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Targeting UCH in *Drosophila melanogaster* as a model for Parkinson's disease

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1. ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease caused by genetic or environmental factors. Among several animal models, the *Drosophila melanogaster* is one of the valuable models widely used in studying genes and proteins implicated in PD. UCH-L1 (Ubiquitin carboxyl-terminal hydrolase L1) which is involved in formation of Lewy bodies, shows loss of function mutations in PD causing degeneration of dopaminergic neurons in mice. Here, we summarize the results from studying the UCH-L1 and its knockdown in *Drosophila* model of PD with respect to movement, degeneration of dopamine producing neurons, dopamine deficiency and age dependent dependency of progression of the disease. The knockdown of the UCH-L1 in *Drosophila* can be used in studying the epidemiology of the disease as well as in drug screening for finding therapeutic targets for PD.

2. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide and although its etiology is considered to involve both genetic and environmental factors, the underlying molecular mechanisms remain unclear (1,2). Several experimental models have been utilized to study PD, such as cellular models, animal models (the roundworm *Caenorhabditis elegans* and the fly *Drosophila melanogaster*), *Teleost* fish (zebrafish and medaka), and mammalian models (rodents and non-human primates) (3-5). Among these models, *D. melanogaster* is regarded as a valuable model to examine the mechanisms responsible for PD and the

screening of drugs for its treatment. *Drosophila* possesses many homologue genes of human PD-related genes, such as Dardarin/LRRK2, parkin, PINK1, Omi/HtrA2, DJ-1, UCH-L1, GIGYF2, PLA2G6, and GBA (6). A detailed understanding of dopaminergic (DA) neurons, which play an important role in the pathogenesis of PD, from the early larval to adult stages makes *Drosophila* a powerful organism for modelling and investigating progressive neurodegeneration in PD (7,8). The *Drosophila* model also mimics most of the main symptoms of PD, including impaired locomotion and the degeneration of DA. Furthermore, *D. melanogaster* has a short life span, which is advantageous for epidemiological studies on PD (6).

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is an abundant protein of approximately 24,824 Da with 223 amino acids in neurons and accounts for 1-2% of proteins in the human brain. UCH-L1 functions in the ubiquitin proteasome system by a hydrolase peptide bond between two ubiquitins when in mono-form (9,10). However, when UCH-L1 is in its dimer form, it forms a polyubiquitin chain linked through lysine 63 (K63) (11). Since UCH-L1 plays an important role in maintaining a pool of free monomeric ubiquitin, it is also important for the function of the ubiquitin proteasome system, through which UCH-L1 is assumed to play a role in many biological processes, such as DNA repair, cell signaling, trafficking, endocytosis, and degradation (10). However, the functions of UCH-L1 in living systems currently remain unclear. In PD, UCH-L1 was initially identified as a PD-related protein when a mutation (I93M) was detected in PD patients (12). UCH-L1 has also been implicated in the accumulation of α-synuclein and Lewy body formation in PD (11).

3. *DROSOPHILA* MODEL OF PD TARGETING UCH-L1

3.1. Molecular competence of the *Drosophila* model of PD with a focus on UCH-L1

In *Drosophila*, the protein CG4265 was identified as a homologue of human UCH-L1. The identity of human UCH-L1 (hUCHL1, P09936), mouse UCH-L1 (mUCHL1, Q9R0P9), and *Drosophila* UCH-L1 (dUCH) was 42.7%, while that of hUCHL1 and dUCH was 43.7%. Two active sites (C90 and H161) (14-16) and four important sites for hydrolytic activity (E7, H97, D176, and F204) (15-17) were in identical positions and the remaining site for hydrolytic activity, I93 was in a highly conserved position. In contrast, S18, which was previously suggested to be an important site for dimerization and ligation, was in a poorly conserved position. Furthermore, ubiquitin-interacting sites and peptide-binding sites, which are inferred from the cysteine peptidase C12 containing ubiquitin C-terminal hydrolase (UCH) families L1 and L3 domain conservation (18) were observed in highly conserved regions. The majority of inhibitor-binding sites were accommodated in highly conserved residues..

3.2. PD symptoms and PD–like phenotypes in the dUCH knockdown fly model

3.2.1. The dUCH knockdown fly model exhibited the PD-like phenotype of movement

Locomotive impairment is one of the main symptoms of PD. PD patients have difficulties walking, move slowly, and have stiff and trembling limbs as well as balance disorders (19). In the fly model of PD, locomotive impairment has been analyzed based on crawling ability in the larval stage and climbing ability in the adult stage (20-22). The *Drosophila* model of PD with a focus on UCH-L1 exhibited marked impairments in locomotion when dUCH was knocked down in DA neurons. dUCH knockdown larvae displayed a shorter moving path and slower mean velocity than the control (Figure 1). In the adult stage, heterozygous dUCH knockdown flies showed a decreased climbing ability. The decline observed in locomotion in the adult stage was age-dependent, which is a characteristic of PD (Figure 2).

3.2.2. dUCH knockdown fly model displayed the PD-like phenotype of DA neuron degeneration

In *D. melanogaster*, the majority of DA neurons are generated at embryogenesis, mature, and then gather into clusters during the first larval stage. *Drosophila* larvae have 21 DA neurons

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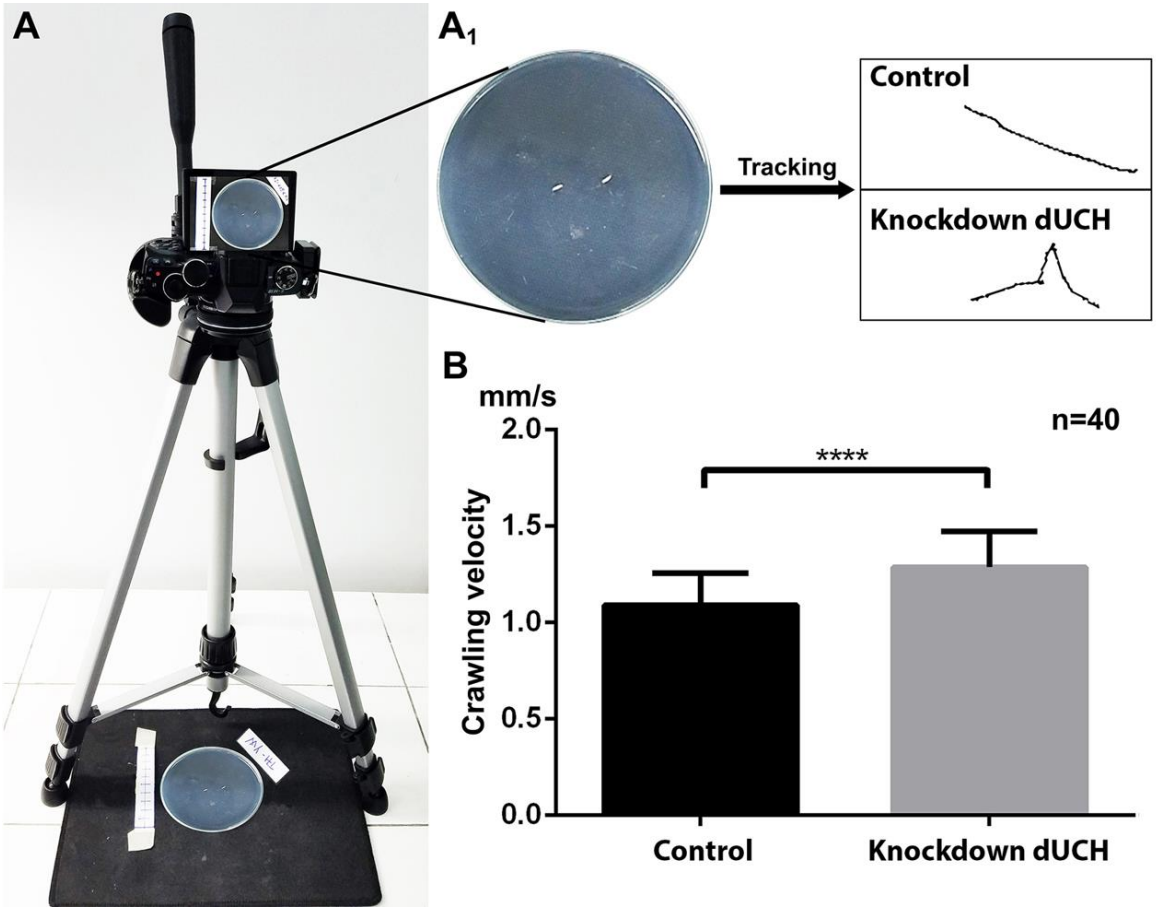

Figure 1. Dysfunction in the locomotor behaviors of dopaminergic neuron-specific dUCH knockdown larvae. (A): Model of crawling assay. (B) Crawling velocity of driver control (TH), dUCH knockdown (TH>dUCH-IR), n=40, one-way ANOVA with Tukey's multiple comparisons test, ****p< 0.0001, data are presented as mean ± SD.

grouped into seven DA neuron clusters per hemisphere. DA neuron clusters are called DM1a, DM1b, DM2, DL1a, DL1b, DL2a, and DL2b (7). In adult *Drosophila*, DA neurons are classified into nine clusters: PAM, PAL, PPM1, PPM2, PPM3, PPL1, PPL2ab, PPL2c, and VUM (23,24). The nine DA neuron clusters may be distinguished based on the position of the neuron cell body and dendrites as well as the number of DA neurons, and the effects of environmental or genetic factors on DA neurons may be examined according to number, morphology, and location (7,25). Therefore, the fly model emulates the PD symptom of DA neuron degeneration. In the fly model, DA neurons may be visualized by immunostaining with anti–tyrosine hydroxylase (anti-TH), an enzyme that plays a key role in the dopamine synthesis pathway. In the fly PD model with a focus on UCH-L1, the specific knockdown dUCH in DA neurons caused a defect in neuron clusters in *Drosophila* larvae. The DA cluster showed a significant reduction in the number of DA neurons between dUCH knockdown and driver control flies. This effect was efficiently rescued when flies were treated with vitamin C (VitC) at a dose of 0.5 mM (Figure 3).

The brains of dUCH knockdown adult flies exhibited the prominent loss of DA neurons in the PPM2, PPM3, PPL2ab, and VUM clusters (Figure 4). In PPM1/2, one or some DA neurons in the dUCH knockdown brain had degenerated relative to those in control flies, whereas others still remained. These

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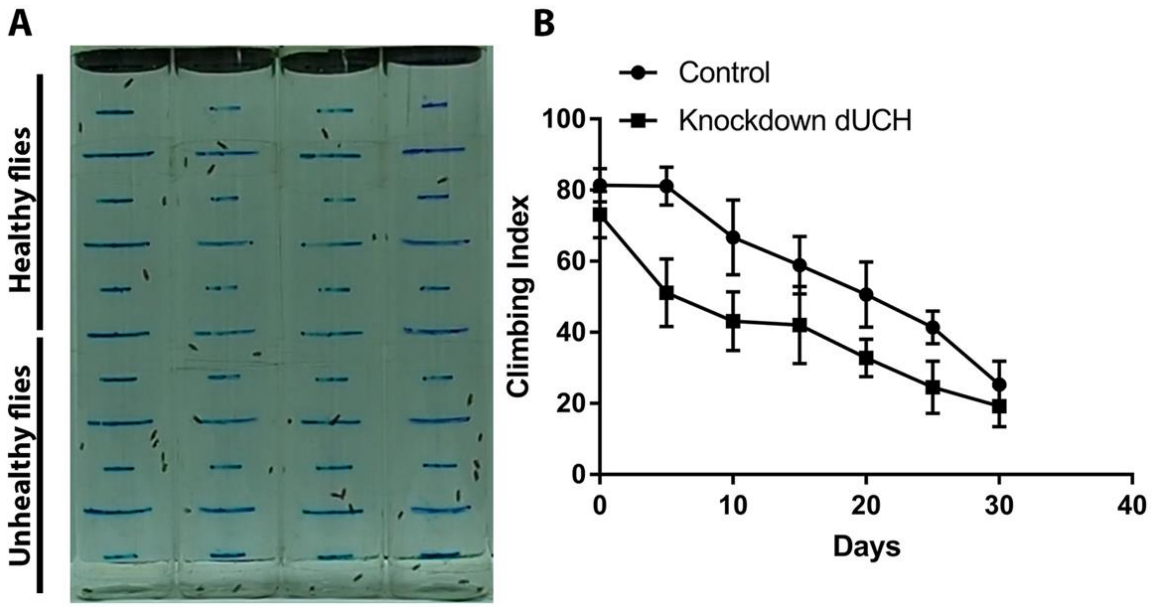

Figure 2. Dysfunction in the locomotor behaviors of dopaminergic neuron-specific dUCH knockdown adult flies. (A): Model of climbing assay. (B) Climbing index of driver control (TH), dUCH knockdown (TH>dUCH-IR), n=80, data are presented as mean ± SD.

patterns may be explained by the random loss of DA neurons based on differences in the susceptibility of neurons to the lack of dUCH. The loss of DA neurons was also observed in other DA clusters (such as PPM3, PAL, PPL1, and PPL2) in the dUCH knockdown brain. These phenotypes indicate that the lack of dUCH leads to the loss of DA neurons, which consequently causes locomotor dysfunction in flies.

3.2.3. The PD-like phenotype of aging-dependent progression in the dUCH knockdown fly model

Since PD is characterized not only by DA neuron degeneration, but also by the progressive loss of DA neurons in the course of aging, the *Drosophila* model of PD is advantageous for investigating aging-dependent PD characteristics due to its short life span (5,7). Another advantage of the *Drosophila* model for examining PD is the ease with which numerous samples may be handled at one time; therefore, the *Drosophila* model provides reliable data for statistical analyses without bias (5). DA neurons were observed in 1- to 40-day-old dUCH knockdown fly brains and the results obtained revealed that these brains had significantly lower numbers of DA neurons in PPM2, PPM3, PPL2ab, and VUM than those in control flies at 40 days old. The age at which dUCH knockdown flies exhibited a significant degeneration in DA neurons varied from cluster to cluster. The reduction in DA neurons was initially observed in PPM3 at 10 days old. PPM2 and VUM showed significant losses starting at 30 days old, whereas degeneration in PPL2ab started at 40 days old. These results indicated that the degeneration of DA neurons in dUCH knockdown brains did not occur immediately at a certain time point, but proceeded gradually at different time points with aging. Degeneration began in PPM3, followed by PPM2 and VUM, and the most severe degeneration occurred in all four clusters, including PPL2ab, in the oldest flies in the population examined (40 days old). These results also implied a difference in the susceptibility of DA neuron clusters when individual flies exhibited a lack of dUCH with aging.

3.2.4. The PD-like phenotype of a dopamine shortage in the dUCH knockdown fly model

A reduction in the neurotransmitter dopamine has been reported in PD patients and declared a PD clinical symptom (26). The production of dopamine mainly occurs in DA neurons via the catecholamine biosynthesis pathway (27). In the

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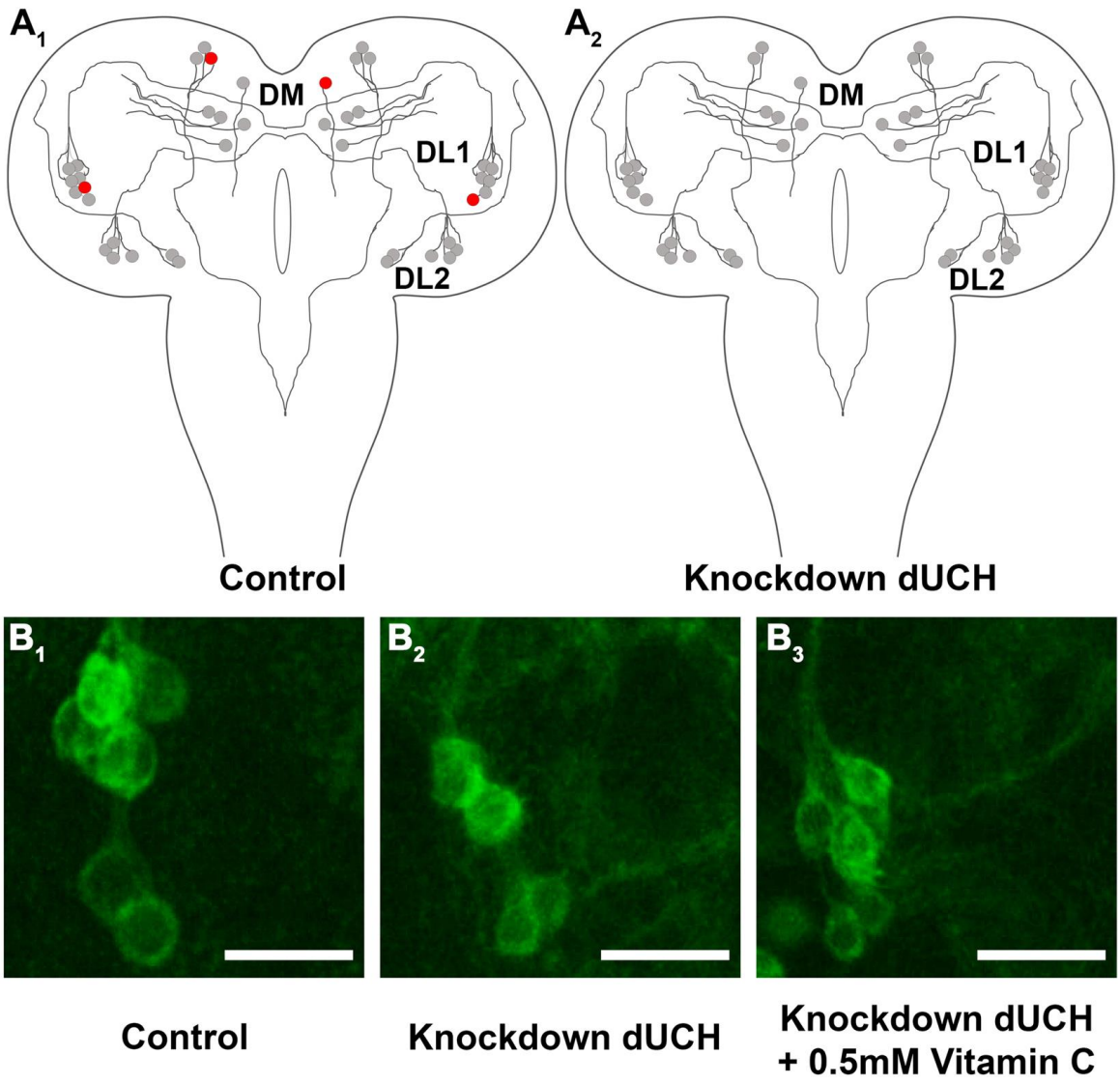

Figure 3. Abnormalities in the number of DL1 dopaminergic neurons in the dUCH knockdown larval brain. (A) A schematic representation of DA neuron clusters in larvae. (B) Representative images show DA neuron clusters in the third instar larval central brain immunostained with the anti-TH antibody (TH, green). Driver control flies (+; +; TH-GAL4/+) are shown in the panel B1, the dUCH knockdown flies (+; +; TH-GAL4/UAS-dUCH-IR) in the panel B2 and the VitC treated dUCH knockdown flies was showed in panel B3.

brains of dUCH knockdown flies, dopamine levels were lower on every day of the examination (1, 10, 15, 20, and 25 days after eclosion) than those in the brains of control flies. In the period from 1 to 10 days old, dUCH knockdown and control flies exhibited significant reductions in dopamine levels, with fold differences of 19.5 and 24.7%, respectively. Control flies did not show any significant differences in dopamine levels from the period of 10 to 25 days old with a fold difference of 8.1% (20 versus 25 days old), whereas knockdown flies exhibited significant reductions in dopamine levels from 10-, 15-, and 20-day-old flies to 25-day-old flies with a fold difference of 18% (20 versus 25 days old). This contributed to the high fold difference observed between 1- and 25-day-old dUCH knockdown flies of 37.3 to 22.7% in driver control flies. These results are consistent with previous findings on climbing ability and DA neuron

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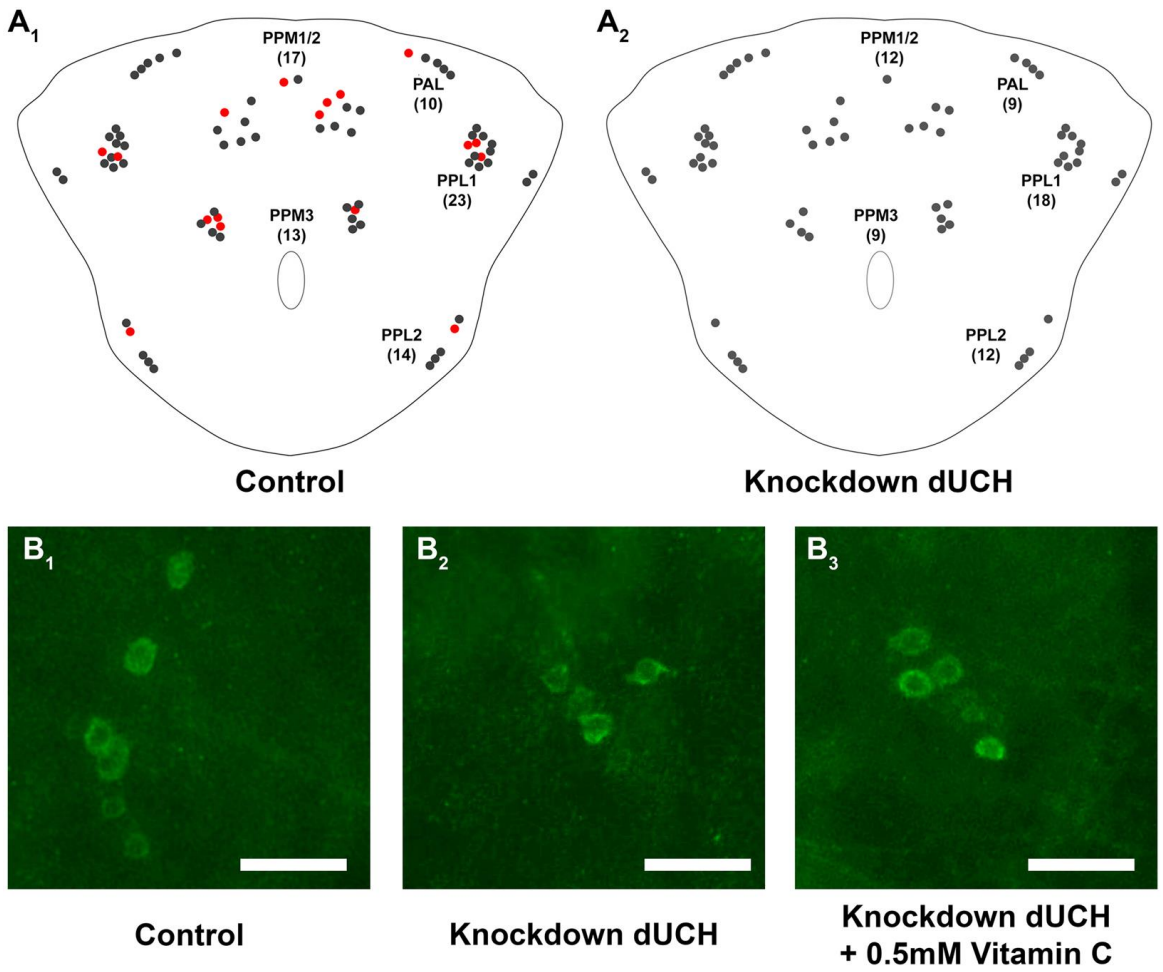

Figure 4. The degeneration of DA neurons in the dUCH knockdown adult brain. (A) A schematic representation of DA neuron clusters in adult fly. (B) Representative images show DA neuron clusters in the adult fly brain immunostained with the anti-TH antibody (TH, green). Driver control flies (+; +; TH-GAL4/+) are shown in the panel B1, the dUCH knockdown flies (+; +; TH-GAL4/UAS-dUCH-IR) in the panel B2 and the VitC treated dUCH knockdown flies was showed in panel B3.

integrity. A significant reduction in climbing ability began in 25-day-old flies and most DA neuron clusters (PPM2, PPM3, and VUM) exhibited degeneration in 20- to 30-day-old flies. These events perfectly matched the marked reduction observed in dopamine levels in 25-day-old dUCH knockdown flies with a fold difference of 18% to 8.1% in driver control flies. The reduction in dopamine in dUCH knockdown flies suggested a relationship between DA neuron impairments by the dUCH knockdown and locomotor deficits. These results may be modeled as a reduction in dUCH causing impairments in DA neurons, which result in decreases in dopamine levels followed by dysfunctional locomotor behaviors.

4. CONCLUSION AND PERSPECTIVES

UCH-L1 is a protein that has been implicated in the pathogenesis of cancer, diabetes, and neurodegenerative diseases, particularly PD. However, the role of UCH-L1 is still being investigated. With a close link to PD from clinical features to genetic factors, UCH-L1 is regarded as an interesting target that has attracted the attention of many scientists. Since *Drosophila* possesses many useful features, it is utilized as a powerful model of PD for genetic study and drug screening. These useful features include: 1) the reproduction of neuropathological and clinical features; 2) the conservation of basic biological

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processes and PD-related genes; 3) the availability of genetic tools for gene manipulation and 4) a short life cycle. The *Drosophila* model has been used to investigate the role of UCH-L1 in PD. The tissue-specific knockdown of human homologue UCH-L1 in the fly (dUCH) resulted in phenotypic abnormalities in locomotion in the larval and adult stages. dUCH knockdown L3 larvae exhibited a decline in crawling ability that appeared to be a consequence of a lack of DL1 DA neurons in the larval central nervous system. The defect in climbing ability is a progressive decline during the course of aging due to DA neuron degeneration. This process mimics important symptoms and pathogenic events in PD patients, thereby demonstrating that the dUCH knockdown fly has potential as a model for studying the pathogenesis of PD. The prevalence of PD increases with aging in dUCH knockdown flies, which indicates that the dUCH knockdown fly displays the epidemiological characteristics of PD and also that it is not only a suitable model for studying the pathogenesis of PD, but also a promising model for investigating its epidemiology.

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