

VPS35 Protects Against *TMEM230*-Mutation-Induced Progressive Locomotor Deficits in *Drosophila*

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Abstract

Background

Recently, four Parkinson's disease (PD)-linked mutations (*Y92C*, *R141L*, *184PGext*5* and *184Wext*5*) in transmembrane protein 230 (*TMEM230*) were identified in PD patients, and these mutations have implications in protein trafficking and neurodegeneration. However, there is a lack of in vivo studies on the roles of PD-related variants of *TMEM230* in PD pathogenesis.

Methods

In this study, we generated human wild-type (WT) and mutant *TMEM230* (*Y92C*, *R141L*, *184PGext*5* and *184Wext*5*) transgenic *Drosophila* using isoform α cDNA.

Results

We found that the expression of *TMEM230 184PGext*5* in pan-neurons or dopaminergic neurons in *Drosophila* induced PD-like phenotypes, which included impaired locomotor ability, a shortened lifespan, reduced TH levels, and increased phosphorylated JNK and cleaved caspase-3 levels. Moreover, rotenone, a common pesticide, enhanced *TMEM230-184PGext*5*-induced PD-like phenotypes. In contrast, the overexpression of wild-type (WT) *VPS35* rescued *TMEM230-184PGext*5*-induced PD-like phenotypes, while the knockdown of *VPS35* by RNA interference (RNAi) or the expression of mutant *VPS35 D620N* worsened PD-like phenotypes.

Conclusion

These results indicate that *VPS35*, as a downstream effector of *TMEM230*, plays a critical role in *TMEM230*-linked JNK/caspase-3 signalling pathways and that mutations in *TMEM230* and *VPS35* disrupt these pathways, resulting in dopaminergic neurodegeneration and PD-like phenotypes. These findings provide novel insight into the molecular mechanisms of mutant *TME230*- and *VPS35*-induced abnormalities underlying the pathogenesis of PD.

Background

Parkinson's disease (PD) is the second most common neurodegenerative disease, with classic motor features of resting tremor, rigidity, bradykinesia and postural instability. Selective loss of dopaminergic neurons (DNs) in the substantia nigra pars compacta (SN) and the presence of Lewy bodies that include insoluble α -synuclein aggregates are hallmarks of PD pathology. A total of 5–10% of PD patients suffer from a monogenic form of the disease with Mendelian inheritance. However, the underlying mechanism of PD pathogenesis remains largely unknown[1].

Recently, a new PD-related gene, transmembrane protein 230 (*TMEM230*), was identified in PD patients [2]. Four PD-linked *TMEM230* mutations were identified [2], including *Y92C*, *R141L*, *184PGext*5* (replacing

the stop codon with proline and glycine, thus adding seven amino acids, PGHPPHS, to the C-terminus) and *184Wext*5* (replacing the stop codon with six amino acids, WHPPHS). *Y92C*, *R141L* and *184Wext*5* are found in North American patients with PD, whereas the *184PGext*5* mutation is identified in Chinese families with PD [2]. Subsequently, studies have suggested that *TMEM230* variants are related to PD pathogenesis in vitro [3–5]. However, there is a lack of in vivo studies on the roles of PD-related variants of *TMEM230* in PD pathogenesis.

The *TMEM230* gene encodes two protein isoforms [2], the 183-amino-acid isoform-1 and the 120-amino-acid isoform-2. Isoform-2 accounts for more than 95% of the *TMEM230* protein isoforms in humans (Figure 1A). The physiological and pathological cellular functions of *TMEM230* in PD are still elusive [6]. Increasing evidence suggests that *TMEM230* may be involved in various cellular functions, such as vesicle trafficking [2, 3], trans-Golgi network secretion [2, 3], Rab family proteins in the endosomal pathway [2, 3, 7], cell toxicity [4, 5, 7], protein aggregation and the autophagy lysosomal pathway [2, 3, 7]. Studies suggest that mutant *TMEM230* may alter these cellular pathways and has implications in PD and other related disorders.

VPS35 protein is a major component of the retromer complex involved in endosome-to-Golgi retrieval and membrane-protein recycling [8]. The autosomal-dominant mutation *D620N* in *VPS35* has been identified in PD patients [9, 10]. Studies have demonstrated that the *VPS35 D620N* mutation disrupts the association of *VPS35* with other trafficking proteins, leading to protein trafficking defects [11, 12]. *TMEM230* has been shown to colocalize with *VPS35* and the retromer complex [2]. *TMEM230 R141L* and *184Wext*5* variants disrupt *VPS35* cytoplasmic distribution in a cell [3]. However, whether there is physiological interplay between *VPS35* and *TMEM230* in vivo is still unclear.

In this study, we generated wild-type (WT) and mutant *TMEM230* transgenic *Drosophila* to study the roles of *TMEM230* and its interplay with *VPS35* using genetic tools. Our studies provide novel insight into the roles of *TMEM230* and its interaction with rotenone (Rot) and *VPS35* in PD pathogenesis.

Materials And Methods

Drosophila genetics

All *Drosophila* stocks were maintained with a 12 hours light/dark cycle at 25°C and 55% humidity, raised in non-crowded conditions on standard cornmeal medium. Fly food consisted of (per 1 L) 10 g agar, 7.25 g sucrose, 30 g glucose, 24.5 g yeast, 50 g corn meal, 17.5ml methyl 4-hydroxybenzoate, 4ml propionic acid. The control flies used for this study were W1118 strain. We generated *UAS-TMEM230-WT*, *UAS-TMEM230- Y92C*, *-R141L*, *-184Wext*5* and *-184PGext*5* transgenic flies as described previously [13]. Briefly, *UAS-TMEM230-WT*, *UAS-TMEM230-Y92C*, *-R141L*, *-184Wext*5* and *-184PGext*5* isoform cDNA were cloned between EcoRI and KpnI sites of pUAST vector (Figure 1A) and verified by sequencing. The resulting constructs were microinjected in *w¹¹¹⁸* fly embryos. *TH-GAL4* and *elav-GAL4* were obtained from Bloomington *Drosophila* Stock Center (BDSC), *UAS-VPS RNAi* was obtained from **Vienna *Drosophila***

Resource Center (VDRC), *UAS-VPS35*, and *UAS-VPS35-D620N* were generated by our lab. The following genetic combinations were used to express transgenes in adult neurons: (1) *elav-GAL4/+; UAS-TMEM230-WT/+*, (2) *elav-GAL4/+; UAS-TMEM230-Y92C/+*, (3) *elav-GAL4/+; UAS-TMEM230-R141L/+*, (4) *elav-GAL4/+; UAS-TMEM230-184PGext*5/+*, (5) *elav-GAL4/+; UAS-TMEM230-184Wext*5/+*. The following genetic combinations were used to express transgenes in adult dopaminergic neurons: (1) *TH-GAL4/+; UAS-TMEM230-WT/+*, (2) *TH-GAL4/+; UAS-TMEM230-184PGext*5/+*, (3) *TH-GAL4/+; UAS-VPS35-WT/+*, (4) *TH-GAL4/+; UAS-VPS35-RNAi/+*, (5) *TH-GAL4/+; UAS-VPS35-D620N/+*, (6) *TH-GAL4/+; UAS-VPS35-WT/+; UAS-TMEM230-184PGext*5/+*, (7) *TH-GAL4/+; UAS-VPS35-RNAi/+; UAS-TMEM230-184PGext*5/+*, (8) *TH-GAL4/+; UAS-VPS35-D620N/+; UAS-TMEM230-184PGext*5/+*.

Western blot analysis

Adult fly heads were homogenized using RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% SDS, 1% NP-40, supplemented with complete protease inhibitor mixture; Roche). The homogenates were boiled with SDS loading buffer, were separated on SDS-PAGE gels and were transferred to PVDF membranes (GE Healthcare). Blots were incubated with the primary antibodies overnight at 4°C, and then HRP-conjugated secondary antibodies at room temperature for 1h. Primary antibodies were used including anti-tyrosine hydroxylase (TH) (ab112, Abcam Inc; 1:1000), anti-phospho-ERK (Thr202/Tyr204), anti-ERK, anti-phospho-SAPK/JNK (Thr183/Tyr185) or anti-JNK (1:2000, Cell Signaling Technology), anti-cleaved human caspase 3 (1:2000, Cell Signaling Technology), and mouse anti- β -tubulin antibody (1:5,000).

Fly climbing assay

Adult male flies were put into a 50 mL measuring cylinder. Cohorts of 60 flies from each genotype were subjected to assay every 10 days from 5 days after eclosion. The flies were gently tapped to the bottom of a vial and allowed to climb for 30 seconds. The climbing ability of flies was quantified as number of animals that reach the 10cm of cylinder in 15 seconds. The test was repeated 3 times for each group. The number of flies that reached the 10cm was converted into a percentage value, the Turkey's test after one-way analysis of variance (One-way ANOVA) was used for data analysis.

Flight assay

The flight assay was performed as described in [14]. Briefly, adult flies at age of 5, 15, or 25 days, were subjected to flight assays. Flies were anaesthetized on ice for 5 minutes, then a thin metal wire was glued between the neck and thorax region with the assist of nail polish. After allowing them to recover for ~5-7 minutes, a gentle mouth-blown air puff then was given and flight time was recorded using a stop watch. Flight duration was recorded for a maximum of fifteen minutes for each fly in batches of 5-8 flies at a time. For each genotype, each experimental group, a minimum of 20 flies were tested.

Life span assay

Adult male flies (60 flies per group) from each group were used for survival analyses. Flies were grown at 25 °C and moved to fresh medium every 4 days. Death was recorded every other day until all flies from the experimental groups were dead. Log-rank tests were performed for statistical analysis via Prism GraphPad software.

Rotenone exposure method

The flies were exposed to varying concentrations (0.25mM, 0.5mM and 0.75M) of rotenone (Rot) for 7 days in order to assess the lethality responses to choose the effective dose for LD₅₀ [15]. After segregation of 20 flies per vial into four groups (three vials in each group). Group I control flies fed with cornmeal medium. Group II to IV flies received ingestion of rotenone (0.25mM, 0.5mM and 0.75M) through the medium for seven consecutive days. On the end of experiment (8th day), flight assay and western blot analysis were performed as described above.

Results

Expression of TMEM230 184PGext*5 and 184Wext*5 in pan-neurons induces locomotor deficits and shortens the fly lifespan

To study the role of *TMEM230* in neurodegenerative pathology in the fly model, we generated human WT and mutant *TMEM230* (*Y92C*, *R141L*, *184Wext*5* and *184PGext*5*) transgenic flies using *TMEM230 isoform-2 cDNA* as described previously[5]. We observed the lifespan of *elav-GAL4; TMEM230* flies (*WT*, *Y92C*, *R141L*, *184PGext*5* and *184Wext*5*), in which the *TMEM230* protein is expressed in all neurons. We found that *elav-GAL4;184Wext*5* and *elav-GAL4;184PGext*5* flies had significantly shortened lifespans compared with non-transgenic normal control flies (Fig. 1C). The lifespan of *elav-GAL4; Y92C* and *elav-GAL4; R141L* flies was slightly decreased (but not significantly) compared with that of *elav-GAL4; TMEM230* flies. (Figure 1C).

Flight and climbing assays are often used to assess motor functions in flies, as described previously [13, 14]. The climbing and flight abilities of WT and mutant *TMEM230* flies were evaluated at time points after eclosion (climbing days: 5, 15, 25, 35, 45, 55; flight days: 5, 15, 25). We found that *elav-GAL4;184PGext*5* and *elav-GAL4;184Wext*5* flies displayed age-related locomotor impairment, while *elav-GAL4; Y92C* and *elav-GAL4; R141L* flies exhibited slightly decreased climbing activities but no significant difference compared with non-transgenic flies (Figure 1D). Similarly, the flight ability of *elav-GAL4;184PGext*5* and *elav-GAL4;184Wext*5* flies was significantly impaired, while the flight ability of *elav-GAL4; Y92C* and *elav-GAL4; R141L* flies was only slightly impaired compared with that of non-transgenic flies (Figure 1B). These results suggest that the *TMEM230 184PGext*5* and *184Wext*5* mutations induced more severe PD-like behavioural impairment in the fly models. Since *elav-GAL4;184PGext*5* was more neurotoxic than *elav-GAL4;184Wext*5* in flies (Figure 1), we then prioritized

our investigation of the *184PGext*5* mutation in this study; thus, only *TMEM230 WT* and *184PGext*5* data are reported in the rest of this study.

Expression of *TMEM230 184PGext*5* in DNs evokes locomotor deficits

Given that PD is characterized by a loss of DNs in the brain [16], we expressed WT and mutant *TMEM230* in DNs using TH driver flies. We found that *TH-GAL4;184PGext*5* flies displayed a shorter lifespan than *TH-GAL4; TMEM230* flies (Fig. 2A). Homozygous *TH-GAL4;184PGext*5* flies died earlier than heterozygous *TH-GAL4;184PGext*5* flies (Figure 2A). Moreover, *TH-GAL4;184PGext*5* flies showed locomotor deficits that were comparable with those of *TH-GAL4; TMEM230* flies (Figure 2C). Homozygous *TH-GAL4;184PGext*5* flies had more severe locomotor impairment than heterozygous *TH-GAL4;184PGext*5* flies (Figure 2C). Similarly, *TH-GAL4;184PGext*5* flies showed flight ability impairment that was comparable with that of *TH-GAL4; TMEM230* flies (Figure 2B).

Tyrosine hydroxylase (TH), a marker of DNs [17], is often used to test the lesion and/or loss of DNs in *TH-GAL4;184PGext*5* flies. There was a significant decrease in TH protein levels in *TH-GAL4;184PGext*5* flies compared with *TH-GAL4; TMEM230* flies (Figure 2D). Homozygous *TH-GAL4;184PGext*5* flies had lower TH protein levels than heterozygous *TH-GAL4;184PGext*5* flies (Figure 2D). These results demonstrated that *TMEM230 184PGext*5* induced DN degeneration in an age- and dose-dependent manner.

184PGext*5 accelerates Rot-induced neurotoxicity in flies

Rot, a mitochondrial complex I inhibitor, is often used to induce oxidative damage and dopaminergic neurodegeneration to model parkinsonism in *Drosophila* models [18]. Consistent with our previous studies, flies exposed to 0.75 mM Rot for 8 days showed increased mortality (up to 50%) compared with flies not exposed to Rot (Figure 3A). Rot-exposed flies also showed decreased TH protein levels and flight abilities compared with non-Rot-exposed flies (Figure 3B and C). Most interestingly, only *TH-GAL4;184PGext*5* flies exposed to 0.25 mM Rot showed significantly increased mortality compared with *TH-GAL4; TMEM230* flies (Fig. 3D). In addition, *TH-GAL4;184PGext*5* flies exposed to 0.50 mM Rot showed significantly exacerbated locomotor impairment compared with *TH-GAL4; TMEM230* flies exposed to 0.50 mM Rot; however, there was no difference in locomotor impairment between these two transgenic fly groups in the absence of Rot exposure (Figure 3E). These results demonstrate that the *TMEM230 184PGext*5* mutation leads to increased sensitivity to Rot.

***TMEM230 184PGext*5* increases JNK phosphorylation and caspase-3 activation in flies**

Oxidative stress plays a major role in mediating DN pathology [19], in which the c-Jun N-terminal kinase (JNK) pathway is activated and subsequently promotes inflammation and cell death [20, 21]. A previous study demonstrated that *184West** and *184PGext** increase reactive oxygen species (ROS) levels in mitochondria and induce caspase 3/7 activation and poly (ADP-ribose) polymerase (PARP) cleavage in vitro [5]. We were interested in studying whether the *TMEM230 184PGext*5* mutation alters the JNK

pathway and caspase-3 activation. We found that *TH-GAL4;184PGext*5* flies showed significantly increased JNK (p-JNK) phosphorylation (Figure 4A-B) and increased cleaved caspase-3 levels (Figure 4C) compared with non-transgenic and *WT-TMEM230* flies.

Expression of VPS35 reduces TMEM230-184PGext*5-induced JNK phosphorylation and caspase-3 cleavage in flies

Retromer dysfunction plays a critical role in neurodegeneration in some PD models [22]. VPS35 is the core of the retromer complex, and mutations in *VPS35* disrupt protein accumulation and clearance and have implications in PD [22]. Our results showed that VPS35 expression decreased p-JNK protein levels in *TH-GAL4; VPS35:184PGext*5* flies (Fig. 5A and 5B). In contrast, the knockdown of VPS35 by RNA interference (RNAi) further elevated *184PGext*5*-induced JNK phosphorylation in *TH-GAL4; VPS35RNAi:184PGext*5* flies compared with *TH-GAL4;184PGext*5* flies (Figure 5B). Moreover, *VPS35* decreased *184PGext*5*-induced Caspase-3 cleavage in *TH-GAL4; VPS35:184PGext*5* flies compared with that in *TH-GAL4;184PGext*5* flies (Figure 5C).

VPS35 alleviates TMEM230-184PGext*5-induced PD-like phenotypes in flies

The overexpression of *VPS35* in DNs by TH driver flies increased TH protein levels (Figure 6A), increased flight duration time (Figure 6B), enhanced locomotor ability (Figure 6C) and extended longevity (Figure 6D) in *TH-GAL4; VPS35:184PGext*5* flies compared with *TH-GAL4;184PGext*5* flies. Previous studies showed that the PD-like mutation *VPS35-D620N*, a point mutation, disrupts VPS35 function[11]. We found that the overexpression of *VPS35-D620N* worsened the PD-like phenotypes in *TH-GAL4; VPS35-D620N:184PGext*5* flies compared with *TH-GAL4;184PGext*5* flies (Figure 6). These results suggest that VPS35 may act as a downstream effector of TMEM230 and play an important role in dopaminergic neurodegeneration and PD phenotypes.

Discussion

In this study, we generated human *WT* and mutant *TMEM230* (*Y92C*, *R141L*, *184PGext*5* and *184Wext*5*) transgenic *Drosophila*. We found that the expression of either *TMEM230 184PGext*5* or *184Wext*5* in pan-neurons or in DNs in *Drosophila* induced PD-like phenotypes, which included impaired locomotor ability, a shortened lifespan, reduced TH levels, and increased phosphorylated JNK and cleaved caspase-3 levels. *TMEM230 184PGext*5* caused more severe PD-like phenotypes than other mutations. Moreover, Rot exposure enhanced *TMEM230-184PGext*5*-induced PD-like phenotypes. In contrast, the overexpression of *WT VPS35* rescued *TMEM230-184PGext*5*-induced PD-like phenotypes, while the knockdown of VPS35 by RNAi or the expression of mutant *VPS35 D620N* worsened PD-like phenotypes. These results indicate that VPS35, as a downstream effector of TMEM230, plays a critical role in TMEM230-linked JNK/caspase-3 signalling pathways and that mutations in TMEM230 and VPS35 disrupt these pathways, resulting in dopaminergic neurodegeneration and PD-like phenotypes.

Dopaminergic neurodegeneration is a key hallmark of PD pathology. We and others previously found that the expression of PD-linked *TMEM230* variants induces cell death in vitro [5, 7]. In this study, we found that the expression of mutant *TMEM230 184PGext*5* or *184Wext*5* in pan-neurons or DNs decreased TH levels, impaired locomotor activation and shortened the lifespan in flies compared with *WT TMEM230* or non-transgenic flies, which resembled the key features of human PD. However, flies expressing the *TMEM230* variant (Y92C or R141L) showed no changes compared with non-transgenic flies. This is consistent with the in vitro finding that *TMEM230 184PGext*5* or *184Wext*5* caused more severe neurotoxicity than the *TMEM230 Y92C* or *R141L* variants. Our studies provide the first in vivo *TMEM230*-based PD *Drosophila* model for further pathogenesis studies.

In this study, we first found that *TMEM230 184PGext*5* and *184Wext*5*, but not *WT*, *Y92C* and *R141L*, shortened longevity and reduced locomotive ability in flies, in which *184PGext*5* induced more neurotoxicity than *184Wext*5*. We thus expressed *184PGext*5* in DNs and found decreased locomotive abilities and lifespan and reduced TH protein levels in *TMEM230 184PGext*5* DNs.

Oxidative damage caused by an increase in ROS suggests that ROS play a critical role in DN degeneration [23, 24]. An increase in oxidative stress activates the JNK-MAPK signalling pathway and induces apoptosis [25, 26]. Our results showed that exposure to Rot, which increased oxidative damage, enhanced mutant *TMEM230-184PGext*5*-induced PD-like phenotypes, suggesting that oxidative stress converges with genetic mutations in *TMEM230*-linked PD pathology.

Previously, we found that the expression of *WT TMEM230* and the variants *184Wext*5* and *184PGext*5* increased mitochondrial ROS levels, activated caspase 3/7 and induced PARP1 cleavage in cultured cells [5]. Consistent with in vitro studies, we found that p-JNK-MAPK and cleaved Caspase-3 were increased in *TMEM230 184PGext*5* flies. Moreover, the expression of *WT VPS35* suppressed *TMEM230-184PGext*5*-induced dopaminergic neurodegeneration and reduced JNK activation and caspase-3 cleavage. These results further demonstrated that the JNK/caspase-3 pathway mediates *TMEM230-184PGext*5*-linked neurodegeneration and PD-like phenotypes.

TMEM230 colocalizes with *VPS35* in synaptic vesicles (SVs), which is related to vesicle trafficking in trans-Golgi network secretion and has implications for Rab family proteins in the endosomal pathway, protein aggregation and the autophagy lysosomal pathway. The *VPS35 D620N* mutation has been identified in late-onset PD patients [9, 10]. Previous studies showed that mutant *VPS35* interacts with the retromer cargo and disrupts cellular trafficking [22]. Our results showed that *VPS35* suppressed mutant *TMEM230*-induced PD-like phenotypes in flies. In contrast, the expression of mutant *VPS35 D620N* had no effect on mutant *TMEM230*-induced PD-like phenotypes. These findings indicated that *VPS35* acts as a downstream effector and interacts with *TMEM230*. Mutations in either *VPS35* or *TMEM230* may disrupt this interplay, leading to neurodegeneration and PD-like phenotypes.

Conclusions

Cumulatively, we generated the first TMEM230-based PD *Drosophila* model in which the expression of human *TMEM230 184PGext*5* or *184Wext*5* in pan-neurons or DNs induced parkinsonism-like behaviours. *184PGext*5* activated the JNK/caspase-3 pathway and increased dopaminergic neurodegeneration, while VPS35 attenuated *184PGext*5*-PD-like phenotypes by reducing JNK activation and caspase-3 cleavage. These findings provide novel insight into the mechanisms underlying mutant-TMEM230- or mutant-VPS35-linked PD-like pathology. This TMEM230-based PD *Drosophila* model may provide a platform for further study of PD pathogenesis and therapeutics.

Abbreviations

PD: Parkinson's disease

TMEM230: transmembrane protein 230

VPS35: Vacuolar protein sorting-35

WT: wild-type

RNAi: RNA interference

DNs: dopaminergic neurons

SN: substantia nigra pars compacta

Rot: rotenone

TH: Tyrosine hydroxylase

JNK: c-Jun N-terminal kinase

ROS: reactive oxygen species

PARP: poly (ADP-ribose) polymerase

SVs: synaptic vesicles

Declarations

Ethics declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Soochow University.

Consent for publication

All authors have given their consent for publication.

Availability of data and materials

Please contact the corresponding author for all data requests.

Competing interests

The authors declare that no competing interests exist.

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Authors' contributions

Data curation and Methodology, C.M.; Data analysis and Software, X.W.; Project administration, Z.L and W.W.S.; Funding acquisition and Supervision, Z.L.; Writing original draft, C.M. and Z.L.; Writing—review & editing, W.W.S. All authors have read and agreed to the published version of the manuscript.

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Figures

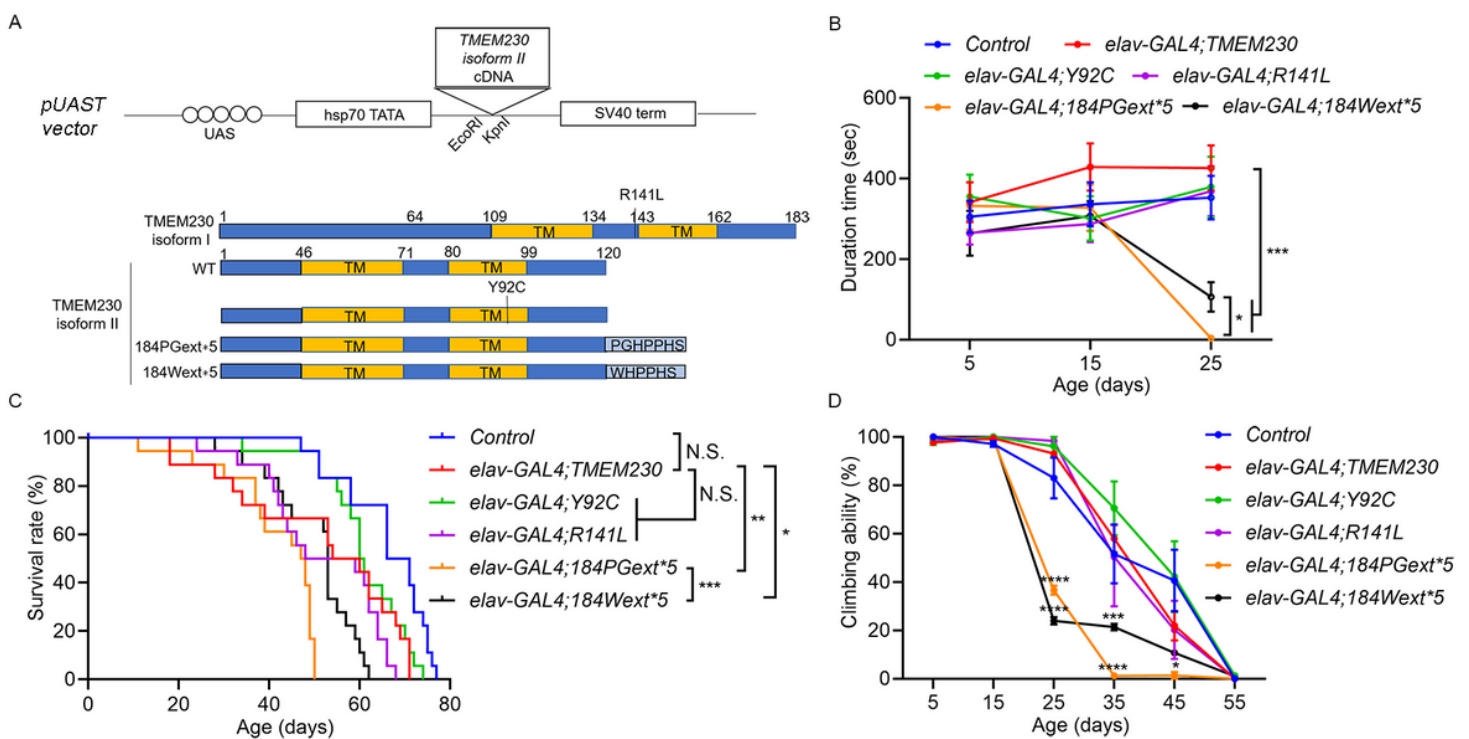


Figure 1

Expression of TMEM230 184PGext*5 and 184Wext*5 in neurons induces locomotor deficits and shortens the fly lifespan. A. Vector for UAS-TMEM230 (WT, R141L, Y92C, 184PGext*5 and 184Wext*5) gene expression is indicated upper in A, TMEM230 structure and the tested mutants in isoforms 1 and 2, the locations of two putative transmembrane domains (TM), R141L, Y92C, 184PGext*5 (PGHPPHS) and 184Wext*5(WHPPHS) are indicated below in A; B. flight assays. Elav-184PGext*5(orange) and elav-

184Wext*5(black) showed flight deficit as compared to elav-WT flies (red); C. lifespan assays. Elav-184PGext*5 and elav-184Wext*5 shortened lifespan (by log-rank test); D. climbing assays. Elav-184PGext*5 and elav-184Wext*5 displayed locomotor impairment. One-way ANOVA, Turkey's test with multiple comparisons. N.S. no significance, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.001). There were at least 60 flies in each experimental group.

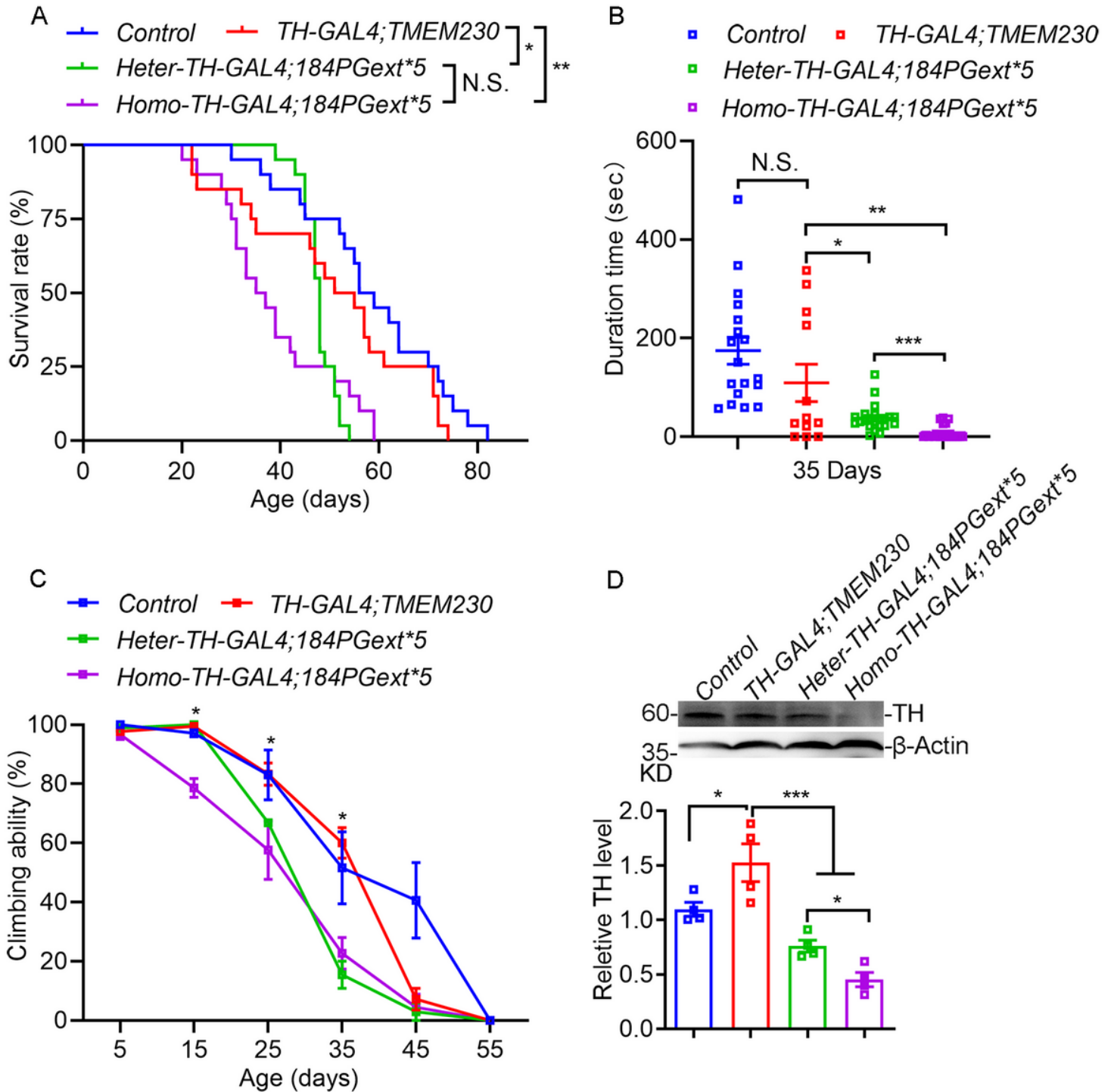


Figure 2

Expression of TMEM230 184PGext*5 in DNs evokes locomotor deficits. A. Lifespan assays. TH-GAL4;184PGext*5 (green) shortened lifespan as compared to TH-GAL4; WT flies (red). Log-rank test was used to analyse the data. B. Flight assays. TH-GAL4;184PGext*5 flies displayed the impaired flight ability at the age of 35 days; C. Climbing assays. TH-GAL4;184PGext*5 displayed age-related locomotor decline; D. TH-GAL4;184PGext*5 decreased TH protein level in brains. The expression of TH in fly brains were measured using western blot analysis with an-TH antibody. β -actin was used as loading control. The quantity of TH proteins was normalized to β -actin. One-way ANOVA, followed by post Turkey's test for multiple group comparison * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There were at least 60 flies in each experimental group.

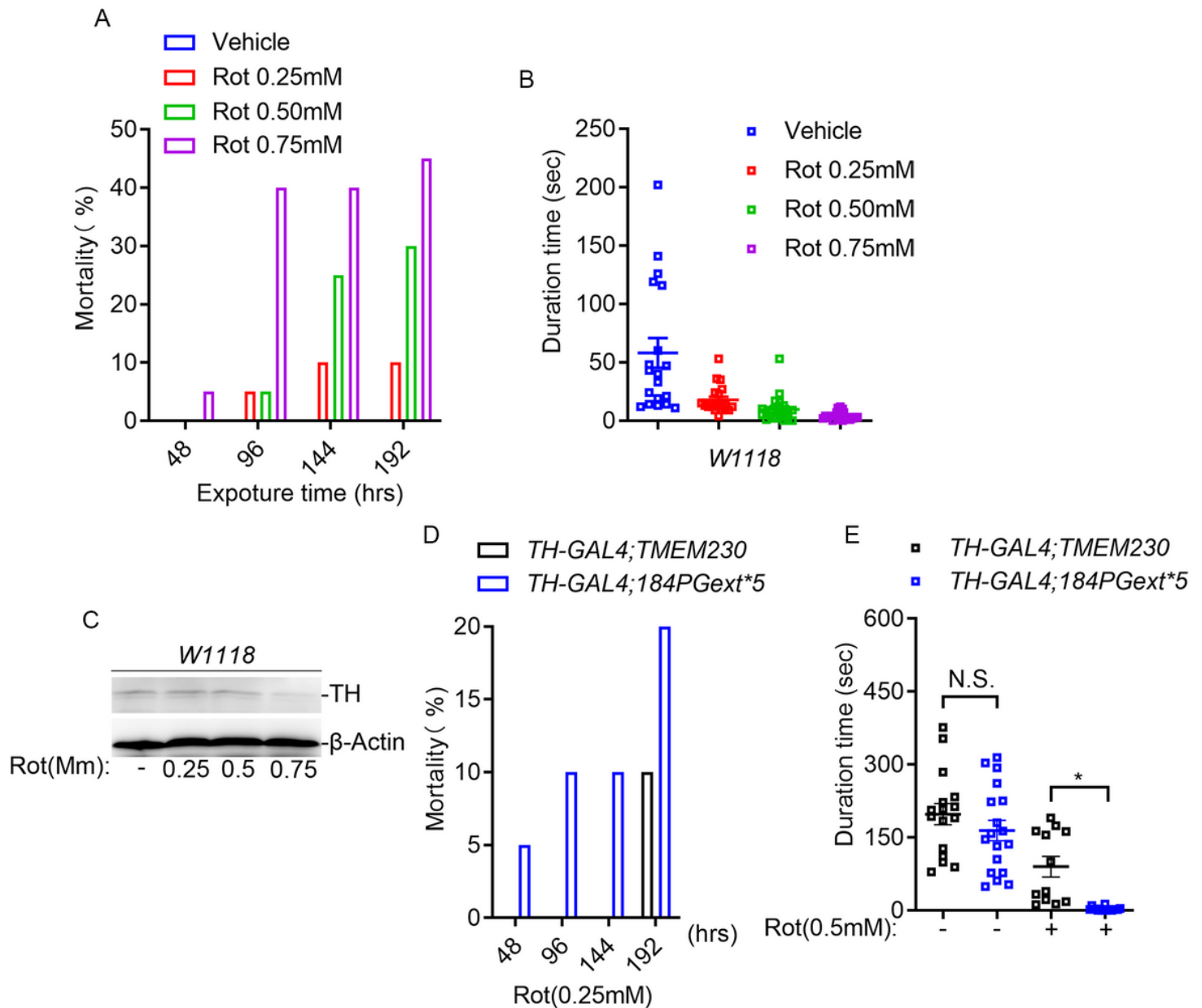


Figure 3

Expression of TMEM230 184PGext*5 in DNs enhances Rotenone-induced toxicity in flies. A. Mortality of adult flies exposed to Rotenone (0.25, 0.5 and 0.75mM) was calculated; B. Adult flies exposed to Rotenone were subjected to flight assays; C. The expression of TH in fly brains after exposed to Rotenone was measure by anti-TH immunoblotting assays. Anti- β -actin immunoblotting was used as protein loading control; D. Mortality of TH-GAL4;184PGext*5 flies increased after exposing Rotenone at 0.25 mM concentration; E. Expression of TH-GAL4;184PGext*5 exacerbated Rotenone-induced locomotor impairment; N.S. no significance, * $P < 0.05$, One-way ANOVA, Turkey's test with multiple comparisons. There were at least 60 flies in each experimental group.

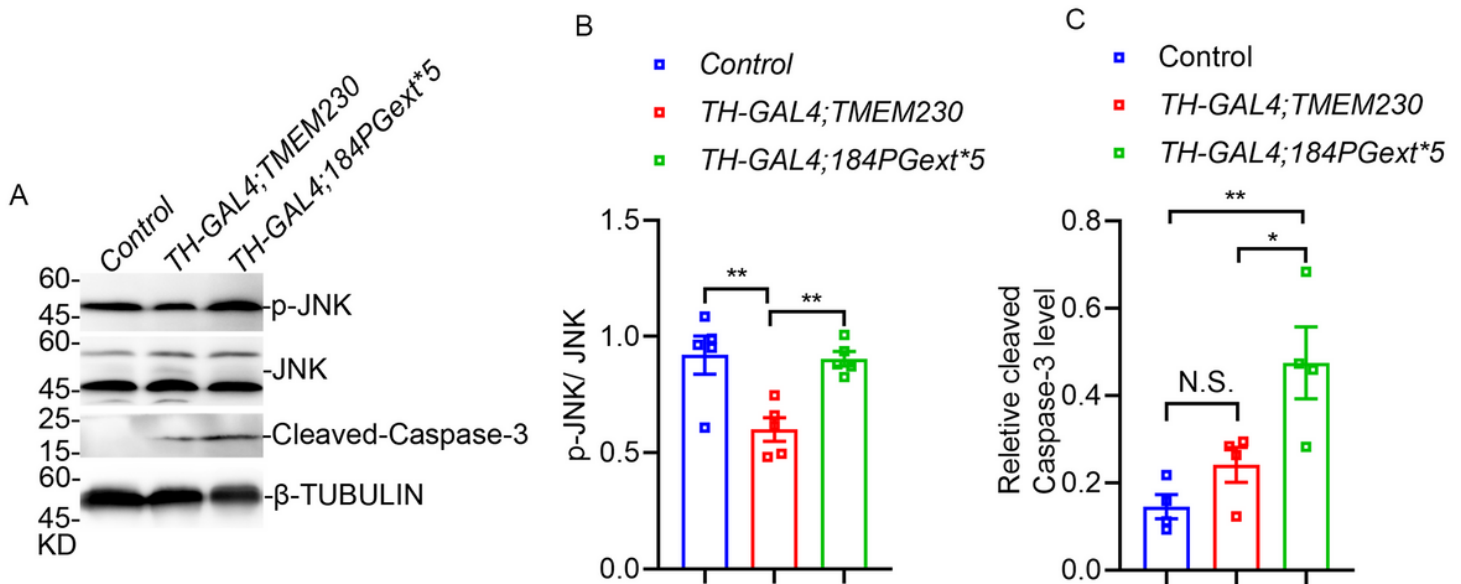


Figure 4

TMEM230 184PGext*5 induces JNK phosphorylation and caspase-3 cleavage. A. TH-GAL4;184PGext*5 induced JNK/caspase-3 activation compared with WT-TMEM230 transgenic flies. The expression of p-JNK, JNK and cleaved-Caspase-3 in fly brain tissue from adult flies were measured by western blot analysis with antibodies against p-JNK, JNK, cleaved-Caspase-3 antibody and β -tubulin; B. Quantification of p-JNK, JNK in A; C. Quantification of cleaved-Caspase-3 in A; One-way ANOVA, Turkey's test with multiple comparisons. N.S. means no significance, * $P < 0.05$, ** $P < 0.01$.

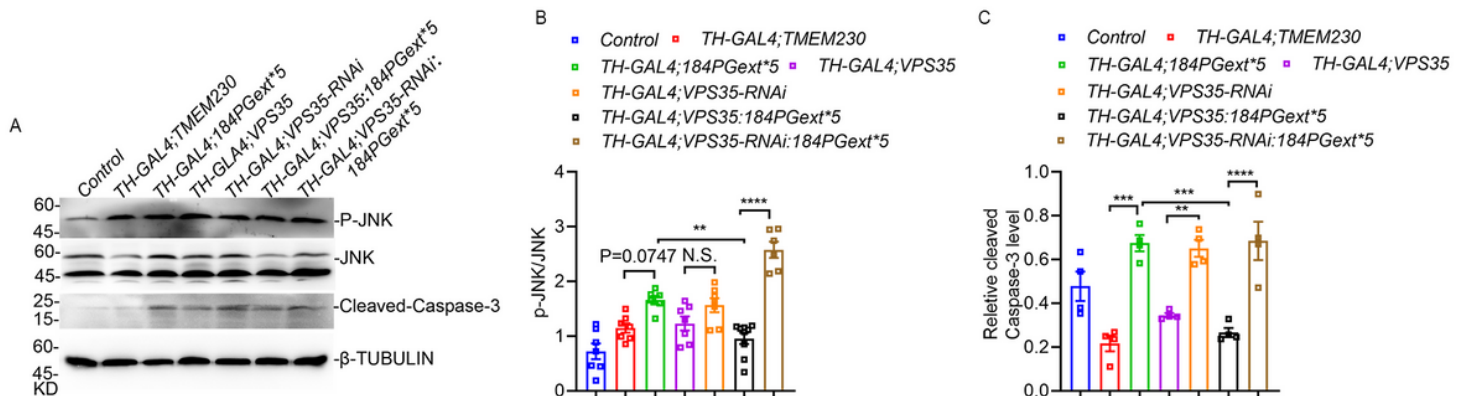


Figure 5

Expression of VPS35 in DNs reduces TMEM230-184PGext*5-induced JNK phosphorylation and caspase-3 activation. A. The levels of p-JNK, JNK, cleaved-Caspase-3 were measured by western blot analysis using antibodies against p-JNK, JNK, cleaved-Caspase-3 and β -tubulin; B. TH-GAL4; VPS35 decreased phosphorylation level of JNK in TH-GAL4; VPS35:184PGext*5 flies. p-JNK was measured by western blot analysis; C. TH-GAL4; VPS35 decreased Caspase-3 cleavage in TH-GAL4; VPS35: 184PGext*5 flies. Cleaved-caspase-3 was measured by western blot analysis. The quantity of proteins was normalized to β -tubulin; One-way ANOVA, Turkey's test with multiple comparisons. N.S. means no significance, ** $P < 0.01$, *** $P < 0.001$ **** $P < 0.0001$.

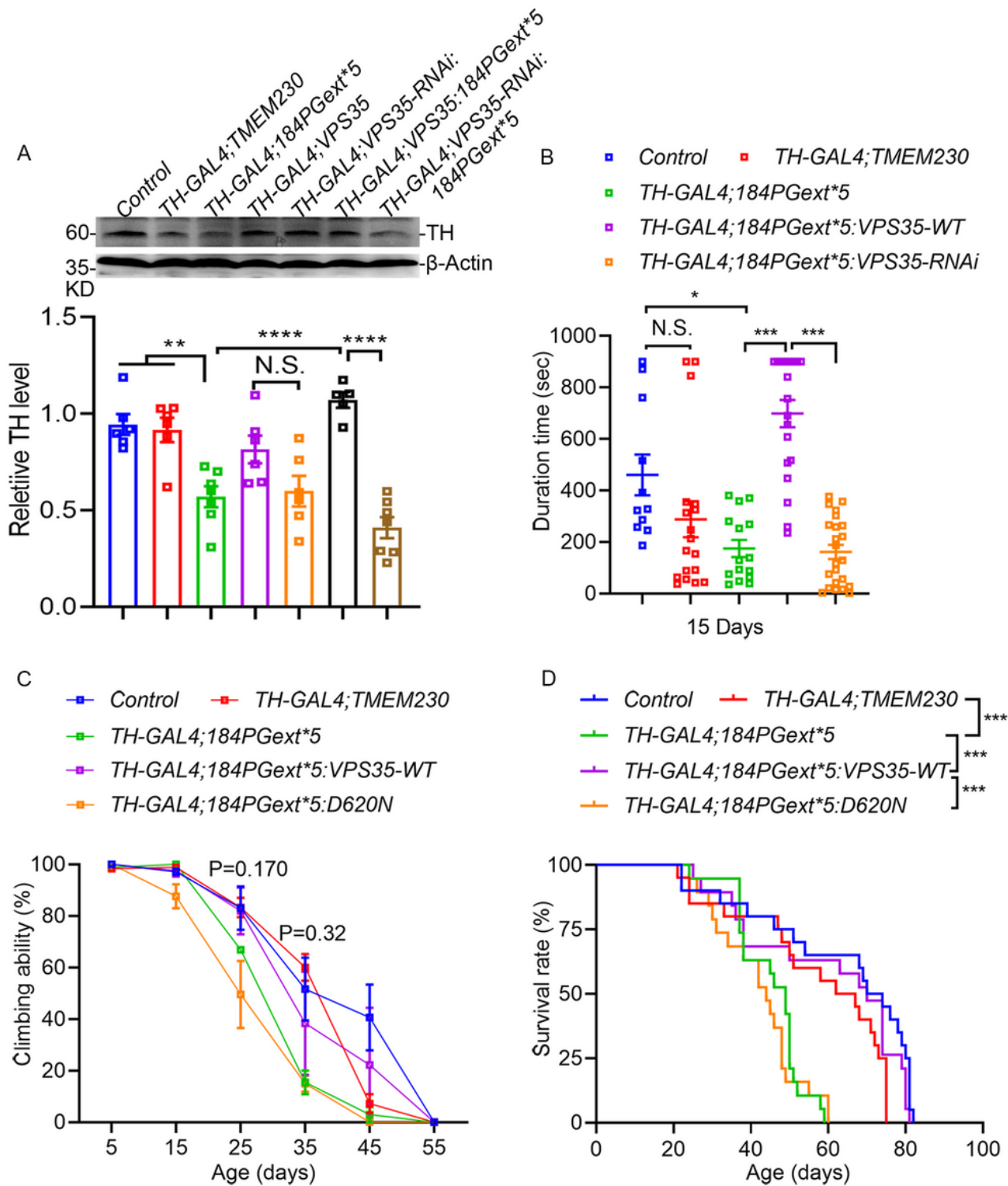


Figure 6

Expression of VPS35 in DNs attenuates TMEM230-184PGext*5-induced PD-like phenotypes in flies. A. TH-GAL4; VPS35 increased TH in TH-GAL4; VPS35: 184PGext*5 flies. TH was measured by western blot analysis. The quantity of TH proteins was normalized to β -actin; B. Flight assays. TH-GAL4; VPS35 extended the duration time of flight in TH-GAL4; VPS35:184PGext*5 flies; C. Climbing assays. TH-GAL4; VPS35 improved 184PGext*5-induced locomotor impairment in TH-GAL4; VPS35: 184PGext*5 flies; D.

Survival assays. TH-GAL4; VPS35 extends the survival time of TH-GAL4; VPS35: 184PGext*5 flies. Survival curves of indicated genotypes are shown and the statistical significance was analysed by log-rank test. Data from flight assays and climbing assays were analysed by One-way ANOVA, Turkey's test with multiple comparisons. N.S. means no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ **** $P < 0.0001$.