

## **Inactivation of *Drosophila* Huntingtin affects long-term adult functioning and the pathogenesis of a Huntington's disease model**

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## ABSTRACT

A polyglutamine expansion in the *huntingtin (Htt)* gene causes neurodegeneration in Huntington's disease (HD), but the *in vivo* function of the native protein is largely unknown. Numerous biochemical and *in vitro* studies have suggested a role in neuronal development, synaptic function, and axonal trafficking. To test these models, we generated a null mutant in the putative *Drosophila Htt* homolog (*dhtt*), and, surprisingly, found that *dhtt* mutant animals are viable with no obvious developmental defects. Instead, *dhtt* is required for maintaining the mobility and long-term survival of adult animals, and in modulating axonal terminal complexity in the adult brain. Further, removing endogenous *dhtt* significantly accelerates the neurodegenerative phenotype associated with a *Drosophila* model of polyglutamine Htt toxicity (HD-Q93), providing *in vivo* evidence that disrupting the normal function of Htt might contribute to HD pathogenesis.

## INTRODUCTION

Huntington's disease (HD) is an autosomal-dominant, progressive neurodegenerative disorder characterized clinically by deteriorating choreic movements, psychiatric disturbances and cognitive deficits (Gusella and MacDonald, 1995; Martin and Gusella, 1986; Vonsattel et al., 1985). HD is caused by an abnormal expansion of a polyglutamine (polyQ) tract at the N-terminus of a large cytoplasmic protein, Huntingtin (Htt) (The Huntington's Disease Collaborative Research Group. ). In the wildtype Htt protein, the

polyQ tract is between 6 to 35, whereas is expanded beyond 36 repeats in HD (The Huntington's Disease Collaborative Research Group). Numerous studies have demonstrated that mutant Htt containing an expanded polyQ-tract is toxic to neurons (Cattaneo et al., 2001; Gusella and MacDonald, 2000). PolyQ expansion is also linked to at least 8 other neurodegenerative disorders, collectively referred to as polyQ diseases (Riley and Orr, 2006; Zoghbi and Orr, 2000). Although Htt is ubiquitously expressed in the brain, HD mainly affects medium-sized spiny neurons in the striatum and to a lesser extent cortical pyramidal neurons that project to the striatum, suggesting other cellular factors also contribute to pathogenesis (Cattaneo et al., 2001; Vonsattel and DiFiglia, 1998). Recent studies indicate that alteration of wildtype Htt function might contribute to this specificity and subsequent disease progression (Cattaneo et al., 2001). For example, mutant Htt can sequester wildtype Htt into insoluble aggregates, thereby exerting a dominant negative effect (Huang et al., 1998; Kazantsev et al., 1999; Narain et al., 1999; Preisinger et al., 1999; Wheeler et al., 2000). In addition, wildtype Htt can suppress cell death induced by the mutant polyQ-expanded Htt in vitro (Leavitt et al., 2001; Van Raamsdonk et al., 2005). Further, wildtype Htt is proposed to have a neuroprotective role, as expression of Htt can protect cultured striatal neurons from stress- and toxin-mediated cell death (Rigamonti et al., 2000).

Since its identification, the normal function of Htt has been subjected to extensive investigation (Cattaneo et al., 2001; Harjes and Wanker, 2003). The murine Htt homolog (Hdh) is essential during early mouse development, as *Hdh*-null mice die during gastrulation at embryonic day 7.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Chimeric analysis demonstrated that the early embryonic lethality is due to a

critical role of Hdh in extraembryonic membranes , as this lethality can be rescued by providing wildtype Hdh function in extraembryonic tissue (Dragatsis et al., 1998). Conditional knockout of Hdh in the mouse forebrain at postnatal or late embryonic stages causes a progressive neurodegenerative phenotype, lending support to the hypothesis that depletion of normal Htt activity during disease progression contributes to HD pathogenesis (Dragatsis et al., 2000). A more recent study in Zebrafish, which used morpholino oligos to transiently knock down endogenous Huntingtin, suggests a role of Huntingtin in normal blood function and iron utilization (Lumsden et al., 2007). Currently, little is known about the normal biological function of wildtype Htt (Cattaneo et al., 2005).

Htt encodes a large cytoplasmic protein of 350 kDa. Structural analysis of Htt proteins identified the presence of many HEAT (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats, a 40 amino acid (a. a.) long, anti-parallel helices structural motif of unknown function (Andrade and Bork, 1995). No other identified domain exists in Htt to suggest a biological function. Functional studies in mammalian systems, mainly from protein interaction assays, have associated Htt with diverse cellular processes, including endocytosis, modulation of synapse structure and synaptic transmission, transcriptional regulation, especially the brain-derived neurotrophic factor (BDNF) which is essential for the survival of the striatal neurons affected in HD, axonal transport of BDNF and vesicles, and apoptosis (Cattaneo et al., 2001; Cattaneo et al., 2005; Harjes and Wanker, 2003; Zuccato et al., 2001; Zuccato and Cattaneo, 2007). Importantly, few of these proposed functions of Htt have been directly tested *in vivo* due to the early embryonic lethality associated with *Hdh* null mutant mice.

In an extensive search for Htt homologs in other species, Li et al. (1999) identified a single HD homolog in *Drosophila* (*dhtt*). By sequence comparison, the homologous regions between *Drosophila* and human Htt proteins are mainly located within five discrete areas, including 3 relatively large continuous regions and two small segments, that cover about one third of total protein length (Fig. S1A). At the amino acid level, these homologous regions, which are comprised of about 1,200 a.a. in dHtt, share ~ 24% identity and 49% similarity between the fly and human sequences (Li et al., 1999). In addition to the sequence similarity, other shared features support that the identified gene is indeed the *Drosophila* homolog of human Htt, as noted in the study of Li et al. (Li et al., 1999). For example, in term of the protein size, both *Drosophila* dHtt and mammalian Htt proteins are unusually large proteins, with Htt containing 3,144 a.a. residues in humans and 3,583 a.a. in *Drosophila* (Li et al., 1999). In addition, the regions with relatively high level of conservation are not only clustered in large continuous stretches, but are also located in the same order and distributed over the entire length of the proteins. Moreover, *dhtt* and mammalian HD genes share similar patterns of gene expression ((Li et al., 1999) and also see Fig. 1). Interestingly, although a Htt homolog exists in *Drosophila*, no Htt-like gene has been found in other less complex eukaryote species such as yeast *S. cerevisiae* or *C. elegans* (Li et al., 1999).

Identification of a *Drosophila* Htt homolog provides a unique opportunity to evaluate the role of Htt in this well-established genetic model system. Several cellular processes implicated in Htt function, including axonal transport and synapse formation, have been well-characterized in *Drosophila*, allowing *in vivo* evaluation of their relationship with Htt. Further, as fly models of HD have been well established, it allows

*in vivo* examination the function of endogenous Htt in HD pathogenesis (Marsh and Thompson, 2006; Steffan et al., 2001). In this study, we report the isolation of a *dhtt* mutant and describe its phenotype. Further, we examine the effect of removing endogenous *dhtt* on several Htt-associated cellular processes implicated from earlier studies and test the effect of loss of endogenous *dhtt* on the pathogenesis associated with an established *Drosophila* model of polyglutamine toxicity (HD-Q93).

## RESULTS

### HEAT repeats in *Drosophila* Htt

Considering the limited sequence homology between mammalian and fly Htt, it is important to examine the extent of structural similarity between these proteins. In Htt family proteins, the HEAT repeat is the only identifiable structural motif (Andrade and Bork, 1995; Cattaneo et al., 2005). A previous phylogenetic study identified 16 HEAT repeats in human Htt, and notably, 14 of these 16 HEAT repeats were also found in insect Htt proteins including *Drosophila* dHtt (Tartari et al., 2008). A less-stringent structural analysis predicted up to 40 HEAT repeats (including the AAA, ADB and IMB subgroups) in human Htt (see Methods). Interestingly, using the same parameter, 38 HEAT repeats could be identified in *Drosophila* dHtt (Supplemental file S1 for details of the predicted HEAT repeats). Further, these HEAT repeats span the entire-length of each protein and have similar distribution, clustering as 4 groups at the N-, middle- and C-terminal regions that largely overlap with their sequence-homologous segments (Supplemental Fig. S1B). Although further studies are needed to elucidate the structure of

Htt proteins, this rather remarkable similarity raises the possibility of a conserved secondary structure among Htt family proteins and that both human and *Drosophila* Htt proteins are composed largely of repeated HEAT motifs.

### **Ubiquitous expression of *dhtt* in *Drosophila***

Previous analysis has shown that *dhtt* is widely expressed during all developmental stages from embryos to adults (Li et al., 1999). We confirmed the expression of *dhtt* transcript in adults using RT-PCR (data not shown, see also Fig 5C). To examine tissue-specific *dhtt* expression, we performed whole-mount RNA *in situ* hybridization. Staining with two Dig-labeled antisense probes targeting different regions of *dhtt* both revealed similar, ubiquitous *dhtt* expression at different stages of fly embryogenesis and in larval tissues (Figs. 1A-F and data not shown), while a positive control performed in parallel gave rise to robust *in situ* signals (Fig. S2). Importantly, two negative controls included in the assay, one with sense *dhtt* RNA probes on wildtype samples (Fig. 1B), and the other with the same set of antisense *dhtt* RNA probes against tissues from a *dhtt* deletion mutant subsequently generated (Fig. 1F), both produced much weaker background signals. Together, these data indicate that *dhtt* is expressed widely at low levels during all stages of *Drosophila* development.

### **dHtt is a cytoplasmic protein**

Mammalian Htt proteins are largely cytoplasmic with a widespread expression pattern (DiFiglia et al., 1995; Gutekunst et al., 1995; Sharp et al., 1995). To determine the expression and sub-cellular localization of endogenous dHtt protein, we developed and

affinity-purified polyclonal antibody against dHtt. The specificity of this antibody is confirmed by its ability to recognize ectopically expressed dHtt protein in transfected *Drosophila* S2 cells (Figs. 1G and 1H) and larval tissues (Figs. 1I-K). When dHtt protein was ectopically expressed from a UAS-*dhtt* transgene by a *patched*-Gal4 driver, our anti-dHtt antibody could easily detect the striped pattern of dHtt expression in the middle of imaginal discs, the characteristic domain of Patched expression (Fig. 1I). In wildtype animals, the anti-dHtt antibody revealed a low-level, ubiquitous staining in embryos, larval and adult tissues with no specific pattern of protein expression (data not shown). At the subcellular level, ectopically expressed dHtt was found predominantly in the cytoplasm in transfected S2 cells and in larval tissues (Figs. 1G-K and data not shown), suggesting that *Drosophila* dHtt, similar to its human counterpart, is mainly a cytoplasmic protein.

### **Creating a *dhtt* deletion**

Similar to human Htt, the *Drosophila* homolog (*dhtt*) encodes an unusually large protein of 3583 a.a.. The 11, 579 base-pair (bp) cDNA of *dhtt* gene, is derived from 29 exons in a 38 kb transcribed genomic region at cytological interval 98E2 (Li et al., 1999) (Figs. 2A and C). No null mutations in *dhtt* have been previously isolated. To generate a null mutant for *dhtt*, we selected two FRT-bearing insertion lines surrounding *dhtt*: P-element d08071 inserted at the 5' end of the neighboring CG9990 gene, and piggyBac insertion f05417 located near the 3' end of *dhtt*, inside the intron between *dhtt* exon 27 and exon 28 (Fig. 2A). Using Flipase-FRT mediated recombination (Parks et al., 2004), we generated a precise deletion of 55 kb between the two insertions (See Experimental



Procedures). This deletion allele, termed *Df(98E2)*, removed most of the CG9990 gene and 34 kb of *dhtt*'s 38kb genomic-coding region, with only the last two exons of *dhtt* gene remaining (Figs. 2A-C). The deletion was confirmed by inverse PCR from extracted genomic DNA and by DNA sequencing (data not shown).

*Drosophila* containing the *Df(98E2)* deletion, which removes both CG9990 and *dhtt*, are homozygous lethal at the embryonic stage. CG9990 encodes a previously uncharacterized protein belonging to the ABC transporter superfamily (Dean et al., 2001). To separate the mutant phenotype of *dhtt* from CG9990, we generated transgenic flies carrying a CG9990 genomic rescue in the *Df(98E2)* background (see Methods). These lines, referred to as *dhtt-ko* (*dhtt knock-out only*), carry both the CG9990 rescue transgene and the *Df(98E2)* deletion, and thus are mutant only for *dhtt* (Figs. 2B-D).

### ***dhtt* is dispensable for *Drosophila* development**

The *dhtt-ko allele* removes 27 of the 29 exons of *dhtt* (Fig. 2). Since *Hdh* homozygous knock-out mutant mice die during early embryogenesis (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995), we expected that loss of *dhtt* in the fly would be associated with prominent developmental defects. However, *dhtt-ko* flies are homozygous viable, demonstrating the lethality observed in *Df(98E2)* is caused by loss of CG9990. To verify that the *dhtt* gene is indeed deleted in *dhtt-ko*, we extracted genomic DNA from homozygous *dhtt-ko* adults and performed Southern Blot analysis. As shown in Fig. 2D, all genomic DNA containing the *dhtt* gene was removed in *dhtt-ko* flies except the final two 3'exons.

Further analyses demonstrated that homozygous *dhtt-ko* flies develop at a similar

rate as wildtype and give rise to fertile adults with no discernable morphological abnormalities. Progeny derived from homozygous *dhtt-ko* flies did not show a reduction in viability or display other obvious developmental defects, suggesting there is no significant effect on animal development or function by maternally contributed *dhtt*.

To determine if developmental defects are present in *dhtt-ko* animals, we characterized *dhtt-ko* mutants using a variety of cellular and neuronal markers. These studies failed to reveal any obvious developmental abnormalities during embryogenesis, or during larval and adult stages (Fig. 3 and data not shown). In particular, the embryonic CNS (Fig. 3A) and muscles, larval muscles, CNS, eye and other imaginal discs all appeared normal (Fig. 3B and data not shown). Further, in aged adults (40-day-old), the external eye morphology was normal and the eight neuronal photoreceptor cells in each ommatidium with their accessory cells clearly present (Figs. 3C and 3D). We note that our result is different from a recent study that examined *dhtt* function using RNA interference (RNAi) that implicated a role for *dhtt* in axonal transport and eye integrity (Gunawardena et al., 2003). The exact nature of this phenotype discrepancy is not clear. It is possible that the observed RNAi phenotypes are due to cellular toxicity caused by the high-level expression of Gal4, given the experiment was carried out at 29°C (Gunawardena et al., 2003). The more severe phenotypes might also be due to the non-specific RNAi off-target effects caused by the knockdown of unrelated genes (Kulkarni et al., 2006). Nevertheless, our results suggest that *dhtt* is dispensable for normal *Drosophila* development. This result is in contrast to the essential role of *Hdh* in mouse (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). These phenotypic differences are likely due to distinct mouse and fly embryogenesis, as the early lethality of *Hdh*-null

mice is due to function of Hdh in extraembryonic membranes for which there are no equivalents in *Drosophila* ((Dragatsis et al., 1998), see Discussion)).

### **Normal synapse organization in *dhtt-ko***

Htt has been reported to interact with a diverse group of proteins whose functions have been directly or indirectly linked to synapse organization and synaptic activity (Harjes and Wanker, 2003), including proteins that regulate cytoskeleton dynamics and clathrin-mediated endocytosis (*e.g.*, Hip1 (Sla2p), Hip12, PACSIN/Syndapin 1, and Endophilin 3) (Chopra et al., 2000; Higgins and McMahon, 2002; Kalchman et al., 1997; Modregger et al., 2002; Seki et al., 1998; Singaraja et al., 2002; Sittler et al., 1998; Wanker et al., 1997), axonal vesicle transport (*e.g.*, HAP1) (Engelender et al., 1997; Gunawardena et al., 2003), as well as dendritic morphogenesis and synaptic plasticity (*e.g.*, post-synaptic density protein PSD95, adaptor proteins Grb2 and CIP4) (Holbert et al., 2003; Liu et al., 1997; Sun et al., 2001). To test whether *dhtt* plays a role in synapse organization, we examined the formation of glutamatergic neuromuscular junctions (NMJs) in 3<sup>rd</sup> instar larvae, a well-characterized system for studying synapse formation and function in *Drosophila* (Budnik and Gramates, 1999). Examination with a panel of synapse markers showed that axonal pathfinding, muscle innervation, and overall synapse structure are normal in *dhtt* mutants (Figs. 3, and data not shown). Double-labeling with the axonal membrane marker anti-HRP and the post-synaptic density marker anti-Dlg (the *Drosophila* PSD-95 homolog) revealed well-organized pre- and post-synaptic structures (Figs. 3E1-E8), as well as an enrichment of synaptic vesicles at synapses, with no obvious synapse retraction phenotype (data not shown). Moreover, *dhtt* mutants showed

normal organization of the presynaptic microtubule (MT) cytoskeleton, with the presence of stable MT bundles traversing the center of NMJ branches and a dynamically reorganized MT network at distal boutons (data not shown). Quantification of NMJ bouton number and axonal branching did not reveal a significant difference between wildtype control and *dhtt* mutants (Figs. 3G-I). Finally, *dhtt* mutants displayed the stereotypical complementary pattern of active zones surrounded by the honeycomb-like organization of periaxial zones, and examination of multiple synaptic proteins such as nc82 (an active zone marker) and Fascicilin II (FasII, a periaxial zones marker) showed normal synaptic localization (Figs. 3F1-F8). As a control, we also examined synapse organization in CG9990 transgenic animals, which displayed similar well-organized NMJ structures (Fig. S3 and data not shown). Together, these results suggest that *dhtt* is not essential for synapse formation and organization at NMJs.

### ***dhtt* is not essential for axonal transport**

Htt has also been proposed to regulate axonal vesicle transport, as one of its binding partners, HAP1, interacts directly with p150Glued, which is an essential subunit of dynactin complex involved in regulating the motor protein dynein-mediated retrograde axonal transport (Engelender et al., 1997; Gunawardena et al., 2003; Harjes and Wanker, 2003). In *Drosophila*, individuals defective for essential components of the axonal transport machinery often display characteristic mobility phenotypes, such as tail flipping during larva crawling, and progressive lethargy and paralysis (Gindhart et al., 1998; Gunawardena and Goldstein, 2001; Martin et al., 1999). Such mutant animals also develop an axonal swelling phenotype due to the abnormal accumulation of synaptic

vesicles along axons, and the failure to properly deliver and localize synaptic components to the termini (Gindhart et al., 1998; Gunawardena and Goldstein, 2001; Martin et al., 1999). *dhtt* mutants showed normal crawling behavior during larval stages. Furthermore, the distribution of the synaptic vesicle markers anti-Synaptotagmin (Syt) or anti-Cysteine String Protein (CSP) were normal, revealing no obvious accumulation or axonal swellings (Figs. 4 and data not shown). Further, immunostaining with other synaptic components, including Synapsin (a reserved vesicle pool marker), FasII (a periaxonal zone marker) and Dlg, demonstrated that synaptic components were properly delivered to synapses (Figs. 3E-3F and data not shown). Similarly, examination of CG9990 transgenic animals failed to reveal discernable larval mobility or axonal transport defects (Fig. S3). Together, these results suggest that *dhtt* does not play an essential role in axonal transport.

### ***dhtt* is critical for aged adults**

We next investigated whether *dhtt* may function in adult animals. We examined whether newly emerged *dhtt* adults are hypersensitive to stress tests, including prolonged heat and cold exposure, vortexing, and feeding with the oxidative stress compound paraquat. In these tests, *dhtt* mutants showed similar responses as wildtype controls (data not shown). Thus, unlike flies mutated for Parkinson's disease genes, such as *parkin* and *pink1*, which are sensitive to multiple stress challenges (Clark et al., 2006; Greene et al., 2003; Park et al., 2006), loss of *dhtt* does not render young adult animals more vulnerable to environmental stresses.

Next, we followed the activity and viability of *dhtt* animals throughout the adult lifecycle. Although no discernable difference in the activity and survival rate was observed between *dhtt-ko* and wildtype young adults, we observed striking defects in older adult *dhtt-ko* flies. *dhtt-ko* animals showed similar spontaneous locomotion to that of wildtype controls at day 15 and earlier (Fig. 5A, see Movie S1). However, as flies aged, *dhtt-ko* mutants displayed a rapidly declining mobility, which was evident by day 25. By day 40, almost all *dhtt* mutants showed severely-compromised mobility (Fig. 5A, see Movie S2) and their viability declined quickly. While on average half of wildtype controls died around day 59 but could live up to 90 days, half of *dhtt-ko* died around day 43 and almost all by day 50 (Fig. 5B).

To verify that late-onset mobility and viability defects were due to loss of *dhtt*, we constructed a *dhtt* mini-gene that expressed full-length *dhtt* under the control of its endogenous regulatory region (see Experimental Procedures). In the presence of this mini-*dhtt* transgene, the expression of *dhtt* gene was restored in *dhtt-ko* mutants, as confirmed by RT-PCR (Fig. 5C) Both the late-onset mobility and viability phenotypes observed in *dhtt-ko* mutants were rescued by the *dhtt* transgene (Fig. 5A and 5B, also see Movie S3). Importantly, introduction of an unrelated transgene construct into the *dhtt-ko* background such as *elav-Gal4* (Fig. 5B) or *uas-eGFP* (data not shown) could not rescue the mobility and viability phenotypes of *dhtt-ko* mutants, confirming the rescue was due to the restored expression of *dhtt*. Thus, although *dhtt* is not essential for normal *Drosophila* development, its function is important in maintaining the long-term mobility and survival of adult animals.

### ***dhtt-ko* is dispensable for normal neurotransmission**

The observed mobility and viability phenotype could be due to an underlying neurotransmission defect in *dhtt* mutants, as several proposed Htt functions, such as axonal vesicle transport and clathrin-mediated endocytosis in neurons, are essential for delivery and recycling of synaptic vesicles at nerve terminals to ensure effective neurotransmission (Cattaneo et al., 2001; Eaton et al., 2002; Harjes and Wanker, 2003; Hinshaw, 2000; Slepnev and De Camilli, 2000). To test neuronal communication in *dhtt* mutants, we measured synaptic physiology at the well-characterized 3<sup>rd</sup> instar larval NMJ. We quantified the amplitude of evoked excitatory junctional potentials (EJPs), resting membrane potential, and paired-pulse facilitation (Figs. 6A-6D and data not shown). In adult animals, we recorded electroretinogram (ERG) responses in the eye and quantified DLM (dorsal longitudinal flight muscle) bursting activity in the giant fiber flight circuit (Fig. 6E and data not shown). Following exhaustive analysis, we found no significant defect in either synaptic transmission or short-term plasticity in *dhtt* mutants. Together, these data suggest that *dhtt* is not essential for neurotransmission.

To investigate whether the observed mobility and viability phenotypes in aged *dhtt* mutants are correlated with neuronal communication defects, we screened for electrophysiological phenotypes in animals aged 40-45 days, when behavioral motor abnormalities are prominent. Aged *dhtt* mutant adults did not display abnormal seizure activity in extracellular recordings from DLM flight muscles in the giant fiber escape pathway, as has been observed in *Drosophila* temperature-sensitive mutants that alter synaptic transmission (Guan et al., 2005). Aged mutants also showed normal visual transduction and synaptic transmission in the visual system at room temperature (Fig. 6E,

and data not shown), consistent with the observation that *dhtt* does not play a critical role in neurotransmission.

Previous studies have shown that mutations affecting synaptic function often manifest a more prominent phenotype under temperature-induced stress (Atkinson et al., 1991; Coyle et al., 2004). To assess whether phototransduction can be maintained under elevated temperature in *dhtt* mutants, we performed ERG recordings at 37°C. Although *dhtt* mutants aged 1-3 days displayed normal ERGs at both 20°C and 37°C, aged *dhtt* mutants displayed abnormal sensitivity to 37°C, with over 60% of aged mutants losing light-induced phototransduction and the on/off transients at 37°C, as compared to only 20% in control aged adults (Figs. 6E and 6F), indicating an important role of *dhtt* in maintaining phototransduction under temperature-induced stress. Given that aged *dhtt* mutant adults did not show additional motor defects when placed at 37°, we hypothesize that the loss of photoreceptor depolarization found in ERG recordings reflects temperature-sensitive defects in the phototransduction cycle, rather than in synaptic transmission. These results suggest that aged *dhtt* mutants are more sensitive to stress than are young *dhtt* animals, or aged controls.

### **Reduced axon terminal complexity in *dhtt-ko* brains**

To further test for *dhtt* function in the adult brain, we examined *dhtt* brain morphology using a series of cellular markers. The overall patterning and gross morphology of *dhtt-ko* brains appeared normal, as shown by staining with antibodies such as glia cell marker anti-Repo, the pan-neuronal marker anti-Elav, and the synaptic vesicle marker anti-CSP (data not shown). In addition, within the ventral nerve cord, the neuropile was similarly



enriched with synaptic vesicles. Further, the overall axonal morphology of *dhtt-ko* mutants appeared normal as well, with no clear axonal blebbing or defasciculation phenotypes (data not shown). Interestingly, examination of the mushroom bodies (MB), which are involved in learning and memory in flies, by anti-FasII staining, revealed that the signal intensity was weaker in *dhtt* mutants than in wildtype controls, despite appearing morphologically normal (Figs. 7J and 7M). Quantification of MB size and FasII staining signals revealed that, while there was a slight reduction of the average size of MB in *dhtt-ko* mutants as compared to wildtype controls (Fig. 7P, average area cover by each MB: wildtype (WT)=13,352±156  $\mu\text{m}^2$ ; *dhtt-ko* mutant=11,927±310  $\mu\text{m}^2$ ; p=0.0003), the average signal intensity of FasII signals in MB of *dhtt-ko* mutants was decreased by ~50% (Fig. 7Q, relative signal intensity of MB: wildtype=100±5.8/ $\mu\text{m}^2$ ; *dhtt-ko* mutant=47.7±6.1/ $\mu\text{m}^2$ ; p< 0.0001. Total number of MB quantified: wildtype control, n=13; *dhtt-ko* mutants, n=10).

To examine the effect of *dhtt* loss-of-function on the detailed structure of individual neurons in the brain, we used the A307-Gal4 line, which labels the pair of Giant Fiber (GF) neurons and a small number of other neurons of unknown identity in adult brain, to examine axonal projection patterns and fine axonal terminal structure of individual neurons (Phelan et al., 1996) (Fig. 7). Among the A307-Gal4 labeled neurons, one pair, located at the dorsal-lateral edge of the brain, projects a prominent axon tract along the dorsal-posterior surface to the dorsal-central region of the brain, forming extensive dendritic connections (Fig. 7B-D and Fig. S4). These neurons further extend their projections anteriorly, establishing a complex axon terminal structure with extensive varicosities and fine branches above the antennal lobe region of the brain (Figs. 7E, 7F,

7K and Fig S4). In *dhtt-ko* mutants, the axonal projections of A307-positive neurons follow the same path and their axons terminate at similar location as that in wildtype, consistent with the observation that *dhtt* does not affect axonal integrity or pathfinding (Fig. 7G and H). Interestingly, axonal termini from both wildtype and *dhtt-ko* show a similar age-dependent maturation process: whereas axonal termini in young adult brains are mainly composed of a network of variable thin branches with no clearly recognizable synaptic boutons (Figs. S4C and F, 3-day-old), mature boutons develop as animals age, with many prominent boutons being easily identifiable in 40-day-old brains (Fig. 7F and I. Compare to Figs. S4C and S4F). Due to the significant variation in their structure and the lack of recognizable boutons, it is difficult to directly quantify and compare the size of these axonal termini in young adults. However, in aged *dhtt-ko* mutants, it is apparent that the axonal termini contain a significantly reduced number of varicosities and branches (Figs. 7H, 7I and 7N). Quantification of total area covered by each axonal terminus revealed that A307-positive axonal termini in 40-day-old *dhtt-ko* mutants cover about half of the area compared to controls (Fig. 7R, average area covered by each axonal terminus: wildtype (WT) control= $168.7 \pm 8.0 \mu\text{m}^2$ ; *dhtt-ko* mutant= $86.1 \pm 7.2 \mu\text{m}^2$ ;  $p < 0.0001$ ). Total number of A307-positive axonal termini quantified: wildtype control,  $n=18$ ; *dhtt-ko* mutants,  $n=17$ ). To rule out the possibility that such reduced complexity was due to the accelerated aging process or a secondary effect associated with the reduced mobility of *dhtt-ko* mutants, we examined the axonal termini of the A307-positive neurons in 83-day-old wildtype flies, as animals at this age are near the end of their lifespan and have severely reduced mobility. The structure of axonal termini in these

flies is similar to that of 40-day-old flies, showing no obvious reduction of terminal complexity (Fig. S5).

Using the membrane-bound mCD8-eGFP reporter driven by the GF-specific A307 Gal4 line, we further analyzed the axonal projection and terminal morphology of the GF neurons in *dhtt-ko* mutants. The GF neurons are a pair of large interneurons located in the central brain and project their prominent axons unbranched to the mesothoracic neuromere (T2) in the ventral nerve cord, where they bend laterally and synapse with other inter- and motorneurons (Phelan et al., 1996). In both young (3-day-old) and aged (40-day-old) *dhtt-ko* mutants, GF neurons project normally to the T2 neuromere and form the characteristic terminal bends, resembling that observed in wildtype controls (Fig. S6). In young (3-day-old) animals, the signal intensity of the mCD8-eGFP reporter at the GF axonal termini was similar between *dhtt-ko* mutants and wildtype controls (Fig. S6B and C. Total number of 3-day-old GF axonal termini examined: wildtype control, n=10; *dhtt-ko* mutants, n=12). However, in old (40-day-old) animals, the GF axonal termini in wildtype controls showed a much stronger enrichment for the mCD8-eGFP reporter than that in *dhtt* mutants (Fig. S6D-G. Total number of 40-day-old GF axonal termini examined: wildtype control, n=16; *dhtt-ko* mutants, n=20). The exact nature behind such a difference remains to be clarified, but might represent subtle alterations in axonal transport of membrane proteins in aged neurons. Nevertheless, these results suggest that *dhtt* does not affect axonal pathfinding and overall brain organization, but has a functional role in regulating the complexity of axonal termini in the adult brain.

### **Loss of *dhtt* enhances the pathogenesis of HD flies**

Earlier studies in cell culture and in *Hdh* mutant mice have shown that wildtype Htt has a protective role for CNS neurons (Dragatsis et al., 2000; O'Kusky et al., 1999; Rigamonti et al., 2000; Van Raamsdonk et al., 2005). Further, existing evidence suggests that normal Htt activity can be inactivated by mechanisms such as abnormal sequestration into insoluble aggregates (Huang et al., 1998; Kazantsev et al., 1999; Narain et al., 1999; Preisinger et al., 1999; Wheeler et al., 2000). These and other observations have led to the hypothesis that the perturbation of endogenous Htt function, such as late-onset inactivation of endogenous Htt, contributes to HD pathogenesis (Cattaneo et al., 2001). Attempts to test this directly in mouse *Hdh* mutants have been complicated by the critical role of *Hdh* both in early mouse embryogenesis and in later brain development (Auerbach et al., 2001; Dragatsis et al., 2000; Duyao et al., 1995; Leavitt et al., 2001; Nasir et al., 1995; Van Raamsdonk et al., 2005; White et al., 1997; Zeitlin et al., 1995). Since *dhtt* is not essential for fly embryogenesis and *dhtt* mutants appear to behave normally at young ages with only a mild axon terminal defect in the brain, we have a unique opportunity to examine this in *Drosophila*. To this end, we used a well-established fly HD model for polyQ toxicity (HD-Q93), in which a human Htt exon 1 with 93 glutamine repeats is expressed in all neuronal tissues (genotype: *elav-Gal4/+; UAS-Httexon1-Q93/+*) (Steffan et al., 2001). These HD-Q93 flies develop age-dependent neurodegenerative phenotypes in adults, manifested as initial hyperactivity followed by a gradual loss of coordination and a decline in their locomotor ability, with eventual death around 20 days (Figs. 8F-8H). The HD-Q93 flies also develop a progressive degeneration of both the brain and

other neuronal tissues, most prominently in the photoreceptor cells in the eye (Steffan et al., 2001).

After introducing HD-Q93 into the *dhtt* mutant background (“*HD-Q93; dhtt-ko*”; genotype: *elav-Gal4/+; UAS-Httexon1-Q93/+; dhtt-ko/dhtt-ko*), we examined eye degeneration phenotype by quantifying the number of rhabdomeres in each ommatidia as animals age. The loss of photoreceptor cells in HD-Q93 flies were not significantly enhanced in the absence of endogenous *dhtt* (Fig. 8A-8E). For example, at 11 days of age, ~40% of ommatidia lost 3 photoreceptors in both HD-Q93 and “*HD-Q93; dhtt-ko*” flies (Fig 8E. Number of ommatidia with 4 photoreceptors: HD-Q93= 42.6±2.9%, n=562, adult eyes analyzed: n=8; “*HD-Q93; dhtt-ko*”= 37.6±4.1%, n=266, adult eyes analyzed: n=7. The difference is statistically insignificant:  $p>0.5$ ). Further, the overall profile of remaining photoreceptors per ommatidia was also similar between these flies (Fig. 8E).

Interestingly, although these “*HD-Q93; dhtt-ko*” flies showed normal mobility at the beginning of their adult life, they displayed a declining mobility that could be detected as early as 5 days, and which rapidly deteriorated further over the next few days (Movie S4-S8). During this time, the flies become progressively uncoordinated, showing an increasing frequency of faltering while walking, and a higher frequency of falling during climbing (Figs. 8F and 8G, Movie S4-S8). In a standard climbing assay on day 3, almost all of the “*HD-Q93; dhtt-ko*” flies could successfully climb to the top of a vial, similar to that observed for wildtype controls. However, by day 11, only ~10% of viable “*HD-Q93; dhtt-ko*” could make to the top, while the success rate was 61% for HD-Q93 flies and more than 94% for wildtype and *dhtt-ko* flies (Fig. 8F, average of at least 5 independent assays of 20 flies for each genotype at a given age; for test results on day 11,

the difference of wildtype controls with *dhtt-ko* flies was not significant ( $p=0.30$ ); the difference with HD-Q93 and “HD-Q93; *dhtt-ko*” were significant ( $p<0.0001$ ). Similarly, when spontaneous locomotion was tested, “HD-Q93; *dhtt-ko*” flies showed a more rapid decline of their motility over time, and by day 9 were less than half as active as other controls (Fig 8G, activity was measured as the number of spontaneous turns every 4 minutes; on day 9, “HD-Q93; *dhtt-ko*”=26.4±5.9, n=9, difference with “*uas-Httex1Q93; dhtt-ko*” control is statistically significant,  $p=0.00002$ ; HD-Q93=66.5±5.0, n=8; “*elav-Gal4; dhtt-ko*” control=60.6±4.3, n=9; “*uas-Httex1Q93; dhtt-ko*” control=66.2±3.0, n=9). Furthermore, the lifespan of “HD-Q93; *dhtt-ko*” flies was also shortened significantly, with half of them dying by day 8 and almost all by day 14. In contrast, only about 7% of the HD-Q93 flies died at day 8 and half of them by day 14 (Fig. 8H; viability at day 8: “HD-Q93; *dhtt-ko*”=45.0±3.2%, n=409 (from 4 different crosses); HD-Q93=93.1±1.7%, n=1082 (from 8 different crosses); the difference between HD-Q93 and “HD-Q93; *dhtt-ko*” flies was statistically significant:  $p=0.0003$ ). Notably, *dhtt-ko* mutants alone at day 15 were healthy and displayed similar mobility as that of wildtype controls (Figs. 5A and 5B).

To understand the underlying pathology of these phenotypes, we further examined the structure of the brains of these flies. Compared to HD-Q93 flies of the same age, the brains of “HD-Q93; *dhtt-ko*” flies had already developed a more severe pathology at 5 days. Notably, the MB appeared less organized: the characteristic bulged tip of the vertical  $\alpha$ -axonal lobes, which were prominent in HD-Q93 (white arrowheads in Fig. 9C. n=14) and other controls, were largely unrecognizable in “HD-Q93; *dhtt-ko*” flies (white arrowheads in Fig. 9H. n=18; 1/18. Also see Figs S7 and S8). The clear

separation between the pair of medially projected  $\beta$ -lobe along the midline, which was obvious in HD-Q93 (white arrow in Fig. 9C. Also see Figs S7 and S8. n=14) and wildtype (Fig. 7J), also became less distinct and often appeared merged (white arrow in Fig. 9H. Also see Figs S7 and S8; n=18; 5/18). In addition, the MB did not stain as strongly with anti-FasII as did the MB of HD-Q93 flies (Figs. 9C and 9H, also see S7 and S8). Quantification of MB size and FasII staining signals revealed that there was a  $\sim 7\%$  reduction in the average size of MB in “*HD-Q93; dhtt-ko*” mutants as compared to HD-Q93 controls (Fig. 9K,  $\gamma$ -lobe signals were too weak to be reliably tracked in both HD-Q93 and “*HD-Q93; dhtt-ko*” flies, and only the  $\alpha$ - and  $\beta$ -lobes in each MB were measured; average MB size: HD-Q93=11,137 $\pm$ 314  $\mu\text{m}^2$ , n=6; “*HD-Q93; dhtt-ko*”=10,412 $\pm$ 212  $\mu\text{m}^2$ , n=7; p= 0.04). The average signal intensity of FasII signals in MB of *dhtt-ko* mutants was also decreased by  $\sim 31\%$  (Fig. 9L, relative signal intensity of  $\alpha$ - and  $\beta$ -lobes in MB: HD-Q93=100 $\pm$ 6.7/  $\mu\text{m}^2$ ; *dhtt-ko* mutant=69.3 $\pm$ 4.0/  $\mu\text{m}^2$ ; p=0.0004). Further, “*HD-Q93; dhtt-ko*” brains showed large areas devoid of neuronal cells anteriorly (Figs. 9A and 9F, also see S7 and S8), while areas lacking neuronal cells were present in the posterior brain (Figs. 9D and 9I, also see S7 and S8). Quantification of brain size suggested that although the overall size of the brains were similar between HD-Q93 and “*HD-Q93; dhtt-ko*” flies (Fig. 9M; average brain size: HD-Q93=119,279 $\pm$ 2,015  $\mu\text{m}^2$ , n=7; “*HD-Q93; dhtt-ko*”=115,389 $\pm$ 2,412  $\mu\text{m}^2$ , n=7; p=0.003), the total area that was devoid of neuron cells were increased by  $\sim 25\%$  (Fig. 9N; the total area that were devoid of neurons cells: HD-Q93=26,324 $\pm$ 1,869  $\mu\text{m}^2$ , n=7; “*HD-Q93; dhtt-ko*”=32,945 $\pm$ 2,494  $\mu\text{m}^2$ , n=7; p= 0.03). Together, these results suggest an increased disorganization and

neuronal loss in “*HD-Q93; dhtt-ko*” brains. Thus, loss of endogenous *dhtt* renders animals more vulnerable to the toxicity associated with polyQ-expanded Htt.

## **DISCUSSION**

Htt has been characterized extensively in mammalian cell culture and in mouse systems (Cattaneo et al., 2001; Harjes and Wanker, 2003). However, few functional studies on its homologs in other model organisms have been performed. By deleting the *dhtt* gene, we demonstrate that Htt is not required for normal *Drosophila* development, but instead has an essential role for the long-term mobility and survival of adult animals. Subsequent analyses revealed that loss of *dhtt* mildly affects the integrity of adult brains. Further, in the absence of endogenous *dhtt*, the neurodegenerative phenotypes associated with a *Drosophila* model of polyQ toxicity were significantly enhanced.

### **The role of *Htt* homologs in animal development**

Earlier studies showed that mice lacking *Hdh* die during early embryogenesis (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). It is surprising to find that *dhtt* is dispensable during *Drosophila* development. As no other Htt homolog exists in the fly genome (Li et al., 1999), such a mild phenotype is unlikely caused by a functional redundancy by another Htt-like gene in *Drosophila*. Considering the evolutionary distance between *Drosophila* and mammals, such an observation might indicate that *Drosophila* and mammalian Htts, with their relatively restricted sequence homology, are not functionally conserved. However, this phenotypic discrepancy may also reflect intrinsic differences during mouse and fly embryogenesis. In a chimeric analysis of *Hdh*



mutant mice, Zeitlin's group showed that the early embryonic lethality of *Hdh*-null mice was primarily due to a critical role of Hdh in extraembryonic membranes (Dragatsis et al., 1998). It is likely that as *Drosophila* does not require equivalent tissues such as extraembryonic membranes to support its early development, and its embryogenesis can proceed normally in the absence of *dhtt*. Although an Htt homolog is found in *Drosophila* and vertebrates, no Htt homolog has been found in yeast or *C. elegans* (Li et al., 1999). The absence of an Htt homolog in *C. elegans* suggests that Htt does not have a function essential for the development of invertebrates in general, which is in agreement with our observation that *dhtt* is dispensable for normal *Drosophila* development. Interestingly, a study on phylogenetic comparison of Htt proteins from different species postulates that Htt in the protostome (which includes *Drosophilids*) might be dispensable, since compared to the deuterostome branch, evolution of Htt genes along the protostome branch is more heterogeneous (Tartari et al., 2008). Further studies will be required to determine if the mammalian Htt gene can rescue *Drosophila dhtt* null phenotypes.

Analyses of *dhtt-ko* mutants suggest that loss of *dhtt* does not affect synapse formation, neurotransmission and axonal transport (Figs 3-6), which are also in agreement with the absence of an Htt homolog in the worm, in which the essential components of these cellular processes are conserved. It is possible that Htt still regulate these cellular processes, but with a minor role. In an extrapolation, there is also a possibility that function of Htt is associated with a novel cellular process and/or animal function acquired during evolution. For example, although *dhtt* is dispensable for *Drosophila* development, it is important for maintaining the long-term mobility and viability of adult animals (Figs. 5 and 6). Compared to the worm, *Drosophila* has a

relatively longer lifespan, a more complex nervous system and a more active life cycle, raising an intriguing possibility that the function of Htt might be directly related to these higher functions in adult animals.

### **The role of Htt in adult brain**

Although *dhtt* is dispensable for normal *Drosophila* development, *dhtt* mutants show significantly reduced mobility and viability as they age, indicating an important role of *dhtt* in maintaining the long-term functioning and survival of adult animals (Fig. 5). Analysis of *dhtt* mutants revealed a mild abnormality in mushroom body structure and a reduced complexity of axonal termini in the brain (Fig. 7). Similarly, *Hdh* mutant mice with a reduced level of Htt expression display severe brain abnormalities even though non-neuronal tissue forms normally (White et al., 1997). Targeted inactivation of *Hdh* in the mouse forebrain also causes a progressive neurodegeneration phenotype, suggesting a requirement for Htt in the development and survival of neuronal cells (Dragatsis et al., 2000). It remains to be determined whether common underlying molecular mechanisms are responsible for the observed brain phenotypes in fly and mouse. Nonetheless, considering the evolutionary distance between *Drosophila* and mouse, the existence of structural defects in the adult brain in both species could indicate a conserved role of Htt in maintaining neuronal integrity.

The axonal terminal phenotype in *dhtt* mutants is also reminiscent of that observed in a mouse knockout model for Htt (*Hdh<sup>ex5</sup>*) which contains a deleted exon 5 (Nasir et al., 1995). While mice homozygous for this *Hdh* deletion are early embryonic lethal, *Hdh<sup>ex5</sup>* heterozygotes survive to adulthood and display increased neuronal loss, motor and cognitive deficits, and a significant loss of synapses in specific regions of the

brain (Nasir et al., 1995; O'Kusky et al., 1999). Synapse complexity can be modulated by many factors, among them neuronal activity and membrane and cytoskeleton dynamics. Many questions, such as the significance of this observation, the exact function of Htt in axonal termini complexity, and a possible causal link between this brain defect and the observed adult mobility and viability phenotypes, remain to be answered in the future.

### **The role of endogenous Htt in HD pathogenesis**

Extensive studies on HD and other polyQ disease have demonstrated that an expanded polyQ tract itself can be neurotoxic (Zoghbi and Orr, 2000). Given the distinctive neuronal loss observed in each polyQ disease caused by otherwise unrelated disease genes that are widely expressed, it has been hypothesized that other cellular factors affect disease pathogenesis (Cattaneo et al., 2001; Zoghbi and Orr, 2000). In HD, accumulating evidence from cell culture and mutant mouse studies suggest that wildtype Htt has a neuroprotective function (Cattaneo et al., 2001). When endogenous wildtype Hdh is replaced by YACs containing full-length human Htt with expanded polyQ (YAC46, YAC72 and YAC128) (Leavitt et al., 2001; Van Raamsdonk et al., 2005), the mice develop massive cell death in the testes, which can be suppressed by the wildtype Hdh gene, suggesting that normal function of Htt may mitigate cellular toxicity associated with polyQ-expanded mutant Htt protein (Leavitt et al., 2001; Van Raamsdonk et al., 2005). In addition, a genome-wide study of HD animal models and postmortem tissues has shown that neuronal genes regulated by the transcriptional repressor REST/NRSF, including BDNF, were similarly repressed by the presence of polyQ expanded Htt protein

as by the depletion of endogenous wildtype Htt (Zuccato et al., 2007). These and other studies together support that loss of normal Htt function affects HD pathogenesis.

We were able to examine the effect of removing endogenous *dhtt* on the pathogenesis of an established *Drosophila* HD model for polyQ toxicity (HD-Q93). Our results show that phenotypes of HD-Q93 flies, including mobility, viability and brain pathology, are significantly exacerbated in the absence of endogenous *dhtt*, providing *in vivo* evidence that loss of normal Htt function can accelerate HD pathogenesis. It should be noted that our results do not directly demonstrate that loss of endogenous Htt specifically affects HD pathogenesis, as the enhanced pathology may be due to an additive effect of two detrimental factors in the animals: the presence of a toxic polyQ tract and the disturbance of Htt's normal function. Although *dhtt*-ko mutants alone exhibit only a mild age-dependent adult phenotype, it is possible that in the absence of endogenous *dhtt*, some undetected cellular defects might develop which render the animals more susceptible to other cellular attacks. In the presence of a toxic polyQ tract, this vulnerability may become further exposed, leading to additive phenotypes. Our result is in agreement with the early hypothesis that HD might be caused by the combination of an acquired toxicity conferred by the expanded polyQ in the mutated Htt and an incurred neuronal vulnerability due to the loss of endogenous Htt function (Cattaneo et al., 2001). It is important to note that previous studies have demonstrated that expansion of polyQ tracts within the Htt protein does not abolish its endogenous function, as full-length Htt with expanded polyQ can fully support the development of *Hdh* null mice (Leavitt et al., 2001; Van Raamsdonk et al., 2005; White et al., 1997).

Recently, RNAi-mediated depletion of polyQ-expanded Htt protein has been proposed as a therapeutical approach against HD (Farah, 2007). Our data, together with previous mammalian studies, suggest that the normal function of Htt has a conserved neuroprotective role in the brain and that its depletion could render neuronal cells more vulnerable to the toxicity associated with the polyQ tract (Auerbach et al., 2001; Cattaneo et al., 2001; Dragatsis et al., 2000; Leavitt et al., 2001). Application of such RNAi-based strategy requires the consideration that normal Htt function be preserved. Given the observations that *Hdh*-null neurons can develop and survive in adult mouse brain and that *dhtt-ko* animals are largely normal with only mild adult brain defects, together with the devastating consequence of fully progressed HD, our data indicate that benefit gained from a balanced-administration of RNAi knockdown therapy in adult brain may justify the relatively mild loss caused by depletion of Htt-associated neuroprotective function.

## **METHODS**

### ***Drosophila* stocks and genetics**

Flies were maintained at 25°C and raised on standard *Drosophila* medium unless otherwise specified. To establish transgenic animals, DNA constructs were injected into *w1118* embryos together with p $\pi$ 25.7WC helper plasmid to generate germline transformation, and transformants were selected in the next generation according standard procedures. Flies of the genotype “*w1118/w1118*” were used as controls in all assays unless otherwise specified, as the *dhtt-ko* mutant allele was generated from this *w1118* genetic background during the genetic crosses.

P-element line d08071, piggyBac insertional line f05417 and FLP recombinase transgene line (genotype: *y,w, pCaspaseR-(hs-flipase)*) were from Exelixis (Parks et al., 2004).

To analyze the A307-Gal4 labeled neurons in adult brains, flies with genotypes of “A307-Gal4/+; *dhtt-ko /TM6C, Tb*” and “UAS-*mCD8-eGFP/+; dhtt-ko /TM6C, Tb*” were generated and crossed together, their adult progenies carrying “A307-Gal4/UAS-*mCD8-eGFP; dhtt-ko /dhtt-ko* ” were selected and analyzed. Controls were progenies from the cross of A307-Gal4 line with the UAS-*mCD8-eGFP* line.

HD-Q93 flies were from crosses of pan-neuronal line *elav-Gal4* (C155) with UAS-*Httexon1Q93* (line P468) generously provided by Drs. L. Thompson and J.L. Marsh (Steffan et al., 2001). To generate “HD-Q93; *dhtt-ko* ” flies, flies with genotypes of “*elav-Gal4(c155)/+; dhtt-ko /TM6C, Tb*” and “UAS-*Httexon1Q93/Cyo; dhtt-ko /TM6C, Tb*” were established and then crossed together, adult progenies with the genotype of “*elav-Gal4/+; UAS-Httexon1Q93/+; dhtt-ko /dhtt-ko* ” were selected and analyzed. Controls flies were of genotypes “*elav-Gal4* (C155)/+; ” and “UAS-*Httexon1Q93/+*”, “*elav-Gal4* (C155)/+; *dhtt-ko /dhtt-ko* ” and “UAS-*Httexon1Q93/+; dhtt-ko /dhtt-ko* ”.

### **Genetics and molecular clonings to generate *dhtt-ko* mutant allele**

**Generating *Df(98E2)* deletion:** *Df(98E2)* deficiency, which deleted both CG9990 and *dhtt*, was generated following the Flp-FRT based procedure as described (Parks et al., 2004). Briefly, P-element line d08071 (inserted at the 5' end of neighboring CG9990 gene,) was crossed to virgins carrying a FLP recombinase transgene (genotype: *y,w, pCaspaseR (hs-flipase)*). In the next generation (F1), the male progenies carrying both the

d08071 and the hs-Flipase lines were selected and mated with virgins carrying the f05417, a piggyBac insertional line inserted near the 3' end of *dhtt* gene. The progenies from the above crosses (F1) were heat-shocked 48 hours after egg-laying at 37°C for 1 hour and further heat-shocked for 1 hour in each of the following 4 days. In the third generation (F2), the virgin females were collected and crossed to males carrying balancer chromosomes (w; TM3, Sb/TM6B, Tb, Hu). In the fourth generation, progenies carrying the *Df(98E2)* deletion from the above crosses (F2) were selected based on their darker eye color, and further crossed to balancer to establish individual fly lines. The presence of deletion in these lines was further confirmed by PCR analysis of extracted genomic DNA and DNA sequencing.

**Genomic rescue construct for CG9990 gene:** To generate the genomic rescue construct for CG9990 gene, a 24.7kb genomic DNA fragment covering the CG9990 genomic region was isolated from BAC clone BACR10P23 (CHORI) following double digestion with restriction enzymes XbaI and XmaI. This 24.7kb genomic DNA fragment starts at an XbaI site near the end of neighboring CG9989 gene, 2.66 kb proximal from the inserted site of P-element line d08071, and ends at the XmaI site within the 2<sup>nd</sup> exon of *dhtt* gene, thus covering the whole genomic region of CG9990 gene (Fig. 1B). To generate the CG9990 rescue transgene, this 24.7kb genomic DNA fragment for CG9990 gene was cloned into the NotI and SmaI sites in the pCasper-4 transgenic vector; DNA for pCasper-4-CG9990 genomic rescue construct was injected into *w1118* embryos and transformants were selected following standard procedures. 3 independent CG9990 transgenic lines were established and tested by crossing into the *Df(98E2)* deletion (see description below).

**Generating *dhtt-ko* mutant allele** Flies with the *Df(98E2)* deletion, which removes both CG9990 and *dhtt*, are homozygous lethal at embryonic stage. Through genetic crossings, we reintroduced the CG9990 genomic rescue transgene back into the *Df(98E2)* deletion background, generating fly lines that are defective at the molecular level only for the *dhtt* gene (subsequently referred as *dhtt-ko* (*dhtt knock-out only*)). 3 independent genomic CG9990 transgenic lines were tested by crossing into the *Df(98E2)* deletion; all of them can rescue the embryonic lethality of the *Df(98E2)* deletion, producing viable adults. Thus, *dhtt-ko* flies, which carry both the *Df(98E2)* and the CG9990 transgene, are homozygous viable and can fully develop into adulthood (see text for details), demonstrating that the lethality observed in *Df(98E2)* deletion is caused by the loss of CG9990. To further verify that the *dhtt* gene is indeed deleted as expected in this *dhtt-ko* allele, we extracted genomic DNA from the homozygous *dhtt-ko* and wildtype control adults and performed Southern Blot (see description below).

**Southern analysis:** genomic DNA were extracted from the *dhtt-ko* adults or from control *w<sup>1118</sup>* adults as and digested with restriction enzyme BamHI. DNA fragments were separated by 1% agarose gel and transferred onto nitrocellular membrane according to standard Southern protocol. DNA fragments specifically targeting all exons of *dhtt* gene was labeled by P<sup>32</sup>-dCTP radioactives with Klenow polymerase and random-hexamer primer method (Amersham) and used as probe. Hybridization was performed at 65°C overnight and subsequently washed with SDS/SSC buffers following standard Southern procedures.

***dhtt* Mini-gene rescue construct:** The size of the genomic region covering *dhtt* is about 43kb (Li et al., 1999), which is too large to be cloned into a transgenic vector by



conventional approaches. Accordingly, we engineered a *dhtt* mini-gene rescue construct which express full-length *dhtt* under the control of its own endogenous regulatory region. In *Drosophila*, the regulatory elements controlling a gene's endogenous expression pattern normally are located at the 5' region of the gene, within the 5' upstream untranscribed region and in the first few introns. In addition, a gene's expression level is also affected by its 3' end untranslated region. Accordingly, we isolated a 14.9kb genomic DNA fragment from BAC clone BACR10P23 (CHORI) following double digestion with restriction enzymes XbaI and AscI, with the XbaI site at the end of CG9990 gene and the AscI site within the 10<sup>th</sup> exon of the *dhtt* coding region, thus covering all the 5' untranscribed region of *dhtt* gene and its first 10 introns and exons. We also isolated a 1.2kb genomic DNA fragment by double digestion with StuI/EcoRI that covers the 3' end of *dhtt* gene, including *dhtt*'s last exon and the remaining of its 3' end polyA sites and untranscribed region. We next assembled and isolated a 6.6kb *dhtt* cDNA fragment double digested by AscI/StuI restriction enzymes that covers the most of *dhtt*'s 3' cDNA region, from the single AscI site within the 10<sup>th</sup> exon to the StuI site within its last exon. The *dhtt* mini-gene rescue construct was generated by ligating and fusing in-frame the 3 fragments that cover all of the *dhtt* regulatory and coding regions, including the 5' 14.9kb genomic DNA fragment covering all the 5' untranscribed region of *dhtt* gene as well as its first 10 introns and exons, the middle 6.6 kb *dhtt* cDNA fragment covering from exon 10 to exon 29, and the 3' 1.2kb genomic DNA fragment covering exon 29 and 3' end polyA sites as well as the remaining 3' untranscribed region. The 22.7kb *dhtt*-minigene was cloned into the NotI and XbaI sites in the pCasper-4 transgenic vector. After generating the corresponding transgenic animals for this *dhtt*

mini-gene rescue construct, we crossed these transgenic animals into the *dhtt-ko* mutant to test its ability to rescue the mobility and viability phenotypes of *dhtt-ko* mutants.

**UAS-*dhtt* construct and transgenic animals:** Based on the published *dhtt* cDNA sequence (Li et al., 1999), we isolated overlapping fragments covering the full-length of *dhtt* cDNA by PCR amplification using Pfu polymerase. We then assembled a full-length *dhtt* cDNA construct using these sequencing-verified *dhtt* fragments, cloned it into pUASP vector, and generated transgenic fly lines following standard protocols.

**RT-PCR analysis:** Total RNA samples were isolated using Trizol reagent (Invitrogen) from adult animals of the following genotypes: *w1118* wildtype control, homozygous *dhtt-ko* mutants and “*dhtt-ko* Rescue”. RT-PCR reactions were performed using SuperScript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen) following manufacturer’s instruction. PCR primers are designed as following: *rp49* control: forward (in *rp49* gene’s exon 1)-5’ *accatccgccagcatacagg* 3’; reverse (in *rp49* gene’s exon 2)-5’ *ttggcgcgctcgacaatctcc* 3’. *dhtt-N*: forward (in exon 5 of *dhtt* gene)- 5’ *gccaatgtagccagagtctg* 3’; reverse (in exon 6 of *dhtt* gene)- 5’ *cgcattcgtgatgctgcgtg* 3’; *dhtt-M*: forward (in exon 13 of *dhtt* gene)- 5’ *aagctattcgagccgatggtc* 3’; reverse (in exon 15 of *dhtt* gene)-5’ *gcaccaggaatctcagcatgg* 3’; *dhtt-C*: forward (in exon 23 of *dhtt* gene)- 5’ *tcgggaattgactttcgagc* 3’; reverse (in exon 24 of *dhtt* gene)-5’ *tgagtttgaggcagcgttcc* 3’.

**Analysis of HEAT repeats in Htt proteins** Using the motif-predicting program developed by Dr. M. A. Andrade at the EMBL (<http://www.embl-heidelberg.de/~andrade/papers/rep/search.html>), both human Htt and *Drosophila* dHtt proteins were analyzed for the presence of HEAT repeats. Using the default parameters at

the site and including the AAA, ADB and IMB groups of HEAT repeats (Andrade et al., 2001), a total of 40 HEAT repeats for human Htt and 38 for *Drosophila* dHtt were identified.

**Immunohistochemistry and imaging analysis** Sample preparation and staining for embryos, larvae tissues and adult fly eyes were as described (Sullivan et al., 2000). To analyze NMJ, wandering 3<sup>rd</sup> instar larvae were dissected in Ca<sup>2+</sup>-free saline (128 mM NaCl, 2mM KCl, 0.1mM CaCl<sub>2</sub>, 4mM MgCl<sub>2</sub>, 35.5 mM Sucrose, 5mM Hepes at PH 7.2, 1mM EGTA) and stained as described (Sullivan et al., 2000). 1-2µM thin sections of embedded adult eyes were cut using Microtome and imaged directly without further dye staining.

To stain and image adult brains, adult flies were dissected in 1xPBS or in *Drosophila* M3 medium to remove cuticles and external eye tissues, fixed in 4% formaldehyde in 1xPBS for 1 hour at room temperature, washed for 6 times with 1xPBT for one hour, then stained with primary and secondary antibodies as indicated.

**Whole-mount RNA in situ:** *Drosophila* embryos collection and fixation, larvae tissue dissection and fixation as well as RNA *in situ* hybridization were carried out according to standard procedures (Hauptmann and Gerster, 2000). The Digoxigenin-labeled RNA probes were generated according to the manufacturer's instructions (Roche, Indianapolis, IN). Both exon 8 and exon 20 sequences of *dhtt* gene were used to generate *dhtt* specific *in situ* probes, which gave rise to similar *in situ* results. The samples for *in situ* were analyzed with a Zeiss Axiophot 2 compound microscope.

**Antibodies** To generate anti-dHtt antibody, a cDNA fragment corresponding to the N-terminal 459 amino acids of dHtt protein was cloned into the pGEX-4T1 vector, the corresponding GST-fusion protein was purified according to manufacturer's instruction (Promega) and used to produce polyclonal antibody in a rabbit (Convance). The antiserum was affinity-purified and used at a final dilution of 1:200.

Primary antibodies were applied at 4°C overnight at the following dilutions: rabbit-anti-GFP (1:1,000, Molecular Probe), rabbit anti-Fasciclin II (1: 400) and anti-Dlg (1:40,000) were generously provided by Dr. Mary Packard and Vivian Budnik (Budnik et al., 1996; Koh et al., 1999); rabbit anti-Synaptotagmin (1:500) (Littleton et al., 1993). Mouse anti- $\alpha$ -Tubulin (1:10,000, Sigma). Monoclonal mouse anti-Fasciclin II (1D4, 1:20), anti-Dlg (1:20), anti-CSP (1:20), anti-Synapsin (1:50), anti- $\alpha$ -Spectrin (1:20), anti-Armadillo (1:100), nc-82 (1:50), anti-Futsch (22C10, 1:100) (Hummel et al., 2000; Roos et al., 2000) were from DSHB (Developmental Studies Hybridoma Bank); goat-anti-HRP (1:500, Jackson labs). Alex488-, Alex-594, and Alex-647-conjugated secondary antibodies were used at 1:500 (Molecular Probes). Rhodamine Red-X-conjugated goat-anti-HRP and CY5-conjugated secondary antibodies were used at 1:200 (Jackson labs). DAPI (0.2  $\mu$ g/ml final concentration, Molecular Probes) and Tritc-conjugated Phalloidin (10ng/ml final, Sigma) were applied in PBST for 30 minutes to label nuclei and F-Actin, respectively.

### **Image analysis**

To quantify mushroom bodies (MB) in adult brains, adult flies were dissected in 1xPBS to remove cuticles and external eye tissues, fixed in 4% formaldehyde in 1xPBS for 1

hour at room temperature, then washed for 6 times with 1xPBT for one hour. Dissected adult brains of both the wildtype controls and *dhtt-ko* mutants were stained side-by-side in a 12-well plate with mouse anti-Fasciclin II antibody (1D4, 1:20 in 1xPBT) overnight at 4°C. After washing with 1xPBT 6 times for 2hour, samples were stained with Alex-594-conjugated secondary antibodies (1:500 in 1xPBT) at room temperature for 2 hrs, followed by extensive washing with 1xPBT for 2 hours at room temperature. To ensure that the stained samples had the similar signal background during imaging analysis, brains of both the wildtype controls and *dhtt-ko* mutants were mounted onto the same slide but at the opposite ends. The samples were photographed at 20x magnification under the same optical parameters by a Zeiss fluorescent microscopy (Axioskop 2 Mot Plus). The boundary of MBs were traced manually with Zeiss's AxioVision Rel 4.5 software, and both the overall size of each MB and the total intensity of FasII staining signals within the MB boundary were then computed using the same Zeiss's AxioVision Rel 4.5 software. The FasII staining signals outside the MB boundary was also measured and used as reference to subtract out the background signals. Since we could not confidently measure the thickness of the MB in these samples, only the overall size of the area covered by each MB were measured (in  $\mu\text{m}^2$ ). The average Fas II signal intensity in each MB was calculated by dividing the "total intensity of FasII staining signals within each MB boundary" by "the overall size of the same MB". To calculate the relative signal intensity, the average signal intensity of FasII signals from all wildtype brains was calculated, and its mean was set as the reference point of 100 for wildtype brains.

To measure the overall brain size and the regions that were devoid of neurons in the brain, adult flies were similarly dissected and stained with rat anti-Elav antibody

(1:40) which labels all neuronal cells. The boundary of the whole protocerebrum in the brain and regions devoid of neurons were tracked manually and quantified with Zeiss's AxioVision Rel 4.5 software, as described above.

To quantify the size of A307-positive axonal terminals, adult brains of both the wildtype controls and *dhtt-ko* mutants were similarly dissected, fixed and stained side-by-side with anti-GFP antibody as described above, then mounted on the two sides of the same slide, so as to ensure they had the similar background for imaging analysis. Both the wildtype controls and *dhtt-ko* mutants samples were imaged by confocal microscopy at 40x magnification under the same optical parameters. A Z-serial section of confocal images covering the entire depth of each axonal terminus were collected using the Leica TCS confocal microscopy, and then projected into one merged image using the Leica software to generate the whole picture of each axonal terminus. Since the branches and boutons in the brain were too small to clearly distinguish, only the overall size of the area covered by each axonal terminus was measured (in  $\mu\text{m}^2$ ).

Fluorescent images were analyzed and captured by a Zeiss fluorescent Microscopy (Axioskop 2 Mot Plus) or a Leica confocal microscopy (Leica TCS SP2 AOBS system). Confocal images were analyzed and projected using Leica Confocal software (LCS) in the Leica TCS confocal system.

### **Viability assay**

*Drosophila* viability was measured by placing 30 newly hatched female flies of each genotypes into individual vials containing fly food at 25°C. The number of dead flies were recorded daily. Flies were transferred every 3-4 days into a new food vial to prevent

them from sticking to old food. For each genotype, at least 2 independent cohorts of flies raised at different times from independent crosses were tested, and the results were averaged.

### **Mobility Tests of Adult Flies and Videos**

Female flies were chosen in all the mobility tests. Fly videos were captured using the Sony Digital Handycam camera (DCR-TRV140 NTSC, Digital 8, SteadyShot Digital Effect) and edited in Apple's iMovie program.

***Climbing assay:*** Climbing assays were performed as described (Ganetzky and Flanagan, 1978; Le Bourg and Lints, 1992). Briefly, 20 flies at the specific age in a plastic vial were knocked down to the bottom of the vial. The number of flies that can climb to the top after 18 seconds were counted. The test was repeated 6-7 times for each genotype at the specified age.

***Spontaneous locomotion assay:*** The assay were performed as described (Feany and Bender, 2000; Joiner and Griffith, 1999; Wang et al., 2004). For each genotype, 6-14 flies at the specific ages test were tested.

**Paraquat Test** Flies were fed 30 mM of methyl viologen (Sigma) in instant *Drosophila* medium (Carolina), a dosage of paraquat that would kill about 50% of the wildtype flies after 48 hours of exposure. Control flies were fed instant medium only. Adult flies that have eclosed within the last 24 hours were kept in 30 mM paraquat or in drug-free medium for 48 hours after eclosion. The number of survivors was counted 48 hours after the start of paraquat treatment.

**Electrophysiology Analysis:** Electrophysiological analysis of wandering stage 3<sup>rd</sup> instar larva was performed in *Drosophila* HL3.1 saline (NaCl, 70 mM; KCl, 5; MgCl<sub>2</sub>, 4; CaCl<sub>2</sub>, 0.2; NaHCO<sub>3</sub>, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) using an Axoclamp 2B amplifier (Axon Instrument) at 22°C. Recordings were performed at muscle fiber 6/7 of segments A3 to A5 under current clamp. Paired-pulse facilitation was measured by determining the peak amplitude responses (P2/P1) following stimulation pairs of the indicated latency. All error bars are standard error of the mean (SEM). In adults, extracellular field potentials were recorded by placing a sharp glass electrode near the longitudinal flight muscles after piercing the cuticle, with a reference electrode in the fly head. Electroretinograms were performed as previously described (Rieckhof et al., 2003). Temperature shifts were performed by heating mounting clay encompassing the fly to the desired temperature with a peltier heating device.

For measurements of evoked EJP amplitude, (Figs. 6A and 6B), the number of NMJs examined were: control, n=8; *dhtt-ko*, n=23. Voltage traces of evoked EJPs were recorded from muscle fiber 6 in 3<sup>rd</sup> instar larvae in 0.2 mM extracellular calcium. Average resting potential: 60.2 +/- 1.2 mV in control animals and 62.4 +/- 0.8 mV in *dhtt* mutants. Average EJP amplitude: 19.5 +/- 1.2 mV in control animals and 17.8 +/- 1.0 mV in *dhtt* mutants.

Measurements of voltage traces of paired pulse facilitation (PPF) (Fig. 6C) were performed at 25 msec intervals in control (rescued) and *dhtt* mutant 3<sup>rd</sup> instar larvae in 0.2 mM extracellular calcium. Quantification of paired pulse facilitation (amplitude of EJP 2/amplitude of EJP 1) were performed in control and *dhtt* mutants for 25 msec, 50 msec



and 75 msec intervals (Fig. 6D). The number of preparations analyzed was: control, n=9; *dhtt-ko*, n=8.

The giant fiber flight circuit can be activated by stimulation of the brain and extracellular recordings can be made from the dorsal longitudinal flight muscles (DLMs). Neither control nor *dhtt-ko* mutants displayed abnormal activity in the DLM flight muscles (data not shown).

To measure Electroretinograms (ERGs) at the elevated temperature (37°C), flies were rapidly heated from 20°C to 37°C, with test light pulses (black bar below trace in Fig. 6E) given at regular intervals. A total of 10 *dhtt-ko* mutants aged 1-3 days were tested and all showed normal ERGs at 20°C and 37°C (Fig. 6E). The number of preparations for animals aged 40-45 days analyzed was: control, n= 10; *dhtt-ko*, n=16.

**Supplementary Information** is attached to the paper.

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### **AUTHOR CONTRIBUTIONS**

S.Z., M.B.F., J.T.L., and N.P. conceived the experiments; S.Z. performed and analyzed most of the experiments with the exception of electrophysiological studies reported in Fig. 6, which were executed and interpreted by S.S. and J.T.L.; M.B.F. performed histology analyses and assessment of *dhtt-ko* mutants and “HD-Q93; *dhtt-ko*” adults; S.Z. drafted most parts of the manuscript except for those reported in Fig. 6, which were prepared primarily by S.S. and J.T.L. All authors reviewed and discussed the manuscript.

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## Figure legends

### Figure 1. Ubiquitous expression of *dhtt* in *Drosophila*

(A-F) *dhtt* is widely expressed at low level during *Drosophila* development as revealed by whole-mount *in situ* hybridization.

(A and B) Stage 15 *Drosophila* embryos stained with (A) dig-labeled *dhtt* anti-sense probes revealed the low-level and ubiquitous expression of *dhtt* transcript. (B) Control embryo at the corresponding stage, which were stained with *dhtt* sense probes, showed only minimal background signals. All embryos are lateral views, anterior to the left and dorsal side up.

(C-F) Third instar larval tissues hybridized with *dhtt* antisense probes. Low-level and ubiquitous *dhtt* expression were observed in (C) the brain, (D) wing and leg and (E) eye imagal discs. (F) A negative *in situ* control, an eye imaginal disc from a *dhtt* deletion mutant stained in parallel, showed only minimal background signals.

(G-K) dHtt protein predominantly localizes to cytoplasm. (G and H) In transfected *Drosophila* S2 cell, ectopically expressed dHtt protein (green) was found predominantly in cytoplasm and also on cellular protrusions, but was mostly excluded from the nucleus.

(G) Overlaying images of the S2 cells co-stained with Phalloidin for F-Actin (red) and DNA dye DAPI (blue) to reveal the overall cell morphology and the cell nuclei, respectively.

(I-K) Cytoplasmic localization of dHtt protein (green) ectopically expressed in *Drosophila* 3<sup>rd</sup> instar larval imaginal disc tissues. (I) Anti-dHtt antibody can recognize dHtt overexpressed in the *patched* expression domain driven by *patched*-Gal4, which showed the characteristic striped pattern in the middle of the wing and leg imaginal discs.

Genotype: *patched*-Gal4/+ > UAS-*dhtt*/+.

imaginal disc with ectopically expressed dHtt protein (J, green), which showed a mainly cytoplasmic localization. (K) Overlaying images of the same eye disc region co-stained with DAPI (red) to reveal the cell nuclei. Genotype: GMR-Gal4/+ > UAS-*dhtt*/+.

**Figure 2. Genomic organization of *dhtt* locus and *dhtt-ko* deletion**

(A) Genomic structure of cytological region 98E2. The scale bar on top indicates the gene size (in base pairs). The position and transcriptional direction of *dhtt* and nearby genes (open boxes) is diagramed, with introns (dashed lines) and exons (colored filled boxes) labeled, as well as FRT insertions (triangles) used for generating the *Df(98E2)* deficiency.

(B) *dhtt-ko* was generated by FRT-mediated precise deletion (*Df(98E2)*) of a 55kb genomic region covering most of *CG9990* and *dhtt*. A genomic transgene covering the entirety of *CG9990* (open box) was reintroduced as a transgenic construct in the *Df(98E2)* background.

(C) Detailed genomic structure of the *dhtt* gene. The scale bar, with predicted BamHI fragments, is drawn at the top. Exons for *dhtt* gene were depicted as red arrows and squares, while introns were highlighted as red dashed lines, both were drawn in scale. (D) *dhtt-ko* removes all but the last two of *dhtt*'s 29 exons as verified by Southern blotting using BamHI digestion of genomic DNA. DNA extracted from controls and *dhtt-ko* adult animals was hybridized with a DNA probe targeting all exons of *dhtt* (see Experimental Procedures for details). Two BamHI fragments (1.95 kb and 1.59 kb) containing the two last remaining exons in *dhtt-ko* mutants are highlighted in (C).

**Figure 3. *dhtt* is dispensable for *Drosophila* development**

(A) Normal development of the CNS during embryogenesis in (A3 and A4) *dhtt-ko* compared with (A1 and A2) wildtype controls as revealed by anti-Armadillo staining. (A2 and A4) Enlarged views of the ventral nerve cord in wildtype (A2) and *dhtt-ko* (A4) embryos show its regular ladder-like structure.

(B) Differentiation and patterning of the eye during the 3<sup>rd</sup> instar larval stage in (B3 and B4) *dhtt-ko* is indistinguishable from (B1 and B2) wildtype controls as revealed by anti-Elav staining (red) to label differentiated neurons, and phalloidin staining for F-Actin (green) to reveal overall cytoskeleton organization, respectively.

(C & D) Eye images of 40-day-old *dhtt-ko* mutants. Both the overall (C) external eye morphology and the (D) internal organization of neuronal photoreceptors are normal, even in the 40-day-old *dhtt-ko* adults.

(E-I) Synaptic development is normal in *dhtt* mutants. (E1 and E5) Low and (E2-4, E6-8, and F1-8) high magnification confocal images of glutamatergic NMJs in abdominal segment A3 of 3<sup>rd</sup> instar muscles 6 and 7. (E) NMJs are double labeled with anti-HRP (red) and anti-Dlg (green), which reveal the well-defined pre- and post-synaptic NMJ structures in (E1-E4) wildtype and (E5-E8) *dhtt-ko* mutants. (E2-E4) and (E6-E8) are magnified views of areas highlighted in (E1 and E5). (F) NMJs double labeled with the neuronal membrane marker anti-HRP (white), the periaxial zone marker anti-FasII (green) and the active zone marker nc82 (red), reveal normal periaxial zone and active zone organization in (F1-F4) wildtype and (F5-F8) *dhtt-ko*. (F4 and F8) are overlaid images of F1-3 and F5-7, respectively. WT: *w1118* wildtype control. Scale bar equals 5µm in all panels.

**(G-I)** Quantitative analysis of NMJs at muscle 6 and 7 of segment A3 for wildtype controls (WT, blue) and *dhtt-ko* mutants (red). (G) Average number of type 1b boutons: WT control=34.5±1.6 (n=24); *dhtt-ko* mutants=33.5±2.4 (n=21). The difference is statistically insignificant, p=0.72 (Student's t test). (H) Average number of total boutons: WT control=63.1±1.8 (n=24); *dhtt-ko* mutants=62.3±2.0 (n=21). p=0.76. (I) Total number of branches: WT control=18.0 ±0.6 (n=28); *dhtt-ko* mutants=17.4±0.6 (n=34). p=0.52. The data in G-I are presented as the means±SEM (Standard Error of the Mean).

#### **Figure 4. Normal axonal transport in *dhtt-ko* mutants**

Confocal images of **(A-C)** wildtype control and **(D-F)** *dhtt-ko* NMJs and (white arrows) neighboring axons of the larval peripheral nervous system (anti-HRP, red). Synaptic vesicles (green, anti-Syt) are properly delivered to NMJs and show no obvious accumulation in axons of **(D-F)** *dhtt-ko* mutants similar to **(A-C)** wildtype controls. Signals for Syt were overexposed in (B, C, E, and F) in order to reveal any possible abnormal accumulations of synaptic vesicles within axons (white arrows). **(C and F)** are overlaid images of anti-HRP and anti-Syt double-stainings. WT: *w1118* wildtype control. Scale bar equals 10µm in all panels.

#### **Figure 5. Compromised mobility and viability of aging *dhtt-ko* mutants**

**(A)** Spontaneous locomotion assay. *dhtt-ko* mutants show normal mobility at day 15 but significantly reduced mobility in older animals. **(B)** Age-dependent survival rate of adult animals. *dhtt-ko* mutants have reduced lifespan. Both the mobility and viability defects in *dhtt-ko* mutants were rescued by the presence of a *dhtt* genomic-minigene construct

("dhtt-ko Rescue"). Flies were collected from at least 3 different batches. Total number of flies counted for viability quantification: wildtype n=659; dhtt-ko n=1,573. The difference is statistically significant. Student's t-test: p=0.0001. "dhtt-ko Rescue" n=804. The difference with wildtype control is not statistically significant: p=0.36. "elav-Gal4/+; dhtt-ko" n=550. The difference with wildtype control is statistically significant: p<0.00001. The data in (A and B) are presented as the means±SEM (Standard Error of the Mean). (C) RT-PCR analysis confirmed that expression of endogenous *dhtt* was lost in *dhtt-ko* mutants (lane "3") but was restored by the presence of *dhtt* genomic-minigene rescue construct (lane "4"), similar to that in wildtype controls (lane "2"). RT-PCR was performed on total RNA samples extracted from adult animals of each indicated genotypes. Primers for RT-PCR were located in adjacent exons in control *rp49* gene (the 4 left wells indicated), or in neighboring exons at the N-terminal (targeting exons 5 and 6, *dhtt-N*), middle- (targeting exons 13 and 15, *dhtt-M*) and C-terminal (targeting exons 23 and 24, *dhtt-C*) regions of *dhtt* gene (see Experimental Procedures for details). Lanes "1" are controls of PCR products from wildtype genomic DNA template using these primer pairs, which are longer than RT-PCR product generated by the same pair of primers due to the spliced out introns, thus confirming that RT-PCR products were indeed amplified from transcribed RNA templates. *w1118*: wildtype control.

**Figure 6. Normal synaptic transmission in *dhtt-ko* mutants**

(A-F) Electrophysiological analyses of *dhtt* mutants (see Experimental Procedures for details). (A) Voltage traces of evoked EJPs recorded from muscle fiber 6 in *dhtt* mutant or control 3<sup>rd</sup> instar larvae. (B) Measurements of evoked EJP amplitude. The *dhtt-ko*

mutants exhibited no significant difference in synaptic vesicle release following simulation ( $p=0.18$ , Student t-test). Resting membrane potential was unchanged in animals lacking *dhtt*. **(C)** Voltage traces and **(D)** quantification of paired pulse facilitation (PPF) in control and *dhtt* mutant 3<sup>rd</sup> instar larvae. No change in the amplitude of PPF was observed in *dhtt* mutants, indicating short-term plasticity is intact. **(E)** Electroretinograms (ERGs) recorded from controls and *dhtt-ko* mutants aged 40-45 days, or from *dhtt-ko* mutants aged 1-3 days at (top graphs) 20°C or at (bottom graphs) 37°C heat pulse given at regular intervals. Black bar below trace indicated the test light pulses. **(F)** Percent of adult animals aged 40-45 days with a loss of phototransduction at 37°C. *dhtt-ko* mutants showed a more severe temperature-induced loss of phototransduction than controls, suggesting photoreceptors in aged *dhtt-ko* mutants were stress-sensitive compared to controls. The “*dhtt-ko* Rescue” animals were used as controls in the above electrophysiological analyses to ensure consistent genetic background (A-F). The data in B and D are presented as the means $\pm$ SEM.

**Figure 7. Reduced complexity of axonal termini in *dhtt-ko* brains**

**(A-C)** Brain morphology of a 40-day-old wildtype adult fly as revealed by anti-FasII staining (A, red) that strongly labels the mushroom bodies (MB). A307-positive neurons and their axonal projections in the same brain are revealed by a membrane-bound mCD8-eGFP reporter (B, green). **(C)** Overlaying of images (A) and (B) to show the relative positions of MB and A307-positive neurons in the brain. A307-positive neurons with prominent axonal projections are highlighted (white arrows in C). White-dashed lines delineate the region magnified in the following pictures **(D-O)**.

**(D-I)** Axonal projection patterns and axon terminal structures of A307-positive neurons in 40-day-old (D-F) wildtype and (G-I) *dhtt-ko* mutant brains. For clear visualization, the (D and G) posterior and (E and H) anterior of the brains are projected separately.

**(D and G)** A307-positive neurons have a similar axonal projection pattern (white arrows) in (G) *dhtt-ko* mutant and (D) control brains.

**(E and H)** Anterior views of the same brain regions to show axon terminal structure. White dashed lines highlight one axon terminus in each brain, which is further magnified in **(F and I)**, respectively. Notice the significant reduction of branching and varicosities at the axonal termini of the *dhtt-ko* mutant (H and I).

**(J-O)** The MBs (J and M) and axonal termini of A307-labeled neurons (K and N) in another pair of 40-day-old (J-L) wildtype and (M-O) *dhtt-ko* mutant brains. The overall morphology of MB is normal but the signal intensity is weaker in the (M) *dhtt-ko* mutant. One axon terminus in each brain is highlighted. Note the reduced complexity of axonal termini in the *dhtt-ko* mutant.

Dorsal is up in all images. WT: *w1118* wildtype control. Scale bars equal 10 $\mu$ m in (F and I) and 30 $\mu$ m in all other panels.

**(P and Q)** Quantification of (P) average size of area covered by each MB and (Q) relative signal intensity of MB between wildtype controls (WT, blue) and *dhtt-ko* mutants (red), as revealed by anti-FasII staining. See text for details. Relative signal intensity of MB: value for average wildtype control was set as 100, (SEM=5.8); *dhtt-ko* mutant=47.7 $\pm$ 6.1;  $p < 0.0001$ .



**(R)** Quantification of average total area covered by each A307-positive axonal terminus. See text for details. Genotypes of each fly line tested are indicated under each chart. WT: *w<sup>1118</sup>* wildtype control. The data in (P-R) are presented as the means $\pm$ SEM.

**Figure 8. Loss of endogenous *dhtt* enhances the mobility and viability phenotypes of HD flies**

**(A-D)** Retinal organization as revealed by pseudopupil imaging of 7-day-old adult eyes. Both (A) wildtype and (B) *dhtt*-ko flies had well-patterned seven rhabdomeres in each ommatidium. (C) HD-Q93 alone or (D) with *dhtt*-ko both showed extensive loss of photoreceptors (arrows highlight ommatidia with only 3 or 4 rhabdomeres). (E) Histograms showing the number of remaining photoreceptors per ommatidium in 11-day-old adults. Similar profile of degeneration was observed for HD-Q93 flies alone (red) or “HD-Q93; *dhtt*-ko” (blue). See text for details. **(F-H)** Quantification of (F) climbing ability, (G) spontaneous locomotion and (H) age-dependent survival rate. Genotypes of each fly line tested are described within each chart. HD flies with a background *dhtt*-ko mutation show an (F and G) accelerated loss of mobility and (H) earlier lethality. The data in (E-H) are presented as the means $\pm$ SEM.

**Figure 9. Loss of endogenous *dhtt* affects the pathogenesis of HD flies**

**(A-J)** Loss of endogenous *dhtt* causes an enhanced brain pathology in HD flies. Confocal images of adult brains to show the distribution of neuronal cells (anti-Elav, green), cell nuclei (DAPI, white), and MB (anti-FasII, red) in 5-day-old (D-H) HD-Q93 and (I-M) “HD-Q93; *dhtt*-ko” brains. As cells in the fly brain are mainly localized at its surface, the

(A, B, F and G) anterior and (D, E, I and J) posterior halves of the brains are projected separately for better visualization of the distribution pattern. Note the enlarged regions devoid of neuronal cells in the (F) “*HD-Q93; dhtt-ko*” brain as compared to the corresponding region in the (A) HD-Q93 brain (highlighted in white dashed lines). Arrows in (I) indicate the area lacking neuronal cells at the posterior of the brain. (C and H) The MB in the (H) “*HD-Q93; dhtt-ko*” brain is also less well-organized and shows weaker signal intensity than that in (C) HD-Q93 brain. Arrows highlight the clear separation between the two medial  $\beta$ -lobes along the midline, which is less distinct and appears merged in (H). Arrowheads indicate the bulged tip of vertical  $\alpha$ -lobes, which become less distinct in (H) “*HD-Q93; dhtt-ko*” brains. WT: *w1118* wildtype control. Scale bars equals 50 $\mu$ m in all panels.

**(K and L)** Quantification of (K) average MB size and (L) relative signal intensity of MB between HD-Q93 (blue) and “*HD-Q93; dhtt-ko*” mutants (red), as revealed by anti-FasII staining. In both both HD-Q93 and “*HD-Q93; dhtt-ko*” flies, FasII signals for  $\gamma$ -lobe in MBs were too weak to be reliably tracked (see also Fig. S7 and S8), and thus only the  $\alpha$ - and  $\beta$ -lobes in each MBs were measured. See text for details.

**(M and N)** Quantification of (M) average brain size and (N) total size of the regions devoid of neuronal cells in the anterior brain, as revealed by neuronal-specific anti-Elav antibody stainings. See text for details. The data in (K-N) are presented as the means $\pm$ SEM.