acid have been shown to be activators of HSEs (Carratu *et al.*, 1996). Thus, the modest induction of *HO-1* by SA may be through modulation of HSE as opposed to ARE. It was also interesting that



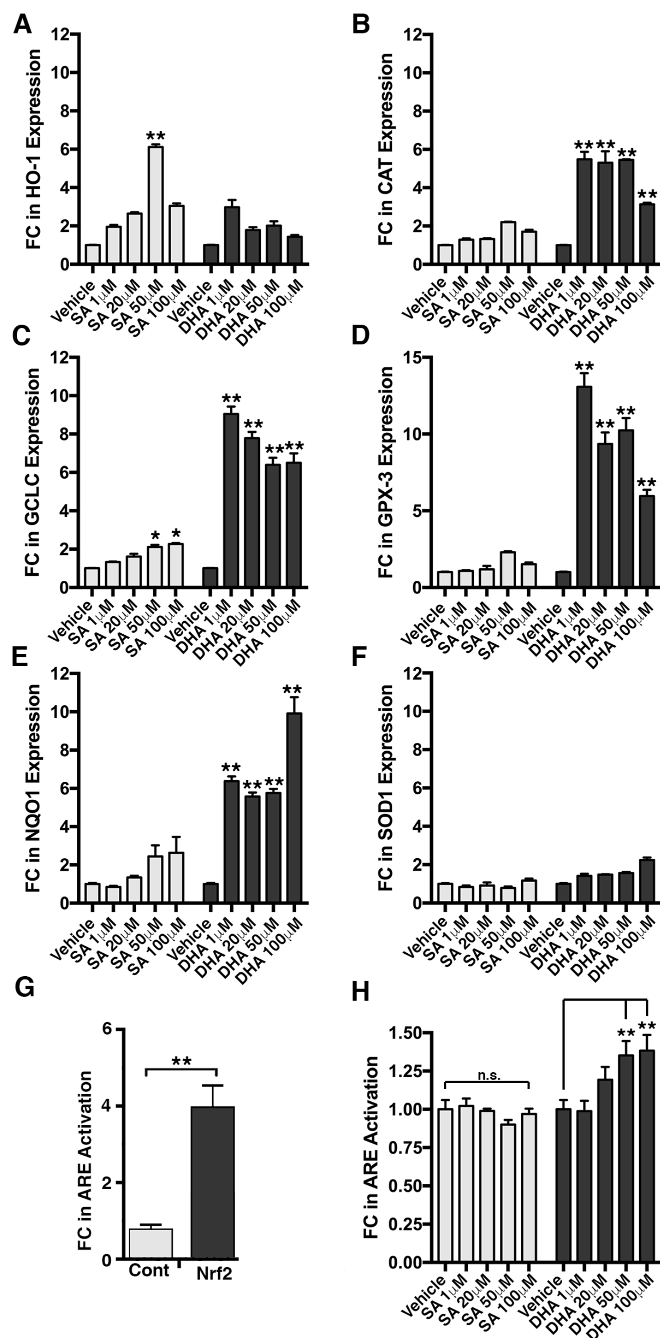


FIGURE 2: DHA treatment of neurons increases Nrf2-transcriptional activity and expression of Nrf2-target genes. (A–F) Primary rat cortical neurons were treated with increasing concentration of DHA or SA and expression of Nrf2-target genes was assessed by qPCR. $n = 6$ independent cultures; *, $p < 0.05$; **, $p < 0.01$; two-way ANOVA; post hoc Dunnett's test. (G) Luciferase reporter assay of ARE activation following Nrf2 overexpression in SH-SY5Y 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-SY5Y neuroblastoma cells. $n = 6$ independent cultures; *, $p < 0.05$; **, $p < 0.01$; two-way ANOVA; post hoc Dunnett's test.

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.

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 in 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

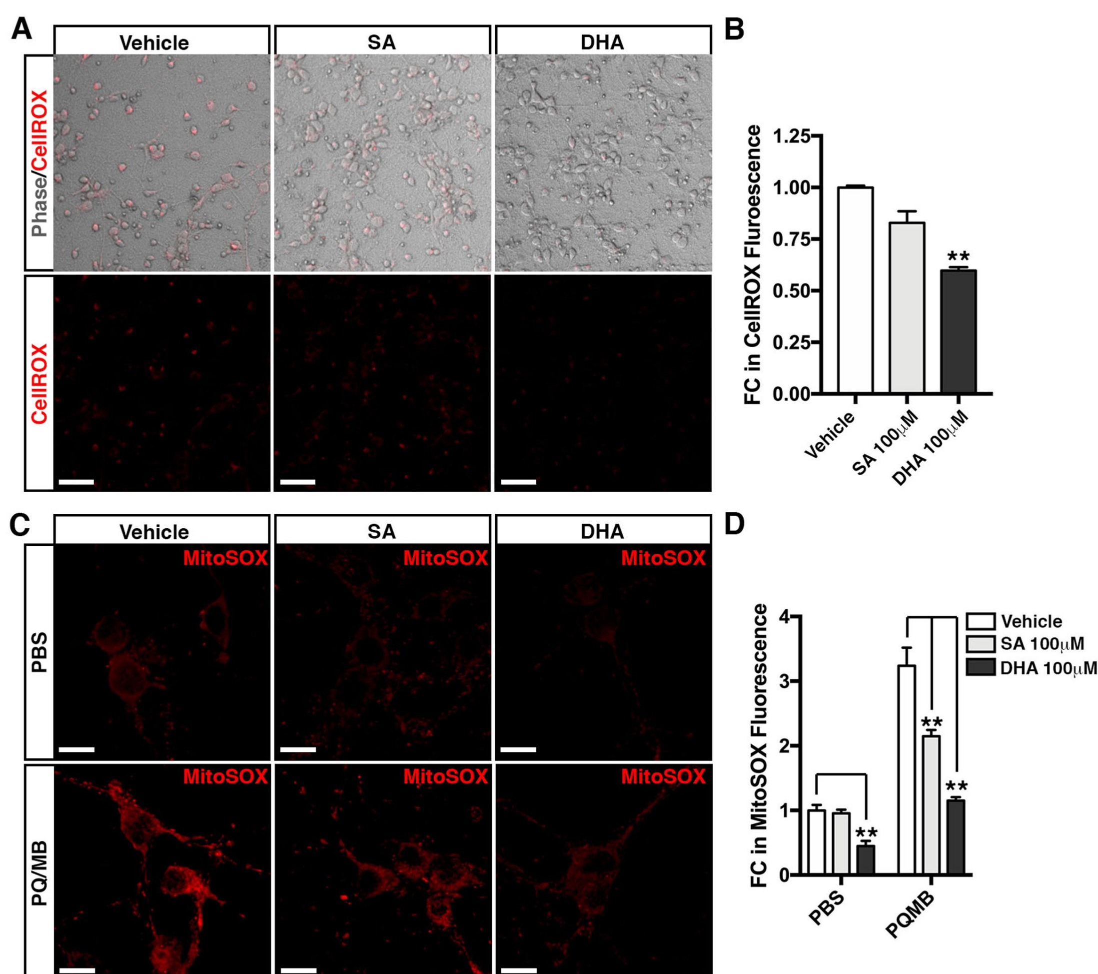


FIGURE 3: DHA treatment of neurons inhibits baseline redox stress and protects from mitochondrial stress evoked by agrochemical exposure. (A, B) Primary rat cortical neurons were treated with 100 μM DHA, SA, or vehicle for 24 h before staining with CellROX to measure cytosolic ROS levels. Representative 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-dentritic 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 Tuj1 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

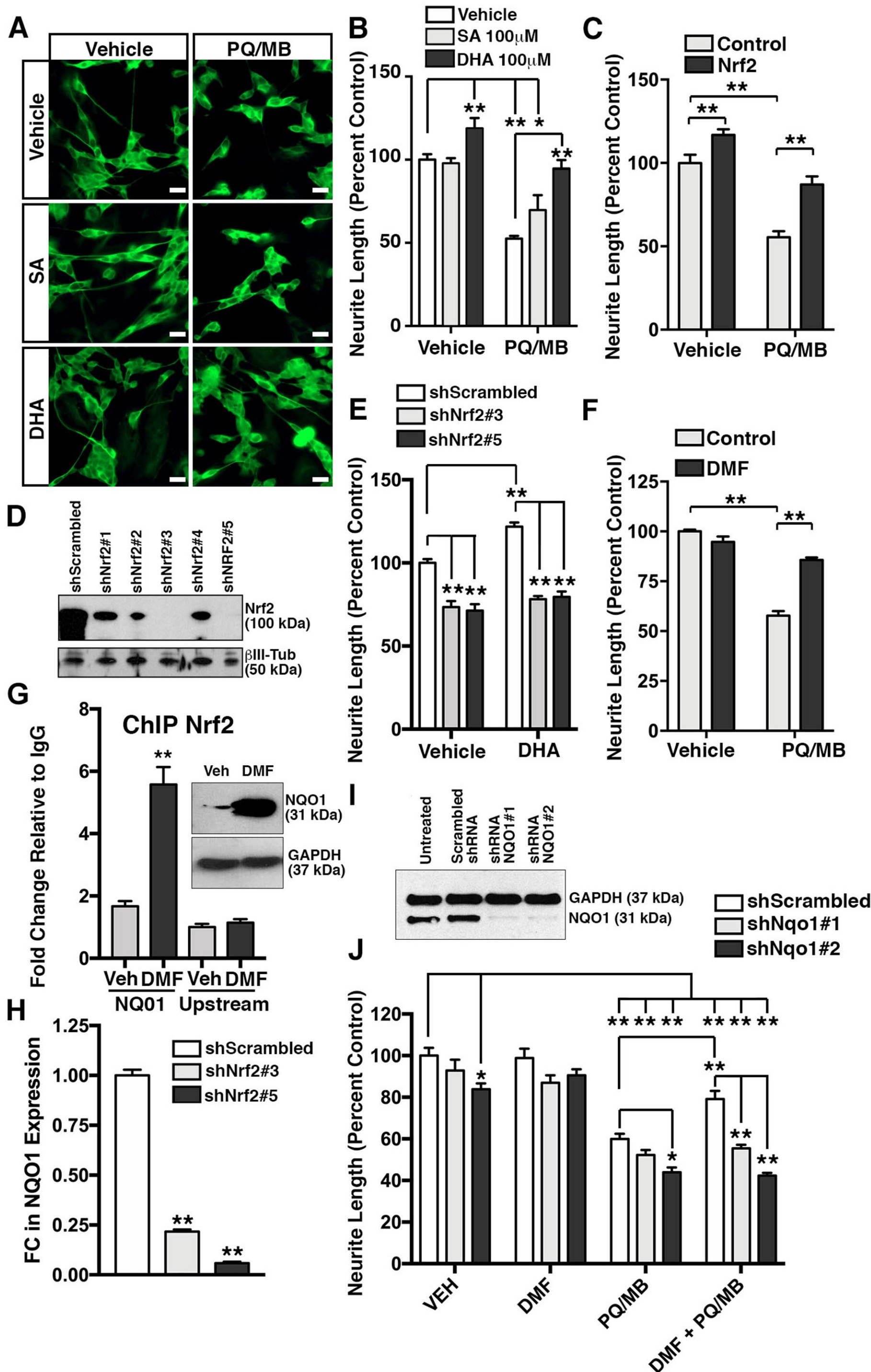


FIGURE 4: Nrf2 transcriptional activation is required for DHA-mediated neurite outgrowth. (A, B) SH-SY5Y cells were treated with 28 μM PQ and 10 μM MB for 15 min before the addition of 100 μM DHA, SA, or vehicle for 24 h and subsequently labeled for Tuj1. Representative micrographs are depicted in A 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-SY5Y 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 (15 neurons per culture); *, $p < 0.05$; **, $p < 0.01$; two-way ANOVA; post hoc Tukey test. (F) SH-SY5Y were treated with 10 μM DMF and either 28 μM PQ/10 μM MB or vehicle and neurite length assessed. $n = 6$ independent cultures (45 neurons per culture); **, $p < 0.01$; two-way ANOVA; post hoc Tukey test. (G) SH-SY5Y were treated with 10 μM DMF before Nrf2 ChIP. Recruitment of Nrf2 to the *Nqo1* promoter and subsequent induction 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 two unique *Nqo1*-targeted shRNAs coexpressing GFP were validated by Western blot analysis of Nqo1 expression. (J) Neurite length of GFP fluorescent cells transduced with *Nqo1*-targeted shRNAs or control was assessed following treatment with 10 μM DMF 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 4B), consistent with the expression profiling of *Fat-1* animals that showed induction of genes involved in neurite outgrowth. To determine whether the inhibition of PQ/MB-induced neuritic retraction could be attributed to Nrf2, we carried out a series of gain of function–loss of function experiments in SH-SY5Y neuroblastoma cells. We first overexpressed Nrf2 in SH-SY5Y cells to determine whether Nrf2 protected from the deleterious effect of PQ/MB exposure. We found that forced expression of Nrf2 significantly inhibits PQ/MB-evoked neuritic retraction and similarly promotes baseline neurite outgrowth (Figure 4C). Using lentiviral transduction, we next expressed *Nrf2*-targeted shRNAs (Figure 4D) in combination with DHA treatment to determine whether Nrf2 was required for DHA-mediated neurite outgrowth. Loss of Nrf2 significantly reduced neurite length consistent with our previous reports (Czaniecki *et al.*, 2019) and treatment with DHA had no effect on neurite length in the context of Nrf2 knockdown (Figure 4E). These data support the notion that DHA mediates its effects, in part, through Nrf2.

We next sought to gain further insight into what Nrf2 targets were critical in counteracting the effects of agrochemical exposure. Given that in both *Fat-1* brain tissue and primary neurons, *Nqo1* was the Nrf2-target gene most responsive to DHA, and that DHA has previously been reported to activate Nrf2 and induces the expression of Nqo1 in a dose-dependent manner (Bang *et al.*, 2017), we began by examining the effect of *Nqo1* on PQ/MB-evoked neuritic retraction. NQO1 is a well characterized quinone reductase but has also been ascribed roles in superoxide reduction and in NAD+ generation, thereby supporting fatty acid β-oxidation (Ross and Siegel, 2017). These roles suggest that NQO1 can both counteract PQ/MB-evoked redox stress and maintain energy homeostasis during neurite outgrowth. We therefore sought to establish whether NQO1 can counteract the effects of PQ/MB on axodendritic pathology. To this end, we made use of a well characterized Nrf2 transcriptional activator, dimethyl fumarate (DMF). DMF treatment results in S-alkylation of Keap1, which destabilizes the Keap1-Nrf2 complex, preventing Nrf2 degradation and permitting Nrf2 nuclear translocation, thereby activating the antioxidant response (Linker *et al.*, 2011). We first confirmed that DMF treatment prevents neuritic retraction evoked by PQ/MB (Figure 4F). Nrf2 binds a well characterized ARE in the NQO1 promoter (Nioi *et al.*, 2003). We therefore performed chromatin immunoprecipitation (ChIP) of Nrf2 following DMF treatment and showed that DMF 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 PQ/MB-evoked neuritic retraction, we silenced *Nqo1* (Figure 4I) and assessed whether DMF could still be rescued from the effects of PQ/MB. Loss of Nqo1 significantly reduced neurite length (Figure 4J). Analysis of neuritic retraction following PQ/MB exposure in the context of *Nqo1* knockdown showed that DMF no longer had any protective effect, suggesting that protection from PQ/MB was dependent, in part, on *Nqo1* expression. Collectively, these data demonstrate that DHA-mediated protection from PQ/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 axodendritic 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 axodendritic 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,

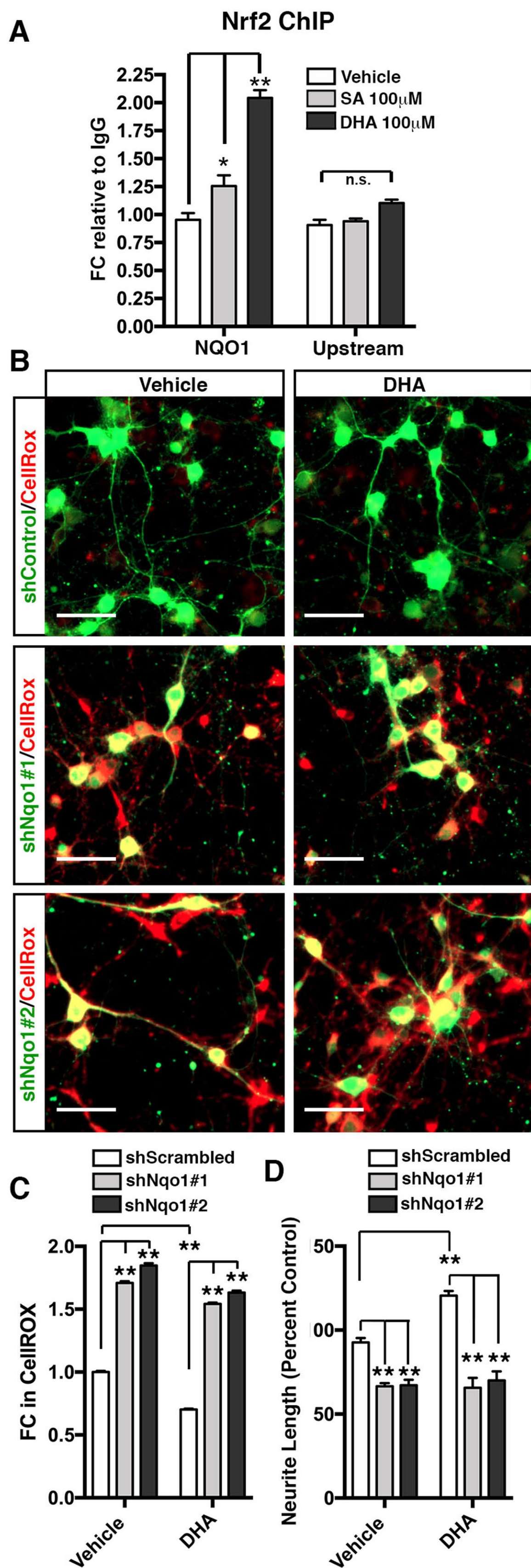


FIGURE 5: NQO1 is a critical mediator of DHA signaling. (A) SH-SY5Y were treated with 100 µM DHA, SA, or vehicle for 24 h before Nrf2 ChIP and recruitment of Nrf2 to the *Nqo1* promoter was assessed; $n = 3$ independent samples; *, $p < 0.05$; **, $p < 0.01$; ANOVA; post hoc Dunnet's test. (B–D) Lentiviral 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.

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 isofluorene 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 (ThermoFisher) 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

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 (VWR), 1% MEM nonessential amino acids, 10 U/ml penicillin/streptomycin (GE LifeSciences), 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% CO_2. 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* (Nrf2) 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 (Biotium) 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 Nrf2 overexpression was employed, pcDNA3-Myc3-Nrf2 (Addgene) or pcDNA3 vector control (Addgene) was cotransfected with the ARE constructs.

qPCR

RNA was extracted from samples using the RNeasy 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-Nrf2 antibody (Abcam) as previously described (Czaniecki *et al.*, 2019). The AREs within the *Nqo1* enhancer that were amplified for determination of Nrf2 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), Nrf2 (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 Nrf2 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 Neurolucida 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 Neurolucida 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 texts were employed.

REFERENCES

Adams JD, Mukherjee SK, Klaidman LK, Chang ML, Yasharel R (1996). Apoptosis and oxidative stress in the aging brain. Ann NY Acad Sci 786, 135–151.

Alam J, Cook JL (2003). Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 9, 2499–2511.

Alam J, Cook JL (2007). How many transcription factors does it take to turn on the heme oxygenase-1 gene? Am J Respir Cell Mol Biol 36, 166–174.

Bang HY, Park SA, Saeidi S, Na HK, Surh YJ (2017). Docosahexaenoic acid induces expression of heme oxygenase-1 and NAD(P)H:quinone oxidoreductase through activation of Nrf2 in human mammary epithelial cells. Molecules 22, doi: 10.3390/molecules22060969.

Bousquet M, Calon F, Cicchetti F (2011). Impact of omega-3 fatty acids in Parkinson's disease. Ageing Res Rev 10, 453–463.

Bousquet M, Saint-Pierre M, Julien C, Salem N Jr., Cicchetti F, Calon F. (2008). Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease. FASEB J 22, 1213–1225.

Calderon F, Kim HY (2004). Docosahexaenoic acid promotes neurite growth in hippocampal neurons. J Neurochem 90, 979–988.

Cao D, Kevala K, Kim J, Moon HS, Jun SB, Lovinger D, Kim HY (2009). Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. J Neurochem 111, 510–521.

Cardoso HD, dos Santos Junior EF, de Santana DF, Goncalves-Pimentel C, Angelim MK, Isaac AR, Lagranha CJ, Guedes RC, Beltrao EI, Morya E, *et al*. (2014). Omega-3 deficiency and neurodegeneration in the substantia nigra: involvement of increased nitric oxide production and reduced BDNF expression. Biochim Biophys Acta 1840, 1902–1912.

Carratu L, Franceschelli S, Pardini CL, Kobayashi GS, Horvath I, Vigh L, Maresca B (1996). Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. Proc Natl Acad Sci USA 93, 3870–3875.

Chao XD, Ma YH, Luo P, Cao L, Lau WB, Zhao BC, Han F, Liu W, Ning WD, Su N, *et al*. (2013). Up-regulation of heme oxygenase-1 attenuates brain damage after cerebral ischemia via simultaneous inhibition of superoxide production and preservation of NO bioavailability. Exp Neurol 239, 163–169.

Coyle JT, Puttfarcken P (1993). Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689–695.

Czaniecki C, Ryan T, Stykel MG, Drolet J, Heide J, Hallam R, Wood S, Coackley C, Sherriff K, Bailey CDC, Ryan SD (2019). Axonal pathology in hPSC-based models of Parkinson's disease results from loss of Nrf2 transcriptional activity at the Map1b gene locus. Proc Natl Acad Sci USA 116, 14280–14289.

de Lau LM, Bornebroek M, Witteman JC, Hofman A, Koudstaal PJ, Breteler MM (2005). Dietary fatty acids and the risk of Parkinson disease: the Rotterdam study. Neurology 64, 2040–2045.

Deshane J, Kim J, Bolisetty S, Hock TD, Hill-Kapturczak N, Agarwal A (2010). Sp1 regulates chromatin looping between an intronic enhancer and distal promoter of the human heme oxygenase-1 gene in renal cells. J Biol Chem 285, 16476–16486.

Dinkova-Kostova AT, Talalay P (2010). NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Biochem Biophys 501, 116–123.

Floor E, Wetzel MG (1998). Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. J Neurochem 70, 268–275.

Gao L, Wang J, Sekhar KR, Yin H, Yared NF, Schneider SN, Sasi S, Dalton TP, Anderson ME, Chan JY, *et al*. (2007). Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. J Biol Chem 282, 2529–2537.

Gatto NM, Rhodes SL, Manthripragada AD, Bronstein J, Cockburn M, Farrer M, Ritz B (2010). α-Synuclein gene may interact with environmental factors in increasing risk of Parkinson's disease. Neuroepidemiology 35, 191–195.

Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM (2000). Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. Science 290, 985–989.

Gomez C, Bandez MJ, Navarro A (2007). Pesticides and impairment of mitochondrial function in relation with the parkinsonian syndrome. Front Biosci 12, 1079–1093.

Gruber F, Ornelas CM, Karner S, Narzt MS, Nagelreiter IM, Gschwandtner M, Bochkov V, Tschachler E (2015). Nrf2 deficiency causes lipid oxidation, inflammation, and matrix-protease expression in DHA-supplemented and UVA-irradiated skin fibroblasts. Free Radic Biol Med 88, 439–451.

Gutman M, Singer TP, Beinert H, Casida JE (1970a). Reaction sites of rotenone, piericidin A, and amytal in relation to the nonheme iron components of NADH dehydrogenase. Proc Natl Acad Sci USA 65, 763–770.

Gutman M, Singer TP, Casida JE (1970b). Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. XVII. Reaction sites of piericidin A and rotenone. J Biol Chem 245, 1992–1997.

He C, Qu X, Cui L, Wang J, Kang JX (2009). Improved spatial learning performance of fat-1 mice is associated with enhanced neurogenesis and neuritogenesis by docosahexaenoic acid. Proc Natl Acad Sci USA 106, 11370–11375.

Hopperton KE, James NCE, Mohammad D, Irfan M, Bazinet RP. (2019). Dietary fish oil, and to a lesser extent the fat-1 transgene, increases astrocyte activation in response to intracerebroventricular amyloid-β1-40 in mice. Nutr Neurosci 22, 418–424.

Hopperton KE, Trepanier MO, James NCE, Chouinard-Watkins R, Bazinet RP. (2018). Fish oil feeding attenuates neuroinflammatory gene expression without concomitant changes in brain eicosanoids and docosanoids in a mouse model of Alzheimer's disease. Brain Behav Immun 69, 74–90.

Huang da W, Sherman BT, Lempicki RA (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37, 1–13.

Huang da W, Sherman BT, Lempicki RA (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44–57.

Itoh K, Nakamura K, Iijima M, Sesaki H (2013). Mitochondrial dynamics in neurodegeneration. Trends Cell Biol 23, 64–71.

Jia Z, Zhu H, Misra HP, Li Y (2008). Potent induction of total cellular GSH and NQO1 as well as mitochondrial GSH by 3H-1,2-dithiole-3-thione in SH-SY5Y neuroblastoma cells and primary human neurons: protection against neurocytotoxicity elicited by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or hydrogen peroxide. Brain Res 1197, 159–169.

Jo HS, Kim DS, Ahn EH, Kim DW, Shin MJ, Cho SB, Park JH, Lee CH, Yeo EJ, Choi YJ, *et al*. (2016). Protective effects of Tat-NQO1 against oxidative stress-induced HT-22 cell damage, and ischemic injury in animals. BMB Rep 49, 617–622.

Kang JX, Wang J, Wu L, Kang ZB (2004). Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. Nature 427, 504.

Kang JX (2007). Fat-1 transgenic mice: a new model for omega-3 research. Prostaglandins Leukot Essent Fatty Acids 77, 263–267.

Kim J, Kim SK, Kim HK, Mattson MP, Hyun DH (2013). Mitochondrial function in human neuroblastoma cells is up-regulated and protected by NQO1, a plasma membrane redox enzyme. PLoS One 8, e69030.

Langston JW, Ballard P, Tetrud JW, Irwin I (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979–980.

Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, Zeng W, Hronowsky X, Buko A, Chollate S, *et al*. (2011). Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. Brain 134, 678–692.

Luo S, Kang SS, Wang ZH, Liu X, Day JX, Wu Z, Peng J, Xiang D, Springer W, Ye K (2019). Akt phosphorylates NQO1 and triggers its degradation, abolishing its antioxidative activities in Parkinson's disease. J Neurosci 39, 7291–7305.

Luo S, Lei K, Xiang D, Ye K (2018). NQO1 is regulated by PTEN in glioblastoma, mediating cell proliferation and oxidative stress. Oxid Med Cell Longev 2018, 9146528.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA (2002). Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 10, 119–127.

McMurray CT (2000). Neurodegeneration: diseases of the cytoskeleton? Cell Death Differ 7, 861–865.

Milani P, Gagliardi S, Cova E, Cereda C (2011). SOD1 transcriptional and posttranscriptional regulation and its potential implications in ALS. Neurol Res Int 2011, 458427.

Mildenberger J, Johansson I, Sergin I, Kjobli E, Damas JK, Razani B, Flo TH, Bjorkoy G (2017). N-3 PUFAs induce inflammatory tolerance by formation of KEAP1-containing SQSTM1/p62-bodies and activation of NFE2L2. Autophagy 13, 1664–1678.

Murphy TH, So AP, Vincent SR (1998). Histochemical detection of quinone reductase activity in situ using LY 83583 reduction and oxidation. J Neurochem 70, 2156–2164.

Nioi P, McMahon M, Itoh K, Yamamoto M, Hayes JD (2003). Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. Biochem J 374, 337–348.

Okinaga S, Takahashi K, Takeda K, Yoshizawa M, Fujita H, Sasaki H, Shibahara S (1996). Regulation of human heme oxygenase-1 gene expression under thermal stress. Blood 87, 5074–5084.

Pezzoli G, Cereda E (2013). Exposure to pesticides or solvents and risk of Parkinson disease. Neurology 80, 2035–2041.

Rasheed MSU, Tripathi MK, Patel DK, Singh MP (2020). Resveratrol regulates Nrf2-mediated expression of antioxidant and xenobiotic metabolizing enzymes in pesticides-induced Parkinsonism. Protein Pept Lett 27, 1038–1045.

Ross D, Siegel D (2017). Functions of NQO1 in cellular protection and CoQ10 metabolism and its potential role as a redox sensitive molecular switch. Front Physiol 8, 595.

Ryan SD, Dolatabadi N, Chan SF, Zhang X, Akhtar MW, Parker J, Soldner F, Sunico CR, Nagar S, Talantova M, *et al*. (2013). Isogenic human iPSC Parkinson's model shows nitrosative stress-induced dysfunction in MEF2-PGC1α transcription. Cell 155, 1351–1364.

San Gil R, Ooi L, Yerbury JJ, Ecroyd H (2017). The heat shock response in neurons and astroglia and its role in neurodegenerative diseases. Mol Neurodegener 12, 65.

Son HJ, Choi JH, Lee JA, Kim DJ, Shin KJ, Hwang O (2015). Induction of NQO1 and neuroprotection by a novel compound KMS04014 in Parkinson's disease models. J Mol Neurosci 56, 263–272.

Stykel MG, Humphries K, Kirby MP, Czaniecki C, Wang T, Ryan T, Bamm V, Ryan SD (2018). Nitration of microtubules blocks axonal mitochondrial transport in a human pluripotent stem cell model of Parkinson's disease. FASEB J 32, 5350–5364.

Sykiotis GP, Bohmann D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*. Dev Cell 14, 76–85.

van Muiswinkel FL, de Vos RA, Bol JG, Andringa G, Jansen Steur EN, Ross D, Siegel D, Drukarch B (2004). Expression of NAD(P)H:quinone oxidoreductase in the normal and Parkinsonian substantia nigra. Neurobiol Aging 25, 1253–1262.

Wignall SM, Gray NS, Chang YT, Juarez L, Jacob R, Burlingame A, Schultz PG, Heald R (2004). Identification of a novel protein regulating microtubule stability through a chemical approach. Chem Biol 11, 135–146.

Yan MH, Wang X, Zhu X (2012). Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic Biol Med 62, 90–101.

Yoo HY, Chang MS, Rho HM (1999). The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. J Biol Chem 274, 23887–23892.

Zafar KS, Inayat-Hussain SH, Siegel D, Bao A, Shieh B, Ross D (2006). Overexpression of NQO1 protects human SK-N-MC neuroblastoma cells against dopamine-induced cell death. Toxicol Lett 166, 261–267.

Zhang M, Wang S, Mao L, Leak RK, Shi Y, Zhang W, Hu X, Sun B, Cao G, Gao Y, *et al* (2014). Omega-3 fatty acids protect the brain against ischemic injury by activating Nrf2 and upregulating heme oxygenase 1. J Neurosci 34, 1903–1915.

Zhao F, Wu T, Lau A, Jiang T, Huang Z, Wang XJ, Chen W, Wong PK, Zhang DD (2009). Nrf2 promotes neuronal cell differentiation. Free Radic Biol Med 47, 867–879.

Zhu W, Ding Y, Kong W, Li T, Chen H (2018). Docosahexaenoic acid (DHA) provides neuroprotection in traumatic brain injury models via activating Nrf2-ARE signaling. Inflammation 41, 1182–1193.