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Research article

Expression of human Ras-related protein Rab39B variant T168K in *Caenorhabditis elegans* leads to motor dysfunction and dopaminergic neuron degeneration

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ABSTRACT

Human RAB39B gene is related to familial early-onset Parkinson disease. In early adulthood, men with the RAB39B c.503C > A (Thr168Lys, p. T168K) mutation develop typical tremor, bradykinesia, and alpha-synuclein accumulation. We investigated the pathological mechanism of RAB39B T168K in a *Caenorhabditis elegans* model. In early adult *C. elegans*, RAB39B T168K led to dopaminergic neuron degeneration that presented as disrupted dendrites and blunt neuronal cells. Abnormal dopamine secretion was inferred from a decline in motor function and a positive basal slowing phenotype. Dopamine-associated tests confirmed that synthesis and recycling of dopamine were normal. The RAB39B T168K mutation might impair dopamine vesicular transmission from the presynaptic membrane to the synaptic gap in dopaminergic neurons. The release-dependent feedback mechanism in neurotransmitters regulates the balance of receptor activities. Protein-protein interactions network analysis revealed that RAB39B may also function in lysosomal degradation and autophagy. Impaired disposal of misfolded α-synuclein eventually leads to protein aggregation. Thus, like other members of the Rab family, RAB39B may be involved in vesicular transport associated with dopamine secretion and α-synuclein clearance.

1. Introduction

The human RAB39B gene encodes a neuronal-specific small monomeric GTPase of the Ras GTPase superfamily [1]. Because the Ras superfamily has vital functions in intracellular vesicle trafficking [2], Rab39B is suggested to be an important protein in vesicular transport. Rab39B localizes at the Golgi and Golgi-related compartment. Mutant Rab proteins are associated with multiple central nervous system disorders and multifactorial diseases [3,4]. Most Rab mutations are associated with overall cellular dysfunction in vitro [5,6]. To date, most Rab39B mutations have been associated with mental retardation, intellectual and developmental disability, and epilepsy [7–10]. Carriers with mutations near the C-terminus are more likely to develop parkinsonism symptoms [11]. Wilson et al.

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reported that the missense mutation RAB39B c.503C > A (Thr168Lys, p. T168K) was a virulence gene in X-linked, early onset Parkinson's disease (PD) [12]. Furthermore, male patients with duplicated RAB39B mutations also exhibit a PD phenotype. Thus, RAB39B mutations may present a dominant negative effect [13]. Male individuals with this mutation develop typical tremor and bradykinesia in early adulthood, and most have good response to levodopa. In addition, brain autopsies show that patients with RAB39B T168K had typical PD pathological changes. Interestingly, a recent study showed that females with missense mutation RAB39B develop later-onset parkinsonism without intellectual disability, suggesting a link of heterozygous RAB39B mutation to PD.

Studies in vitro showed that Rab39B was localized with the cis-Golgi, Golgi complex, VAMP4, STX16, STX13, and RAB11, which are all essential in the *trans*-Golgi network and the endosome recycling network [14–16]. Rab39B also assists in transporting the GluA2-AMPAR subunit for membrane protein balance [17], and Rab39B is a cargo recognition site in myosin Va in the post-Golgi secretory system. In all, Rab39B has a key function in the regulation of intracellular vesicle trafficking [18,19]. However, the function of Rab39B in membrane transport is not fully understood.

Compared to vertebrates, *Caenorhabditis elegans* has a remarkably similar dopamine system and a RAB39B ortholog. Thus, we used *C. elegans* as a model to examine whether the heterozygous T168K mutation induce PD-related phenotypes, we expressed human wild type and mutant RAB39B T168K in *C. elegans* dopaminergic neurons. The transgenic *C. elegans* showed early neuronal degeneration. Dopamine-related behavior assays confirmed a disturbance in dopamine signaling, which was only partly rescued by exogenous, but not endogenous, dopamine. Other behavior assays revealed disturbed vesicular release from the presynaptic membrane and a release-dependent feedback mechanism of neurotransmitters.

2. Materials and methods

2.1. Molecular biology

Caenorhabditis elegans (*C. elegans*) was used as model organism, and all the methods followed tenants of the Declaration of Helsinki.

2.1.1. C. elegans strains and plasmid construction

Cultures of *C. elegans* strains and microinjection were performed according to standard methods. *Escherichia coli* strain OP50 was precultured overnight at 37 °C after spreading on the surface of *C. elegans* growth medium. *C. elegans* were then cultured on the prepared medium in a 20 °C incubator. *C. elegans* strains N2, UA57, CB111 , CB112 , KP1097 , RM2072 , LX645, and LX702 used in the behavior assays were purchased from the Caenorhabditis Genetic Center (Minneapolis, MN, USA; Table 1).

Transgenic *C. elegans* were created by microinjection. We added the dat-1 promoter to the *C. elegans* expression plasmid pSM to ensure that the target gene was expressed in only dopaminergic neurons. The wild type RAB39B gene (642bp) was inserted into the plasmid between BamHI and KpnI sites adjacent to the dat-1 promoter. Then, the plasmid containing RAB39B was subjected to PCR mutagenesis to create the T168K mutation. We co-injected the plasmid mix containing both pSM-RAB39B gene and neural-specific red fluorescein (mCherry) into the gonad of *C. elegans* N2 to generate transgenic *C. elegans* with a model marker. Single worm PCR and western blot were used to test the expression of RAB39B (Fig. 1 D). UA57 strain, with dat-1 promoter and fused GFP protein was used to observe the dopaminergic neurons directly by fluorescence in an optical microscope.

2.1.2. Behavior assays

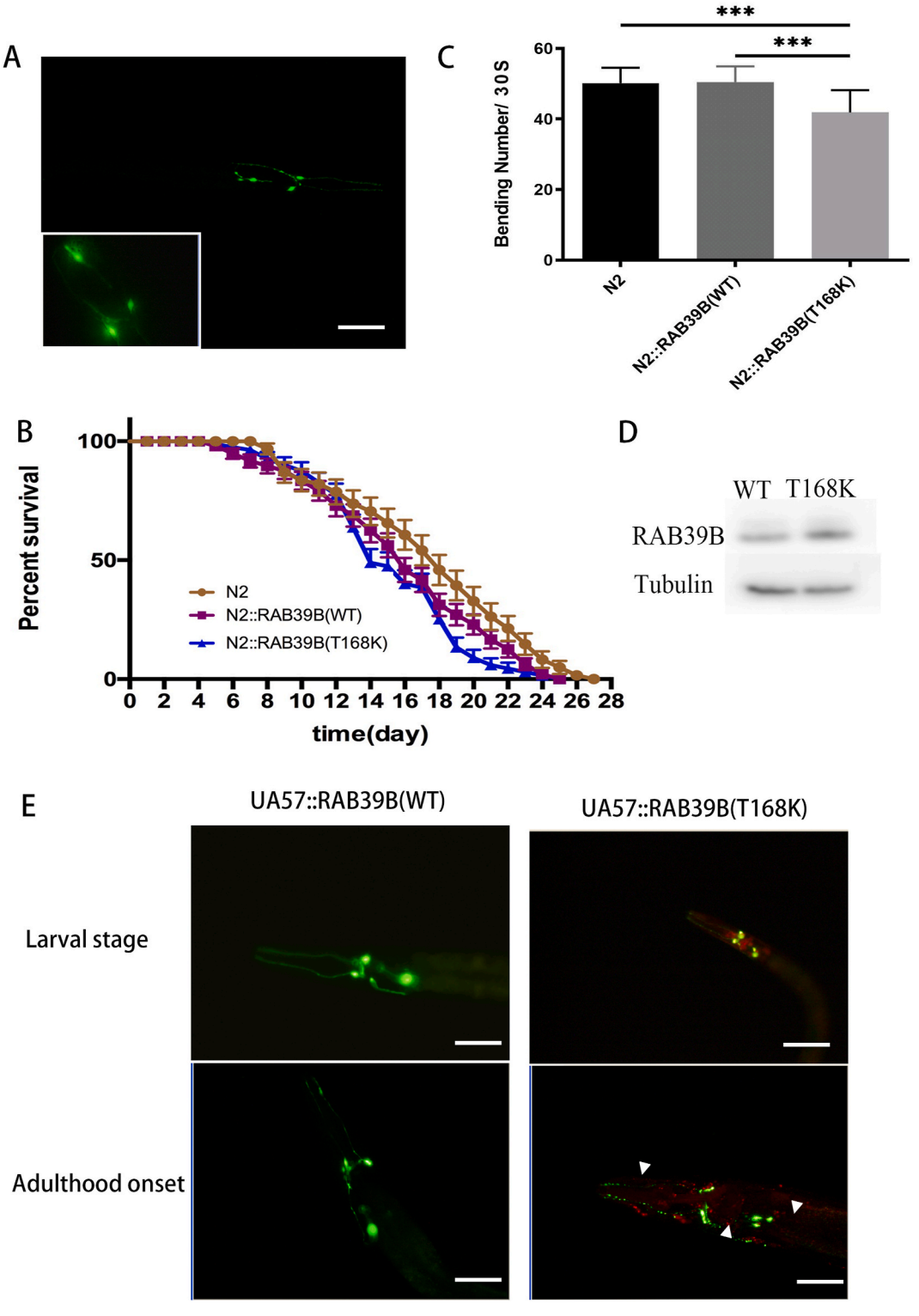
All behavior experiments with *C. elegans* were consistent and conservative. All behavior tests focused on function of dopamine in the synaptic gap.

Basal slowing response is a modest slowdown in response to food used as a behavior test for *C. elegans*. The behavior is mediated by a dopamine-containing neural circuit that senses a mechanical attribute of food (*E. coli*). This basal slowing response is an adaptive mechanism to increase the amount of time animals spend in the presence of food. Movement in the environment is inversely related to the amount of nearby food.

Swimming-induced paralysis (SWIP) is a specific experiment to test the function of presynaptic dopamine transporter receptor. Loss of function mutations in the DAT-1 gene that encodes DAT-1 receptor result in a rapid paralysis when animals are placed in M9 buffer. Loss of function mutations in genes that support dopamine biosynthesis, dopamine vesicular packaging, and dopamine action at the extra-synaptic D2-type dopamine receptor DOP-3 suppress SWIP in dat-1 animals, consistent with paralysis arising from excessive dopamine signaling.

Table 1
C. elegans strains' genotype and phenotype list.

Strains	Genotype	Phenotype
N2	Wild type	Normal
UA57	bals 4 [dat-1p:GFP + dat-1p:CAT2]	GFP expression in DAnergic neurons, easy to observe dopamine neurons with a microscope
CB111	△cat-1: [cat-1 deletion]	Deletion of cat-1 gene, lack of endogenous dopamine synthesis, exhibited dopamine deficiency phenotypes.
CB112	△cat-2: [cat-2 deletion]	Deletion of cat-2 gene, lack of Carnitine acetyl-CoA transferase, exhibited dopamine deficiency phenotypes
LX645	△dop1: [dop-1 deletion]	Deletion of D-1 like receptors.
LX702	△dop3: [dop-3 deletion]	Deletion of D-2 like receptors.
KP1097	△dgk-1: [dgk-1 deletion]	Deletion of Diacylglycerol kinase (dgk-1), hypersensitive to aldicarb.
RM2072	△dat1: [dat-1 deletion]	Deletion of Dopaminergic transporter (DAT), easy to paralysis in SWIP test.



(caption on next page)

Fig. 1. (A) Image of transgenic UA57RAB39B (WT) *C. elegans* by fluorescence microscopy. Six anterior dopamine neurons in the head show green fluorescence under optical imaging (two anterior dendrites and four cephalic neurons) (Scale bar = 75μm). (B) Life span of N2, N2RAB39B(WT), and N2RAB39B(T168K). ($n > 65$ per strains; Log-Rank test; $^{**}p < 0.01$) (C) Bar graph of body bend assays (in 30 s) of N2, N2RAB39B(WT), and N2RAB39B(T168K). (mean ± SD; $n \geq 53$ per strains; ANOVA analysis; $^{***}p < 0.001$). (D) Western blot was used to test the expression of RAB39B. The protein α tubulin was used as a loading control. (E) Dopaminergic neurons visualized by fluorescence microscopy of UA57RAB39B (WT) and UA57RAB39B (T168K). Picture showed blebbing/discontinued dendrites and circinal cell body in adulthood onset stage (Arrows). ($n \geq 30$ per strains). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Aldicarb-induced paralysis is an experiment to determine defects in synaptic transmission. Acetylcholine released from cholinergic motor neurons activates acetylcholine receptors on the muscle membrane, leading to muscle excitation and contraction. Acetylcholinesterase hydrolyzes acetylcholine in the synaptic cleft and terminates neurotransmission. The accumulation of acetylcholine in the synaptic cleft leads to sustained muscle activation and eventually paralysis.

Age-matched synchronized *C. elegans* were used for bioactivity assays. Synchronized 2- and 5-day-old *C. elegans* were cultured for analysis of dopaminergic neuron degeneration. All observations were performed with epi-fluorescence microscopy or confocal microscopy (Leica SP5II). The anterior dopaminergic neurons in the head consisted of two anterior dendrites and four cephalic neurons. A degenerative phenotype of dopaminergic neurons was considered as blebbing dendrites, disrupted dendrites, rounded cell body, and loss of neurons.

At least 50 *C. elegans* per group were randomly selected for life-span and bending assays. In the life-span assay, *C. elegans* were fed ample *E. coli* after being synchronized. The viability and mortality numbers were counted every day until no *C. elegans* remained. Death was defined as a halt of food intake or non-responsiveness to gentle mechanical touch. The body bending frequency in a 30-s period in liquid medium was recorded. A bend was considered from mid-body and return to original position.

C. elegans prepared for swimming induced paralysis (SWIP), basal slowing, and aldicarb paralysis assays were washed in M9 buffer to remove *E. coli* from their surface. *C. elegans* were placed in culture medium with or without food to measure their mobility. In the SWIP assay, *C. elegans* were placed in M9 buffer in 96-well plates and the paralysis rate was measured within 10 min. In the dopaminergic neuron resistance assay, *C. elegans* were placed on agar plates containing 40 mM dopamine, and the spontaneous movement ability was measured within 20 min. In the aldicarb paralysis assay, *E. coli* was mixed with an aldicarb solution to 1 mM and spread on the surface of growth medium. *C. elegans* were transferred to plates containing aldicarb and the paralysis rate was measured within 120 min.

Dopamine solution was prepared 1 h just before use, protected from light, and used within 4 h (Dopamine hydrochloride, Sigma-Aldrich). The dopamine was mixed at various concentrations with *E. coli* and spread on the culture medium.

3. Statistics

All data were analyzed using SPSS Statistics 18 software. Survival data were analyzed by the Kaplan-Meier method (log-rank test). One-way analysis of variance (ANOVA) was followed by a Tukey's post hoc test for statistical comparisons. Significance of difference was determined on the basis of a $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ respectively).

4. Results

4.1. RAB39B T168K leads to motor dysfunction and dopaminergic neuron degeneration in C. elegans

Men carriers of the RAB38B T168K mutation exhibit early-onset parkinsonism symptoms with typical tremor and bradykinesia. *C. elegans* could be observed directly in an optical microscope as fluorescent proteins were tagged into dopaminergic neurons (Fig. 1A). To investigate the effect of the T168K variant, we assessed development and behavior of transgenic *C. elegans* that expressed wild type and mutant T168K RAB39B (*Homo sapiens*) genes in dopaminergic neurons. Compared with *C. elegans* that expressed RAB39B wild type, we did not detect any difference in development and reproduction in *C. elegans* that expressed the T168K mutant. The median life spans of N2, N2RAB39B(WT), and N2RAB39B(T168K) were 18, 16 and 14 days. The average body bends in 30s were 50.17 for N2, 51.56 for N2RAB39B(WT) and 41.88 for N2RAB39B(T168K). Results revealed that RAB39B T168K *C. elegans* had a slightly diminished life-span, with an accelerated death rate in the late one-third of their life-span (Fig. 1B). In addition, adult *C. elegans* showed a mild decline (~20%) in motor function in bending ability in liquid medium (Fig. 1C).

We examined by epi-fluorescence microscopy the morphology of dendrites and cephalic cell body of dopaminergic neurons. In the early adult stage, 2 days after hatching, blebbing appeared along the dendrites from head to nose. At day 5, dendrites became discontinuous with suspended fluorescent light along the path. The cell bodies of dopaminergic neurons became circular instead of fusiformis, and the edges of the cells faded. In sum, we observed overall degeneration from dendrites to cell body (Fig. 1E). The results indicated that four dendrites were more vulnerable than cephalic cell body. Thus, RAB39B T168K *C. elegans* exhibited both motor dysfunction and obvious dopaminergic neuronal degeneration at an early adult stage.

4.2. Endogenous dopamine supplementation did not reverse insufficient dopamine supply in synaptic gap

The major direct cause of parkinsonism symptoms is lack of sufficient dopamine in the central nervous system. Thus, we assessed further the relationship between dopamine supply and motor dysfunction in the transgenic *C. elegans*. First, we used the basal slowing

test to assay the basic dopaminergic supply in RAB39B T168K *C. elegans*. Dopaminergic receptors in the postsynaptic membrane act antagonistically in mobility of the *C. elegans*. Activation of the D1-like receptor (encoded by dop1 gene in *C. elegans*) leads to activation of locomotion, food sensation, and learning. The D2-like receptor (encoded by dop3 gene) acts in the opposite manner. As a result, *C. elegans* LX702 (dop3 deletion) had a positive basal slowing phenotype, whereas LX645 (dop1 deletion) *C. elegans* did not exhibit this phenotype.

Wild type (N2) and RAB39B WT-expressing *C. elegans* had decreased motor ability while food was abundant. But RAB39B T168K *C. elegans* had a positive basal slowing phenotype, with no difference in activity with or without food (Fig. 2A). Then, we tested *C. elegans* with an endogenous dopamine supply in the basal slowing assay. The *C. elegans* CAT-2 gene is responsible for synthesis of dopamine in dopaminergic neurons; co-expression of CAT-2 and RAB39B T168K in dopaminergic neurons increased the endogenous dopaminergic level. The elevated endogenous dopamine did not rescue the basal slowing phenotype, and exogenous dopamine only partly rescued (Fig. 2B and C). These results implied that the increased endogenous level of dopamine in the dopaminergic neurons did not increase the amount of effective dopamine in the synaptic gap. In the basal slowing trial with food, RAB39B T168K *C. elegans* slowed on media containing dopamine; thus, the basal slowing phenotype was partly rescued by increasing dopamine directly to synapses. This rescue may have occurred because exogenous dopamine increased dopamine concentration in synapses and then continued the signaling by dopaminergic receptors to conduct motor activity. Thus, we hypothesized that RAB39B T168K affected dopaminergic vesicular release or dopamine recycling in the presynaptic membrane.

4.3. Relationship between RAB39B T168K and dopamine receptors in pre- and postsynaptic membrane

We have shown that RAB39B T168K inhibited the dopamine pathway. Because the mutant gene was expressed only in dopaminergic neurons, theoretically, receptors in the postsynaptic membrane would not be affected. The mutant protein may have affected only the release/transport/recycling of dopamine. Recycling of dopamine back to dopaminergic neurons depends on DAT receptors. Thus, we tested *C. elegans* with the swimming induced paralysis (SWIP) test. The SWIP phenotype of *C. elegans* occurs with dat-1-deficient, dopamine recycles receptor-deficient mutants. In M9 buffer, *C. elegans* must exert maximal motor activity, which demands efficient dopamine clearance mediated by the dopamine transporter. Normal *C. elegans* exhibits paralysis within 10 min after swimming in M9 buffer. Mutant *C. elegans*, such as DAT receptor deletion strain RM2702, would become paralyzed because dopamine had a sharp increase in synaptic gap. RAB39B T168K did not become paralyzed compared with the positive control RM2702. (Fig. 3A). These findings indicated that the forced swimming behavior did not lead to abnormal accumulation of dopamine in synapses because the function of DAT receptors was normal in RAB39B T168K *C. elegans*.

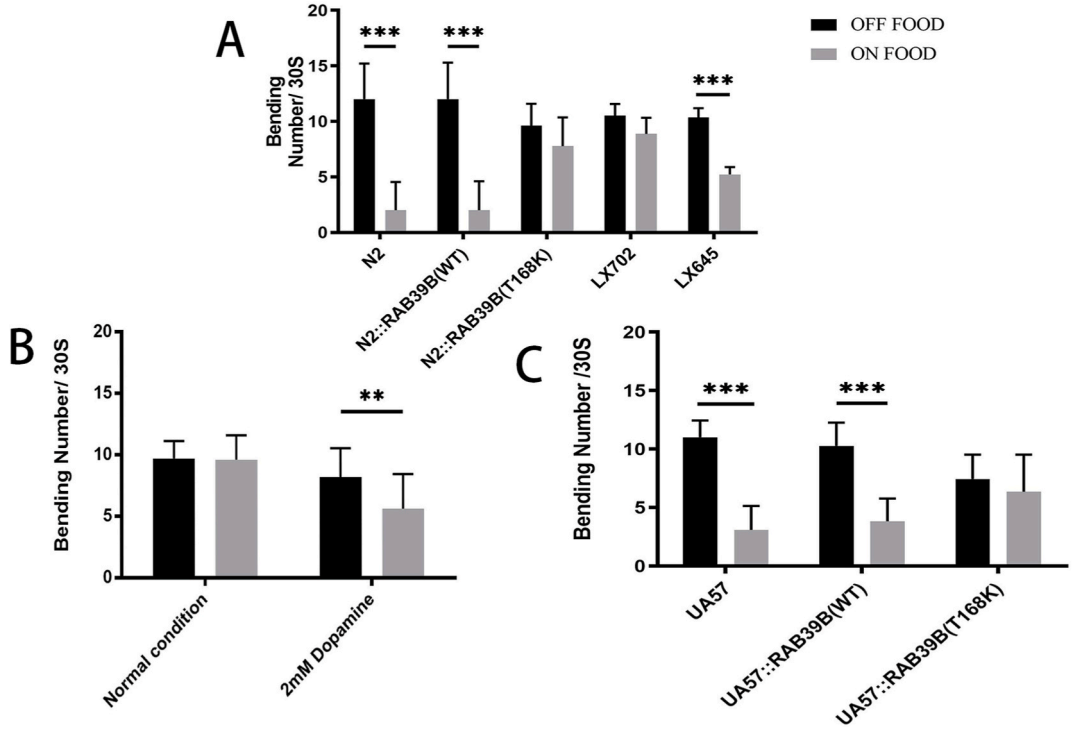


Fig. 2. Bar graphs of basal slowing assays (nematode bends in 30 s, with or without food). (A) Basal slowing assays of N2, N2RAB39B(WT), N2RAB39B(T168K), LX702, and LX645. (B) Basal slowing phenotype of N2RAB39B(T168K) mutant *C. elegans* after 2 mM dopamine treatment. (C) Basal slowing assays of UA57, UA57RAB39B(WT), UA57RAB39B(T168K). (All graph date are presented mean ± SD; n ≥ 30 per conditions/strains; paired Student's t-test; ***p < 0.001; **p < 0.01).

C. elegans has a complete dopamine synthesis, secretion, and recycling system, the same system as in vertebrates. The cat-2 gene is responsible for the synthesis of dopamine and the dat-1 gene is responsible for dopamine recycling. Different food circumstances lead to movement patterns, driven by the balance of synaptic post-membrane D1/D2 receptors. Whether there is sufficient dopamine in synaptic gaps can be judged by comparing movement patterns using different behavior experiments. UA57RAB39B(T168K) *C. elegans* can increase the synthesis of dopamine in cells as it overexpressed cat-2 in RAB39B(T168K) *C. elegans*. However, the increased of intracellular dopamine did not increase dopamine in the synaptic gap, which indicated that dopamine secretion might have been blocked in the presynaptic membrane or that dopamine recycling was overactive from gap back to cell. Although results from SWIP indicated a normal recycling system, the results suggested that Rab39B may be responsible for transport or release of dopamine vesicles from the pre-membrane of dopaminergic neurons.

In addition, in the dopamine resistance test, *C. elegans* that expressed RAB39B (T168K) protein were more likely to become paralyzed in the presence of a high concentration of dopamine (Fig. 3B). The positive dopamine resistance paralysis suggested an overactive state of D1 receptors, which was illustrated by comparing the performance of the dop-1 deletion strain (LX645), dop-3 deletion strain (LX702), cat-2 deletion strain (CB112), and cat-1 deletion strain (CB111). Because the mutant protein was expected to be expressed only in dopaminergic neurons, the result indicated an abnormal balance between D1-D2 receptors in the postsynaptic membrane. Conversely, LX702RAB39B (T168K) *C. elegans* showed a semi-paralysis phenotype caused by lack of dopamine, and this phenotype was reversed by postsynaptic receptor adjustment.

4.4. Effect of RAB39B T168K on acetylcholine in C. elegans

A positive aldicarb paralysis test confirmed vesicular release dysfunction in *C. elegans*. Aldicarb, a cholinesterase inhibitor, prevents hydrolysis of acetylcholine in the synapse. Acetylcholine released from cholinergic motor neurons activates acetylcholine receptors on the muscle membrane, causing muscle excitation and contraction. Acetylcholinesterase hydrolyzes acetylcholine in the synaptic cleft, thereby terminating neurotransmission. The accumulation of acetylcholine in the synaptic cleft causes sustained muscle activation and eventually paralysis. The dgk-1 gene encodes a diacylglycerol kinase, which regulates the formation and release of the synaptic vesicles. Deletion of dgk-1 (KP1097) led to hypersensitivity to aldicarb. As an excitatory neurotransmitter to dopaminergic neurons,

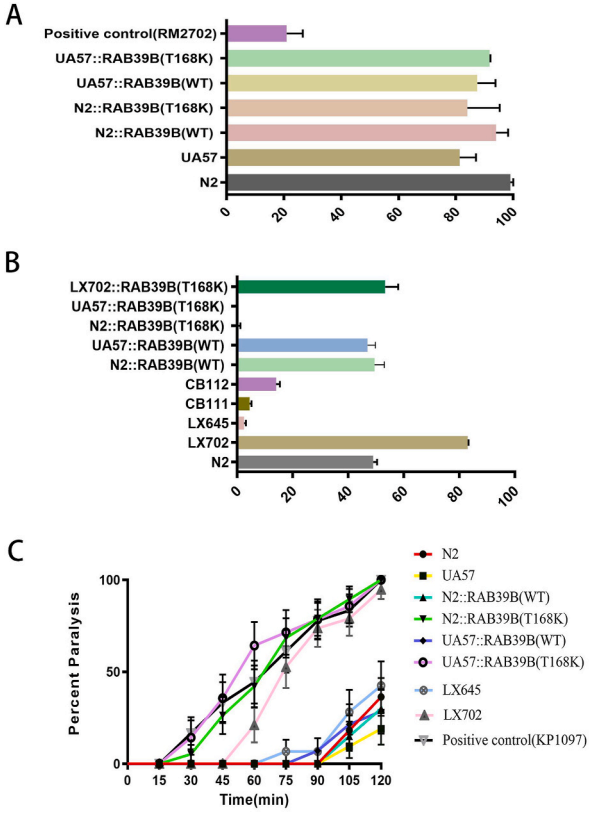


Fig. 3. (A) Bar graph of Swimming induced paralysis (SWIP) assays. Strains N2, UA57, positive control RM2702, N2RAB39B(WT), N2RAB39B (T168K), UA57RAB39B(WT), UA57RAB39B(T168K) were used. Graph shows percent of paralysis rate in SWIP within 10 min ($n \geq 76$ per strains) (B) Bar graph of Dopamine resistance assays. Graph shows percent of *C. elegans* that did not become paralyzed within 20 min after high concentration dopamine (40 mM) exposure. ($n \geq 68$ per strains) (C) Survival analyses graph of aldicarb paralysis assays. Graph shows precent of *C. elegans* that become paralyzed after exposure to aldicarb. ($n \geq 28$ per strains).

dopamine also affects motor function. And unbalanced expression of dopaminergic receptors in the postsynaptic membrane affects acetylcholine release. RAB39B T168K *C. elegans* treated with aldicarb exhibited a faster paralysis, which meant that the mutant animals had diminished synaptic transmission. Half of the RAB39B T168K *C. elegans* were paralyzed within 66 min. This paralysis rate was similar to the positive control diacylglycerol kinase deletion strain (KP1097) and D-2-like receptor strain (LX702) (Fig. 3C).

5. Discussion

The small Rab GTPase Rab39B is associated with development of PD [20]. Previous in vitro studies showed that Rab39B localized to the Golgi, early and recycling endosome, and the plasma membrane [15,21]. This small protein has a crucial function in intracellular transport. The RAB39B T168K variant can lead to X-linked recessive early-onset PD, with typical pathological changes in dopaminergic neurons. In addition, the mutant protein is suggested to have effects beyond simply a loss of function [22]. In this study, we found that RAB39B T168K expressed in *C. elegans* was associated with dopaminergic neuron degeneration. We used dopamine-related behavior tests to identify a cause for this degeneration. Any impediment in synthesis, transport, or secretion of dopamine in *C. elegans* would have had an adverse effect on dopamine signaling to downstream cells.

5.1. RAB39B T168K caused insufficient release of dopamine

Related studies have shown that disrupting dopaminergic signaling by mutations linked to PD can change the locomotor behavior of *C. elegans* [23]. Our study indicated that, in RAB39B T168K *C. elegans*, expression of the mutant gene only in dopaminergic neurons significantly decreased the thrashing frequency of *C. elegans* in liquid medium, whereas the wild type RAB39B gene did not produce any effect. In the *C. elegans* model, we expected postsynaptic membrane function to be normal because the mutant gene was expressed only in dopaminergic neurons. We supposed that RAB39B T168K may cause a deficiency of dopamine in the synaptic gap by disrupting dopamine synthesis, packaging into vesicles, transport in the cell body, or vesicle release from the presynaptic membrane. Moreover, exogenous, but not endogenous, dopamine rescued the basal slowing phenotype, which also indicated that reduced dopamine release from the dopaminergic neurons was responsible for this abnormal behavior. Because RAB39B was also reported as a cargo combination site in myosin, RAB39B might be responsible for protein transport form the nucleus to the plasma membrane, associated with dopamine vesicle transport to the end of dendrites.

Sawin et al. showed that a defective basal slowing response was associated with CAT-1 and CAT-2 mutations in *C. elegans*. CAT-2 is a tyrosine hydroxylase homologue, and CAT-1 is a vesicular monoamine transporter 2 (VMAT2) homologue [24]. VMAT2 mediates the packaging of dopamine into synaptic vesicles and the traffic from the cell body to nerve terminals in a UNC-104-dependent mechanism. The absence of UNC-104, a kinesin motor protein, may lead to CAT-1 vesicle retention in the cell body and a decrease in synaptic signaling. We then supposed that mutant Rab39B may reduce dopamine release by affecting VMAT2-mediated vesicle transport. However, the mechanism needs to be identified by determining the relationship between RAB39B and CAT-1/2.

5.2. Negative swimming induced paralysis phenotype of RAB39B T168K C. elegans

Dopamine transporter DAT-1, expressed in *C. elegans* dopaminergic neurons, is responsible for re-uptake of synaptic dopamine and limiting dopamine spillover to extra-synaptic sites. When *C. elegans* exerted maximal physical activity in M9 buffer, a SWIP phenotype emerged in DAT-1-deficient *C. elegans* because of over-activation of dop-3 by an excessive amount of dopamine. The negative SWIP phenotype in Rab39B-expressing *C. elegans* seemingly reflected an intact DAT-1 function. However, Hardaway et al. found that the SWIP phenotype of DAT-1-deficient *C. elegans* could be rescued when vesicular stores of dopamine were depleted by reserpine [25]. We then concluded that there were relatively enough DAT-1 receptors on pre-synaptic membranes compared to reduced level of dopamine.

5.3. Lack of downstream dopamine stimulated acetylcholine release upstream by release-dependent feedback

DOP-1 and DOP-3 receptors are co-expressed in the motor neurons of ventral cord and junction [26]. These two receptors antagonistically modulate locomotion of the *C. elegans* [27]. Dopamine acts through the D2-like receptor DOP-3 to inhibit locomotion in response to food, which enables *C. elegans* to remain in a food-rich environment. DOP-3 signaling also inhibits acetylcholine release, whereas DOP-1 signaling enhances acetylcholine release; these opposing activities indicate a coordinating mechanism among all neurotransmitters [28]. Although massive exogenous dopamine inhibited locomotion in *C. elegans* that expressed RAB39B T168K, we concluded that dop-3 receptor mutants, or mutants in postsynaptic membrane related to dop-3 receptor signaling, were resistant to dopamine paralysis. In addition, mutations in glr-1/and ace/1 cause dopamine resistance; glr-1/and ace/1 encode an AMPA-type glutamate-gated cation channel subunit and Class A acetylcholinesterase, respectively [29]. Together with the fact that the RAB39B T168K mutant *C. elegans* model expressed the mutant protein only in dopaminergic neurons, we assumed that there is a neurotransmitter feedback mechanism in synapses [30,31].

Although the RAB39B T168K mutation would reduce the level of dopamine in the synaptic gap, *C. elegans* had only mild motor dysfunction in the early adult-stage. This finding may be explained by a cell-autonomous-compensatory mechanism. The finding also demonstrates that the effect of the mutation was more than just a loss-of-function mechanism. Because dopamine and acetylcholine function as neuromodulators in the central neural circuity to balance the bending activity of *C. elegans*, the lack of sufficient dopamine may stimulate cells upstream of dopaminergic neurons to release more dopamine for partial compensation. As a result, more

acetylcholine was needed to activate the dopaminergic neurons, which resulted in the positive aldicarb paralysis behavior test. Conversely, the lack of dopamine secretion may lead to hypersensitivity to dopamine of postsynaptic cells, which may explain why *C. elegans* that expressed the mutant protein were easy to paralyze with a hyper-exogenous dopamine exposure.

5.4. RAB39B's function in dopaminergic neuron degeneration

Several studies showed that the accumulation of cytosolic dopamine can be neurotoxic by generating reactive oxygen species and/or quinones. The reduced vesicular release of dopamine and accumulation of intracellular dopamine would cause progressive nigrostriatal neurodegeneration [32]. Cytosolic dopamine will be sequestered by VMAT2 in small synaptic and dense core vesicles for subsequent release [33]. Released dopamine will be taken back into dopaminergic neurons by dopamine transporters, which represents another source of cytosolic dopamine. Kanaan et al. found that increased DAT, VMAT, and DAT/VMAT ratios were associated with increased 3-nitrotyrosine in aging rhesus monkey dopaminergic neurons [34]. The increased functional DAT/VMAT ratios may have been responsible for dopaminergic neuron degeneration, which would be consistent with our results. Conversely, in the protein-protein interaction network analysis, nine genes were divided into two major categories: (1) formation and fusion of transport vesicles and (2) autophagy and α-synuclein homeostasis. RAB8A an regulate intracellular membrane trafficking, from the formation to fusion of md MYO5Vembranes of transport vesicles. Here we proved that RAB39B (T168K) determined dopaminergic neurons degeneration. Further, because RAB39B is highly enriched in brain neurons with a typical phenotype of synaptic loss and Lewy body pathology in vivo, RAB39B may have broader pathological activities in PD. RAB39B is involved in regulating the intracellular trafficking of vesicles, particularly the transport and recycling of synaptic vesicles in neurons. Specifically, the RAB39B(T168K) mutant may interfere with the normal recycling of synaptic vesicles in neurons, which is necessary for proper neurotransmission. In this study, all these results revealed that the RAB39B T168K mutation interrupts DA signaling, most likely in the process of vesicular release from the pre-synaptic membrane. It also interacts with proteins involved in vesicle trafficking, such as KIF1B and MYOV. Indicating that RAB39B can bind cargo proteins to trafficking-related proteins. On the other hand, the level of α-synuclein was reduced with RAB39B silencing in vitro, which indicated a different pathological change in RAB39B (T168K) mutant individuals. And the cargo proteins related to RAB39B might go far beyond neurotransmitters. The specific mechanism by which RAB39B regulates the docking and fusion of synaptic vesicles with the presynaptic membrane needs more experiments.

There are several limitations in the present study. First of all, *C. elegans* have endogenous RAB39B. Thus, the model *C. elegans* created in the present study own human mutated RAB39B(T168K) and endogenous *C. elegans* RAB39B. In this regards, the current results may only recapitulate the phenotypes in female patients with heterozygous T168K mutation. It should be noticed that RAB39B (T168K) is mainly associated with an X-linked PD. Therefore, future study is warranted to conducted in RAB39B knockout *C. elegans* strains to address the underlying mechanisms by which RAB39B(T168K) mediates X-linked PD.

6. Conclusion

The RAB39B T168K mutation, at least in a heterozygous form, can specifically lead to dopaminergic neuron degeneration by disturbing the release of vesicles. RAB39B may be involved in vesicular release from the pre-synaptic membrane. Overall, the understanding RAB39B in PD is still in its infancy and more investigations are needed.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yixuan Zeng: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Funding acquisition, Formal analysis. **Tengteng Wu:** Visualization, Investigation, Data curation. **Fengyin Liang:** Software, Resources, Methodology. **Simei Long:** Validation, Project administration, Methodology. **Wenyuan Guo:** Methodology, Investigation, Formal analysis. **Yi Huang:** Software, Resources. **Zhong Pei:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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