Mutant human torsinA, responsible for early-onset dystonia, dominantly suppresses GTPCH expression, dopamine levels and locomotion in Drosophila melanogaster

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ABSTRACT

Dystonia represents the third most common movement disorder in humans with over 20 genetic loci identified. TOR1A (DYT1), the gene responsible for the most common primary hereditary dystonia, encodes torsinA, an AAA ATPase family protein. Most cases of DYT1 dystonia are caused by a 3 bp (ΔGAG) deletion that results in the loss of a glutamic acid residue (ΔE302/303) in the carboxyl terminal region of torsinA. This torsinAΔE mutant protein has been speculated to act in a dominant-negative manner to decrease activity of wild type torsinA. Drosophila melanogaster has a single torsin-related gene, dtorsin. Null mutants of dtorsin exhibited locomotion defects in third instar larvae. Levels of dopamine and GTP cyclohydrolase (GTPCH) proteins were severely reduced in dtorsin-null brains. Further, the locomotion defect was rescued by the expression of human torsinA or feeding with dopamine.

Here, we demonstrate that human torsinAΔE dominantly inhibited locomotion in larvae and adults when expressed in neurons using a pan-neuronal promoter Elav. Dopamine and tetrahydrobiopterin (BH4) levels were significantly reduced in larval brains and the expression level of GTPCH protein was severely impaired in adult and larval brains. When human torsinA and torsinAΔE were co-expressed in neurons in dtorsin-null larvae and adults, the locomotion rates and the expression levels of GTPCH protein were severely reduced. These results support the hypothesis that torsinAΔE inhibits wild type torsinA activity. Similarly, neuronal expression of a Drosophila DtorsinΔE equivalent mutation dominantly inhibited larval locomotion and GTPCH protein expression. These results indicate that both torsinAΔE and DtorsinΔE act in a dominant-negative manner. We also demonstrate that Dtorsin regulates GTPCH expression at the post-transcriptional level. This Drosophila model of DYT1 dystonia provides an important tool for studying the differences in the molecular function between the wild type and the mutant torsin proteins.

KEY WORDS: Dystonia, Drosophila, GTP cyclohydrolase, TorsinA, Movement disorder

INTRODUCTION

Dystonia is the third most common movement disorder in humans, after essential tremor and Parkinson’s disease (Defazio, 2010). Dystonia comprises a group of movement disorders that are characterized by involuntary movements and abnormal postures. It is a complex disease involving at least 20 genetic loci in humans (Tarsy and Simon, 2006; Breakefield et al., 2008; Brüggemann and Klein, 2010).

One of the loci, TOR1A/DYT1, is responsible for most cases of early-onset dystonia and has been the most studied form of dystonia (Breakefield et al., 2001; Atai et al., 2012; Bragg et al., 2011). It is an autosomal dominant syndrome with onset between 5 to 28 years of age and low penetrance. The TOR1A gene encodes torsinA, a 332 amino acid protein from the AAA ATPase family. The torsinA protein is widely expressed in the body and is localized within the lumen of the endoplasmic reticulum and the nuclear envelope (Breakefield et al., 2008), but its function is still under study. A 3-bp (ΔGAG) deletion that removes one of a pair of glutamic acid residues (ΔE302/E303) in the carboxyl terminal region of torsinA causes the autosomal dominant dystonia phenotype (Breakefield et al., 2008; Bragg et al., 2011). TorsinA displays LAP1 and LULL1-dependent ATPase activity, while the torsinAΔE protein is defective in this activation (Zhao et al., 2013). The torsinAΔE (ΔE302/303) mutant protein has been speculated to act in a dominant-negative manner, so that the wild type function is reduced but not eliminated in the cells expressing both torsinA and torsinAΔE, although this has never been clearly demonstrated (Breakefield et al., 2001; Breakefield et al., 2008).

Most AAA ATPase proteins form oligomeric complexes and use energy from ATP hydrolysis to regulate protein folding, membrane trafficking, and vesicle fusion (Neuwald et al., 1999; Vale, 2000; Hanson and Whiteheart, 2005; Zhao et al., 2013). Although torsinA is widely expressed in human tissue, it is considered to have a critical role in the central nervous system, where it is present in neurons at high levels during development and in adult life (Augood et al., 2003; Xiao et al., 2004; Vasudevan et al., 2006). In homozygous torsinA-knock-out mice, abnormal nuclear membrane morphology was observed in neurons, suggesting a functional role of torsinA in maintaining the normal structure of the nuclear envelope in the central nervous system (Goodchild et al., 2005). TorsinA has been shown to interact with nesprins, which are anchored in the outer nuclear envelope and form bridges to the cytoskeleton (Nery et al., 2008; Jungwirth et al., 2011; Atai et al., 2012), suggesting an important functional role of torsinA at the nuclear envelope, including nuclear polarization during cell migration (Nery et al., 2008). Recent studies also implicate torsinA in egress of Herpes simplex
virus capsids (Maric et al., 2011) and large ribonucleoprotein particles (Jokhi et al., 2013) out from the nucleus into cytoplasm.

The fruit fly, Drosophila melanogaster, provides an excellent model system to study functions of human disease genes and has contributed to better understanding of many human diseases (Bellin et al., 2010). Drosophila has a single TOR1A-related gene, dtorsin (Torsin), at position 4C11 on the X chromosome (Ozelius et al., 1999; Breakefield et al., 2001; Wakabayashi-Ito et al., 2011). The dtorsin-encoded protein, Dtorsin, comprises 339 amino acids with 31.9% identity to human torsinA and also displays the characteristic features of the AAA ATPase gene family members (supplementary material Fig. S1) (Ozelius et al., 1999). We recently isolated dtorsin-null mutants and showed that hemizygous mutant third instar male larvae exhibited locomotion defects that were rescued by feeding dopamine (Wakabayashi-Ito et al., 2011). The dtorsin-null mutation was semi-lethal at the pupal stage with only less than 1% reaching adult stage. The dtorsin mutant exhibited a very strong genetic interaction with Pu (Punch: GTP cyclohydrolase: GTPCH), the ortholog of the human gene underlying dopa-responsive DYT5a dystonia (GCH1) (Segawa, 2009). Moreover, biochemical analysis revealed a severe reduction of GTPCH protein and activity in dtorsin-null adults and larvae, as well as marked reduction in tetrahydrobipterin (BH4), the terminal product of the GTPCH pathway. In contrast, levels of tyrosine hydroxylase (TH) protein, which catalyzes the rate limiting step in dopamine production, were not affected, although dopamine pools were reduced (Wakabayashi-Ito et al., 2011). Since GTPCH is rate limiting for the synthesis of BH2, and BH4 is required by TH as a rate-limiting cofactor for dopamine synthesis in flies as in mammals (Krishnakumar et al., 2000), these data suggested that dtorsin plays a novel role in dopamine metabolism as a positive-regulator of GTPCH protein levels in Drosophila. Moreover, the wild type human torsinA cDNA expressed with the pan-neuronal promoter elavGAL4 rescued dtorsin-null male larval mobility with marked significance. These results demonstrated that the function of torsin in regulating larval locomotion is conserved between the fly and the human proteins (Wakabayashi-Ito et al., 2011). However, the fly dtorsin-null mutant is not an authentic DYT1 disease model system, since the dtorsin-null mutant line does not express any functional Dtorsin protein, while mutated torsinA protein is expressed together with normal torsinA in the DYT1 patients (Breakefield et al., 2001).

To investigate the molecular mechanism underlying the human disease caused by mutated torsinA protein using the fly system, we expressed human wild type torsinA and/or torsinAAE cDNA using the pan-neuronal GAL4 driver, elavGAL4, in fly brains. We report here that expression of the human mutant form caused larval and adult locomotion defects, and severe reduction of GTPCH protein, dopamine, and BH4 levels in larval brains and adult heads. Moreover, co-expression of human torsinAAE and the wild type human torsinA in dtorsin-null male larvae resulted in similar larval/adult locomotion and neurochemical defects, suggesting that the human torsinAAE exerts dominant-negative effects on human wild type torsinA protein in Drosophila neurons, as in human tissues. Furthermore, a comparable mutation in the Drosophila gene, dtorsinAAE also had a dominant-negative effect on larval locomotion and GTPCH protein level, as did human torsinAAE. Finally, we report that the relative amount of GTPCH RNA was similar in wild type and dtorsin-null adult male heads, suggesting that GTPCH protein levels depend on wild type dtorsin-activity at the post-transcriptional level. Our findings establish conclusively that torsinAAE dominantly inhibits the normal function of torsinA and Dtorsin including the regulation of GTPCH expression. These results demonstrate that Drosophila provides a powerful system for studying the molecular abnormalities caused by the torsinAAE mutation.

**RESULTS**

**Human torsinAAE dominantly inhibits larval locomotion**

In the previous study, we analyzed the peristaltic frequency of third instar larvae to quantify the difference in locomotion between wild type and mutant. The wild type third instar larvae show approximately 55 muscle contraction cycles per minute when placed on 0.7% agarose plates at room temperature. These peristaltic rates are relatively easy to monitor and provide a sensitive and reliable way of quantifying larval locomotion (Song et al., 2007; Wakabayashi-Ito et al., 2011). Males of the null mutant, dtorsinKO13, exhibit approximately a ~50% decrease in peristaltic rates, 22.9±2.5 (n=28, p<0.0001) (Fig. 1A, column 5), compared to wild type (55.2±2.5, n=15) (Fig. 1A, column 1). As previously observed, the wild type human torsinA cDNA expressed with the pan-neuronal driver elavGAL4 rescued dtorsinKO13 male larval mobility to a very significant level (56.3±3.7, n=14, p<0.0001) (Fig. 1A, column 7) (Wakabayashi-Ito et al., 2011), compared to dtorsinKO13 male larvae with the elavGAL4 transgene (27.2±1.1, n=39) (Fig. 1A, column 6). By way of controls, the pan-neuronal expression of the wild type human torsinA cDNA in wild type flies had no effect on larval mobility (54.3±2.3, n=15, p=0.7) (Fig. 1A, column 3), compared to male larvae with elavGAL4 transgene alone (53.0±1.8, n=9) (Fig. 1A, column 2). Similarly, the presence/absence of the elavGAL4 transgene had no effect on mobility in wild type (Fig. 1A, columns 1, 2) and dtorsinKO13 larvae (Fig. 1A, columns 5, 6).

To examine the effect of mutated human torsinAAE protein in flies, we expressed human torsinAAE cDNA with the pan-neuronal elavGAL4 driver in wild type males (w dtorsinA). ElavGAL4/UAS-htorsinAAE males exhibited a severe locomotion deficit, approaching that of the dtorsin-null mutant (26.7±3.4, n=9, p<0.0001) (Fig. 1A, column 4, compared to column 2). This result demonstrates that pan-neuronal expression of human torsinAAE protein has a negative effect on larval locomotion, similar to the dtorsin-null state in flies, and that it interferes with the function of endogenous Dtorsin.

While pan-neuronal expression of human wild type torsinA could rescue the locomotion deficit phenotype of dtorsinKO13 males (Fig. 1A, columns 6, 7), human torsinAAE was unable to do so (20.5±2.0, n=21) (Fig. 1A, column 8). To determine whether the human torsinAAE could inhibit wild type human torsinA, we co-expressed the human torsinAAE cDNA with the wild type human torsinA cDNA in dtorsinKO13 male using the same elavGAL4 driver. Co-expression of human wild type torsinA and human torsinAAE resulted in a significant inhibition of mobility (25.3±2.8, n=14, p<0.0001) (Fig. 1A, column 9), compared to the rescue by human torsinA alone (Fig. 1A, column 7).

**Human torsinAAE dominantly inhibits adult locomotion**

We have also analyzed the locomotion activities in the adult stage to examine whether they were similarly affected by the neuronal expression of human torsinAAE. Adult flies, aged 3–5 days after eclosion, were placed in vials, subjected to a gentle mechanical disturbance, and then locomotion activities were quantified as the number of seconds each fly spent in motion during a 45 second
period (Carbone et al., 2006). Adult wild type male flies (Canton S-B) spent approximately 21.8±0.9 seconds in motion (n=47) (Fig. 1B, column 1). Adult male flies that were heterozygous for a lethal Punch (GTPCH gene) null mutation, Pu/+
(Mackay et al., 1985), exhibit a significant reduction of locomotion activities with 16.7±0.8 seconds spent in motion (n=44, p<0.0001) (Fig. 1B, column 2), compared to wild type (Fig. 1B, column 1). Similarly, adult males of the null mutant, dtorsinKO13, exhibit a significant reduction of adult locomotion activities, (17.5±0.7 seconds, n=64, p=0.0002) (Fig. 1B, column 3), as observed in the third instar larvae. The wild type human torsinA cDNA expressed with the pan-neuronal driver elavGAL4 strongly rescued dtorsinKO13 male adult locomotion activities (21.4±0.9 seconds, n=77) (Fig. 1B, column 4), compared to dtorsinKO13 adult males (Fig. 1B, column 3) (p=0.01). The mutant form of torsinAAE was unable to rescue the adult locomotion defect (16.9±0.9 seconds, n=69) (Fig. 1B, column 5) (p=0.603, compared to column 3). Co-expression of the human torsinAAE with the wild type human torsinA cDNA in dtorsinKO13 adult male resulted in a significant reduction of locomotion activities (14.6±0.9, n=63) (Fig. 1B, column 6) compared to the rescue by human torsinA alone (Fig. 1B, column 4) (p<0.0001). These results demonstrate that adult locomotion activities in flies are dominantly inhibited by the neuronal expression of the mutant form of human torsinA.

**Human torsinAAE dominantly suppresses GTPCH expression**

We have previously shown, and confirm here, that dtorsinKO13 males have a severe reduction of both the 45 kDa (Pu-RA) and 43 kDa (Pu-RC) isoforms of GTPCH protein in adult brains (Fig. 2A, lane 1 and 2; supplementary material Fig. S3A, columns 43 kDa (Pu-RA) isoforms of GTPCH protein in adult brains and actin (42 kDa) are indicated. Thirty μg of proteins were loaded in each lane. (B) Adult head extracts were analyzed by western blots. The membrane was probed with rabbit anti-GTPCH A/C (upper panel) and reprobed with rabbit anti-actin (lower panel). The genotypes are: (1) y w/Y (wild type) male (n=47), (2) w elavGAL4Y; UAS-htorsinA/+ male (n=44), (3) w elavGAL4Y; UAS-htorsinAAE/+ male (n=64), (4) w elavGAL4Y; UAS-htorsinA/+ male (n=39), (5) w elavGAL4 dtorsinKO13/Y (dtorsin-null) male (n=14), (6) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA/+ male (n=14), (7) w elavGAL4 dtorsinKO13/Y; UAS-htorsinAAE/+ male (n=21), (9) w elavGAL4 dtorsinKO13/Y; UAS-htorsinAAE/+ male (n=14). Results are expressed as the mean±S.E.M. ***p<0.0001. (A) Adult locomotion activities were measured for the adults of the genotype: (1) Canton S-B (wild type) male (n=47), (2) Pu/22/+ (Pu null mutation) male (n=44), (3) w elavGAL4 dtorsinKO13/Y (dtorsin-null) male (n=64), (4) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA/+ male (n=39), (5) w elavGAL4 dtorsin+/Y; UAS-htorsinAAE/+ male (n=69), (6) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA, UAS-htorsinAAE/+ male (n=63). Results are expressed as the mean±S.E.M. ***p<0.0001, **p<0.01.
1, 2) (Wakabayashi-Ito et al., 2011). Males heterozygous for the embryo-lethal Pu null mutation, PuZ22/+ (Mackay et al., 1985), had a severe reduction of both Pu-RA and Pu-RC isoforms (supplementary material Fig. S2, lanes 1, 2), confirming that these two polypeptides are encoded by the GTPCH gene. To investigate whether human torsinAΔE has a similar effect on GTPCH, we prepared extracts from heads of dtorsinKO13 adult males expressing human torsinAΔE in neurons, dtorsinKO13 adult males expressing wild type torsinA in neurons, and dtorsinKO13 adult males expressing both human torsinA and human torsinAΔE in neurons, and compared GTPCH protein levels by western blot analysis (Fig. 2A, lanes 3–5; supplementary material Fig. S3A, columns 3–5). Pan-neuronal expression of human torsinAΔE in dtorsinKO13 adult males, confirmed by immunoblotting using an antibody specific to human torsinA (Bragg et al., 2004) (Fig. 2B, lane 3), revealed that the mutant human torsinA protein was unable to rescue GTPCH protein levels when expressed alone in dtorsinKO13 adult males (Fig. 2A, lane 4 compared to lane 2). In contrast, neuronal expression of wild type human torsinA alone (Fig. 2B, lane 2) strongly rescued both isoforms of GTPCH in dtorsin-null males (compare Fig. 2A, lanes 2 and 3). Severe reduction of GTPCH was observed in dtorsinKO13 adult males expressing human torsinA and human torsinAΔE together (Fig. 2A, lane 5 compared to lane 3), even though the expression of torsinAΔE with the wild type form does not diminish the total level of human torsinA expressed in fly neurons (Fig. 2B, lane 4; supplementary material Fig. S3B).

We obtained comparable results using brain extracts from third instar larvae of the corresponding genotypes (Fig. 3; supplementary material Figs S4, S5) as those of adult head extracts (Fig. 2; supplementary material Fig. S3). That is, the htorsinAΔE transgene fails to rescue either isoform of brain GTPCH, both of which are affected by complete knockout of the dtorsin gene (Fig. 3, lane 3 and 4; supplementary material Fig. S4, columns 3, 4). Expression of wild type htorsinA (Fig. 3, lane 5; supplementary material Fig. S4, column 5) rescues expression of both isoforms (Pu-RA and Pu-RC) of GTPCH expression with Pu-RA rescue slightly more effectively than Pu-RC. The basis for this slight difference is unclear at this time. Nevertheless, these results confirm that wild type human torsinA is capable of rescuing neuronal expression of Drosophila GTPCH and demonstrate that the human torsinAΔE, when co-expressed with the wild type human transgene, dominantly suppresses GTPCH protein levels in both larval and adult brains without negatively affecting the expression of wild type human torsinA.

**Human torsinAΔE dominantly reduces BH4 and dopamine level**

Tyrosine hydroxylase is the rate limiting enzyme in dopamine synthesis (Friggi-Grelin et al., 2003) and its activity is limited by the availability of the BH4 cofactor (Kumer and Vrana, 1996). In flies and mammals, activity of GTPCH, the first enzyme in the BH4 biosynthesis pathway, controls the intracellular concentration of the cofactor (Kumer and Vrana, 1996; Krishnakumari et al., 2000; Thöny et al., 2000). Thus, dopamine pools are subject to regulation by protein levels and catalytic activity of GTPCH. We have previously reported that there is a significant reduction of GTPCH activity and dopamine levels in larval and adult head of heterozygous dtorsinKO13/+ and dtorsinKO13/+ females (Wakabayashi-Ito et al., 2011). To investigate whether expression of human torsinAΔE could also reduce the dopamine pool level, we measured BH4 levels and dopamine levels in extracts from brains of wild type male larval and adult brains expressing wild type human torsinA, dtorsinKO13 male larvae expressing human torsinAΔE, and dtorsinKO13 male larvae expressing both human torsinA and human torsinAΔE (Fig. 4A,B). The level of BH4 in dtorsinKO13 male brains was significantly lower (0.098±0.007 ng/brait, n=3 replicate samples, each sample=75 brains, p<0.001) (Fig. 4A, column 2) compared to wild type brains (0.300±0.010, n=3 replications) (Fig. 4A, column 1). The BH4 level in dtorsinKO13 male brains expressing wild type human torsinA (0.341±0.009, n=3, p<0.001) (Fig. 4A, column 3) was significantly higher compared to those in dtorsinKO13 male brains (Fig. 4A, column 2). Neuronal expression of human torsinAΔE further decreased BH4 levels (0.018±0.002, n=3, p<0.01) (Fig. 4A, column 4 compared to column 2). Co-expression of human torsinAΔE with wild type human torsinA blocked the rescue by human torsinA (0.030±0.004, n=3, p<0.001) (Fig. 4A, column 5 compared to column 3).

Similarly, the level of dopamine in dtorsinKO13 male larval brains was significantly lower (0.021±0.002 ng/brait, n=3, p<0.001) (Fig. 4B, column 2) as compared to wild type brains (0.062±0.002, n=3) (Fig. 4B, column 1). The dopamine level in dtorsinKO13 male brains expressing wild type human torsinA (0.073±0.001, n=3, p<0.001) (Fig. 4B, column 3) was significantly higher compared to that in dtorsinKO13 male brains (Fig. 4B, column 2). Neuronal expression of human torsinAΔE further decreased dopamine levels (0.011±0.001, n=3, p<0.01) (Fig. 4B, column 4 compared to column 2). Co-expression of human torsinAΔE with wild type human torsinA blocked the rescue by human torsinA (0.007±0.0008, n=3, p<0.001) (Fig. 4B, column 5 compared to column 3).

**DtorsinE dominantly inhibits larval locomotion**

Dtorsin protein has conserved amino acids E306/D307, compared to E302/E303 in human torsinA (supplementary material Fig. S1). To determine whether Dtorsin with either ΔE306 or ΔD307...
deleted would have a similar dominant-negative activity on the wild-type Dtorsin protein as observed for the human torsinA\(D\)\(E\) mutation, we made two deletion mutant constructs of the \(dtorsin\) cDNA in E306 (UAS-\(dtorsin\)\(E\)) and D307 (UAS-\(dtorsin\)\(A\)) and expressed them with the elavGAL4 driver. Although pan-neuronal expression of wild-type Dtorsin did not affect larval locomotion (peristaltic frequency 53.0\(\pm\)1.5, \(n=8\), not significant) (Fig. 5, column 2) compared to wild type (53.0\(\pm\)1.8, \(n=9\)) (Fig. 5, column 1), wild type male larvae expressing Dtorsin\(E\) exhibited a significant locomotion deficit (38.7\(\pm\)2.5, \(n=23\), \(p=0.002\)) (Fig. 5, column 3). Male larvae co-expressing Dtorsin\(E\) with wild-type Dtorsin also exhibited a locomotion deficit (38.5\(\pm\)2.8, \(n=15\), \(p=0.001\)) (Fig. 5, column 4 compared to column 1) similar to the deficit caused by expression of Dtorsin\(E\) only (column 3).

Mutant male larvae (\(dtorsin^{KO13}\)) expressing wild type Dtorsin showed much improved larval locomotion. We tested two independent transgenic lines expressing wild type Dtorsin.

Results are expressed as the mean\(\pm\)S.E.M. ***\(p<0.001\). (C) Peristaltic frequencies were counted for the wandering stage third instar larvae. The genotypes are: (1) \(w^{1}Y\) males without dopamine supplementation (\(n=12\)), (2) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=11\)), (3) \(w^{1}Y\) males without dopamine supplementation (\(n=11\)), (4) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=11\)), (5) \(w^{1}Y\) males without dopamine supplementation (\(n=11\)), (6) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=8\)), (7) \(w^{1}Y\) males without dopamine supplementation (\(n=14\)), (8) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=5\)), (9) \(w^{1}Y\) males without dopamine supplementation (\(n=11\)), (10) \(w^{1}Y\) males without dopamine supplementation (\(n=11\)), (11) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=10\)), (12) \(w^{1}Y\) males with 20 mM dopamine supplementation (\(n=5\)). Results are mean\(\pm\)S.E.M. ***\(p<0.0001\), very significant difference between without and with 20 mM dopamine supplementation.
in male dtorsinKO13 larvae resulted in a peristaltic frequency of 50.5±2.5, n=20, p<0.0001 (Fig. 5, column 6), while expression of another transgene UAS-dtorsin(A11), on the third chromosome, rescued the peristaltic frequency to 48.2±1.1, n=9, p<0.0001 (Fig. 5, column 7) compared to the dtorsin-null (dtorsinKO13) males (Fig. 5, column 5). The presence of two copies of UAS-dtorsin transgenes (B5 and A11) together in the dtorsin-null background did not elevate locomotion further (peristaltic frequency: 50.2±2.1, n=23, p<0.001) (Fig. 5, column 8).

In striking contrast to the rescuing effect of wild type Dtorsin expression, DtorsinΔE expression in male dtorsinKO13 larvae failed to rescue the locomotion deficit (22.9±2.8, n=13, not significant) with a slight reduction of peristaltic rate (Fig. 5, column 9), relative to dtorsinKO13 males (Fig. 5, column 5). Similarly, mutant males co-expressing DtorsinΔE and wild type Dtorsin transgenes exhibited a locomotion deficit that was not significantly different from that of the dtorsinKO13 larvae (UAS-dtorsin(A11) and UAS-dtorsinΔE(#12); 32.4±4.2, n=7, not significant) (Fig. 5, column 5); UAS-dtorsin(B5) and UAS-dtorsinΔE(#21); 29.9±3.6, n=14, not significant) (Fig. 5, column 12).

Interestingly, pan-neuronal expression of DtorsinΔD in dtorsinKO13 males rescued the larval mobility (45.2±2.9, n=11, p<0.0001) (Fig. 5, column 10 compared to column 5). Similarly, co-expression of DtorsinΔD with the wild type Dtorsin in dtorsinKO13 males had no effect on locomotion (peristaltic frequency: 49.4±3.2, n=20) (Fig. 5, column 13). These results indicate that E302/303 of human torsinA protein and E306 of Drosophila Dtorsin protein are functionally similar and that deletion of these glutamates both cause reduced locomotion in Drosophila larvae, presumably due to the same functional abnormality, while DtorsinΔD appears similar to wild type Dtorsin.

DtorsinΔE dominantly suppresses GTPCH expression

These studies described above demonstrate a striking similarity in the dominant inhibition of larval locomotion by DtorsinΔE and human torsinΔD. Since we found that human torsinΔE dominantly inhibited GTPCH protein expression, we next examined the protein levels of GTPCH in adult male heads expressing wild type Dtorsin and DtorsinΔE in the dtorsin-null background (Fig. 6). The expression of endogenous GTPCH in the dtorsinKO13 mutant line and in the dtorsinKO13 elavGAL4 transgene line revealed similar patterns of reduced GTPCH expression of both RA and RC isoforms (Fig. 6, lane 2 and 3). Dtorsin expressed in dtorsinKO13 males, under the control of elavGAL4, rescued GTPCH expression substantially (Fig. 6, lane 4, compared to lane 3; supplementary material Fig. S6 column 4, compared to column 3).

DtorsinΔE expressed in dtorsinKO13 neurons failed to affect the GTPCH protein level (Fig. 6, lane 5, compared to lane 3; supplementary material Fig. S6, column 5, compared to column 3). Severe reduction of GTPCH was also observed in adult males co-expressing Dtorsin and DtorsinΔE in dtorsinKO13 (Fig. 6, lane 6).
6, compared to lane 4; supplementary material Fig. 6C, column 6, compared to column 4). These results demonstrate that DtorsinΔE and human torsinΔAΔE have indistinguishable effects on the expression of GTPCH protein in Drosophila brains, both dominantly inhibiting GTPCH protein expression. In contrast, DtorsinAD expressed in dtorsinKO13 neurons moderately rescued GTPCH protein level (supplementary material Fig. S7), consistent with the results of larval locomotion assays (Fig. 5).

The mobility defect of larvae expressing either human torsinΔAΔE or DtorsinΔE can be rescued by dopamine supplementation

In Drosophila, ingestion of dopamine increases dopamine pools in the fly head, though in mammals peripher al dopamine does not enter the brain (Chaudhuri et al., 2007). We previously showed that the locomotor deficit phenotype in dtorsinKO13 mutant male was partially rescued by dopamine supplementation to the larval growth medium, but not by serotonin or octopamine (Wakabayashi-Ito et al., 2011). Since we observed in the current study a very similar reduction of dopamine levels in larval brains expressing human torsinΔAΔE, we hypothesized that dopamine supplementation to the larval growth medium could also restore the locomotion defect of larvae expressing human torsinΔAΔE (or Drosophila DtorsinΔE). To test this hypothesis, we added 20 nM dopamine in the food of larvae with different dtorsin genotypes (Fig. 4C). Dopamine supplementation had no effect on the locomotion of wild type (elavGAL4/Y) larvae (56.3 ± 1.0, n = 11, p = 0.562) (Fig. 4C, column 2) compared to the larvae of the wild type without dopamine (55.4 ± 1.0, n = 12) (Fig. 4C, column 1). In contrast, dopamine supplementation substantially rescued the locomotion defect of dtorsinKO13 larvae (49.3 ± 1.8, n = 11, p < 0.0001) (Fig. 4C, column 4) compared to the larvae of the same genotype without dopamine (27.7 ± 2.8, n = 11) (Fig. 4C, column 3) confirming our previous results (Wakabayashi-Ito et al., 2011). Dopamine supplementation also rescued the locomotion defect of dtorsinKO13 larvae expressing human torsinΔAΔE and wild type torsinA (52.2 ± 3.6, n = 5, p < 0.0001) (Fig. 4C, column 8) compared to the larvae of the same genotype without dopamine (25.3 ± 2.8, n = 14) (Fig. 4C, column 7). These results demonstrate that locomotor defects caused by the pan-neuronal expression of human torsinΔAΔE orDtorsinΔE, measured by our larval locomotion assay, can be substantially rescued by dopamine supplementation. As a control, knockdown of GTPCH (Pu) mRNA expression levels was accomplished by neuronal expression of GTPCH RNAi, a short-hairpin specific for GTPCH (Pu) gene (Fig. 7), which was accompanied by a moderate reduction of larval locomotion (38.7 ± 1.1, n = 11) (Fig. 4C, column 9). Dopamine supplementation almost completely rescued locomotion defect of wild type larvae expressing GTPCH RNAi (57.5 ± 1.0, n = 10, p < 0.0001) (Fig. 4C, column 10). Dopamine supplementation also substantially rescued the locomotion defect of dtorsinKO13 larvae expressing GTPCH RNAi (42.3 ± 1.7, n = 10, p < 0.0001) (Fig. 4C, column 12) compared to the larvae of the same genotype without dopamine (24.4 ± 3.1, n = 11) (Fig. 4C, column 11).

The expression level of GTPCH is regulated at the post-transcriptional level

Having found that Dtorsin/torsinA regulates GTPCH protein, we next tested whether torsin regulates GTPCH expression at the transcriptional or post-transcriptional level. In order to analyze these alternative possibilities, we prepared total RNA from adult brains and analyzed GTPCH (Pu) mRNA levels by quantitative RT-PCR (qRT-PCR). The relative amount of GTPCH mRNA was determined by normalizing to mRNA for the housekeeping gene RpL32 (p49) as an internal control (see Materials and Methods). A significant increase, rather than reduction of GTPCH mRNA levels was observed in the brains of elavGAL4 dtorsinKO13 (B5)(II)/+ males (4.52 ± 0.20, n = 3, p < 0.0001) (Fig. 7, column 2) compared to those in wild type: elavGAL4/Y (1.00, n = 3) (Fig. 7, column 1). Expression of wild type human torsinA (2.57 ± 0.24, n = 3, **p < 0.001) (Fig. 7, column 3) was rescued to wild type levels by knockdown of GTPCH expression (1.00, n = 3) (Fig. 7, column 4). To confirm our qRT-PCR results, we also analyzed GTPCH protein levels using western blot (see Supplementary material). The adults heads were collected and homogenized in Laemmli buffer supplemented with 2% SDS. Total protein was loaded in each lane and the membranes were probed with rabbit anti-GTPCH and actin antibodies. As expected, the expression level of endogenous GTPCH was rescued in larvae expressing GTPCH RNAi (57.5 ± 0.75) compared to larvae expressing wild type GTPCH (Pu) (1.00, n = 3). Results are expressed as the means ± S.E.M. **p < 0.0001, ***p < 0.0001.
Neuronal expression of GTPCH RNAi substantially reduced GTPCH RNA levels (0.27±0.01, n=3, p<0.0001) (Fig. 7, column 5), validating our quantification of GTPCH mRNA by qRT-PCR. There were some variations in the relative abundance of GTPCH mRNA, but the reason is not clear at this moment. These results, however, indicate that dorsin/torsin mutant brains do not have decreased levels of GTPCH mRNA and therefore have a defect in GTPCH expression at the post-transcriptional level.

**DISCUSSION**

*Drosophila* has a single torsin-related gene, *dtorsin* (*Torsin*), with 31.9% amino acid identity to human torsinA (supplementary material Fig. S1). *dtorsin*-null animals have reduced locomotion at the third instar larval stage and reduced pigmentation in the adult stage (Wakabayashi-Ito et al., 2011). The dopamine levels and GTPCH activity/protein levels are severely reduced in *dtorsin*-null animals, suggesting GTPCH deficiency is responsible for dopamine depletion since TH protein is unaffected in the mutant (Wakabayashi-Ito et al., 2011). The pan-neuronal expression of wild type *Drosophila* Dtorsin or human torsinA rescued the locomotion defect in *dtorsin*-null larvae and adults, suggesting that human torsinA and *Drosophila* Dtorsin are functionally conserved (Figs 1, 5) (Wakabayashi-Ito et al., 2011). Pan-neuronal expression of human torsinAΔE protein alone did not rescue the locomotion defect, or the depletion of GTPCH protein, BH4, and dopamine (Figs 1–4) in *dtorsin*-null larvae and adults, demonstrating that human torsinAΔE protein is inactive. Further, co-expression of human wild type torsinA and torsinAΔE did not rescue the defects (Figs 1–4), demonstrating a dominant-negative effect of torsinAΔE on wild type torsinA activity. These results, for the first time, clearly show that torsinAΔE inhibits wild type torsinA activity in neurons, resulting in reduced locomotion and dopamine levels in *Drosophila*.

*Drosophila* Dtorsin has similar types of amino acids E306-D307 compared to human torsinA E302-E303 in the conserved location near the C terminal region of the protein (supplementary material Fig. S1). *Dorsophila* D306, when expressed in neurons, had a similar dominant-negative effect on locomotion and GTPCH protein levels as human torsinAΔE (Figs 5, 6), while DorsinAD307 was still active as it could rescue the *dtorsin*-null locomotion defect as well as GTPCH protein expression, and had no inhibitory effect on wild type Dtorsin (Fig. 5; supplementary material Fig. S7). Furthermore, neuronal expression of DorsinAD306 inhibited locomotion of wild type larvae, demonstrating a dominant-negative effect on the wild type protein. The phenotypes caused by neuronal expression of human torsinAΔE or *Drosophila* DtorsinΔE are indistinguishable from those of *dtorsin*-null larvae or adults, resulting in co-reduction of locomotion, dopamine levels, and GTPCH protein levels. These results strongly support the hypothesis that torsinAΔE acts as a dominant-negative molecule that suppresses the wild type protein activity (Breakfield et al., 2001).

We have demonstrated that *dtorsin*-null larvae and DtorsinΔE (or human torsinAΔE) expressing larvae have very similar phenotypes, resulting in the severely decreased level of GTPCH. Rates of dopamine synthesis depend on the activity of TH, which in turn depends on the amount of BH4 produced by GTPCH (O’Donnell et al., 1989; Thöny et al., 2000). Severe reduction of GTPCH protein levels results in a shortage of BH4 and decreased activity of TH, thereby leading to decreased dopamine pool levels in brains of *dtorsin*-null animals (Wakabayashi-Ito et al., 2011), as well as in brains expressing torsinAΔE (Fig. 4A,B). This defect, however, is unlikely to be the only defect in the dopamine signal transduction system in *dtorsin*-null or torsinAΔE-expressing animals. Although feeding dopamine could partially rescue the locomotion defect in our assay in *dtorsin*-null larvae (Wakabayashi-Ito et al., 2011) or in DorsinAΔE (or human torsinAΔE)-expressing larvae (Fig. 4C), very few larvae of *dtorsin*-null or torsinAΔE-expressing animals survived until the late third instar larval stage (data not shown). Early lethality could be the result of earlier developmental requirements for dopamine since strong loss-of-function mutations in the TH-encoding gene cause embryonic lethality in *Drosophila* (Neckameyer and White, 1993). Alternatively, Dtorsin may be affecting other neurotransmitter signaling systems directly or indirectly through dysfunction in dopaminergic circuitry. In the case of DYT1 dystonia patients, L-dopa is not therapeutic, suggesting that dopamine cannot compensate for defects resulting from mutant torsinA (Breakfield et al., 2008).

Recent publications in mouse DYT1 model systems demonstrated defective dopamine D2 receptor signaling in the striatal cholinergic neurons (Sciama et al., 2009; Sciama et al., 2011; Sciama et al., 2012). The lack of responsiveness of DYT1 patients to L-dopa treatment would be expected if the dopamine D2 receptor signaling or other component of the dopaminergic system is defective in addition to defects in dopamine synthesis.

Translational control of localized mRNA is a common mechanism for regulating protein expression in specific subdomains of a cell, in processes such as body axis formation, asymmetric cell division and synaptic plasticity (St Johnston, 2005; Holt and Bullock, 2009; Medioni et al., 2012). These localized mRNAs are often transported in large ribonucleoprotein particles (RNPs) or RNA granules (Kiebler and DesGroseilliers, 2000; Kiebler and Bassell, 2006; Holt and Bullock, 2009; Medioni et al., 2012). We have recently shown that *dtorsin* is involved in export of large RNPs out of nuclei on the way to the neuromuscular junction (Jokhi et al., 2013). Here, we have shown that the mRNA levels of GTPCH/Punch gene were not significantly decreased in *dtorsin*-null adult brains (Fig. 7), suggesting that the regulation of GTPCH/Punch expression is at the post-transcriptional level. This is consistent with a model in which GTPCH mRNA is transported through the nuclear membrane as a part of a large RNP complex whose transport depends on Dtorsin. If this hypothesis is correct, the Dtorsin protein could regulate the nuclear export and subsequent transport of large RNP complexes with subsequent compromise of the translation of the GTPCH mRNA. This nuclear export of mRNAs within RNPs could explain the mechanism by which torsin regulates expression of multiple proteins such as GTPCH and dopamine D2 receptor at the same time and thereby modulate synaptic plasticity (Sciama et al., 2012). Further testing of this hypothesis will be very important for understanding the molecular function of torsin proteins and the pathophysiology of DYT1 disease in human patients. The *Drosophila* system with its abundant genetic tools provides us an excellent model system to probe this hypothesis.

**MATERIALS AND METHODS**

**Fly stocks**

Flies were grown on standard medium containing cornmeal, yeast and agar at 25°C in fly incubators with a constant humidity of 70%
(Ashburner and Roote, 2007). ElavGAL4 transgenic strain was obtained from the Drosophila Stock Center (Bloomington, IN USA). The d torsin null lines, y w d torsin KO(FM7), Act-GFP and w elavGAL4 d torsin KO(FM7). Act-GFP were described previously (Wakabayashi-Ito et al., 2011). The Punch-null line, PyZ2, was previously described (Mackay et al., 1985). The RNAi line for GTPCH (Piu) gene, v107296 (KK107763) (Dietzl et al., 2007), was obtained from Vienna Drosophila RNAi Stock Center (Vienna, Austria). This RNAi line has 514 nt hairpin sequences that target all three isoforms (Pu-RA, Pu-RB, and Pu-RC) of GTPCH (Piu) transcripts.

**UAS lines**

dtorsinAe and dtorsinAa cDNA constructs were made from the wild type d torsin cDNA using QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA USA). Briefly, a 1.2 kb wild type d torsin cDNA was cut from pUAST-dtorsin with EcoRI and NotI (Wakabayashi-Ito et al., 2011) and cloned between the EcoRI and NotI sites of pBlueScript II KS (Agilent Technologies). Mutagenesis strand synthesis was done following the manufacturer’s protocol using two primers torpa4E3 (5’-CTAATGGGAGGTTATATGATAATGTTCATTTTGTGTTCGC-3’) and torpa4E5 (5’-GCGAACAACAAAAAATTCTGCAATAAATACCTCCCTAG-T3’) to make dtorsin cDNA that lacks GAC (E306), and torpa4D3 (5’-CTAATGGGAGGGTTATATCTCAATGATTTTTTGTGTTCGC-3’) and torpa4DS (5’-GGCGAACAACAAAAAACATCTGAGATTAAATACCTCCCTAG-T3’) to make d torsin EDNA that lacks GAC (D307), respectively. After confirming mutated sequences, the insert was again cut out with EcoRI and NotI and inserted between EcoRI and NotI sites of pUAST to produce pUAST-dtorsinAe and pUAST-dtorsinAa. The transgenic lines E12 (pUAST-dtorsinAe transgene on the second chromosome), E21 (pUAST-dtorsinAe on the third chromosome) and D19 (pUAST-dtorsinAa on the third chromosome) were used for the experiments.

A 1.0 kb human torsinAe EDNA was amplified from pcDNA3-htorM (Hevett et al., 2000) by PCR using the following primers htor5 (5’-CAGCGATCCATCCATGAGTCCGGCGCGGCTGCGCTGCGTGCGCTGTCG-3’) and htor3 (5’-TCCGACGCGCCGCTCATTACATCTGATGTAATACCTACTGGTG-3’). The PCR product was digested with Acc65I and NotI and inserted between Acc65I and NotI sites of pUAST. Injections were performed by Genetic Services, Inc. (Cambridge, MA USA). The transgenic line #24 with UAS-htorsinAe transgene on the second chromosome was used for the experiments.

**Larval locomotion assay**
The larval locomotion assay was done as described previously (Wakabayashi-Ito et al., 2011). Briefly, a wandering third instar larva of a particular genotype was individually picked from the vial with a bamboo stick and placed at the center of a 100 mm petri dish containing 0.7% agarose at room temperature placed on a light box. Larval locomotion was recorded for one minute using a Canon Powershot G7 digital camera attached to a stereoscopic microscope. Peristaltic frequency was counted manually using the Quicktime movie. The experiments were done in a double-blinded manner with only numbers assigned for each genotype. Peristaltic rates are usually highly reproducible with little variation for each genotype with relatively small SEM values. Since we were unable to get reproducible with little variation for each genotype with relatively small SEM values.
Isolation of RNA
Total RNA was extracted from thirty adult male fly heads suspended in 100 μl PBS/0.1% Triton X-100 using 800 μl TRI reagent (Molecular Research Center, Inc., Cincinnati, OH USA) and 80 μl of BCP (Molecular Research Center, Inc.) following the manufacturer’s protocol.

qRT-PCR analysis
qRT-PCR was performed as described previously with minor modifications (Balaj et al., 2011). Total RNA (2 μg) was converted into cDNA with the Omniscript reverse transcription kit (Qiagen, Valencia, CA USA) using random primers, according to manufacturer’s recommendations, and a 1:10 fraction (corresponding to 2.5 ng reverse transcribed RNA) was used for qRT-PCR. All reactions were performed in a 20 μl reaction using Power SYBR Green PCR Master Mix (Life Technologies) and 320 nM of each primer. Amplification conditions consisted of: 1 cycle of 50°C; 2 minutes; 1 cycle of 95°C, 10 minutes; 40 cycles of 95°C, 15 seconds; and 60°C, 1 minute followed by a dissociation curve analysis of each amplicon on the 7000 ABI Prism system (Life Technologies). Ct values were analyzed in auto mode. The Ct-values were normalized to the housekeeping gene RpL32 (tp49) in each sample (Brown et al., 2009; Willis et al., 2010). The following primers were used for qRT-PCR: RpL32: F:5’-CTACAGAATTATCCCAATG-3’; R:5’-GTTCGTATGAAACTGCTG-3’; Pu: F:5’-CGGA TAGGTGATGCGAAGG-3’; R:5’-AGTAGAGCATGACGAGCTGCC-3’.

Dopamine feeding assay
The dopamine feeding assay was done with some modification, as described previously (Wakabayashi-Ito et al., 2011). Fifty females of w, elavGAL4, dtorsinKO13, 594 work was provided by Dystonia Medical Research Foundation (to N.I. and X.O.B.); the University of Alabama at J.M.O., R.R.A., B.W.H., and O.M.D.; NINDS [the National Institutes of Health (NIH)] [R15 NS078728 to J.M.O.; NIH/ NINDS P50 NS037490 to N.I. and X.O.B.]; and Department of Defense (Peer Reviewed Medical Research Program) [W81XWH-12-1-0380 to N.I.].

Author contributions

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