A Drosophila model of early onset torsion dystonia suggests impairment in TGF-β signaling

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To investigate the cellular and molecular etiology of early onset torsion dystonia, we have established a Drosophila model of this disorder. Expression of mutant human torsinA deleted for a single glutamic acid residue (ΔE HtorA), but not normal HtorA, elicits locomotor defects in Drosophila. As in mammalian systems, ΔE HtorA in flies forms protein accumulations that localize to synaptic membranes, nuclei and endosomes. Various morphological defects at the neuromuscular junction in larvae expressing ΔE HtorA were observed at the EM level, some of which resemble those recently reported for mutants with defects in TGF-β signaling. These results together with the distribution patterns and localizations of ΔE HtorA accumulations suggested that ΔE HtorA could interfere with some aspect of TGF-β signaling from synapses to endosomes or nuclei. Consistent with this possibility, neuronal overexpression of Drosophila or human Smad2, a downstream effector of the TGF-β pathway, suppressed the behavioral and ultrastructural defects of ΔE HtorA flies. These results raise the possibility that a defect in TGF-β signaling might also underlie early onset torsion dystonia in humans.

INTRODUCTION

Dystonia, the third most common movement disorder in humans, is characterized by sustained involuntary muscle contractions resulting in severe twisting movements and abnormal postures. Although there are a number of heritable forms of dystonia, the most common and severe form is early onset torsion dystonia, a neurological disorder affecting the central nervous system. It is inherited in autosomal dominant fashion with variable penetrance. About 30–40% of heterozygotes manifest the disease with onset between 1 and 28 years of age (1–3). Although the cellular and molecular etiology of torsion dystonia is unknown, it is not associated with any obvious neurodegeneration (4,5). Recently, this form of dystonia has been linked to the DYT1 gene (6). The causative mutation is a 3 bp deletion that results in deletion of a glutamic acid (ΔE302/303) residue near the C-terminus of the encoded protein, Torsin A (HtorA). Another study in a patient with early onset dystonia and early onset Parkinson disease with myoclonic features revealed the presence of an 18 bp deletion mutation in the DYT1 gene that resulted in deletion of six amino acids (ΔF323–Y328) in HtorA (7).

Sequence comparisons demonstrate that HtorA is a member of the HSP100/Clp family of ATP-dependent chaperones that functions in the assembly, disassembly and operation of a variety of protein complexes including those involved in many types of vesicular transport (8). Immunolocalization of HtorA expressed in transfected cell lines suggests that it is a lumenally oriented protein localized to the intracellular membranes of the endoplasmic reticulum (ER). ΔE HtorA has been found in large spheroid intracellular structures that do not colocalize with ER markers (9). At the EM level, it was reported that ΔE TorsinA was found in membrane whorls that may derive from ER (10). In addition, HtorA is localized in Lewy bodies in postmortem Parkinson disease patients (11,12). Expression of normal HtorA in cultured cells resulted in colocalization with various heat shock proteins and suppressed α-synuclein aggregation. However, expression of ΔE HtorA did not suppress α-synuclein aggregation (13). Similarly, expression of normal HtorA suppressed polyglutamine-induced protein aggregation in Caenorhabditis elegans (14). These observations suggest that HtorA could function as a chaperone involved in the folding or processing of secreted or membrane proteins. The effects of the ΔE mutation on HtorA functions are unknown although the dominant phenotype together with other observations suggests that the mutant protein has a dominant negative or gain-of-function mode of action. The basis of the variable penetrance of early onset torsion dystonia caused by ΔE HtorA is still...
unclear. A ‘second-hit’ model in which other genetic mutations or environmental factors increase the susceptibilities of individuals heterozygous for the \( \Delta E \) HtorA mutation has been proposed (9,15,16).

As *Drosophila* offers many experimental advantages for genetic and molecular studies, while the basic molecular components and neuronal signaling mechanisms are evolutionarily conserved (17–19), it is increasingly being used as a model system to study human neurological disorders. Here, we have established a *Drosophila* model of early onset torsion dystonia that may increase our understanding of the molecular and cellular mechanisms responsible for this disorder. Several lines of evidences from this study suggest that the behavioral and ultrastructural abnormalities observed in \( \Delta E \) HtorA-expressing flies involve impaired TGF-\( \beta \) signaling. Therefore, we propose that a defect in TGF-\( \beta \) signaling may also contribute to the etiology of early onset torsion dystonia in humans.

**RESULTS**

Transgenic expression of human *TorsinA* in *Drosophila*

To develop a model of early onset torsion dystonia in *Drosophila*, we used the Gal4/UAS system (20) to express human cDNAs encoding wild-type (WT) or \( \Delta F323–Y328 \) (\( \Delta FY \)) (7) or \( \Delta E302/303 \) (\( \Delta E \)) (6) mutant forms of HtorA in transgenic flies. We used the C155-Gal4 driver (21) and the 3,4-dihydroxyphenylalanine-\( \cdot \)-decarboxylase (Ddc)-Gal4 driver (22), respectively, to drive expression of these constructs either pan-neuronally or specifically in dopaminergic neurons.

To characterize anti-HtorA antiserum and various UAS-HtorA transgenic flies, expression of WT HtorA proteins by Ddc-Gal4 drivers was examined using goat-anti-HtorA antiserum (Fig. 1A). Strong immunolabeling was observed in dopaminergic neurons in ventral nerve cords (Fig. 1A1). No staining was observed in larvae carrying the driver (Ddc-Gal4/+) alone (Fig. 1A2). Preincubation of the anti-HtorA antiserum with HtorA peptides also eliminated staining (Fig. 1A3).

Examples of WT and mutant HtorA expression in individual dopaminergic neurons in the ventral nerve cord are shown in Figure 1B–D. Comparable levels of expression were observed for each of the different HtorA constructs in two independent transgenic lines for each construct (Fig. 1B1–D2). A striking difference was apparent in the immunolocalization patterns of \( \Delta E \) HtorA compared with WT or \( \Delta FY \) HtorA (Fig. 1B–D). WT and \( \Delta FY \) HtorA are distributed diffusely in the cytoplasm in adult neurons, whereas \( \Delta E \) HtorA forms dense accumulations that cluster around membranes (Supplementary Material, Fig. S3A–D).

The same \( \Delta E \) HtorA clusters were observed using two independent anti-HtorA antisera (Supplementary Material, Fig. S1). These distribution patterns are similar to those that have been reported for expression of WT and mutant HtorA in transfected human cells cultured *in vitro* (10,23). Thus, the aberrant localization of the \( \Delta E \) HtorA into clusters can be recapitulated when expressed *in vivo* in *Drosophila*. When expression of WT, \( \Delta FY \) and \( \Delta E \) HtorA constructs was driven postsynaptically by the muscle-specific C57-Gal4 driver (24), the proteins showed distribution patterns similar to those in neurons (Fig. 3A and B). WT and \( \Delta FY \) HtorA proteins localized diffusely in the muscle sarcoplasm. In contrast, the \( \Delta E \) HtorA protein formed protein clusters. Western blot analysis of larval body wall muscle extracts revealed one strong band at the size of 40 kDa (Supplementary Material, Fig. S2). Comparable levels of expression were found for WT and both mutant forms of HtorA. Compared with either WT or \( \Delta E \) HtorA, the \( \Delta 18 \) bp HtorA moved faster consistent with its smaller size (Supplementary Material, Fig. S2). Similar results were reported from human cell culture experiments (23).

**Expression of \( \Delta E \) HtorA in *Drosophila* results in behavioral abnormalities**

Expression of WT, \( \Delta FY \) and \( \Delta E \) HtorA in neurons of the adult CNS gave results similar to those observed in larvae. WT and \( \Delta FY \) HtorA proteins are distributed broadly throughout the cytoplasm in adult neurons, whereas \( \Delta E \) HtorA forms dense accumulations that cluster around membranes (Supplementary Material, Fig. S2). Comparable levels of expression were found for WT and both mutant forms of HtorA. Compared with either WT or \( \Delta E \) HtorA, the \( \Delta 18 \) bp HtorA moved faster consistent with its smaller size (Supplementary Material, Fig. S2). Similar results were reported from human cell culture experiments (23).
Material, Movie A and B). Ubiquitous expression using a Tubulin-Gal4 driver produced very similar results (Fig. 2D).

We observed a similar but somewhat less extreme phenotype when ΔE HtorA was expressed specifically in dopaminergic neurons (Fig. 2B). It was necessary to age the ΔE HtorA-expressing flies for at least 12 days before 90–100% of them lost locomotor control at 38°C. The weaker phenotype of the flies expressing ΔE HtorA in dopaminergic neurons compared with the pan-neurally expressing flies suggests that the early onset and more severe phenotype requires ΔE HtorA expression in multiple types of neurons, which may include motor neurons.

Postsynaptic expression of ΔE HtorA in muscles also resulted in an age- and temperature-dependent loss of locomotor control with 90–100% of the ΔE HtorA-expressing flies remaining on the bottom of the vials at 38°C after 9 days of age (Fig. 2C).

In summary, expression of ΔE HtorA in Drosophila either in neurons or muscles can cause severe motor impairments as a function of age and environmental conditions, and the severity and age of onset of this phenotype vary depending on the spatial pattern of expression.

**ΔE HtorA clusters have distinct subcellular localization**

To further investigate how the ΔE HtorA protein clusters might contribute to the observed behavioral defects, we examined the subcellular distribution patterns of these clusters in well-characterized type I glutamatergic synapses in larval neuromuscular junction (NMJ). At type I glutamatergic synapses, we found that at least some of the postsynaptic ΔE HtorA clusters were localized near synaptic boutons in association with the subsynaptic reticulum (SSR). This is illustrated in Figure 3A and B, which shows three consecutive confocal slices for two sets of synaptic boutons labeled with specific antibodies for presynaptic membranes (anti-HRP) as well as for the SSR (anti-DLG).

Cell bodies of motor neurons in ventral nerve cords of larvae expressing ΔE HtorA in a pan-neural pattern contained HtorA clusters whose subcellular location partially overlapped with endosomes as marked by anti-HRS antibodies (Fig. 3C1–C3). We also observed some overlap between ΔE HtorA clusters and endosomes in the motor neuron nerve bundles (Fig. 3D) and in muscles (data not shown). In contrast, no overlap was observed between WT or Δ18 bp HtorA proteins and the endosomal markers detected by anti-HRA antiserum (data not shown). We examined the localization of ΔE HtorA clusters at higher resolution using silver-enhanced immuno-EM analysis (Fig. 4). In ΔE HtorA-expressing flies, silver-enhanced immunogold particles were observed to be associated with various cellular membranes at nerve terminals and in cell bodies. Examples of immunogold particles localized at a synapse, within the SSR, at the outer periphery of the nucleus, adjacent to

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**Figure 1. Expression of human TorsinA in Drosophila.** Confocal images of ventral nerve cords expressing WT HtorA in dopaminergic neurons from third instar larvae stained with anti-HtorA antibodies. (A1) Ddc-Gal4/UAS-WT HtorA expressing, (A2) control for genetic backgrounds (Ddc-Gal4/+ ) were stained with anti-HtorA antibodies. (A3) Ddc-Gal4/UAS-WT HtorA stained with anti-HtorA antibodies previously incubated with HtorA peptides to demonstrate specificities of immunostaining. (B and C) Confocal images of single dopaminergic neurons from larval ventral nerve cords expressing different HtorA constructs stained with anti-HtorA antibodies. Two independent lines were examined for each construct: (B–B2) WT, (C–C2) ΔFY, (D–D2) ΔE. Note the presence of distinct protein clusters in cells expressing ΔE HtorA. Bar: 60 μm (A1–A3) and 5 μm (B–D2).
mitochondria and inside the lumen of single- and double-membrane endosomes are shown in Figure 4A–C. In control experiments with larvae that did not express ΔE HtorA incubated with anti-HtorA antibodies or with larvae that did express ΔE HtorA incubated in the absence of anti-HtorA antibodies, we observed fewer than 10% of the total particles seen in the experimental analysis, confirming the specificity of silver-enhanced anti-HtorA particles. Similar subcellular distribution patterns were observed with either presynaptic or postsynaptic expression of ΔE HtorA. The localization of ΔE HtorA clusters in synaptic regions, at nuclei and at endosomes suggests that the mutant protein could affect synaptic structure and function by interfering with one or more signaling pathways from synapses to nuclei and/or endosomes or vice versa.

**Expression of ΔE HtorA induces morphological abnormalities at type I glutamatergic synapses**

To determine whether ΔE HtorA expression resulted in any structural defects at synapses, we further examined type I glutamatergic synapses at the larval NMJ. We did not observe any significant differences in numbers of type I synaptic boutons in larvae expressing WT or ΔE HtorA in neurons (WT, 88.7 ± 2.9; ΔE, 79.7 ± 3.9, P > 0.08) or in muscles (WT, 78.0 ± 4.0; ΔE, 87.2 ± 3.6, P > 0.1) (Fig. 5A–D). However, high-power confocal microscopy revealed altered morphologies of synaptic boutons expressing ΔE HtorA motor-neurons or muscles (Fig. 5E–H). Synaptic boutons expressing WT HtorA had a normal, smooth rounded appearance (Fig. 5E and G). In contrast, synaptic boutons expressing ΔE HtorA were noticeably enlarged and many of them had abnormal irregular shapes (Fig. 5F and H).

To further characterize synaptic defects associated with ΔE HtorA expression, we performed an ultrastructural morphometric analysis on serial reconstructions obtained from two independent samples of boutons expressing WT or ΔE HtorA presynaptically. We examined a total of seven controls, 10 WT HtorA and nine ΔE HtorA boutons. There was no significant difference between control synaptic boutons and WT HtorA synaptic boutons (Table 1). However, the length, surface area and volume of ΔE HtorA boutons were all significantly increased compared to WT HtorA boutons (Table 1). This suggests that the mutant protein could interfere with the morphogenesis of synaptic boutons at the larval NMJ.

**Figure 2.** Impaired locomotor behaviors in HtorA-expressing flies. Flies of different ages expressing WT or ΔE HtorA under the control of various Gal4 drivers were placed in vials at 38°C and their behavior was monitored for 10 min. The number of actively walking or climbing flies is plotted at each time point. Time points showing a significant difference (P < 0.05) determined by t-test analysis are marked by an asterisk. (A) C155 (pan-neuronal) driver, (B) Ddc (dopaminergic neuronal)-Gal4 driver, (C) C57 (muscle) driver, (D) Tubulin (ubiquitous) Gal4 drivers. Thirteen groups of C155/+ , 15 groups of C155;UAS-WT HtorA/+ , 15 groups of C155;UAS-ΔE HtorA/+ , 13 groups of DdcGal4/+ , 11 groups of Ddc-Gal4/+ ;UAS-WT HtorA/+ , 10 groups of DdcGal4/UAS-ΔE HtorA, 12 groups of C57/+ , 15 groups of C57/UAS-WT HtorA, 14 groups of UAS-ΔE HtorA/+ ;C57/+ , 12 groups of TubGal4/+ , 12 groups of TubGal4/UAS-WT HtorA, 12 groups of UAS-ΔE HtorA/+ , TubGal4/+ were collected from three different independent crosses and tested.
significantly larger than those of controls or WT HtorA boutons (Fig. 6A1 and A2; Table 1).

In addition, expression of ΔE HtorA also caused ultrastructural abnormalities at regions of synaptic contact (Fig. 6C–F). One such defect was the absence of synaptic vesicle pools near some synapses. Synapses in WT HtorA boutons were always surrounded by a pool of synaptic vesicles regardless of whether one or more presynaptic dense bodies (T-bars or 'active zones') were present (Fig. 6B1–B6). However, about 12% of ΔE HtorA synapses (23/183) were devoid of synaptic vesicle pools and surrounded instead by a discrete clear zone (Fig. 6C1–C6). A second abnormality observed in synaptic regions was the presence of abnormal membrane structures that extended within synaptic vesicle pools (Fig. 6D; Table 1). The abnormal membrane structure extended across the bouton, interconnecting two dense bodies on opposite sides. We observed similar abnormal membrane structures in all ΔE HtorA boutons examined. A third presynaptic abnormality was the presence of free dense bodies (Fig. 6E), which are clear in a high-magnification inset. Despite their morphological resemblance to presynaptic dense bodies, these structures were free at the presynaptic terminals rather than anchored to the synaptic membrane (Table 1). In addition, the boutons expressing ΔE HtorA showed significantly increased synaptic density area compared with boutons expressing WT HtorA (Table 1). These structural defects at the synaptic terminals in ΔE HtorA expressing boutons may account for the locomotor defects observed at elevated temperatures.

**Figure 3.** Immunolocalization of ΔE HtorA protein clusters at synaptic boutons and within neurons (A and B) Two sets of three consecutive Z-series confocal slices (0.3 μm intervals) of type Ib glutamatergic boutons labeled with anti-HRP (blue), anti-DLG (green), and anti-HtorA (red) antisera. White arrowheads point to ΔE HtorA clusters localized near synapses or within SSR. ΔE HtorA proteins were expressed in muscles. (C1–C3) Confocal images of neuronal cell soma expressing ΔE HtorA in all neurons in the larval ventral nerve cord stained with (C1) anti-HtorA antisera (red) and (C2) anti-HRS antisera (green). Note that colocalizations indicated by yellow colors (white arrowheads) of ΔE HtorA clusters with anti-HRS signal, which is a specific marker for endosomes are clearly seen in a superimposed image (C3) and an inset. (D) Confocal image of larval nerve bundles. Again, note overlap between a subset of ΔE HtorA clusters and endosomal markers. Bar: 5 μm (A and B) and 10 μm (C and D).
Expression of dSmad2 or hSmad2 suppresses ΔE HtorA phenotypes

The presence of free dense bodies in presynaptic terminals associated with expression of ΔE HtorA was also recently observed in several Drosophila mutant affecting receptors or ligands of the TGF-β signaling pathway (26–28). The phenotypes are not identical however, as mutations in wishful thinking (wit), which affects a type II BMP receptor causes additional synaptic defects including a decrease in levels of Fasciclin II and a distinctive detachment of synaptic membranes, which are not observed in larvae expressing ΔE HtorA (Fig. 6 and data not shown). Those observations suggest that ΔE HtorA expression might disrupt some part of the TGF-β signaling pathway to interfere with proper synaptic development. As ΔE HtorA clusters localized at synapses, SSR, endosomes and the nuclear periphery, it is possible that they disrupt trafficking of some components of TGF-β signaling from synapses to endosomes or nuclei. Mad and dSmad2 are known transcriptional effectors of the TGF-β pathway that regulates target gene expression to control cell proliferation (29), neuronal remodeling during metamorphosis (30) and synaptic plasticity in type I glutamatergic NMJs (26,27). Studies indicate that dynamic changes in the molecular components of Smad complexes during trafficking from receptors to endosomes or nuclei are essential for eliciting proper responses (31,32). We hypothesized that ΔE HtorA interfered with trafficking of mothers against decapentaplegic (Mad) or Drosophila Smad2 (dSmad2) between synapses and nuclei or endosomes.

To explore the possibility that ΔE HtorA interfered with the TGF-β pathway, we examined the effect of overexpressing Mad or dSmad2 in a ΔE HtorA background. At the behavioral level, pan-neural expression of dSmad2 substantially rescued the locomotor defects caused by ΔE HtorA; at 38°C, these flies remained as active as controls (C155-Gal4/þ) (Fig. 7A).

However, under the same condition, Mad overexpression did not suppress the ΔE HtorA behavioral defects. Pan-neural expression of dSmad2 alone had no effect on the flies compared with the control background (C155-Gal4/þ) (Fig. 7B). Although ΔE HtorA behavioral phenotypes were suppressed by dSmad2 overexpression, mutating one copy of dSmad2 to

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**Figure 4.** Subcellular localization of ΔE HtorA by immuno-EM analysis. ΔE HtorA-expressing larvae were dissected and incubated with anti-HtorA antiserum followed by addition of anti-rabbit IgG-conjugated 1 nm gold particles and processed for EM after silver enhancement. Localization was detected at synaptic densities (A, A1), within SSR (A2), at nuclear membranes (A3), associated with mitochondria (B) and at endosomes (C1 and C2). In (A) and (A1), ΔE HtorA expression was under the control of the C155 pan-neuronal driver. In the remaining panels expression was driven by the C57 muscle driver. n, Nucleus.

**Figure 5.** Altered morphology in ΔE HtorA-expressing larvae. NMJs for muscle 6/7 were stained with anti-HRP to visualize overall morphology and bouton numbers. No major differences between these parameters were observed in comparison between (A) C155-Gal4;UAS-WT HtorA and (B) C155-Gal4;UAS-ΔE HtorA or (C) UAS-WT HtorA/C57-Gal4 and (D) UAS-ΔE HtorA;C57-Gal4. However, closer examination revealed abnormalities (black arrowheads) in the size and appearance of boutons in larvae expressing ΔE HtorA either presynaptically (F) or postsynaptically (H) compared with the corresponding controls (E and G). Bar: 50 μm (A–D) and 10 μm (E–H).
reduce the gene dosage up to 50% did not enhance the phenotypes (Fig. 7B). Expression of dSmad2 also rescued the ΔE HtorA morphological and ultrastructural phenotypes (Fig. 6C; Table 1). The volume, surface area and length of synaptic boutons were completely rescued as was synaptic area. Other presynaptic abnormalities observed in ΔE HtorA animals including synapses with empty synaptic vesicle pools, presence of abnormal extended membrane structures and the presence of free dense bodies were eliminated or significantly reduced by expression of dSmad2 (Table 1). Furthermore, we found that expression of human Smad2, a human homologue of dSmad2 (33), rescued behavioral abnormalities as does dSmad2 (Fig. 7). Rescue of ΔE HtorA phenotypes by expression of human or Drosophila Smad2 was not due to indirect effect on ΔE HtorA expression as the levels of ΔE HtorA remained unaltered in the appeared backgrounds (data not shown).

**DISCUSSION**

The major goal of the present investigation was to develop a Drosophila model for early onset torsion dystonia. Our most significant finding is that expression of ΔE HtorA but not WT HtorA either in the nervous system or in muscles of Drosophila results in severely impaired motor behavior. Affected flies exhibit loss of coordination, inability to stand upright, spasmodic activity, leg kicking and held out wings, all of which are reminiscent of the uncontrolled muscle contractions characteristic of early onset torsion dystonia in humans. Interestingly, the ΔE HtorA-expressing flies manifest these behavioral abnormalities only upon acute exposure to 38°C. This result is consistent with the proposed second-hit model to explain why only about 30–40% of individuals heterozygous for the DYT1 mutation develop the phenotypic manifestations of early onset torsion dystonia (9,15,16). According to this model, expression of the mutated ΔE HtorA protein in heterozygous individuals predisposes them towards the disease state making these individuals susceptible to a second hit, such as exposure to some environmental stress or the presence of another genetic factor that triggers complete onset of the disease.

In fact, we have found several phenotypic abnormalities associated with ΔE HtorA expression that are not temperature-dependent and that could underlie the observed behavioral defects at elevated temperatures. Similar to what has been observed in transfected human cells in culture (9,10), we found that ΔE HtorA forms protein aggregates when expressed in Drosophila neurons or muscles (Figs 1 and 3). Localization of ΔE HtorA protein clusters at the confocal microscopy level revealed that some of these clusters were associated with synaptic boutons particularly in the region of the SSR or colocalized with endosomal markers (Fig. 3). In addition to confirming the presence of ΔE HtorA in SSR and in association with endosomes, immune-EM studies demonstrated that ΔE HtorA was also found associated with synaptic densities and the nuclear envelope (Fig. 4). The subcellular localization of ΔE HtorA raises the possibility that it could interfere with a signal transduction pathway between synapses and nuclei that is required for proper synaptic development and function.
Figure 6. Ultrastructural analysis of synapses at type Ib glutamatergic synapses in NMJs of ΔE HtorA expressing larvae. (A1 – A3) Midline cross sections of synaptic boutons with neuronal expression of (A1) WT HtorA, (A2) ΔE HtorA or (A3) dSmad2 and ΔE HtorA simultaneously. Compared with controls, larvae expressing ΔE HtorA showed increased bouton size and abnormal elongated membrane structures (black arrowheads). Arrows indicate presynaptic dense bodies. (B1 – B6) Consecutive images are taken from serial thin sections of a synapse from a larvae expressing WT HtorA in neurons. Synaptic vesicle pools are present at the presynaptic terminals. Arrows indicate presynaptic dense bodies. (C1 – C6) Consecutive images from serial thin sections from synapses of a larva expressing ΔE HtorA in motor-neurons show empty at synaptic vesicle pools near a synapse indicated by asterisk. (D1 – D3) Serial EM images show extended membrane structures observed from synaptic bouton expressing ΔE HtorA protein in neurons. Arrowheads indicate abnormal extended membrane structures and an arrow indicates a presynaptic dense body. (E1 – E4) Free dense bodies indicated by arrow heads at the presynaptic terminals observed from neuronal expression of ΔE HtorA. Free dense bodies structures are more clearly seen at higher magnification EM in a inset. Mit, mitochondria; SSR, subsynaptic reticulum. Bar: 500 nm (A) and 250 nm (B1 – E4).
Detailed confocal and electron microscopic examination of synaptic boutons revealed a number of structural defects in ΔE HtorA-expressing larvae (Figs 5 and 6). The presence of free dense bodies was particularly intriguing because this phenotype has only previously been reported for mutations that perturb the TGF-β signaling pathway. TGF-β signaling pathways regulate many developmental and physiological processes both in vertebrates and invertebrates and perturbations in these signaling pathways have been implicated in a wide array of pathological conditions (34). Recent studies in Drosophila have demonstrated that proper development and plasticity of synaptic boutons in the larval NMJ is dependent on TGF-β pathway, at least in part, by signaling to endosomes and nuclei—where they could gates are found at precisely those subcellular locations—perturbs synaptic development and function of the synapses (27,36).

The existence of a known signaling pathway from synapse to nucleus via endosomal intermediates that regulates growth and development of synapses was of particular interest in the context of the present work because ΔE HtorA expression perturbs synaptic development and ΔE HtorA protein aggregates are found at precisely those subcellular locations—synaptic terminals, endosomes and nuclei—where they could most readily interfere with such a signaling pathway. This possibility was further strengthened with the finding that the production of free dense bodies in motor terminals is associated with ΔE HtorA expression as it is with mutations of components of the TGF-β signaling (26,27).

To investigate further whether the phenotypic abnormalities associated with ΔE HtorA expression were, in fact, dependent on perturbing the TGF-β signaling, we asked whether these phenotypes could be modified by overexpression of Mad or dSmad2, the transcriptional effectors of the TGF-β pathway (26–29). Remarkably, we found that pan-neural overexpression of dSmad2, but not Mad, substantially suppressed the locomotor defects associated with ΔE HtorA expression.

As the locomotor defects observed in ΔE HtorA flies at elevated temperatures bear some striking resemblance with the phenotype of torsion dystonia patients the possible involvement of common mechanisms does not seem unreasonable.
Moreover, a variety of data demonstrate that, as in Drosophila, TGF-β signaling is important for the development of the mammalian nervous system and, in particular, plays an essential role in synaptic development and plasticity (34,37). We also showed that expression of hSmad2 rescues abnormal behaviors as that of dSmad2 in flies (Fig. 7). Consequently, the possibility that the underlying mechanism of early onset torsion dystonia in humans involves a disruption of TGF-β signaling merits further investigation.

Early onset torsion dystonia is one of many severe complex neurobehavioral disorders caused by the interaction of multiple genes, or by a combination of genetic and environmental risk factors (14,15). As there is significant variation in phenotype manifested by human patients, identification of susceptible genes that may be responsible for triggering the severe symptoms is important in seeking an adequate treatment (1–5). In addition to examining human patients, another strategy is to find susceptible genes by taking advantage of an established animal model. As the studies reported here demonstrate, further studies in this Drosophila model for early onset torsion dystonia should provide unique opportunities to obtain additional novel information about the underlying cellular and molecular mechanisms of this disorder.

MATERIALS AND METHODS

Fly genetics

Flies were raised on standard medium at 25°C unless otherwise specified.

Generating human TorsinA transgenic flies. The WT HtorA open reading frame was amplified by polymerase chain reaction (PCR) from a human fetal brain cDNA library (Clontech, CA, USA). Amplified PCR products were subcloned into pBlueScript II KS+ (Stratagene, CA, USA) and DNA sequences were confirmed. For generating ∆E302/303 forms of HtorA, Kunkel mutagenesis was employed (38). Briefly, a ΔGAG primer (5′-TGA AGC AGA GTG GCT GAG ATG ACA TTT TTC CCC-3′) missing a glutamic acid codon at amino acid position 302/303 (indicated by asterisk) was designed. Single-stranded DNAs of pBlueScript II KS+ containing HtorA were generated, purified and annealed with ΔGAG primers. Plasmids containing ΔGAG were confirmed by DNA sequencing. For generating 18 bp deletion mutant forms (∆F323-Y328) of HtorA, three rounds of PCR were performed using three different overlapping primers (5′-CTT TAT CTC AGA AAA CTC TCT CTT TGG GGA AA-3′, 5′-GTT GAA CAC CGT TTT GCA GCC TTT ATC GCC TTT ATC TGA GAA AA-3′, 5′-TGG TAG TAA TAA TCA ATC TGG GTG AAC ACC GTT TT-3′). Final PCR products were deleted for the desired 18 bp were confirmed by DNA sequencing. Open reading frames of WT, ∆E302/303 and ∆F323-Y328 of HtorA were cloned into pUAST-germline transformation vectors. DNA sequences were confirmed again and typical germline transformation technique procedure was followed (19). Several independent fly lines were generated for each construct. Similar levels of expressions and phenotypic properties were observed for independent isolates.

Antibodies and immunocytochemistry

His-tagged human TorsinA recombinant peptides (residues 61–321) were expressed in Escherichia coli M15 carrying the pREP4 plasmid (Qiagen). The fusion proteins were purified using Ni-NTA beads (Qiagen) and SDS–PAGE and then injected into rabbits for antibody production.

Procedures for immunocytochemistry are as described in literature (24). The following antibodies were used: (1) anti-HRP-FITC and anti-HRP-CY5 (Jackson Labs; Bouin’s, 1:80); (2) guinea pig anti-hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (40) (Bouin’s, 1:4000); (3) mAb anti-DLG (41) (Bouin’s, 1:200); (4) rabbit anti-human TorsinA (Bouin’s, 1:2000); and (5) goat anti-human TorsinA (Santa Cruz Biotechnology, Inc., CA, USA; Bouin’s, 1:200). For blocking experiments in Figure 1A3, rabbit or goat anti-human TorsinA antibodies were incubated with human TorsinA peptides (Santa Cruz Biotechnology, Inc., 200 ng/1 μl). Donkey anti-rabbit Alexa 568, anti-guinea pig Alexa 488 and anti-mouse Alexa 488 were used as secondary antibodies with 1:200 dilutions (Molecular Probes, OR, USA). Confocal images were taken on a Bio-Rad MRC 1024 confocal laser scanning microscope (BioRad, UK) and processed using Adobe Photoshop 5.5 (Adobe, CA, USA).

The number of synaptic boutons in larval muscles 6 and 7 (segment A2) in experimental and control larvae was counted under an epi-fluorescence microscope. Two sample t-tests were performed using Minitab software.

Mad, dSmad2 or hSmad2 overexpression. To test suppression by Mad, hSmad2 or dSmad2, UAS-Mad, UAS-hSmad2 (33) (gifts from Dr Stewart Newfeld, Arizona State University) or UAS-dSmad2 flies (30) (a gift from Dr Tzumin Lee, University of Illinois) were crossed to C155-Gal4; UAS-ΔE HtorA/SM6b to generate C155-Gal4/UAS-Mad; UAS-ΔE HtorA, C155/UAS-hSmad2; UAS-ΔE HtorA/UAS-hSmad2 or C155-Gal4; UAS-ΔE HtorA/UAS-dSmad2 flies, which expressed both ΔE HtorA and Mad, hSmad2 or dSmad2 pan-neuronally. UAS-dSmad2 flies were crossed with C155-Gal4 to generate flies overexpressing dSmad2 in neurons to determine if there were any effects on behavior. To examine whether or not compromising TGF-β pathway yielded any obvious phenotypes, dSmad21/2/FM7c flies (gift from Dr Tzumin Lee, University of Illinois) were crossed with C155-Gal4;UAS-ΔE HtorA flies. C155-Gal4/dSmad21/2;UAS-ΔE HtorA flies were collected and tested for locomotor behavior as described in the following text.

For EM analysis, UAS-dSmad2 females were crossed to C155-Gal4/Y; UAS-ΔE HtorA/SM6b-kr-GFP males to separate C155-Gal4/+; UAS-ΔE HtorA/UAS-dSmad2 larvae, which were identified as female larvae lacking GFP expression.
Behavioral assays
Each group of flies tested for locomotor abilities consisted of five males and five females of the same age. After each group of flies was transferred into a vial, the vial was placed in pre-heated 38°C water bath, and observed continuously over a 10 min period. Flies retaining locomotor abilities were considered to be those walking on the sides or tops of vials, rather than being immobilized or remaining on the bottom, at each time point. Tested flies were collected from three different independent crosses and raised at 30°C to accelerate aging. Genetic backgrounds of Gal4 drivers or ΔE HtorA expressing flies were compared with WT HtorA expression flies. Two sample t-tests were performed using Minitab software.

Silver-enhanced anti-human TorsinA immuno-gold electron-microscopy
Control and experimental larvae were dissected in Ca²⁺ free saline and then fixed with 0.005% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h. Samples were marked and processed in the same well. After washing three times with 0.1 M phosphate buffer (pH 7.2) with 0.2% Triton-X 100 (PBT), samples were incubated with anti-human TorsinA antisera (diluted 1:1000) overnight at 4°C. Samples were washed three times with PBT and then with 4% bovine serum albumin and 4% normal goat serum to prevent non-specific binding. Samples were incubated with 1:200 diluted 1 nm gold conjugated anti-rabbit IgG (BBInternational, UK; diluted 1:200) for 2 h at RT with shaking. After washing three times with PBT, silver enhancement was performed according to manufacturer’s directions for 10 min (BBInternational, UK). Samples were washed with H₂O for 5 min and then postfixed with 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M PB overnight.

Samples were subsequently processed as previously described (24). Two independent controls were performed to confirm the specificity of silver-enhanced particles. Larvae that did not express ΔE HtorA antisera were dissected and ΔE HtorA-expressing larvae incubated without anti-HtorA antisera were processed simultaneously with experimental samples. Thin sections (100 nm) were collected on membrane coated one-slot grids and double stained with lead citrate and uranyl acetate solution. Silver-enhanced anti-HtorA positive particles were photographed at 25,000× by a Megaplus digital camera (Kodak, USA) attached to a Philips CM120 Scanning Transmission Electron Microscope (STEM) at 60 kV.

Transmission electron microscopy
The procedure for TEM and morphometric analysis of synaptic boutons have been previously described (24). For this analysis, only type Ib glutamatergic synaptic boutons on muscles 6 and 7 (segment A2) were serially thin-sectioned (100 nm). Thickness of thin sections was confirmed by examining wrinkles of thin sections under TEM. Serial images of type Ib boutons were taken at 15,000× with a Megaplus digital camera (Kodak, USA) attached to a Philips CM120 STEM at 60 kV. To determine length, surface area and volume of boutons, and area of synapses, serial reconstructions of synaptic boutons were made by carefully aligning digital images. The relevant features of synaptic boutons such as bouton surfaces and synaptic densities were digitalized using Adobe Photoshop and analyzed with NIH image software. Two different samples of each genotype were used for morphometric analysis. Minitab software was used to perform two sample t-tests.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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