Reduced TDP-43 Expression Improves Neuronal Activities in a Drosophila Model of Perry Syndrome

Yuka Hosaka a, Tsuyoshi Inoshita b, Kahori Shiba-Fukushima b, Changxu Cui c, Taku Arano d, Yuzuru Imai a,b,⁎, Nobutaka Hattori a,b,c,⁎

⁎ Corresponding authors at: Department of Neurology, Juntendo University, Graduate School of Medicine, Tokyo 113-8421, Japan. E-mail addresses: yzimai@juntendo.ac.jp (Y. Imai), nhattori@juntendo.ac.jp (N. Hattori).

1. Introduction

Perry syndrome (PS) is an autosomal dominant disorder characterized by parkinsonism with depression, sleep disturbance, weight loss, and central hypventilation (Perry et al., 1975; Wider and Wszolek, 2008; Farrer et al., 2009). Genome-wide linkage analysis has identified disease-segregating missense mutations located in the dynactin (DCTN1) gene (Farrer et al., 2009). The gene product of dynactin, p150Glu, forms a complex with dynein, the microtubule-dependent retrograde motor. Disease-associated missense mutations (G71R, G71E, G71A, T72P, Q74P) are located in the cytoskeleton-associated protein Gly-rich (CAP-Gly) domain of p150Glue, which has been implicated in binding to microtubules recruiting dynein (Farrer et al., 2009; Abbreviations and symbols are defined in Table 1). Mutations associated with PS show milder synaptic phenotypes but cause impaired retrograde flux (Lloyd et al., 2012; Moughamian and Holzbaur, 2012).

The TAR DNA-binding protein of 43 kDa (TDP-43) is a highly conserved heterogeneous ribonucleoprotein (hnRNP) involved in the transcription, splicing, stabilization and transport of specific mRNAs (Volkening et al., 2009; Fiesel et al., 2010; Polymenidou et al., 2011; Ling et al., 2015). TDP-43 has been identified as the key component of intracellular ubiquitin-positive inclusions observed in affected brain areas of patients with amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD) and Alzheimer’s disease (Arai et al., 2006; Neumann et al., 2006; Josephs et al., 2015; Tan et al., 2015; McAleese et al., 2017). A pathological feature of PS is the accumulation of TDP-43 in affected areas (Farrer et al., 2009; Wider et al., 2009).

Mutations associated with PS show milder synaptic phenotypes but cause impaired retrograde flux (Lloyd et al., 2012; Moughamian and Holzbaur, 2012).

The TAR DNA-binding protein of 43 kDa (TDP-43) is a highly conserved heterogeneous ribonucleoprotein (hnRNP) involved in the transcription, splicing, stabilization and transport of specific mRNAs (Volkening et al., 2009; Fiesel et al., 2010; Polymenidou et al., 2011; Ling et al., 2015). TDP-43 has been identified as the key component of intracellular ubiquitin-positive inclusions observed in affected brain areas of patients with amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD) and Alzheimer’s disease (Arai et al., 2006; Neumann et al., 2006; Josephs et al., 2015; Tan et al., 2015; McAleese et al., 2017). A pathological feature of PS is the accumulation of TDP-43 in affected areas (Farrer et al., 2009; Wider et al., 2009).

Increased TDP-43 is toxic to neurons (Li et al., 2010; Swarup et al., 2011), possibly because of its proneness to aggregation and conversion to an abnormal protein structure similar to that of prion and α-Synuclein (Nonaka et al., 2013). On the other hand, TDP-43 is dispensable to mouse development (Chiang et al., 2010; Sephton et al., 2010) and Drosophila survival (Feiguin et al., 2009; Diaper et al., 2013b; Vanden Broeck et al., 2013), thus suggesting that the control of appropriate protein levels is critical for TDP-43 function.
The molecular relationship between p150Glued and TDP-43 is largely unknown. To determine whether TDP-43 contributes to PS phenotypes, we manipulated TDP-43 protein levels in a Drosophila PS model and found that reduced TDP-43 improved defects in axonal transport and the synaptic activity of central dopaminergic neurons, as well as motor neurons, caused by the neuron-specific expression of a PS-associated p150Glued mutation.

2. Materials and Methods

2.1. Drosophila Strains

Fly culture and crosses were performed on standard fly food containing yeast, cornmeal, and molasses. Flies were raised at 25 °C. All other fly stocks and GAL4 lines used in this study were obtained from the Bloomington Drosophila Stock Center and Kyoto Stock Center and have previously been described: UAS-p150WT-HA and UAS-p150G50R-HA (Lloyd et al., 2012); TBPHΔ23 (Feiguin et al., 2009); UAS-GFP-TBPHWT, UAS-RFP-TBPHWT and UAS-RFP-TBPHA315T (Estes et al., 2011); and UAS-VMAT-phluorin (Wu et al., 2013). Stocks were backcrossed to the w1118 wild-type background for six generations, and w1118 was used as a wild-type allele.

2.2. Survival Assay and Climbing Assay

For lifespan studies, approximately 20 adult flies per vial were maintained at 25 °C, transferred to fresh fly food, and scored for survival every 4 days. To control for isogeny, fly lines were backcrossed to the w1118 background for six generations. A climbing assay was performed as previously described (Shiba-Fukushima et al., 2014). For spontaneous locomotor behavior, male flies were preconditioned at 25 °C under a 12-h light: dark cycle for 3 days and were then recorded for another 3 days using a Drosophila activity monitoring (DAM) system (TriKinetics), which monitors the activity of individual flies (16 to 32 flies per experiment) in polycarbonate tubes (length, 65 mm; inside diameter, 3 mm).

2.3. Whole-mount Immunostaining and Transmission Electron Microscopy (TEM) Analysis

The antibodies used in immunocytochemistry were anti-Bruchpilot (1:10, Developmental Studies Hybridoma Bank (DSHB), nc82, RRID:AB_2314867), anti-GluRIIA (1:10, DSHB, B84D2, RRID:AB_528269), anti-Dlg (1:250, DSHB, 4F3, RRID:AB_2528023), anti-GFP (1:500, Thermo Fisher Scientific, A6455, RRID:AB_221570), anti-RFP (1:100, Abcam, ab62341, RRID:AB_945213), and Alexa Fluor594- (1:200) or DyLight649- (1:500) conjugated anti-horseradish peroxidase (HRP) (Jackson ImmunoResearch, 123-585-021, RRID: AB_2338966 and 123-495-021, discontinued). The visualizations of synapse boutons, mitoGFP and active zones (AZs) in larval motor neurons were analyzed by whole-mount immunostaining as previously described (Lee et al., 2010). For image processing of synapse boutons, thirty Z-stack images were taken at 0.15- to 0.30-μm intervals and were reconstituted using ImageJ. TEM images were obtained using an electron microscope (Hitachi, HT7700) at the Laboratory of Ultrastructural Research of Juntendo University.
2.4. Immunoprecipitation and Western Blot Analysis

To analyze the binding between TBPH and p150\textsuperscript{Glued}, eight 3rd-instar larvae (\(\mu\text{g}\)118) from which the digestive organs had been removed were lysed with 200 \(\mu\text{g}\) lysis buffer (Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 5% glycerol, 1 mM EDTA, 1 mM DTI) containing Complete protease inhibitor cocktail (Sigma–Aldrich) with a motor-driven pestle. Supernatant obtained after centrifugation at 16,000 \(\times\) g for 10 min was subjected to immunoprecipitation with anti-p150\textsuperscript{Glued} conjugated with TrueBlot Magnetic Beads (Rockland Immunochemicals Inc., RRID:AB_2610703) and was incubated at 4 °C overnight. The beads washed three times with the lysis buffer were analyzed by subsequent western blotting. For western blotting to determine protein levels, larvae from which the digestive organs had been removed were homogenized in 10 \(\mu\text{l}\) of RIPA buffer (20 mM Tris-HCl, pH 7.2, 70 mM NaCl, 5 mM KCl, 20 mM MgCl\(_2\), 10 mM \(\text{NaHCO}_3\), 115 mM NaCl, 1% Triton and 5.3% (vol/vol) methanol. If a significance result was determined using ANOVA (p < 0.05), the mean values of the control and the specific test group were analyzed using a Tukey-Kramer test. Steel’s test and Dunnnett’s test were used to determine significant differences between two specific groups or among multiple groups of interest. The data distribution was assumed to be normal, although this assumption was not formally tested. Randomization was used in each genotype, and data collection and analysis were not performed blind to the conditions of the experiments. All the data obtained in the experiments were included except for the VMAT-pHluorin assay, for which the samples that showed increased fluorescence signals immediately after photobleaching were excluded.

2.5. Dopamine Measurement

Five adult male fly heads were dissected and homogenized in 50 \(\mu\text{l}\) of 0.1 M perchloric acid by using a motor-driven pestle around 1 p.m. Dopamine levels in Drosophila brain extracts were determined by HPLC coupled to electrochemical detection using a mobile phase containing 50 mM citric acid, 50 mM sodium dihydrogen phosphate, pH 2.5, 0.1 mM EDTA, 4.4 mM 1-heptanesulfonic acid, 2.2% (vol/vol) acetonitrile, and 5.3% (vol/vol) methanol.

2.6. Live Imaging of Axonal Transport

Living 3rd-instar larvae were dissected in HL-3 saline (5 mM HEPES, pH 7.2, 70 mM NaCl, 5 mM KCl, 20 mM MgCl\(_2\), 10 mM \(\text{NaHCO}_3\), 115 mM NaCl, 0.375 mM (for mEJP) or 2 mM (for EJP and PPR) Ca\(_2\)+, and a recording electrode filled with 3 M KCl was inserted into muscle 6 of the A3 or A4 segment containing NMJs. All data were analyzed using Mini-Analysis software (Synaptosoft). PPR was calculated as a ratio of EJP amplitudes with paired stimulation (50 msec interval). QC was calculated as the average EJP amplitude divided by the average mEJP amplitude as previously described (Lee et al., 2010).

2.7. Electrophysiology

Third-instar larvae were dissected in HL-3, and mEPJs from NMJs were recorded using an electrophysiological setup equipped with an Eclipse FN1 microscope (Nikon), a Multiclamp 700B amplifier (Molecular Devices) and a Digidata 1500A data acquisition system (Molecular Devices). Dissected larvae were incubated in HL-3 containing 10 \(\mu\text{g}\)6753, anti-\(\alpha\)-Tubulin (1:50, DSHB, E7, RRID:AB_390919), anti-TBPH (1:1000) and anti-p150Glued (N and C) antibodies were kind gifts from Drs. F. Hirth and V.I. Gelfand, respectively (Siller et al., 2005; Diaper et al., 2013a).

2.8. VMAT-pHluorin Live Imaging

VMAT-pHluorin live imaging was previously described (Shiba-Fukushima et al., 2014). Briefly, the DA release rates of neuronal fibers and terminals in the whole brain region without the subesophageal ganglion were calculated by normalizing the data with the fluorescence intensity just after photobleaching using ImageJ software.

2.9. Statistical Analysis

Error bars in graphs represent the mean ± SEM. A two-tailed Student’s t-test or a one-way repeated-measures analysis of variance (ANOVA) was used to determine significant differences between two or among multiple groups, respectively, unless otherwise indicated. If a significant result was determined using ANOVA (p < 0.05), the mean values of the control and the specific test group were analyzed using a Tukey-Kramer test. Steel’s test and Dunnnett’s test were used to determine significant differences between two specific groups or among multiple groups of interest. The data distribution was assumed to be normal, although this assumption was not formally tested. Randomization was used in each genotype, and data collection and analysis were not performed blind to the conditions of the experiments. All the data obtained in the experiments were included except for the VMAT-pHluorin assay, for which the samples that showed increased fluorescence signals immediately after photobleaching were excluded.

3. Results

3.1. Removal of One Copy of TBPH Improves the Decreased Motor Ability and Lifespan Caused by PS Mutation

The amino acid residues of p150\textsuperscript{Glued} affected in PS are highly conserved between humans and Drosophila (Farrer et al., 2009; Lloyd et al., 2012). To understand how PS-associated mutations of p150\textsuperscript{Glued} affect neurons, we expressed wild-type p150\textsuperscript{Glued} (p150\textsuperscript{WT}) and a mutant, p150\textsuperscript{Glued}, in which Gly50 is replaced with Arg (p150G50R; corresponding to p150G71R in humans) in Drosophila dopaminergic neurons. Forty-five-day-old normal adult flies exhibited an age-dependent decline in compulsive motor activity compared with 5-day-old flies; this decline was exacerbated in flies expressing p150\textsuperscript{Glued} (Fig. 1A). The introduction of a TAR DNA-binding protein of 43 homolog (TBPH) null allele, TBPH\textsuperscript{fl}, caused an approximately 30% reduction in TBPH protein levels (Fig. S1A, B) (Feiguin et al., 2009; Diaper et al., 2013b) and improved motor disturbances caused by p150\textsuperscript{WT} or p150\textsuperscript{G50R} (Fig. 1A, lower). Whereas the lifespan of TBPH\textsuperscript{fl} flies was reduced compared to controls, the combination of TBPH\textsuperscript{fl} with p150\textsuperscript{WT} or p150\textsuperscript{G50R} improved...
the lifespan of p150WT or p150G50R flies (Fig. 1B). We did not observe decreased spontaneous locomotor activity, loss of TH-positive neurons or decreased total amount of brain dopamine in flies expressing p150WT or p150G50R even in 45-day-old flies (Fig. S1C–E). However, the removal of a copy of TBPH stimulated spontaneous locomotor activity (Fig. S1C).
3.2. Axonal Terminals are Affected by PS Mutation

Previous studies have suggested that p150G50R is required for synapse growth and stabilization (Eaton et al., 2002; Chang et al., 2013). These events appear to be regulated by p150G50R-mediated stabilization of microtubule plus ends and to be abrogated in the Perry mutant G74P (Lazarus et al., 2013). The expression of p150G50R in larval motor neurons caused both distal and proximal synaptic boutons to swell with prominent synaptic debris (Fig. 2A, B) (Fuentes-Medel et al., 2009), but the number of synaptic boutons was not significantly changed (Fig. 2C), thus suggesting that the G50R mutation has moderate effects on p150G50R function. The swollen synaptic phenotype of proximal but not distal boutons by p150G50R was suppressed in the TBPH−/− background, whereas the synaptic phenotypes of TBPH−/− were similar to those of controls (Fig. 2A–C). In this context, TBPH−/− did not suppress the synaptic debris observed in p150G50R synapses (Fig. 2A). The number of AZs in p150G50R synapses increased, possibly as a consequence of an alteration to the signaling pathway for AZ development, accompanied by endocytic defects (Dickman et al., 2006). This phenotype was suppressed by the introduction of TBPH−/− (Fig. 2D, E). Alteration of presynaptic structures may affect the maturation and maintenance of the postsynaptic architecture (Romano et al., 2014). We therefore examined the distribution and expression of a postsynaptic scaffold protein, Dlg, and a subunit of the postsynaptic glutamate receptor, GluRIIA, in the neuromuscular junctions (NMJs) of larval motor neurons (Fig. S1A and S2A). Whereas Dlg expression appeared to be unchanged, GluRIIA tended to be reduced by p150G50R expression or removal of a copy of TBPH, thus suggesting that postsynaptic functions are partially affected.

Because mitochondrial dysfunction is thought to be part of the etiology of Parkinson’s disease, we also analyzed mitochondrial distribution in NMJs (Fig. S2B) (Imai and Lu, 2011). Unexpectedly, smaller mitochondria accumulated in the most distal boutons in TBPH−/− larvae. The mitochondrial number of the terminal 6 boutons, especially the proximal 5 boutons, of the NMJs in p150G50R and p150G50R TBPH−/− larvae tended to be lower, whereas the mitochondrial density in the most distant boutons was not significantly changed among genotypes. These observations suggest that TBPH reduction did not reverse the altered mitochondrial distribution caused by p150G50R.

A decrease in dopamine release is an early event of dopaminergic neurodegeneration in PS as well as Parkinson’s disease (Wider and Wszolek, 2008), and monoamine neurotransmitters are thought to be stored in both dense core vesicles (DCVs) and synaptic vesicles in Drosophila (Grygoruk et al., 2014). A uniform supply of DCVs in each bouton is achieved by a circulation of axonal flow and a local capture mechanism. To visualize the distribution of DCVs in the NMJs, emerald GFP-tagged atrial natriuretic factor (ANF-EMD) was expressed in motor neurons (Fig. 2F and Fig. S2C) (Rao et al., 2001). Most DCVs were approximately 0.5 μm2 in dimension in control animals, whereas the expression of p150G50R resulted in the accumulation of aggregated DCVs over 0.5 μm2 in the most distal boutons (Left graph in Fig. 2G and Fig. S2C). In contrast, the density of DCVs was increased in proximal boutons of TBPH−/− larvae (Right graph in Fig. 2G). Importantly, the accumulation of DCVs in the most distal boutons by p150G50R was suppressed, and the uniform distribution of DCVs was observed at a level similar to controls via the TBPH reduction (Fig. 2F, G and Fig. S2C). Although the expression of p150WT mildly promoted DCV aggregation in the most distal boutons, the phenotype was not changed by decreased TBPH, thus suggesting that the beneficial effect on the bouton DCV dynamics by decreased TBPH is p150G50R specific (Fig. 2F, G and Fig. S2C).

3.3. TBPH Contributes to Defects in Axonal Transport of DCVs

We next monitored the movement of DCVs in the axonal transport of larval motor neurons (Fig. 3A and Movies S1, S2, S3, S4). Larger DCVs tended to appear in TBPH−/− animals (Fig. 3A, B and Fig. S3A). The expression of p150WT caused moderate DCV aggregates, which were suppressed by the removal of a copy of TBPH (Fig. 3A, B and Fig. S3A). The p150G50R expression resulted in the marked appearance of large DCV aggregates, which were also alleviated by the decreased TBPH (Fig. 3A, B and Fig. S3A). In sharp contrast, the morphology of axonal mitochondria was mostly unchanged by the expression of p150G50R, and mitochondria were not colocalized with TBPH, even with an ALS-associated mutant (Fig. S3B, C) (Wang et al., 2016). Whereas p150WT affected the retrograde transport of DCVs, which caused increased stationary DCVs, p150G50R markedly impaired DCV movement in both anterograde and retrograde directions and further increased stationary DCVs (Fig. 3C–E and Fig. S3D, E). In addition, the number of retrograde DCVs that reversed to an anterograde flow was significantly increased after expression of p150G50R but not p150WT; thus suggesting that retrograde transport is especially compromised by p150G50R (Fig. 3C, F and Fig. S3F). The introduction of TBPH−/− partially rescued these defects via p150WT or p150G50R (Fig. 3C–F and Fig. S3D, E), whereas the reduction in the velocity of DCVs by p150G50R was not improved (Fig. 3G). Consistently with defects in the axonal transport of DCVs, the decreased dopamine release estimated by VMAT-pHluorin in the adult brain of p150G50R flies was also alleviated by the introduction of TBPH−/− (Fig. 3H and Fig. S3G). However, we did not detect a physical interaction of TBPH with p150G50R (Fig. S3H) or decreased endogenous p150G50R and dynemin after introducing TBPH−/− (Fig. S1A, B). These results suggest that the rescue effects of p150G50R phenotypes associated with decreased TBPH levels are an indirect mechanism.

3.4. Dysfunction of Synaptic Release by the PS Mutation Is Rescued by TBPH Reduction

Given that synaptic activity is impaired by the blockage of DCV transport in p150G50R-expressing neurons, we next analyzed the electrophysiological properties of the NMJ in larval motor neurons. The excitatory junction potential (EJP) and quantal content (QC) were reduced
in p150G50R but not p150WT animals, thus suggesting impaired synaptic release in PS (Fig. 4A). Consistently with results from morphological analyses of synaptic boutons, TBPH reduction improved the EJP and QC of p150G50R animals. However, the miniature excitatory junction potential (mEJP) frequency was increased in p150WT animals, thus suggesting that p150WT overexpression partially affects synaptic activity (Fig. 4A, B).
and Table S1). The p150\textsuperscript{WT} and TBPH\textsuperscript{+/−} animals tended to exhibit larger spikes in mEPs, and the large spikes of TBPH\textsuperscript{+/−} animals were suppressed by p150\textsuperscript{G50R} expression (Fig. 4B, C). The paired-pulse ratio (PPR) was not different among the five genotypes (Table S1).

Consistently with the increased mEP frequency, the number of synaptic vesicles (SVs) was increased in both the AZ regions and the presynaptic cytosol of TBPH\textsuperscript{+/−} NMJs, thus suggesting that TBPH at synapses is important for the regulation of SVs (Fig. 4D, E and Fig. S4). The enlarged boutons of p150\textsuperscript{G50R}-expressing neurons contained fewer SVs docked at AZs, but many DCVs and uncharacterized large vesicular structures with high or low electron density, which probably include aggregated DCVs and lysosomes (Fig. 4D and Fig. S4B) (Lloyd et al., 2012). Again, the appearance of abnormal vesicular structures was suppressed by the decreased TBPH in p150\textsuperscript{G50R}-expressing neurons (Fig. 4D).

4. Discussion

Whereas mutations of p150\textsuperscript{Glued} cause PS, in which TDP-43 pathology has been reported in the basal ganglia, including the substantia nigra, whether TDP-43 contributes to neurodegeneration in PS remained unknown. Here, we analyzed the molecular relationship between p150\textsuperscript{Glued} and TDP-43 in a Drosophila PS model.

Neuronal expression of the p150\textsuperscript{G50R} mutation, located in the CAP-Gly domain, led to swelling of distal boutons and the prominent aggregation of DCVs but not mitochondria in axons and synapses. DCVs travel between proximal axons and terminal boutons via axonal transport, which efficiently delivers neurotransmitters to synaptic boutons (Wong et al., 2012). Although p150\textsuperscript{G50R} disrupts both the anterograde and retrograde flow of DCVs, the anterograde/retrograde ratios of moving DCVs suggested that the retrograde flow is especially affected by p150\textsuperscript{G50R} (Fig. 3D), thus leading to accumulation of organelles and materials at nerve terminals (Fig. 4D). Consistently with the morphological abnormalities in synaptic phenotypes, synaptic strength, dopamine release and motor ability were decreased by p150\textsuperscript{G50R} expression without detectable neuron loss. Although p150\textsuperscript{WT} expression also affected the retrograde flow of DCVs and the DCV distribution at the terminal boutons, the phenotypes were milder than those of p150\textsuperscript{G50R}. Given that p150\textsuperscript{WT} protein was expressed at higher levels than p150\textsuperscript{G50R} in our transgenic flies, whereas p150\textsuperscript{WT} and p150\textsuperscript{G50R} transgenes were inserted in the same genomic locus and both transcript levels were similar, p150\textsuperscript{G50R} exerts highly deleterious effects on neuronal activity despite its unstable expression (Lloyd et al., 2012). Thus, our data obtained with p150\textsuperscript{G50R} expression would reflect synaptic dysfunction as an early neurodegeneration event in PS.

Altered mitochondrial distribution at the boutons by p150\textsuperscript{G50R} was not significantly rescued by TBPH reduction, thus suggesting that the improvement of DCV phenotypes markedly contributes to the rescue effects in neuronal functions and survival. However, we cannot exclude the possibility that mitochondrial functions may be affected in p150\textsuperscript{G50R} flies, because the fluorescence intensity of mitoGFP (cytochrome C oxidase subunit VIII-GFP fusion protein), which inserts in the mitochondrial inner membrane in a membrane potential-dependent manner, was somewhat reduced by p150\textsuperscript{G50R}. Ultrastructural analysis of synaptic mitochondria also suggested that mitochondrial cristae were partly damaged in p150\textsuperscript{G50R} flies (Fig. 4D). Because mitochondrial shuttling between cell bodies and nerve terminals in neurons would be important to maintain mitochondrial proteins derived from the nuclear genome, which include the respiratory complex I, III, IV and V subunits, the effects of PS mutations on mitochondrial functions at nerve terminals should be examined in future studies (Abe et al., 1995).

TDP-43 accumulation in affected regions is a prominent feature of PS pathology. TDP-43 forms cytoplasmic messenger ribonucleoprotein (mRNP) granules, which also move via axonal transport to synaptic boutons to deliver mRNA for synaptic activities (Alami et al., 2014). Our discovery in Drosophila that the ablation of a copy of the TBPH gene improves the axonal aggregation of DCVs and synaptic defects provides three possible molecular mechanisms: First, a reduced concentration of TBPH in axons and nerve terminals improves axonal flow, suppressing the aggregation of TBPH. Second, TBPH reduction alters the expression of proteins regulated by TBPH at transcript levels, thus alleviating synaptic dysfunctions. Third, the above two mechanisms contribute to rescue effects. We prefer the third mechanism for the reasons listed below.

The DCV aggregates in axons and synapses were suppressed by TBPH reduction without improving the velocity of axonal transport, thus suggesting that aggregation-prone TBPH promotes DCV aggregation when the axonal flow stagnates. However, the numbers of DCVs and SVs were increased in the synaptic boutons of TBPH\textsuperscript{+/−} flies, thus suggesting that TBPH negatively regulates DCV and SV production. A variety of TBPH target mRNAs have been reported in Drosophila (Hazelett et al., 2012; Vanden Broeck et al., 2013; Coyne et al., 2014), and further studies may reveal a target(s) to regulate DCVs and SVs. Regarding synaptic stabilization, microtubule-associated MAP1B/Futsch is an evolutionarily conserved target of TDP-43/TBPH, which negatively or positively regulates synaptic MAP1B/Futsch expression (Sephton et al., 2010; Godena et al., 2011; Coyne et al., 2014) and might maintain axonal transport through regulating microtubule dynamics. Although we did not detect obvious changes in the levels of Futsch protein in TBPH\textsuperscript{+/−} flies (data not shown), the downregulation of Futsch/MAP1B may explain the observation that some phenotypes in TBPH\textsuperscript{+/−} flies were reversed by p150\textsuperscript{G50R} expression. Because p150\textsuperscript{Glued} stabilizes the plus ends of microtubules, the ectopic expression of p150\textsuperscript{G50R} may partially alleviate the microtubule destabilization caused by Futsch/MAP1B downregulation (Lazarus et al., 2013). Alternatively, the decreased axonal flow resulting from p150\textsuperscript{G50R} expression may enable efficient synaptic capture of TDP-43-mRNP granules, which regulate local protein translation for synaptic activity including Futsch/MAP1B (Diaper et al., 2013b; Alami et al., 2014).

Autoregulation of TDP-43 mRNA has been demonstrated in mammals (Ayala et al., 2011; Polymenidou et al., 2011) and has been suggested in Drosophila (Hazelett et al., 2012; Vanden Broeck et al., 2013). Consistently with these reports, the decrease in TBPH protein was at most 30% in TBPH\textsuperscript{+/−} flies. Although the genetic ablation of a copy of the TBPH gene in itself produced some synaptic phenotypes and decreased the lifespan in flies, the transient knockdown of TDP-43 would be a suitable strategy for therapeutic intervention in PS. It has been demonstrated that the transient inhibition of a truncated N-terminal huntingtin with an abnormal polyQ stretch improves the neuropathology and the motor phenotype in a Huntington's disease mouse model (Yamamoto et al., 2000). Thus, disease phenotypes caused by

Fig. 4. Reduced TBPH rescues synaptic strength at the NMJs of p150\textsuperscript{G50R} flies. (A) Representative electrophysiological traces of EJP and mEJP in the larval NMJs. Scale bar for EJP (mEJP): y = 10 mV (3 mV), x = 50 msec (1 sec). EJP amplitudes (EJP) and quantal content (QC) are reduced in p150\textsuperscript{G50R} flies. *p < 0.05 vs. Control by Steel's test (Dunnnett's test). Black dots indicate large spikes (>1.5 mV in EJP). See also Table S1 for further electrophysiological information. (B, C) Cumulative spike numbers (B) and number of large spikes (C) during 60 s. n = 4–10 NMJs in 4–6 flies per genotype. The number of large spikes in (C) was not significantly altered among the genotypes, on the basis of Dunnett's test. The sample size is indicated in the graph (Fig. S4A). (D) Quantification of the SV number in the unit area containing an AZ (defined in Fig. S4A), n = 8–23 AZ neighboring regions in 3 flies. *p < 0.05 vs. Control by Dunnett's test. The sample size is indicated in the graph. See also Table S1 and Fig. S4. Genotypes used: OK6-Gal4, UAS-mitoGFP + (Control); OK6-Gal4, UAS-mitoGFP/UAS-p150\textsuperscript{G50R}-HA (p150\textsuperscript{G50R}), OK6-Gal4, UAS-mitoGFP, TBPH\textsuperscript{+/−} + (TBPH\textsuperscript{+/−}); OK6-Gal4, UAS-mitoGFP, TBPH\textsuperscript{+/−}/UAS-p150\textsuperscript{G50R}-HA (p150\textsuperscript{G50R}, TBPH\textsuperscript{+/−}).
TDP-43 proteinopathies may be reversible under appropriate levels of TDP-43 control.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.06.002.

Funding Sources

This study was funded by Grants-in-Aid for Scientific Research (26293070 [Y.I.], 15H04842 [N.H.]) from MEXT in Japan, a Grant-in-
Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Y.I. conceived and designed the study, Y.H. and T.I. performed most of the experiments and analyzed the data with support from K.S., C.C., and T.A.Y.H., T.I., and N.H. wrote the manuscript with input from the other authors.

Acknowledgments

We thank Drs. F. Feiguin, F.E. Baralle, T.E. Lloyd, A. Kolodkin, D.C. Zarnescu, V.I. Gelfand, C.Q. Doe, F. Hirth, T. Chihara and M. Miura for providing materials, and we thank T. Imura, T. Kanao, Y. Umezaki, M. Yoshida and S. Kakuta for their technical assistance.

References

Abe, K., Aoki, M., Kawagoe, J., Yoshida, T., Hartori, A., Kogure, K., Itoyama, Y., 1995. Ischemic delayed neuronal death. A mitochondrial hypothesis. Stroke 26, 1478–1489.

Alami, N.H., Smith, R.B., Carrasco, M.A., Williams, L.A., Winborn, C., S., Calne, D.B., Lechevalier, B., Chapon, F., Tsuboi, Y., Yamada, T., Gutmann, L., Elibol, N., 2015. TAR DNA-binding protein 43 and pathological subtype of Alzheimer’s disease impact clinical features. Ann. Neurol. 78, 697–709.

Aid for Scientiﬁc Research on Innovative Areas (23111003 [N.H.], a Health Labor Sciences Research Grant 201324123A) (N.H. and Y.I.), and a grant from Otsuka Pharmaceutical (N.H. and Y.I.).
Tan, R.H., Kril, J.J., Fatima, M., McGeachie, A., McCann, H., Shepherd, C., Forrest, S.L., Affleck, A., Kwok, J.B., Hodges, J.R., Kiernan, M.C., Halliday, G.M., 2015. TDP-43 proteinopathies: pathological identification of brain regions differentiating clinical phenotypes. Brain 138, 3110–3122.

Vanden Broeck, L., Naval-Sanchez, M., Adachi, Y., Diaper, D., Dourlen, P., Chapuis, J., Kleinberger, G., Gistelinck, M., Van Broeckhoven, C., Lambert, J.C., Hirth, F., Aerts, S., Callaerts, P., Dermaut, B., 2013. TDP-43 loss-of-function causes neuronal loss due to defective steroid receptor-mediated gene program switching in Drosophila. Cell Rep. 3, 160–172.

Volkening, K., Leystra-Lantz, C., Yang, W., Jaffee, H., Strong, M.J., 2009. TAR DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). Brain Res. 1305, 168–182.

Wang, W., Wang, L., Lu, J., Siedlik, S.L., Fujioka, H., Liang, J., Jiang, S., Ma, X., Jiang, Z., da Rocha, E.L., Sheng, M., Choi, H., Lerou, P.H., Li, H., Wang, X., 2016. The inhibition of TDP-43 mitochondrial localization blocks its neuronal toxicity. Nat. Med. 22, 869–878.

Wider, C., Wszolek, Z.K., 2008. Rapidly progressive familial parkinsonism with central hypoventilation, depression and weight loss (Perry syndrome)—a literature review. Parkinsonism Relat. Disord. 14, 1–7.

Wider, C., Dickson, D.W., Stoeissl, A.J., Tsuboi, Y., Chapon, F., Gutmann, L., Lechevalier, B., Calne, D.B., Personett, D.A., Hulihan, M., Kachergus, J., Rademakers, R., Baker, M.C., Grantier, L.L., Sujith, D.K., Brown, L., Calne, S., Farrer, M.J., Wszolek, Z.K., 2009. Pallidonigral TDP-43 pathology in Perry syndrome. Parkinsonism Relat. Disord. 15, 281–286.

Wong, M.Y., Zhou, C., Shakhireanova, D., Lloyd, T.E., Deitcher, D.L., Levitan, E.S., 2012. Neuropeptide delivery to synapses by long-range vesicle circulation and sporadic capture. Cell 148, 1029–1038.

Wu, T.H., Lu, Y.N., Chuang, C.L., Wu, C.L., Chiang, A.S., Krantz, D.E., Chang, H.Y., 2013. Loss of vesicular dopamine release precedes tauopathy in degenerative dopaminergic neurons in a Drosophila model expressing human tau. Acta Neuropathol. 125, 711–725.

Yamamoto, A., Lucas, J.J., Hen, R., 2000. Reversal of neuropathology and motor dysfunction in a conditional model of Huntington’s disease. Cell 101, 57–66.