Molecules and Cells

Mitochondrial Uncoupling Attenuates Age-Dependent Neurodegeneration in *C. elegans*

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http://dx.doi.org/10.14348/molcells.2017.0172
www.molcells.org

The uncoupling protein 4 (*ucp-4*) gene is involved in age-dependent neurodegeneration in *C. elegans*. Therefore, we aimed to investigate the mechanism underlying the association between mitochondrial uncoupling and neurodegeneration by examining the effects of uncoupling agents and *ucp-4* overexpression in *C. elegans*. Treatment with either DNP or CCCP improved neuronal defects in wild type during aging. Uncoupling agents also restored neuronal phenotypes of *ucp-4* mutants to those exhibited by wild type, while *ucp-4* overexpression attenuated the severity of age-dependent neurodegeneration. Neuronal improvements were further associated with reductions in mitochondrial membrane potentials. However, these age-dependent neuroprotective effects were limited in mitophagy-deficient mutant, *pink-1*, background. These results suggest that membrane uncoupling can attenuate age-dependent neurodegeneration by stimulating mitophagy.

Keywords: aging, *C. elegans*, mitophagy, neurodegeneration, uncoupling

INTRODUCTION

Age-related diseases represent a major public health issue worldwide, as the overall incidence of such diseases has increased due to increases in the average lifespan (Prince et al., 2013). Among such diseases are neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease, and Huntington's disease—all of which are associated with mitochondrial dysfunction (Bossy-Wetzel et al., 2008; Kerr et al., 2017; Villace et al., 2017). Moreover, recent research has revealed that accumulation of dysfunctional mitochondria in neurons is associated with neurodegeneration (Kerr et al., 2017). Increased levels of reactive oxygen species (ROS) and ATP deficits are prominent features of dysfunctional mitochondria in aged neurons (Grimm and Eckert, 2017). Since neuronal cells require high amounts of energy to perform their various functions, maintaining a healthy mitochondrial population is critical for cell survival (Rugarli and Langer, 2012).

In order to maintain healthy mitochondrial population, mitochondria contain several quality control mechanisms including mitophagy, an autophagy for mitochondrial removal (Ashrafi and Schwarz, 2013). Mitophagy is activated when mitochondrial damage cannot be repaired, or when the number of mitochondria in a cell surpasses the amount required (Ashrafi and Schwarz, 2013; Palikaras et al., 2015). Recent studies involving manipulation of uncoupling proteins in *C. elegans* have revealed that mitochondrial uncoupling improves age-related neurodegeneration (Cho et al., 2016). Moreover, neurons are protected by dissipation of the mitochondrial membrane potential via overexpression of uncoupling proteins in transgenic mice or the use of chemical uncouplers in cultured neurons (Geisler et al., 2017). Several studies have further documented that depolarization of the mitochondrial membrane activates mitophagy (Narendra et al., 2008; 2010), while additional research has

Received 16 August, 2017; revised 8 September, 2017; accepted 21 September, 2017; published online 27 October, 2017

eISSN: 0219-1032

demonstrated that chemical uncouplers such as 2,4-dinitrophenol (DNP) or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) induce mitophagy in cell culture systems (Georgakopoulos et al., 2017; Lazarou et al., 2015).

C. elegans is an excellent tool to study neurodegeneration due to the ease of neuronal manipulation and observation. In addition, there are no studies to date that have investigated the association between age-dependent neurodegeneration and mitophagy in *C. elegans*. Therefore, the present study aimed to determine whether age-dependent neuronal degeneration in *C. elegans* can be alleviated by stimulating mitophagy via mitochondrial uncoupling, by application of chemical uncouplers and genetic manipulations.

MATERIALS AND METHODS

Strains and constructs

Wild type of the Bristol N2 strain, *ucp-4* deletion mutant (*ok195*), and CZ10175 *zdls5* [$p_{mec\text{-}4}$GFP] animals were provided by the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota. *pink-1* deletion mutants (*tm1779*) were provided by the Mitani lab through the National Bio-Resource Project of the NEXT, Japan. We crossed *zdls5* and *ucp-4* mutants to acquire *zdls5;ucp-4*, while *zdls5* and *pink-1* were crossed to obtain *zdls5;pink-1*. To obtain constructs overexpressing *ucp-4*, the amplified 1.6 kb *ucp-4* gene region was cloned into the Fire vector, pPD49.83: [p_{hs}UCP-4]. The heat-shock promoter of the vector was then replaced with the *mec-4* promoter region (0.6 kb) to generate $p_{mec\text{-}4}$UCP-4. Microinjection was performed as previously described (Mello and Fire, 1995) and the plasmid pRF4 containing a dominant gene (*rol-6*) was co-injected. Culture conditions were in accordance with standard protocols (Brenner, 1974).

Observation of neuronal defects

Individual neurons were categorized as defective when they exhibited a single structural abnormality, such as an outgrowth in anterior lateral microtubule (ALM) cells or wavy, neuronal sprout, and branching in posterior lateral microtubule (PLM) cells. All observations were performed using a fluorescent microscope (80i-DS-Fi1, Nikon). Unpaired *t*-tests were performed for a comparison between *ucp-4* overexpression animals and its controls. The one-way analysis of variance (ANOVA) was used for multiple comparisons.

DNP and CCCP treatments

DNP and CCCP in absolute ethanol were added at the desired final concentration before pouring the plates: 100 μM DNP and 10 μM CCCP. 500 μM DNP and 50 μM CCCP were added after bacteria had been seeded onto the plates due to inhibition of bacterial growth. Worms in the plates were continuously exposed to DNP and CCCP throughout the lifetime.

Tetramethylrhodamine (TMRE) experiments

Tetramethylrhodamine (TMRE, Molecular Probes) is a cell-permeable and cationic fluorescent dye that is commonly used as an indicator of mitochondrial membrane potential (Ψ_m) (Ehrenberg et al., 1988; Farkas et al., 1989). TMRE in DMSO (50 μM) was applied to worm plates at a final concentration of 0.1 μM. The plates were then incubated for 16 h, following which they were washed with M9 buffer and prepared for observation in accordance with previously described methods (Yoneda et al., 2004). For ectopic overexpression experiments, heat shock was performed for 2 h at 25°C, 12 h prior to observation.

RESULTS AND DISCUSSION

Uncoupler-treated animals showed less neurodegeneration in ALM and PLM during aging

In our previous study, we demonstrated that neuronal defects are associated with increased mitochondrial membrane potentials in *C. elegans* (Cho et al., 2016). Some studies have indicated that moderate uncoupling can attenuate neurodegeneration in rat neurons and improve neuronal activity in mice (Geisler et al., 2017; Wu et al., 2011). Therefore, we aimed to confirm whether age-related neurodegeneration can be delayed in *C. elegans* via treatment with uncouplers such as DNP and CCCP.

In the present study, mechanosensory touch receptor neurons, ALM and PLM cells, were labeled in the *zdls5* [$p_{mec\text{-}4}$GFP] transgenic strain. ALM and PLM cells exhibited obvious neuronal defects such as soma outgrowth, wavy processes, neuronal sprouting and branching during aging (Chen et al., 2013; Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). First, control animals (*zdls5*) were treated with 100 μM DNP and 10 μM CCCP. At day 1, the ALM cells of young adult animals in the control group exhibited healthy soma (Fig. 1A), while extensive outgrowths and severe neuronal defects were observed in ALM cells of day-10 control animals (Fig. 1B). ALM defect scores exhibit progressive increases throughout the aging process in control animals (Fig. 1C). On day 15, the ALM outgrowth rate was 4.6 times higher than that on day 1 (Fig. 1C). On day 1, control and uncoupler-treated animals exhibited similarly minimal ALM defects. However, animals treated with 100 μM DNP exhibited 32%, 32%, and 33% decreases in neuronal defects relative to controls on days 5, 10, and 15 (Fig. 1C), respectively, while those treated with 10 μM of CCCP exhibited 41%, 37%, and 27% decreases in neuronal defects on days 5, 10, and 15 (Fig. 1C), respectively. Figure 1D depicts a healthy PLM cell, while Fig. 1E depicts a PLM cell exhibiting wavy processes and neuronal sprouting (arrows and arrowheads, respectively). Progressive, age-dependent increases in PLM defects were also observed in control animals (Fig. 1F). 72% of PLM showed defective morphology by day 15 in controls. On days 5, 10, and 15, animals treated with DNP exhibited 36%, 40%, and 19% decreases in neuronal defects relative to controls (Fig. 1F), respectively. Similarly, CCCP-treated animals exhibited 42%, 48%, and 22% decreases in neuronal defects relative to controls on days 5, 10, and 15 (Fig. 1F), respectively. Such decreases in ALM and PLM defects in uncoupler-treated animals indicate that age-dependent neurodegeneration was attenuated by treatment with 100 μM DNP and 10 μM CCCP. Initially, we examined animals treated with a range of concentrations of DNP (10

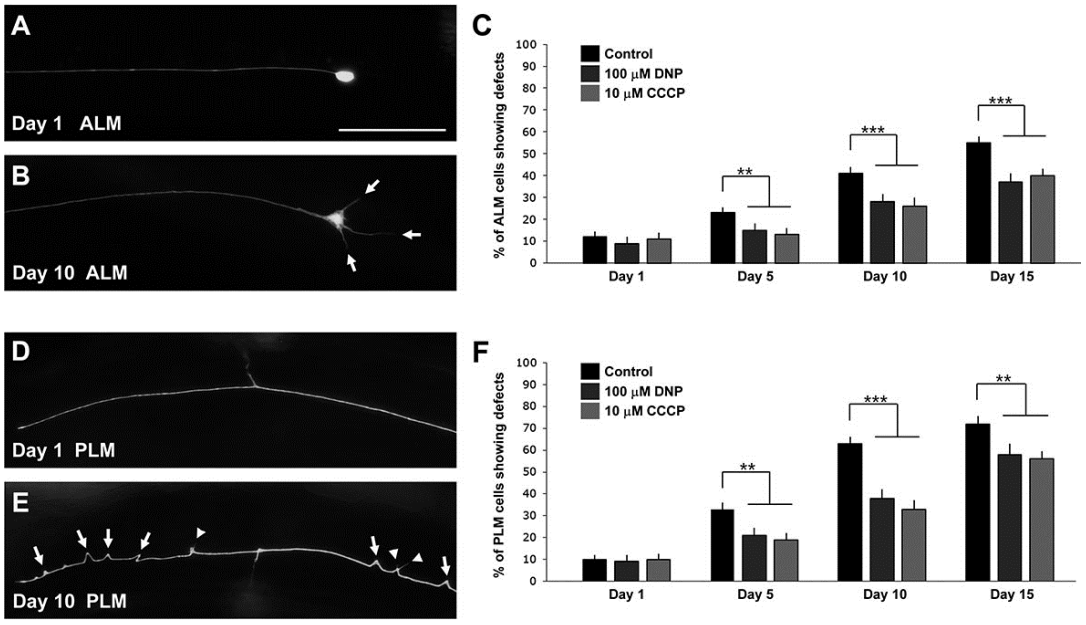

Fig. 1. Age-dependent neuronal defects were attenuated following treatment with DNP or CCCP. Representative images of mechanosensory neurons in a control worm on days 1 and 10: ALM (A and B, arrows indicate outgrowth); PLM (D, E). (E) Arrows and arrowheads indicate wavy processes and neuronal sprouting in the PLM, respectively. Scale bar = 50 μm. (C and F) ALM and PLM neuronal defects are presented as a percentage of the number of defective neurons out of all the ALM and PLM neurons observed for each condition in controls [$p_{mec\text{-}4}$GFP] on days 1, 5, 10, and 15. n > 100 worms per line in each experiment. Five independent experiments were performed. Error bars represent the SE. **p < 0.05; ***p < 0.001, one-way analysis of variance (ANOVA). DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; ALM, anterior lateral microtubule; PLM, posterior lateral microtubule; SE, standard error.

to 500 μM) and CCCP (1 to 50 μM). In those trials, the degree of neuronal defects decreased in a concentration-dependent manner (data not shown). However, 500 μM DNP and 50uM CCCP inhibited bacterial growth, so we chose 100 μM DNP and 10 μM CCCP to observe neurons. Moreover, such findings indicate that concentration dependence should be considered during the development of treatment strategies involving uncouplers. In early studies, a high dose of DNP was toxic or caused adverse effects, but recently a low dose of DNP was beneficial for neuroprotection in experimental models of stroke and Parkinson's disease (Geisler et al., 2017).

Neuronal defects decreased during aging in *ucp-4* overexpressing animals and uncoupler-treated *ucp-4* mutants

If membrane uncoupling attenuates neurodegeneration, the effects of chemical uncouplers should be observed in animals with relevant genetic modifications. Thus, we examined the effects of such treatment in animals overexpressing *ucp-4* and in *ucp-4* mutants to verify this hypothesis. Animals overexpressing *ucp-4* exhibited the lowest rate of ALM defects throughout aging: 12%, 18%, 29%, and 44% neuronal defects on days 1, 5, 10, and 15 (Fig. 2A), respectively. Improvements in neuronal phenotypes were also consistently observed for PLM cells during aging in *ucp-4* overexpressing animals (Fig. 2B). However, *ucp-4* mutants characterized by relative increases in mitochondrial membrane potential exhibited more neuronal defects even at day 1, 21% in ALM and 22% in PLM (Figs. 2A and 2B). Progressive, age-dependent ALM defects were observed in mutants: 36%, 54%, and 73% on days 5, 10, and 15 (Fig. 2A), respectively. However, at day 5, both 100 μM DNP and 10 μM CCCP treatment improved ALM defect phenotypes in mutants whose defects were decreased by 33% and 25%, respectively (Fig. 2A). These neuronal improvements were sustained throughout the aging process.

The *ucp-4* mutants exhibited consistent increases in PLM defects, which were more severe than ALM defects, on days 10 and 15 (72% and 88%, respectively) (Fig. 2B). However, DNP and CCCP treatments had attenuated severe PLM defects to levels observed in animals overexpressing *ucp-4* during aging. For instance, 49% and 55% decrease in DNP and CCCP treated mutants were observed on day 5. These findings suggest that age-dependent neurodegeneration can be attenuated by treatment with chemical uncoupling agents or overexpression of the *ucp-4* gene. In the present study, we utilized the *mec-4* promoter to develop the *ucp-4* overexpression. This neuron-specific *ucp-4* overexpression allows for minimization of indirect effects and misinterpretation associated with non-specific overexpression (Moriya, 2015; Prelich, 2012). In addition, the consistent results between *ucp-4* gene manipulation and treatment with chemical uncoupling agents indicate that DNP/CCCP were not significantly associated with non-specific side effects (Berezhnov et al., 2016).

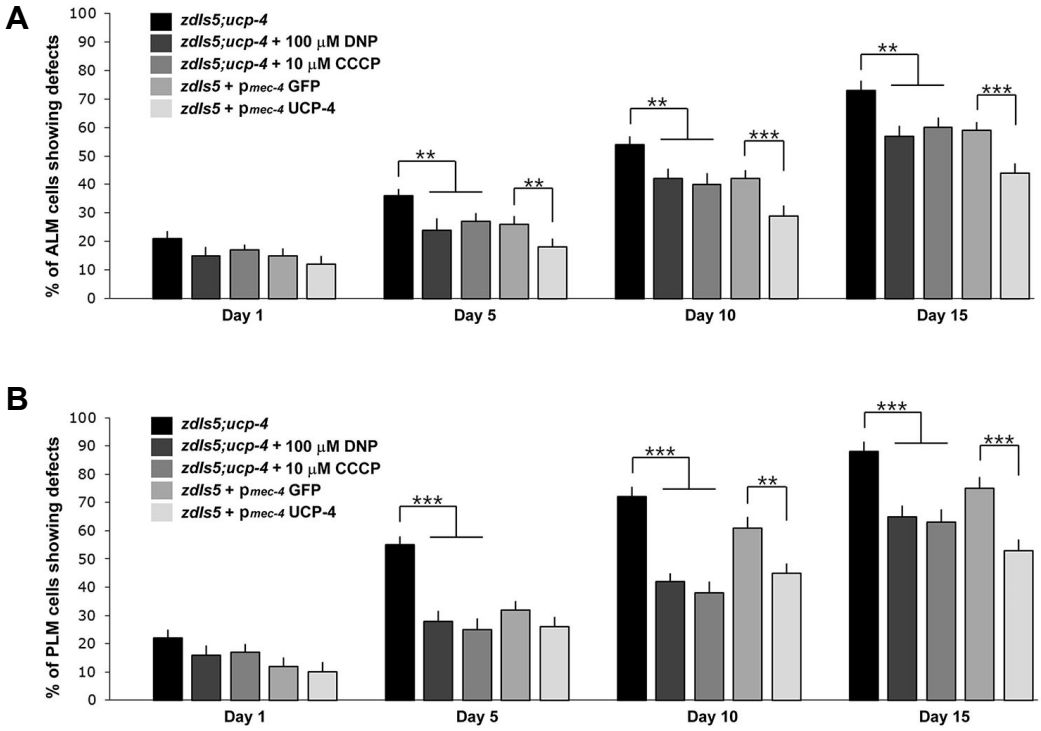

Fig. 2. Neuronal defects in *ucp-4* mutants were attenuated following treatment with DNP or CCCP. (A, B) ALM and PLM neuronal defects are presented as a percentage of the total ALM and PLM scores in neurons in each group of treated animals on days 1, 5, 10, and 15. *zdls5;ucp-4*, ucp-4 in *zdls5* [p_{mec-4}GFP] ; *zdls5;ucp-4* + 100 μM DNP, *zdls5;ucp-4* treated with 100 μM DNP; *zdls5;ucp-4* + 10 μM CCCP, *zdls5;ucp-4* treated with 10 μM CCCP; *zdls5* + p_{mec-4}GFP, p_{mec-4}GFP in *zdls5*; *zdls5* + p_{mec-4}UCP-4, p_{mec-4}UCP-4 in *zdls5*. n > 100 worms per line in each experiment. Five independent experiments were performed. Error bars represent the SE. $^{**}p < 0.05$; $^{***}p < 0.001$, One-way analysis of variance (ANOVA). Unpaired *t*-test between p_{mec-4}GFP and p_{mec-4}UCP-4 in *zdls5*. DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; ALM, anterior lateral microtubule; PLM, posterior lateral microtubule; SE, standard error.

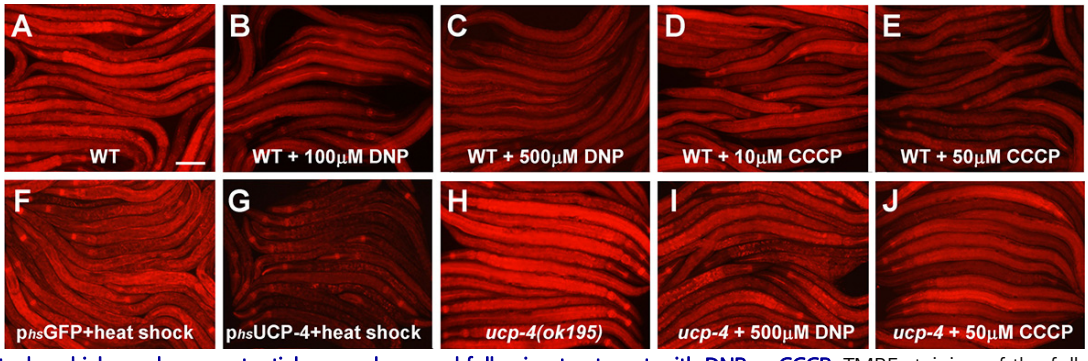

Fig. 3. Mitochondrial membrane potentials were decreased following treatment with DNP or CCCP. TMRE staining of the following animals at day 1: wild type (A); wild type with 100 μM or 500 μM DNP (B, C); wild type with 10 μM or 50 μM CCCP (D, E); p_{hs}GFP in wild type (F); p_{hs}UCP-4 in wild type (G); *ucp-4* (*ok195*) (H); *ucp-4* with 500 μM DNP (I); *ucp-4* with 50 μM CCCP (J). Scale bar = 100 μm. DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; TMRE, tetramethylrhodamine.

Decreased mitochondrial membrane potentials were observed in animals treated with uncouplers and those overexpressing *ucp-4*

To determine whether phenotypic improvements observed in the present study resulted from reductions in mitochondrial membrane potentials, TMRE was used as an indicator of mitochondrial membrane potential (Yoneda et al., 2004). As shown in Fig. 3, animals treated with DNP (100 μM and 500 μM) or CCCP (10 μM and 50 μM) exhibited decreased TMRE intensity relative to that observed in wild type controls

on day 1 (Figs. 3A-3E). A positive association was observed between TMRE intensity and chemical concentration in uncoupler-treated animals: TMRE intensity in 500 μM DNP or 50 μM CCCP-treated animals was lower than the intensity in 100 μM DNP or 10 μM CCCP-treated animals (Figs. 3B-3E). Substantially lower TMRE staining was observed in *ucp-4* overexpressing animals, indicating the *ucp-4* overexpression indeed depolarizes the mitochondrial membrane (Fig. 3G). In addition, intense TMRE staining in *ucp-4* mutants was restored to the level observed in wild type controls following treatment with DNP or CCCP (Figs. 3H-3J). These findings confirmed the previous study that *ucp-4* plays a key role in decreasing mitochondrial membrane potential during aging (Cho et al., 2016). The current results further suggest that chemical uncouplers and the *ucp-4* gene represent potential targets for attenuating age-related neurodegeneration.

TMRE staining patterns in aged animals were similar to those observed on day 1, lower intensity in *ucp-4* overexpressing animals and *ucp-4* mutants treated with DNP or CCCP (data not shown). However, the intensity of fluorescence in aged worms was lower than that observed on day 1. The low TMRE intensity in aged animals may be due to reduced TMRE uptake, its accumulation in only active mitochondria (Farkas et al., 1989).

Neurons of mitophagy-deficient mutant *pink-1* exhibited limited response to either uncoupler treatment or *ucp-4* overexpression

Recent research has indicated that depolarization of the mitochondrial membrane potential activates mitophagy, especially via the PINK1/Parkin pathway (MacVicar and Lane, 2014; Narendra et al., 2008; 2010). Furthermore, age-related impairments in mitophagy have been associated with neurodegenerative symptoms in patients with Alzheimer's, Parkinson's, and Huntington's disease (Itoh et al., 2013). Therefore, we investigated whether mitophagy plays a role in age-dependent neurodegeneration via reductions in mitochondrial membrane potential. Experiments in mitophagy-deficient mutants, *pink-1*, revealed both ALM and PLM defects during aging. On days 1 and 5, there were no significant differences in the number of ALM or PLM defects among all animals tested: *pink-1*, DNP or CCCP-treated *pink-1, ucp-4* overexpression in *pink-1* (Figs. 4A and 4B).

Progressive increases in the number of ALM defects were observed in *pink-1* mutants during the aging process (24%, 43%, and 60% on days 5, 10, and 15, respectively) (Fig. 4A).

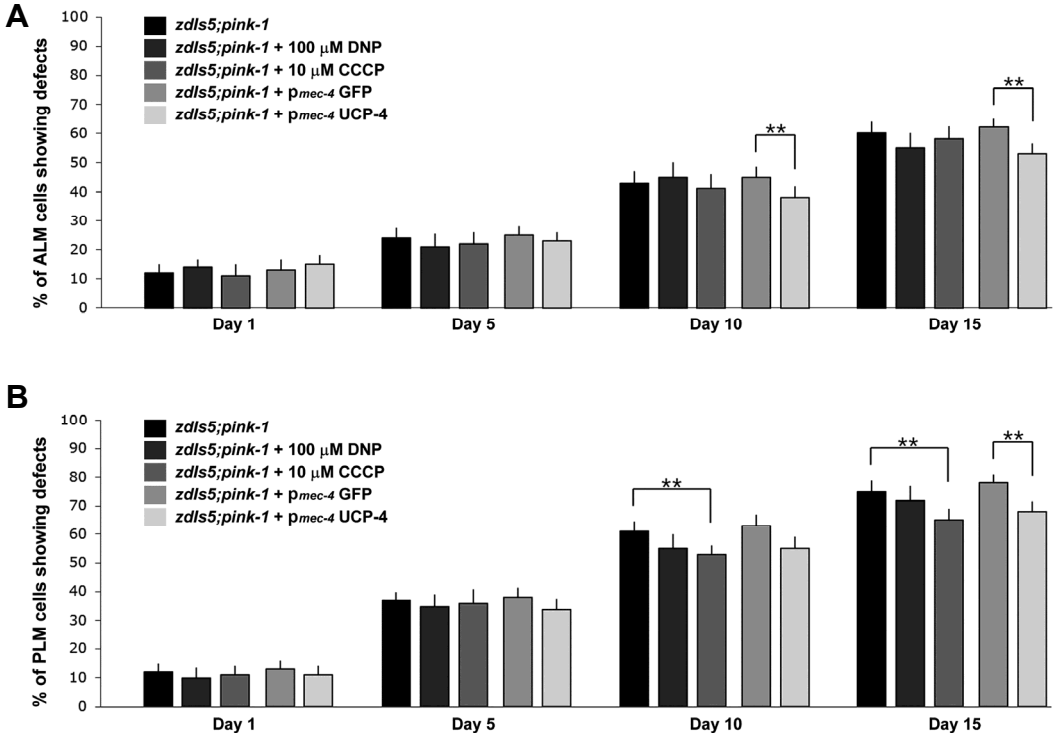

Fig. 4. Age-dependent neuronal defects in *pink-1* mutants were not significantly attenuated following DNP or CCCP treatment. (A, B) ALM and PLM neuronal defects are presented as a percentage of the total ALM and PLM scores for neurons in controls (p_{mec-4}GFP in *pink-1*) on days 1, 5, 10, and 15. *zdls5;pink-1*, *pink-1* (*tm1779*) in *zdls5* [p_{mec-4}GFP]; *zdls5;pink-1* + 100 μM DNP, *zdls5;pink-1* treated with 100 μM DNP; *zdls5;pink-1* + 10 μM CCCP, *zdls5;pink-1* treated with 10 μM CCCP; *zdls5;pink-1* + p_{mec-4}GFP, p_{mec-4}GFP in *zdls5;pink-1*; *zdls5;pink-1* + p_{mec-4}UCP-4, p_{mec-4}UCP-4 in *zdls5;pink-1*. n > 100 worms per line in each experiment. Five independent experiments were performed. Error bars represent the SE. **p < 0.05, One-way analysis of variance (ANOVA). DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; ALM, anterior lateral microtubule; PLM, posterior lateral microtubule; SE, standard error.

However, DNP and CCCP treatments did not reduce ALM defects in *pink-1* mutants during aging and only slight reductions in ALM defects were observed in *pink-1* mutants with *ucp-4* overexpression on days 10 and 15 (16% and 15% decreases respectively, Fig. 4A). Similar to ALM observation, progressive increases in rates of wavy processes, neuronal sprouts and branching defects were observed among PLM cells in *pink-1* mutants, and these defects were especially severe on days 10 and 15 (61% and 75%, respectively) (Fig. 4B). Consistent with ALM defects, there was no significant difference in PLM defects between *pink-1* mutants with and without DNP treatment during aging (Fig. 4B). Different to ALM, the PLM defects showed limited difference between *pink-1* mutants with CCCP and without CCCP treatment (19% defect-reduction on day 15). *ucp-4* overexpression in *pink-1* mutant worms caused slight reduction in PLM defects compared to the control animals (13% on day 15, Fig. 4B). These results suggest age-dependent neuroprotective effects due to mitochondrial uncoupling are associated with activation of mitophagy. However, the observed reductions in *pink-1* mutants with uncoupling indicate that it can be the results of uncoupling effect and/or more than one mitophagy pathway such as PINK1/Parkin independent pathways (Allen et al., 2013). There are studies that suggest membrane uncoupling reduces ROS generation in mitochondria, which can improve neurodegeneration (Caldeira da Silva et al., 2008; Kalogeris et al., 2014). Therefore, modifying membrane potential may improve age-dependent neurodegeneration by two factors: reduction of ROS generation and stimulation of mitophagy via membrane potential reduction.

In summary, the findings of the present study indicate that mitochondrial uncoupling attenuated neuronal defects during aging in *C. elegans*. Moreover, we observed that treatment with uncoupling agents in mitophagy-deficient mutants did not result in significant improvements of neuronal defects, suggesting that mitophagy plays a key role in mediating the protective effect of mitochondrial uncoupling against age-dependent neurodegeneration. However, since there is no direct evidence that PINK1/Parkin mitophagy pathway is triggered by reduced mitochondrial membrane potentials in *C. elegans*, further studies are required in order to elucidate the molecular mechanisms underlying the association between mitophagy and age-dependent neurodegeneration.

ACKNOWLEDGMENTS

We thank the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, USA, for the worm strains and Andrew Fire for the Fire vectors. We thank the Mitani lab through the National Bio-Resource Project of the NEXT, Japan for *pink-1* strain. The study was supported by a research fund from Chosun University, 2014.

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