https://doi.org/10.5607/en.2018.27.5.377
Exp Neurobiol. 2018 Oct;27(5):377-386.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

Experimental Neurobiology

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Ubiquitin C-terminal Hydrolase L1 Regulates Lipid Raft-dependent Endocytosis

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Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that is highly expressed in neurons, and gathering evidence indicates that UCH-L1 may play pathogenic roles in many neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (PD). Additionally, lipid rafts have attracted interest in neurodegeneration as playing a common role in many neurodegenerative diseases. In the present study, we demonstrated that UCH-L1 associates with lipid rafts as with other PD-associated gene products. In addition, UCH-L1 regulates lipid raft-dependent endocytosis and it is not dependent on the expression and degradation of caveolin-1 or flotillin-1. Finally, UCH-L1 regulates cell-to-cell transmission of α-synuclein. This study provides evidence that many PD-associated gene products share common signaling pathways to explain the pathogenesis of PD.

Key words: alpha-synuclein, UCH-L1, Parkinson's disease, prion disease

INTRODUCTION

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that is highly expressed in neurons, comprising 1~2% of total neuronal proteins [1]. Accumulating evidence indicates that UCH-L1 may be involved in the pathogenesis of many neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). UCH-L1 is detected in cortical Lewy bodies and neurofibrillary tangles in patients with diffuse Lewy body disease and AD, respectively [2]. Down-regulation and extensive oxidative modification of UCH-L1 have been observed in the brains of AD and PD patients [3]. A missense mutation, I93M, in UCH-L1 has been identified in patients with autosomal dominant familial PD [4], and an S18Y mutation in UCH-L1 has been reported to exert a neuroprotective effect against PD [5], although these genetic studies are controversial [6, 7].

Recently, lipid rafts have attracted interest in neurodegeneration. Lipid rafts are specialized membrane microdomains that are enriched in cholesterol and glycosphingolipids. They serve as platforms for the assembly of signaling molecules and regulate receptor-mediated signal transduction and endocytosis [8, 9]. Changes in lipid rafts content are present in many neurodegenerative diseases such as AD, PD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and prion disease [10]. Also, significant alteration in numerous proteins in lipid rafts has been reported in AD and ALS mouse models [11, 12]. Aβ, α-synuclein, mutant huntingtin and prion, which are key players of AD, PD, HD and prion disease, respectively, have been found in lipid rafts [13-16] and the main proteins responsible for Aβ generation such as amyloid precursor protein, β-secretase [17, 18], and presenilin-1 [19]

Received May 8, 2018, Revised September 17, 2018,
Accepted September 18, 2018

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are present in lipid rafts. Many PD-associated gene products such as parkin, PINK1, DJ-1, and LRRK2 [20-23] also associates with lipid rafts, implying that lipid rafts may play a common role in many neurodegenerative diseases.

Additionally, recent evidence indicates that regional and intercellular spreading of tau and α-synuclein are significant in the pathogenesis of AD and PD, respectively. It has attracted much attention because intercellular spreading may explain unveiled pathogenesis and help with developing novel therapeutic interventions such as blocking secretion and reuptake [24, 25]. Previously, we demonstrated that α-synuclein is internalized into microglia in a lipid raft-dependent manner [26]. In addition, parkin, which has also been known to associate with lipid rafts, regulates caveolin-1 expression, which alters lipid rafts and the cell-to-cell transmission of α-synuclein [27], implying that lipid raft components may be involved in cell-to-cell transmission of α-synuclein.

Although UCH-L1 is mainly cytosolic protein, 20~50% of UCH-L1 is membrane-associated [28-30]. However, association of UCH-L1 with lipid rafts has not been explored. In the present study, we explored whether UCH-L1 associates with lipid rafts and whether altered UCH-L1 expression is involved in lipid raft function. Additionally, we explored the role of UCH-L1 in cell-to-cell transmission of α-synuclein.

MATERIALS AND METHODS

Reagent and antibodies

Antibodies against flotillin-1 and flotillin-2 were purchased from BD Bioscience (Fraklin Lakes, NJ, USA). Antibodies against GAPDH, CD71 (transferrin receptor) and UCH-L1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Methyl-β-cyclodextrin and LDN-57444 were purchased from Sigma-Aldrich (St Louis, MO, USA). Rhodamine-conjugated transferrin and BOIPY® FL C5-Lactosylceramide were purchased from Molecular Probes (Leiden, the Netherlands).

Cell culture and transfection

SH-SY5Y, Hela, and human alpha-synuclein overexpressing SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Primary cortical neurons were cultured from Sprague-Dawley rat embryos at embryonic day 18 and maintained in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) with L-glutamine and B-27 supplement (Invitrogen). Hela cells were transfected using lipofectamine 2000 (Invitorgen) according to the manufacturer's instruction. After 24 hr of transfection, the cells were used for further experiments. The plasmid for UCH-L1-myc was kindly provided by Prof. K. J. Lee at Ewha Womans University, Korea. UCH-L1 knockdown SH-SY5Y cells were prepared using lentiviral constructs that expressed shRNA for UCH-L1 (Sigma-Aldrich) as described previously [31] and selected using puromycin.

Western blot

Cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and protease inhibitor mixture (GenDEPOT, Barker, TX)) for 20 min on ice after sonication for 3 s. The lysates were cleared by centrifugation at 13,000×g for 30 min at 4°C. The supernatants were collected and mixed with sample buffer, resolved by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies. They were then detected using an enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, USA).

Isolation of lipid rafts

Cell were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold PBS containing 1% Triton X-100 and protease inhibitor mixture. After the lysates were incubated for 10 min at 4°C, they were centrifuged at 13,000×g for 15 min at 4°C. Supernatants were used as the soluble fraction. The pellets were washed with ice-cold PBS, solubilized with 1 x sample buffer and used as the insoluble fraction. These individual fractions were analyzed by SDS-PAGE and Western blot. For sucrose density gradient centrifugation fractionation analysis, cells were harvested in lysis buffer (25 mM MES, pH 6.5, 50 mM NaCl, 1 mM Na_3VO_4 and 1% Triton X-100) with protease inhibitor mixture and phosphatase inhibitor added, and incubated for 30 min at 4°C with Dounce homogenization every 10 min. The lysates were adjusted to 42.5% sucrose, overlaid with 35 and 5% sucrose in lysis buffer without Triton X-100. The mixed lysates were centrifuged at 275,000×g for 20 hr at 4°C. From the top of the gradient, eleven 1-ml fractions were collected, and some volumes of each fraction were analyzed by Western blot.

Endocytosis assay

Endocytosis assay was performed as described previously [22]. SH-SY5Y cells were incubated with 50 nM BOIPY® FL C5-Lactosylceramide and 2.5 μg/ml rhodamine-conjugated transferrin for indicated times. The cells were then fixed and observed by confocal microscopy. The intensity was analyzed by ImageJ (http://imagej.nih.gov/ij/).

Cell-to-cell transmission assay using dual chamber

Cell-to-cell transmission assay was performed as described pre-

https://doi.org/10.5607/en.2018.27.5.377

viously [27, 32]. α-Synuclein-overexpressing SH-SY5Y cells [26] as donor cells were differentiated by treatment with 50 μM retinoic acid for 5 days. Then, SH-SY5Y cells cultured on coverslips on 12-well plates as recipient cells were cocultured with differentiated α-synuclein-overexpressing SH-SY5Y cells cultured on the insert for 12 h. The recipient cells were prepared for staining with the anti-α-syn antibody (BD Bioscience, Franklin Lakes, NJ, USA).

Confocal microscopy

Cells cultured on coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature; the fixed cells were then washed with PBS and incubated with PBS containing 0.1% Triton X-100 for 10 min at room temperature. After they were washed with PBS, the cells were blocked with PBS containing 1% bovine serum albumin (GenDEPOT, Barker, TX, USA) for 1 hr at room temperature, and then incubated overnight with primary antibodies at 4°C. Preparations were then stained with fluorescence-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) for 2 h, mounted, and observed using a model LSM510 or LSM710 confocal microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

All values are expressed as the mean±SEM. Statistical significance was evaluated using Graphpad Prism 5 (San Diego, CA, USA).

RESULTS

UCH-L1 associates with lipid rafts

To explore whether UCH-L1 associated with lipid rafts in the same way as other PD-associated proteins, we first isolated lipid rafts of SH-SY5Y cells based on their solubility in 1% Triton X-100 on ice [33] and performed Western blot. As shown in Fig. 1A, we observed about ~20% of total UCH-L1 in the cold Triton X-100 insoluble fraction, and we obtained similar results in the primary

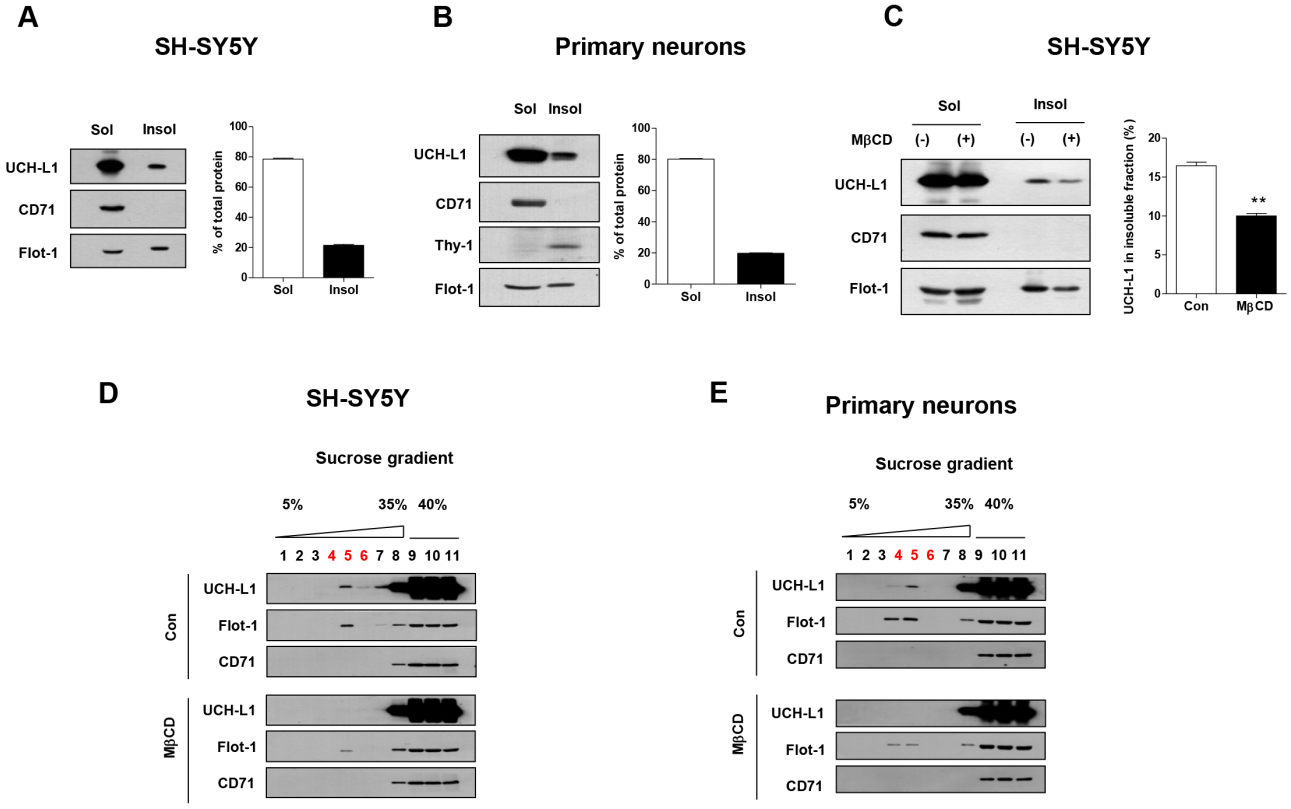

Fig. 1. UCH-L1 associates with lipid rafts. (A, B) SH-SY5Y cells (A) and rat primary neurons (B) were lysed in ice-cold 1% Triton X-100 buffer and fractionated as soluble or insoluble fractions, and then the samples were analyzed by Western blot. (C) After the treatment of SH-SY5Y cells with 10 mM MβCD for 1 h, the cells were lysed in ice-cold 1% Triton X-100 buffer and fractionated; the soluble and insoluble fractions were then analyzed by Western blot. Values obtained are from three independent experiments. *p<0.05, **p<0.01 against control by unpaired t-test. (D, E) SH-SY5Y (D) and rat primary neurons (E) were fractionated by sucrose gradient centrifugation fractionation assay in the presence or absence of 10 mM MβCD, and the lysates were then analyzed by Western blot.

neurons (Fig. 1B). When SH-SY5Y cells were treated with methyl-β-cyclodextrin (MβCD), a cholesterol-depleting agent, to exclude the possibility that UCH-L1 was isolated as a contaminant of lipid raft preparations [34], UCH-L1 was translocated into the Triton X-100 soluble fraction (Fig. 1C). To further confirm these observations, we isolated lipid rafts using sucrose density gradient centrifugation fractionation [22], and we also observed a small portion of UCH-L1 in the low-density lipid raft fractions (fractions #4~#6) of SH-SY5Y cells and primary neurons. Additionally, treatment with MβCD induced the translocation of UCH-L1 into non-lipid rafts fractions such as flotillin-1 (flot-1), a lipid raft marker (Fig. 1D and 1E), suggesting that UCH-L1 associates with lipid rafts as a lipid raft protein and not as a contaminant.

UCH-L1 regulates lipid raft-dependent endocytosis

In our previous report, DJ-1, a PD-associated genes, associate with lipid rafts and regulates lipid raft-dependent endocytosis in astrocytes [22] by regulating flot-1 and caveolin-1 (cav-1) expression [35]. Parkin, which has been known to associate with lipid rafts [20], also regulates lipid raft-dependent endocytosis in MEF cells through regulating cav-1 expression [27]. To explore whether altered UCH-L1 expression was involved in lipid raft function, we

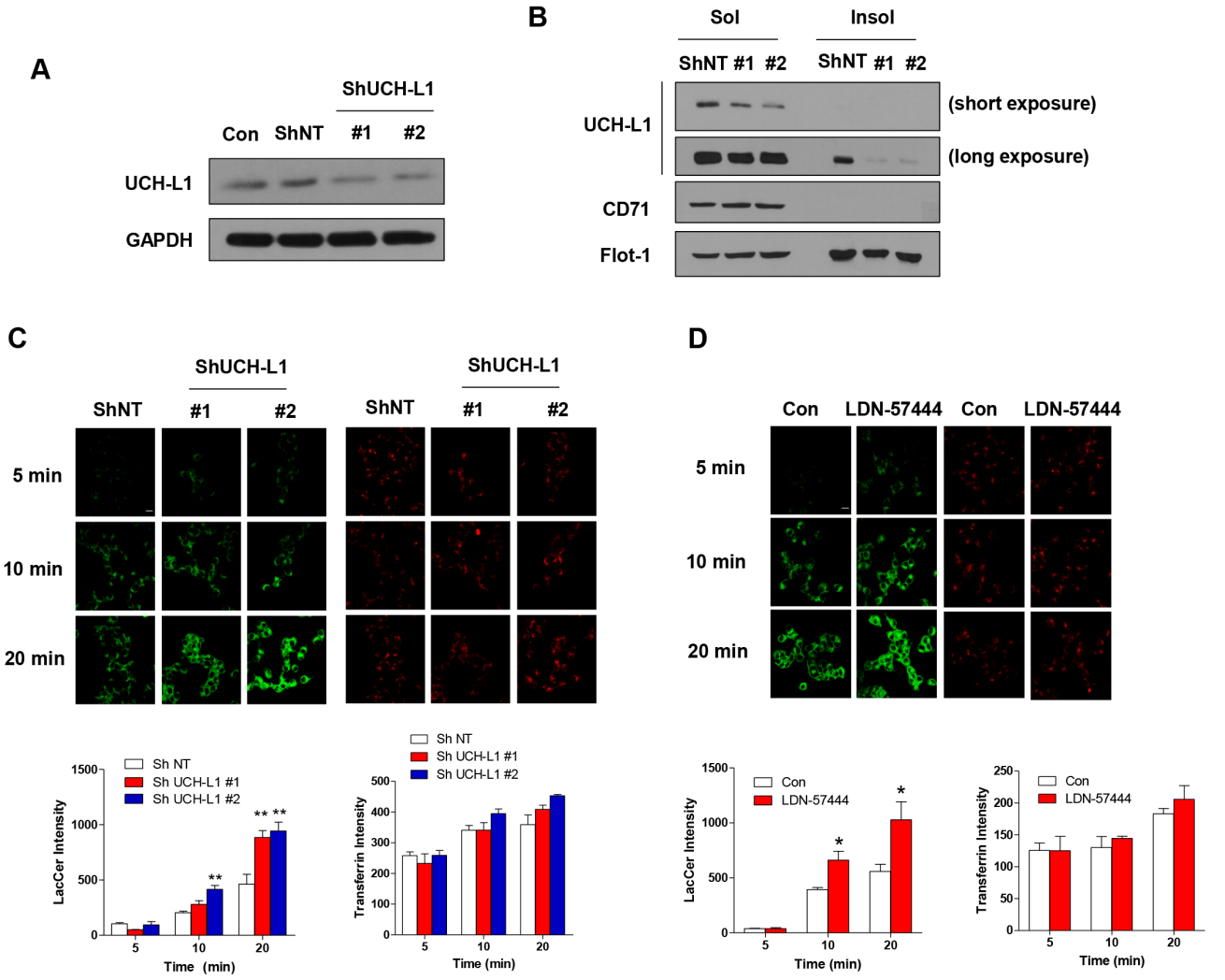

Fig. 2. UCH-L1 regulates lipid raft-dependent endocytosis. (A) Western blot was performed using lysates of NT and UCH-L1 KD #1 and #2 SH-SY5Y cells. (B) The cells were lysed in ice-cold 1% Triton X-100 buffer and fractionated as soluble or insoluble fractions, and then the samples were analyzed by Western blot. (C) The cells were incubated with 50 nM BOIPY® FL C5-Lactosylceramide and 2.5 μg/ml rhodamine-conjugated transferrin for the indicated times. (D) SH-SY5Y cells were treated with LDN-57444 for 24 h, and then incubated with 50 nM BOIPY® FL C5-Lactosylceramide and 2.5 μg/ml rhodamine-conjugated transferrin for the indicated times. The cells were then fixed and observed by confocal microscopy. The intensity was analyzed with the ImageJ program. Values are representative of three independent experiments. Scale bar indicates 20 μm. *p<0.05, **p<0.01 against control by one-way ANOVA.

https://doi.org/10.5607/en.2018.27.5.377

generated stable cell lines with downregulated UCH-L1 expression. UCH-L1 expression was efficiently downregulated in both fractions (Fig. 2A, B), and then performed general endocytosis assay using these cell lines. We used lactosylceramide as a marker for lipid raft-dependent endocytosis [36, 37] and transferrin as a marker for clathrin-dependent endocytosis [38, 39]. As shown in Fig. 2C, downregulation of UCH-L1 in SH-SY5Y cells enhanced lipid raft-dependent endocytosis, but not clathrin-dependent endocytosis. The treatment with LDN-57444, a UCH-L1 inhibitor that inhibits UCH-L1 hydrolase activity [40, 41], also enhanced lipid raft-dependent, but not clathrin-dependent endocytosis, suggesting that UCH-L1 regulates lipid raft-dependent endocytosis (Fig. 2D). Next, to explore whether UCH-L1 regulated lipid raft-dependent endocytosis by regulating the expression of flot-1 or cav-1 in the same way as DJ-1 or parkin, we performed Western blot analysis. We observed no changes in flot-1 or -2 expression in UCH-L1 knock-down (KD) cell lines (Fig. 3A) or with LDN-57444 treatment (Fig. 3B). Overexpression of UCH-L1 did not alter the expression of flot-1 and cav-1 or -2 (Fig. 3C), suggesting that lipid raft-dependent endocytosis changes with UCH-L1 do

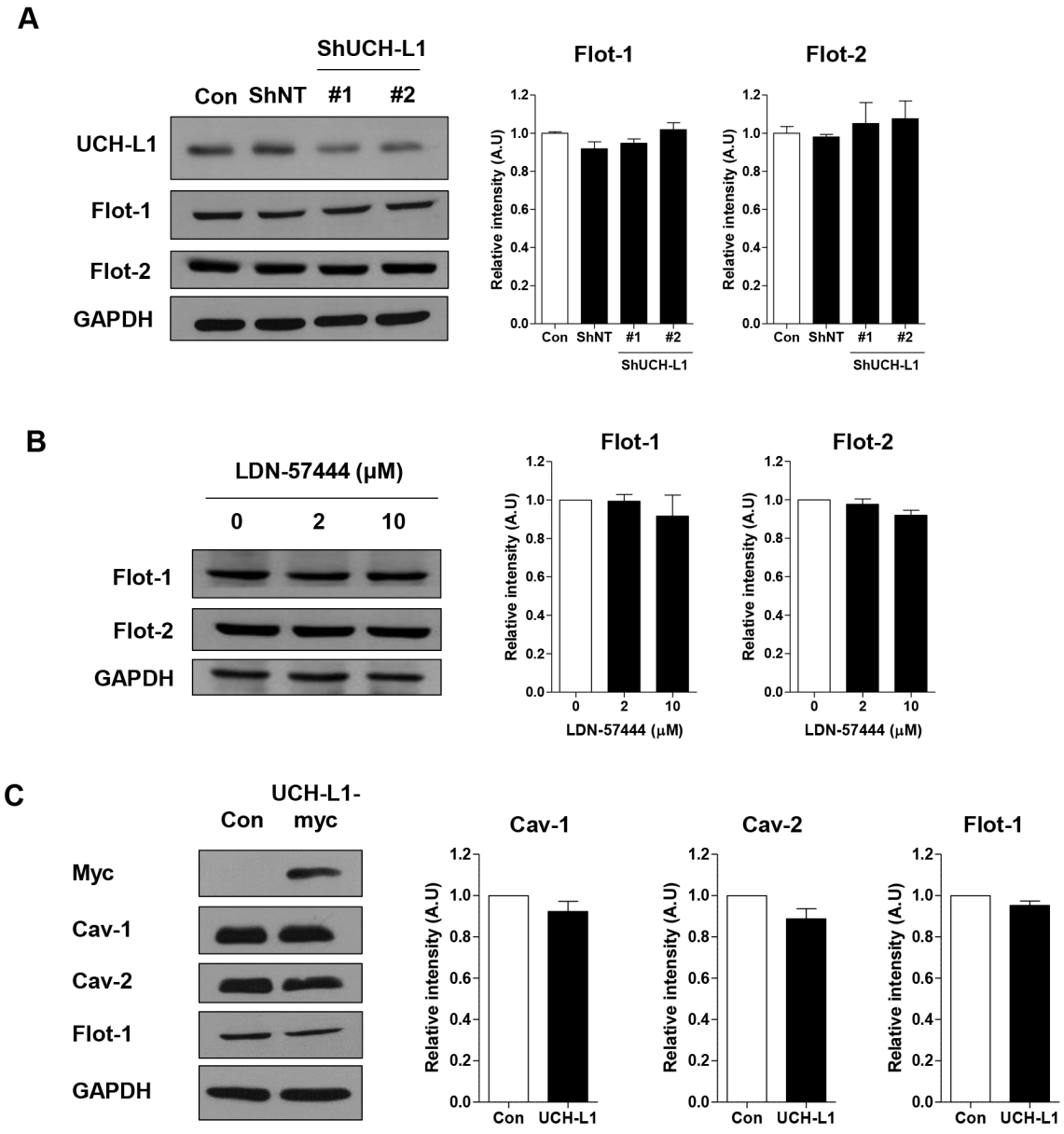

Fig. 3. UCH-L1 does not regulate the expression of caveolins and flotillins. (A) NT and UCH-L1 KD #1 and #2 SH-SY5Y cells were lysed. (B) SH-SY5Y cells were treated with LDN 57444 for 24 h, and the cells were lysed. (C) Hela cells were transfected with myc-tagged UCH-L1, and the cells were lysed. The lysates were then analyzed by Western blot. Values obtained are from three independent experiments.

not depend on the expression of flotillins and caveolins.

UCH-L1 regulates cell-to-cell transmission of α-synuclein

Previously, we demonstrated that extracellular α-synuclein is internalized into cells in a lipid raft-dependent manner [26], and also, signaling for lipid raft-dependent endocytosis is involved in cell-to-cell transmission of α-synuclein [32]. Accordingly, we explored whether UCH-L1 regulates cell-to-cell transmission of α-synuclein. As shown in Fig. 4A, cell-to-cell transmission of α-synuclein was greater in UCH-L1 KD cell lines than in the controls. In addition, UCH-L1 inhibition by LDN-57444 treatment also enhanced cell-to-cell transmission of α-synuclein (Fig. 4B). Endogenous α-synuclein expression did not altered by down-regulation of UCH-L1 (Fig. 4C). These data suggest that UCH-L1 regulates cell-to-cell transmission of α-synuclein via lipid raft-dependent endocytosis.

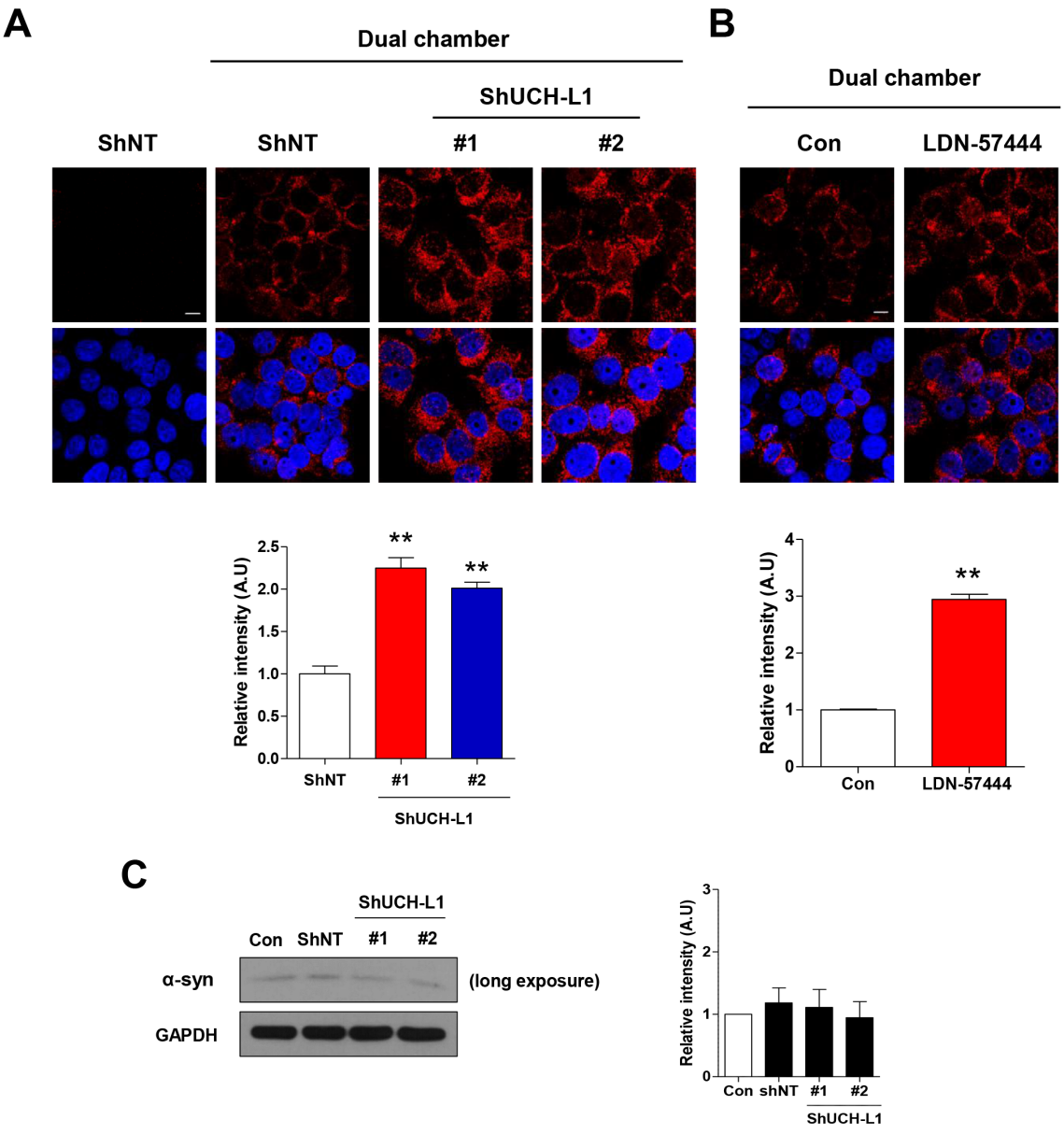

Fig. 4. UCH-L1 regulates cell-to-cell transmission of α-synuclein. (A) NT and UCH-L1 KD #1 and #2 SH-SY5Y cells were co-cultured with differentiated α-synuclein overexpressing SH-SY5Y cells for 12 hr using dual chamber. (B) SH-SY5Y cells were treated with LDN-57444 for 24 h, and further co-cultured with differentiated α-synuclein overexpressing SH-SY5Y cells for 12 hr using dual chamber. The cells were then fixed and immunostained with the anti-α-synuclein antibody. The intensity was analyzed with the ImageJ program. Values are representative of three independent experiments. *p<0.05, **p<0.01 against control by one-way ANOVA (A) and unpaired t-test (B). (C) NT and UCH-L1 KD #1 and #2 SH-SY5Y cells were lysed. The lysates were then analyzed by Western blot. Values obtained are from three independent experiments.

https://doi.org/10.5607/en.2018.27.5.377

DISCUSSION

The accumulating evidence indicates that the molecular pathways of neurodegeneration triggered by each mutation may be shared by several genetic forms of PD and may also play a role in the common sporadic disease [42-46]. Considering that the sub-localization of proteins is very important for protein function and that many PD-associated proteins, including a-synuclein, parkin, PINK1 and LRRK2, associate with lipid rafts, it is very informative to know whether other PD-associated proteins also associate with lipid rafts to elucidate the common molecular pathways involved in lipid rafts.

In the present study, we demonstrated that UCH-L1 also associates with lipid rafts. Lipid modifications of proteins such as palmitoylation and farnesylation facilitate the association of soluble proteins with cell membranes. Palmitoylation has been known to play important roles in trafficking into lipid rafts [47]; for instance, DJ-1 has been known to associate with lipid rafts via palmitoylation [22]. On the contrary, farnesylation drives membrane proteins to non-rafts domains [48], and UCH-L1 has been known to associate with membranes via farnesylation [30]. Nevertheless, UCH-L1 also associates with lipid rafts; FTI-277, a farnesylation inhibitor [49], and 2-bromopalmitate, a palmitoylation inhibitor [22] did not affect the association of UCH-L1 into lipid rafts (data not shown), suggesting that UCH-L1 associates with lipid rafts in palmitoylation- and farnesylation- independent manners, which is also supported by the finding that UCH-L1 membrane association is neuron specific and not dependent on farnesylation [50]. UCH-L1 also lacks obvious lipid interaction domains, but many deubiquitinases operate as part of larger protein complexes, which may induce the association of UCH-L1 with lipid rafts [1]. Farnesylated H-ras also dynamically associates with lipid rafts by bound nucleotides, regulating downstream signals [51]. More studies will be needed to explore how UCH-L1 associates with lipid rafts.

We also demonstrated that UCH-L1 regulates lipid raft-dependent endocytosis, but it is not dependent on the expression and degradation of caveolin-1 and flotillin-1, unlike DJ-1 and parkin. UCH-L1 is intimately involved in regulating protein ubiquitination, which has emerged as a common element in the internalization of plasma membrane proteins. Cell surface turnover of GLT-1 is mediated by ubiquitination [41], and ubiquitinated EGFR has been reported to be endocytosed through a lipid raft-dependent route [52]. Although we could not identify the exact substrates of UCH-L1 for regulating lipid raft-dependent endocytosis, UCH-L1 may participate in lipid raft-dependent endocytosis by regulating the ubiquitination or deubiquitination of lipid raft proteins.

Interestingly, loss of UCH-L1 enhanced lipid raft-dependent endocytosis and then, cell-to-cell transmission of α-synuclein. Prion-like propagation of protein inclusions such as α-synuclein, tau, and mutant huntingtin in many neurodegenerative diseases has received a great deal of attention. Although the detailed mechanism of intercellular propagation of protein inclusions has not been well understood, this propagation has been anticipated to explain the unveiled pathogenesis of many neurodegenerative diseases [25]. Given that the molecular pathways of neurodegeneration triggered by each mutation may be shared by several genetic forms of PD, it is unsurprising that many PD-related genes have been shown to affect one or more of these processes. Loss of parkin has been reported to be involved in lipid rafts-dependent endocytosis and cell-to-cell transmission of α-synuclein [27]. Glucocerebrosidase depletion, which is strongly associated with PD, promotes propagation of α-synuclein aggregates [53]. The knock-down of some orthologs of some PD-related genes such as catp-6 (*ATP13A2*), djr-1.2 (*DJ-1*), lrk-1 (*LRRK2*), pdr-1 (*Parkin*) has been also reported to increase cell-to-cell transmission of α-synuclein in a *C. elegans* BiFC model [54].

Decreased UCH-L1 expression and oxidative modification of UCH-L1 have been observed in the brains of AD and PD patients [3]. The overexpression of UCH-L1 reduces the number of amyloid beta plaques and improves memory deficits in AD mice [55], suggesting that UCH-L1 has a protective effect in AD models. Accordingly, enhancing UCH-L1 activity may become a useful target against AD and PD progression.

In summary, we demonstrated that UCH-L1 associates with lipid rafts in the same way as other PD-associated gene products, and also that UCH-L1 regulates lipid raft-dependent endocytosis and cell-to-cell transmission of alpha-synuclein. This study provides more evidence that many PD-associated gene products share common signaling pathways to explain the pathogenesis of PD and evidence of UCH-L1 activity as a useful target against the progression of PD.

ACKNOWLEDGEMENTS

The authors thank Dr. K. J. Lee (Graduate School of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, Seoul, Korea) for providing plasmids for UCH-L1-myc. This research was supported by the National Research Foundation of Korea (NRF) grants funded by the Korean government (Ministry of Science and ICT) (Grant No. 2017R1E1A1A91973713, NRF-2012R1A5A2048183).

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https://doi.org/10.5607/en.2018.27.5.377