Chapter

Ubiquitin Carboxyl-Terminal Hydrolase L1 in Parkinson's Disease

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Abstract

Ubiquitin plays the crucial roles to maintain the ubiquitin proteasome system (UPS) functions, which were suggested that involved in Parkinson's diease (PD). Ubiquitin C-terminal hydrolase L1 (UCHL1), which was detected in Lewy bodies of nerve cells in PD brains, plays an important role for maintaining ubiquitin pool in UPS. The first UCHL1 mutation (UCHL1I93M) was found in two siblings of a PD family. By contrast, UCHL1S18Y mutation was recognized to reduce the risk of developing PD by its specific antioxidant protective function. The studies of UCHL1 in mouse models showed that lack of UCHL1 resulted in motor ataxia, degeneration of axons, and instability of free ubiquitin level. Transgenic mice expressing UCHL1I93M mutant exhibited dopaminergic neuron (DA) degeneration in MPTP-treated conditions. In this chapter, we provide a summary on recent findings related to roles of UCH-L1 in PD. Knockdown dUCH, a homolog of human UCHL1, in fly dopaminergic neuron resulted as some Parkinson's disease—like phenotype such as: (1) the underdevelopment and/or degeneration of DA neurons; (2) the shortage of dopamine in the brain; and (3) the locomotor dysfunctions. Those finding indicated that dUCH (ortholog of human UCH-L1 in *Drosophila*) plays an important role in Parkinson's disease.

Keywords: UCH-L1, Parkinson's diseases, PD model

1. Introduction

Parkinson's disease (PD) was first described in 1817 by Dr. James Parkinson. PD is considered as the second most common neurodegenerative disease which impacts 1% of the population over 60 years old [1]. The basic symptoms of Parkinson's disease are difficulty walking, slow movement, stiff and trembling limbs, balance disorders, and facial paralysis. Symptoms appear gradually and are not marked; it is difficult to recognize and often may be confused with other diseases [2]. Causes are attributed to lack of dopamine, a chemical that plays an important role in nerve signal transmission, due to degeneration/loss of dopaminergic neurons. Besides, the presence of Lewy body was also reported as one of PD symptoms although it is not clear to be a cause or a result of PD [2–4]. The complex interaction between environmental and genetic factors is also thought to be a cause of PD. However, the interaction between these factors in the PD remains unclear [5]. Previous studies have shown that mitochondrial dysfunction, oxidative stress, altered protein proteolysis, and inflammation are responsible for PD pathogenesis [6–8]. In addition, the

relation to PD of many genes and their variants such as α-synuclein, PINK-1, DJ-1, LRRK2, and UCH-L1 has been reported [9, 10].

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is an abundant protein in neurons. The UCH-L1 polypeptide is 24,824 Da, contains 223 amino acids, and accounts for 1–2% of brain protein in humans [11]. In addition to the brain, UCH-L1 is also expressed strongly in the peripheral nervous system, including sensory and nervous system activity. UCH-L1 functions as an important enzyme in ubiquitin proteasome system. In a form of monomer, UCH-L1 hydrolyzes the peptide bond between two ubiquitin molecules [12]. In dimer form, it plays a function as a ligase [13]. However, the functions of UCH-L1 in living cell and tissue still remain unclear. UCH-L1 has been suggested to have its functions via the role of ubiquitin proteasome system by maintaining a pool of free ubiquitin molecules [14]. Dysfunction of UCH-L1 resulted in reduction of protein degradation, consequenced by the accumulation of ubiquitinated proteins which has been believed as the cause of cell degeneration [15–17]. UCH-L1, therefore, involves in many biological processes such as cell signaling, cell cycle, DNA repair, and other ubiquitination-dependent biological processes [14–16]. Consequently, UCH-L1 had been reported as close relevant to neurodegenerative diseases, diabetes, as well as cancer [14–16, 18].

2. UCH-L1 in Parkinson's disease

In PD, there are some evidences which reveal that ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1 or PGP9.5) is associated with PD. First, an UCH-L1I93M mutant was identified in two siblings from a German family with autosomal dominant PD in 1998 [18]. After that, UCH-L1S18Y mutant was discovered by Lincoln et al. [19]. UCH-L1S18Y mutant, in some cases, has been believed to have the potential in decreasing the risk of developing PD [20, 21] by its specific antioxidant protective function [22]. Moreover, UCH-L1 is also localized in Lewy bodies [23]; inclusions were found in nerve cells of PD. Although UCH-L1 had shown to have close link to PD, roles of the protein in PD are still controversial. Previous studies showed that not all mutant carriers manifest the phenotype of PD or show the protective effect to PD. The homozygous mutation of UCH-L1 (UCH-L1E7A), which also shows the decrease in hydrolytic activity, was found in three siblings of a Turkish family with progressive visual loss due to optic atrophy but neither the patients homozygous for UCH-L1E7A nor their heterozygous parents or siblings exhibited PD features on neurological examination [24]. In addition, Healy et al. and Ragland et al. showed that UCH-L1S18Y does not exhibit any protective effects against PD [25, 26].

Recently, research on UCH-L1 cellular and animal models has revealed many important findings of UCH-L1 functions in PD. An in-frame deletion of UCH-L1 gene encoding a truncated UCH-L1 lacking catalytic residue [17] in gracile axonal dystrophy (gad) mouse exhibits some PD pathogenesis such as locomotor ataxia, tremor, and difficulty in moving, and these symptoms are progressively severe [27]. Analysis of transcriptomic, proteomic, and histochemical in the brain of gad mouse revealed some prominent genes and proteins, which contribute to PD pathogenesis [28–30].

In PD research, *Drosophila* melanogaster has served as a valuable model to get insight into important features of PD pathogenesis [31–33]. The *Drosophila* model of PD provided a useful tool for tracking the integrity of the whole dopaminergic neuron system, analyzing neurodegeneration with a large number of animals to study PD in the population level, high-throughput genetic and drug screening.

In our study, knockdown *Drosophila* homolog of human UCH-L1 (dUCH) in dopaminergic neuron system of the fly brain exerted a fly model of Parkinson's disease [34]. The fly model mimics all of the main PD-related symptoms including locomotor behaviors, dopamine production, DA neuron integrity, as well as the progression of DA neuron degeneration.

2.1 Loss function of UCH-L1 homolog in *Drosophila melanogaster* resulted in locomotor dysfunction, one of most important PD phenotypes

Parkinson's disease is the most common movement disorder which is normally featured by motor symptoms. These symptoms include tremor, rigidity, bradykinesia, and postural instability. In the early stage of *Drosophila* development, the effects of dUCH knockdown on the third instar larval wandering behavior were examined by crawling assay. Heterozygous dUCH knockdown larvae displayed a tremor-like behavior which was tracked as tight wavy line when moving horizontally on agar plates. Additionally, these larvae accomplished a shorter moving path (**Figure 1A**, right panel) comparing to driver controls (**Figure 1A**, left panel) in an identical interval of time. The mean velocity of knockdown larvae was reduced to 62% of the controls (**Figure 1B**). The reduction was statistically significantly different when comparing two means (Student's t test with Welch's correction, N= 10, p< 0.01). In the adult stage, dUCH knockdown resulted in decline locomotion of *Drosophila*. Both knockdown and control flies showed age-related decline in the climbing ability (**Figure 1C**). However, the climbing ability of knockdown flies dropped sharply at day 25 and led to the difference between the control and knockdown to statistically significance at this time

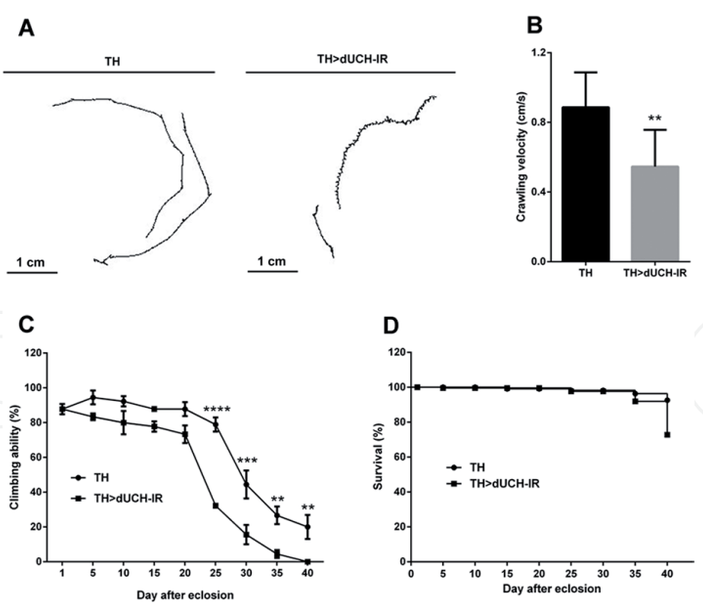

Figure 1.
*The dysfunction in locomotor behavior of dopaminergic neuron-specific dUCH knockdown flies. (A) Motion paths of control larvae (TH) and dUCH knockdown larvae (TH>dUCH-IR). Knockdown larvae exhibit shorter and disorder crawling paths (right panel) compared to control (left panel). (B) Crawling velocity of control (TH) and knockdown larvae (TH>dUCH-IR). Knockdown larvae show the reduction in crawling pace and parametric unpaired t test with Welch's correction: ** p < 0.01, error bars present SD. (C) The climbing ability of control (TH) and dUCH knockdown adult flies (TH>dUCH-IR). Knockdown flies start to exhibit the decline in the climbing ability at day 25 after eclosion (repeated measures two-way ANOVA with Bonferroni's post hoc test, **p < 0.01, ***p < 0.001, ****p < 0.0001; error bars present SEM). (D) Survival curve of control (TH) and dUCH knockdown (TH>dUCH-IR). Control and knockdown flies do not show the difference in survival (Kaplan-Meier method with log-rank test, p > 0.05. TH (+; +; TH-GAL4/+), and TH>dUCH-IR (+; +; TH-GAL4/UAS-dUCH-IR)).*

point (repeated measures two-way ANOVA with Bonferroni's post hoc test, $p < 0.0001$). The decline in climbing function of knockdown flies still sustained on day 30 onward (repeated measures two-way ANOVA with Bonferroni's post hoc test, $p < 0.001$ at day 30 and $p < 0.01$ at days 35 and 40) and was struck down to 0% at day 40 in which no fly can climb across 10 ml mark in 10 s (**Figure 1C**). Furthermore, the survival analysis was carried out to determine the toxicity of the reduction of dUCH. There is no significant difference in survival curve of control and knockdown flies (Kaplan-Meier method with log-rank test, $p > 0.05$) (**Figure 1D**). It illustrated that the knockdown of dUCH played no effect on *Drosophila* life span. The analysis also proved that there were no effects of death events in climbing analysis. Taken together, those data demonstrated that the reduction of dUCH specifically in dopaminergic neurons of *Drosophila melanogaster* leads to the disorder in crawling behavior and decline in locomotor ability but does not affect *Drosophila*'s life span.

2.2 Loss function of UCH-L1 homolog in *Drosophila melanogaster* exerted PD phenotype of dopaminergic neuron degeneration

Forno [35] and Thomas [2] have shown that locomotor dysfunction in PD patients may be caused by the degeneration of dopaminergic neurons (DA neurons) [2, 35]. These neurons play important roles in dopamine production for central nervous system and control multiple functions of the brain including voluntary movement. In *Drosophila*, the locomotor deficit was observed in many PD-related genes such as SNCA [36], LRRK2 [37], and PARKINR275W [37] ectopic expression followed by the degeneration of DA neurons. The study of Budnik and White showed that DA neurons assembled into some different clusters with the differences in projection and number of DA neurons [38]. In addition, DA neurons in PPL2 cluster in adult brain were demonstrated to originate from DL2a cluster [39]. It seems to be that the development of DA neurons not only occurs in embryonic to larval stages but also in larval to adult stages. In *Drosophila* model of PD, dopaminergic neurons in both larval and adult dUCH knockdown brains showed its degeneration. The DA neuronal system in the third instar larval brain lobe was classified into six clusters: DM1a, DM1b, DM2, DL1, DL2a, and DL2b (**Figure 2A**) [38, 39]. The pattern, shape, and number of DA neurons in most of clusters in dUCH knockdown and control are similar except on DL1 cluster (**Figure 2B**). In DL1 cluster, dUCH knockdown brain (TH>dUCH-IR) exhibited the reduction in numbers of DA neurons compared to driver control (TH). It indicates that the reduction of dUCH may cause the incomplete loss or underdevelopment of DA neurons in DL1 clusters of the third instar larval central brain.

On the other hand, in the adult *Drosophila*, when dUCH was specifically knocked down in dopaminergic neurons, the PPM2 dopaminergic cluster lost its neurons (**Figure 3A2**, **A2'** and **A3**, **A3'**). This loss occurred in 1-day-old flies, increased progressively by age, reached 50% on age 20, and affected all the individuals of 40-day-old flies (**Figure 3A4**). In PPM3 dopaminergic cluster, the neuron loss occurred in 1-day-old flies with highly prevalence proportion nearly 40% of the population (**Figure 3B2**, **B2'**, **B3**). The number of PD disease—like flies—reached 50% as early as 10 days old and got nearly maximum prevalence on the age of 20 (**Figure 3B3**). In PPL2 dopaminergic cluster, the loss of a specific neuron was seen in knockdown brain TH>dUCH-IR (**Figure 3C2**, **C2'**) compared to TH-GAL4 control TH (**Figure 3C1**, **C1'**). DA neuron in PPL2 cluster was lost in highly prevalence proportion; 40% of knockdown flies exhibited DA loss at 1 day old. However, unlike PPM3 cluster the number of disease flies increased steadily through the age

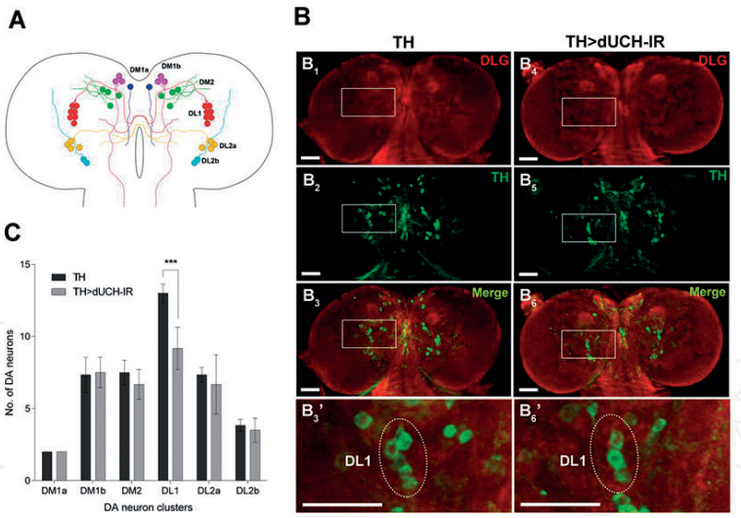

Figure 2.
*The abnormality in the number of DL1 dopaminergic neurons in dUCH knockdown larval brain. (A) A schematic representation of six DA neuron clusters DM1a, DM1b, DM2, DL1, DL2a, and DL2b and projection in Drosophila larval central brain were redrawn based on the study of Blanco et al. [39]. (B) Representative confocal images show that DA neuron clusters in the third instar larval central brain were stained with anti-TH (green). The whole brain was counterstained with anti-DLG (red). The heterozygous driver control TH-GAL4/+ (TH) on the left panel (B1, B2, B3, B3') and heterozygous dUCH knockdown TH-GAL4/UAS-dUCH-IR (TH>dUCH-IR) on the right panel (B4, B5, B6, B6'). The boxed area in merge image (B3, B6) marks that DL1 cluster was magnified in (B'3, B'6), respectively. The number of DA neurons in DL1 clusters in dUCH knockdown brain was less than those in driver control (B'3, B'6). (C) Quantification of DA neurons in each cluster in driver control (black bars) and dUCH knockdown (gray bars). Only the difference in the number of DA neurons in DL1 clusters between dUCH knockdown and driver control was significantly different (parametric unpaired Student's t-test with Welch's correction, ***$p < 0.001$, $n = 6$). Scale bars, 50 μm. DA neuron, dopaminergic neuron; DM, dorsal medial; DL, dorsal lateral; TH, tyrosine hydroxylase; DLG, Drosophila discs large.*

and reached maximum prevalence on the age of 40 (**Figure 3C3**). The DA neuron susceptibility to dUCH reduction depended on the age and neuronal type of *Drosophila* adult brain.

2.3 Loss function of UCH-L1 homolog in *Drosophila melanogaster* resulted in dopamine shortage

The reduction of neurotransmitter, dopamine, was observed in PD patients' brain which has been thought to be a direct cause leading to PD symptoms. The production of dopamine mainly occurs in DA neurons according to catecholamine biosynthesis pathway (**Figure 4A**). In addition, studies on *Drosophila* model have demonstrated that some *Drosophila* life activities such as locomotor activity [33, 40], olfactory conditioning [41], sleep and arousal regulation [42–44], and memory and learning process [45, 46] involve dopamine (**Figure 4B**). In the *Drosophila* model of PD, the underdevelopment or degeneration of DA neurons was detected in the brain of dUCH knockdown flies. Therefore, the dopamine level in the brain of knockdown flies may be affected by these impairments. Quantification of dopamine in dUCH knockdown brain showed that dopamine was reduced at every time point of examination (1, 10, 15, 20, and 25 days after eclosion) in dUCH knockdown flies compared to driver control (**Figure 4C**) (ordinary two-way ANOVA with Tukey's multiple comparisons test, ****$p < 0.0001$). The statistical analysis indicated that there is no significant difference in the amount of dopamine at every time point in the driver control flies. However, there are significant differences in the dopamine level from days 1 to 10 and 20 to 25 in the knockdown flies (ordinary two-way ANOVA with Tukey's multiple comparisons test, ***$p < 0.001$, *$p < 0.05$). The data

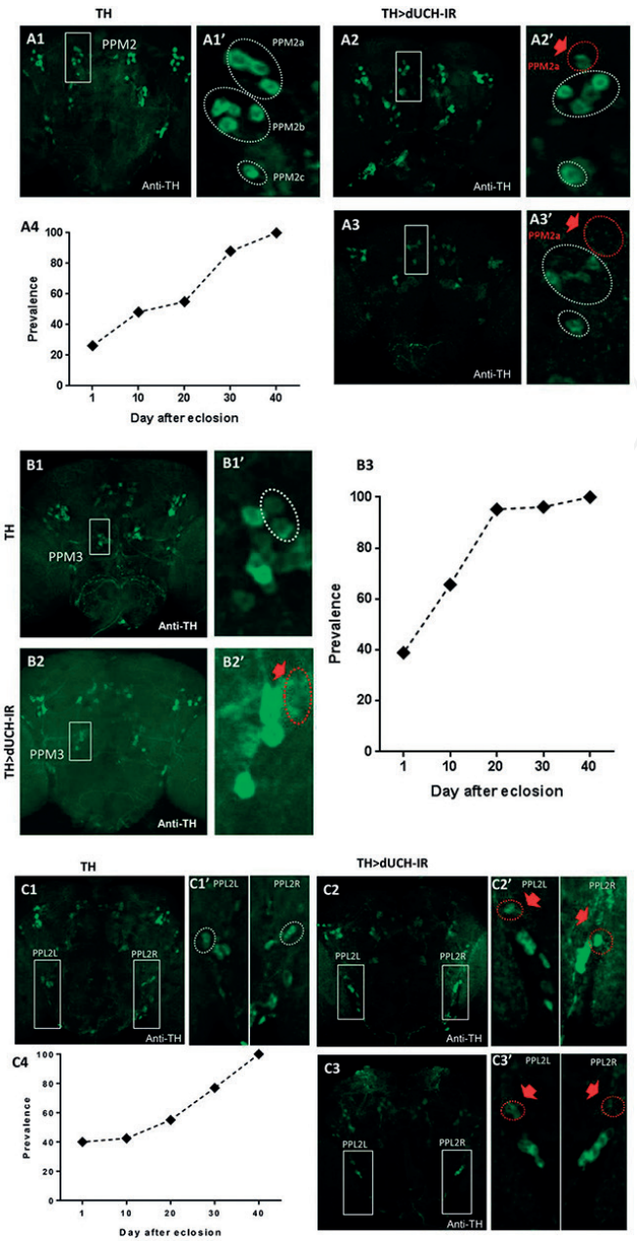

Figure 3.
The susceptibility of DA neurons in each cluster depends on age and neuronal type. Confocal images showed TH-positive neurons in PPM2 (A1–A3), PPM3 (B1–B2), and PPL2 (C1–C3) clusters in adult central brain. The prevalence proportion of dUCH knockdown flies on each cluster was described as a progressive graph, PPM2 (A4), PPM3 (B3), and PPL2 (C4). In PPM2 cluster, two kinds of partial loss of DA neurons (two to three neurons) were observed in heterogeneous dUCH knockdown flies TH>dUCH-IR (A2, A2'; A3, A3') compared to heterogeneous driver control TH-GAL4 (TH) (A1, A1'). The prevalence of PPM2 in dUCH knockdown flies increased with age; 20-day-old flies reached nearly 50% of population. In PPM3 cluster, the loss of two DA neurons was specifically seen in knockdown flies (B2, B2') compared to control (B1, B1'). However, the number of flies with this loss rose dramatically and reached 50% of population before 10 days old (B3). The loss also occurred partially in a specific DA neurons in PPL2 cluster in knockdown flies (C2, C2'; C3, C3') compared to TH-GAL4 control (C1, C1'). Loss of DA neurons in PPL2 cluster happened steadily through aging brain was described in C4; 50% of population suffered from PPL2 DA neuron loss around 20 days old (C4).

indicated that knockdown of dUCH leads to the reduction of dopamine beginning at the first day of eclosion and continuing on the following days. Interestingly, there are two significant periods (1 to 10 and 20 to 25 days) showed the reduction of dopamine in dUCH knockdown brain which may involve in the DA neuron integrity. The reduction of dopamine in dUCH knockdown flies suggested the connection between DA neuron impairment and locomotor deficit. These results can be modeled as the reduction of dUCH caused the impairment of DA neurons which leads to the reduction of dopamine followed by the dysfunction in locomotor behaviors (**Figure 4D**).

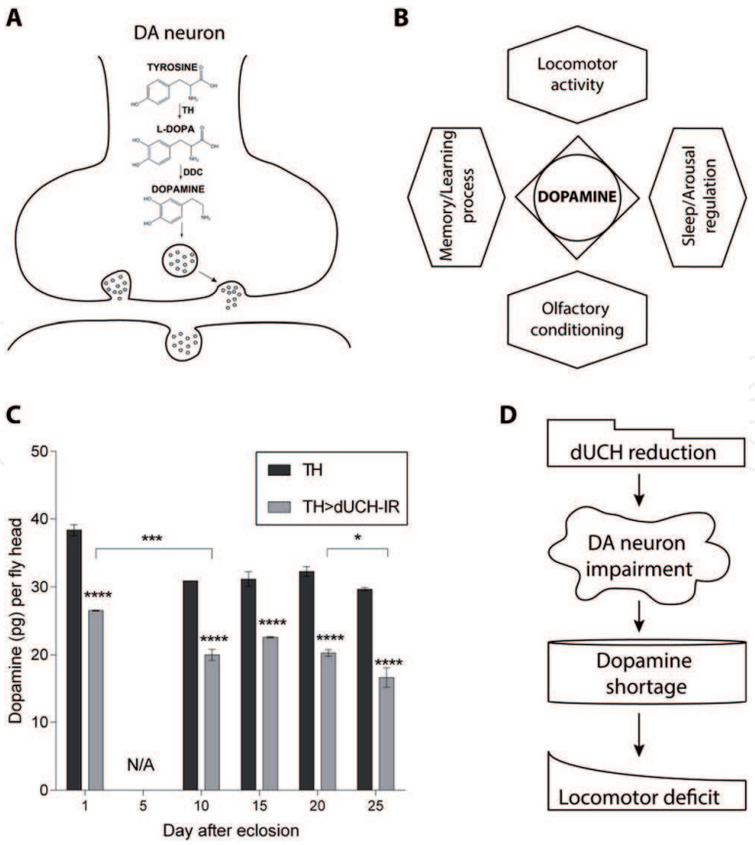

Figure 4.
*Dopamine shortage in adult dUCH knockdown brain. (A) The production of dopamine through catecholamine biosynthesis pathway in DA neuron. (B) The functions of dopamine in life activities of fruit fly [40, 41, 43–46]. (C) The quantity of dopamine per fly head in dUCH knockdown (TH>dUCH-IR) and driver control (TH). Knockdown flies show the reduction of dopamine in the brain in every time point compared to driver control (two-way ANOVA with Tukey's multiple comparisons test, ****$p < 0.0001$). Knockdown flies also show the reduction of the dopamine level in 1- to 10- and 20- to 25-day period (two-way ANOVA with Tukey's multiple comparisons test, ***$p < 0.001$, *$p < 0.05$). (D) The intermediate role of dopamine in the process of DA neuron impairment to locomotor deficit.*

3. Material and methods

3.1 Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose, and 7% dry yeast. Wild-type strain Canton-S was obtained from the Bloomington Drosophila Stock Center (BDSC). RNAi lines carrying UAS-dUCH-IR fusion (GD#26468) for knockdown *Drosophila* ubiquitin carboxyl-terminal hydrolase (dUCH, CG4265) were received from the Vienna *Drosophila* Resource Center (VDRC). GAL4 drivers were used to perform the targeted knockdown of dUCH in dopaminergic neuron of *D. melanogaster*: TH-GAL4 (BDSC#8848).

3.2 Immunostaining

Larval and adult brains were dissected in cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at 25°C for 15 min. After washing with 0.3% PBS-T (PBS containing 0.3% Triton-X100) twice, the samples were blocked in blocking solution (0.15% PBS-T containing 10% normal goat serum) at 25°C for 20 min. Samples were then incubated with the following primary antibodies diluted in blocking solution: rabbit anti-*Drosophila* ubiquitin carboxyl-terminal hydrolase (anti-dUCH; 1:500) at 4°C for 16 h or rabbit anti-tyrosine hydroxylase (anti-TH; 1:250; Millipore, AB152) at 4°C for 20 h. After washing with 0.3% PBS-T, samples

were incubated with secondary antibodies conjugated with Alexa 488 or FITC (1,500; Invitrogen) at 25°C for 2 h and then washed and mounted in Vectashield Antifade Mounting Medium (Vector Laboratories, Japan). Finally, the samples were inspected by a confocal laser scanning microscope (Olympus Fluoview FV10i) or Olympus BX41 Microscope.

3.3 Crawling assay

Male larvae in the early third instar stage were collected randomly and washed with PBS to discard food traces. After that, larvae were transferred to agar plates containing 2% agar with a density of two to four larvae per plate. The movement of larvae was recorded by a digital camera for 60 s. The recorded videos were then converted into the AVI type by MOV to AVI converter (Pazera Jacek, Poland) and then analyzed by ImageJ (NIH, USA) with wrMTrck plugin (developed by Dr. Jesper Søndergaard Pedersen) to track larval movement and draw motion paths.

3.4 Climbing assay

Newly eclosed adult male flies were collected and transferred to conical tubes which have heights of 15 cm and diameters of 2 cm. After that, the tubes were tapped to collect the flies to the bottom, and the length of time to record the movement of flies was 30 s. The procedures were repeated five times and recorded by a digital camera. For all of the climbing experiments, the height which each fly climbed to was scored as follows: 0 (less than 2 cm), 1 (between 2 and 4 cm), 2 (between 4 and 6 cm), 3 (between 6 and 8 cm), 4 (between 8 and 10 cm), and 5 (more than 10 cm). The climbing assay was performed every 5 days until all flies lose their locomotor abilities.

3.5 Dopamine quantification

Dopamine quantification procedure was performed as described [45] with the following modifications. Thirty fly heads were homogenized in 600 μl homogenization buffer (0.1 M perchloric acid/3% trichloroacetic acid) on ice and sonicated 5 times for 30 s each and then placed on ice for 30 min. Debris were removed by centrifugation at 15,000 g for 15 min at 4°C. Fifty microliter of supernatant was utilized for HPLC analysis using Nanospace SI-2 (Shiseido, Japan) with running buffer containing 180 mM chloroacetic acid, 50 μM EDTA, 160 mM sodium hydroxide, and 8.5% acetonitrile. Sample was separated in CapCell Pak C18 UG120 column (Shiseido, Japan) at 0.5 ml/min flow rate. Dopamine was electrochemically detected by Electrochemical Detector 3005 (Shiseido, Japan). Dopamine (H8502, Sigma-Aldrich) was used to build the standard curve at 0.0025, 0.005, 0.01, 0.02, and 0.04 μM. The differences in the dopamine level of examined samples were statistically analyzed using ordinary two-way ANOVA with Tukey's multiple comparison test and graphed by GraphPad Prism 6.0 (GraphPad Software, USA).

4. Conclusion and perspective

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a protein that may play multiple roles in the cell through the effect on ubiquitin system. UCH-L1 had been found as a PD-related protein. However, the exact mechanism remains unclear. In the *Drosophila* model, specific knockdown dUCH in dopaminergic neuron exerted PD-lie phenotypes including locomotor dysfunctions, DA degeneration, and dopamine

shortage. Interestingly, the degeneration of DA neurons in dUCH knockdown adult brain which occurred progressively and severely during the course of aging mimics the epidemiology of PD. These results provided one more evidence of the UCH-L1 in PD and suggest that the dUCH knockdown *Drosophila* is a promising model for studying both PD pathogenesis and epidemiology. The major advantages of the *Drosophila* model are a complex nervous system with DA neuron clusters and a conservation of the basic biological process and PD-related genes and can exhibit many PD features. The *Drosophila* model also can be utilized for either high-throughput screen.

Acknowledgements

I am grateful to Professor Yamaguchi Masamitsu and Professor Tran Linh Thuoc for their great supports to our research. I would also like to thank my students Tran Hoang Hiep, Dang Ngoc Anh Suong, and Huynh Man Anh for their great contribution in the research.

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