

DEGENERATION OF NEURONAL MITOCHONDRIA IN PARKINSON'S DISEASE:

Mitochondrial Turnover in Neuromuscular Junctions of Parkin Mutants

Student Author



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Mentors



Peter J. Hollenbeck is a cellular neurobiologist whose laboratory has for many years studied the life cycle of mitochondria in the nervous system. He is a professor of biological sciences and is the associate vice provost for faculty affairs. He received his PhD from

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Nguyen, K. (2016). Degeneration of neuronal mitochondria in Parkinson's disease: Mitochondrial turnover in neuromuscular junctions of Parkin mutants. *Journal of Purdue Undergraduate Research*, 6, 41–48. <http://dx.doi.org/10.5703/1288284316155>

Abstract

The accumulation of dysfunctional or damaged mitochondria in neurons has been linked to the pathogenesis of many neurodegenerative diseases, including Parkinson's disease. It has been proposed that the Parkinson's-related proteins PINK1 and Parkin regulate mitochondrial quality control by selectively targeting depolarized mitochondria for autophagic degradation, a process known as mitophagy. The compartmentalization of mitochondrial turnover in neurons still remains unclear, but evidence suggests that mitochondria are locally degraded in the distal axon and perhaps in the neuromuscular junctions (NMJs). To study this, intact *Drosophila* nervous systems were analyzed in vivo by performing gentle dissections on third instar larvae to expose the ventral ganglia and segmental nerves with their NMJs. Both control larvae and *parkin* mutants (*parkin*²⁵) were genetically modified to mark mitochondria via mitoGFP expression in their motor neurons, with *park*²⁵ being additionally modified by deletion of the *parkin* gene. The physiological states of mitochondria were quantified through measurements of mitochondrial membrane potential ($\Delta\psi_m$), and the density of mitochondria in NMJs were analyzed through comparing α HRP and mitoGFP stain intensities in synaptic boutons. Unexpectedly, *parkin*²⁵ mitochondria displayed normal $\Delta\psi_m$ readings in NMJs, indicating that mutant nerve terminals do not accumulate senescent mitochondria. In addition, reduced mitochondrial density was observed in synaptic boutons of *parkin*²⁵ animals. These results argue against the hypothesis that loss of Parkin results in the accumulation of depolarized mitochondria, instead suggesting a reduction of organelle density in synaptic boutons as a result of Parkin deficiency. By elucidating the role of Parkin in the synapse of neurons, the pathogenic mechanism of Parkinson's and other neurodegenerative diseases will be better understood.

Keywords

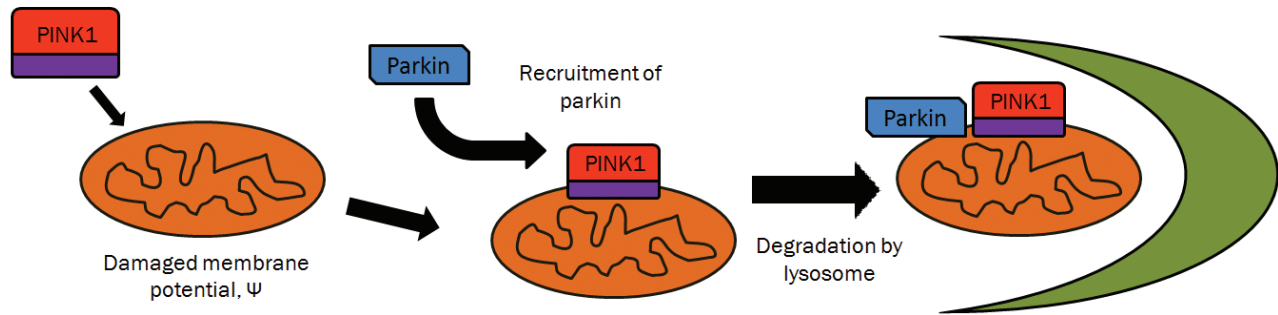
Parkin, mitophagy, mitochondrial turnover, mitochondrial membrane potential, *Drosophila*, synaptic bouton, neuromuscular junction

INTRODUCTION

Health of neurons depends critically on the function of their mitochondria, which mediate ATP production, cytosolic calcium homeostasis, and apoptosis. This is most evident in the manifestation of neurodegenerative diseases due to mitochondrial dysfunction (Shidara & Hollenbeck, 2010). Mitochondria exhibit a complex life cycle that is characterized, in part, by morphological changes through fission and fusion (Okamoto & Shaw, 2005) and turnover through autophagic degradation, known as mitophagy (Rubinsztein et al., 2012; Terman et al., 2010). The regulation and performance of these life cycle features in a systematic order, known as mitochondrial quality control, is essential for proper maintenance of mitochondrial integrity and neuronal health (Devireddy et al., 2015). Due to the large and asymmetric structure of neurons, the compartmentalization and mechanisms of mitochondrial quality control within neurons remain poorly understood. The encoding of mitochondrial proteins in the nuclear genome and presence of lysosomal compartments suggests that the cell body is the major location for mitochondrial turnover. And evidence also suggests that these processes may occur in the axons (Amiri & Hollenbeck, 2008). However, it is unknown whether mitochondrial turnover occurs in the nerve terminals.

It has been proposed that two Parkinson's disease-related proteins, PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin, regulate mitochondrial quality control (Narendra et al., 2012). Recent studies postulate three possible functions for the PINK1/Parkin pathway: tagging of dysfunctional mitochondria for mitophagy (Geisler et al., 2010), prevention of fusion between senescent and healthy mitochondria (Yang et al., 2008), and the arrest of mitochondrial motility to facilitate their degradation (Wang et al., 2011). Figure 1 outlines the proposed process of mitophagy. Though the precise mechanisms are not fully understood, it is believed that PINK1 is first translocated to the surface of dysfunctional, depolarized mitochondria (Verburg and Hollenbeck, 2008); PINK1 then recruits Parkin to the mitochondrial membrane, which tags the mitochondrion with ubiquitin, marking it for mitophagy (Jin & Youle, 2012). Parkin then mediates the mitochondria's engulfment via autophagosomes and its subsequent degradation.

In this study, we tested the widely held hypothesis that mitochondria in NMJs of *parkin*²⁵ will exhibit deficient $\Delta\psi_m$ states and increased mitochondrial density in synaptic boutons. Since previous studies



■ **Figure 1.** The proposed mechanism of the PINK1-Parkin pathway for mitochondrial turnover. See text for details.

have shown that mitophagy occurs primarily in the cell body (CB), we proposed that there is a “barrier” between the CB and axon, which allows only healthy mitochondria to pass through. If this holds true, it is believed that mitochondria in the synapses exhibit normal conditions rather than varying membrane potential states. To quantify $\Delta\psi_m$ states of mitochondria, a mitochondrial-to-cytoplasmic TMRM dye fluorescence ratio (F_m/F_c) was measured in various mitochondria within synaptic boutons of NMJs. A mean measurement per sample was found, and a mean of means was calculated to compare the F_m/F_c ratio between control and *parkin*²⁵ animals. We found that, unexpectedly, there was no significant difference between the F_m/F_c states of mitochondria from control and mutants, indicating that the physiological states of mitochondria in NMJs are seemingly independent of Parkin. Furthermore, Parkin mutants show reduced mitochondrial density in nerve terminals, suggesting that senescent mitochondria do not accumulate in neurons.

MATERIALS AND METHODS

Drosophila lines. All fly stocks were maintained on standard cornmeal agar medium with 12-hour light/dark cycles at 25° C. Two fly sets, with a wild type control and *parkin*²⁵ mutant line, were used for the mitochondrial $\Delta\psi_m$ and density data. The wild type control fly line was (W; +/+; D42 > UAS-mitoGFP/+) and *parkin*²⁵ line was (W; +/+; *parkin*²⁵, D42-Gal4/*parkin*²⁵, UAS-mitoGFP). To visualize mitochondria with green fluorescent protein (mitoGFP) in the motor neurons, the GAL4-UAS system was used. In this system, the GAL4 protein is driven by D42 promoter to bind to the central control region UAS, located upstream from the inserted mitoGFP gene. This activates gene transcription of mitochondria with GFP specifically in motor neurons.

Larval dissection for live confocal imaging.

To observe *Drosophila* nervous systems in vivo, late third instar larvae were collected, dissected, and prepared following previously described methods (Devireddy et al., 2014). Refer to Figure 2 for the step-by-step method of dissection. Larvae were pinned down and longitudinally incised along their dorsal sides in HL6 buffer, which contains 0.6 mM CaCl₂ and 4 mM L-glutamate. Fat bodies and intestines were gently removed, and larval body walls were slightly pushed back to reveal the ventral ganglion (VG) and intact segmental nerves (SNs) with NMJs. The dissected larva was transferred to a glass slide, covered with a glass coverslip using double-sided tape as a spacer, and filled with HL6 buffer before being sealed with VALAP wax on all sides to form a chamber. Mitochondria were imaged in distal axons, synapses, and NMJs of motor neurons using the Nikon C-1 laser scanning confocal system (LSCM) mounted on an Eclipse 90i microscope, and analysis was conducted with EZ-C1 and Elements software (Figure 3). The entire dissection and imaging procedure was completed within 25 minutes to ensure normal physiological in vivo conditions.

Measurement of mitochondrial inner membrane potential ($\Delta\psi_m$).

To quantify membrane potential, images from the NMJs of control and *parkin*²⁵ were taken between muscles 6 and 7 (M6–7). Samples were fixed with a red tetramethylrhodamine methyl ester (TMRM, Life Technologies Molecular Probes; Cat. No. T668) stain for 20 minutes before imaging. The dye does not penetrate well into SNs, but it does equilibrate into the larval body wall. Therefore, $\Delta\psi_m$ of samples was estimated by comparing a mitochondrial-to-cytoplasmic dye fluorescence ratio of TMRM signal intensity (F_m/F_c). One hundred ninety-seven mitochondria from 13 control animals and 159 mitochondria from 12 mutant animals were analyzed in the thin axons adjacent to A4 SNs. The mean F_m/F_c ratio was calculated per animal, and a

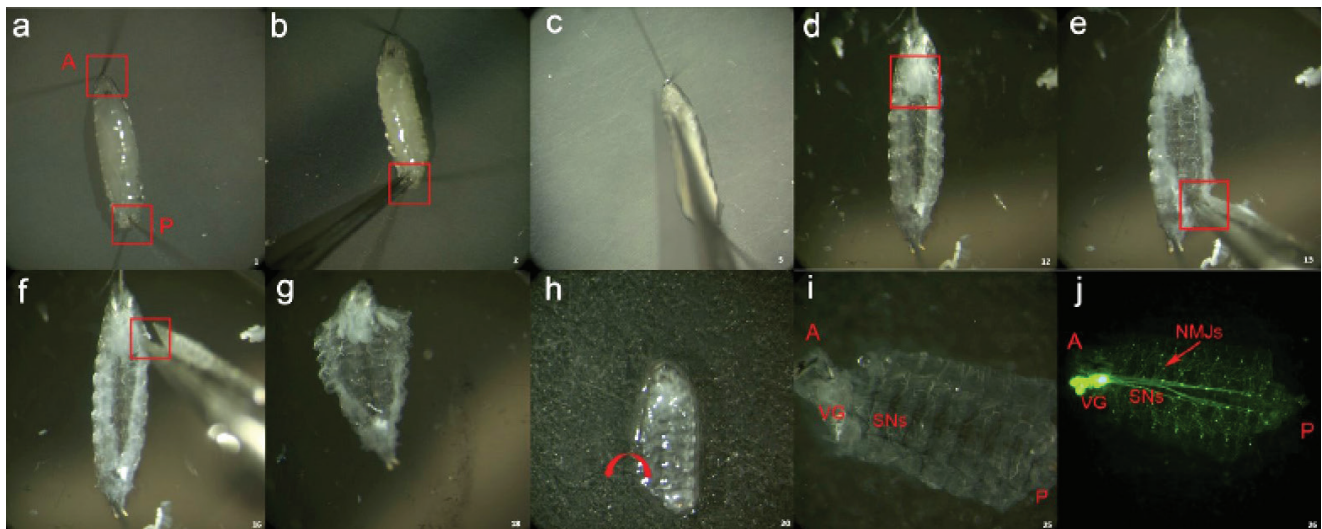


Figure 2. *Drosophila* third instar larvae dissection. Pin the larvae and make an incision down its abdomen, opening gently to remove all intestines and fat. Make four small horizontal cuts to further open the body, revealing its intact VG and SNs. Image under a confocal microscope. Dissections must be conducted gently and within 20 minutes to ensure an in vivo system.

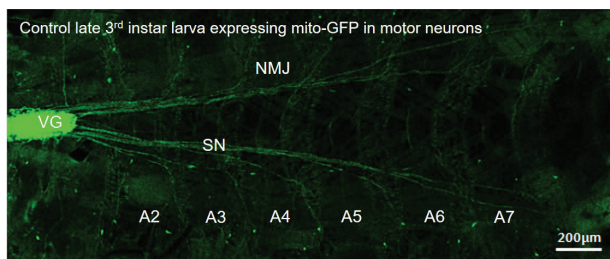


Figure 3. To observe *Drosophila* nervous systems in vivo, third instar larvae were collected, dissected, and prepared. All images were taken via confocal microscopy. In each sample, mitochondria were analyzed in NMJs of middle A4 axons of SNs.

mean of means was calculated per control and *park*²⁵ animal. F_m is determined by subtracting and thresholding the background to determine the mean mitochondrial fluorescent intensity using the Elements software. F_c is determined by using two hand-drawn regions of $\sim 0.5 \mu\text{m}^2$ to find the mean signal intensity of the two boxes in the cytoplasmic region. The mean is taken as the F_c of that particular mitochondrion. The F_m/F_c ratio has a logarithmic relationship to the inner mitochondrial membrane potential (Verburg & Hollenbeck, 2008).

Quantification of mitochondrial density in synaptic boutons. Samples exhibiting mitoGFP were stained with horseradish peroxidase (α HRP) and bruchpilot (α BRP) stains. α HRP highlights synaptic boutons while α BRP highlights calcium channels of nerve

terminals to reflect NMJ activity. Density was determined using hand-drawn polygonal ROI regions around clear synaptic boutons to find the relative signal intensity of α HRP. This process was repeated to find the relative signal intensity of mitoGFP. The two signal intensities were then compared in order to determine the integrated intensity of mitochondria within each bouton. This value served as a method to quantify mitochondrial density. Density determination was conducted using the Elements software.

RESULTS

Parkin Mutants Exhibit Normal Mitochondrial $\Delta\psi_m$

Control and *park*²⁵ samples were stained with TMRM lipophilic fluorescent cationic dye for 20 minutes before imaging. Figure 4 shows representative control and *park*²⁵ images in NMJs at A4 SNs. Mitochondria in synaptic boutons were selected for quantification. Samples were compared with a mitochondrial matrix/nerve axon ratio of TMRM signal intensity (F_m/F_c) in order to standardize the signal from the TMRM dye in comparison to mitochondrial intensity. Mean F_m/F_c ratios from 197 mitochondria from 13 control and 159 mitochondria from 12 *park*²⁵ animals were collected, and a mean of means was calculated. From the results, it is clear that the $\Delta\psi_m$ state of mitochondria in NMJs is normal in both control and mutants. The differences between control and *park*²⁵ were not statistically significant, indicated by the t-test p value of 0.2764 (Figure 5). p value > 0.05 ; data is not statistically significant.

In Figure 5, the error bars indicate the mean \pm standard error of the mean (SEM). In the total control mitochondria analyzed, the mean of means TMRM intensity was 17.32 ± 0.81 . For *park²⁵*, TMRM intensity was 19.09 ± 1.40 . The error bar margins in both control and *park²⁵* show relatively consistent data across all mitochondria samples from the calculated mean. However, the *p* value of 0.2764 indicates that the mean of means F_m/F_c ratios between control and *park²⁵* are too similar and do not have a statistically significant difference. This indicates that synaptic boutons in both control and *park²⁵* exhibit normal $\Delta\psi_m$ states.

In order to determine whether there was a variance in the distribution of mitochondria between control and *park²⁵* and whether this contributed to the obtained F_m/F_c results, a two-sample Kolmogorov-Smirnov test was conducted (Figure 5). A side-by-side comparison of mitochondria in the synaptic boutons of control and *park²⁵* is shown on the bar graph. The test showed that there was no significant difference in the distribution of synaptic mitochondria between control and mutant animals. This validates the mean of means quantification method that was used for F_m/F_c $\Delta\psi_m$ analysis.

Park²⁵ Animals Show Reduced Mitochondrial Density in Nerve Terminals

Samples exhibiting mitoGFP were additionally stained with α HRP and α BRP in order to further outline the synaptic boutons and quantify NMJ nerve activity. As shown in Figure 6, the three different stains on each control and *park²⁵* sample can be

viewed separately from a superimposed image. Figure 7 shows representative density images for control and *park²⁵*. The red circular outlines represent individual synaptic boutons; these were analyzed for mitochondrial density analyses. The entire synaptic bouton was selected, and the mitoGFP signal from within each synaptic bouton was selected as well. These two intensities of α HRP and mitoGFP were then compared by integrated intensity to determine the relative density of mitochondria present in the respective synaptic bouton.

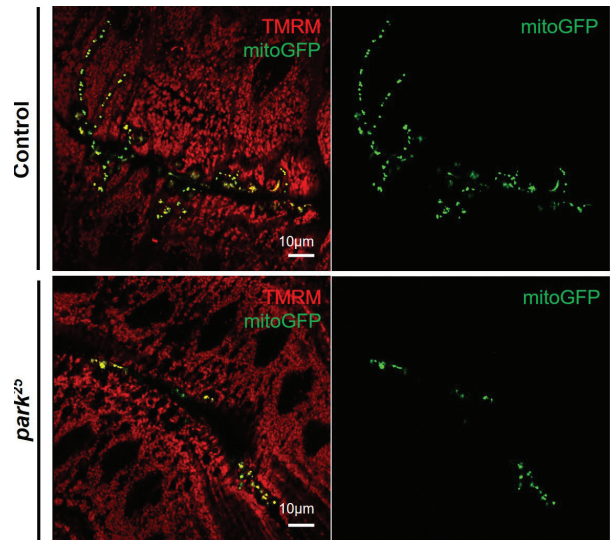
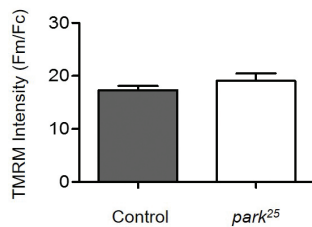


Figure 4. Representative TMRM images for control and *park²⁵* in NMJs at A4 SNs. Mitochondria in synaptic boutons were selected for quantification.



Statistical values
Mean \pm SEM
Control : 17.32 ± 0.81
park²⁵ : 19.09 ± 1.40
t-test : *p* value, 0.2764

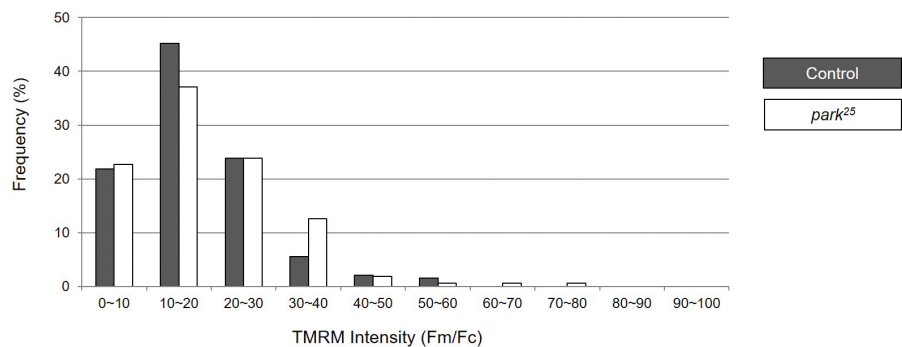


Figure 5. Quantitative analyses of F_m/F_c mean of means method showed that there is no significant difference in $\Delta\psi_m$ states between control and mutant animals. Error bars indicate mean \pm SEM. *p* value 0.2764 > 0.05; data is *not* statistically significant. Two-sample Kolmogorov-Smirnov test for potential differences in mitochondrial distribution between control and *park²⁵* shows no difference in distribution across all samples. One hundred ninety-seven mitochondria from 13 control animals and 159 mitochondria from 12 *park²⁵* animals were analyzed for $\Delta\psi_m$ data.

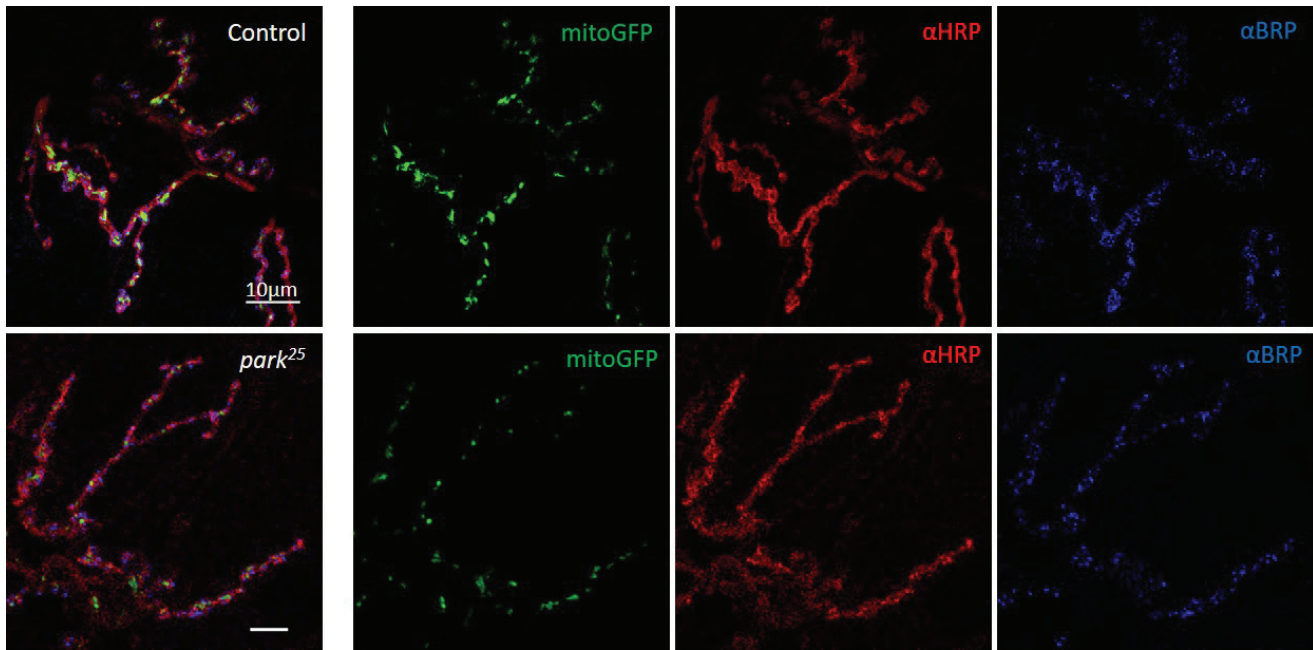


Figure 6. Control and *park²⁵* samples exhibiting mitoGFP were also stained with α HRP and α BRP. The left-most neurons show the superimposed image of the three signals. α HRP highlights of the synaptic boutons were used for mitochondrial density quantification.

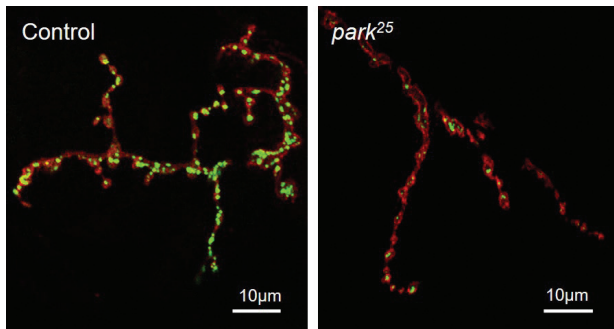


Figure 7. Representative density images for control and *park²⁵* animals.

Density differences between control and *park²⁵* were found to be statistically different. However, instead of *park²⁵* exhibiting an increased mitochondrial density, they displayed a statistically significant decreased density in comparison to control synapses (Figure 8). Density was analyzed in 61 synaptic boutons from 9 control and 61 synaptic boutons from 10 *park²⁵* animals. In control, synaptic boutons have approximately 44% coverage with mitochondria while *park²⁵* only has 30% coverage. Data indicates that mutants show reduced mitochondrial density. The *t*-test *p* value was also calculated to be < 0.001 , indicating a clear statistical difference between control and *park²⁵*.

DISCUSSION

Mitochondrial integrity plays a pivotal role in the normal functioning of neurons; therefore, defects in the mitochondrial life cycle are frequently associated with many diseases of the nervous system. But in most cases, it is still unclear which of these potential defects underlie these disorders, nor are the relationships between specific defective processes and neuropathology known. In this study, we modeled the neurodegenerative disease Parkinson's, which is characterized by a progressive loss in muscle control. It is believed that one of the most frequent causes of Parkinson's disease is the accumulation of dysfunctional or damaged mitochondria in the neurons, meaning that the pathogenesis of Parkinson's may arise in part from the ineffective turnover of mitochondria (Winklhofer & Haass, 2010). A plethora of previous work (e.g., Ashrafi et al., 2014; Amiri & Hollenbeck, 2008; Rugarli & Langer, 2012; Schwarz, 2013) has studied the turnover of mitochondria in the CB and axons, but no work has been done to analyze mitophagy in the nerve terminals. We aimed to elucidate the mechanisms of Parkin in mitochondrial turnover in the NMJs in vivo in intact nervous systems of *Drosophila*.

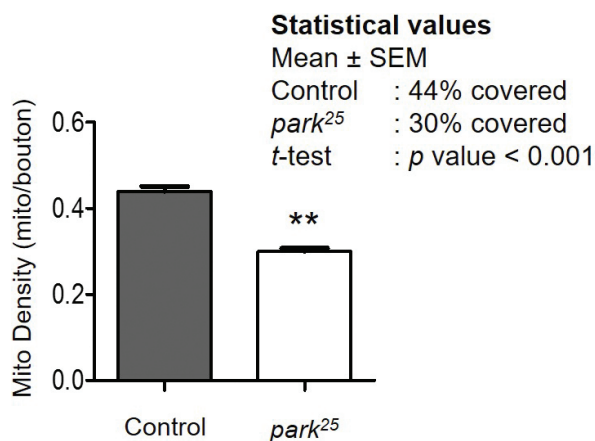


Figure 8. Density differences between synaptic bouton mitochondria of control and *park*²⁵ are statistically significant. Error bars indicate mean \pm SEM. *p* value < 0.001, data is statistically significant. In control 44% of synaptic boutons are covered by mitochondria while *park*²⁵ has 30% coverage. Sixty-one boutons from 9 control and 61 boutons from 10 *park*²⁵ animals were analyzed.

Mitochondria use their membrane potential for a variety of functions, such as the synthesis of ATP. Therefore, decreased $\Delta\psi_m$ readings in mitochondria are associated with unhealthy mitochondria that will undergo turnover. In *park*²⁵ mutant animals, which exhibited Parkin null expression, we expected to see disruption to the PINK1-Parkin mitophagy process. This, we believed, would cause the accumulation of senescent mitochondria in neurons. From this, we hypothesized that *park*²⁵ NMJ mitochondria would exhibit overall deficient $\Delta\psi_m$ states and increased mitochondrial density in comparison to control animals.

Results from $\Delta\psi_m$ data seems to show that there is no statistical significance in $\Delta\psi_m$ readings between control and *park*²⁵ animals (Figures 4–5). This goes against our hypothesis, for we predicted that Parkin null mutants would exhibit accumulation of senescent mitochondria in nerve terminals. However, as indicative from our results, this does not appear to be the case. The density results further show that mitochondria are not accumulating within neurons, for *park*²⁵ exhibited overall decreased mitochondrial density within synaptic boutons (Figures 6–8).

From our results, we believe that there is a “barrier” of some sort between the CB and the axon that is selectively permeable only to healthy mitochondria; this is consistent with previous findings that the

majority of mitochondria in axons seemed to exhibit normal physiological states, and that there were noticeably fewer mitochondria in axons than CBs. As mitochondria are mostly synthesized in the CB to be shipped out to the rest of the neurons, the proposed hypothesis appears consistent with this finding as well. Or, here is a potential second mechanism for how the obtained results are possible; unhealthy mitochondria may be anterogradely shipped to the cell body for turnover. If the mitochondria are shipped out of the synaptic boutons, our readings on normal $\Delta\psi_m$ would indicate that we only measured the membrane potentials of the remaining, healthy mitochondria. And since mitochondria would be returning to the CB, there would be reduced density as well.

Essentially, we believe that there are two possible explanations for our results of normal $\Delta\psi_m$ but reduced density in *park*²⁵ animals. (1) There is a “barrier” between the CB and axon that is only permeable to healthy mitochondria, or (2) unhealthy mitochondria may be anterogradely shipped to the CB for turnover. Potential future studies could aim to elucidate the proposed mechanisms further by utilizing α BRP to quantify nerve activity. While implicated to play an important role in the mitochondrial life cycle, the role of Parkin still widely remains unknown.

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