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Electron microscopy analysis of alpha-synuclein and LRRK2 transgenic C. elegans

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ELECTRON MICROSCOPY ANALYSIS OF ALPHA-SYNUCLEIN AND LRRK2 TRANSGENIC *C. ELEGANS*

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ABSTRACT

Mutations in alpha-synuclein and leucine-rich repeat kinase 2 (LRRK2) have been implicated in the cause of Parkinson's disease (PD). These two proteins have been the targets of a great deal of recent research that has transformed our understanding of this disorder. Recent research using *C. elegans* as a model species has shown that alpha-synuclein expression and the LRRK2-G2019S mutation potentiate neurodegeneration similar to that seen in cases PD. Further exploration revealed that defects in autophagy of dopaminergic neurons may be the cause for the observed pathology.

In the current study, the confirmation of autophagy as a possible cause of pathology due to the expression of alpha-synuclein and the LRRK2-G2019S mutation is completed through the use of electron microscopy. We observed that large vacuoles had formed in the cephalic dopaminergic neurons of alpha-synuclein + LRRK2 transgenic samples not seen in wild-type samples. Further, large morphological changes in the nerve ring area of the transgenic nematodes were also observed that may implicate that alpha-synuclein expression in conjunction with the LRRK2-G2019S mutation may have a widespread effect on many neurons that was not previously expected.

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C. elegans	*Caenorhabditis elegans*
CEPDL	left dorsal cephalic
CEPDR	right dorsal cephalic
DDSA	dodecenylsuccinic anhydride
HPF	high pressure freezing
LRRK2	leucine-rich repeat kinase 2
NMA	methyl-5-norbornene-2,3-dicarboxylic anhydride
PD	Parkinson's disease

INTRODUCTION

In 1817, Dr. James Parkinson first described several clinical cases and symptoms of a disease that would later be named in his honor. In *An Essay on the Shaking Palsy*, he describes this shaking palsy or paralysis agitans as "[i]nvoluntary tremulous motion, with lessened muscular power, in parts not in action and even when supported […]" (1817) (Parkinson 1817). Today, Parkinson's disease (PD) is the world's second most common neurodegenerative disorder affecting an estimated 5 million people, and the number of people that will develop PD is expected to increase dramatically in the coming decades (Olanow et al. 2009).

Symptoms

The four cardinal motor symptoms of PD are resting tremor, rigidity, postural instability, and bradykinesia (Jankovic 2008). Tremor at rest is the most common symptom of PD and is almost always prominent in the most distal part of an extremity (Jankovic 2008). Rigidity refers to increased resistance during the movement of a limb and may also be associated with pain (Jankovic 2008). This rigidity of the neck and truck often causes postural instability and deformities that usually occurs late in the disease (Jankovic 2008). Bradykinesia is the most characteristic clinical feature of PD and is characterized by slowness of movement that is the result of disorders of the basal ganglia (Jankovic 2008). Out of the four cardinal motor symptoms of PD, bradykinesia was found to correlate best with the degree of dopamine deficiency of the patient (Vingerhoets et al. 1997).

Non-motor symptoms of PD are often underappreciated. They include autonomic dysfunction, neurobehavioral abnormalities, and sensory and sleep disorders (Zesiewicz et al. 2006). Autonomic dysfunction may include orthostatic hypotension and sweating dysfunction (Jankovic 2008). Neurobehavioral abnormalities refer to cognitive decline, and studies done by Hely et al. demonstrated that 48% of PD patients met the diagnostic criteria for dementia (2005). Sensory disorders such as olfactory dysfunction, oral pain, and genital pain are frequent but are often not recognized as symptoms of PD (Doty 2007). Sleeping disorders include excessive daytime sleepiness and insomnia (Gjerstad et al. 2006).

Pathology

The neuropathology of PD consists of the degeneration of the dopaminergic neurons in the substantia negra, which is also responsible for the motor symptoms displayed in patients with PD (Fahn & Sulzer 2004). In addition, intracellular eosinophilic inclusions containing aggregations of the misfolded protein alpha-synuclein called Lewy bodies are observed in many of the surviving neurons (Del Tredici & Braak 2012). It is believed that the impairment of protein degradation systems, including the ubiquitin-proteasome system and/or the autophagy-lysosome pathway, leads to the aggregation of proteins resulting in the presence of Lewy bodies and ultimately neurodegeneration in PD (Pan et al. 2008) (Figure 1).

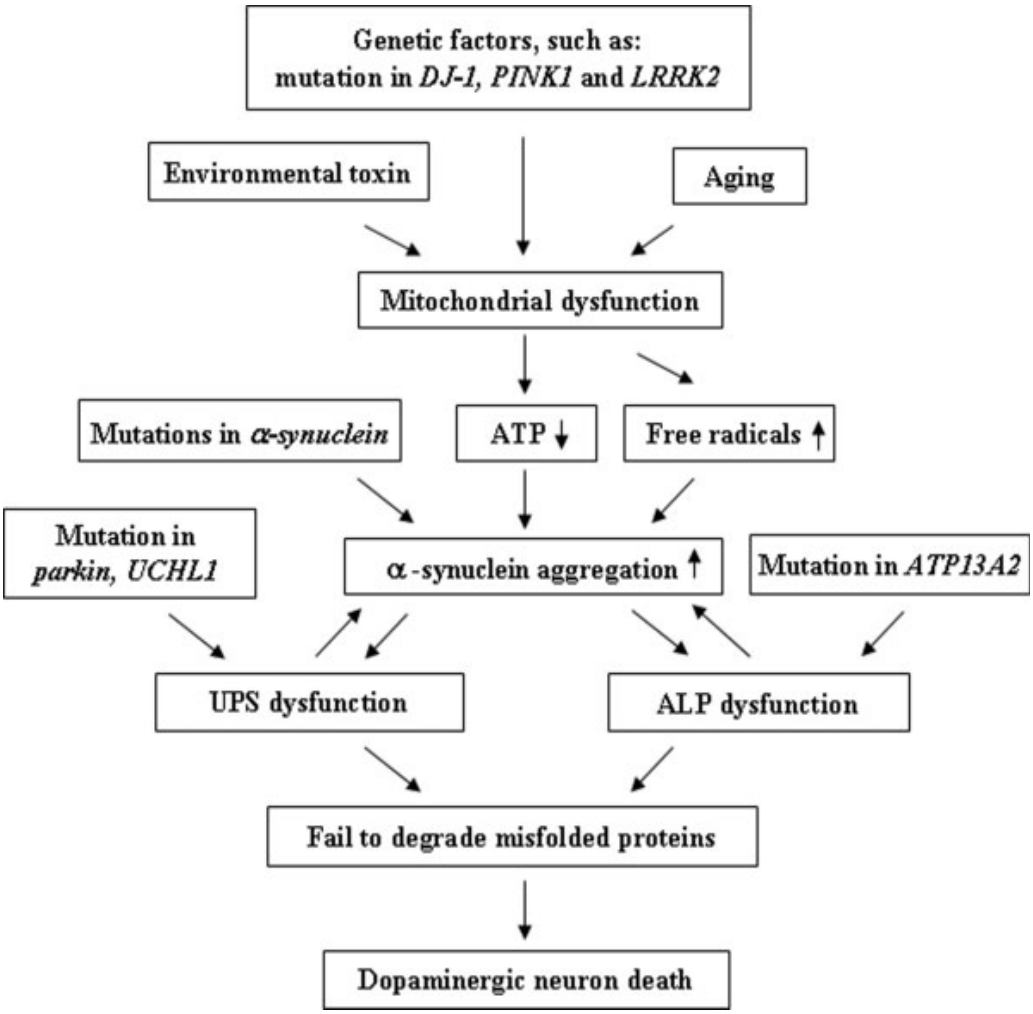


Figure 1 – Causes of protein aggregration directly or indirectly affecting the function of protein degradation systems ultimately leading to dopaminergic neuron death. (Figure taken from Pan et al., 2008)

Leucine-Rich Repeat Kinase 2 (LRRK2)

While the role of genetic factors had long been considered insignificant in the etiology of PD, two papers published in 2006 describing the role of a mutation in the

leucine-rich repeat kinase 2 (LRRK2) as a cause for autosomal dominant PD has shifted the focus of research regarding the etiology of PD towards genetics (Ozelius et al. 2006) (Lesage et al. 2006) (Bonifati 2006). While the aforementioned cases of PD involve only a small subset of patients with PD, it has been shown that the same G2019S substitution in the LRRK2 gene commonly found in North African Arabs and Ashkenazi Jews with PD are also common in sporadic cases PD (Gilks et al. 2005). It is currently the most common genetic cause of PD in Europe, accounting for ~7.14% of familial PD and ~4% of sporadic PD (Paisan-Ruiz 2009).

The LRRK2 gene encodes a 2,257-amino acid protein called dardarin and has 51 exons (Paisan-Ruiz 2009). In addition to the G2019S mutation found in LRRK2, there have been many other variants discovered and these variants are shown in Figure 2.

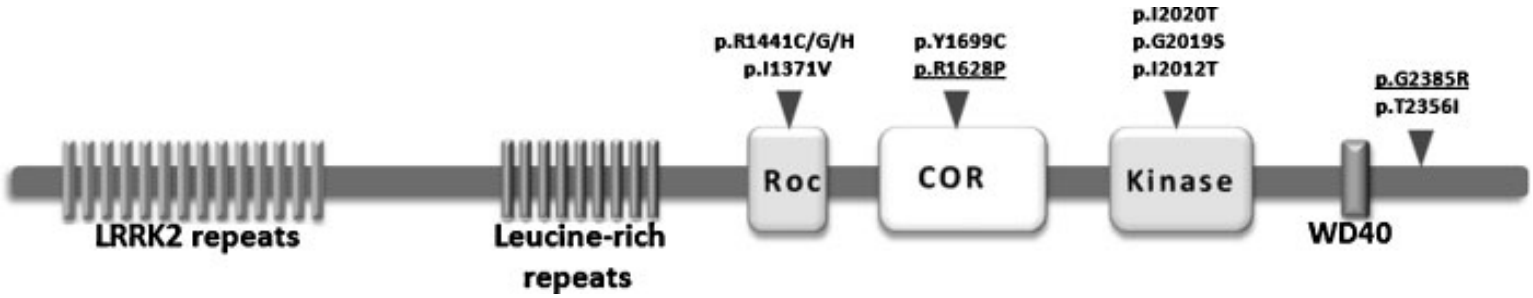


Figure 2 – LRRK2 protein with its functional domains. Figure adapted from Paisan-Ruiz, 2009.

The physiological role of LRRK2 is currently unknown. However, research done by Tong et al. has shown that the loss of LRRK2 in mice dramatically increases apoptotic cell death, inflammatory responses, and oxidative damage in the kidney of the mice (2010). Although there was no finding of neurodegeneration in these mice, the increased alpha-synuclein and ubiquitinated protein aggregation and impairment of the autophagy-lysosomal pathway that was also observed in the kidney of these mice might offer clues to how mutations in LRRK2 may cause PD (Tong et al. 2010).

***Caenorhabditis elegans* (*C. elegans*)**

The use of *C. elegans* as a model species for PD research has been invaluable. While the human brain has over 100 billion nerve cells interacting with one another, this nematode has 302 neurons for which a precise neuronal connectivity map exists (Caldwell & Caldwell 2008). In addition, its physical transparency, the short life span of about 2 weeks, and the ease of maintaining the worms make them an ideal model species for studying neurological disorders and aging (Caldwell & Caldwell 2008). *C. elegans* may also provide insight into the etiology of sporadic PD that is still inexplicably linked to environmental causes and age after studies have demonstrated that the worm is susceptible to toxins commonly used to model neurodegeneration (Nass et al. 2002). Due to the evolutionary conservation of metazoan genomes, all orthologous genes related to familial PD with the exception of the gene encoding for alpha-synuclein have been identified in *C. elegans* genes (Caldwell & Caldwell 2008). The probability of

establishing therapeutic significance increases with these associations to the mammalian genome.

Previous Experiments

Previous experiments done in the Wolozin lab have shown that an *in vivo* model using *C. elegans* expressing a mutant LRRK2 in association with alpha-synuclein potentiates neurodegeneration (Gowda et al. 2012). Green fluorescent protein expression studies of dopaminergic neuron activity in *C. elegans* models have shown that co-expression of LRRK2-G2019S and alpha-synuclein lead to a decrease in dopaminergic neuron activity that can be observed as early as day 7 of the life of the nematode (Gowda et al. 2012). Further, an observation of the increase in Lgg-1, a requirement for the degradation of cellular components by autophagy in *C. elegans*, indicates that the resulting pathology may be caused by deficits in autophagy (Gowda et al. 2012).

Although Lgg-1 is a good indicator of autophagy in *C. elegans*, the gold standard method to confirm autophagy activity is transmission electron microscopy (Singletary & Milner 2008). The presence of double membrane vacuole structures that engulf cytoplasmic components is the strongest indicator that autophagy is occurring (Singletary & Milner 2008). The goal of this project is to find evidence of autophagy in the LRRK2-G2019S alpha-synuclein transgenic *C. elegans* through the use of transmission electron microscopy.

METHODS

The *C. elegans* samples were prepared and mounted onto slotted copper grids at the Albert Einstein College of Medicine by the laboratory of David Hall, Ph. D. The high pressure freezing (HPF) procedure was used as described by Hall et al. (2012):

1. Each sample underwent rapid freezing at high pressure (2100 bar) in a 2% osmium tetroxide in 98% acetone and 2% water solution in order to preserve organelle structure. The HPF apparatus was programmed to hold the temperature at -90°C for 110 hours, warm slowly to -20°C (5°C/hour), and held at -20°C for 15 hours. Then, it was warmed to 0°C slowly (6°C/hour) and held at 0°C.
2. The samples were rinsed in 100% acetone at 0°C for 20 minutes three times.
3. The samples were removed from specimen carrier at room temperature and rinsed three times in 100% acetone for 20 minutes at room temperature.
4. The samples were placed into a Pella Specimen Capsule and submerged in a one part resin, three parts acetone mixture in a capped shell vial. The setup was then placed on a rotator at room temperature for three hours.
5. The mixture was changed to a 1:1 resin:acetone mixture and rotated for three hours at room temperature.

6. The mixture was changed to a 3:1 resin:acetone mixture and rotated for 16 hours at room temperature.
7. The mixture was changed to 100% resin four times over the next 24 hours and rotated at room temperature.
8. The samples were embedded between Aclar films or in an embedding mold and cured at 60°C for two days.

The resin mixture used was composed of 24 g of Embed812, 9 g of dodecenylsuccinic anhydride (DDSA), 15 g of methyl-5-norbornene-2,3-dicarboxylic anhydride (NMA). The resin mixture was then stored at room temperature in a desiccator until ready for use. 0.75 g of DMP-30 resin accelerator was added to the resin mixture only in steps that called for 100% resin.

Each *C. elegans* sample was then sectioned serially using the Hartwieg Method as described by Hall et al. (2012). The block containing the sample was trimmed to a perfect trapezoid, where the longest side of the trapezoid is shorter than the width of the diamond tipped knife. Some sticky wax, Charette ASTM D4236 that can found in graphics arts stores, was applied to the top and bottom of the trapezoid to allow the sections to remain adhered to each other after being cut. Thin sections were then cut to obtain a light gray to white color of the section. Continuous sections were then picked up by a coated slotted grid.

Negative staining was completed after mounting on copper slotted grid using a 2% uranyl acetate solution. This solution was filtered using a 0.2 μm syringe filter before

applying to the grid. After one minute of stain application, filter paper was used to draw off the stain, and the grid was left to dry for several hours.

Imaging was completed using the Zeiss Libra 120 transmission electron microscope at the Harvard University Center for Nanoscale Systems. The samples were imaged at room temperature with a tungsten filament. The omega filter of the microscope was used when possible, and the acceleration voltage used was 80 kV.

RESULTS

The *C. elegans* samples from previous studies prepared for electron microscopy were imaged and analyzed to locate the cephalic dopaminergic neurons. Irregularities in the dopaminergic neurons were found in many samples such as the ones shown in figures 3 and 4.

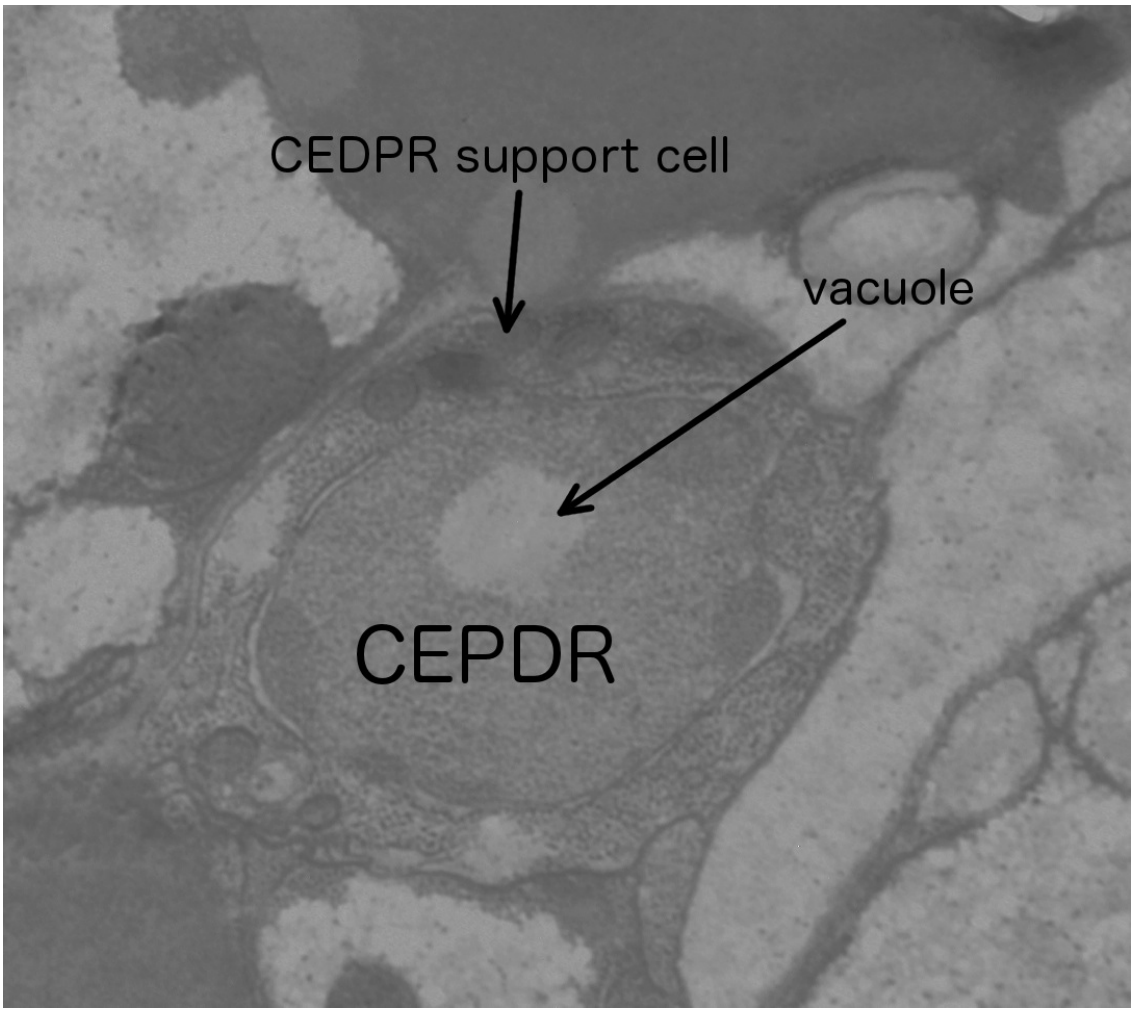
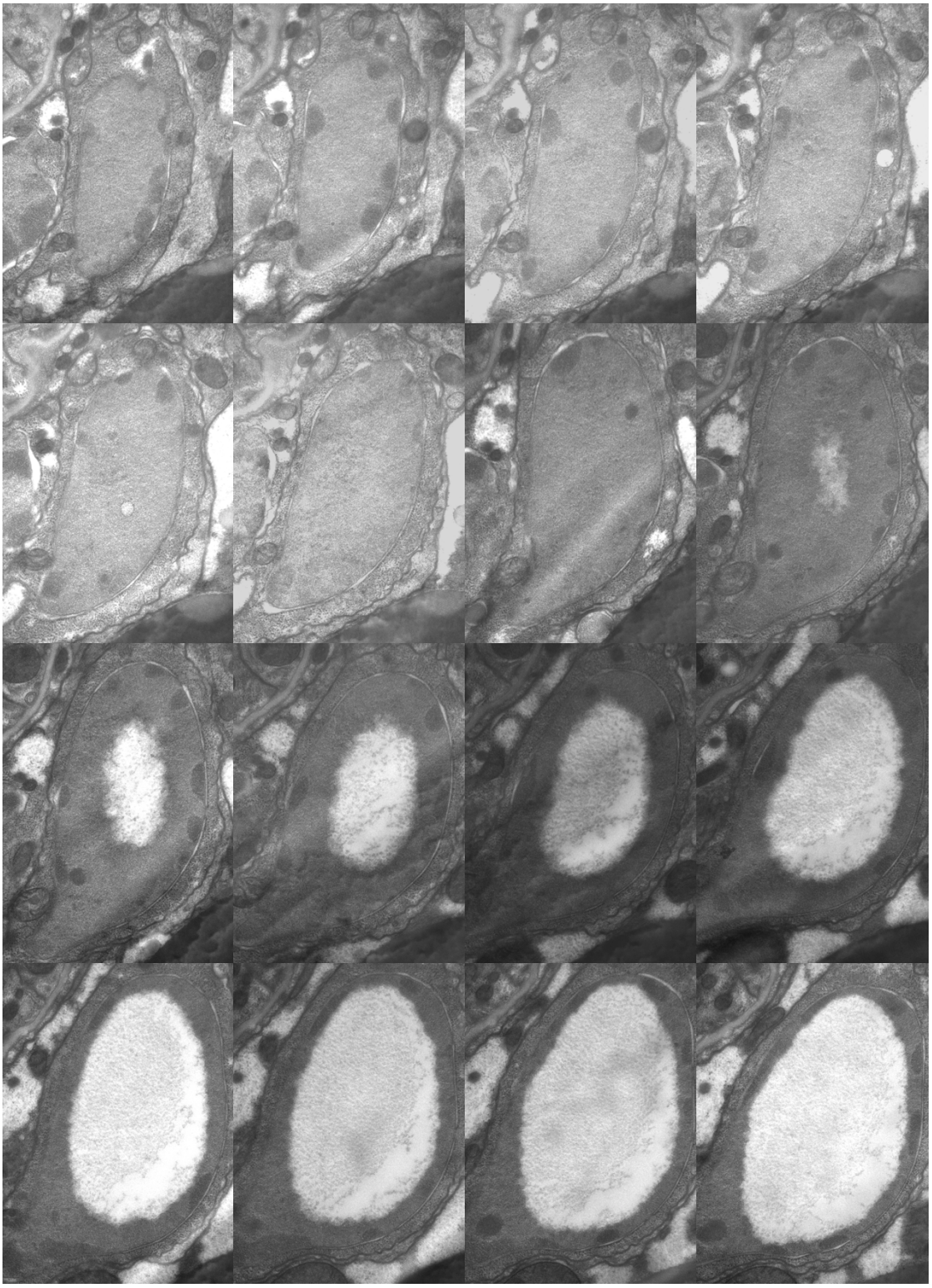


Figure 3 – Transmission electron microscope image of the right dorsal cephalic dopaminergic (CEPDR) neuron of an alpha-synuclein + LRRK2-G2019S transgenic *C. elegans* sample at 8000 times magnification. An unusually large vacuole is seen that is expected in these transgenic *C. elegans* samples due to errors in autophagy leading to an accumulation of cellular debris within the cell body of the neuron. Other smaller vacuoles are present, but it is unknown whether their presence is due to errors in autophagy due to their smaller size.



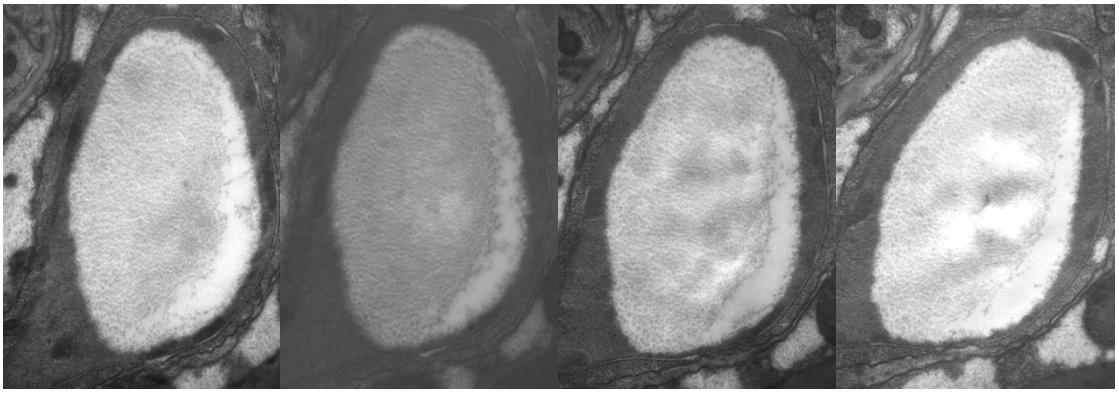


Figure 4 - A series of images depicting serial sections of a left dorsal cephalic (CEPDL) neuron of a transgenic *C. elegans* at 8000x magnification. The neuron has developed a vacuole large enough to assume most of its internal space.

Comparisons between images of a wild-type CEPDR and an alpha-synuclein + LRRK2-G2019S transgenic CEPDR were also completed as shown in figure 5.

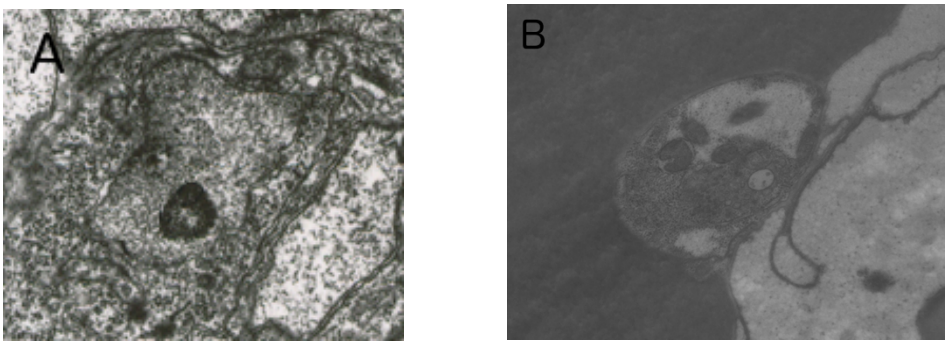


Figure 5 – Comparison between wild-type and alpha-synuclein + LRRK2-G2019S transgenic CEPDR images of *C. elegans* at 8000x magnification. (a) The wild-type CEPDR neuron displays no discernible vacuoles within its cytoplasm. No abnormal phenotype is observed. Image adapted from (Thomson 2011a) (b) The transgenic CEPDR

neuron has developed empty spaces indicative of vacuoles that have developed mostly likely due to the LRRK2 mutation in association with alpha-synuclein expression.

Further comparisons between wild-type and transgenic *C. elegans* of the whole cross-section of the worm at the area where the nerve ring is found was done in order to observe the effects of the mutations in a holistic manner as seen in figure 6. There were significant morphological changes in the transgenic worm sample compared to the wild-type worm. Large empty white spaces and areas of high electron density are seen in the transgenic nematode but not in wild-type. However, the muscular system of the transgenic worm appears to be similar to that of the wild-type worm.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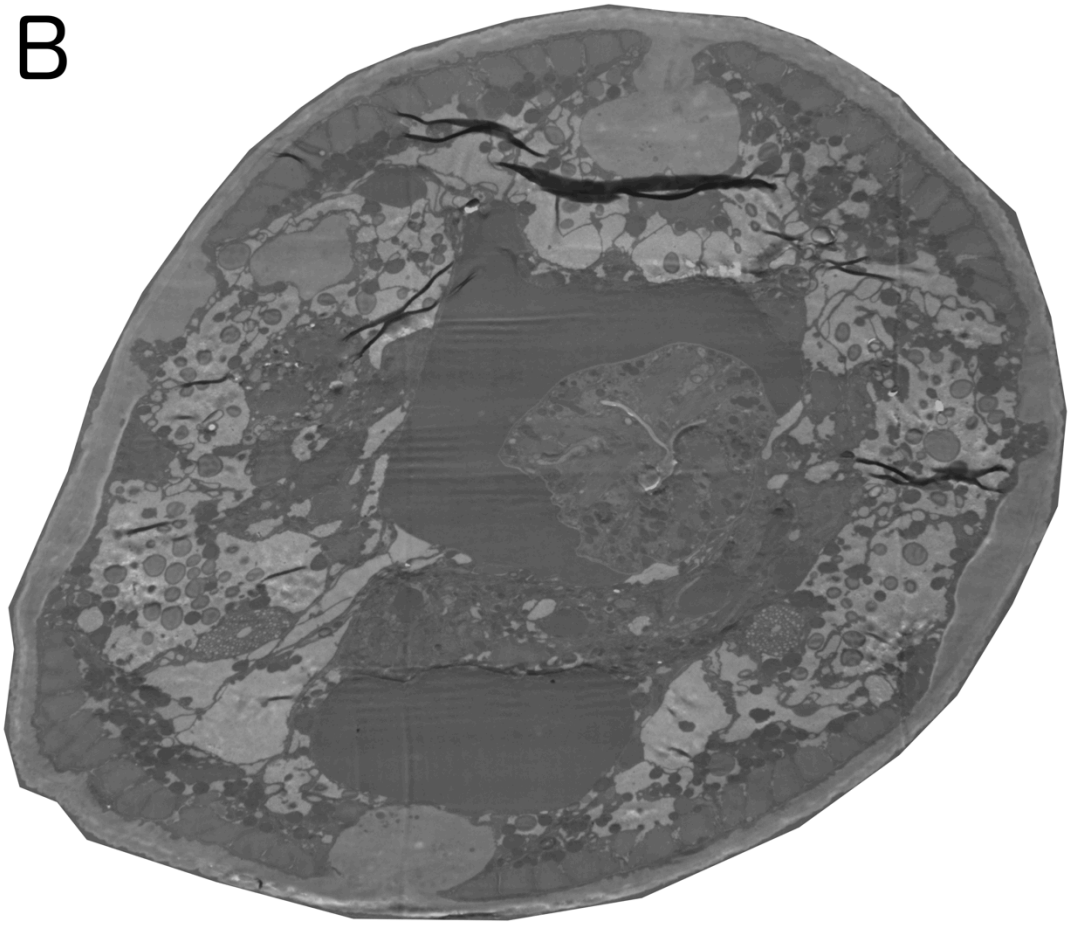

Figure 6 – Comparison of electron microscope images of cross sections of whole nematode samples located around the nerve ring at 2000x magnification. The images have been rotated so that the dorsal side faces up in both images. (a) The image of the cross-section of the wild-type *C. elegans* displays no visible pathology. The pharyngeal valve is found in the center of the worm with its non-striated muscles. Striated muscles used for movement by the nematode are found along the edges of the cross-section. In the area in between these areas are the nerves of the nerve ring and their supporting cells. Image was adapted from (Thomson 2011b) (b) The cross-section of the transgenic *C.*

elegans displays the pharyngeal valve, its muscles, and the striated muscles used for movement without any visible pathology. However, the area in between these areas is markedly different from that of the wild-type *C. elegans*. There are areas of white spaces and areas of very electron dense areas not observed in the wild-type cross section.

DISCUSSION

The results of previous studies in conjunction with this project suggest that defects in autophagy may play a critical role in the pathogenesis of PD. Further, recent research done on LRRK2-G2019S transgenic mice have shown similar defects in autophagy (Orenstein et al. 2013). Strong evidence that disease-linked mutations in LRRK2, such as G2019S, impair catabolic activity within the neuron has also been found in flies (Hindle & Elliott 2013). Enhancement of toxic protein aggregates and alpha-synuclein occurs as a result of this failure of autophagy eventually leading to the neurodegeneration seen in PD.

The comparison of the images of the cross-section of the whole nematode (figure 6) reveals that the LRRK2-G2019S mutation in conjunction with alpha-synuclein expression may have an effect beyond the four sole dopaminergic neuron that was expected. While alpha-synuclein is known to be expressed in only dopaminergic neurons in humans (Lakso et al. 2003), its addition to *C. elegans* may have widespread unknown effects throughout the nerve ring. In addition, the widespread effects seen in the images of the transgenic *C. elegans* may be due to LRRK2-G2019S, a mutation with effects that are known to be widespread throughout many areas of the brain in humans (Paisan-Ruiz 2009). This widespread effect in humans may affect the whole of the nerve ring within *C. elegans* in a similar manner to how pathology is caused in humans. Future imaging of transgenic *C. elegans* samples with only the alpha-synuclein expression and other samples with only the LRRK2-G2019S mutation should be completed to compare any differences in the resulting morphology.

If further imaging of serial sections of *C. elegans* is completed, a 3-dimensional model of the neuronal cell body could be formed in order to further detail the effect that the LRRK2-G2019S mutation and alpha-synuclein expression has on the neuron and the nervous system as a whole. The detail from a 3-dimensional model could aid in the understanding of the pathophysiology of PD due to LRRK2-G2019S and/or alpha-synuclein with more electron microscopy images of serial sections of more transgenic *C. elegans*. In addition, further electron microscopy imaging with immunostaining for Lewy bodies in transgenic *C. elegans* could be performed in order to better understand the progression of PD and add more detail to the 3-dimensional renders.

The limit of *C. elegans* as a model for imaging the effects of PD should be addressed. Because of the lack of endogenous alpha-synuclein expression in wild-type samples, its effects in transgenic samples must be closely examined in order to determine if the results are comparable to the pathology observed in humans or even higher species models such as mice. The use of zebrafish as models for recent PD studies have been successful and appear to be a good compromise between the accessibility and short generation time that *C. elegans* offers and the homology that *Mus musculus* offers.

The role of LRRK2 in autophagy is a strong focus for current research. Although the evidence for disease-linked mutations in LRRK2 impairing catabolic activity is great, the specific function of LRRK2 and the mechanism for its effects on autophagy are still largely unknown. The link between LRRK2 and alpha-synuclein should be explored further as well. It has been theorized that that the defects in autophagy linked to disease-linked mutations of LRRK2 can ultimately lead to the alpha-synuclein protein

aggregation that is a key component of Lewy bodies . How these two proteins interact within the neuron is not well-understood, and future research in this area would allow a greater understanding of the pathogenesis of PD.

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