Neurobiology of Disease 74 (2015) 180–193

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Glutamate excitotoxicity in neurons triggers mitochondrial and endoplasmic reticulum accumulation of Parkin, and, in the presence of N-acetyl cysteine, mitophagy

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ARTICLE INFO

Article history:
Received 9 September 2014
Revised 14 November 2014
Accepted 18 November 2014
Available online 3 December 2014

Keywords:
Parkinson's disease
Parkin
Mitochondria
Mitophagy
Endoplasmic reticulum
Glutamate
NMDA receptor
Excitotoxicity
N-Acetyl cysteine
Antioxidant

ABSTRACT

Disruption of the dynamic properties of mitochondria (fission, fusion, transport, degradation, and biogenesis) has been implicated in the pathogenesis of neurodegenerative disorders, including Parkinson's disease (PD). Parkin, the product of gene *PARK2* whose mutation causes familial PD, has been linked to mitochondrial quality control via its role in regulating mitochondrial dynamics, including mitochondrial degradation via mitophagy. Models using mitochondrial stressors in numerous cell types have elucidated a PINK1-dependent pathway whereby Parkin accumulates on damaged mitochondria and targets them for mitophagy. However, the role Parkin plays in regulating mitochondrial homeostasis specifically in neurons has been less clear. We examined whether a stressor linked to neurodegeneration, glutamate excitotoxicity, elicits Parkin–mitochondrial translocation and mitophagy in neurons. We found that brief, acute exposure to glutamate causes Parkin translocation to mitochondria in neurons, in a calcium- and N-methyl-D-aspartate (NMDA) receptor-dependent manner. In addition, we found that Parkin accumulates on endoplasmic reticulum (ER) and mitochondrial/ER junctions following excitotoxicity, supporting a role for Parkin in mitochondrial–ER crosstalk in mitochondrial homeostasis. Despite significant Parkin–mitochondria translocation, however, we did not observe mitophagy under these conditions. To further investigate, we examined the role of glutamate-induced oxidative stress in Parkin–mitochondria accumulation. Unexpectedly, we found that glutamate-induced accumulation of Parkin on mitochondria was promoted by the antioxidant N-acetyl cysteine (NAC), and that co-treatment with NAC facilitated Parkin-associated mitophagy. These results suggest the possibility that mitochondrial depolarization and oxidative damage may have distinct pathways associated with Parkin function in neurons, which may be critical in understanding the role of Parkin in neurodegeneration.

Introduction

Dysregulation of the homeostatic mechanisms of mitochondrial maintenance (mitochondrial fission, fusion, transport, biogenesis and degradation — collectively termed 'mitochondrial dynamics'), has been increasingly linked to neurodegeneration and Parkinson's disease (PD) neuropathology (Chen and Chan, 2009; Subramaniam and Chesselet, 2013; Van Laar and Berman, 2009). The E3 ubiquitin ligase Parkin, mutations of which cause familial autosomal–recessive juvenile-onset PD, is associated with maintenance of mitochondrial dynamics (de Vries and Przedborski, 2013; Deng et al., 2008; Liu et al., 2012; Narendra et al., 2008; Park et al., 2008; Poole et al., 2008; Scarffe et al., 2014; Wang et al., 2011; Yu et al., 2011). Parkin has been

Abbreviations: APV, (2R)-amino-5-phosphonovaleric acid; C7, 7-chlorokynurenic acid; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DCF, 2′,7′-dichlorofluorescien; DIV, day in vitro; ER, endoplasmic reticulum; GFP-Sec61β, green fluorescent protein-tagged Sec61beta protein; GFP-LC3, green fluorescent protein-tagged LC3 protein; H2-DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; hu-Parkin, full-length human Parkin; mtDsRed2, mitochondrially-targeted DsRed2 protein; MAM, mitochondrial-associated membrane; MDVs, mitochondria-derived vesicles; NAC, N-acetyl cysteine; NMDA, N-methyl-D-aspartate; PA-mtGFP, mitochondrially-targeted photoactivatable green fluorescent protein; PINK1, PTEN induced putative kinase 1; PD, Parkinson's disease; SHM, sucrose homogenization medium; TMRM, tetramethyl rhodamine methyl ester

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2014.11.015
0969-9961/

specifically linked to mitochondrial quality control, whereby damaged, depolarized mitochondria are targeted for autophagic degradation (mitophagy) (Narendra et al., 2008). In this pathway, Parkin translocates to depolarized mitochondria via a PINK1-dependent mechanism, identifying them for mitophagic degradation (Narendra et al., 2008, 2010; Vives-Bauza et al., 2010). This pathway is proposed to be important in PD as regulation of mitochondrial homeostasis is crucial to neuronal survival, and a build-up of damaged mitochondria could be detrimental (de Vries and Przedborski, 2013; Scarffe et al., 2014). However, it is not clear why loss of Parkin function would lead to a selective loss of PD-affected neurons.

Whereas the Parkin-mediated mitophagy pathway has been well described in cancer cell lines and other proliferating cells, its regulation in neurons remains less well defined (Grenier et al., 2013; Van Laar and Berman, 2013). Previously, we showed that the unique bioenergetics of post-mitotic neurons, which are dependent on mitochondrial respiration, appear to downregulate Parkin translocation following global mitochondrial depolarization by the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Van Laar et al., 2011). Under conditions that preserved ATP (by altering neuronal bioenergetics), we did observe CCCP-triggered Parkin–mitochondrial translocation occurring in neurons. Even with these conditions, however, increased mitophagy was not observed (Van Laar et al., 2011). Other studies have since confirmed that Parkin less readily undergoes mitochondrial translocation in neurons, observing increased Parkin–mitochondria association occurring under specific conditions such as long exposures to protonophores/ionophores (Cai et al., 2012; McCoy et al., 2014; Rakovic et al., 2013; Seibler et al., 2011), exposure to selective complex inhibitors (Wang et al., 2011), or exposure to stressors after replacing culture media with media lacking supplements and antioxidants (Joselin et al., 2012). Likewise, after Parkin translocation to mitochondria in neurons, subsequent mitophagic degradation in neurons has more rarely been clearly demonstrated. It has been expressly observed following prolonged CCCP exposure in the presence of anti-apoptotic agents (Cai et al., 2012) and, most recently, following localized mitochondrial damage in distal axons (Ashrafi et al., 2014). It was also suggested to occur after overexpression of an Alzheimer's-linked NH_2-tau fragment (Amadoro et al., 2014). On the other hand, mitophagy did not measurably occur despite induction of Parkin translocation to mitochondria following valinomycin exposure in iPS-derived neurons (Rakovic et al., 2013). Further, the relationship of some previously utilized conditions to in vivo settings is not clear.

We sought to determine whether neurochemically- and PD-relevant triggers of mitochondrial depolarization could affect Parkin translocation to mitochondria and subsequent mitophagy in neurons. One such trigger, glutamate excitotoxicity, has been proposed to play a role in neurodegenerative disease, including PD neuropathology (Blandini, 2010; Mehta et al., 2013). Glutamate, through activation of N-methyl-D-aspartate (NMDA) receptor calcium ion channels, causes an influx of calcium, which is taken up by mitochondria and subsequently causes mitochondrial depolarization (Schinder et al., 1996; Stout et al., 1998; White and Reynolds, 1996). This may be especially important in PD neuropathology, as calcium dysregulation has been linked to the selective vulnerability of neurons in PD (Chan et al., 2007, 2010; Hurley et al., 2013; Mosharov et al., 2009). Related to this, glutamate excitotoxicity has been proposed to play a role in the neurodegeneration observed in some PD models (Kress and Reynolds, 2005; Loschmann et al., 1994; Meredith et al., 2009; Plowey et al., 2014; Wu and Johnson, 2007). In addition, the PINK1/Parkin pathway has been reported to influence cellular response to excitotoxicity in neurons (Yu et al., 2011).

We therefore evaluated the effect of glutamate exposure on Parkin localization as well as mitophagy. To mimic acute excitotoxicity, we used a previously described model of short exposure to glutamate in primary rat neuron cultures (Reynolds and Hastings, 1995; Stout et al., 1998). We report that, in contrast to depolarizing protonophores, glutamate exposure induced Parkin accumulation at mitochondria. Accumulation occurred in a calcium- and NMDA receptor-dependent manner. In addition, glutamate-exposed neurons exhibited non-mitochondrial Parkin accumulations on endoplasmic reticulum (ER), and accumulations between mitochondria and ER. These findings have implications for the shared role of calcium handling between mitochondria and ER. We also investigated the role of reactive oxygen species (ROS) in Parkin-associated mitophagy in neurons. Whereas glutamate alone induced accumulation of Parkin on mitochondria in neurons, it did not induce mitophagy. However, unexpectedly, co-treatment with the antioxidant N-acetyl cysteine (NAC) promoted both glutamate-induced Parkin translocation to mitochondria and Parkin-associated mitophagy. Our results suggest that following physiologically relevant mitochondrial depolarization, both bioenergetic and oxidative stress pathways may regulate Parkin translocation and mitophagy. Elucidating the distinct roles these pathways play may be critical in understanding the role of Parkin in PD-related neurodegeneration.

Materials and methods

Cortical neuron culture, transfection, and treatment

Primary cortical neuron cultures were prepared from E17–18 Sprague–Dawley rats as previously described (Arnold et al., 2011; Van Laar et al., 2011; modified from Ghosh and Greenberg, 1995) and plated onto glass coverslips, glass-bottom MatTek dishes (MatTek Corp.), or plastic culture dishes coated with poly-D-lysine and mouse laminin. Cultures were maintained by feeding with 1/2 media changes every 3 days. Cells were transfected at day in vitro (DIV) 6, utilizing Lipofectamine 2000 by previously described methods (Arnold et al., 2011; Van Laar et al., 2011). Neurons were transfected where noted with plasmids expressing mitochondrially-targeted DsRed2 (mtDsRed2; Clontech), mitochondrially-targeted photo-activatable GFP (PA-mtGFP; (Karbowski et al., 2004)), full-length human Parkin (hu-Parkin; (Petrucelli et al., 2002)), GFP-tagged LC3 (Addgene plasmid 21073; deposited by T. Yoshimori (Kabeya et al., 2000)), and/or GFP-tagged Sec61β (Addgene plasmid 15108; deposited by T. Rapoport (Voeltz et al., 2006)). Of note, we routinely obtain 100% co-transfection of the multiple plasmids (Arnold et al., 2011).

Treatments were performed at DIV15 for all experiments. 1 M stocks of glutamate and glycine (Sigma) were prepared in sterile 1× PBS, aliquoted, and stored at −20 °C until day of use. For the glutamate treatments, media were collected from the cells and saved. Cells were rinsed with HBSS media (1× Hanks Balanced Salt Solution (Sigma, cat# H1641) supplemented with $NaHCO_3$ (4.2 mM), HEPES (10 mM), and D-glucose (35 mM)). Cells were then exposed to HBSS media alone (control) or to HBSS media with glutamate (as indicated) plus glycine (1 μM) for 10 min in a 37 °C, 5% CO_2 tissue culture incubator. All glutamate exposures described included the co-agonist glycine. Calcium-free experiments substituted a Hank's Solution lacking calcium (Sigma, cat# H4641) in the HBSS media. Immediately following treatment, cells were again rinsed with HBSS media. The saved culture media were replaced, and cells returned to the incubator for the indicated times.

For experiments using pretreatments, where indicated, E-64d (Calbiochem; 15 mM stock in DMSO), Pepstatin A (Fisher; 15 mM stock in DMSO), MG132 (Sigma; 10 mM stock in DMSO) and/or NAC (Sigma; 0.5 M stock, prepared fresh on day of use in sterile culture-grade H_2O) were added directly to the culture media, to the desired final concentration, 1 h before treatment. Following glutamate treatment, the saved media containing pretreament compounds were placed back on the respective cells. Thus, these compounds were also present during the post-exposure stage. For NAC conditions, the respective concentration of NAC (500 or 1000 μM) was also present during the 10 min glutamate exposure.