Neurobiology of Disease 111 (2018) 26–35

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Familial knockin mutation of LRRK2 causes lysosomal dysfunction and accumulation of endogenous insoluble α-synuclein in neurons

Jason Schapansky[a], Saurabh Khasnavis[a], Mark P. DeAndrade[a], Jonathan D. Nardozzi[a], Samuel R. Falkson[a], Justin D. Boyd[a], John B. Sanderson[a], Tim Bartels[a], Heather L. Melrose[b], Matthew J. LaVoie[a,*]

[a] Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, United States
[b] Department of Neuroscience, Mayo Clinic Jacksonville, Jacksonville, FL 32224, United States

ARTICLE INFO

Keywords:
LRRK2
α-Synuclein
Tau
Tau phosphorylation
Lysosome
Parkinson's disease

ABSTRACT

Missense mutations in the multi-domain kinase LRRK2 cause late onset familial Parkinson's disease. They most commonly with classic proteinopathy in the form of Lewy bodies and Lewy neurites comprised of insoluble α-synuclein, but in rare cases can also manifest tauopathy. The normal function of LRRK2 has remained elusive, as have the cellular consequences of its mutation. Data from LRRK2 null model organisms and LRRK2-inhibitor treated animals support a physiological role for LRRK2 in regulating lysosome function. Since idiopathic and LRRK2-linked PD are associated with the intraneuronal accumulation of protein aggregates, a series of critical questions emerge. First, how do pathogenic mutations that increase LRRK2 kinase activity affect lysosome biology in neurons? Second, are mutation-induced changes in lysosome function sufficient to alter the metabolism of α-synuclein? Lastly, are changes caused by pathogenic mutation sensitive to reversal with LRRK2 kinase inhibitors? Here, we report that mutation of LRRK2 induces modest but significant changes in lysosomal morphology and acidification, and decreased basal autophagic flux when compared to WT neurons. These changes were associated with an accumulation of detergent-insoluble α-synuclein and increased neuronal release of α-synuclein and were reversed by pharmacologic inhibition of LRRK2 kinase activity. These data demonstrate a critical and disease-relevant influence of native neuronal LRRK2 kinase activity on lysosome function and α-synuclein homeostasis. Furthermore, they also suggest that lysosome dysfunction, altered neuronal α-synuclein metabolism, and the insidious accumulation of aggregated protein over decades may contribute to pathogenesis in this late-onset form of familial PD.

1. Introduction

Parkinson's disease (PD) is an aging-associated neurodegenerative disorder characterized by insoluble proteinaceous Lewy body and Lewy neurite inclusions that accumulate within multiple affected neuronal populations. The primary component of these intracellular inclusions is α-synuclein (α-syn), a small aggregate-prone protein that plays a central role in both idiopathic and familial forms of PD. The broad role of this small protein on the majority of PD pathogenesis makes it critical that we understand how other PD-associated genetic insults influence α-syn homeostasis.

Mutations in leucine rich-repeat kinase 2 (LRRK2) are perhaps the most common genetic cause of PD, with the G2019S mutation being the most prevalent (Healy et al., 2008). Pleomorphic pathology including tauopathy or pure nigral degeneration have been reported in rare cases (Bonifati, 2006; Zimprich et al., 2004). However, these particular monogenic forms of disease most frequently match the pathology observed in sporadic PD with both late onset and neuronal Lewy body formation. LRRK2 is a rather unique protein in that it possesses two functional domains contributing kinase and GTPase activities, as well as numerous protein-binding motifs. The current consensus in the field is that the pathogenic mutations most likely share increased kinase activity as a common gain-of-function.

LRRK2 is implicated in the regulation of Wnt signaling (Berwick and Harvey, 2012; Sancho et al., 2009), synaptic transmission (Arranz et al., 2015; Matta et al., 2012), mitochondrial function (Wang et al., 2012), and cytoskeletal polymerization (Caesar et al., 2015; Caesar et al., 2013). How these functions may contribute to the pathologic accumulation of PD-relevant proteins within neurons is unclear. Some of the earliest insights into LRRK2 neurobiology were gained from the

* Corresponding author at: Building For Transformative Medicine Rm 10016M, 60 Fenwood Road, Boston, MA 02115, United States.
E-mail address: mlavoie@rics.bwh.harvard.edu (M.J. LaVoie).

https://doi.org/10.1016/j.nbd.2017.12.005
Received 10 August 2017; Received in revised form 5 December 2017; Accepted 11 December 2017
Available online 12 December 2017
0969-9961/

overexpression of a PD-linked mutation of LRRK2 in neurons. Ectopic expression of G2019S LRRK2 was shown to increase tau phosphorylation, induce neurite retraction, and lysosome dysfunction and cell death (MacLeod et al., 2006). Further studies then confirmed the neurotoxic consequences of G2019S LRRK2 overexpression, with inhibition of LRRK2 kinase activity rescuing many of these toxic effects (Lee et al., 2010).

However, little data are currently available to explain how endogenous expression of LRRK2 containing disease-linked mutations adversely affect neuron function and whether subtle changes that long precede cell death might relate to the emergence of these more aggressive phenotypes. These questions have grown particularly more complex over the last few years, as the field has come to appreciate two observations: one, LRRK2 is only modestly expressed in neurons but highly expressed in glial and immune cell populations; and two, that dysfunction within these non-neuronal cells is likely to contribute to the pathogenesis of both LRRK2-dependent and idiopathic PD. Therefore, it is particularly important to determine whether neurons expressing endogenous LRRK2 mutation manifest phenotypes associated with known pathologic pathways involved in PD. In the present study, primary cortical cultures from recently characterized G2019S knock-in mice were analyzed. Prior work established an increase in tau phosphorylation in G2019S KI brain tissue (Yue et al., 2015), which was confirmed here and also observed in neurons cultured from these mice. Our subsequent efforts focused on lysosome biology and metabolism of α-syn in G2019S neurons.

The α-syn protein can be degraded by both the proteasome and the lysosome (Klucken et al., 2012; Rideout et al., 2004; Vogiatzi et al., 2008; Webb et al., 2003), suggesting that either pathway could be affected in synucleinopathies, such as PD. Our previous data demonstrated that LRRK2 knockdown results in lysosome dysfunction in myeloid cells (Schapansky et al., 2014). As neuronal α-syn is a key component of disease pathology, it is crucial to determine whether expression of LRRK2 mutations at endogenous levels affect *neuronal* lysosome biology in a meaningful way to sufficiently alter α-syn metabolism. Our data demonstrate that primary cortical neurons from homozygous G2019S KI mice had decreased lysosomal protein expression and altered morphology compared to lysosomes from WT mouse neurons. In addition, G2019S neurons had alkalinized lysosomes and decreased autophagic flux. We also examined for changes in α-syn degradation and neuronal secretion, which we collectively refer to as α-syn metabolism. Importantly, we observed that these lysosomal changes were associated with the accumulation of endogenous, detergent-insoluble α-syn and increased neuronal α-syn release. Finally, pharmacological inhibition of LRRK2 kinase activity decreased the adverse effects on α-syn and rescued the lysosomal deficits exhibited by G2019S cultures. These data suggest a causal link between LRRK2 and α-syn metabolism, whereby subtle lysosomal deficits induced by LRRK2 mutation may compound over decades to contribute to PD pathogenesis and Lewy body and Lewy neurite formation.

2. Methods and methods

2.1. Antibodies/reagents

The 2F12 monoclonal mouse antibody (mAb) against α-syn was generated by immunizing α-syn −/− (KO) mice with α-syn purified from human erythrocytes as described previously (Bartels et al., 2011). Hybridoma cell lines were generated by fusion of mouse splenocytic B lymphocytes with X63-Ag8.653 myeloma cells. Antibody was generated and purified from hybridoma supernatant by Cell Essentials (Boston, MA). Syn-1 is a α-syn mAb purchased from BD Biosciences (Franklin Lakes, NJ, catalog no. 610787), and used for ELISA analysis. Antibodies were purchased from as follows: LRRK2 N241A/34, Antibodies Inc./UC Davis NeuroMab; Rab5, Cell Signaling (2143S); TfR, Invitrogen (136800); p62, Abcam (56416); Actin, Sigma (A5441); LC3, MBL (PM036); VDAC, Abcam (ab34736); LAMP1, Abcam (ab24170); LAMP2, Abcam (ab13524); Tau K9JA, DAKO, AT8 (phospho-specific tau antibody at positions S202, T205), Thermo Fisher (MN1020).

The LRRK2 inhibitor GSK2578215A (GSK) was purchased from Tocris. Cell culture media and lysosomal dyes (LysoTracker, LysoSensor) were obtained from Invitrogen. LRRK2 inhibitor CZC-25146 (CZC), chloroquine, pepstatin A, leupeptin, and DAPI were purchased from Sigma. Both LRRK2 inhibitors were used at 1 μM, with LRRK2 kinase inhibition confirmed via blotting for phospho-S935 (UDD2, Abcam) levels in HEK293 cells transiently transfected to express LRRK2 (Fig. S1).

2.2. Neuronal cell culture

Heterozygous breeding pairs of mice were mated to yield WT and homozygous G2019S progeny that were then bred to obtain homozygous embryonic WT or KI pups. At day E18, cortices were dissected from male and female embryos and cells were plated in DMEM media with 10% FBS. Media was changed to Neurobasal media supplemented with B27 after 3–4 h, and mitotic inhibitors were added with a half-media change at 3 DIV to restrict glial contamination. Half-media changes were performed every three days thereafter until cells were harvested at 10 days in vitro (DIV).

2.3. High content analysis of lysosomal morphology

Neurons were plated 50,000 per well on 96-well imaging plates (Greiner) coated with poly-L-lysine (Sigma). At 10 DIV, neurons were labeled with LysoTracker Red (Invitrogen) according to the manufacturer's specifications, and 20 ng/ml of DAPI. Labeled live cells were imaged at 40 × magnification using the IN Cell Analyzer 2000 (GE Healthcare). Cell count, lysosome count, mean lysosome area and total lysosome area were calculated. Four fields per well were acquired using the DAPI and Cy3 channels for visualizing the DAPI and LysoTracker Red, respectively. Images were analyzed with the IN Cell Workstation software (GE Healthcare) multi target analysis protocol. Briefly, nuclei were segmented by applying a Top Hat algorithm with a minimum area of 50 square μm and a sensitivity level of 50 to the DAPI channel. An 8 μm collar around the nuclei was used to distinguish the perinuclear region and the distal region of the cells (Fig. 3E, F). Lysosomes were defined as objects with a 1 to 3 μm diameter segmented by 2 scales with a sensitivity level of 20 in the corresponding channel. Lysosomes were categorized as perinuclear or distal according to the region defined by the collar. Independent experiments were performed on WT and G2019S KI neurons plated as described above. Cells were fixed and subject to Lamp-2 immunocytochemical staining and high content image analysis.

2.4. LysoSensor analysis

For lysosomal pH analysis, the ratiometric dye LysoSensor Yellow/Blue (Invitrogen) was used. Cells were incubated with dye (1 μM) for 10 min prior to rinsing 2 × with HBSS buffer. Cells were imaged using a Synergy H1 hybrid reader (Biotek; reading at excitation 329/384, emission 440/540). Calibration experiments were conducted in WT and G2019S neurons loaded with 1 μM LysoSensor for 1 h at 37 °C, washed, and then measured for a baseline LysoSensor signal. Then, cells were incubated for 5 min at 37 °C with pH calibration standards (pH of 3.96, 4.46, 4.96, 5.47, and 5.97) prepared in 20 mM 2-(N-morpholino)ethanesulfonic acid, 110 mM KCl, and 20 mM NaCl freshly supplemented with 30 μM nigericin and 15 μM monensin. A pH standard curve was determined for each genotype using GraphPad Prism 7 and individual baseline pH values were interpolated from these standard curves.