**Drosophila** Tubulin-specific chaperone E functions at neuromuscular synapses and is required for microtubule network formation

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Hypoparathyroidism, mental retardation and facial dysmorphism (HRD) is a fatal developmental disease caused by mutations in tubulin-specific chaperone E (TBCE). A mouse Tbec mutation causes progressive motor neuronopathy. To dissect the functions of TBCE and the pathogenesis of HRD, we generated mutations in *Drosophila* tbce, and manipulated its expression in a tissue-specific manner. *Drosophila* tbce nulls are embryonic lethal. Tissue-specific knockdown and overexpression of *tbce* in neuromusculature resulted in disrupted and increased microtubules, respectively. Alterations in TBCE expression also affected neuromuscular synapses. Genetic analyses revealed an antagonistic interaction between TBCE and the microtubule-severing protein Spastin. Moreover, treatment of muscles with the microtubule-depolymerizing drug nocodazole implicated TBCE as a tubulin polymerizing protein. Taken together, our results demonstrate that TBCE is required for the normal development and function of neuromuscular synapses and that it promotes microtubule formation. As defective microtubules are implicated in many neurological and developmental diseases, our work on TBCE may offer novel insights into their basis.

**KEY WORDS:** *Drosophila*, HRD, Spastin, TBCE (CG7861), Tubulin chaperone

## INTRODUCTION

Microtubules (MTs), one of the major building blocks of cells, play a crucial role in a diverse array of biological functions including cell division, cell growth and motility, intracellular transport and the maintenance of cell shape. As MTs are important in all eukaryotes, it is not surprising that defects in MTs are associated with a number of severe human diseases, including Fragile X mental retardation and autosomal dominant hereditary spastic paraplegia (AD-HSP) (Lewis and Cowan, 2002; Penagarikano et al., 2007; Reid, 1997; Roll-Mecak and Vale, 2005; Sherwood et al., 2004; Trotta et al., 2004; Zhang and Broadie, 2005). MTs are formed by polymerization of tubulin heterodimers consisting of one α- and one β-tubulin polypeptide. The formation of α-β tubulin heterodimers is mediated by a group of five tubulin chaperones, TBCA-TBCE (Tian et al., 1996) (for reviews, see Lewis et al., 1997; Nogales, 2000). TBCA and TBCD assist in the folding of β-tubulin, whereas TBCB and TBCE facilitate the folding of α-tubulin (Lewis et al., 1997; Tian et al., 2006).

A group of rare, recessive and fatal congenital diseases, collectively called hypoparathyroidism, mental retardation and facial dysmorphism (HRD), is caused by mutations in the gene encoding TBCE (Parvari et al., 2002). TBCE contains three functional domains: a glycine-rich cytoskeleton-associated protein domain (CAP-Gly) that binds α-tubulin, a series of leucine-rich repeats (LRR), and an ubiquitin-like (UBL) domain; the latter two domains mediate protein-protein interactions (Bartolini et al., 2005; Grynenberg et al., 2003; Parvari et al., 2002). Identification of the HRD disease gene revealed a 12 bp deletion in *tbce* that leads to the expression of a mutated TBCE protein lacking four amino acids in the CAP-Gly domain (Parvari et al., 2002). The mutation causes lower MT density at the MT organizing center, perturbed MT polarity and decreased precipitable MT, while total tubulin remains unchanged (Parvari et al., 2002). Remarkably, overexpression of TBCE in cultured cells also results in disrupted MTs (Bhamidipati et al., 2000; Sellin et al., 2008; Tian et al., 2006). Thus, both loss-of-function mutations and overexpression of *TBCE* disrupt the MT network in mammalian systems.

Two independent studies have demonstrated that a Trp524Gly substitution at the last residue of mouse TBCE results in progressive motor neuronopathy (PMN), which has been widely used as a model for human motor neuron diseases (Bommel et al., 2002; Martin et al., 2002). Similar to what has been reported for cells from human HRD patients, the point mutation in mouse *Tbce* leads to a reduced number of MTs in axons (Bommel et al., 2002). Isolated motor neurons of mutant mice exhibit shorter axons and irregular axonal swellings (Martin et al., 2002). More specifically, axonal MTs are lost progressively from distal to proximal, which correlates with dying-back axonal degeneration in mutant mice (Schaefer et al., 2007). This demonstrates a mechanistic link between TBCE-mediated tubulin polymerization and neurodegeneration.

TBCE is well conserved across species, from yeast to human. Genetic analyses of the TBCE homolog in *S. pombe*, StoIP, showed that it is essential for viability and plays a crucial role in the formation of cytoplasmic MTs and in the assembly of mitotic spindles (Grishchuk and McIntosh, 1999; Radcliffe et al., 1999). *S. cerevisiae* mutants of the *TBCE* homolog * PAC2* show increased sensitivity to the MT-depolymerizing agent benomyl (Hoyt et al., 1997). Similarly, *tbce* mutants of *Arabidopsis* have defective MTs, leading to embryonic lethality (Steinborn et al., 2002).

The *Drosophila* genome contains a TBCE ortholog, listed as CG7861 in FlyBase (http://flybase.org), but no studies of it have been reported. To gain a mechanistic insight into the in vivo functions of TBCE, we introduced different mutations into *Drosophila* tbce. *Drosophila* tbce nulls are embryonic lethal, indicating that it is an essential gene. We also examined the...
developmental, physiological and pharmacological consequences with regard to neuromuscular synapses and MT formation when the expression of TBCE was altered specifically in neurons or muscles using the UAS-Gal4 system (Brand and Perrimon, 1993). We found that TBCE is required for the normal development and function of neuromuscular synapses and that it promotes MT formation in vivo.

MATERIALS AND METHODS

Drosophila husbandry and stocks

Flies were cultured in standard cornmeal media at 25°C, unless specified. w1118 was used as the wild-type control. Other stocks used include muscle-specific C57-Gal4 (Budnik et al., 1996), pan-neuronal elav-Gal4, and deficiencies Df(2R)ED1484 and Df(2R)ED1482, which remove tbce completely (Bloomington Stock Center). The spastin-null mutant spastin75 was from N. Sherwood (Sherwood et al., 2004), and a UAS-spastin line was from K. Brodie (Trotta et al., 2004). The chemical mutagen ethyl methanesulfonate (EMS)-induced nonsense mutation Z0241 in tbce (see Fig. 1B) was obtained from a TILLING (targeting induced local lesions in genomes) service at Seattle (http://tilling.fhcrc.org).

P element-mediated excision was used to generate small deletions in tbce following a standard protocol. The original stock KG09112 from Bloomington has a P element insertion in the intergenic region between CG14591 and tbce (Fig. 1B). Before mobilizing KG09112 as mediated by P transposase A2-3, we inosgenized the original stock. w1 deletion lines with the P insertion excised either precisely (LH198) or imprecisely (LH260 and LH15) were initially screened by PCR followed by DNA sequencing, in conjunction with immunohistochemical analyses to confirm the mutations at the protein level.

Production of UAS and RNAi transgenic flies

For overexpression studies, a UAS-TBCE construct was made by amplifying the full-length tbce cDNA from EST clone GM13256, obtained from the Drosophila Genomics Resource Center (https://dgrc.cgb.indiana.edu/vectors), and cloned into the transformation vector PUASt. For tissue-specific knockdown assays, an RNAi construct was made according to a previously described procedure (Kalidas and Smith, 2002). Specifically, a cDNA fragment from the tbce second exon was fused to the corresponding genomic sequence plus intron 2 as a spacer (see Fig. 1B) to make a hairpin RNAi construct targeting nucleotides 337-842 of GenBank sequence NM_136353. An independent RNAi line targeting a different sequence (nucleotides 919-1280 of NM_136353) of tbce was obtained from the Vienna Stock Center. Although multiple independent lines of transgenic flies carrying UAS-TBCE or RNAi were generated, here we report on UAS-TBCE and UAS-RNAi insertions on the third chromosome with apparent effect. To ensure high efficiency of overexpression or knockdown of TBCE by the UAS-Gal4 system, flies, including wild-type controls, were raised at 28°C instead of 25°C for all the assays involving the UAS and RNAi transgenic lines.

Production of monoclonal antibody against TBCE

His-tagged peptide corresponding to the N-terminal 512 amino acids of TBCE (full-length TBCE is 542 amino acids) produced in E. coli was used as an antigen. Immunization and screening of antibody producing cells were performed according to standard procedures. Several positive clones were identified, but the antibody produced by clone 8E11 is specific for both western and immunostaining.

Behavioral analyses

The larval roll-over assay was performed largely according to published protocols (Bodily et al., 2001; Pan et al., 2008). Before the assay, larval culture and agar plates were placed at room temperature for 2 hours to acclimate. For each assay, an individual animal was placed on a 1% agar plate and allowed to move freely for 2 minutes. The test animal was rolled over using a soft brush to a completely inverted position, indicated by the ventral midline facing up. The time that the animal took to totally right itself was recorded. Three assays were performed continuously without any resting time for each animal, and then averaged to produce one data point.

Immunohistochemical analyses and confocal microscopy

For western analyses, third instar larvae were dissected in PBS with all internal organs removed, followed by homogenization in 2× loading buffer. Half a fillet was used for each loading. Primary antibodies used were anti-TBCE (1:200), anti-α-tubulin (1:50,000; mAb B-5-1-2, Sigma) and anti-actin (1:50,000; mAb1501, Chemicon). The blots were detected with horseradish peroxidase (HRP)-coupled secondary antibodies using a chemiluminescent method (ECL Kit, Amersham).

Whole-mount embryos were fixed and stained with anti-FASII (1:100; Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa) and BP102 antibody (1:200; DSHB) using standard procedures. For immunostaining of first instar larvae, animals were dissected and processed following an established protocol (Budnik et al., 2006). The medical adhesive Compton (Shunkang, Beijing, China) was used to glue the epidermis of larve to Silgard-covered coverslips. Dissection and antibody staining of third instar larvae are described elsewhere (Zhang et al., 2001). Primary antibodies used include: anti-α-tubulin (1:1000; Sigma), anti-TBCE (1:1), Texas Red-conjugated goat anti-HRP (1:50; Jackson Laboratory), anti-Futsch (1:1000; DSHB) and anti-Discs large (DLC) 4-F3 (1:1000; DSHB). All primary antibodies were visualized using Alexa 488- or Cy3-conjugated goat anti-mouse IgG (1:200; Invitrogen). To examine the MT network in muscles, muscle 2 in abdominal segment A4 was analyzed as it has fewer tracheal branches to obscure the observation of MTs. Nuclei were visualized by staining with propidium iodide (PI; 1.25 μg/ml) for 30 minutes at room temperature. Images were collected with a Leica SP5 confocal microscope and processed using Adobe Photoshop.

NMJ quantifications largely followed published procedures (Zhang et al., 2001). All images analyzed were projections from complete z-stacks through the entire NMJ4 of abdominal segment A3. Synthetic boutons were defined according to anti-HRP (presynaptic) and anti-DLG (postsynaptic) staining. Branches originating directly from the nerve entry point were defined as primary branches, and each higher-order branch was counted only when two or more boutons in a string could be observed. For bouton area analyses, ImageJ 3.0 (NIH) was used to define anti-HRP-stained individual boutons. The software output reports the area for each bouton automatically. At least 22 NMJ4 terminals of different genotypes were analyzed.

Futsch staining intensity relative to HRP staining at NMJ synapses was quantified largely according to Trotta et al. (Trotta et al., 2004). Staining intensities of Futsch and HRP from an entire NMJ4 terminal were digitalized automatically using ImageJ 3.0. Synthetic boutons with different Futsch staining patterns were quantified following published procedures (Packard et al., 2002; Sherwood et al., 2004). Synthetic boutons were divided into three types based on the Futsch staining pattern: (1) continuous (bundle or spayed bundle), (2) looped, and (3) diffuse (punctate) or no staining. Terminal boutons were defined as those at the ends of synaptic branches. Fourteen NMJ4 terminals from seven animals for each genotype were statistically analyzed for Futsch expression features (see Fig. 7G-I).

For quantification of tubulin staining in muscles, all images analyzed were three-dimensional projections of serial stacks through the muscle cell. The perinuclear areas were defined as the coverage that spans 10 μm around nuclei, which were stained with PI. Tubulin staining signals within the perinuclear area from muscle 2 of abdominal segment A4 were calculated using ImageJ 3.0. The software output reports the ratio of the tubulin-positive area divided by the total perinuclear area. At least four readings, one from one animal, were analyzed for each genotype.

Physiological assays

Intracellular recordings were carried out at 18°C following a conventional procedure (Jan and Jan, 1976). Specifically, wandering third instar larvae were dissected in Ca2+-free HL3.1 saline (Feng et al., 2004) and recorded in HL3.1 saline containing 0.25 mM Ca2+. Intracellular microelectrodes with a resistance of 10-20 MΩ filled with 3M KCl were used for the assay. Recordings were performed using an Axoclamp 2B amplifier (Axon Instruments) in Bridge mode. Data were filtered at 1 kHz, digitized using a Digitizer 1322A (Axon Instruments) and collected with Clampex 9.1 software (Axon Instruments). EJPs were evoked at 0.3 Hz by a suction electrode with a depolarizing pulse delivered by a Grass S488 stimulator (Astro-Grass). EJPs were recorded from muscle 6 of abdominal segment
A2 or A3, followed by mEJP recording for 120 seconds. EJP and mEJP recordings were processed with Clampfit 9.1 software (Axon Instruments). Quantal content was calculated by dividing the corrected EJP amplitude by the mEJP amplitude according to a classical protocol (Martin, 1955). The EJP correction for nonlinear summation was performed using a reversal potential of 10 mV. At least nine animals were recorded for each genotype.

Pharmacological treatment of dissected larvae with nocodazole

MT dynamics were examined using the MT-depolymerizing drug nocodazole. Nocodazole (Sigma) was prepared as a 16.6 mM stock in dimethyl sulfoxide (DMSO). Third instar larvae were dissected open in Ca²⁺-free HL3.1 buffer and incubated with 30 μM nocodazole in Schneider’s insect medium (Sigma) for 4 hours at 25°C. The drug was then washed out with the medium and animals further incubated for 1, 2, 5 and 20 minutes for recovery of MTs. The control group was mock treated with Schneider’s insect medium containing DMSO. The treated samples were then fixed, stained, and imaged as described.

Statistical analyses

All statistical comparisons were performed using GraphPad InStat 5 software. P-values were calculated by two-tailed Student’s t-test.

RESULTS

Drosophila CG7861 encodes an ortholog of human TBCE

Sequence comparison showed that the Drosophila genome contains an uncharacterized gene, CG7861, that encodes an ortholog of human TBCE. Drosophila TBCE is 30% identical and 49% similar to human TBCE (Fig. 1A). The high degree of conservation between Drosophila and human TBCE suggests that its function is well maintained through evolution.

As a first step to understanding the in vivo functions of TBCE, we generated null mutations of tbce (Fig. 1B). We obtained an EMS-induced nonsense mutation, Z0241 (Fig. 1B), by a TILLING approach. This mutation is likely to be a null, as it is located at the 5’ terminus of the coding region and produced no detectable TBCE as assessed by immunostaining (Fig. 2B). We also generated a deletion, LH15, which removes a large part of tbce (Fig. 1B) via P element-mediated imprecise excision. LH15 is presumably another null allele of tbce, based on its molecular lesion. Hemizygous or heteroallelic Z0241 and LH15 mutants are embryonic lethal, whereas LH198, a precise excision control, is fully viable. Similarly, LH260, which removes the majority of the flanking gene CG14591, is also viable with no detectable phenotype (data not shown) (Fig. 1B). In summary, tbce is essential for viability, as the independent mutations Z0241 and LH15 resulted in embryonic lethality. The embryonic lethality of tbce nulls prevented straightforward developmental and functional analyses. To overcome the limitation of early lethality, we constructed multiple independent UAS and RNAi transgenic lines for tissue-specific overexpression and knockdown of tbce, respectively, using the UAS-Gal4 system (Brand and Perrimon, 1993). The efficacy of the transgenic lines was confirmed by western analysis and immunostaining with an anti-TBCE monoclonal antibody, 8E11, that we generated (Fig. 1C; Fig. 2E-G).

Fig. 1. Drosophila TBCE. (A) Sequence alignment of Drosophila (d) and human TBCE. CAP-Gly, glycine-rich cytoskeleton-associated protein; LRR, leucine-rich repeat; UBL, ubiquitin-like. The CAP-Gly, LRR and UBL domains are delineated as previously published (Bartolini et al., 2005). Dark and light gray shading indicate identical and similar amino acids, respectively. Nonsense mutation Z0241 is indicated. (B) Genomic structure of Drosophila tbce and mapping of mutants. The intron-exon organization of tbce and its flanking gene, CG14591, is shown at the top. Gray boxes, coding regions; white boxes, untranslated regulatory regions; ‘gaps’, introns; horizontal line, intergenic region (2220 bp). The two deletion lines LH260 and LH15, generated by imprecise excision of KG09112, are depicted, as is the precise excision line LH198. The genomic region used to generate RNAi knockdowns is indicated. (C) Western analysis of tbce overexpression and RNAi knockdown transgenic flies. The anti-TBCE monoclonal antibody (8E11) that we generated recognizes a single band of the expected size (60 kDa). No change in α-tubulin expression was detected when tbce expression was altered. Actin was used as a loading control.
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TBCE is cytoplasmic and ubiquitously expressed

Previous TBCE overexpression studies in HeLa cells detected TBCE in the cytoplasm (Bhamidipati et al., 2000; Tian et al., 2006). Mouse TBCE is enriched in motor neurons and localizes in both crude membrane and cytosolic fractions prepared from spinal cord (Schaefer et al., 2007). We investigated the expression pattern and subcellular localization of TBCE in the ventral nerve cord (CNS) and muscles (A), whereas mutant embryos showed no specific expression (B). Immunostaining of larval muscles (C) and epidermal cells (D) showed that TBCE protein is localized in the cytoplasm and excluded from the nucleus. Weak expression of TBCE was observed in the central neurons (asterisk) and peripheral axons (arrow). Cytoplasmic TBCE in the ventral ganglion neurons was clearly seen when TBCE was pan-neuronally overexpressed using elav-Gal4 (F), whereas no appreciable expression of TBCE was observed when RNAi was driven by elav-Gal4 (G). Scale bars: 20 μm.

MT network was greatly decreased, with fewer and shorter MT fibers in mutant muscle cells as compared with the dense and evenly distributed MT network in the WT (compare Fig. 3D with 3B), indicating that TBCE is required for MT formation or maintenance.

A mutated Tbce in mouse leads to retarded axonal growth and axonal degeneration (Bonnell et al., 2002; Martin et al., 2002; Schaefer et al., 2007). To assess the role of TBCE in neuronal development, we stained stage 16 embryos with anti-FASII, which detects a set of three longitudinal axon bundles, and with BP102 antibody, which recognizes the anterior and posterior commissures and longitudinal connectives of the ventral nerve cord (VNC). As shown in Fig. 3E, anti-FASII staining of WT embryos showed three parallel longitudinal axon bundles at either side of the body. Compared with the WT, Z0241/DF [Df(2R)1482] or Df(2R)1484 removes tbce completely) and LH15/DF mutants showed longitudinal axon bundles that crossed at the midline (Fig. 3F,G). Interrupted axon bundles were also observed (Fig. 3G). Heteroallelic Z0241/LH15 had comparable axonal defects (data not shown). WT embryos stained with BP102 antibody showed a regular ladder-like pattern of axon bundles in the VNC (Fig. 3H). However, the regular pattern of axons was grossly disrupted in tbce mutants with interrupted or missing longitudinal bundles (Fig. 3L). The dramatic axonal defects in tbce nulls suggest that TBCE is required for axonal growth in Drosophila.

To understand the physiological functions of TBCE, we performed behavioral assays. As tbce nulls are embryonic lethal, we examined the behavior of larvae in which TBCE expression had been genetically altered in a tissue-specific fashion by the UAS-Gal4 system. Tissue-specific knockdown of tbce in neurons or in muscles by C57-Gal4 or in muscles by C57-Gal4 produced fully developed larvae (they were late pupal lethal and fully viable, respectively) with normal rhythmic peristalsis and crawling activity (data not shown). However, a larval roll-over assay revealed obvious and profound locomotion defects when TBCE expression was altered. As a genetic control, transgenic flies of elav-Gal4, C57-Gal4, UAS-tbce and UAS RNAi without alterations in TBCE expression showed indistinguishable roll-over time from the WT (Fig. 4). However, tbce knockdown in neurons and muscles caused significantly slower locomotion compared with the WT, with the average roll-over time increased to 205% and 246%, respectively (Fig. 4). Similarly, neuronal and muscular overexpression of TBCE also caused a significantly compromised roll-over performance compared with the WT, with average roll-over time increased to 168% and 190%, respectively (Fig. 4). The abnormal behavior of animals with altered TBCE expression indicates that TBCE is required for the physiological function of the neuromusculature.

TBCE regulates the development of neuromuscular junction synapses

Abnormal synapses are associated with misregulated MTs (Roos et al., 2000; Sherwood et al., 2004; Trotta et al., 2004). To understand the molecular pathogenesis of HRD, we examined the development of neuromuscular junction (NMJ) synapses in flies in which tbce expression had been manipulated by the UAS-Gal4 system. Drosophila NMJ synapses are a commonly used system to examine protein function at synapses, as they are large, simple and amenable to various morphological and functional assays.

Three NMJ synapse features – synaptic branching, bouton number and average bouton area – were statistically analyzed (Fig. 5). For synaptic branching, both elav-Gal4-driven presynaptic and

Fig. 2. TBCE is cytoplasmic and ubiquitously expressed in neuromusculature. (A,B) Wild-type (WT) and Z0241 mutant Drosophila embryos stained with the 8E11 anti-TBCE monoclonal antibody. WT embryos showed specific and substantial expression of TBCE in the ventral nerve cord (CNS) and muscles (A), whereas mutant embryos showed no specific expression (B). (C,D) Immunostaining of larval muscles (C) and epidermal cells (D) showed that TBCE protein is localized in the cytoplasm and excluded from the nucleus. (E) Weak expression of TBCE was observed in the central neurons (asterisk) and peripheral axons (arrow). (F,G) Cytoplasmic TBCE in the ventral ganglion neurons was clearly seen when TBCE was pan-neuronally overexpressed using elav-Gal4 (F), whereas no appreciable expression of TBCE was observed when RNAi was driven by elav-Gal4 (G). Scale bars: 20 μm.
TBCE functions at synapses and promotes microtubule formation

C57-Gal4-driven postsynaptic knockdowns of tbce displayed a significant over-branching compared with the WT control (Fig. 5A-C,F). However, overexpression of tbce pre- or postsynaptically did not show the opposite phenotype to RNAi knockdown. Instead, presynaptic overexpression of TBCE resulted in normal NMJ branching, whereas postsynaptic overexpression showed mild but significant over-branching (P=0.04) (Fig. 5D-F). Thus, except for presynaptic overexpression of TBCE, which caused normal branching, all other manipulations of TBCE expression resulted in increased branching of NMJ synapses.

Synaptic bouton number was affected similarly to synaptic branching for the four genotypes assayed. Both the elav-Gal4-driven and C57-Gal4-driven tbce knockdown caused a significant increase in bouton number compared with the control (Fig. 5A-C,G). However, overexpression of TBCE by elav-Gal4 resulted in normal bouton numbers, whereas C57-Gal4-driven overexpression caused a significant increase in bouton number (Fig. 5D,E,G). For synaptic bouton area, C57-Gal4-driven knockdown or overexpression of tbce showed significantly decreased bouton size (Fig. 5A,C,E,H). elav-Gal4-driven knockdown also showed decreased bouton size (P<0.001), but presynaptic overexpression exhibited normal bouton size (Fig. 5D,H). These NMJ phenotypes are not due to the Gal4 driver, UAS or RNAi insertions, as these showed wild-type NMJ synapses (data not shown). In summary, quantification analyses showed that except for presynaptic overexpression of tbce, the remaining three manipulations of tbce caused increased branching number, increased bouton number and decreased bouton size at NMJ synaptic terminals. These results demonstrate that TBCE plays a crucial role in NMJ synapse development.

TBCE regulates neurotransmission at NMJ synapses

As shown above, TBCE regulates the development of NMJ synapses (Fig. 5). We then investigated whether TBCE plays a role in synaptic function. We found no change in the amplitude of excitatory junction potentials (EJPs) in animals in which TBCE had been knocked down or overexpressed postsynaptically (Fig. 6A,D-F). However, compared with the WT, both knockdown and overexpression of TBCE presynaptically elevated the EJP amplitudes significantly, by 29% and 40%, respectively (Fig. 6A-D,F). We also examined the miniature excitatory junction potentials (mEJPs), i.e. the amplitude of the response to a single vesicle release, also known as quantal size. The mEJP for the WT was 0.95±0.05 mV. Knockdown and overexpression of tbce presynaptically increased the mEJP by 35% and 27%, respectively (Fig. 6A-C,G). The increase in EJP and mEJP was not due to increased neurotransmission (Fig. 6A,C,G). As it displayed normal neurotransmissions (data not shown). Alterations in TBCE on the postsynaptic side caused no significant change in mEJP as compared with the WT (Fig. 6D,E,G). The quantal content – the number of vesicles released per evoked event, calculated by dividing the corrected EJP amplitude by the mEJP amplitude – was affected only when tbce was overexpressed presynaptically (an increase of 69%, P<0.05) (Fig. 6H). Presynaptic knockdown of tbce caused a significant increase in mEJP frequency, but other manipulations of tbce expression showed no significant changes (Fig. 6I).
In summary, altering the dosage of tbce on the postsynaptic side had no effect on the neurotransmission parameters we examined. But, a precisely controlled expression of tbce on the presynaptic side was necessary for the normal function of NMJ synapses. Taken together, these analyses demonstrate that TBCE functions presynaptically to control neurotransmission at NMJ synapses.

TBCE is required for MT formation in presynaptic terminals

A previous study revealed decreased MT density in mutant cells from HRD patients (Parvari et al., 2002). In TbcE mutant mice, axonal MT loss proceeds retrogradely in parallel with the axonal dying-back process (Martin et al., 2002; Schaefer et al., 2007). To investigate the effect of TBCE on MTs in the nervous system, we stained third instar larvae with antibodies against α-tubulin, Futsch (the fly ortholog of mammalian MAP1B; MTAP1B) and HRP (Fig. 7). As shown in Fig. 7B, tbce knockdown in presynaptic neurons resulted in obviously decreased, interrupted or even missing MTs at the distal part of the synaptic terminal detected by anti-α-tubulin staining (Fig. 7B-B'). Overexpression of tbce, however, led to smooth and continuous α-tubulin staining, compared with the WT (compare Fig. 7C' with 7A'). Anti-Futsch is a useful marker to reveal stabilized MTs specifically in neurons (Fig. 7D-F). Similar to the α-tubulin staining, a much weaker and thinner staining with anti-Futsch, with weak or no staining in the terminal boutons, was observed when tbce was knocked down (Fig. 7E'). Statistical analyses showed that the Futsch staining intensity relative to that of HRP was significantly decreased in the knockdown and increased upon overexpression of TBCE in presynaptic neurons, as compared with the WT (Fig. 7D-F,G).

To further define the effect of TBCE on MTs, we quantified synaptic boutons based on the Futsch staining pattern. tbce knockdown in presynaptic neurons caused significantly decreased numbers of synaptic boutons that had organized (continuous and looped) Futsch, and increased numbers of boutons with diffuse or no Futsch signals (82.28%), as compared with the WT (23.57%) (Fig. 7H). By contrast, boutons with Futsch loops were significantly increased, and boutons with diffuse or no Futsch staining were decreased, when TBCE was overexpressed (Fig. 7H). These differences were also reflected in terminal boutons. Only 69% of terminal boutons in tbce knockdown animals had Futsch-positive boutons, compared with 98% in the WT, whereas TBCE overexpression showed a similar number of Futsch-positive boutons as the WT (Fig. 7I). In summary, knockdown of tbce in presynaptic neurons resulted in decreased MTs, whereas overexpression of tbce led to increased MTs in synaptic terminals.
TBCE antagonizes Spastin to regulate MT formation

To better understand how TBCE affects MTs, we studied its genetic interaction with Spastin. Spastin severs the MT network in cultured cells and Drosophila neuromusculature (Errico et al., 2002; Roll-Mecak and Vale, 2005; Sherwood et al., 2004; Trotta et al., 2004). We first confirmed that Spastin severs MTs when it is overexpressed in muscles (see Fig. 8D) (see also Sherwood et al., 2004; Trotta et al., 2004), although no obvious abnormality in the MT network was observed in spastin nulls (Fig. 8E).

Compared with the thin presynaptic neuronal terminals (Fig. 7), muscle cells enabled a much higher resolution visualization of MTs (Fig. 8). In the large multi-nucleated muscle cells (80×400 μm) from a third instar larva, a remarkable MT meshwork was revealed by anti-α-tubulin staining, with the highest intensity of staining around the nucleus (Fig. 8A). The intensity of perinuclear MT staining was quantified for various genotypes (see Fig. S1 in the supplementary material). Compared with the WT, overexpression of tbce in muscles increased the MT network dramatically, with a prominent perinuclear enrichment (Fig. 8B). Indeed, the perinuclear MTs had to be overexposed in order to see individual MT fibers in the area distal to the nucleus (Fig. 8C). Conversely, RNAi knockdown of tbce decreased the network, with sparser and shorter MT fibers (Fig. 8D). The decreased MT network was ameliorated, although not completely rescued (compare Fig. 8H with 8C,E), indicating an antagonistic interaction between the two.

TBCE is acutely required for MT polymerization

To further elucidate the requirement for TBCE in MT formation, we treated dissected animals with the MT-depolymerizing drug nocodazole to disassemble the MTs completely, and then followed MT reformation after drug washout. We noticed that after mock treatment for 4 hours with a buffer containing the DMSO solvent, the MT network in muscles, especially in WT and tbce-overexpressing muscles, was not as dense as in the untreated cells (compare Fig. 9A-B with the corresponding Fig. 8A-B). As expected, 30 μM nocodazole treatment of WT animals for 4 hours eliminated the MT network, and only residual MT buds could be seen in muscles (compare Fig. 9Aa with 9A). Appreciable recovery of MTs, represented by denser perinuclear tubulin staining and longer MT fibers, could be detected 2 minutes after drug washout in the WT (Fig. 9Ab). By 5 minutes, near complete recovery of MTs was observed (compare Fig. 9Ac with 9A). tbce-overexpressing animals showed a similar pattern of MT recovery as the WT (compare Fig. 9Ba-Bd with the corresponding 9Ab-Ad). The recovery of MTs was much slower, however, when TBCE was knocked down by RNAi (compare Fig. 9Ca-Cd with the corresponding 9Aa-Ad). By 20 minutes, recovery of MTs was appreciable, but there was still a large number of MT buds or fibers that had not yet formed a MT network (Fig. 9Cd). By 1 hour after washout, the MTs had still not completely recovered (data not shown). For statistical analyses of MT recovery after the drug treatment, see Fig. S2 in the supplementary material. This experiment showed that tbce is acutely required for MT network formation.

DISCUSSION

Misregulated MTs are associated with human diseases such as Fragile X syndrome, hereditary spastic paraplegia and HRD. We have been using Drosophila as a model system to unravel the molecular pathogenesis of Fragile X syndrome (Reeve et al., 2008; Zhang et al., 2001; Zhang and Broadie, 2005), the most common form of inherited mental retardation. Fragile X mental retardation protein (FMRP, encoded by FMR1) plays an important role at synapses (Penagarikano et al., 2007; Zhang and Broadie, 2005). At the molecular level, the MT-associated protein MAP1B is a target of FMRP and is upregulated in Fmr1 knockout mice and mutant flies.
(Iacoangeli et al., 2008; Lu et al., 2004; Zhang et al., 2001). Since defective MTs are implicated in both Fragile X syndrome and HRD, and both diseases show mental retardation, we sought to unravel the in vivo functions of TBCE with regard to synapse development and MT formation. We provide in vivo evidence demonstrating that TBCE and the MTs that it regulates function at NMJ synapses, and that at the molecular level, TBCE is both necessary and sufficient to promote MT formation.

TBCE regulates NMJ synapse development and function

The MT cytoskeleton regulates synapse development and function, but how MT functions at synapses is poorly understood (Ruiz-Canada and Budnik, 2006). Futsch, a MT-associated protein, stabilizes MTs in presynaptic neurons, and futsch mutants show reduced bouton number and increased bouton size (Roos et al., 2000), whereas spastin mutants have the opposite phenotype of increased bouton number but decreased bouton size (Sherwood et al., 2004). Our analyses show that TBCE plays a role at synapses. Except for the presynaptic overexpression of tbce, all other manipulations of tbce at either side of the NMJ synapse caused increased branching number, increased bouton number and decreased bouton size, demonstrating that tbce is required for normal NMJ synapse development (Fig. 5). Given the dramatic MT alterations on both pre- and postsynaptic sides (Figs 7 and 8), the NMJ phenotypes appear subtle (Fig. 5). The seemingly conflicting result that both overexpression and knockdown of tbce on the postsynaptic side led to similar phenotypes in synapse development supports an existing hypothesis that abnormal synaptic growth results from the disruption of MT dynamics, rather than from an alteration in the absolute quantity of MTs (Ruiz-Canada and Budnik, 2006).

Increased neurotransmission, reflected in both EJP and mEJP amplitude, was observed upon presynaptic alteration of tbce expression, whereas postsynaptic manipulations of tbce showed normal neurotransmission (Fig. 6). This suggests that synaptic neurotransmission is sensitive to pre- but not postsynaptic MT alteration, although postsynaptic alterations of tbce had a significant effect on synapse development (Fig. 5). Interestingly, both overexpression and knockdown of tbce on the presynaptic side led to a similar increase in both EJP and mEJP amplitude (Fig. 6). The increased EJP amplitude observed upon presynaptic alterations of TBCE might be accounted for by increased mEJP amplitude (Fig. 6). The increase in mEJP amplitude could be caused by an increase in presynaptic vesicle size, an increase in the concentration of vesicular glutamate, or an increase in postsynaptic glutamate receptor sensitivity. It is interesting to note that the mEJP is also increased in both Fmr1-null and Fmr1-overexpression NMJ synapses (Zhang et al., 2001). However, the exact mechanism by which TBCE, and other MT regulators, affect neurotransmission remains to be elucidated.
TBCE antagonizes Spastin in regulating MT dynamics

Our genetic analyses revealed an antagonistic interaction between TBCE and Spastin. TBCE promotes MT formation, whereas Spastin severs MTs. Autosomal dominant hereditary spastic paraplegia (AD-HSP) is a heterogeneous group of neurodegenerative disorders characterized by progressive and bilateral spasticity of the lower limbs, with specific degeneration of the longest axons in the CNS (Reid, 1997). Forty to fifty percent of all AD-HSP cases are caused by mutations in spastin. However, the MT-related pathology of human patients with spastin mutation has not been documented.

Overexpression of *spastin* in *Drosophila* neuromusculature (Sherwood et al., 2004; Trotta et al., 2004) and in cultured cells (Errico et al., 2002; Roll-Mecak and Vale, 2005) caused dramatically fragmented and reduced MTs. Surprisingly, morphologically normal muscles are present in patients with spastin mutations, although large-scale disruption of MT pathways was detected at the molecular level (Molon et al., 2004). No MT defects were reported in a mouse model in which the endogenous spastin is truncated (Tarrade et al., 2006). Similarly, *spastin*-null mutants of *Drosophila* show no dramatic change in MT appearance in muscles (Fig. 8E), suggesting that Spastin plays a fine-tuning role in MT dynamics. Indeed, *spastin* nulls are late pupal lethal with a few adult escapers, further confirming a subtle role for Spastin in MT regulation. By comparison, *tbce* nulls are embryonic lethal, whereas knockdown of *tbce* leads to a dramatically reduced MT network in *Drosophila* neuromusculature (Figs 7-9). Thus, in contrast to the nuanced role of endogenous Spastin, TBCE plays a crucial role in MT formation.

TBCE promotes MT formation

Although *Drosophila* possesses a TBCE ortholog, no previous studies of it have been reported. Our work shows for the first time that *tbce* is essential for early neuromuscular development in *Drosophila* (Fig. 3). We also provide in vivo evidence demonstrating that *Drosophila* TBCE is both required and sufficient for MT formation.

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**Fig. 8.** *tbce* antagonizes MT-severing *spastin*. (A-H*) *Drosophila* larval muscles were stained with anti-α-tubulin to show the MT network (green) and with PI to show the nucleus (red). A’-H’ are higher magnification views from A-H. The MT network in the muscles is shown for the WT (A) and for *tbce* overexpression (B), *tbce* knockdown (C), *spastin* overexpression (D) and *spastin*-null (E) mutants. Co-overexpression of *tbce* and *spastin* produced a phenotype more like that of overexpression of *spastin* alone (compare F with B and D). Knockdown of *tbce* while concomitantly overexpressing *spastin* led to an enhanced form of the phenotype observed upon *spastin* overexpression alone (compare G with D). Knockdown of *tbce* in the *spastin* mutant background ameliorated the *tbce* RNAi phenotype (compare H with C and E). Scale bars: 10 μm.

**Fig. 9.** TBCE is required for MT network formation. (A-Cd) Muscle MTs were examined after treatment with the MT-depolymerizing drug nocodazole. (A-Ad) WT; (B-Bd) *tbce* overexpressed; (C-Cd) *tbce* knocked down by RNAi. (A-C) Muscle MTs upon mock treatment with DMSO solvent for 4 hours. Note that the MTs in mock-treated cells of WT (A) and *tbce* overexpression (B) flies were consistently less dense than in their untreated counterparts (compare with Fig. 8A,B). (Aa-Ca) MTs in muscle cells treated with nocodazole with no washout. Ab-Cb, Ac-Cc and Ad-Cd show MTs after nocodazole washout for 2, 5 and 20 minutes, respectively. Near complete recovery of MTs by 5 minutes after washout was observed in the WT (Ac), but only weak recovery of MTs was observed in *tbce* knockdown flies (Cc). Even after 20 minutes of washout, the recovery was still not complete in *tbce* knockdown flies (Cd). MTs are labeled with anti-α-tubulin (green); nuclei are stained with PI (red). Scale bar: 10 μm.
formation (Figs 7-9), supporting early in vitro biochemical studies that showed that TBCE assists in α-β-tubulin heterodimer formation (Tian et al., 1996; Tian et al., 1997).

We found that overexpression of tbce produced increased MTs (Figs 7 and 8). To our knowledge, this is the first report of increased MT formation when a tubulin chaperone is overexpressed, and is contrary to reports in other systems. Overexpression of human TBCE in cultured cells leads to complete disruption of MTs (Bhamidipati et al., 2000; Sellin et al., 2008; Tian et al., 2006), as does overexpression of a TBCE-like protein (Bartolini et al., 2005; Keller and Lauring, 2005; Sellin et al., 2008). It was further hypothesized that the UBL domains present in TBCE and the TBCE-like protein might contribute to the degradation of tubulin via the proteasomal pathway (Bartolini et al., 2005). In addition, the overexpression of other tubulin chaperones, such as TBCD, results in a similar disruption of MTs (Bhamidipati et al., 2000; Martin et al., 2000). These in vivo data are consistent with the early in vitro observation that TBCD or TBCE in excess destroys tubulin heterodimers by sequestering the bound tubulin subunit, leading to the destabilization of the freed partner subunit (Tian et al., 1997). It is thus believed that in addition to assisting in the folding pathway, TBCE also interacts with native tubulins to disrupt α-β-tubulin heterodimers (Bhamidipati et al., 2000). The discrepancy between our overexpression result and the findings of others could have several explanations. First, the use of different experimental systems: transgenic animals in this work and cultured cells in other studies (Bhamidipati et al., 2000; Sellin et al., 2008; Tian et al., 2006). Second, different systems might have different expression levels of tbce, leading to varying effects on MTs. Third, Drosophila and human TBCE might have diverged functions. Further analyses are needed to reconcile the conflicts in the effects of TBCE overexpression in these different systems. In general, however, tbce mutant phenotypes are consistent in all species examined so far, from yeast to human, indicating that the function of TBCE in promoting MT formation has been well-conserved throughout evolution.

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