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Tubby domain superfamily protein is required for the formation of the 7S SNARE complex in *Drosophila*

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ABSTRACT

Tubby domain superfamily protein (TUSP) is a distant member of the Tubby-like protein (TULP) family. Although other TULPs play important roles in sensation, metabolism, and development, the molecular functions of TUSP are completely unknown. Here, we explore the function of TUSP in the *Drosophila* nervous system where it is expressed in all neurons. *Tusp* mutant flies exhibit a temperature-sensitive paralysis. This paralysis can be rescued by tissue-specific expression of *Tusp* in the giant fibers and peripherally synapsing interneurons of the giant fiber system, a well-characterized neuronal circuit that mediates rapid escape behavior in flies. Consistent with this paralytic phenotype, we observed a profound reduction in the assembly of the ternary 7S SNARE complex that is required for neurotransmitter release despite seeing no changes in the expression of each individual SNARE complex component. Together, these data suggest TUSP is a novel regulator of SNARE assembly and, therefore, of neurotransmitter release.

1. Introduction

The mammalian tubby-like protein (TULP) family comprises five genes: *Tubby* and four tubby-like protein genes (i.e., *Tulp1* through *Tulp4*) [1]. Mutations in the *Tulp* genes impair neuronal function and survival. Mice carrying *tubby* mutations exhibit late-onset obesity and neurosensory deficits like retinal degeneration and hearing loss [2–4]. Mutations of *Tulp1* produce retinal degeneration in both humans and mice [5,6]. Mutations in *Tulp3* are embryonic lethal in mice [7]. Mutations in *Tulp4* are associated with short stature, cleft lip, and cleft palate [8,9]. The mostly non-overlapping phenotypes associated with the loss of each TULP suggest each TULP family member has a distinct biological function.

These unique molecular functions of the TULP homologues are being studied in multiple model systems. In mice, while Tubby regulates insulin signaling, Tulp3 interacts with intraflagellar transport-A (IFT-A) and regulates the ciliary trafficking of GPR161, modulating Sonic hedgehog (Shh) signaling [10–13]. *Drosophila* Tubby (dTULP) is required for the ciliary localization of IAV and NompC, two ion channels required in chordotonal neurons for hearing [14,15]. In *C. elegans*, tub-1 regulates fat storage via its interaction with RBG-3, a Rab GTPase-activating protein [11]. The two remaining TULPs, Tulp2 and Tulp4, are understudied and have not yet been linked to any specific function. Tulp4 is the most distantly related TULP family member and its C-terminal tubby domain is less conserved than the tubby domain of other TULP members. This suggests that of all the TULPS, Tulp4 likely has the most unique biological function.

Mouse *Tulp4* is expressed mainly in the brain and testis; human Tulp4 is abundant in the brain, skeletal muscle, kidney, and placenta [16]. No Tulp4 loss-of-function experiments have yet been reported, so Tulp4's functions remain unknown. The *Drosophila* genome contains two TULP family genes, *dTulp* and *Tubby domain superfamily protein* (*Tusp*). These are the fly homologues of mammalian Tubby and Tulp4, respectively [17]. The reduced number of TULPs in the *Drosophila* system makes it suitable for discovering the first molecular function for a Tulp4 homologue.

Here, we used the *Drosophila* model system to reveal a novel molecular function for the fly Tulp4 homologue TUSP. We have

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discovered an unexpected role for TUSP in the assembly of the 7S synaptobrevin-syntaxin-SNAP-25 ternary complex that is required for synaptic vesicle exocytosis and neurotransmitter release.

2. Materials and methods

2.1. Fly stocks

Fly stocks were maintained under standard laboratory conditions (25 °C, 60% humidity, 12 h light/12 h dark cycles). The *UAS-mCD8::GFP*, *hs-FLP*, *OK307-GAL4*, *Cha-GAL4*, *12862-GAL4*, *ShakingB-GAL4*, *BG57-GAL4*, *D42-GAL4*, and *OK371-GAL4* strains were obtained from the Bloomington Stock Center (Bloomington, IN). The R17A04_p65ADZp (attP40); 68A06_ZpGdbd (attP2) (*GF-split-GAL4*) strain was a generous gift of Dr. Gwyneth M. Card.

2.2. Generation of Tusp mutants and transgenic flies

We used ends-out homologous recombination to generate the $Tusp^{G}$ mutant allele as previously described [18]. We used the pw35*GAL4* vector to insert the *GAL4* coding sequence with an initiation codon into the *Tusp* locus to generate the *GAL4* reporter $Tusp^{G}$ allele. From w^{1118} genomic DNA, we used PCR to amplify 3 kb homologous arms from the 5′ and 3′ ends of the first coding exon (1–96 residues) of *Tusp*. We then subcloned these arms into the pw35*GAL4* vector. The primer sequences (5′ to 3′) were as follows: 5′ homologous arm (AGATCTATTAGGAAATGATTAGTTA and TTATGGCCCAAAAGCGCCG); 3′ homologous arm (GCGTGAGTACCGTGATTAACTG and AGCTTTCTGATGGCTGG). We generated transgenic flies by germ line transformation (BestGene Inc., Chino Hills, CA) and screened for properly targeted alleles as previously described [19]. These targeted alleles were then subjected to five generations of outcrossing to the w^{1118} genetic background.

To generate the $Tusp^{D}$ allele, we used the FLP/FRT recombination system as previously described [20]. From Exelixis at Harvard Medical School (Boston, MA), we obtained two fly lines containing PiggyBac element insertions near the *Tusp* locus (i.e., PBac{RB}dsd^{e01820} and PBac{WH}Mtl^{f07113}). To induce a recombination event between the FRT sites in each of these transposons, we crossed flies containing the first PiggyBac insertion and a heat shock-driven FLP recombinase to flies containing the second PiggyBac insertion. This induced the activation of the FLP recombinase, resulting in the generation of a deletion between the two transposons. $Tusp^{D}$ has a 31 kb deletion that covers the entire *Tusp* coding sequence.

We confirmed the deletions in each of the $Tusp^{G}$ and $Tusp^{D}$ alleles via genomic PCR. The primer sequences (5′ to 3′) used for this confirmation were: deleted genomic fragment (ATGCATTTACACTTTGAGCGAAACATCAAC and AGTGCCGGACAGTTTAATGGACCT); intact second exon in the $Tusp^{G}$ allele (TGGGTTGATCGGCTCAAAGAGTGA and GTCCATGTTCTTGCGGCAATGTGA); *Mtl* (ATGTCAACCGGAAGGCCCAT and CATGTCGCTTGGCAACTTACC).

To generate the transgenic rescue flies, we used RT-PCR to amplify a *Tusp* cDNA from total w^{1118} RNAs and subcloned it into the pUASTattB vector. We then generated transgenic flies by germ line transformation (BestGene Inc., Chino Hills, CA).

2.3. Expression analysis

To visualize its *GAL4* expression pattern, we crossed flies carrying the $Tusp^{G}$ allele to *UAS-mCD8::GFP* flies. After dissecting the tissues of interest, fixing them in 4% paraformaldehyde/PBS, washing them three times with 0.2% Triton X-100 in PBS (PBS-T), and blocking with 5% goat serum/PBS-T for 30 min, we added rabbit anti-GFP (Invitrogen, 1:200) and mouse nc82 (Hybridoma Bank, 1:50) primary antibodies, and incubated the tissues overnight at 4 °C. Then, after further washing, we incubated the tissues with Alexa 488 anti-rabbit (Molecular Probes) and Alexa 568 anti-mouse (Molecular Probes) secondary antibodies for 2 h at room temperature. After three more washes, we mounted the samples in Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined them using an LSM700 confocal microscope (Zeiss, Jena, Germany).

2.4. Adult paralysis analysis

Before beginning the behavior experiments, we housed 10 flies (10 days old) in plastic vials. After a 5 min soak in a 40 °C water bath, we removed the vials and counted the paralyzed flies. Then, we observed the vials as we maintained them at room temperature (22–24 °C) to quantify their recovery rates. We also placed individual flies into a plastic plate on a 40 °C heat block to visualize their behavior. Flies did not exhibit any seizure activity (i.e., uncontrolled wing flapping) before losing the ability to stand.

2.5. Preparation of 7S complexes

We prepared 7S complexes as previously described from 10 flies of each genotype [21]. After this preparation, we subjected proteins from the fly heads of each genotype to electrophoresis on 9–15% SDS-polyacrylamide gels without boiling (15 mA/gel) and transferred them to polyvinylidene fluoride (PVDF) membranes. We blocked these membranes for 1 h with 5% nonfat milk plus 0.1% Tween-20 and performed immunoblotting with primary antibodies against syntaxin (Hybridoma Bank, 8C3, 1:2000) and α-tubulin (Hybridoma Bank, 12G10, 1:5000). Ratio of 7S complex to α-tubulin were normalized to control per genotypes, quantified using the ImageJ software.

2.6. Real-time quantitative RT-PCR

We extracted total RNA from adult heads using Trizol (Invitrogen, Carlsbad, CA). Then, we generated cDNA from 1 μg of the total RNA from each genotype using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). Finally, we performed the qPCR amplification using the SYBR-Green reagent (Takara Bio). Transcript levels were normalized to *rp49* as an internal control and the $\Delta\Delta C_T$ method was used for comparing relative expression. The specific primer sequences (5′ to 3′) were: *nSyb* (neuronal synaptobrevin; TCGATGAGGTCGTGGACATC and CGCGCTCCAGCACCTT); *Syb* (AGCTGAAGCGCAAGCAATG and CACGCCCAGAATGATCATCA); *SNAP-25* (TTCCTTTCCAGGTCCCATCA and CATTTGTGTTCTTCCGTGCAA); *Syx*, (ATGATCGACAAGGTGCAGGAT and GGATGGCCGAGTGCTTCTT); *Tusp* (ATCGCGACCTGGAGACCTT and ATCTTGCCGCGCTTTAGCT); *rp49* (GACCATCCGCCCAGCATACAG and AATCTCCTTGCGCTTCTTGGAGGAG).

2.7. Data analysis

All data are presented as means ± S.E.M. with differences between groups analyzed using one-way ANOVAs with Tukey *post-hoc* tests. $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Generation of Tusp mutants

Drosophila TUSP shows approximately 51% amino acid similarity with mouse Tulp4. To investigate the biological functions of TUSP, we generated two *Tusp* mutant alleles. We generated $Tusp^{G}$ by replacing the first exon of the *Tusp* coding sequence (residues 1 to 96) with the *GAL4* coding sequence using ends-out homologous

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