Downregulation of eEF1A/EFT3-4 Enhances Dopaminergic Neurodegeneration After 6-OHDA Exposure in C. elegans Model

Pawanrat Chalorak, Permphan Dharmasaroja and Krai Meemon*

Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, Thailand

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by the aggregation of α-synuclein protein and selective death of dopaminergic (DA) neurons in the substantia nigra of the midbrain. Although the molecular pathogenesis of PD is not completely understood, a recent study has reported that eukaryotic translation elongation factor 1 alpha (eEF1A) declined in the PD-affected brain. Therefore, the roles of eEF1A1 and eEF1A2 in the prevention of DA neuronal cell death in PD are aimed to be investigated. Herein, by using Caenorhabditis elegans as a PD model, we investigated the role of eft-3/4, the worm homolog of eEF1A1/eEF1A2, on 6-hydroxydopamine (6-OHDA)-induced DA neuron degeneration. Our results demonstrated that the expressions of eft-3 and eft-4 were decreased in the 6-OHDA-induced worms. RNA interference (RNAi) of eft-3 and eft-4 resulted in dramatic exacerbation of DA neurodegeneration induced by 6-OHDA, as well as aggravated the food-sensing behavior, ethanol avoidance, and decreased lifespan when compared with only 6-OHDA-induced worms. Moreover, downregulation of eft-3/4 in 6-OHDA-induced worms suppressed the expression of the anti-apoptotic genes, including PI3K/age-1, PDK-1/pdk-1, mTOR/let-363, and AKT-1,2/akt-1,2, promoting the expression of apoptotic genes such as BH3/egl-1 and Caspase-9/ced-3. Collectively, these findings indicate that eEF1A plays an important role in the 6-OHDA-induced neurodegeneration through the phosphatidylinositol 3-kinase (PI3K)/serine/threonine protein kinase (Akt)/mammalian target of rapamycin (mTOR) pathway and that eEF1A isoforms may be a novel and effective pro-survival factor in protective DA neurons against toxin-induced neuronal death.

Keywords: eEF1A, Parkinson’s disease, dopaminergic neuron, Caenorhabditis elegans, 6-hydroxydopamine

INTRODUCTION

Parkinson’s disease (PD) is a progressive neurodegenerative disorder of the central nervous system (CNS) resulting from the loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain, causing severe motor deficits such as tremor, rigidity, postural imbalance, and slowness of movement (Davie, 2008). Currently, there is a lack of specific and effective treatment for PD as the commonly used drug, levodopa, only palliates the symptoms and may even exacerbate the
symptoms over long-term treatment. Moreover, long-term use of levodopa can cause dyskinesia in PD patients (Pandey and Srivanitchapoom, 2017). Because of the limitations of the existing PD treatment, molecular mechanisms underlying progressive DA neurons death and the discoveries of novel therapeutic agents for PD are essential for the prevention and cure of the disease.

Although molecular mechanisms of DA neurons death are not completely understood, many studies suggested that the phosphatidylinositol 3-kinase (PI3K)/serine/threonine protein kinase (Akt)/mammalian target of rapamycin kinase (mTOR) pathway is associated with the neuronal proliferation, differentiation, and programmed apoptotic cell death (Hawkins et al., 2006). Moreover, the PI3K/Akt/mTOR pathway is also associated with the neuroprotective signaling pathways in PD (Chen et al., 2013). This evidence has been supported by several studies on pharmacological treatment, such as exenatide and rotigotine, to improve PD via the activation of PI3K/Akt/mTOR pathway (Oster et al., 2014; Hauser et al., 2016; Athauda et al., 2019).

Eukaryotic translation elongation factor 1 alpha (eEF1A) proteins are guanosine triphosphate (GTP)-binding proteins that are transcribed in the nucleus and functions in protein synthesis. During the elongation process of protein translation, eEF1A delivers aminoacyl-tRNAs to the ribosomal A-site for binding of codon and anticodon (Dinman and Kinzy, 1997). Two isoforms, namely, the eEF1A1 and eEF1A2, can be found in mammals that are paralog and share more than 90% of human DNA (Kahns et al., 1998). Interestingly, previous studies reported that the eEF1A declined in the human brain tissues with history of PD disease progression, indicating an association of eEF1A and altered polypeptide synthesis in the corresponding area (Licker et al., 2014; Garcia-Esparcia et al., 2015). In the cellular model, upregulation of genes of eEF1A has been found to be associated with the increased expression of PI3K, AKT, and mTOR in 1-methyl-4-phenylpyridinium (MPP+)-induced cellular PD model (Khwanraj et al., 2016). Since the roles of eEF1A1 and eEF1A2 in the prevention of DA neuronal cell death in PD remain unclear, this study aims to investigate the role of eEF1A isoforms on degenerated DA neurons by using Caenorhabditis elegans as a PD model.

_Caenorhabditis elegans_ is a eukaryotic organism with a short life cycle (Brenner, 1974), which is advantageous to monitor the progression of neurodegenerative PD diseases. _C. elegans_ has been used as a PD model since it demonstrates eight well-mapped DA neurons which are subdivided into two pairs of cephalic neurons (CEP), one pair of anterior deirid neurons (ADE) in the head and the posterior deirid neurons (PDE) in the tail (Nass et al., 2002; Harrington et al., 2010). In comparison with mammals, the DA synthesis, storage, and transport mechanisms, as well as cellular apoptosis and survival PI3K/AKT/mTOR pathways, are conserved in _C. elegans_ (Sanyal et al., 2004; Lant and Storey, 2010; Lord and Gunawardena, 2012). Moreover, _C. elegans_ also share a wide homologous genome with mammals including eEF1A1; _eft-3_ homolog and eEF1A2; _eft-4_ homolog. Therefore, _C. elegans_ is suitable to use as a model in the study of neurodegenerative diseases.

In the present study, we exposed _C. elegans_ to neurotoxin, 6-hydroxydopamine (6-OHDA) to induce degeneration of DA neurons and behavioral characteristics associated with PD. This study provides evidence that the downregulation of eEF1A by RNA interference (RNAi) exhibits the morphological changes of DA neurons, deficits in dopamine-dependent behaviors, and increased lifespan in _C. elegans_. Moreover, the knockdown of eEF1A also accelerated the DA neurodegeneration induced by 6-OHDA in _C. elegans_ through downregulation of the survival pathway.

**MATERIALS AND METHODS**

**C. elegans Strains, Maintenance, and Synchronization**

Wild-type Bristol N2, transgenic BZ555 (dat-1p:GFP; green fluorescent protein expression in the DA neuronal soma and processes), SD1340 [eft-3p:His-24:mCherry; red fluorescent protein (RFP) expression in the most cells], and CU394 (ced-3p:GFP green fluorescent protein expression in the apoptotic cell) were obtained from the Caenorhabditis Genetics Center (University of Minnesota, United States). Strain UA202 (dat-1p:psid-1, myo-2p:mCherry; dat-1p:GFP) which is sensitive to RNAi specifically in DA neurons was kindly provided by Caldwell Laboratory (The University of Alabama, United States). All procedures performed in the _C. elegans_ were according to the protocols approved by the Faculty of Science, Mahidol University Animal Care and Use Committee (MUSC-ACUC). All strains were cultured following standard methods (Brenner, 1974). Large populations of _C. elegans_ can be grown by culturing on solid nematode growth medium (NGM) and fed with _Escherichia coli_ OP50 as a food source at 20°C. Synchronized eggs were isolated from gravid worms by bleaching solution (12% NaClO and 10% 1 M NaOH), plated on NGM without bacteria and then incubated at 20°C overnight to obtain newly hatched animals or L1 larvae. To acquire L3 larvae, synchronized L1 larvae were transferred onto NGM plates containing _E. coli_ OP50 and incubated for 20–24 h at 20°C.

**RNAi Treatment**

Bacterial RNAi feeding constructs of _eft-3_ and _eft-4_ were obtained from the Vidal laboratory library (Source BioScience, Nottingham, United Kingdom). They were isolated and grown overnight in Luria broth medium plus 100 µg/ml ampicillin (Sigma Aldrich, St. Louis, MO, United States). Cells were seeded on 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Aldrich, St. Louis, MO, United States) to induce dsRNA expression, and then, these plates were allowed to dry overnight at room temperature and stored at 4°C.

**C. elegans Dopaminergic Neurodegeneration Assay**

The _C. elegans_ were induced by the neurotoxin 6-OHDA (Sigma Aldrich, St. Louis, MO, United States) to selectively
degenerate the DA neurons. Synchronized L3 larvae were treated in a solution containing diluted OP50 mixed with 10, 25, and 50 mM 6-OHDA and 2, 5, and 10 mM ascorbic acid, respectively. The solution was mixed gently with pipette every 10 min for 1 h at 20°C. After incubation, the worms were washed by M9 buffer for three to four times. Induced worms were then retransferred onto RNAi bacterial plates and 0.04 mg/ml 5-fluoro-2′-deoxyuridine (FUDR, Sigma, St. Louis, MO, United States) for inhibiting progeny development and treated for 72 h before performing in subsequent assays. L4440 [empty vector (EV)] bacteria NGM plates were used as a control. The experiment was performed independently for three times (n = 40–50 animals/group per replicate).

**Imaging and Quantification Analysis**

After treating the worms with various conditions of 6-OHDA, the adult hermaphrodites were washed three times with M9 buffer to remove the bacteria from the nematode cuticle. They were loaded onto 2% agarose-padded slides. Worms were anesthetized by 2% sodium azide and sealed with coverslips. The immobilized worms were observed and photographed under a fluorescence microscope (BX53, Olympus Corp., Tokyo, Japan). Fluorescence intensity was measured in 40–50 randomly selected worms by ImageJ software [National Institutes of Health (NIH), Maryland, United States]. For phenotype analysis of DA neurons, the observer was blinded to the genotype and the treatment of the worms.

**Food-Sensing Behavior**

This assay was performed to test the food-sensing function controlled by DA neurons. Normally, the worms move slowly in bacterial lawn when compared with worms in plates without bacteria (Sawin et al., 2000). In brief, *E. coli* OP50 were spread on the center of NGM plates, and the bacteria-containing plates were incubated at 37°C overnight. Wild-type L3 larvae nematodes were firstly treated with either 6-OHDA with empty vector bacteria or 6-OHDA with RNAi bacteria for 72 h at 20°C and then washed three times with M9 buffer. Worms were transferred to the plates with or without bacterial supply and settled for 5 min. The worm body bending was recorded and counted in 30-s intervals. The body bending in the plates with and without bacteria was counted and compared in each group. Percentage of basal slowing rate was calculated as the difference in the rate of movement on bacterial lawn versus outside bacterial lawn divided by the rate of movement outside bacterial lawn (n ≥ 30 animals/group per replicate).

**Ethanol Avoidance Assay**

Adult treated worms were washed three times with M9 buffer and transferred to the center of NGM assay plates. Assay plates were divided into four quadrants; two quadrants seeded with 50 µl ethanol and two quadrants seeded with 50 µl distilled water. After 30 min, the numbers of worms were scored in each quadrant. Ethanol avoidance index was calculated as the difference in number of worms in control quadrants versus in ethanol quadrants divided by the total number of worms (Cooper et al., 2015; Maulik et al., 2017) (n ≥ 30 worms/group per replicate).

**Lifespan Assay**

After being treated with RNAi or EV bacteria, synchronized L3 stage N2 were induced with 6-OHDA for 1 h and transferred to RNAi plates containing various conditions, EV bacteria, and 0.04 mg/ml FUDR plates at 20°C. The numbers of live and dead worms were counted and recorded daily until all worms died, then the mean lifespan and percent survival were calculated. Dead worms were determined when they did not respond to touch by a platinum wire and showed no pharyngeal pumping. Censored worms were the worms with internally hatched progeny or extruded gonad which were excluded from the experiment. Three independent replicates were conducted for each treatment with approximately 30–40 animals per replicate, so more than 100 animals were analyzed.

**Quantitative RT-PCR**

Total RNA was synthesized using the RNA extraction kit (Qiagen, Germany) following the manufacturer’s protocol. The RNA samples were stored at −80°C until use. For quantitative gene expression analyses, high-capacity complementary DNA (cDNA) was generated from 2 µg of RNA using the iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Foster City, CA, United States). Then, cDNA was diluted to a ratio of 1:10 with SsoFastTM EvaGreen® Supermix with Low ROX qRT-PCR (Bio-Rad, Foster City, CA, United States), mixed with forward and reverse primers of specific genes (Table 1). Real-time PCR was first performed by holding the sample at 95°C for 30 s. Then, the PCR sample was set for denaturing at 95°C for 5 s and to annealing/extension at 60°C for 30 s. After 44 cycles repeat, the sample was then heated up to 95°C for melt curve analysis. Eventually, EvaGreen fluorescence was detected by the CFX96 Touch Real-time PCR detection system (Bio-Rad, Foster City, CA, United States), and Cq values were obtained. All targeted genes were measured in triplicate, and three independent biological triplicates were detected in each condition. The Cq values were then calculated via 2−(ΔΔCq) equation representing relative fold change in the expression of each gene. Relative mRNA expression levels were normalized using reference internal control gene, act-1.

**Statistical Analysis**

All assays were completed with a minimum of three biological replicates. All data were presented as mean ± standard error of the mean (SEM). Differences among groups were determined by one-way analysis of variance (ANOVA) following the Tukey-Kramer test for multiple comparisons. For grouped analyses, a two-way ANOVA series was used with Tukey’s multiple comparison for post hoc test. Survival plots were compared using the log-rank test. Probability levels (p-value) of <0.05 were considered statistically significant. All statistical analyses were determined by GraphPad Prism software 7 (GraphPad Software Inc.).
TABLE 1 | Primer lists used in this study.

| Primer | Sequence |
|--------|----------|
| Forward (5′—3′) | Reverse (5′—3′) |
| eft-2 | TTATCCTGTGGCAAGCGCTT | TCCATAAGCCACAGATCTC |
| eft-3 | GGAACGTTAGAGCAAGCGAT | TTAGCAAGAGCATTCGAGG |
| eft-4 | CATGTTCAAGGAGTGAGGGC | ATTCGGTTCACACACGTT |

RESULTS

Dopaminergic Neurons Loss and Reduction of eft-3 and eft-4 Induced by 6-Hydroxydopamine Exposure

In transgenic BZ555 strain worms, morphological patterns of DA neurons were fluorescently labeled by the expression of the dopamine transporter marker Pdat-1::GFP (Figure 1A, control). Selective degeneration of C. elegans DA neurons could be accomplished by the exposure of 6-OHDA. Results showed that the exposure to 10, 25, and 50 mM of 6-OHDA gradually damaged cell bodies in CEs and ADEs and fragmented neuronal processes of anterior CEP in a dose-dependent manner (Figure 1A). We defined the DA neurodegeneration by determining GFP expression as the following indexes: (1) ADE + CEP, (2) ADE + partial CEP, and (3) only ADE, and (4) no ADE + CEP. We found that the percent of worms possessing all ADE and CEP significantly reduced to 64.8% ± 4.97% and 34.8% ± 4.75% when exposed to 25 and 50 mM 6-OHDA, respectively (Figure 1B). Moreover, the DA neuron viability in BZ555 was also evaluated by analyzing the mean fluorescence intensity (MFI) of GFP expression and the morphology of DA neurons. Our results showed that the percent relative MFI of 25 and 50 mM 6-OHDA-treated group significantly reduced to 73.96% ± 7.51% and 62.23% ± 2.12%, respectively (Figure 1C). However, in 10 mM 6-OHDA exposure, there were non-significant changes in both GFP fluorescence and apparent dopaminergic–neuronal morphological changes of ADE and CEP compared to the control group.

To further determine the effect of 6-OHDA exposure on the expression of eEF1A isoforms in the C. elegans PD model, we investigated the mRNA expression changes of eft-3 and eft-4 (homolog of mammalian EEF1A1 and EEF1A2, respectively) after exposure to 50 mM 6-OHDA. Quantitative RT-PCR showed that eft-3 and eft-4 mRNA expression levels were significantly reduced to 0.76 ± 0.07 fold and 0.51 ± 0.12 fold in 6-OHDA-treated worms when compared with normal worms (Figure 1D). Moreover, using transgenic strain SD1340 for the expression of fusion eft-3::RFP, we found obvious downregulation of RFP expression in the 6-OHDA-treated group (Figure 1E), with MFI significantly reduced to approximately 72.17% ± 4.34% (P < 0.05) when compared to the untreated worms (Figure 1F).

RNAi Knockdown of eft-3 and eft-4 Accelerated Dopamine Neurodegeneration Caused by 6-Hydroxydopamine

Caenorhabditis elegans eft-3 or eft-4 homozygous null mutant generated an abnormal development involving embryonic viability, fertility, and germline maintenance (Gönczy et al., 2000; Maeda et al., 2001). Therefore, to determine the function of eft-3 and eft-4 on DA neurons without interfering with the normal cellular process, we performed the RNAi knockdown in C. elegans UA202 strain to assay for DA neurodegeneration. After RNAi knockdown, the levels of eft-3 or eft-4 mRNA expression were significantly reduced, while eft-2 mRNA level did not change, indicating successful knockdown experiments in these treated worms (Figure 2A). Decreasing of eft-3 or eft-4 expression generated fragmented DA neuronal processes, blebbing neurites, and shrinkage of cell bodies (Figure 2B). Quantification analysis of GFP expression on DA neurons revealed that knocking down eft-3 and eft-4 caused a significant decrease of the percentage of worms carrying normal DA neurons at 56.00% ± 7.97% and 50.40% ± 6.54%, respectively (Figure 2C). Meanwhile, the knockdown of eft-3 and eft-4 also resulted in a significant decrease of the MFI of DA neurons at 78.36% ± 7.26% and 73.04% ± 7.68%, respectively, when compared with EV control (Figure 2D).

To determine the roles of eft-3 and eft-4 in DA neurodegeneration in PD, we further examined the effects of eft-3 or eft-4 RNAi on 6-OHDA vulnerability in C. elegans. As expected, worms treated with 50 mM 6-OHDA and EV exhibited DA neurons loss (Figure 2B). Moreover, co-treatment of eft-3 or eft-4 RNAi with 6-OHDA worsened DA neuronal losses (Figure 2B). By quantifying the morphology of DA neurons, the results showed that 6-OHDA co-treatment with eft-3 or eft-4 RNAi caused greatly significant decreases of the percentage of worms carrying normal DA neurons at 9.20% ± 1.90% and 9.60% ± 2.92%, respectively (Figure 2C). The MFI of DA neurons in 6-OHDA co-treatment with eft-3 or eft-4 RNAi condition also significantly decreased in the mean intensity at 35.51% ± 3.80% and 33.31% ± 2.98%, respectively, when compared with 6-OHDA treatment alone (Figure 2D).

Decreasing of eft-3 or eft-4 Caused Deficits in Food Sensing and Ethanol Avoidance Behaviors

We further examined the effects of eft-3 and eft-4 RNAi on the dopamine-dependent behaviors: food-sensing behavior or...
basal slowing rate (Sawin et al., 2000) and ethanol avoidance (Cooper et al., 2015) in C. elegans which are controlled by DA neurons. Normally, when worms come across the bacteria lawn or food source, they reduce body bending frequency to feed themselves. UA202 worms revealed 56.66% ± 3.63% reduction in bending frequency when in contact with bacteria lawn. 6-OHDA-treated worms, however, failed to reduce the bending frequency due to the loss of the food-sensing system. For
the UA202 treated with 6-OHDA, the basal slowing rate was significantly reduced to 28.79% ± 2.78% when compared with the untreated N2 group (Figure 3A). The single eft-3 or eft-4 RNAi-treated worms also displayed a significant reduction in slowing response to 42.88% ± 3.51% and 41.63% ± 3.98%, respectively. Moreover, the combination of 6-OHDA and eft-3 or eft-4 RNAi treatment significantly reduced the basal slowing behavior to 13.39% ± 2.29% and 13.61% ± 2.35%, respectively, compared with normal and 6-OHDA treatment alone (Figure 3A). Similarly, eft-3 or eft-4 RNAi-treated worms also exhibited the significant ethanol avoidance deficit by decreasing the ethanol avoidance index to -0.01 and 0.03 when compared to normal worms. The combined eft-3 or eft-4 RNAi and 6-OHDA exposure also caused more significant decrease to −0.64 and −0.61 compared with both EV control and only 6-OHDA exposure (Figure 3B).
Decreasing of eft-3 or eft-4 Enhanced 6-Hydroxydopamine-Induced Shortening of Lifespan

We further examined the effects of eft-3 or eft-4 RNAi and co-treatment with 6-OHDA on the lifespan of *C. elegans*. The mean lifespan of N2 worms was approximately 12.54 ± 0.20 days. In 6-OHDA-induced worms, the mean lifespan was significantly reduced to 10.74 ± 0.18 days and shortened by 14.35% from normal worms (Figure 4A). Besides, knocking down eft-3 or eft-4 combined with 6-OHDA treatment caused a further shortening lifespan with 12.98 and 12.20% reduction compared with 6-OHDA treatment only (Figure 4B and Table 2). On the other hand, the eft-3 or eft-4 RNAi-treated worms showed a non-significant increase of mean lifespan at 3.76%, 4.84% compared with normal worms, respectively (Figure 4C and Table 2).

Decreasing of eft-3 or eft-4 Affected the Apoptotic Gene Expression

Previously, apoptotic cell death has been linked with DA neuronal loss and the expression of eEF1A (Li et al., 2010; Zeng et al., 2018), hence we sought to determine whether the underlying mechanisms of DA neuron loss is mediated by apoptosis signaling pathway. mRNA transcript levels of genes involved with apoptosis were determined in normal, eft-3 or eft-4 RNAi-exposed, 6-OHDA-induced, and combined-treated worms. We used qPCR to analyze the mRNA levels of egl-1, ced-3, ced-4, and ced-9, which are associated with apoptosis in *C. elegans*. We found that the expression levels of egl-1, ced-3, ced-4, and ced-9 were not significantly changed in 6-OHDA-treated worms compared to those in untreated worms (Figure 5A). The level of egl-1 mRNA was upregulated following the knockdown of eft-3 or eft-4 with 1.48 ± 0.14 fold and 1.52 ± 0.30 fold compared with control. In co-treatment of eft-3 or eft-4 RNAi and 6-OHDA-treated worms, the expression of egl-1 showed more significant increase when compared with both EV worms and only 6-OHDA-treated worms. Moreover, the expression level of ced-3 was slightly increased in eft-3 or eft-4 RNAi-treated worms with 1.31 ± 0.24 fold and 1.42 ± 0.31 fold, respectively. The combined eft-3 or eft-4 RNAi and 6-OHDA treatment caused a significant increase of ced-3 mRNA level compared with both EV control and only 6-OHDA exposure (Figure 5A).

To confirm the apoptosis process in the worms, we further analyzed the CED-3 expression using transgenic CU394 strain (Z-ced-1::ced-3:gfp). We found that the eft-3/eft-4 RNAi- treated worms did not show the change in the MFI of CED-3 expression in *C. elegans* when compared with the EV RNAi (Supplementary Figures S1A,B). However, the MFI of CED-3 was upregulated in combined eft-3 or eft-4 RNAi and 6-OHDA-treated worms compared with both EV control and 6-OHDA treatment alone (Supplementary Figures S1A,B).

Decreasing of eft-3 or eft-4 Affected the Survival Gene Expression

Having revealed a role for eEF1A in PI3K/AKT/mTOR mechanism in several cellular models (Amiri et al., 2006; Khwanraj et al., 2016), we sought to examine whether the eft-3 and eft-4 mediate neuroprotection through the cell survival signaling, PI3K/AKT/mTOR, in 6-OHDA-induced *C. elegans* PD model. We performed real-time qPCR to analyze the mRNA levels of daf-2, age-1, let-363, pdk-1, akt-1, and akt-2, which are homologs with cell-survival signaling genes in mammals. Results showed that knockdown of eft-3 or eft-4 significantly lowered only the akt-1 mRNA expression compared with control. However, the mRNA expression of only let-363 was significantly reduced in the 6-OHDA-treated group.
FIGURE 4 | Effects of eft-3/eft-4 RNA interference (RNAi) and/or 6-hydroxydopamine (6-OHDA) exposure on lifespan in N2 Caenorhabditis elegans. Cumulative survival plots of 6-OHDA-treated (A), co-treatment of eft-3/eft-4 RNAi and 6-OHDA-treated worms (B), and empty vector (EV) RNAi-treated and eft-3/eft-4 RNAi-treated worms (C). Quantitative data representing mean lifespan, maximum lifespan, percentage of increased lifespan, and significance are shown in Table 2.

TABLE 2 | Mean lifespan, maximum lifespan, percentage of increase lifespan and significance P-values.

| Treatment | Mean lifespan (day) | Maximum lifespan | % increase lifespan | Significant (P-value) |
|-----------|---------------------|------------------|---------------------|----------------------|
| N2 + EV   | 12.54 ± 0.20        | 19               | –                   | –                    |
| N2 + eft-3 RNAi | 13.01 ± 0.29    | 19               | 3.76 (compared with N2) | *P < 0.05 |
| N2 + eft-4 RNAi | 13.14 ± 0.30    | 19               | 4.84 (compared with N2) | *P < 0.05 |
| N2 + 6-OHDA | 10.74 ± 0.18       | 14               | –14.35 (compared with N2) | ***P < 0.001 |
| N2 + 6-OHDA + eft-3 RNAi | 9.35 ± 0.17   | 15               | –12.98 (compared with N2 + 6-OHDA) | ***P < 0.001 |
| N2 + 6-OHDA + eft-4 RNAi | 9.43 ± 0.22   | 16               | –12.20 (compared with N2 + 6-OHDA) | ***P < 0.001 |

compared with normal. The co-treatment condition of 6-OHDA and eft-3 or eft-4 knockdown caused significant decreases in mRNA expression of age-1, let-363, pdk-1, akt-1, and akt-2 compared with both EV control and 6-OHDA treatment alone (Figure 5B).

DISCUSSION

In the present study, we reported for the first time the roles of eft-3/4 (homolog of mammalian eEF1A1/2) in C. elegans model of PD. Our data demonstrated that the nematodes exposed to 50 mM 6-OHDA display PD-like phenotype together with the decreased expression of EFTs. Downregulation of eft-3 or eft-4 enhanced 6-OHDA-induced DA neurodegeneration, aggravated food-sensing and ethanol avoidance behaviors, and shortened lifespan. Moreover, the molecular signaling analysis showed that the combination of EFT downregulation and 6-OHDA exposure could increase the expression of egl-1 and ced-3 in the apoptosis pathway and also greatly decrease the expression of genes in the PI3K/AKT/mTOR survival pathway, age-1, let-363, pdk-1, akt-1, and akt-2.

Parkinson’s disease is the neurodegenerative disease associated with the selective loss of DA neurons in the substantia nigra (Hartmann, 2004). It has been proposed that the major loss of DA neurons may come from chronic exposure to neurotoxins (Bové and Perier, 2012). In the present study, we showed that the exposure to 6-OHDA induced the PD-like phenotypes in a dose-dependent manner in C. elegans. 6-OHDA caused degeneration in DA neurons which led to abnormality in the physiological function correlated with the...
FIGURE 5 | Effects of eft-3/eft-4 RNA interference (RNAi) and/or 6-hydroxydopamine (6-OHDA) exposure on apoptosis and survival modulation. (A) Fold change of mRNA expression levels of apoptosis signaling genes including egl-1, ced-3, ced-4, and ced-9 in empty vector (EV) RNAi-treated, eft-3/eft-4 RNAi-treated, 6-OHDA-treated, and co-treatment of eft-3/eft-4 RNAi and 6-OHDA-treated groups. (B) Fold change of mRNA expression levels of survival signaling genes including daf-2, age-1, let-363, pdk-1, akt-1, and akt-2 in EV RNAi-treated, eft-3/eft-4 RNAi-treated, 6-OHDA-treated, and co-treatment of eft-3/eft-4 RNAi and 6-OHDA-treated groups. All data represent the mean ± SEM of three independent biological replicates. The asterisk (*) indicates a significant difference between the EV RNAi-treated and eft-3/eft-4 RNAi and/or 6-OHDA-treated worms (p < 0.05). The hash (#) indicates a significant difference between only 6-OHDA-treated and combined 6-OHDA with eft-3 or eft-4 RNAi-treated worms (p < 0.05).

degeneration of the DA neuron in C. elegans, including food-sensing behavior and the shortened lifespan. These results are consistent with the previous studies that have reported 6-OHDA-induced parkinsonism in both in vitro and in vivo models (Latchoumycandane et al., 2011; Bagga et al., 2015; Chalorak et al., 2018). 6-OHDA selectively induced the toxicity in the DA neurons by entering the cell through dopamine transporter which expressed exclusively in the DA neuronal cell surface (Nass et al., 2002). Once entering into the neurons, 6-OHDA increased oxidative stress and triggered the signaling pathways that eventually led to cell death (Offenburger et al., 2018). Furthermore, in this study, we found that 6-OHDA also decreased the expression of eft-3 and eft-4 in both protein and RNA expression levels. Similarly, decreasing of eEF1A expression has been found in PD brain patients (Garcia-Esparcia et al., 2015). Alteration of protein translation has been proposed to be a contributing factor to PD pathogenesis (Moreno et al., 2012; Taymans et al., 2015; Zhou et al., 2019). Thus, our results encourage the possibility that downregulation of protein translation regulators, eEF1As, is associated with PD and toxin-induced PD model.

eEF1A1 and eEF1A2 proteins are GTP binding proteins that deliver the amino acids during protein translation (Zhu et al., 2001). The downregulation of eEF1As has been previously
found in the substantia nigra and frontal cortex of the PD patients, although the involvement of eEF1A and PD incidence remains unclear (Licker et al., 2014; Garcia-Esparcia et al., 2015). To understand the role of eEF1A in PD pathophysiology, we investigated the effects of eft-3 or eft-4 knockdown on C. elegans PD model. In this study, we found that the downregulation of eft-3 and eft-4 caused the degenerative and misplaced DA neurons in C. elegans. Knocking down these genes in C. elegans also exhibited the dysfunction of the DA neurons as seen in the food-sensing behavior assay. Our results are consistent with the previous reports that the downregulation of eEF1A1 causes the degenerative and misplaced DA neurons as seen in the food-sensing behavior assay.

Moreover, knocking down eft3/4 in 6-OHDA-exposed nematodes worsened the DA neuronal degeneration caused by 6-OHDA exposure alone and greatly aggravated the dopamine-dependent behaviors. These results suggested that eft3/4 may play an important role to sustain DA neurons and their function against neurotoxin.

Although knocking down either eft-3 or eft-4 has similar effects to 6-OHDA-induced DA degeneration, the lifespan of eft-3 or eft-4 RNAi-treated worms was slightly increased compared with the normal worms. However, combined knocking down eft3/4 and 6-OHDA exposure could shorten the lifespan compared with 6-OHDA-induced alone. Previous studies on the interaction between genetic and environmental exposure has shown that many gene mutations could increase the sensitivity to the neurotoxins and cause more severe defects in animal models (Meulener et al., 2005; Ray et al., 2014; Zeng et al., 2018). From these data, it might be possible that the eft3/4 knockdown enhanced the 6-OHDA-induced DA neurodegenerations in C. elegans.

There are two signaling pathways associated with 6-OHDA-induced DA neuron degeneration in C. elegans: (i) the apoptotic signaling molecules and (ii) the anti-apoptotic molecules (Nass et al., 2002). Previously, the microarray analysis of DA neurons from PD patients showed that several signaling pathways are dysregulated. Among these pathways, PI3K/Akt/mTOR signaling pathway was found to be associated with all dysregulation (Elstner et al., 2011). Here, we revealed that 6-OHDA downregulates the mTOR-like let-363 gene expression without alteration of BH3-like egl-1, Apaf-1-like ced-4, and caspase-9-like ced-3 expressions. This observation is similar to previous reports indicating that the 6-OHDA-induced DA cell death is associated with the downregulation of mTOR (Zhang et al., 2017). Moreover, previous studies suggested that 6-OHDA induced DA neuron death independently from apoptosis signaling, ced-3 and ced-4, in C. elegans (Nass et al., 2002). Our study showed that the knockdown of eft3/4 suppressed the akt-1 but not akt-2 expression and promoted the gene expression of egl-1 and ced-3 which are the key apoptotic signaling proteins (Amiri et al., 2006). Previously, eEF1A2 has been shown to possess the anti-apoptosis effect in mouse plasmacytoma cell lines via activation of PI3K/Akt (Li et al., 2010). Although the effects on gene expression of 6-OHDA and eft-3/4 gene silencing were different, the anti-apoptotic genes were strongly suppressed when both treatments were combined. The previous findings showed that the downregulation of eEF1A causes the neurons to be more vulnerable to the external insults (Vera et al., 2014; Davies et al., 2017), and our results further showed that the downregulation of eEF1A2 also increases sensitivity to external insults. Our data encourage the fact that the downregulation of eft-3/4 attenuates the neurons to be more vulnerable to neurotoxin by suppression of the anti-apoptotic genes as well as the promotion of some apoptotic gene expressions.

CONCLUSION

Our study reported that the downregulation of eft3 or eft-4 by RNAi impairs the survival of DA neurons and food-sensing and ethanol avoidance behaviors in C. elegans. The downregulation promoted a pro-apoptotic gene egl-1 expression and decreased expression of pro-survival gene akt-1. Moreover, eft3/4-knocked down worms displayed more severely in DA neuronal system when being exposed in combination with 6-OHDA. Gene expression analysis showed that the combination of eft3/4 downregulation and 6-OHDA decreases several pro-survival gene expressions including age-1, akt1/2, and let-363. These results suggested that the downregulation of eft3/4 may facilitate the devastating effects of the neurotoxin 6-OHDA, and this can be a contributing factor of toxin-induced PD in humans.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All animal procedures used in the present study were performed in the C. elegans model which were approved by the Faculty of Science, Mahidol University Animal Care and Use Committee (MUSC-ACUC).

AUTHOR CONTRIBUTIONS

All authors listed contributed immensely to this study. PC performed the experiments, conducted the data analysis, and wrote the manuscript. KM and PD interpreted the data, suggested and put forward the idea, and reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This study was supported by grants from the Thailand Research Fund and Mahidol University (IRG5780011 to KM and PD) and the Royal Golden Jubilee Ph.D. scholarship (PHD/0137/2559).
to PC). The study was also partially supported by a CIF grant, Faculty of Science, Mahidol University. Some C. elegans strains used in this study were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00303/full#supplementary-material

REFERENCES

Amiri, A., Noei, F., Jeganathan, S., Kulkarni, G., Pinke, D. E., and Lee, J. M. (2006). eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration. Oncogene 26:3027. doi: 10.1038/sj.onc.1210101

Athauda, D., Gulyani, S., Karnati, H. K., Li, Y., Tweedie, D., Mustapic, M., et al. (2019). Utility of neuronal-derived exosomes to examine molecular mechanisms that affect motor function in patients with Parkinson disease: a secondary analysis of the exenatide-PD trial. JAMA Neurol. 76, 420–429. doi: 10.1001/jamaneurol.2018.4304

Bagga, V., Dunnett, S. B., and Fricker, R. A. (2015). The 6-OHDA mouse model of Parkinson’s disease – Terminal striatal lesions provide a superior measure of neuronal loss and replacement than median forebrain bundle lesions. Behav. Brain Res. 288, 107–117. doi: 10.1016/j.bbr.2015.03.058

Bové, J., and Perier, C. (2012). Neurotoxin-based models of Parkinson’s disease. Neurosci. Biobehav. Rev. 36, 281–293. doi: 10.1016/j.neubiorev.2011.10.007

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics 77, 71–94.

Chalorak, P., Jattuian, P., Nobasithan, S., Poomtong, T., Sobhon, P., and Meemon, K. (2018). Holothuria scabra extracts exhibit anti-Parkinson potential in C. elegans: a model for anti-Parkinson testing. Nutr. Neurosci. 21, 427–438. doi: 10.1080/1028415X.2017.1299437

Chen, A., Xiong, L. J., Tong, Y., and Mao, M. (2013). Neuroprotective effect of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway. Mol. Med. Rep. 8, 1011–1016. doi: 10.3892/mmr.2013.1628

Cooper, J. F., Due, D. J., Spielbauer, K. K., Machiela, E., Senchuk, M. M., and Van Raamsdonk, J. M. (2015). Delaying aging is neuroprotective in Parkinson’s disease: a genetic analysis in C. elegans models. NPJ Parkinsons Dis. 1, 15022–15022. doi: 10.1038/npjparkd.2015.22

Davie, C. A. (2008). A review of Parkinson’s disease. Br. Med. Bull. 86, 109–127. doi: 10.1093/bmbld/ldn013

Davies, F. C. J., Hope, J. E., McLachlan, F., Nunez, F., Doig, J., Bengani, H., et al. (2013). Neuroprotective effect of translation elongation factor eEF1A1: a target for antiplatelet agent cilostazol. PLoS One 8:e10755. doi: 10.1371/journal.pone.0010755

Lord, C. E. N., and Gunawardena, A. H. L. A. N. (2012). Programmed cell death signaling with the mammalian system. Acta Neuropathol. Commun. 3:76. doi: 10.1186/s40478-015-0257-4

Maeda, I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001). Large-scale analysis of gene function in Caenorhabditis elegans by high-throughput RNAi. Curr. Biol. 11, 171–176. doi: 10.1016/S0960-9822(01)00052-5

Maulik, M., Mitra, S., Bult-Itso, A., Taylor, B. E., and Vayndorf, E. M. (2017). Behavioral phenotyping and pathological indicators of Parkinson’s disease in C. elegans models. Front. Genet. 8:77. doi: 10.3389/fgene.2017.00077

Meulener, M., Whitworth, A. J., Armstrong-Gold, C. E., Rizzu, P., Heath, R., et al. (2017). Biallelic mutations in the gene encoding eEF1A2 cause seizures and sudden death in F0 mice. Acta Neuropathol. 133, 379–392. doi: 10.1007/s00401-016-1734-5

Moreno, J. A., Radford, H., Peretti, D., Steinert, J. R., Verity, N., Martin, M. G., et al. (2012). Sustained translational repression by eIF2α-P mediates prion neurodegeneration. Nature 485, 507–511. doi: 10.1038/nature11058

Nass, R., Hall, D. H., Miller, D. M. III, and Blakely, R. D. (2002). Neurotoxin-induced degeneration of dopamine neurons in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U.S.A. 99, 3264–3269. doi: 10.1073/pnas.0249799

Nass, R., Hall, D. H., Miller, D. M. III, and Blakely, R. D. (2002). Neurotoxin-induced degeneration of dopamine neurons in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U.S.A. 99, 3264–3269. doi: 10.1073/pnas.0249799
Chalorak et al. eEF1A1 and eEF1A2 in DA Neurodegeneration in PD

Ray, A., Zhang, S., Rentas, C., Caldwell, K. A., and Caldwell, G. A. (2014). RTCB-1 mediates neuroprotection via XBP-1 mRNA splicing in the unfolded protein response pathway. *J. Neurosci.* 34, 16076–16085. doi: 10.1523/JNEUROSCI.1945-14.2014

Sanyal, S., Wintle, R. F., Kindt, K. S., Nuttley, W. M., Arvan, R., Fitzmaurice, P., et al. (2004). Dopamine modulates the plasticity of mechanosensory responses in *Caenorhabditis elegans*. *EMBO J.* 23, 473–482. doi: 10.1038/sj.emboj.7600057

Sawin, E. R., Ranganathan, R., and Horvitz, H. R. (2000). *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron* 26, 619–631. doi: 10.1016/S0896-6273(00)81199-X

Sun-Jung, C., Lee, H., Dutta, S., Seog, D. H., and Moon, I. S. (2012). Translation elongation factor-1A1 (eEF1A1) localizes to the spine by domain III. *BMB Rep.* 45, 227–232. doi: 10.5483/bmbrep.2012.45.4.227

Taymans, J.-M., Nkiliza, A., and Chartier-Harlin, M.-C. (2015). Deregulation of protein translation control, a potential game-changing hypothesis for Parkinson's disease pathogenesis. *Trends Mol. Med.* 21, 466–472. doi: 10.1016/j.molmed.2015.05.004

Vera, M., Pani, B., Griffiths, L. A., Muchardt, C., Abbott, C. M., Singer, R. H., et al. (2014). The translation elongation factor eEF1A1 couples transcription to translation during heat shock response. *eLife* 3:e03164. doi: 10.7554/eLife.03164

Zeng, X.-S., Geng, W.-S., and Jia, J.-J. (2018). Neurotoxin-induced animal models of parkinson disease: pathogenic mechanism and assessment. *ASN Neuro* 10:1759091418777438. doi: 10.1177/1759091418777438

Zhang, J., Cai, Q., Jiang, M., Liu, Y., Gu, H., Guo, J., et al. (2017). Mesencephalic astrocyte-derived neurotrophic factor alleviated 6-OHDA-induced cell damage via ROS-AMPK/mTOR mediated autophagic inhibition. *Exp. Gerontol.* 89, 45–56. doi: 10.1016/j.exger.2017.01.010

Zhou, Z. D., Selvaratnam, T., Lee, J. C. T., Chao, Y. X., and Tan, E.-K. (2019). Molecular targets for modulating the protein translation vital to proteostasis and neuron degeneration in Parkinson's disease. *Transl. Neurodegener.* 8:6. doi: 10.1186/s40035-019-0145-0

Zhu, J., Hayakawa, A., Kakegawa, T., and Kaspar, R. L. (2001). Binding of the La autoantigen to the 5' untranslated region of a chimeric human translation elongation factor 1A reporter mRNA inhibits translation in vitro. *Biochim. Biophys. Acta* 1521, 19–29. doi: 10.1016/S0167-4781(01)00277-9

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Chalorak, Dharmasaroja and Meemon. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.