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# The critical role of Nramp1 in degrading α-synuclein oligomers in microglia under iron overload condition

Kuo-Chen Wu [a], Horng-Huei Liou [b], Yu-Han Kao [a], Chih-Yu Lee [a], Chun-Jung Lin [a,*]

[a] School of Pharmacy, National Taiwan University, Taipei, Taiwan
[b] Department of Neurology, National Taiwan University Hospital, Taipei, Taiwan

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ABSTRACT

Oligomeric α-synuclein is a key mediator in the pathogenesis of Parkinson's disease (PD) and is mainly cleared by autophagy-lysosomal pathway, whose dysfunction results in the accumulation and cell-to-cell transmission of α-synuclein. In this study, concomitant with the accumulation of iron and oligomeric α-synuclein, higher expression of a lysosomal iron transporter, natural resistance-associated macrophage protein-1 (Nramp1), was observed in microglia in post-mortem striatum of sporadic PD patients. Using Nramp1-deficient macrophage (RAW264.7) and microglial (BV-2) cells as *in-vitro* models, iron exposure significantly reduced the degradation rate of the administered human α-synuclein oligomers, which can be restored by the expression of the wild-type, but not mutant (D543N), Nramp1. Likewise, under iron overload condition, mice with functional Nramp1 (DBA/2 and C57BL/6 congenic mice carrying functional Nramp1) had a better ability to degrade infused human α-synuclein oligomers than mice with nonfunctional Nramp1 (C57BL/6) in the brain and microglia. The interplay between iron and Nramp1 exhibited parallel effects on the clearance of α-synuclein and the activity of lysosomal cathepsin D *in vitro* and *in vivo*. Collectively, these findings suggest that the function of Nramp1 contributes to microglial degradation of oligomeric α-synuclein under iron overload condition and may be implicated in the pathogenesis of PD.



## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by a chronic degeneration of dopaminergic neurons in the substantia nigra and axonal degeneration in the striatum. The presence of intracellular inclusions, namely Lewy bodies and Lewy neurites, composed of aggregated α-synuclein is a pathological hallmark of PD. Several post-translational modifications of α-synuclein have been observed in the inclusion bodies (reviewed by Oueslati et al., 2010). Among these, phosphorylation of α-synuclein at serine 129 (ser-129 phosphorylated α-synuclein) is noteworthy for its connection to oligomerization (Chen et al., 2009a) and neurotoxicity (Chen and Feany, 2005). Cell-to-cell transmission of soluble oligomeric α-synuclein rather than insoluble aggregates plays a more important role in disease pathogenesis (Paleologou et al., 2009) and progression (Kalia et al., 2013), and the transmission process can be increased by lysosomal dysfunction (Alvarez-Erviti et al., 2011).

While both autophagy-lysosome and ubiquitin-proteasome systems are responsible for the degradation of α-synuclein, autophagy-lysosomal pathway is the preferential mechanism to clear oligomeric α-synuclein (Lee et al., 2004). Dysregulation of autophagy-lysosome system has been implicated in impaired α-synuclein clearance and the occurrence of PD (Usenovic et al., 2012; Rothaug et al., 2014). Cathepsins are the main lysosomal proteases involved in the degradation of α-synuclein (Sevlever et al., 2008; McGlinchey and Lee, 2015). The activities of lysosomal cathepsins have been demonstrated to be inhibited by iron in rat liver (Misaka and Tappel, 1971) and in human retinal pigment epithelial cells (Chen et al., 2009b). While abnormal high levels of iron are found in certain brain regions in PD, being associated with protein aggregation and neurotoxicity (Berg et al., 2001), the interaction between iron and lysosomal cathepsins has never been demonstrated in the brain.

Intracellular iron levels are maintained by a number of proteins, including iron transporters that belong to SLC11 family, Nramp1 (natural resistance-associated macrophage protein-1; SLC11A1) and DMT1 (divalent metal transporter 1; SLC11A2; Nramp2), that deliver iron across the membrane (Montalbetti et al., 2013). The cellular localization of DMT1 is in the plasma membrane and endosomal membrane,

*Abbreviations:* Cg, congenic; DMT1, divalent metal transporter 1; GFAP, glial fibrillary acidic protein; iba-1, ionized calcium-binding adapter molecule 1; LAMP1, lysosomal-associated membrane protein-1; Nramp1, natural resistance-associated macrophage protein-1; PD, Parkinson's disease; P-Ser129 α-synuclein, serine-129 phosphorylated α-synuclein; WT, wild-type.

* Corresponding author at: School of Pharmacy, National Taiwan University, 33 Linsen south Road, Taipei 100, Taiwan.
*E-mail address:* clementumich@ntu.edu.tw (C.-J. Lin).

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responsible for cellular uptake of iron and the efflux of iron out of the endosomal compartment, respectively (Tabuchi et al., 2000; Mackenzie and Hediger, 2004). The expression of DMT1 was found to be higher in the substantia nigra of PD patients and DMT1-mediated iron transport is involved in neuronal degeneration in PD (Salazar et al., 2008). Different from DMT1, Nramp1 is expressed in the lysosomal compartment (Gruenheid et al., 1997), whereas its role in the occurrence of neurodegenerative disease is unclear. In the brain, Nramp1 has been identified in the neuron and microglia of mice (Evans et al., 2001), whereas its expression in human brain has never been reported.

Compared to neurons and astrocytes, microglia seem to be the most efficient scavenger to clear extracellular α-synuclein in brain parenchyma (Lee et al., 2008a). Given that lysosomes play an important role in degrading α-synuclein, whose function can be affected by iron, we hypothesized that Nramp1-mediated iron homeostasis is crucial for the lysosomal degradation of α-synuclein, particularly in the microglia. In the present study, the proteins of Nramp1 and oligomeric α-synuclein were examined in the microglia of human brain. The interplay between iron and Nramp1 on the degradation of α-synuclein oligomers was examined *in vitro* and *in vivo*. Our results showed that the function of Nramp1 is important for the degradation of oligomeric α-synuclein in microglia, especially under iron overload condition.

## 2. Material and methods

### 2.1. Human brain tissues

Post-mortem brain tissues of PD patients and age-matched controls were obtained from the National Institute of Health NeuroBioBank. The demographic data of these subjects were summarized in Table 1. The use of human brain tissues was approved by the Research Ethics Committee Office at National Taiwan University.

### 2.2. Animals

Male C57BL/6 mice that carry a naturally occurring mutant allele (G169D) of Nramp1 (*Nramp1*$^{s}$) and DBA/2 mice that carry a wild-type allele of Nramp1 (*Nramp1*$^{r}$) (both 6–8 weeks old) were purchased from National Laboratory Animal Center of Taiwan and BioLasco Taiwan corporation (Taipei, Taiwan), respectively. Male C57BL/6 congenic mice expressing functional Nramp1 (B6J.129S1-*Slc11a1*$^{r}$/GbrtJ; *Nramp1*$^{r}$) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). The protocol of animal use was approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine and College of Public Health.

### 2.3. Stereotaxic injections

Mice were anesthetized (80 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal administration) and received intrastriatal infusion of ferric ammonium sulfate (5 μg per brain in saline) or saline (vehicle control) at the following coordinates: A-P +0.2 mm from the bregma, M-L −2 mm from the midline and D-V −3 mm below the dura according to the stereotaxic atlas of Paxinos and Franklin (2004) *via* a Hamilton syringe at a flow rate of 0.5 μL per minute. Recombinant human α-synuclein (200 μM in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.7 mM $KH_2PO_4$, 0.01 mM $Na_2HPO_4$, pH 7.4); Millipore, Temecula, CA, USA) was aged at 37 °C for 7 days to generate α-synuclein oligomers (Zhang et al., 2005) and was verified by the method described previously (Brännström et al., 2014). At 24 h after intrastriatal infusion of iron or vehicle, the assembled human oligomeric α-synuclein (5 μg per brain) was infused to the same position and the amount of oligomeric α-synuclein in the striatum was analyzed by Western blotting and immunohistochemistry. For Western blotting, striatal tissues were obtained right after α-synuclein infusion and at 0.5, 1, 2, 4, and 6 h post-infusion. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C until protein extraction; for immunohistochemistry, mice were transcardially perfused with saline and 4% paraformaldehyde in 0.1 M phosphate buffer at 0.5 and 2 h after the infusion of α-synuclein.

### 2.4. Protein extraction and Western blotting

Samples were lysed in RIPA buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein concentrations were measured by the Bio-Rad DC™ protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as the standard. The immunoblotting was performed according to the procedure described previously (Wu et al., 2015a), using mouse antibody specific for human α-synuclein (LB509; 1:200; Santa Cruz Biotechnology; Supplementary Table 1) with rabbit antibody against β-actin (1:500; Abcam, Cambridge, MA, USA) as the loading control.

### 2.5. Immunohistochemistry and immunofluorescence of human and mouse tissues

For the preparation of human brain tissues, 5 μm-thick paraffin embedded striatal and substantia nigra sections were dewaxed and incubated with target retrieval solution (Dako, Tokyo, Japan) for 30 min at 98 °C. To identify insoluble α-synuclein aggregates, after antigen retrieval, sections were treated with 50 μg/mL proteinase K (Thermo Fisher Scientific, Waltham, MA, USA) in a buffer containing 10 mM Tris HCl, 100 mM NaCl and 0.1% NP-40 for 2 min at 37 °C as described previously (Roberts et al., 2015). Frozen mouse brain sections were prepared according to the procedures described previously (Salazar et al., 2008).

For immunohistochemistry, brain sections were incubated with PBS containing 0.3% hydrogen peroxide for 30 min at room temperature (RT) and then with 4% normal goat serum or 2.5% skim milk in PBS

**Table 1**
Demographic data of post-mortem human subjects.

| Subject | Race (gender) | Brain area | Age (yr) | Cause of death | Other pathology | PMI (hr) |
|---|---|---|---|---|---|---|
| Non-PD-1 | Caucasian (M) | Striatum | 71 | Cardiac arrest | n.a. | 16 |
| Non-PD-2 | Caucasian (F) | Striatum | 76 | Complications of cancer | n.a. | 3 |
| Non-PD-3 | Caucasian (M) | Striatum | 79 | COPD/PVD | n.a. | 5 |
| Non-PD-4 | Caucasian (M) | Striatum; substantia nigra | 71 | ASCVD | n.a. | 18 |
| Non-PD-5 | Caucasian (M) | Substantia nigra | 71 | CAD | n.a. | 23 |
| Non-PD-6 | Caucasian (M) | Substantia nigra | 70 | ASCVD | n.a. | 12 |
| PD-1 | Caucasian (M) | Striatum | 70 | PD | n.a. | 17 |
| PD-2 | Caucasian (F) | Striatum; substantia nigra | 84 | ASCVD | Hypothyroidism and insomnia | 11 |
| PD-3 | Caucasian (M) | Striatum | 74 | Complications of disorder | n.a. | 3 |
| PD-4 | Caucasian (M) | Striatum; substantia nigra | 79 | Complications of disorder | Mild paranoia and dementia | 17 |
| PD-5 | Caucasian (M) | Substantia nigra | 79 | n.a. | Anxiety and dementia | 11 |

F, female; M, male; n.a., not applicable; PMI, post-mortem interval; PD, Parkinson's disease; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; PVD, peripheral vascular disease; ASCVD, arteriosclerotic cardiovascular disease.