Biochemical Society Transactions (2020) **48** 709–717
https://doi.org/10.1042/BST20191283

PORTLAND PRESS

Review Article

Exaggerated mitophagy: a weapon of striatal destruction in the brain?

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Mechanisms responsible for neuronal vulnerability in the brain remain unclear. Striatal neurons are preferentially damaged by 3-nitropropionic acid (3-NP), a mitochondrial complex-II inhibitor, causing striatal damage reminiscent of Huntington's disease (HD), but the mechanisms of the selectivity are not as well understood. We have discovered that Rhes, a protein enriched in the striatum, removes mitochondria via the mitophagy process. The process becomes intensified in the presence of 3-NP, thereby eliminating most of the mitochondria from the striatum. We put forward the hypothesis that Rhes acts as a 'mitophagy ligand' in the brain and promotes mitophagy via NIX, a mitophagy receptor. Since Rhes interacts and promotes toxicity in association with mutant huntingtin (mHTT), the genetic cause of HD, it is tempting to speculate on whether the exaggerated mitophagy may be a contributing factor to the striatal lesion found in HD. Thus, Rhes-mediated exaggerated mitophagy may act as a weapon of striatal destruction in the brain.

Introduction

In the brain, particularly with regard to neurodegenerative diseases, some neurons are more vulnerable to neuronal death than others, but the molecular mechanisms of this selective susceptibility remain unclear [1]. Models that replicate a selective and reproducible brain lesion may facilitate the identification of the mechanisms involved in selective vulnerability. One such model is the plant-derived mitochondrial toxin, 3-nitropropionic acid (3-NP), which, mysteriously, elicits neuronal lesions selectively in the striatum but not in the other regions, such as the cortex or the cerebellum [2]. We found that Rhes, a striatal-enriched protein, is a critical mediator of 3-NP-induced selective lesions involving mitophagy mechanisms. When animals are exposed to 3-NP, which irreversibly inhibits succinate dehydrogenase (SDH, mitochondrial complex-II), a major metabolic and respiratory regulator, it leads to a continued accumulation of dysfunctional mitochondria throughout the brain and the peripheral system. In the striatum, Rhes removes dysfunctional mitochondria via mitophagy, which under normal circumstances may be beneficial to the striatum. However, in the presence of 3-NP, mitophagy by Rhes is exaggerated, a process that eliminates most of the dysfunctional mitochondria from the striatum. Ultimately, striatal neurons become devoid of mitochondria and consequently succumb to demise. Thus, what was initiated as a beneficial process to protect neurons, mitophagy by the Rhes protein, turns into a weapon of striatal destruction.

BNIP3 and BNIP-like molecules as mitophagy receptors

Two broad types of mitophagy pathways have been described [3]: the PINK/Parkin-dependent pathway and the independent mitophagy pathway, which consists of BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) and BNIP3-like (BNIP3L) proteins, also known as NIX (NIP3-like protein X) members. BNIP3 and NIX are homologous with Bcl2 outer mitochondrial proteins in the BH3 domain. They are localized in the outer membranes of ER and mitochondria. On mitochondria, both BNIP3 and NIX (which are 50% identical) associate with Bcl2 via the C-terminal transmembrane

Received: 20 December 2019
Revised: 13 February 2020
Accepted: 14 February 2020

Version of Record published:
4 March 2020

Biochemical Society Transactions (2020) 48 709–717
https://doi.org/10.1042/BST20191283

domain and promote cell death, via mechanisms that are not completely understood [4]. Although these molecules are originally implicated in the cell death pathway, they have an important regulatory role in mitophagy under different conditions [5–8]. It has been proposed that the mechanisms that promote cell death and mitophagy are quite distinct. During mitophagy, BNIP3 and NIX act as mitophagy receptors and prime the damaged mitochondria to autophagosomes for the degradation in lysosomes. Hypoxia conditions are known to induce BNIP3 and NIX. Both contain identical LC3-interacting regions that promote mitophagy by interacting with LC3 on the autophagsomes. How does NIX trigger mitophagy? It was shown that NIX induces mitochondrial depolarization caused by the mitochondrial permeability transition pore (MPT) to initiate mitophagy as well as to recruit autophagosomes, depending upon the cell types [9]. BNIP3 and NIX can interact with Rheb, an activator of the mammalian target of rapamycin (mTOR), to mediate mTOR-dependent cell growth or mTOR-independent mitophagy [10]. But the exact mechanisms of how BNIP3 or NIX promotes mitophagy under different cellular conditions and their role in neuronal mitophagy are unclear [9].

Excessive mitophagy in peripheral and nervous system defects

Diminished mitochondrial mass, an indication of mitophagy, has been found in neurodegenerative diseases, including HD. In the spinal cords of amyotrophic lateral sclerosis (ALS) patients, the activity of citrate synthase (CS), which is often used as a marker of mitochondrial mass and decreased activity of complexes I+ III, II+ III, and IV, is indicative of loss of mitochondria [11]. Diminished mitochondrial mass as measured by CS activity was also found in Alzheimer's disease (AD) patients and animal models [12,13]. Diminished mitochondrial mass was also found in the brains of sporadic Parkinson's disease (PD) patients [14]. Severe loss of mitochondrial activities, such as reduction in the activities of complex II/III and aconitase, was found in postmortem samples of the caudate/putamen in HD patients [15]. However, defects in mitochondrial mass are not very common in HD animal models and were found only in selected models [16–18].

As a process that is potentially deleterious to neurons, excessive mitophagy has been found in various experimental neuronal and non-neuronal models [19–21]. In an ALS mutant $SOD1^{G93A}$ mouse model of the disease, administration of rilmenidine, an antihypertensive agent with selectivity for I_1 imidazoline receptors, up-regulated mitophagy, severely depleted mitochondria in motor neurons, and worsened the disease progression and neurodegeneration [22]. In a neonatal stroke mouse model, BNIP3-mediated excessive mitophagy triggers delayed neuronal death [23]; interestingly, the authors of this study demonstrated that NIX levels are up-regulated in the stroke model but are not involved in activating excessive mitophagy [23]. Excessive mitophagy is implicated in chronic cerebral hypoperfusion (CCH), a chronic state of cerebral blood flow reduction that induces neuronal apoptosis, cognitive impairment, and neuroinflammatory responses. CCH is found in many cerebrovascular diseases, including AD. Excessive BNIP3-cyt C- and parkin-mediated mitophagy was found in CCH, and the fatty acid amide hydrolase inhibitor URB597 (URB) promotes neuroprotection by inhibiting abnormal excessive autophagy as well as mitophagy [24]. Intriguingly, excessive mitophagy was found in a genetic mouse model of HD [25]. The valosin-containing protein (VCP), a member of the AAA family of ATPase, is aberrantly translocated to the mitochondria and bound to mHTT and promotes excessive mitophagy and subsequent striatal neuron degeneration [25]. A study showed that blocking the interaction of mHTT and VCP with a peptide HV-3 inhibits VCP-mediated mitophagy impairment and reduces HD-associated neuropathology and motor deficits in HD transgenic mouse models. That study demonstrated there is a link for excessive mitophagy as a cause of HD pathogenesis [25]. However, VCP is a ubiquitously present protein [26]; how it promotes striatal neuronal degeneration and whether Rhes may participate in that process remain unknown.

Excessive removal of mitochondria via mitophagy can lead to the loss of cardiac myocytes and the development of heart failure. The mitochondrial division inhibitor (Mdivi-1) inhibits abnormal mitophagy and ameliorates the heart failure condition [27,28]. Anticancer agents such as AT-101 act as triggers of lethal mitophagy to promote cell death [29]. The agonist 1-(3,4,5-trihydroxyphenyl) nonan-1-one of orphan nuclear receptor TR3/Nur77 induces autophagy-dependent excessive removal of mitochondria and cell death [30]. Ceramide induces acute myeloid leukemia cell death by lethal mitophagy [31]. Excessive mitophagy, because it promotes cell death and tissue injury, is considered an important modulator in human pulmonary diseases and a potential therapeutic target [32]. In nasal epithelial cell inflammatory injury, mitophagy is associated with chronic obstructive pulmonary disease; phosphatase and tensin homolog inhibits increased mitophagy via the Toll-like receptor (TLR)4-c-Jun kinase (JNK)-Bnip3 pathway and prevents cell death [33]. Excessive mitophagy is implicated in clinical acute lung injury (ALI), a common complication that occurs following sepsis in human

patients. Lipopolysaccharide (LPS), a major endotoxin component of gram-negative bacteria, plays an essential role in the development of ALI. LPS promotes excessive mitophagy in ALI, and Mdivi-1, a mitochondrial division inhibitor, prevents LPS-induced mitophagy and cell death [34]. Thus, as a deleterious process, excessive mitophagy has been found in diverse systems, indicating its prevalent role in cell death, but the mechanisms remain unclear.

Excessive mitophagy and mitochondrial biogenesis

As a type of a feedback regulation in response to mitophagy, mitochondrial biogenesis is well documented. mHTT interacts with GTPase Drp1 and inhibits mitochondrial biogenesis and promotes synaptic degeneration in HD [35]. It was demonstrated that enhanced mitochondrial biogenesis in an HD mouse model reduces HD phenotype [36]. mHTT down-regulates PGC-1α, a major regulator of mitochondrial biogenesis, in HD, and PGC-1α overexpression promotes neuroprotection [37]. Pharmacological modulation of mitochondrial biogenesis or up-regulation of PGC-1α, has been shown to protect neuronal damage in HD [38,39]. Enhanced mitochondrial biogenesis, paradoxically, worsens the neuropathological and behavioral deficits in AD mice, attributed to excessive reactive oxygen species (ROS) production [40]. In immune cells, the excessive mitochondrial biogenesis is linked to ROS production and cell death [41,42]. These studies indicate that mitochondrial biogenesis is critically linked to survival and death. We propose that despite enhanced mitochondrial biogenesis, in a chronic neurodegenerative state, the mitochondria are constantly being damaged by mutant protein or mitochondrial toxin, 3-NP. Once the damage reaches a certain threshold, it will gain an upper hand over mitochondrial biogenesis and lead to an excessive removal of mitochondria and lesions.

We speculate that in presence of 3-NP, neurons may activate feedback mechanisms to promote mitochondrial biogenesis in order to replenish mitochondria damaged by 3-NP. Rhes will eliminate damaged mitochondria via mitophagy, further enhancing the mitochondrial biogenesis process. However, the continued exposure to 3-NP damages the newly born mitochondria, generating more ROS and neuronal death [43]. Hence, excessive mitophagy might promote ROS generation because Rhes works in a positive feedback loop in which it promotes mitophagy as well as produces ROS due to the damage of residual and newly formed mitochondria by 3-NP. But this critical control will not be sustained for long, because continued and chronic exposure to 3-NP will ultimately damage all the mitochondria; thus, no new mitochondria will be produced, setting the stage for 'total mitochondrial destruction.'

Striatum degeneration in Huntington's disease is induced by the mitochondrial toxin 3-NP

The mechanisms of neuronal vulnerability in the brain remain unclear. 3-NP found in plants is known to produce severe toxic symptoms, such as dystonia in both cattle and humans [44,45]. Studies using animal models such as mice, rats, and nonhuman primates have established that 3-NP promotes age-dependent striatal lesions and motor abnormality symptoms reminiscent of Huntington's disease (HD), a genetic disorder that results from expanded poly-Q-dependent huntingtin protein (mHTT) [46–49]. Mechanistically, 3-NP is a highly specific, time-dependent, and irreversible inhibitor of SDH that inhibits the Krebs cycle as well as the mitochondrial complex-II of the electron transport chain. 3-NP blocks SDH ubiquitously in regions such as the cortex, as well as in the liver, yet the striatum is the region that most prominently shows neuronal lesions [50–53]. Excitotoxicity and the generation of ROS have been implicated in 3-NP-induced lesions [54–56]. Accordingly, agents that alter excitotoxicity have been shown to prevent 3-NP-induced lesions in the striatum [57–62]. However, the question as to why 3-NP promotes ROS generation and excitotoxicity-mediated lesions specifically in the striatum remains unanswered. The existence of striatal-specific modulators and the mechanisms that mediate 3-NP-induced lesions are unclear.

Rhes, a striatal-enriched protein, mediates striatal vulnerability in HD

We have reported a novel Rhes-based striatal-specific mechanism that might account for 3-NP-induced striatal lesions in the brain. Rhes is enriched in the striatum, the cortex, and the olfactory bulb [63,64]. Rhes is induced by thyroid hormones and can inhibit the cyclic AMP/protein kinase A pathway and N-type Ca^{2+} channels (Cav 2.2) [65–68]. Over the years, we have identified several new roles for the Rhes protein in the striatum. The Rhes protein can directly bind to, and activate, the mTOR in a guanosine triphosphate (GTP)-dependent manner, and this promotes levodopa-induced dyskinesia in a preclinical model of PD [69].

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https://doi.org/10.1042/BST20191283

Rhes consists of a C-terminal, a small-ubiquitin-like modifier (SUMO) E3-like domain, and it physiologically regulates SUMO modification via 'cross-SUMOylation' of E1 (Aos1) and E2 (Ubc9) enzymes [70]. Rhes affects autophagy via Beclin1, independent of mTOR signaling [71]. Recently, we found that Rhes inhibits striatal motor activity through a 'Rhesactome,' a protein network in the striatum [72].

Rhes mediates 3-NP-induced striatal vulnerability via mitophagy

We have now found that Rhes selectively interacts with damaged mitochondria and removes them via mitophagy, wherein the damaged mitochondria are degraded via lysosomes [73]. Normally, the Rhes protein does not interact with elongated or 'healthy-looking' mitochondria, but it appears to surround the globular or 'unhealthy' mitochondria. Thus, the Rhes protein may regulate mitochondrial homeostasis (the numbers) by eliminating unhealthy mitochondria in the striatum (Figure 1A).

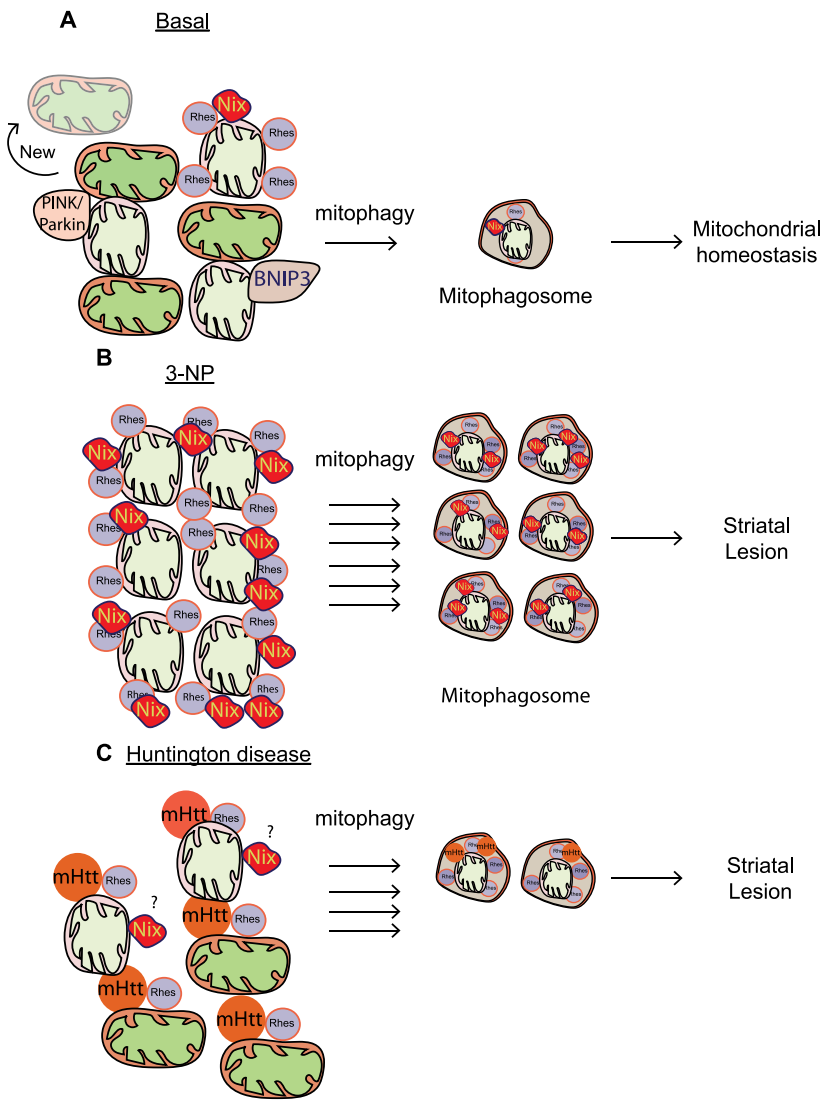

Figure 1. Model for Rhes-mediated mitophagy and neuronal lesion.

(**A**) Under basal condition, Rhes/Nix and other regulators of mitophagy (example, PINK/Parkin, BNIP3) interacts with damaged mitochondria and removes them via mitophagy, which may generate new mitochondria. (**B**) In the presence of 3-NP that irreversibly damage mitochondria, Rhes intensifies mitophagy together with Nix resulting in neuronal lesion. (**C**). In HD, Rhes and mHTT interaction may trigger mitophagy albeit at slow rate, compared with 3-NP, leading to progressive striatal lesion. Whether Nix is involved in mitophagy in HD is unknown. Refer texts for details.

When striatal neurons encounter 3-NP, which irreversibly damages mitochondria, the Rhes protein intensifies the process of removing damaged mitochondria via enhanced mitophagy (Figure 1B). This excessive-mitophagy process will continue until most of the mitochondria are removed from the cells, which will eventually die (Figure 1B). This process requires NIX, a mitophagy receptor. 3-NP-induced glutamate excitotoxicity may further worsen Rhes-induced mitophagy. Excitotoxicity can also occur in the cortex [53,73]; however, 3-NP does not elicit lesions in the cortex. Parkin, a ubiquitous mitophagy regulator, was shown to mediate NMDA receptor excitotoxicity-induced mitophagy in cortical neurons [74]. We speculate that 3-NP-induced excitotoxicity in the striatum may further enhance the Rhes and NIX interaction with one or more components of the damaged mitochondria to exacerbate mitophagy. A previous study showed that extrasynaptic NMDA receptor activation induces Rhes expression [75]. Whether or how 3-NP-induced excitotoxicity is linked to Rhes-mediated mitophagy remains unknown.

Previously, we found that Rhes interacts with mHTT and promotes cellular toxicity by increasing the soluble forms of mHTT via SUMOylation [76]. In animal models, we found that Rhes depletion diminishes and Rhes overexpression worsens, exhibiting HD-related motor behavior and striatal pathology [77]. Independent studies have demonstrated a toxic link for Rhes in HD in various cell and mouse models of HD [75,78–82]. The precise mechanisms by which Rhes increases mHTT toxicity, however, remain unclear. mHTT is known to interact with mitochondria and to disrupt mitochondrial complex II [83], but the mechanisms are not clearly understood. mHtt can be imported to the intermembrane space and binds to TIM23, the inner mitochondrial protein [84]. But whether mHtt interacts with complex II is unknown. We propose that Rhes and mHTT may disrupt mitochondrial complex II and exacerbate mitophagy together with NIX, albeit at a slow rate, compared with 3-NP, leading to a progressive lesion of the striatum in HD (Figure 1C). Because Rhes also robustly transports mHTT between cells via tunneling-like cellular protrusions, whether such intercellular processes are altered by excessive mitophagy and contribute to striatal lesions in HD remains unknown [85].

Rhes as an intercellular mitochondrial surveillant in 3-NP-induced toxicity

We found that Rhes is a potent inducer of cellular protrusion (Rhes tunnel) and transports endosomes and lysosomes, but not mitochondria, to the neighboring cells; however, we found that Rhes specially associates with the damaged mitochondria in the neighboring cells via NIX [73,85]. This indicates that Rhes has the capacity of intercellular mitochondrial surveillance *in vitro*. This finding raises the question of whether Rhes can travel to neighboring neurons whose mitochondrial functions are compromised *in vivo*. This is particularly interesting because the 3-NP-induced lesions in the striatal regions are not random. After 3-NP administration, the lesion appears at a small and specific region and then progresses throughout the striatum. What contributes to such a region-specific lesion? Does cell-to-cell interaction play a role? We propose that such anatomically distinct regions with different cell types and cell-to-cell interactions are the first ones to become vulnerable to the insult by 3-NP. When such hypothetical regions are exposed to 3-NP, Rhes would exacerbate the intercellular mitophagy that may disrupt the anatomical integrity, in turn propagating the lesion throughout the striatum. This hypothesis needs to be tested in the future.

Rhes may act as a mitophagy ligand via NIX to induce mitophagy

Our study demonstrates that Rhes requires the mitophagy receptor NIX to clear damaged mitochondria [73]. In cells that lack the NIX receptor, the Rhes protein fails to induce mitophagy. The NIX receptor alone cannot induce mitophagy in the presence of 3-NP. Levels of the NIX receptor in Rhes-KO striatum are comparable to those of WT, yet Rhes-KO striatum does not show signs of mitophagy upon 3-NP treatment [73]. This raises the possibility that the Rhes protein is a putative 'mitophagy ligand' in the striatum that requires a NIX mitophagy receptor to promote mitophagy. Interestingly Rhes binds to NIX via the C-terminal SUMO E3 ligase domain and to mTOR via the N-terminal region [73], suggesting that ligand-like properties of Rhes are in the C-terminal region. We propose a working model: Under the condition of stress (starvation or 3-NP or mHTT), Rhes decreases mitochondrial membrane potential ($\Delta\Psi_m$), acting as a ligand in association with the NIX receptor. This ligand–receptor association between Rhes and NIX then triggers mitophagy via the recruitment of autophagosomes, by either NIX or Rhes or both. In our model, Nix alone or Rhes alone cannot induce mitophagy. Accordingly, we found that in presence of 3-NP, the interaction between Rhes and NIX increases robustly [73]. However, several questions remain: (a) How does the Rhes protein remove mitochondria that is damaged by 3-NP, but not, for example, by rotenone, a complex-I inhibitor? (b) How does the Rhes protein decrease mitochondrial membrane potential ($\Delta\Psi$m)? and (c) Where does the Rhes protein act on

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https://doi.org/10.1042/BST20191283

mitochondria? Does it interact with dysfunctional SDH and then recruit NIX receptors and lysosomes to initiate mitophagy? Whether there is any cross-talk between Rhes-Beclin1 autophagy and Rhes-NIX mitophagy? Finally, how do the Rhes protein's unique properties, which initiate tunneling-like membranous protrusions and intercellularly transport cargoes [85], regulate mitophagy?

Conclusion

Our study identifies the cause of 3-NP-induced lesions in the striatum: the Rhes protein tries to remove damaged mitochondria in a process that becomes uncontrollably excessive. This is not an active pathway meant for inducing cell death; it is rather a 'side effect' of protecting cells from the damaged mitochondria. This notion raises a philosophical question: Does selective neuronal vulnerability exist because the vulnerable tissue induces the up-regulation of a protective pathway, such as mitophagy, which in turn becomes suicidal for the cells? As with the Rhes protein in 3-NP-induced striatal lesions, it remains unclear whether there are tissue-specific molecules that promote the degeneration of the entorhinal cortex/hippocampal neurons in AD, the loss of substantia nigral neurons in PD, and the loss of motor neurons in ALS. Thus, identifying putative tissue-specific modulators and mechanisms will undoubtably enhance strategies for neurodegenerative disease therapies and drug development.

Perspectives

- Importance: The mechanisms that promote selective neuronal lesions in the brain remain unclear. The idea that 'excessive' mitophagy as a potential cause of selective neurodegeneration is a relatively novel concept. Although it is known that the mitochondrial toxin 3-NP promotes striatal lesions, the molecular causes have remained obscure. We have demonstrated that Rhes, a striatal-enriched protein, associates selectively with 3-NP-induced damaged mitochondria and continues to eliminate them via mitophagy. Accordingly, Rhes-KO mice are protected from 3-NP-induced striatal lesions and mitophagy. Thus, Rhes-mediated excessive mitophagy in the presence of 3-NP regulates striatal lesions in the brain. Such mechanisms may participate in striatal damage in genetic disorders such as HD.

- Summary of current thinking: Lack of clearance of mitochondria via mitophagy has been implicated as a potential cause of neuronal lesions, for example, in Parkin-linked PD models. Yet, Parkin is a ubiquitous protein. How it affects substantia nigra in the brain remains unclear [1]. The neuronal machinery that regulates mitophagy is not fully understood. Within the neurons, the localized elimination of mitochondria may be a faster way to induce tissue-specific neuronal lesions.

- Comment on future directions: The up-regulation of autophagy is known to be generally protective, but it can also be toxic to cells. This principle can be extended to mitophagy. For example, during aging due to excessive ROS production, mitochondria may be eliminated via mitophagy, leading to energy deficit and neuronal dysfunction. New models are required to further address the role of excessive mitophagy as a cause of selective vulnerability in the brain.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

Funding

This research that lead to this review article was supported by grant awards from NIH/NINDS R01-NS087019-01A1, NIH/NINDS R01-NS094577-01A1, and partly from the Cure for Huntington Disease Research Foundation (CHDI).

Abbreviations

3-NP, 3-nitropropionic acid; AD, Alzheimer's disease; ALI, acute lung injury; ALS, amyotrophic lateral sclerosis; CCH, chronic cerebral hypoperfusion; CS, citrate synthase; GTP, guanosine triphosphate; HD, Huntington's disease; LPS, Lipopolysaccharide; PD, Parkinson's disease; Rhes, Ras homolog enriched in striatum; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SUMO, small-ubiquitin-like modifier; VCP, valosin-containing protein.

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