Published in final edited form as:

J Neurosci Methods. 2019 September 01; 325: 108351. doi:10.1016/j.jneumeth.2019.108351.

Methods to Detect Mitophagy in Neurons During Disease

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Abstract

Mitophagy is the selective degradation of mitochondria by autophagy. Methods to study mitophagy in neurons is of increasing importance as neurodegenerative diseases such as Parkinson's and Alzheimer's display disrupted mitophagy as part of their pathogenesis. Since the last decade, researchers have determined how selective mitophagy pathways such as PINK1/Parkin and Mul1 function at the cellular level. Thus, advances in techniques to study these pathways specifically in neurons and glia have arisen. This review will introduce mitophagy pathways studied in neurons and evaluate current techniques available to investigate mitophagy.

Keywords

mitophagy; PINK1; Parkin; Mul1; neurons; neurodegenerative diseases

Introduction

Mitochondria are pivotal in providing energy by generating ATP through cellular respiration and because of this mitochondrion are often referred to as the powerhouse of the cell. The mitochondria are important for many other cellular functions such as but not excluded to apoptosis, calcium regulation, reactive oxygen species (ROS) handing, and iron metabolism (Eisner et al., 2018; Spinelli and Haigis, 2018). Neurons solely rely on oxidative metabolism for energy production, unable to meet their bioenergetic demands through glycolysis (Hall et

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Author contributions

FEC wrote the first draft of the manuscript. M.E.M. drew figures and revised the manuscript. AMP wrote and revised the manuscript. All authors reviewed and edited the manuscript before submission.

Conflict of Interest

The authors declare that they have no competing interests.

al., 2012; Zheng et al., 2016). Neurodegenerative diseases can arise when neurons have a decline in oxidative phosphorylation (OXPHOS) and mitochondrial function (Pickrell et al., 2011; Pinto et al., 2012). Therefore, mitophagy, or the selective autophagic degradation of mitochondria, is needed to properly control mitochondrial homeostasis and quality for properly functioning neurons.

Macroautophagy or autophagy is defined as a type of quality control mechanism for proteins and organelles by targeting cytosolic components to the lysosome for degradation (Mizushima, 2018). Mitophagy selectively degrades and eliminates the whole or parts of damaged mitochondria, while general autophagy unselectively removes mitochondria (Youle and Narendra, 2011). In addition, mitophagy works in conjunction with mitochondrial biogenesis to maintain cellular homeostasis (Palikaras et al., 2015). Neuron-specific conditional ATG5 (autophagy-related protein 5) and ATG7 (autophagy-related protein 7) knockout (KO) mice displayed an absence of autophagy causing progressive neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). ATG5 and ATG7 KO neurons accumulated abnormal mitochondria indicating neurons rely heavily on autophagic pathways for mitochondrial quality control (Hara et al., 2006; Komatsu et al., 2006).

Parkin/PINK1

Mutations in PINK1 (PTEN –induced putative kinase protein 1) (a mitochondrial targeted serine/threonine kinase) and Parkin (an E3 ubiquitin ligase) are known to cause autosomal recessive forms of Parkinson's disease (PD) (Kitada et al., 1998; Matsumine et al., 1997; Valente et al., 2001; Valente et al., 2002). Epistasis experiments in *Drosophila* demonstrated that both of these proteins reside in the same pathway (Clark et al., 2006; Poole et al., 2008; Yang et al., 2006). Parkin normally is in an inactive conformation in the cytosol (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). PINK1 is constitutively imported and degraded inside polarized, functional mitochondria (Silvestri et al., 2005; Yamano and Youle, 2013).

In 2008, Youle and colleagues discovered Parkin specifically translocated to damaged mitochondria facilitating mitophagy (Narendra et al., 2008) (Figure 1), and over the last decade his group and others elucidated mechanistically the Parkin-mediated mitophagy pathway (Pickrell and Youle, 2015). Mitochondrial damage that disrupts mitochondrial membrane potential or misfolded mitochondrial protein stress inhibits PINK1 import, allowing for its accumulation on the outer mitochondrial membrane (OMM) (Burman et al., 2017; Jin et al., 2010; Jin and Youle, 2013; Narendra et al., 2010; Vives-Bauza et al., 2010). PINK1 associates with the translocase of the outer membrane (TOM) to form a complex on the OMM (Lazarou et al., 2012). PINK1 recruits and activates Parkin from the cytosol to the outer mitochondrial membrane by two simultaneous processes: (1) the phosphorylation of Ser65 located in the ubiquitin-like domain of Parkin (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012) and (2) through phosphorylating ubiquitin (UB) and ubiquitin chains at Ser65 on resident OMM proteins (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Ordureau et al., 2015). Activated Parkin in a feedforward manner ubiquitinates resident OMM proteins (Chan et al., 2011; Sarraf et al., 2013; Tanaka et al., 2010; Ziviani et al., 2010). The ubiquitin signal stimulates the UPS (ubiquitin-proteasome

system) to degrade OMM proteins (Chan et al., 2011; Tanaka et al., 2010) and also acts as a scaffold for ubiquitin binding autophagy adaptor proteins to tether mitochondria to the developing autophagophore (Heo et al., 2015; Lazarou et al., 2015; Richter et al., 2016; Wong and Holzbaur, 2014).

The spatial localization of where Parkin-mediated mitophagy occurs within the neuron has been debated. Parkin-mediated mitophagy was originally shown to mostly localize to the cell soma (Cai et al., 2012). Other data demonstrated mitophagy occurs locally in distal axons rather than translocating to the soma (Ashrafi et al., 2014). However, antibody detection of phosphorylated Ser65 UB found an accumulation of signal in the soma of aged healthy and PD human postmortem dopaminergic (DA) neurons (Fiesel et al., 2015). Regardless, Parkin-mediated mitophagy appears important for the pathogenesis of PD in humans and is active in neurons.

Mul1

Mul1 (mitochondrial ubiquitin ligase 1) works in concert with the Parkin/PINK1 mitophagy pathway. The protein was original discovered to reside on the outer mitochondrial membrane as a SUMO and ubiquitin targeted E3 ligase (Braschi et al., 2009; Li et al., 2008; Neuspiel et al., 2008). In mouse embryonic fibroblasts (MEFs) forced to utilize OXPHOS in culture, Mul1 was shown to work in parallel with Parkin triggering mitophagy (Rojansky et al., 2016). Epistasis experiments confirmed that Mul1 acts in parallel to the PINK1/Parkin pathway (Figure 1), and knockdown of Mul1 in Parkin KO cortical neurons caused mitochondrial defects and neuronal death (Yun et al., 2014).

Transgenic Mouse Models of Mitophagy

Transgenic mouse models have been generated to help investigators evaluate mitophagy in a cell-type, tissue-specific manner *in vivo* (Figure 2, Table 1). Each mouse model relies on the expression or overexpression of a different fluorescent reporter protein to detect the autophagosome or autophagolysosome. Researchers also use these reviewed constructs or derivatives to virally overexpress or transfect neurons or glia *in vitro* and *in vivo.*

GFP-LC3 Mouse

The first transgenic mouse generated to probe autophagy was the GFP-LC3 reporter line under the CAG promoter by Mizushima's group (Mizushima and Kuma, 2008; Mizushima et al., 2004). Microtubule-associated protein light chain 3 (LC3) is the mammalian homolog of yeast Atg8, which is conjugated to phosphatidylethanolamine and targeted to autophagic membranes (autophagophore and autophagosome)(Kabeya et al., 2003). When LC3 is tagged with GFP, its punctate or ring-shaped morphology indicates active autophagosomes (Kabeya et al., 2003; Mizushima et al., 2003). Transgenic GFP-LC3 mice will not specifically label whether the autophagic cargo are mitochondria, but with immunostaining to detect mitochondrial proteins, this reporter line can be used to determine if mitophagy is present or perturbed in neurons. There are other forms of mitophagy that require a receptor with an LC3-interacting region (LIR) motif not explicitly stressed in this review. The GFP-LC3 transgenic mouse would be able to detect mitophagy in cases where these receptors

such as FUNDC1 (Liu et al., 2012), BNIP3/Nix (Sandoval et al., 2008; Schweers et al., 2007), and BCL2L13 (Murakawa et al., 2015) may act. However, the role these receptors play in relation to neuronal mitophagy is still under investigation.

There are potential drawbacks using these mice when interested in mitophagy. As alluded to above, the GFP-LC3 mouse was first developed to look at autophagy, so it is not specific for mitophagy detection. Even with immunostaining to detect mitochondrial proteins, this does not rule out general autophagy's role in organelle clearance. Increased GFP-LC3 punctae must also be carefully interpreted. Increases in the number of GFP-LC3 punctae per cell could indicate an upregulation of autophagy/mitophagy; however, it could also indicate a disruption with autophagosome degradation or decreased fusion to the lysosome (Mizushima et al., 2010).

Mito-Keima Mouse

The mito-Keima mouse model is a knock-in pH-dependent fluorescence reporter mouse where the reporter protein is targeted to mitochondria to evaluate whether the organelle resides in the cytosol or the lysosome (Sun et al., 2015). The fluorescent Keima protein is naturally derived from coral with a pH dependent excitation spectrum where it excites at a short wavelength at 440nm in a neutral pH and a long wavelength at 568nm in an acidic environment and is resistant to lysosomal proteases (Katayama et al., 2011). The mito-Keima protein is under the ROSA26 promoter with ubiquitous expression (Sun et al., 2015). When evaluating these mice, basal mitophagy levels were increased in the dentate gyrus, lateral ventricles, and Purkinje cell layers of the cerebellum compared to levels visualized in the cortex, striatum and substantia nigra (Sun et al., 2015). This same reporter protein has also been used to generate transgenic mito-Keima *Drosophila* to detect Parkin-mediated mitophagy dopaminergic neurons (Cornelissen et al., 2018).

There are potential drawbacks that need consideration when evaluating mitophagy in neurons with mito-Keima mice. Tissues need to be freshly isolated and imaged as aldehyde fixation and cryogenic storage of tissues either alters the pH of the lysosome or causes decreased signal for detection (Sun et al., 2017). The ubiquitous nature of the transgene knocked into the ROSA26 locus also causes a heterogenous cell-type populated, densely packaged tissue like brain to be difficult to evaluate in terms of identifying the cell of interest or determining which processes originate from which individual cell. Transgenic cardiac specific mito-Keima mice have recently been reported and were used to determine mitophagy during ischemia and high fat diet (Saito et al., 2019; Tong et al., 2019). Based on position-effect variegation during transgene integration (Feng et al., 2000), sparse oligodendrocyte labeling with membrane bound GFP has been used previously to parse out individual oligodendrocytes (Chong et al., 2012). Instead of knocking in the mito-Keima reporter protein, a transgene approach may be more applicable to CNS cell labeling.

Mito-QC Mouse

The knock-in mito-QC mouse model was developed as an alternative reporter animal to monitor mitochondrial turnover *in vivo* (McWilliams et al., 2016). A binary-based

fluorescence reporter uses a tandem mCherry (red)-GFP (green) fusion protein selectively targeted to the OMM (Allen et al., 2013). In the cytosol, both mCherry and GFP remain stable, but the GFP fluorescence quenches in low pH conditions inside the lysosome (Allen et al., 2013). When evaluating the CNS with the mito-QC mouse, basal mitochondrial turnover levels were high in Purkinje cells, DA neurons and microglia (McWilliams et al., 2016; McWilliams et al., 2018)

There are potential benefits and drawbacks that need consideration when evaluating mitophagy in neurons with mito-QC mice. Live and fixed tissue can be used to measure and quantify mitophagy because this model is unaffected by aldehyde fixation (McWilliams and Ganley, 2019). This allows for the antibody identification of specific cell types. This model provides greater experimental flexibility to researchers with the tools that they have available for imaging. However, this transgene is also knocked into the ROSA26 locus with similar technical difficulties when studying the CNS as discussed above. Thick tissue optical clearing and imaging can overcome some of these difficulties (see Porter and Morton in this special issue of *J. Neuroscience Methods*). Another drawback is the potential for the tandem mCherry-GFP to become degraded during mitophagy by the UPS. This could cause mitophagy events to be missed. Improvements to reporter mice when studying mitophagy in neurons would enable researchers to better examine mitochondrial quality control *in vivo*.

Mass Spectrometry

Advances in proteomics have allowed researchers to detect with more sensitivity the identity of proteins, discover post-translational modifications to peptides, and quantitate proteins and post-translationally modified peptides. Mass spectrometry has been performed to quantify mitophagy and assess general mitochondrial turnover by comparing autophagy and mitophagy knockout fly lines (Vincow et al., 2013). Due to the ubiquitin status of the OMM being a key driver in mitophagy initiation, ubiquitination and post-translational modification of mitochondrial proteins can accurately detect and measure quantitively mitophagy using mass spectrometry (Sarraf et al., 2013).

Absolute quantification (AQUA) mass spectrometry uses isotope labeled peptides as a standard to monitor ubiquitin kinetics, relay information about chain formation, and detect post-translational phosphorylation events on ubiquitin (Gerber et al., 2003; Harper et al., 2018; Kirkpatrick et al., 2005). AQUA proteomics applied to detect mitophagy can evaluate the ubiquitination status after Parkin activation (Ordureau et al., 2014; Rose et al., 2016) or although not evaluated yet, Mul1. With this type of proteomic approach, ubiquitin’s phosphorylation status can also be identified to detect PINK1 activity by the detection of phosphorylated ubiquitin (Ser65 UB)(Ordureau et al., 2014). This method has been shown to work successfully in the central nervous system of mice to detect phospho-ubiquitin (mitophagy) in a mouse model of mitochondrial dysfunction recapitulating some features of PD (Pickrell et al., 2015), and ubiquitin phosphorylation kinetics have also been worked out for IPSC (induced pluripotent stem cell) derived neurons (Ordureau et al., 2018).

Mass spectrometry approaches provide a quantitative and sensitive method for mitophagy detection prior to autophagosome formation, but drawbacks could hinder the interpretation

of the data. Whole cortical tissue fractions that are made of heterogenous CNS cell populations make it difficult to determine what neuronal/cell types are being examined. Immuno-based cell sorting with magnetic microbeads could purify cell types without removing cellular processes as in FACS (fluorescent activated cell sorting) (Holt and Olsen, 2016). It is also possible that PINK1 independent mitophagy pathways in neurons exist making Ser65 UB a poor marker to define all neuronal mitophagic pathways. This has been hinted at previously by groups that examined PINK1 KO flies and mice where mitophagy was prevalent but seemed less dependent on PINK1 basally (Lee et al., 2018; McWilliams et al., 2018). Evaluation of basal levels of mitochondrial ubiquitination in the presence and absence of mitophagy correlated with mitophagy fluorescent reporters would enhance our understanding of the complete process.

Correlative Light and Electron Microscopy (CLEM)

CLEM combines the power of fluorescent microscopy to localize proteins/structures/events of interest with the resolution of electron microscopy (de Boer et al., 2015), which recently this technique has been used to study mitophagy. Using a combinatory approach using cryo-fluorescent, room temperature-fluorescent, and electron microscopy (triCLEM), GFP-Parkin recruitment on depolarized mitochondria, identified by absence of MitoTracker Deep Red, can be visualized in mammalian cell culture to observe autophagosome formation around damaged mitochondria (Ader and Kukulski, 2017). In *Drosophila* skeletal muscle, CLEM utilizing mito-Keima detected mitophagic events as well (Cornelissen et al., 2018). This technique hasn't been utilized to examine neuronal mitophagy; however, CLEM detection of autophagic events for aggregates have been studied in BV2 microglia-derived cell lines (Bussi et al., 2018). Primary neurons from the above-mentioned transgenic models or ISPC derived neurons would be suitable for this type of analysis.

Mitophagy in Other Neurodegenerative Diseases

Age-related neurodegenerative diseases besides PD such as Alzheimer's disease (AD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) have recently begun to be investigated to determine if mitophagy contributes to the pathogenesis of these diseases. All of the most common age-related neurodegenerative diseases are associated with mitochondrial dysfunction (Pinto et al., 2012), so perturbations in mitochondrial quality control could be an underlying reason for these observations.

Alzheimer's disease is the most prevalent age-related neurodegenerative disease characterized by irreversible dementia (Scheltens et al., 2016). Mitochondrial deficits appear early in the disease and contribute to synaptic failure, which is linked to cognitive deficits and memory loss (Du et al., 2010; Maurer et al., 2000; Yao et al., 2009). The defining pathology of AD is the increase in amyloid beta ($A\beta$) aggregates and deposited hyperphosphorylated tau (p-tau) throughout the brain (Masters et al., 2015). AD patients, IPSC derived neurons, and animal models have shown signs of mitophagy impairment (Fang et al., 2019). PINK1 overexpression in AD mouse models reduced $A\beta$ accumulation ameliorating mitochondrial and synaptic dysfunction (Du et al., 2017).

Recent data has also pointed to potential mitophagy defects in ALS, an age-related motor deteriorating neurodegenerative diseases that attacks both upper and lower motor neurons. Mutations in optineurin (a mitophagy adaptor protein) and Tank binding kinase 1 (TBK1) (a kinase that phosphorylates mitophagy adaptors) contribute to ALS (Cirulli et al., 2015; Freischmidt et al., 2015; Maruyama et al., 2010). ALS optineurin mutations were found to inhibit Parkin-mediated mitophagy in cell culture (Lazarou et al., 2015; Wong and Holzbaur, 2014). In an ALS transgenic mouse model expressing the familial SOD1G93A mutation, evidence of active mitophagy occurred but surprisingly found that chronic Parkin activation was detrimental (Palomo et al., 2018).

Huntington's disease (HD) is a genetic polyglutamine neurodegenerative disease with excessive CAG repeats in the gene huntingtin causing motor deterioration and death due to the loss of medium spiny neurons of the striatum. Huntingtin, with increased polyglutamine repeats responsible for HD, interacts with a AAA+ ATPase protein, p97, on mitochondria possibly causing mitophagy defects (Guo et al., 2016; Hosp et al., 2015). Overexpression of PINK1 in an HD *Drosophila* model decreased neurodegenerative and dysfunctional mitochondrial phenotypes to increase survival (Khalil et al., 2015).

Future Directions for Mitophagy in Neurons

Alternative mitophagy pathways play physiological roles in other cell types for processes such as differentiation and development. Mitophagy eliminates mitochondria in developing reticulocytes to mature red blood cells as well as aid in brown and white adipocyte differentiation (Baerga et al., 2009; Schweers et al., 2007; Zhang et al., 2009). Mitophagy is required for proper differentiation and cell fate but do not necessary remove only damaged mitochondria. It is unclear what mitophagy pathways are important for basal mitophagy or if mitochondrial damage is the only trigger for mitophagy in neurons. Evidence also suggests that damaged mitochondria may bypass these types of selective mitophagy pathways in certain cell types (Ahlqvist et al., 2015; Li-Harms et al., 2015). Neuronal mitophagy is clearly present; however, it is still unclear to what extent mitophagy and autophagy converge for physiological homeostasis. The development of new techniques to study mitochondrial quality control in disease and in physiological conditions will help researchers understand how mitochondrial function and dysfunction affects neuronal cell biology.

Acknowledgements

This work was supported by the National Institute of General Medical Sciences Fellowship (2R25GM066534-14A1) (FEC) and departmental start-up funds (AMP).

Abbreviations

Aβ	amyloid beta
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ATG	autophagy-related protein

ATP	adenosine triphosphate
AQUA	absolute quantification
CLEM	correlative light electron microscopy
CNS	central nervous system
DA	dopaminergic
FACS	fluorescent activated cell sorting
GFP	green fluorescent protein
HD	Huntington's disease
IPSC	induced pluripotent stem cell
KO	knockout
LC3	microtubule-associated protein light chain 3
LIR	LC3-interacting region
MEFs	mouse embryonic fibroblasts
mtDNA	mitochondria DNA
Mul1	mitochondrial ubiquitin ligase 1
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PINK1	PTEN–induced putative kinase protein 1
p-tau	phosphorylated tau
QC	quality control
ROS	reactive oxygen species
TOM	translocase outer membrane
UB	ubiquitin
UPS	ubiquitin-proteasome system

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Highlights

- PINK1/Parkin and Mul1 are two main mitophagy pathways in neurons.
- Advances in transgenic mouse models can be used to visualize mitophagy in neurons.
- Mass spec and CLEM detect early stages of mitophagy in neurons.
- Defects in mitophagy contribute to the pathogenesis of neurodegenerative diseases.

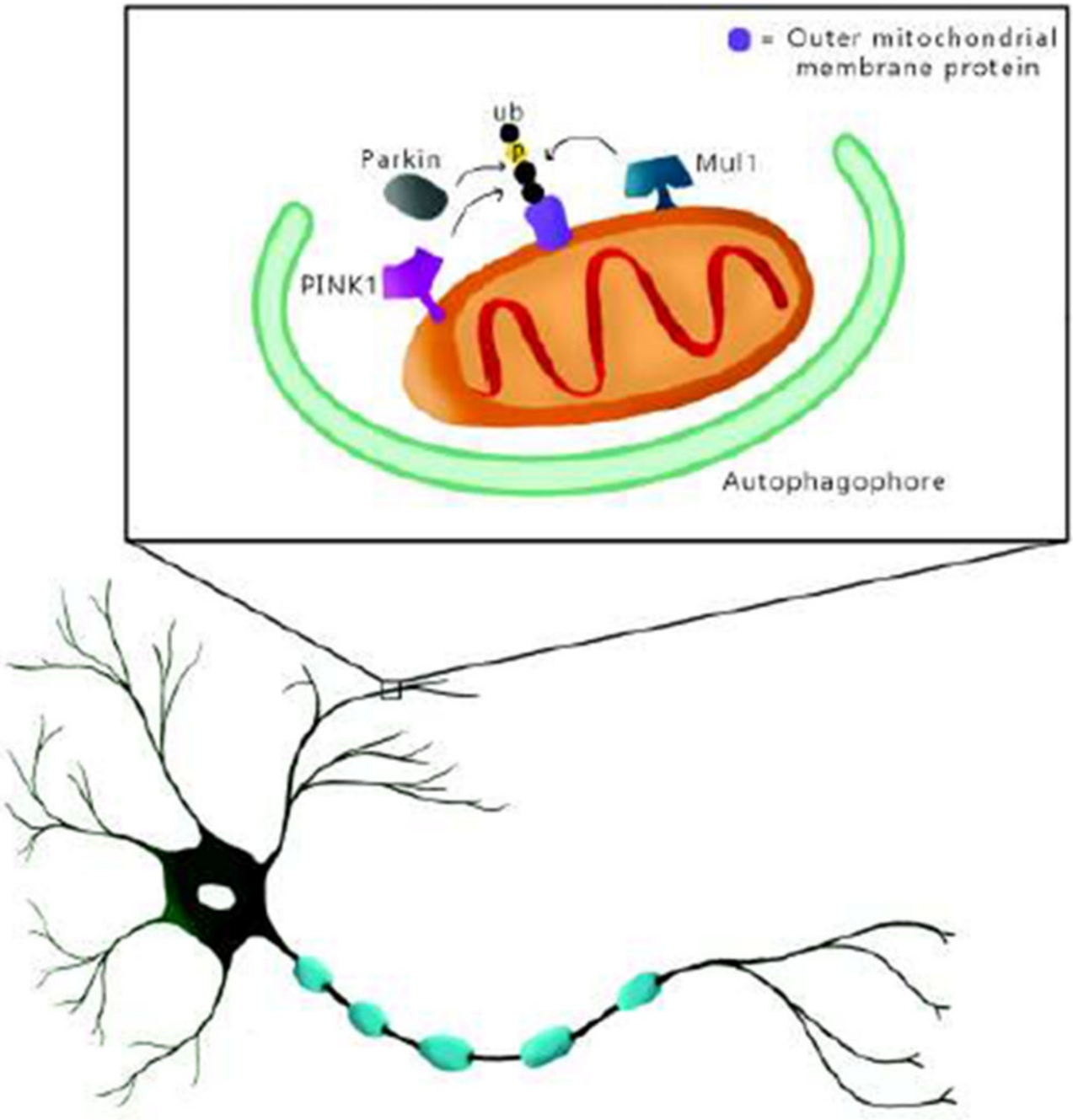

Figure 1: PINK1/Parkin and Mul1 are parallel selective mitophagy pathways in neurons.
Cartoon depiction of neuronal mitophagy. Mul1 and Parkin work in conjunction to tag the OMM proteins with ubiquitin for lysosomal degradation. PINK1 phosphorylates ubiquitin and Parkin to activate Parkin and allow for autophagy adaptor binding (not depicted) to facilitate autophagosome formation around the targeted mitochondrion.

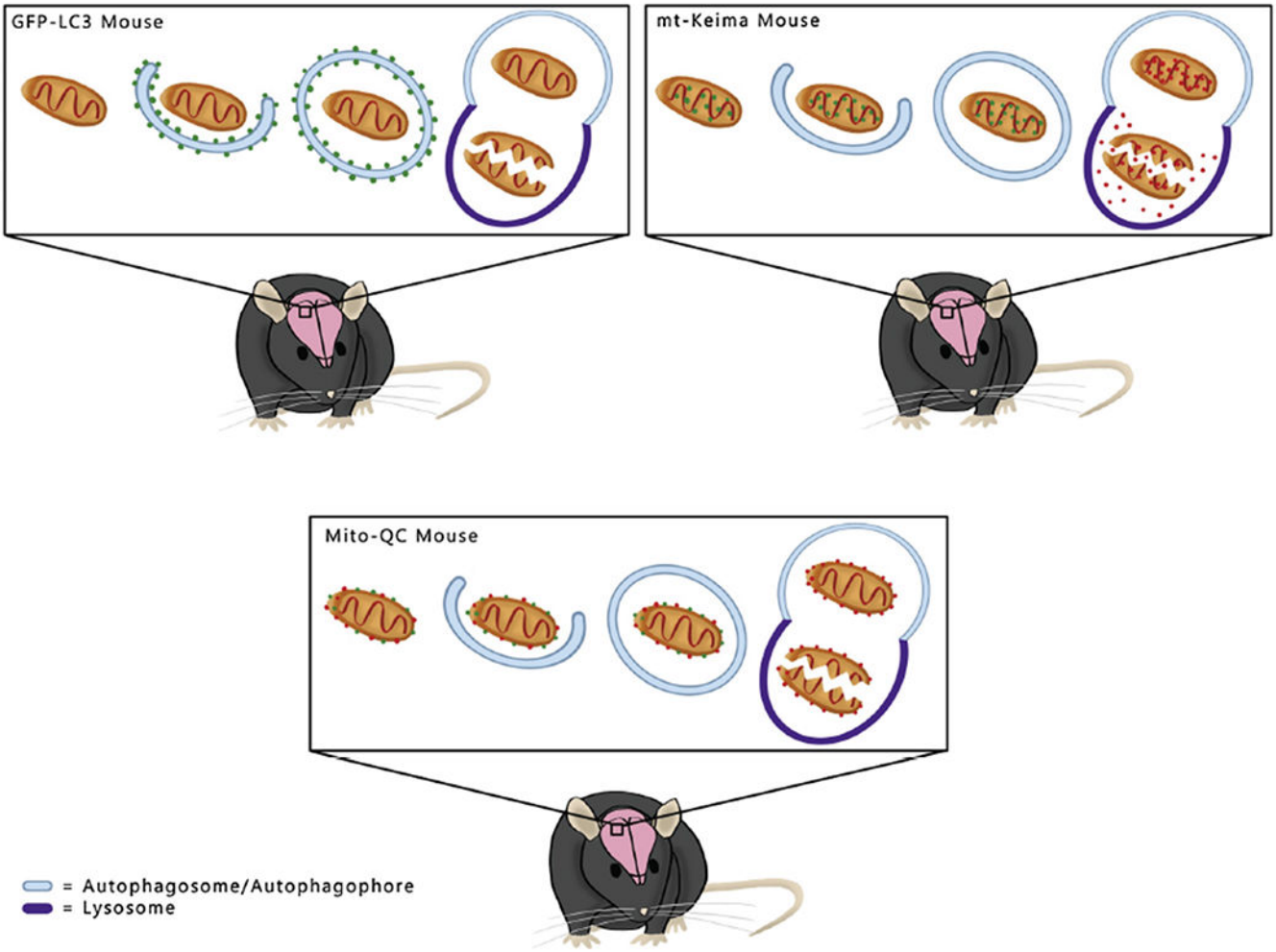

Figure 2: Transgenic reporter mouse models to evaluate mitophagy *in vivo*.

Cartoon illustrating the transgenic mouse models used to detect mitophagy in neurons. GFP – LC3 transgenic mice utilize GFP to visualize the autophagosome. Mito-Keima mice express a pH sensitive fluorescent protein residing within the mitochondrial matrix. Differences in the excitation of this fluorescent protein reflects whether mitochondria are in the cytosol or lysosome. The mito-QC reporter mouse targets a tandem mCherry-GFP fluorescent protein to the outer mitochondrial membrane. GFP is extinguished in acidic conditions inside the lysosome to infer whether the mitochondria are in the cytosol or lysosome.

Table 1:
Summary of mouse models used to examine mitophagy in neurons.

Select examples of the mouse models used in the literature with corresponding references and brief description of their usage.

Mouse model	Usage (Neuron specific)	Reference
GFP-LC3 (Autophagy/Mitophagy)	Generated model	Mizushima et al. MBC 2004
	Lurcher model	Wang *et al.* J Neuro 2006
	DRG primary neurons	Maday *et al.* JCB 2012
	Primary neurons	Maday *et al.* J Neuro 2016
Mito-Keima	Generated model	Sun et al. Mol Cell 2015
	Protocol for usage	Sun et al. Nat Protc 2017
	ALS model	Palomo et al. EMBO Mol Med 2018
	GBA mutant model	Li *et al.* Autophagy 2019
Mito-QC	Generated model	McWilliams et al. JCB 2016
	PINK1 KO model	McWilliams et al. Cell Met 2018
	Parkin S65A model	McWilliams et al. Open Bio 2018
	Rential ganglion cells *in vivo*	McWilliams et al. Autophagy 2019