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Molecules and Cells

ΔFY Mutation in Human Torsina Induces Locomotor Disability and Abberant Synaptic Structures in *Drosophila*

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We investigate the molecular and cellular etiologies that underlie the deletion of the six amino acid residues (ΔF323-Y328; ΔFY) in human torsin A (HtorA). The most common and severe mutation involved with early-onset torsion dystonia is a glutamic acid deletion (ΔE 302/303; ΔE) in HtorA which induces protein aggregates in neurons and cells. Even though ΔFY HtorA forms no protein clusters, flies expressing ΔFY HtorA in neurons or muscles manifested a similar but delayed onset of adult locomotor disability compared with flies expressing ΔE in HtorA. In addition, flies expressing ΔFY HtorA had fewer aberrant ultrastructures at synapses compared with flies expressing ΔE HtorA. Taken together, the ΔFY mutation in HtorA may be responsible for behavioral and anatomical aberrations in *Drosophila*.

INTRODUCTION

Early-onset torsion dystonia (EOTD) is the most severe and common inheritable form of dystonia (Bressman et al., 1989; 2002; Ozelius et al., 1997). The mutation most often associated with this disorder is an in-frame deletion in the *DYT1* gene that removes one of a pair of glutamic acid residues (ΔE302/303; ΔE) near the carboxy terminus of the human torsinA (HtorA) (Kock et al., 2006; Koh et al., 2004; Kustedjo et al., 2000; Ozelius et al., 1997). Although this disorder is transmitted in an autosomal dominant manner, only 30-40% of heterozygous individuals are afflicted with variable onset ages and dystonia symptoms, indicating that EOTD is a complex disorder whose pathogenesis requires a predisposing mutation in combination with other genetic or environmental risk factors for disease manifestation (Bressman et al., 1989; Fahn et al., 1987; Goodchild et al., 2005; Ozelius et al., 1997; Risch et al., 1995). Recent studies showed that asymptomatic ΔE HtorA carriers exhibited impaired learning and performance in sequential motor tasks (Edwards et al., 2003; Ghilardi et al., 2003) and early-onset recurrent major depression (Heiman et al., 2004). These studies confirm that EOTD is one of many complex neurological disorders and that the existence of a ΔE mutation in HtorA may induce certain defects that are insufficient to induce obvious dystonic symptoms, which require another genetic disturbance or environmental stress (Edwards et al., 2003; Sharma et al., 2005).

For a better understanding of the unknown pathophysiology of EOTD, ΔE HtorA-expressing transgenic mice (Sharma et al., 2005; Shashidharan et al., 2005) and ΔE torsin1A (tor1A) knock-in mice (Dang et al., 2005; Goodchild et al., 2005) were investigated. Several key cellular and behavioral features such as the formation of ΔE HtorA aggregates (Shashidharan et al., 2005) and mild motor learning defects (Sharma et al., 2005) are recapitulated in ΔE HtorA mice. Furthermore, heterozygote ΔE tor1A mice having a similar molecular defect as heterozygote ΔE HtorA carriers in humans show defects in nuclear membranes (Goodchild et al., 2005), hyper-motor activities, and abnormal beam walking, reminiscent of mild dystonic symptoms observed from human patients (Dang et al., 2005).

Another mutation is an 18 bp deletion that causes loss of amino acid residues 323-328 (ΔF323-Y328; ΔFY) in HtorA (Leung et al., 2001). Because the ΔFY mutation was reported from one family with additional missense mutation in the epsilon-sarcoglycan gene (SGCE) (Doheny et al., 2002) and the distribution patterns of ΔFY HtorA in human cells were similar to those of wild-type (WT) HtorA, it is still unclear whether ΔFY HtorA is directly associated with dystonia (Kock et al., 2006; Leung et al., 2001; O'Farrell et al., 2002).

Drosophila has been extensively used as a model organism to investigate human neurological disorders. When the causative mutations associated with human movement disorders were introduced to *Drosophila*, locomotor defects and neurodegenerative phenotypes observed from human patients were faithfully recapitulated. Thus, basic neural signaling mechanisms that regulate locomotor capabilities in mammals are evolutionarily conserved in *Drosophila* (Bielen and Bonini, 2005; Koh, 2006). In addition, the molecular components of glutatmatergic synapses in *Drosophila* larval neuromuscular junctions share a high degree of evolutionary conservation with those of excitatory synapses in the human central nervous system (Collins and DiAntonio,

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2007; Featherstone and Broadie, 2000; Koh et al., 1999; Packard et al., 2003).

In a previous study, we have shown that the ΔE HtorA expressed in *Drosophila* neurons induced aberrant synaptic ultrastructures. Here, we address two questions: Is the ΔFY mutation in HtorA responsible for locomotor disabilities in *Drosophila*? Also, is the presence of ΔFY HtorA sufficient to induce aberrant synaptic ultrastructures? Compared with ΔE HtorA flies, ΔFY HtorA flies have similar but delayed onset of locomotor disabilities when placed at elevated temperatures. We also observed predisposed ultrastructural defects such as free dense bodies within synaptic vesicle pools, empty postsynaptic pockets, and increased areas of functional synapses in *Drosophila* larval NMJs that express ΔE or ΔFY HtorA in muscles.

MATERIALS AND METHODS

Fly genetics

Flies were raised on standard medium at 25°C unless otherwise specified. Previously characterized Dopa decarboxylase-Gal4 driver (Ddc-Gal4), neuronal specific Gal4 driver (C155-Gal4), and muscle specific Gal4 driver (C57-Gal4) (Koh et al., 2004; Packard et al., 2002; Pielage et al., 2005; 2006) along with UAS-WT Human TorsinA (HtorA), UAS-an 18 bp deletion (ΔF323-Y328; ΔFY) HtorA, and UAS-a glutamic acid deletion (ΔE 302/303; ΔE) HtorA (Koh et al., 2004) were employed. The expression levels of UAS-WT HtorA, UAS-ΔFY HtorA, and UAS-ΔE HtorA had been shown to be comparable (Koh et al., 2004).

Behavioral analysis

Koh et al. (2004) described procedures for behavioral analysis. Briefly, we tested the group that consisted of age matching five males and five females for locomotor abilities in a water bath at 38°C for ten minutes. At each minute, we classified the flies that could walk or stay on the sides or tops of vials as having retained locomotor abilities and those that were immobilized or remained on the bottom of vials as having lost locomotor abilities. 10-12 groups of flies for each genotype from three independent crosses were tested. Each group was kept at a temperature of 30°C to accelerate aging. Minitab software (Minitab, USA) was used to perform two sample *t*-tests.

Immunocytochemistry

Koh et al. (2004) describes detailed procedures for immunocytochemistry. The following primary antibodies were used:

- rabbit anti-HtorA (Bouin's 1:2,000) (Koh et al., 2004)
- goat anti-HtorA (Santa Cruz Biotechnology, Inc. CA: Bouin's 1:200)
- goat anti-Horseradish peroxidase (HRP)-FITC (4% Paraformaldehyde (PCHO): 1:100) (Koh et al., 2004)
- monoclonal mouse anti-FasII (4% PCHO: 1:2) (Thomas et al., 1997)
- rabbit anti-*Drosophila p*-21 activated serine/threonine kinases (*D*pak, 4% PCHO, 1:4000) (Sone et al., 2000)
- rabbit anti-*Drosophila* synaptotagmin (Dsypt 4% PCHO, 1:1000) (Koh et al., 2004)

Donkey anti-rabbit Alexa-568, donkey anti-goat Alexa-488, and goat anti-mouse Alexa-488 were used as secondary antibodies with a 1:400 dilution (Molecular probes, Or). A Zeiss LSM510 confocal laser scanning microscope (Zeiss, Germany) was used to take serial or single slice confocal immunofluorescence images. Acquired confocal images were processed by an LSM5 image browser and Adobe Photoshop 6.0 (Adobe, USA). An Olympus IX71 epifluorescence microscope (Olympus, Japan) was used to count the number of synaptic boutons in NMJs in muscles 6 and 7 in the second abdominal segment in larvae expressing various forms of HtorA. Two sample t-tests were performed using Minitab software.

Quantification of the area of anti-FasII and anti-*D*pak immunofluorescence signals in boutons expressing various forms of HtorA

Single slice confocal images were taken from the midline cross of several type Ib boutons that were double-labeled with anti-FasII and anti-*D*pak antibodies in larvae expressing various forms of HtorA. To measure the areas of anti-FasII immunofluorescence signals at type Ib boutons, the threshold and the analyze particle functions in Scion Image software (version 4.0.2) (Scion, USA) were used. The threshold for the FasII signals, which was based on an examination of the background intensities of *fasII*^{e76} homozygous mutant muscles, was set at 100 (the relative intensity value between 0-255). The threshold for the *D*pak analysis was calculated from *dpak*^{11} homozygous mutant muscles and set at 160 (the relative intensity value between 0-255). There was no significant difference in anti-FasII or anti-*D*pak intensities among the WT, the ΔFY, and the ΔE HtorA-expressing boutons (data not shown). For this analysis, we used the boutons acquired from four different samples of each genotype. Minitab software was used for the two sample *t*-tests.

Serial TEM reconstructions

The procedure for TEM sample preparation and morphometric analysis in boutons and synapses has been described in Koh et al. (2004). In this experiment, serially thin-sectioned (100 nm) -only type Ib glutamatergic boutons on muscles 6 and 7 (segment 2) were used. EM912Ω (Leo Zeiss Inc., Germany) at the Korea Basic Science Institute and Jeol 1011 (Jeol Inc., Japan) at the ILSONG Institute of Life Sciences, Hallym University were used to take high resolution serial TEM images. Adobe Photoshop 6.0 was used to digitize the relevant features of boutons such as junctional membrane, synapses, and T-shaped active zones so that the length, surface area, and volume of the boutons as well as the area of synapses and the number of T-shaped active zones per bouton could be determined by analyzing data with Scion Image software. Two different preparations of each genotype were used for the quantification analysis. Minitab-software was used for the two sample *t*-tests.

RESULTS

Normal distribution of ΔF323-Y328 HtorA in *Drosophila*

To understand any defect caused by the expression of ΔFY HtorA in neurons or muscles, dopaminergic neuronal Gal4 (Ddc-Gal4) and muscle specific Gal4 (C57-Gal4) drivers (Koh et al., 2004; Packard et al., 2002; Pielage et al., 2005) were employed to transgenically express WT, ΔFY or ΔE HtorA. We used anti-HtorA immuno-fluorescence microscopy (Fig. 1) to examine the distribution patterns of the various HtorA forms in dopaminergic and neuron muscles. The ΔE HtorA induced large protein clusters in neurons and muscles (Figs. 1I-1L). In contrast, the WT and the ΔFY HtorA were evenly distributed as small specks (Figs. 1A-1H). The differences in muscles were much clearer in higher magnification confocal images (Figs. 1E and 1F). An optical section along the Z-axis at the mid-nuclear level showed various HtorA forms localized outside of the muscle nuclei (Figs. 1D, 1H and 1L). Similar localization patterns

Table 1. The number of boutons in larval NMJs expressing various forms of HtorA. The numbers of bouton in type 1 glutamatergic NMJs expressing WT, ΔFY, or ΔE HtorA in neurons or muscles are not statistically different from each other. The number in parenthesis indicates examined preparations.

Genotypes	Neuronal expression			Muscular expression		
	WT HtorA	ΔFY HtorA	ΔE HtorA	WT HtorA	ΔFY HtorA	ΔE HtorA
No. of synaptic boutons	95.9 ± 2.8 (14)	95.6 ± 4.0 (13) p > 0.9	88.1 ± 3.9 (12) p > 0.1	79.0 ± 3.0 (10)	80.5 ± 2.6 (10) p > 0.5	76.0 ± 3.1 (8) p > 0.2

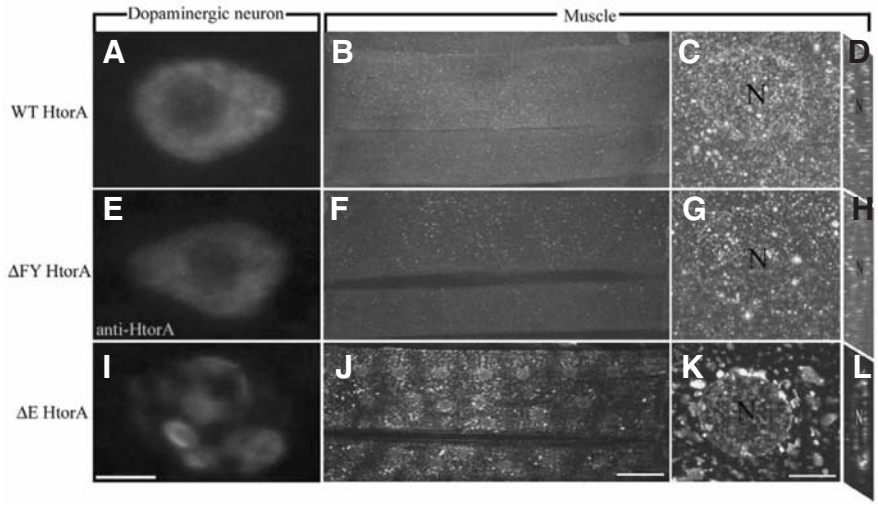

Fig. 1. The localization patterns of various forms of HtorA in *Drosophila* larval dopaminergic neurons and muscles. (A-D) WT HtorA, (E-H) ΔFY HtorA, and (I-L) ΔE HtorA-expressing dopaminergic neurons (A, E, and I) or muscles (B-D, F-H, and J-L) were stained with anti-HtorA antibodies and examined with a LSM 510 confocal laser scanning microscope. Various HtorA forms were expressed and showed distinctive distribution patterns in neurons or muscles. The WT (A-D) and ΔFY HtorA (E-H) specs are evenly distributed all over the neurons or muscles. High power confocal microscopy clearly shows that ΔE HtorA forms protein aggregates in neurons or muscles (I and K). The *Z*-axis slicing at the middle of nuclei shows that various HtorA forms do not localize within a nucleus (D, H, and L). The small specs of WT (A and C) and ΔFY Htor (E and G), as well as the large protein clusters of ΔE HtorA (I and K) are localized outside of nuclear membranes. N, nuclei.

were reported from neurons in the *Drosophila* central nervous system (CNS) (Koh et al., 2004), in the CNS of ΔE HtorA-expressing transgenic mice (Shashidharan et al., 2005) or ΔE tor1A knock-in mice (Dang et al., 2005), as well as in human brains (McNaught et al., 2004) and in cultured human cells (Kustedjo et al., 2000; O'Farrell et al., 2002). Even though ΔFY and ΔE HtorA were expressed and widely localized in muscle sarcoplasm or dopaminergic neurons, there was no obvious developmental or structural defect in muscles or neurons at light or electron microscopic levels (Fig. 1) (Koh et al., 2004).

Locomotor disability in flies expressing ΔFY HtorA

To understand whether ΔFY HtorA expression in flies is sufficient to increase their susceptibility to acute exposure to 38°C that trigger debilitating movement disorders, we examined the locomotor abilities of the Gal4 driver flies (C155/+ or C57/+) as the control flies and the transgenic flies that express various HtorA forms at neurons or body wall muscles after immersion in a 38°C water bath for 10 min. As shown in Fig. 2, after 6 days in neuronal expression or 9 days in muscular expression, 90% of the ΔE HtorA-expressing flies became severely incapacitated within about 1 min of exposure to 38°C, whereas the majority of the control flies or the WT HtorA-expressing flies remained active under the same conditions for up to 12 days. We tested whether neuronal or muscular expression of ΔFY HtorA also caused behavioral defects even though ΔFY HtorA did not form aggregates. As with the ΔE HtorA-expressing flies, the impaired ΔFY HtorA-expressing flies exhibited abnormal leg and wing movements that were suggestive of aberrant muscle contractions. Thus, despite the absence of protein aggregates, muscular expression of ΔFY HtorA caused behavioral abnormalities which, though delayed, were similar to the behavioral abnormalities caused by ΔE HtorA expression. The behavioral data also indicated that the ΔFY and the ΔE HtorA expression in neurons or muscles were sufficient to increase the susceptibility of flies to acute exposure to elevated temperatures.

Aberrant morphology of boutons expressing ΔFY HtorA

To investigate whether ΔFY HtorA expression in neurons or muscles induced predisposed phenotypical abnormalities at synapses, morphological alterations at type I glutamatergic synapses in larval NMJs were examined by performing anti-Horseradish peroxidase (HRP) immunocytochemistry (Fig. 3). Even though the numbers of type I boutons in the larval NMJ expressing WT or mutant HtorA in neurons or muscles were not significantly different from each other (Figs. 3A and 3F; Table 1), the boutons expressing mutant HtorA showed abnormal morphologies such as enlarged sizes of boutons with irregular neuronal membrane protrusions (Figs. 3H, 3I, 3K, and 3L).

Because synapses are the basic units underlying synaptic plasticity and the proper distribution of synaptic molecular components is important for the normal functions of synapses (Kim and Sheng, 2004; Koh, 2006; Koh et al., 2000; Packard et al., 2003), we investigated whether synapses that express mutant HtorA harbor phenotypic changes of two key synaptic molecular components, Fasciclin II (FasII) - *Drosophila* cell adhesion molecules (Koh et al., 1999; Sone et al., 2000) and *Drosophila* p21-activated serine/threonine kinase (*D*pak) (Parnas et al., 2001; Sone et al., 2000). FasII is important for maintaining the synaptic strength (Thomas et al., 1997) and encoding, sta bilizing, or retrieving short term memories (Davis, 2005). *D*pak is important for differentiation of the postsynaptic terminal structures, the Subsynaptic reticulum (SSR) (Sone et al., 2000).

Table 2. The area of the Fas II or the *D*pak signals at boutons in NMJs expressing various forms of HtorA. The average area of the FasII signals at boutons in NMJs expressing ΔFY or ΔE HtorA in muscles is around 50% of those of WT HtorA boutons. In contrast, the average area of the *D*pak signal in mutant HtorA boutons is about twice the size of WT HtorA boutons. The numbers in parenthesis indicate examined FasII and *D*pak labeled structures.

Genotypes	C57-Gal4/WT HtorA	C57-Gal4/ΔFY HtorA	ΔE HtorA; C57-Gal4
Area of FasII signal	48.00 ± 11.1(233)	20.02 ± 3.05 (219) p < 0.05	20.92 ± 3.73 (276) p < 0.05
Area of *D*pak signal	24.45 ± 1.58 (207)	42.22 ± 2.64 (182) p < 0.005	45.57 ± 2.22 (202) p < 0.005

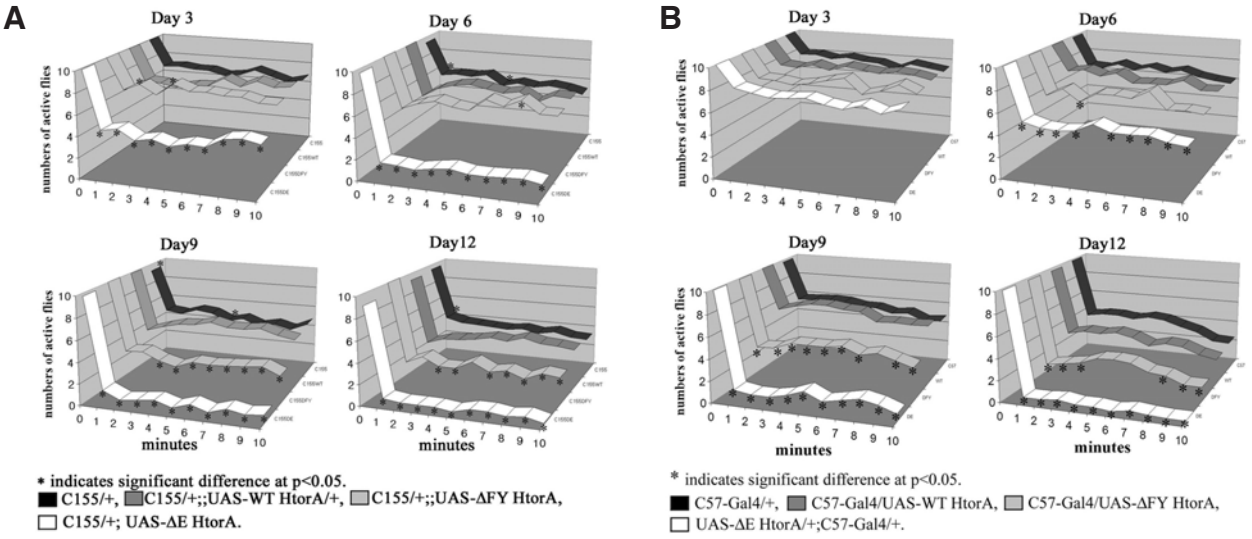

Fig. 2. Impaired locomotor ability in the ΔFY and the ΔE HtorA-expressing flies. The locomotor ability of flies that transgenically express WT, ΔFY, or ΔE HtorA in neurons and muscles was examined. The flies were kept in a water bath at 38°C for 10 min. The number of active flies that dynamically moved at the side or top of vials was counted at each minutes and plotted. The minutes showing a significant difference ($p < 0.05$) determined by two-sampled t-test are marked with an asterisk. (A) Twelve groups of C155-Gal4/+, 12 groups of C155-Gal4/+;;UAS-WT HtorA/+, 10 groups of C155-Gal4/+;;UAS-ΔFY HtorA/+, and 10 groups of C155/+;UAS-ΔE HtorA/+ were collected from three different independent crosses and tested. (B) Twelve groups of C57-Gal4/+, 12 groups of C57-Gal4/UAS-WT HtorA, 12 groups of C57-Gal4/UAS-ΔFY HtorA, and 12 groups of UAS-ΔE HtorA/+; C57-Gal4/+ were collected from three different independent crosses and tested. Three-day-old, 6-day-old, 9-day-old, and 12-day-old flies were tested. Flies expressing ΔE HtorA in all neurons manifested locomotor defects after 3 days, and ΔFY HtorA flies revealed locomtor incapacities after 9 days (A). Similarly, flies expressing ΔE HtorA in muscles showed behavioral deficits after 6 days and ΔFY HtorA flies manifested locomotor incapacities after 9 days (B).

The average occupying area of anti-FasII (green) and anti-*D*pak (red) immunofluorescence signals was altered in the ΔFY or the ΔE HtorA boutons (Fig. 4). In mutant HtorA-expressing boutons, the average area of the FasII signal was significantly diminished to around ~50% of that of WT HtorA boutons (Fig. 4 and Table 2). In contrast, the average area of the *D*pak signal in the mutant HtorA boutons was about twice the size of that of the WT HtorA boutons (Fig. 4 and Table 2). Since *D*pak is exclusively localized at the postsynaptic pockets of synapses (Sone et al., 2000), the increased area of the *D*pak signal suggests that the synaptic area in boutons that express mutant HtorA in muscles might be increased.

Aberrant presynaptic and postsynaptic ultrastructures at ΔFY or ΔE HtorA boutons

We performed ultrastructural morphometric analysis on serial transmission electron microscopy reconstructions obtained from two independent samples of boutons expressing WT, ΔFY, or ΔE HtorA in muscles. Consistent with the confocal microscopic results, high-resolution TEM images taken from the midline cross sections of typical type 1b boutons that expressed ΔFY HtorA or ΔE HtorA showed that the bouton size was larger than that of typical WT HtorA (Fig. 5A), and aberrant membrane protrusions were present (Figs. 5A-5C). The average length of bouton in the WT HtorA NMJs did not differ from that of either the ΔFY or the ΔE HtorA-expressing NMJs (Fig. 6A). However, the average surface area (Fig. 6B), the average volume (Fig. 6C), and the average number of T-shaped active zones per bouton (Fig. 6D) in the ΔFY or ΔE HtorA boutons were 50-100% larger than those of the WT HtorA boutons.

Several aberrant ultrastructures were not observed at the WT HtorA synapses but were present at the ΔFY and the ΔE HtorA synapses (Fig. 7). One of these aberrant ultrastructures, which comprised abnormal free dense bodies at the presynaptic terminals (Fig. 7B), was similar to the aberrant ultrastructures observed in synapses with a mutation in the TGF-β signaling pathway (Ablerle et al., 2002; McCabe et al., 2003) and in synapses expressing ΔE HtorA in neurons (Koh et al., 2004). Despite their

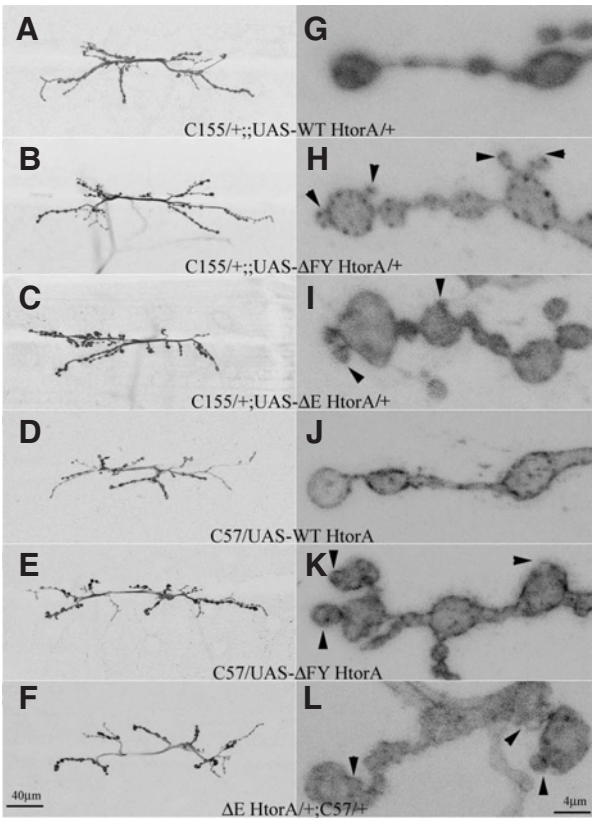

Fig. 3. Aberrant bouton morphologies of ΔFY or ΔE HtorA-expressing NMJs. The NMJs for muscle 6 and 7 were stained with anti-HRP-FITC to investigate general morphology and synaptic bouton numbers. There was no major difference in the number of Type1 synaptic boutons between (A) C155/+;;UAS-WT HtorA/+, (B) C155/+;;UAS-ΔFY HtorA/+, (C) C155/+;UAS-ΔE HtorA/+, (D) C57-Gal4/UAS-WT HtorA, (E) UAS-ΔE HtorA/+; C57-Gal4/+, and (F) C57-Gal4/UAS-ΔFY HtorA. However, high power confocal microscopy revealed that boutons in NMJs that express either ΔFY (H and K) or ΔE HtorA (I and L) were bigger and had more abnormal protrusions than the boutons in WT HtorA-expressing NMJs (G and J). Black arrow heads indicate abnormal membrane structures at boutons. Bar: 40 μm (A-F), 4 μm (G-L).

ultrastructural similarities to T-shaped active zones at synapses, these structures were not anchored to synaptic membranes. The number of free dense bodies of the ΔFY HtorA boutons was only ~25% of that of the ΔE HtorA boutons (Table 3).

Another aberrant ultrastructure was an empty postsynaptic pocket at the synapses (Figs. 5C, 7C1 to 7C4). Normally, the muscle forms a subsynaptic reticulum (SSR), which consists of layers of highly folded and convoluted extensions of the membranes that surround and envelop postsynaptic densities (Fig. 7A). Although its exact function is unknown, the SSR appears to be important in the localization and clustering of important components of postsynaptic machinery such as glutamate receptors, local protein synthesis machineries, or ion channels (Koh, 2006; Koh et al., 2000; Packard et al., 2003; Sone et al., 2000). The SSR was uniformly dense, and the postsynaptic densities were intact at the synapses in the WT HtorA boutons. In contrast, the SSR or the postsynaptic densities of the ΔFY and the ΔE expressing boutons were often interrupted by small clearings (Fig. 7C). This phenotype, which occurred in ~20% of the examined synapses in the ΔFY and the ΔE HtorA boutons, was not observed in the WT HtorA boutons (Fig. 8 and Table 3).

Table 3. The number of free dense bodies at the presynaptic terminals or synapses having empty postsynaptic pockets in boutons expressing various forms of HtorA. Neither free-electron dense bodies nor postsynaptic defects were observed at WT HtorA synapses. The number of free dense bodies of ΔFY HtorA boutons is only around 25% of those of ΔE HtorA boutons. The number of empty postsynaptic densities has an equal frequency in synapses that express either the ΔFY or the ΔE HtorA in muscles. Nine WT, eight ΔFY, or eight ΔE HtorA-expressing boutons were examined. ND, Not-detected.

Genotypes	C57-Gal4/ WT HtorA	C57-Gal4/ ΔFY HtorA	ΔE HtorA; C57-Gal4
No. of free-electron dense bodies/bouton	ND	0.5 ± 0.27	2.12 ± 0.40 (ΔFY, $p < 0.01$)
No. of synapses with a postsynaptic defect/ bouton	ND	4.25 ± 1.18	4.25 ± 0.796 (ΔFY, $p > 0.5$)

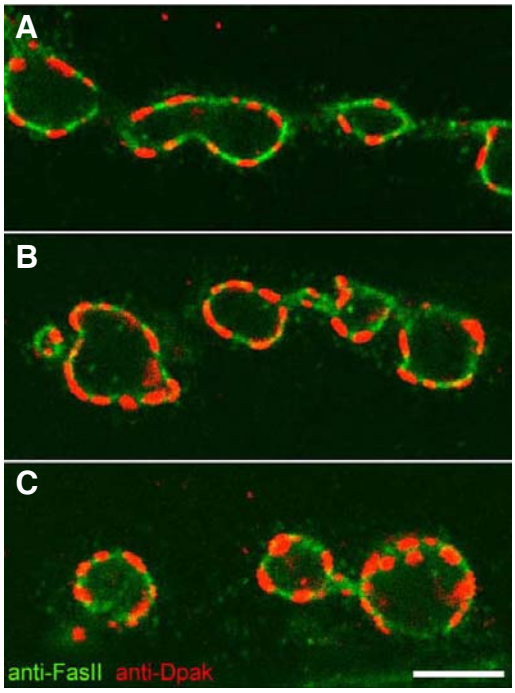

Fig. 4. The occupying area of FasII and *D*pak immunofluorescence signals at boutons expressing the ΔFY and the ΔE HtorA in muscles was altered. (A-C) Single slice confocal images were taken from midlines of several boutons that express WT (A), ΔFY (B), or ΔE HtorA (C) in muscles labeled by monoclonal FasII antibodies (green) or *D*pak antibodies (red). Anti-FasII and anti-*D*pak signals were not colocalized at boutons. The area of the FasII signals in the ΔFY HtorA- or the ΔE HtorA-expressing boutons was significantly less than that of the FasII signals in WT HtorA-expressing boutons. However, the area of the *D*pak signals in the ΔFY HtorA- and the ΔE HtorA-expressing boutons was greatly increased. Bar, 5 μm.

The increased average synapse area in the ΔFY and the ΔE HtorA-expressing boutons

In this study, synapses with a postsynaptic defect were consid-

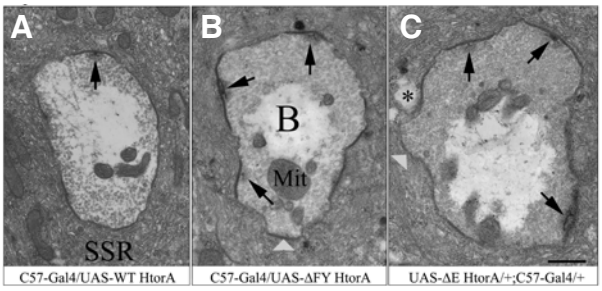

Fig. 5. Abnormalities at boutons expressing ΔFY or ΔE HtorA. (A-C) TEM images were obtained from the midline of thin cross sections of boutons that express WT (A), ΔFY (B), or ΔE HtorA (C) in muscles. In contrast to WT HtorA-expressing boutons, ΔFY or ΔE HtorA-expressing boutons had enlarged bouton cross-sectional areas with irregular shapes or an abnormal postsynaptic terminal structure (a black asterisk). The black arrows indicate the T-shaped active zones; asterisks indicate empty postsynaptic pockets; white arrow heads indicate abnormal protuberant membrane structures. B, boutons; Mit, mitochondria; SSR, subsynaptic reticulum. Bar, 500 nm.

ered to be defective synapses; otherwise, synapses were considered to be normal synapses (Fig. 7). The average areas of the normal and the defective synapses in the ΔFY and the ΔE HtorA boutons were significantly larger than those of the normal synapses in the WT HtorA boutons (Fig. 8A). Synapses are ever-changing structures that undergo emergence, growth, strengthening, weakening, and elimination. Recent studies in T-shaped active zones in *Drosophila* synapses showed that synapses without a T-shaped active zone did not participate in generating evoked synaptic transmissions and were considered to be silent synapses. These silent synapses may exist in the middle of either an emerging process or an eliminating process. In contrast, synapses with one or more T-shaped active zones were functional (Atwood, 2006; Kittel et al., 2006; Wagh et al., 2006). We then further analyzed synapse areas according to the number of T-shaped active zones together with the existence of defects at the postsynaptic densities. When the silent synapses were compared, only the average areas of defective synapses in the ΔE HtorA boutons were significantly enlarged compared to those of the WT boutons (Fig. 8B). We also found that the average areas of the functional synapses in the ΔE and the ΔFY HtorA boutons were significantly increased (Figs. 8C and 8D). The increased area of the *D*pak signals (Fig. 4 and Table 2) and the functional synapses (Fig. 8C), together with unique ultrastructural defects at the pre- and the post-synaptic terminals in the ΔFY and the ΔE HtorA boutons (Figs. 7 and 8) suggest that mutant HtorA expressed in muscles are sufficient to induce aberrant synaptic architecture.

DISCUSSION

Expression of mutant HtorA moved flies' states toward being susceptible to acute exposure to 38°C

The high expression levels of HtorA transcripts and proteins in dopaminergic neurons in substantia nigra in human brains suggested that EOTD may be a disorder caused by defects in dopaminergic transmission in human CNS (Augood et al., 1999; Ozelius et al., 1997). However, more widespread electrophysiological deficits in cortical and spinal circuits in EOTD patients compared with those in ΔE HtorA carriers suggested that additional defects in other neurotransmission systems or another genetic or environmental insult may be critical to manifesting clinical symptoms in EOTD patient (Edwards et al., 2003). In a previous study, we have shown that expression of ΔE HtorA in all neurons or mainly at dopaminergic neurons in *Drosophila* made flies' states toward being susceptible to another environmental stress, indicative of evolutionarily conserved neural pathways perturbed by ΔE mutations in HtorA in *Drosophila* and mammals (Koh et al., 2004).

Interestingly, expressions of ΔFY or ΔE HtorA in *Drosophila* neurons or muscles were enough to confer adult locomotor disabilities and ultrastructural alterations at synapses. The expression of ΔFY HtorA in *Drosophila* neurons or muscles induced adult locomotor disabilities that were similar but delayed compared to ΔE HtorA flies upon acute exposure to 38°C. Even though a similar amount of WT HtorA was expressed in flies (Fig. 1) (Koh et al., 2004), WT HtorA flies did not show any locomotor disability in the same stressful condition (Fig. 2). Thus, behavioral defects observed from the ΔFY and the ΔE HtorA flies were not caused by expression level differences.

Delayed onset of locomtor defects in ΔFY HtorA flies

One intriguing observation in this study is the delayed onset of locomotor disabilities in ΔFY HtorA flies compared with ΔE HtorA flies (Fig. 2). Those differences could be explained by normal distribution patterns of ΔFY HtorA in neurons and muscles (Fig. 1) (Koh et al., 2004) and less severe ultrastructural defects at synapses in ΔFY HtorA boutons (Fig. 7 and Table 3). Despite the

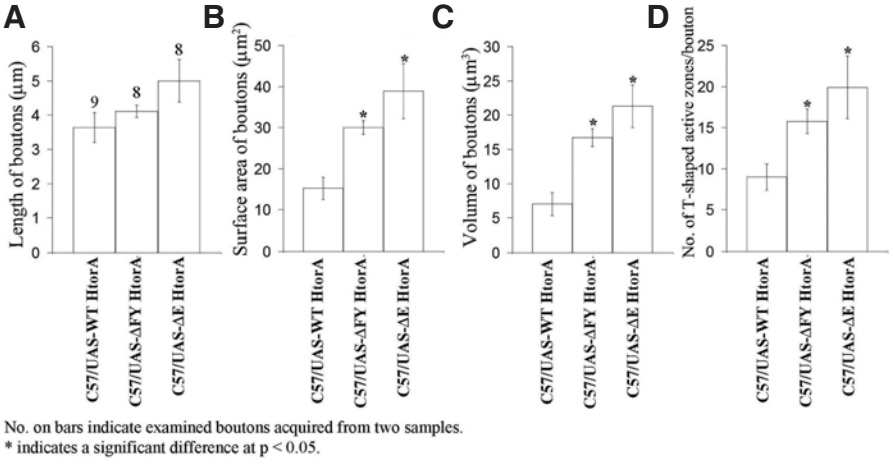

Fig. 6. Morphometric analysis of boutons expressing WT, ΔFY, or ΔE HtorA in muscles. Serial reconstructions of boutons expressing WT, ΔFY, or ΔE HtorA in muscles were used to examine (A) the average length, (B) the average surface area, (C) the average volume, or (D) the number of active zones per synaptic bouton. Aside from the average length of the boutons, all other parameters of the ΔFY and ΔE HtorA-expressing boutons differs significantly ($p < 0.05$) from those of WT HtorA. * indicates a significant difference at $p < 0.05$.

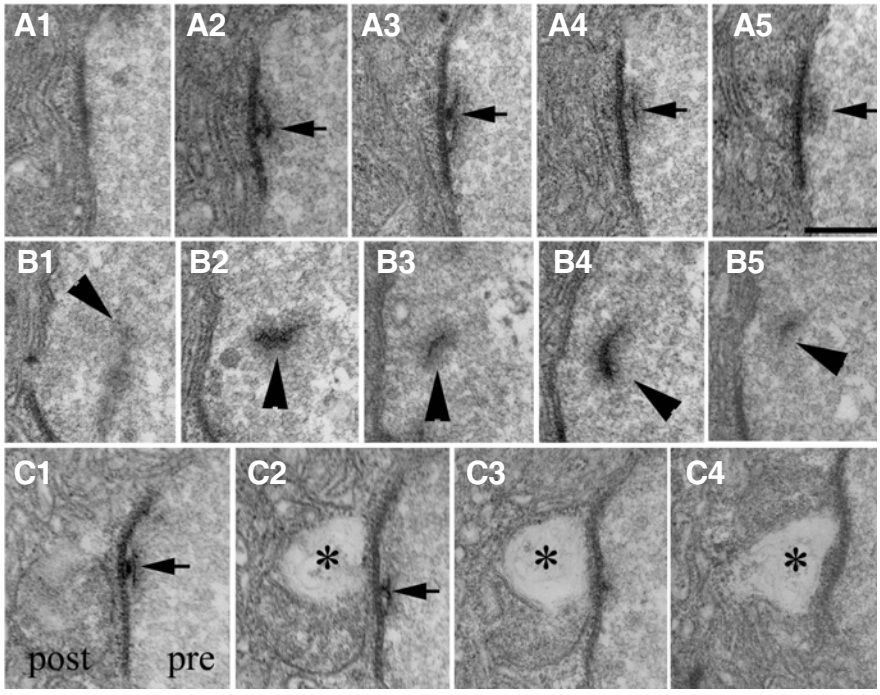

Fig. 7. Ultrastructural defects at synapses expressing ΔFY HtorA or ΔE HtorA. (A1-A5) Serial TEM images were photographed from consecutive thin sections of a synapse from WT HtorA boutons in muscles. The synapses indicated by parallel electron dense membrane structures harbored a T-shaped active zone pointed to by black arrows. T-shaped active zones were usually surrounded by a pool of synaptic vesicles. The postsynaptic pockets surrounded by a convoluted, elaborated membrane structure called an SSR were located at the left side of the synapses. (B1-B5) Abnormal free-electron dense bodies within synaptic vesicle pools indicated by black arrow heads are extended over five serial thin sections taken from ΔFY HtorA-expressing synapses. (C1-C5) Consecutive TEM images taken from serial thin sections of synapses expressing ΔFY HtorA in muscles showed an empty postsynaptic terminal structure indicated by an asterisk. Neither presynaptic nor postsynaptic abnormalities were observed at WT HtorA-expressing synapses. Pre, a pre-synaptic terminal; post, a postsynaptic terminal. Bar, 250 nm.

paucity of our understanding about the biochemical and molecular basis of ΔE HtorA-dependent protein clusters, the suppression of behavioral and ultrastructural phenotypes in ΔE HtorA flies by overexpression of *Drosophila* or human Smad2 suggested that the ΔE HtorA protein aggregates possibly interrupted the TGF-β signaling (Koh et al., 2004). Note, however, that the ΔFY HtorA specks are evenly distributed like the WT HtorA specks (Fig. 1) (O'Farrell et al., 2002). The ΔFY deletion in HtorA, which eliminates much of the helix α-7 domain (amino acid 321-332) in HtorA, may not be sufficient to induce the protein clusters observed in ΔE HtorA. ΔE mutation in HtorA is a glutamic acid deletion (302/303) within the helix α-6 domain (amino acid 302-312 in HtorA) (Kock et al., 2006). Nevertheless, ΔFY mutation in HtorA may be enough to induce behavioral defects that were similar to those of ΔE mutation in HtorA in *Drosophila*. The helix α-6 and α-7 domains of HtorA are not highly conserved in the carboxy terminus of Torp4a, even though they share 34% overall identity (Muraro and Moffat, 2006).

Several ultrastructual defects such as free electron dense bodies, postsynaptic density defects, and increased sizes of synapses that were not associated with exposure to 38°C were observed from the ΔFY and the ΔE HtorA boutons but not from the WT HtorA boutons (Figs. 7 and 8). Among them, the number of free electron dense bodies at the presynaptic terminals and sizes of defective silent synapses in the ΔE HtorA boutons were significantly more or bigger than those in the ΔFY HtorA flies (Table 3), which is indicative of many severe ultrastuctural defects in contribution to early-onset of locomotor incapacity in ΔE HtorA flies upon acute exposure to 38°C.

FasII and *D*pak in the ΔFY and ΔE HtorA synapses

Even though mutant HtorA flies had several predisposing defects at synapses, their locomotor abilities were not discernable from those of WT HtorA flies at the normal condition (Koh et al., 2004). Recent studies stated that asymptomatic ΔE HtorA carriers did not show any difference in movement time and spatial accuracy measured during each task compared with healthy control groups in humans. They only exhibited mildly impaired learning and performance in sequential motor tasks (Ghilardi et al., 2003). Although we have not yet examined whether expressions of mutant HtorA in flies induced defects in any form of learning and memory capabilities, a plethora of studies has shown that the mutations which altered synaptic structures or functions in *Drosophila* larval NMJs were associated with defects in certain forms of learning and memory (Davis, 2005; Margulies et al., 2005). One example is FasII. FasII hypomorphic mutant flies showed defects in encoding, stabilizing, and retrieving short-term memories (Cheng et al., 2001). The FasII occupying areas were diminished in mutant HtorA boutons, an indicator of defects in short-term learning and memory in mutant HtorA flies.

In addition, we also found an increased area of *D*pak signals at mutant HtorA boutons (Fig. 4 and Table 2). This result raised the possibility of activation of certain homeostatic regulating mechanisms that could compensate for the pre- and the postsynaptic defects at mutant HtorA boutons. The increased area of *D*pak signals (Fig. 4) may induce the increased area of functional synapses in mutant HtorA boutons (Fig. 8) that may make flies behave normally under permissive conditions. Under non-permissive conditions, however, the increased sizes of synapses and other ultrastructural abnormalities may be contributed to altered synaptic transmissions, resulting in locomotor defects such as loss of motor control, leg kicking, or upright wings in mutant HtorA flies. The increased *D*pak signals in mutant HtorA flies are important since the roles of Pak-dependent signaling pathways at synapses are evolutionarily conserved. Their known roles include cortical spinogenesis and synaptic transmissions in mice brains (Hayashi et al., 2004; Zhou, 2007), the regulation of dendritic terminal development at mammalian excitatory neuronal cell cultures (Park et al., 2003), the clustering of acetylcholine receptors at the mammalian NMJs (Luo et al., 2002), as well as the development of the

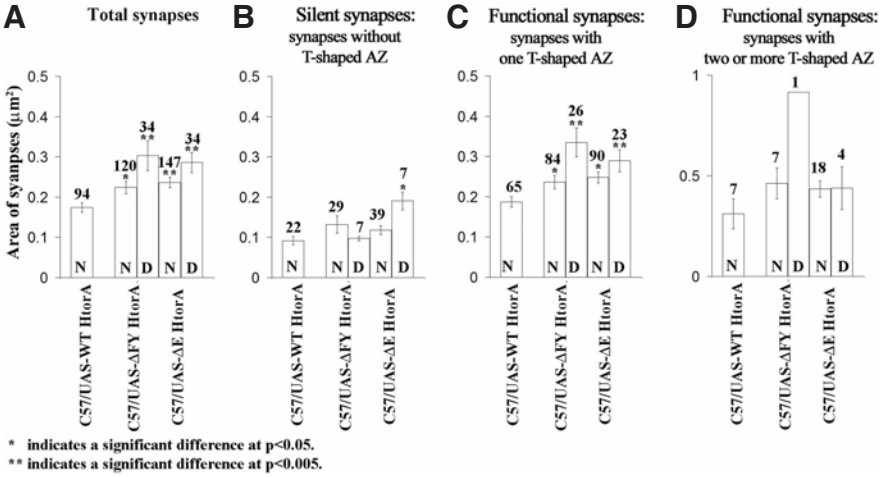

Fig. 8. Morphometric analysis of synapses expressing WT, ΔFY, or ΔE HtorA in muscles. (A) The average areas of normal and defective synapses of the ΔFY or the ΔE HtorA-expressing boutons were significantly increased compared with the average areas of synapses of WT HtorA-expressing boutons. Defective synapses were only observed from mutant HtorA-expressing boutons. In WT HtorA-expressing boutons, all examined synapses were normal. Examined synapses were further divided by the number of T-shaped active zones. (B-D) Synapses without T-shaped active zones were also known as silent synapses. In addition, synapses with one or more T-shaped active zones were considered to be functional synapses. The areas of functional synapses in ΔFY HtorA-expressing boutons were altered compared with those of WT HtorA-expressing synapses (C). However, the defective silent or the defective and the normal functional synapses in ΔE HtorA-expressing boutons were significantly enlarged compared with the normal silent or the normal functional synapses of WT HtorA-expressing synapses, respectively (B and C). Too less numbers of synapses with two or more T-shaped active zones were examined to compare (D). Numbers on bars indicated examined synapses. AZ, active zone; D, defective synapses; N, normal synapses.

postsynaptic terminal structures in glutamaterigc synapses in *Drosophila* NMJs (Parnas et al., 2001; Sone et al., 2000). Thus, it will be an intriguing question that examines distribution of Pak in brains and NMJs of ΔE HtorA-expressing mice, ΔE tor1a knock-in mice, ΔE HtorA carriers, or EOTD patients.

In conclusion, the correlative observation of severe locomotor defects and aberrant ultrastructures at the synapses in flies expressing ΔFY or ΔE HtorA in neurons or muscles strongly suggests that ΔFY HtorA may contribute to the onset and progression of locomotor disabilities in *Drosophila*.

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