Loss and gain of *Drosophila* TDP-43 impair synaptic efficacy and motor control leading to age-related neurodegeneration by loss-of-function phenotypes

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ABSTRACT

Cytoplasmic accumulation and nuclear clearance of TDP-43 characterize familial and sporadic forms of amyotrophic lateral sclerosis and frontotemporal lobar degeneration, suggesting that either loss or gain of TDP-43 function, or both, cause disease formation. Here we have systematically compared loss- and gain of function of Drosophila TDP-43, TAR DNA Binding Protein Homolog (TBPH), in synaptic function and morphology, motor control, and age-related neuronal survival. Both loss and gain of TBPH severely affect development and result in premature lethality. TBPH dysfunction caused impaired synaptic transmission at the larval neuromuscular junction (NMJ) and in the adult. Tissue-specific knockdown together with electrophysiological recordings at the larval NMJ also revealed that alterations of TBPH function predominantly affect presynaptic efficacy, suggesting that impaired presynaptic transmission is one of the earliest events in TDP-43 related pathogenesis. Prolonged loss and gain of TBPH in adults resulted in synaptic defects and age-related, progressive degeneration of neurons involved in motor control. Toxic gain of TBPH did not downregulate or mislocalize its own expression, indicating that a dominant-negative effect leads to progressive neurodegeneration also seen with mutational inactivation of TBPH. Together these data suggest that dysfunction of Drosophila TDP-43 triggers a cascade of events leading to loss-of-function phenotypes whereby impaired synaptic transmission results in defective motor behavior and progressive deconstruction of neuronal connections, ultimately causing age-related neurodegeneration.
INTRODUCTION

Tar DNA binding protein of 43 kDa (TDP-43) has been identified as the major disease protein present in cytoplasmic inclusions in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD). Full length TDP-43 protein as well as 25kDa, C-terminal fragments were found to be hyper-phosphorylated and ubiquitinated in aggregates present in affected brain and spinal cord of ALS and FTLD cases (1, 2). TDP-43 inclusions are found as secondary histopathological feature in other neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's disease, thereby defining a novel proteinopathy (3, 4). Dominant missense mutations in the TARDBP gene have been identified in familial and sporadic cases of ALS and FTLD (5, 6, 7, 8), indicating that TDP-43 dysfunction may cause disease formation.

TDP-43 encodes an evolutionarily conserved protein comprising a bipartite nuclear localization signal, two RNA recognition motifs (RRMs), a nuclear export signal, a glycine and glycine/glutamine/asparagine-rich C-terminal region. The C-terminus resembles a prion-like domain (9, 10) predominantly mutated in TDP-43-related ALS and FTLD cases (11, 12). In vitro studies showed that TDP-43 is involved in transcriptional regulation, RNA biogenesis and splicing (13). The C-terminal domain of TDP-43 regulates tissue-specific gene expression, transcriptional repression, and alternative splicing, and is essential for binding to heterogeneous nuclear ribonucleoproteins involved in microRNA and mRNA biogenesis, as well as RNA turnover (12, 13).

The pathophysiological role of TDP-43 has been addressed in cell culture and animal models using mutation, alteration or expression of disease-specific variants of TDP-43 and its homologs in worms, flies, zebrafish, mice and rats (11, 12, 14). These studies revealed that post-translational modification (phosphorylation, ubiquitylation), truncation, mislocalization, nuclear clearance, cytoplasmic accumulation, and defective autoregulation can all impact either
directly or indirectly onto TDP-43 toxicity (12). The resulting phenotypes include lethality or reduced lifespan, defects in axogenesis and neurite branching, altered synaptic morphology, axon and neuron degeneration, astrogliaosis, defective fat metabolism, impaired motor behavior and cognitive deficits (11, 14). The available data suggest that TDP-43 toxicity is either directly or indirectly related to disease formation. So far, however, no consensus has emerged as to whether loss (i.e. nuclear clearance) or toxic gain (i.e. cytoplasmic accumulation) of TDP-43, or both, are causally related to disease onset and progression. Moreover, despite the fact that a large number of TDP-43 RNA targets have been identified (15, 16, 17, 18), the initiating events and pathogenic pathways underlying TDP-43 mediated neurodegeneration remain to be identified.

Here we show that similar to its human homolog, *Drosophila* TDP-43, TAR DNA Binding Protein Homolog (TBPH), is expressed in neurons, glia and muscle cells. Both loss of function and overexpression of TBPH result in decreased viability, impaired synaptic transmission, defective locomotion, and age-related progressive neurodegeneration. Together these data suggest that both increased and decreased levels of TDP-43 initiate a loss-of-function phenotype, resulting in decreased neuronal viability and subsequent degenerative cell loss that characterize motor neuron diseases like ALS and FTLD.

**RESULTS**

*Drosophila* TDP-43, TBPH, is expressed in neurons, glia and muscle cells

BLAST search of the annotated *Drosophila melanogaster* genome identified two genes with sequence similarities to TARDBP/TDP-43, namely TBPH (19) and CG7804. Genomic sequence analysis revealed that the predicted 6 isoforms of TBPH are encoded by 5-6 exons, whereas the coding region of CG7804 is intronless with sequence similarity to exon 1-4 of TBPH. The differences in exon-intron structure and coding sequence similarities between
CG7804 and TBPH suggest that CG7804 is paralogous to TBPH and likely to be a newly evolved retrogene that derived from TBPH rather than TARDBP (20). Moreover, in contrast to TBPH, CG7804 is not expressed in CNS and lacks a prion-like C-terminus that is predominantly mutated in ALS and FTLD (Figure S1). We therefore focused on TBPH.

We generated polyclonal antibodies against TBPH-specific peptide sequences. Western blot analysis of control tissue identified a 58kDa protein encoded by TBPH which was absent in deletions uncovering the genomic locus (Figure S2); we did not detect any cleavage products, TBPH fragments, dimers nor band that indicate post translational modifications (Figure S2). Whole-mount immunocytochemistry revealed that, similar to human TDP-43, TBPH is expressed throughout development and adulthood in the nucleus of neurons, glia and muscle cells (Figure 1). Perinuclear, cytoplasmic TBPH expression was detectable in postmitotic neurons (Figures 1L-1O), although immunoreactivity was less intense than nuclear anti-TBPH labelling. These data suggest that similar to TDP-43, and predicted by nuclear localization and export signals (21, 22), TBPH is predominantly localized to the nucleus but also shuttles into the cytoplasm.

**Both loss and gain of TBPH affect survival and motor behavior**

To study TDP-43 dysfunction in *Drosophila*, we generated specific loss and gain-of-function alleles of TBPH. Imprecise P-element excision resulted in two independent deletions that removed the promoter region and start codon, or the promoter region, start codon, exons 1-3 and parts of exon 4 coding for the TBPH RRM3 (Figure 2A). RT-PCR and Western blot analyses determined the absence of TBPH RNA and protein (Figures 2B and 2C). For tissue-specific TBPH LOF, we generated two independent hairpin-loop UAS-RNAi lines that target exon 5 of all predicted TBPH isoforms (Figure S3). BLAST search confirmed that our chosen target sequences do not detect off-targets, in contrast to other available TBPH RNAi lines, and
Western blot analysis revealed that *tub-Gal4* driven *UAS-TBPH-RNAi* is able to knock down endogenous TBPH protein expression below detection level (Figure S3).

For gain of TBPH function (GOF), several independent UAS lines were generated with single inserts on different chromosomal locations. Expression efficacy was tested using eye-specific *GMR-Gal4* activation. Western blot analysis revealed that *GMR>TBPH* showed increased amounts of TBPH protein (Figure 2D) comparable among different insertion lines (Figure 2E). Targeted misexpression resulted in excessive accumulation of TBPH both in the nucleus and cytoplasm (Figure S4).

Analysis of zygotic TBPH LOF (*TBPH-/-*) and Gal4-mediated GOF mutants revealed that both affect development and lifespan: only 10-20% of progeny eclosed as adult while the majority of mutant cases died during late larval/pupal stages (Figure 3A). Adult *TBPH-/-* mutant and panneuronal Gal4-specific *ELAV>TBPH* GOF flies were characterized by shortened lifespan, with *TBPH-/-* adults dying around day 7 and panneuronal Gal4-specific *ELAV>TBPH* flies dying around day 35, as compared to wild-type controls with an average lifespan of 80 days (23, 24) (data not shown). Affected larvae exhibited impaired peristalsis and defective locomotion, which was more severe in *TBPH-/-* LOF mutants compared to panneuronal Gal4-specific *ELAV>TBPH* GOF (Figure 3B). Adult *TBPH-/-* LOF mutant escapers and panneuronal Gal4-specific *ELAV>TBPH* GOF flies were characterized by inexistent or severely impaired innate escape and climbing behaviors (Figure 3C).

To further investigate motor behavior, we used an open-field paradigm together with video-assisted motion tracking (23, 24) (Figure 3D) and compared adult *TBPH-/-* mutant LOF escapers with *EB1-Gal4*-mediated *UAS-TBPH* GOF flies targeting TBPH to ellipsoid body neurons of the central complex that are upper motor neurons due to their prominent role in the
higher control of locomotion (25). Analysis of adult $TBPH^{-/-}$ mutant LOF escapers and $EB1>TBPH$ GOF flies revealed severely reduced walking activity and distance travelled; motor activity remained low over time with only $TBPH^{-/-}$ flies walking slower (Figures 3D-3H). In addition, both LOF and GOF flies were characterised by severe gait abnormalities (Figure 3I). These data suggest that TBPH dysfunction affects lifespan and motor behavior.

To confirm that the phenotypes observed in homozygous $TBPH^{-/-}$ LOF flies were indeed due to the absence of TBPH, we carried out a complementation test using a deficiency line uncovering the genomic region of chromosome 2R ($Df(2R)L06$) including the TBPH locus, which revealed early larval lethality suggesting non-complementation. In addition and to rule out hidden second-site mutations, we first analysed heteroallelic combinations using our $TBPH^{DD96}$ and $TBPH^{DD100}$ alleles together with a recently reported deletion (26), which also revealed non-complementation. Second, we generated a genomic TBPH construct covering the entire coding region of $TBPH$ as well as 3' - and 5' - regions upstream of the $TBPH$ locus (Figure S5). To analyse its rescue potential, we integrated $TBPH^{genomic}$ on 3R using the attP86Fb landing site and crossed it into the $TBPH^{DD96}$ mutant background. Analysis of homozygous $TBPH^{DD96/-}$ flies carrying one or two copies of $TBPH^{genomic}$ revealed full rescue. Thus, development, larval locomotion, eclosion, adult climbing, walking and tripod gait phenotypes of homozygous $TBPH^{DD96}$ mutants were all restored in $TBPH^{DD96/-}$; $TBPH^{genomic}$ flies (Figure S6). Together, these data suggest that the observed loss-of-function phenotypes are indeed due to loss of $TBPH$, and hence our $TBPH^{-/-}$ mutants represent bona fide $TBPH$ null alleles.

**TBPH dysfunction does not affect synapse morphology but function**

To determine the cause of TBPH-mediated behavioral phenotypes, we analyzed whether TBPH dysfunction results in muscle defects. Phalloidin staining of either larval L3 or adult day 2 $TBPH^{-/-}$ muscle preparations revealed a muscle pattern indistinguishable of wild-type or
heterozygous TBPH+/- controls (data not shown). Next we investigated whether TBPH dysfunction may cause alterations of NMJ synapse structure and morphology, as previously suggested (27, 28, 29, 30). We used a collection of synaptic markers (31) including Fasciclin II (FasII), involved in synapse development and plasticity; Bruchpilot (NC82), a component of the synaptic active zone of neurotransmitter release; synaptotagmin (Syt) involved in membrane traffic and calcium dependent neurotransmitter release; Futsch, a pre-synaptic cytoskeletal protein that binds microtubles and regulates synaptic growth and dendritic morphology; and Neuroglian (Nrg), a cell surface, transmembrane adhesion molecule involved in motor neuron pathfinding. Larval NMJ preparations were double-labeled for synaptic proteins and Horse Radish Peroxidase (HRP) that visualizes axons, dendrites and synaptic boutons in Drosophila. However, for all of the synaptic proteins examined, none were found to be mis-localized or absent in TBPH-/- LOF larvae compared to age-matched w1118 controls (Figure S7). A quantitative analysis of HRP-labeled synaptic boutons and active zone puncta immunolabeled by anti-Bruchpilot, did not reveal any indication for alterations in the number of synaptic boutons or anti-Bruchpilot positive active zones in homozygous TBPHDD96-/- LOF larvae compared to age-matched w1118 controls (Figure S8). These data suggest that the observed TBPH-related larval motor phenotypes are not caused by morphological defects, mislocalization or loss of selected proteins involved in synapse formation and maintenance.

We then carried out electrophysiological recordings of cases and controls to address whether TBPH-mediated behavioral phenotypes might be caused by defects in synaptic efficacy and function. Electrophysiological recordings made at the NMJ of homozygous TBPHDD96-/- LOF and panneuronal Gal4-mediated ELAV> TBPH GOF L3 larvae revealed a decrease in both the amplitude and frequency of miniature excitatory junction potentials (mEJPs) in the homozygous loss of function background. Low frequency (0.2Hz) stimulation of the NMJ showed no difference in evoked excitatory junction potential (EJP) amplitudes. As a
consequence of the changed mini amplitude but unaltered EJP amplitude there was an increase
in the quantal content in TBPH LOF but not in ELAV> TBPH GOF (Figures 4A and 4B). No
differences were seen during high frequency stimulation between cases and controls (data not
shown).

We also recorded electroretinograms (ERGs) from adult TBPH LOF and GOF cases compared
to appropriate controls. Both white-eyed TBPH--/ LOF and w^{118} control revealed similar
responses to blue light pulses with no differences in peak-peak amplitude of the ERG.
Moreover, the ERG amplitude of red-eyed GMR>TBP-H-RNAi and OregonR were
indistinguishable, also when compared between 1 and 5 day–old flies (Figures 4C and 4D). We
then examined the off-transient and plotted their size as a function of the maintained
photoreceptor response. On average, data points from both TBPH LOF strains, TBPH--/ and
GMR>TBP-H-RNAi flies, were above those from controls (Figure 4E). We tested the null
hypothesis that data points were distributed evenly above and below the control regression line
between maintained ERG potential and off transient, which revealed significant deviation for
both TBPH--/ and GMR>TBP-H-RNAi. Moreover, we compared the size of the off-transient
recorded in both TBPH LOF lines with that estimated from the control regression line which
revealed 15% and 25% reduction in the off-transient in TBPH--/ and GMR>TBP-H-RNAi,
respectively. As positive control, we blocked synaptic transmission in the retina using
GMR>Shibire-RNAi, the Drosophila homolog of Dynamin involved in synaptic transmission
(32), which resulted in completely abolished off-transient (Figure 4C) with data points
significantly above the wild-type regression line (Figure 4E).

We also recorded ERGs from TBPH GOF flies where Drosophila TDP-43 was over-expressed
in the eye. TBPH/w-- controls showed an ERG in response to blue light pulses like wildtype,
whereas GMR/w-- flies showed a smaller response. In contrast, GMR>TBP-H GOF flies showed
a very small response, with no off/on transients visible (Figures 4F and 4G), suggesting reduced synaptic signaling. Taken together these data suggest that both TBPH LOF and GOF affect synaptic efficacy and function, which is also supported by an impaired innate escape response (Figures 3C and S9). The observed differential effects on synaptic efficacy and function in TBPH LOF and GOF suggest that Drosophila TDP-43 dysfunction either impairs postsynaptic efficacy or alters the size or loading of synaptic vesicles, which is consistent with the observed behavioral phenotypes of TBPH LOF and GOF (Figure 3).

**Presynaptic deficits are early phenotypes of dysfunctional TBPH**

To gain further insights into TBPH-related synaptic transmission defects, we wondered whether TBPH dysfunction at the larval NMJ affects pre- or postsynaptic efficiency, or both. To address this question, we manipulated TBPH levels in a tissue-specific manner and focused on Gal4-mediated TBPH knockdown by using UAS-TBPH-RNAi in combination with UAS-Dicer-2 expression, which enhances knockdown efficiency (33). For presynapse-specific knockdown at the larval NMJ, we carried out panneuronal Gal4-mediated ELAV>Dcr2, TBPH-IR knockdown, and for postsynaptic TBPH knockdown, we used muscle-specific, Gal4-mediated BG57>Dcr2, TBPH-IR knockdown, in both cases utilizing reportedly strong and specific Gal4 drivers (34, 35). Effects of pre- or postsynaptic TBPH-RNAi were analysed using electrophysiological recordings at the L3 larval NMJ (Figure S10).

Recordings at the NMJ of panneuronal Gal4-mediated ELAV>Dcr2, TBPH-IR knockdown larvae revealed a decrease in both the amplitude and frequency of mEJPs. Low frequency (0.2Hz) stimulation of the NMJ showed no difference in evoked EJP amplitudes. As a consequence of the changed mini amplitude but unaltered EJP amplitude, we observed an increase in the quantal content in ELAV>Dcr2, TBPH-IR larvae (Figures S10A and S10B). These data suggest that, similar to recordings at the NMJ of homozygous TBPH<sup>dd96</sup>−/− L3 loss
of function larvae, panneuronal Gal4-mediated ELAV>Dcr2, TBP-H-IR knockdown leads to impaired synaptic transmission (compare Figure S10A, S10B with Figure 4A, 4B).

In contrast to presynaptic knockdown of TBPH, recordings at the NMJ of muscle-specific Gal4-mediated BG57>Dcr2, TBP-H-IR L3 knockdown larvae did not reveal any significant alterations in either amplitude or frequency of mEJPs, when compared with controls (Figure S10C, S10D). Low frequency (0.2Hz) stimulation of the NMJ revealed a small but significant difference in evoked EJP amplitudes. However, we did not detect any significant differences in quantal content in BG57>Dcr2, TBP-H-IR L3 knockdown larvae (Figures S10C and S10D).

Together, these data demonstrate that altered TBPH function causes defective presynaptic transmission at the larval NMJ, suggesting that impaired presynaptic efficacy is one of the earliest events in the aetiology of Drosophila TDP-43 related pathogenesis.

TBPH is essential for neuronal activity underlying motor behavior

To further dissect TBPH dysfunction, we focused on cell type-specific LOF and GOF and carried out TBPH-RNAi together with Dcr2 targeted by Tubulin-Gal4, which is ubiquitously and constitutively active during development, adulthood and ageing (24), as well as pan-neuronal Elav-Gal4, and EB1-Gal4 specific to upper motor neurons. Tub>Dcr2, TBP-H-IR mimicked TBPH mutations, causing larval/pupal lethality with few adult escapers (data not shown) and a disturbed tripod gait (Figure 3I). To rule out that UAS-Dcr2 itself does cause a walking phenotype, we analysed tub>Dcr2 flies which revealed no differences in walking activity, distance or activity over time when compared to controls (Figure S11). Adult Tub>Dcr2, TBP-H-IR flies displayed impaired motor behavior comparable to TBPH<sup>DD96-/</sup> null mutants with severely reduced walking activity and distance travelled; their motor behavior remained limited over time with significantly reduced walking speed (Figure 5), and walking was characterised by severe gait abnormalities (Figure 3I). We observed similar motor
phenotypes with Gal4-mediated UAS-TBPH-RNAi LOF targeted either to postmitotic neurons (Elav>Dcr2, TBPH-IR) or to upper motor neurons (EB1>Dcr2, TBPH-IR) (Figure 5).

Moreover, analysis of open-field locomotion revealed that upper motor neuron-specific TBPH GOF (EB1>TBPH) and upper motor neuron-specific TBPH-RNAi knockdown (EB1>Dcr2, TBPH-IR), resulted in similar phenotypes characterised by severely impaired motor behavior (Figures 3E-3H and 5). These data suggest that both tissue-specific loss and gain of Drosophila TDP-43 function impair neuronal activity underlying essential motor behavior.

**TBPH dysfunction differentially affects synaptic integrity in an age-related manner**

Next we investigated whether impaired synaptic activity caused by TBPH dysfunction may lead to synaptic alterations in aging flies. We therefore analyzed brains of aged flies with or without either UAS-TBPH-IR LOF or UAS-TBPH GOF targeted to EB1-Gal4 specific upper motor neurons. The EB1-Gal4 driver becomes active during late pupal stages and remains active during adulthood and ageing in a population of roughly 80 upper motor neurons (ellipsoid body ring neurons). In contrast to severe motor phenotypes (see Figures 3E-3H and 5G-5J), dysfunction or loss of this small population of upper motor neurons does not significantly alter the lifespan of flies and hence is an ideal assay system to study Drosophila TDP-43 dysfunction at the cellular and synaptic level (see Figures 6 and 7). We used EB1-Gal4 driven UAS-TBPH-IR LOF or UAS-TBPH GOF and co-expressed a membrane-bound form of GFP (UAS-mCD8::GFP) that also visualizes axons and synaptic terminals, and co-labeled whole mount brains with anti-Fasciclin 2 (Fas2) that is also expressed at pre- and postsynaptic sites of the ellipsoid body ring neuropil (Figure S12).

Analysis of brains of Gal4-mediated upper motor neuron-specific EB1>Dcr2, TBPH-IR at day 5, 20 and 40 did not reveal any significant differences at synaptic termini of the EB ring neuropil based on distribution, intensity, and shape of mCD8::GFP or Fas2 expression (Figures
S12A-S12J); however, by day 40, but not at day 5 or day 20, we consistently observed misshapen, fragmented and scattered cell bodies of EB ring neurons, together with a severe reduction in number and fasciculation of axonal projections, when compared to age-matched controls (Figures S12E-S12H, compare to S12A-12D). In contrast, we observed strong reduction already by day 5 of mCD8::GFP labeling in cell body membranes, axons and synaptic termini of TBPH GOF brains (EB1>mCD8::GFP, TBPH), which was more pronounced by day 40; Fas2 immunoreactivity, however, appeared unaffected (Figures S12K-S12T).

To determine in more detail whether the age-related synaptic alterations were either pre- or postsynaptic, we made use of the pre-synaptic marker synaptotagmin fused to GFP (UAS-Syt::GFP) in combination with the post-synaptic marker DenMark, a fusion protein of telencephalin and mCherry, which labels the somatodendritic compartment in Drosophila (36) (Figure 6). Analysis of day 5, day 40 and day 50 adult EB1>Syt::GFP, DenMark controls revealed that expression of these markers alone did not cause obvious phenotypes (Figures 6A-6C, 6I - 6K, and data not shown). However, when co-expressed together with UAS-TBPH-IR and UAS-Dcr2 in upper motor neurons, Gal4-mediated upper motor neuron-specific EB1>Dcr2, TBPH-IR RNAi-mediated LOF specifically caused down-regulation of both pre-synaptic Syt::GFP and dendritic DenMark at day 50 (Figures 6D-6F). To rule out preparation or immunolabeling artifacts, independent replicates of fly brains were similarly processed and analyzed with identical confocal microscopy settings, which established significant reduction of reporter gene expression in pre-synaptic (Figure 6H) and post-synaptic compartments when compared to age-matched controls (Figure 6G). Similar analyses were carried out on day 5 and day 40 Gal4-mediated upper motor neuron specific GOF flies, with adult EB1>Syt::GFP, DenMark, TBPH flies showing a down-regulation of both pre-synaptic Syt::GFP and dendritic DenMark at day 5 and day 40 (Figure 6L-N) compared to EB1>Syt::GFP, DenMark controls
Independent replicates of brains were similarly analysed and showed a significant reduction of reporter gene expression in pre-synaptic (Figure 6P) and post-synaptic compartments when compared to age-matched controls (Figure 6O and data not shown). Together these findings suggest that upper motor neuron-specific TBPH GOF and RNAi LOF affect synaptic integrity; in both cases, phenotypes occur in a progressive and age-related manner, and are associated with severely impaired motor behavior (Figures 3D-3H and 5).

**TBPH dysfunction results in age-related and progressive neurodegeneration**

Synaptic deficits and subsequent neurodegeneration are considered to be hallmarks of ALS and other neurodegenerative diseases (37, 38). We therefore investigated whether TBPH LOF and GOF can lead to degenerative cell loss. To visualize and number targeted neurons, we analyzed brains of aged flies that co-express *UAS-mCD8::GFP* with or without either *UAS-TBPH-IR* LOF or *UAS-TBPH* GOF again targeted to *EB1-Gal4* specific upper motor neurons (Figure 7).

Analysis of *EB1>mCD8::GFP, Dcr2, TBPH-IR* brains at day 5 and 20 did not reveal any significant differences in neuron numbers; however, by day 40 and 50, we observed a significant reduction of targeted EB neurons (Figure 7G), showing a worsening of the phenotype over time. For the remaining EB neurons, mCD8::GFP labeling revealed misshapen, fragmented and scattered cell bodies when compared to age-matched controls (Figures 7D-7F, compare to 7A-7C). A comparable but more severe degenerative phenotype was observed in TBPH GOF. Brains of 5-day old *EB1>mCD8::GFP, TBPH* flies showed reduced mCD8::GFP expression in perikarya as well as axonal extensions and synaptic arborizations of ellipsoid body ring neurons. GFP expression was further reduced by day 40 (Figures 7K-7M, compare with 7H-7J) and almost undetectable in day 60 old *EB1>mCD8::GFP, TBPH* flies when compared to age-matched controls. Analysis of independent replicates of brains of age-matched *EB1>mCD8::GFP, TBPH* and *EB1> mCD8::GFP* flies that were similarly processed and
analysed with identical confocal microscopy settings, established significant and progressive, age-related loss of upper motor neurons (Figure 7N).

To independently confirm age-related and progressive loss of neurons, we studied *pox neuro* (*poxn*) expression, which is specific to ellipsoid body ring neurons (39). Notably, loss of poxn-expressing cells progressed between day 5 and day 40 in *EB1>mCD8::GFP, TBPH* flies (Figure S13). In addition to the decrease and subsequent loss of mCD8::GFP and poxn expression, degenerative cell loss was confirmed by the specific and age-related decrease in the number of ellipsoid body ring neurons that expressed excessive amounts of TBPH in *EB1>mCD8::GFP, TBPH* flies (Figure S4 and data not shown). TUNEL labelling of brains of aged *EB1>mCD8::GFP, TBPH* flies did not indicate increased apoptotic activity (Figure S14), suggesting that progressive loss of upper motor neurons was not caused by programmed cell death. Together these data suggest that prolonged dysfunction of *Drosophila* TDP-43 causes age-related and progressive neurodegeneration.

**Gain of TBPH does not downregulate or mislocalize endogenous TBPH**

The phenotypic similarities between tissue-specific TBPH LOF and GOF may suggest that the observed synaptic deficits, impaired motor behavior and age-related neurodegeneration might be due to a common pathogenic mechanism. Previous *in vitro* and *in vivo* studies indicated that TDP-43 regulates its own expression by a negative feedback loop (17, 40, 41) that may participate in a feed-forward mechanism whereby cytoplasmic aggregation depletes nuclear TDP-43 function, essentially leading to a loss of function phenotype. We tested this hypothesis and over-expressed UAS constructs coding for tagged or untagged full length TBPH (Figure 8). We utilized *GMR-Gal4* and specifically targeted TBPH GOF to differentiating photoreceptor cells which resulted in a rough eye phenotype due to photoreceptor cell degeneration (42, data not shown). Western blot analysis of heads of 1-3 day old cases and controls identified a 58kDa
band in each condition similar to the control (Figure 8A). Quantification of independent replicates confirmed that GMR-Gal4 specific TBPH GOF with C- and/or N-terminal tags did not alter endogenous levels of TBPH expression (Figure 8B).

We then tested whether GMR-Gal4 specific TBPH GOF may lead to aggregate formation and/or deplete nuclear expression, hence affecting the function of endogenous TBPH without changing overall TBPH protein levels. Third instar eye imaginal discs were immunolabelled with anti-TBPH and anti-FLAG or anti-HA and counterstained with DAPI to determine potential aggregates and nuclear localization. In none of the cases examined did we detect aggregate formation, nor did we observe loss or alteration of nuclear TBPH expression (Figures 8C-8N and data not shown). We also tested whether GMR-Gal4 specific TBPH GOF may result in ubiquitinated TBPH and thus render the endogenous protein non-functional even though expression levels and nuclear localization appeared unaltered. However, immunolabeling with anti-FK2, which recognizes poly-ubiquitination, did not reveal any differences to controls (data not shown). Together these data suggest that gain of Drosophila TDP-43 function does not alter its own expression level or localization, albeit it results in severe synaptic, behavioral and neurodegenerative phenotypes.

**DISCUSSION**

Here we have systematically compared the phenotypic consequences of loss and gain of TDP-43 function in Drosophila. Our results demonstrate that deregulation of TBPH affects neuronal function and viability. In either case, deregulated TBPH initiates synaptic deficits and impaired motor behavior, ultimately causing age-related and progressive neurodegeneration. Our findings indicate that common loss of function phenotypes underlie TDP-43 dysfunction in Drosophila, which has implications for understanding TDP-43-mediated pathogenesis in ALS and FTLD.
Both loss and gain of TDP-43 trigger disease formation

TDP-43 related ALS and FTLD cases are characterized by both, nuclear clearance and the cytoplasmic accumulation of full length, truncated and modified forms of TDP-43 (1). TDP-43 has been shown to autoregulate its own expression (17, 41), and its ubiquitylation, phosphorylation, aggregation, truncation, mislocalization, nuclear clearance, and defective autoregulation, can contribute either directly or indirectly to disease formation (12). However, no consensus has emerged as to whether loss or toxic gain of TDP-43 function, or both, are causally related to disease onset and progression.

In our study, we systematically compared loss- and gain of TBPH, the Drosophila homolog of TDP-43, and analyzed their effect on synaptic function and morphology, motor control, and age-related neuronal survival. Our results demonstrate that loss of function, RNAi-mediated downregulation and overexpression of wildtype Drosophila TDP-43 are sufficient to cause age-related neurodegeneration. In the case of TDP-43 overexpression, we did not detect alterations in expression levels or mislocalization of the endogenous protein; we also found no evidence for ubiquitylation, aggregation, truncation, or autoregulation, and based on Western blot data, excessive phosphorylation appears unlikely. Our data thus argue that TDP-43 truncation, modification, altered autoregulation or aggregates are not a prerequisite for toxicity and age-related neurodegeneration, which is also supported by data in other model systems (28, 41, 43, 44, 45, 46).

Previous studies showed that TDP-43 can form homodimers (47) and functions in multiprotein/RNA complexes (15, 48, 49) where multiple TDP-43 molecules are incorporated into each ribonucleoprotein complex (50). These data suggest that equilibrated, physiological levels of TDP-43 and a stoichiometric relationship with ribonucleoprotein complex
components are essential for proper biogenesis, spatio-temporal expression and hence, regulation of TDP-43 target genes. Our results establish that deregulation of TDP-43 levels is a core event underlying disease formation, which can be triggered by either decreasing or increasing levels of TDP-43. This suggests that de-regulation of TDP-43 levels, by loss of function, downregulation, or overexpression, alters the stoichiometry of participating components and, hence, ribonucleoprotein complex formation required for proper physiological function. In either condition, the net result of de-regulated TDP-43 is impaired physiological function which, when continuing, affects neuronal survival, ultimately causing progressive cell loss. In support of this notion, we determined synaptic deficits as one of the earliest phenotypes of TDP-43 dysfunction.

**Presynaptic deficits are early, initiating events of TDP-43 related pathogenesis**

In our study we found that both loss and gain of *Drosophila* TDP-43 result in synaptic deficits and impaired motor behavior, followed by progressive, age-related neurodegeneration. In contrast to previous reports in *Drosophila* (27, 28, 29), we did not detect any obvious structural defects at the larval neuromuscular junction or mislocalization of synaptic proteins that might explain the observed behavioral phenotypes. This discrepancy might be attributable to differences in genetic background and/or methods used. Motor terminals are dynamic structures that show developmental and circadian fluctuations in shape and size (51). In our study, we used *bona fide* TBPH null alleles together with genomic rescue, as well as overexpression alleles. Instead of structural defects at the larval NMJ, our experiments reveal that affected flies are characterized by defective synaptic transmission, a phenotype detectable both at the larval NMJ and in the adult. In addition, our tissue-specific RNAi-mediated knockdown experiments identified presynaptic, rather than postsynaptic deficits as the earliest detectable phenotypes at the larval NMJ, suggesting that presynaptic deficits are initiating events in disease formation.
Our observations therefore allow for the first time the description of a sequence of events whereby TDP-43 dysfunction causes impaired synaptic transmission and motor abnormalities, followed by the subsequent loss of neuronal connections that precede degenerative cell death in an age-related and progressive manner. Such a sequence of events suggests that TDP-43 related pathogenesis progresses from the synapse and axon to the neuronal cell body, thereby resembling affinities to the "dying back" phenomena, a hallmark of several neurodegenerative diseases (37). Comparable events have been, at least to some extent, reported for Wallerian degeneration (52), and recent genetic evidence indicates that neuronal "dying back" underlies an active process of self-destruction distinct from apoptotic cell death (53). A "dying back" process also characterizes human ALS patients and mouse superoxide dismutase 1 (SOD1) models of ALS (38, 54). Like TDP-43 inclusions, SOD1 mutations are commonly identified in familial ALS (55). Although further, conclusive experiments are required, it is tempting to speculate that TDP-43 might not only regulate RNAs related to synaptic function (15, 17), but potentially also negatively regulates the transcription or translation of target genes involved in the active self-destruction of neuronal connections.

**TDP-43 dysfunction results in loss of function phenotypes**

In addition to similarities between loss and gain of function phenotypes, we also observed phenotypic differences, including electrophysiology at the larval NMJ but also at the level of synaptic integrity and neurodegeneration in adult brain, where GOF typically caused more severe phenotypes than LOF. While some of these differences might be attributable to differing efficiencies of UAS/Gal4-mediated LOF and GOF, or a possible gain of toxic function, it is striking that both conditions lead to comparable, detrimental phenotypes with similar endpoint: age-related and progressive neurodegeneration.
It should be emphasized, however, that the similar phenotypic outcomes of loss and gain of
*Drosophila* TDP-43 function, including age-related neurodegeneration, do not necessarily
implicate similar mechanisms related to TDP-43 mediated pathogenesis. It is conceivable but
remains to be shown that loss, downregulation or gain of TDP-43 function could trigger
distinct pathogenic mechanisms that all converge on a functional node which when defective
initiates disease formation. Previous studies suggest that such a functional node is likely the
stochiometric activity of TDP-43 in ribonucleoprotein complexes that are essential for the
regulation and correct processing of multiple target genes, including those related to synaptic
function (15, 17). This model is consistent with familial cases of ALS and FTLD, where TDP-43
is predominantly mutated in the C-terminal, prion-like domain (5, 6, 7, 8) that regulates tissue-
specific gene expression, transcriptional repression, and alternative splicing, and is essential for
binding to ribonucleoproteins involved in microRNA and mRNA biogenesis, as well as RNA
turnover (9, 10, 12, 13). According to this model, C-terminal, but also RRM mutations may
hinder the participation of TDP-43 in ribonucleoprotein complex formation and hence, their
function, leading to physiological deficits and eventually neurodegeneration.

In summary, our findings demonstrate that both loss and gain of TDP-43 function result in
functional deficits whereby impaired synaptic transmission and defective motor behavior
precede progressive deconstruction of neuronal connections, ultimately causing age-related
neurodegeneration. Our data therefore provide *in vivo* evidence for common loss of function
phenotypes underlying TDP-43 dysfunction that likely also characterize TDP-43 mediated ALS
and FTLD.

**EXPERIMENTAL PROCEDURES**

**Fly stocks.** Fly stocks were maintained at 25°C on standard cornmeal food, unless for aging
experiments where aged flies were maintained on 15% sugar/yeast medium (23). The
following strains were used: *Oregon R* (wild type); *w^{118}*, *w*; *TBPHnull/CyO,GFP*; + (26) *Df(2R)106/SM5*; *w*; +; *Elav-Gal4*; *GMR-Gal4*; and *Tub-Gal4*; *UAS-mCD8::GFP* (56); *UAS-Dcr2*; *UAS-Shibire-RNAi* (Bloomington Stock Centre); p{Gawb}C57 (BG57, ref. 34); *EB1-Gal4* (57); *UAS-DenMark*, *UAS-Syt::GFP* (36); *UAS-RFP-TBPH* (58).

**Transgenic constructs.** The *TBPH* gene was amplified by PCR from cDNA clone GH09868 (DGRC). PCR primers to amplify TBPH in pUAST vector are

forward primer: 5'-GGAGATCTATGGATTTCGTTCAAGTGTCGG-3';
reverse primer: 5'-GGCTCGAGTTAAAGAAAGTTTGACTTCTCCGC-3'.

Primers used to generate Flag-TBPH-HA are:

forward primer:

5'-GGAGATCTATGGACTACAAGGACGACGATGACAAGGATTTCGTTCAAGTGTCGG-3';

reverse primer: 5'-GGCTCGAGTTAGGCATAGTCTGGGACGTCATATGGATAAAGAAAGTTTGACTTCTCCGC-3'.

Bgl II and Xho I sites were used to insert TBPH into pUAST vector. DNA sequence was confirmed by sequencing. The *UAS-TBPH^{A765}* line was used for all behavioural, electrophysiology and immunohistochemistry experiments. To specifically knockdown TBPH, the target region was chosen by E-RNAi web service (59). TBPH RNAi construction was carried out as previously described (33). PCR primers with EcoRI (forward) and XbaI (reverse) sites were designed to subclone TBPH RNAi construct into pMF3 vector (33).

**TBPH-RNAi**

forward primer: 5'-GGGAATTCCACTCATACCACCCACAGGG-3';
reverse primer: 5'-GGTCTAGAGTTATGCGGCTGGTTCATTC-3'.

forward primer: 5'-GGGATCTATGGACTACAAGGACGACGATGACAAGGATTTCGTTCAAGTGTCGG-3';
reverse primer: 5'-GGCTCGAGTTAGGCATAGTCTGGGACGTCATATGGATAAAGAAAGTTTGACTTCTCCGC-3'.

Bgl II and Xho I sites were used to insert TBPH into pUAST vector. DNA sequence was confirmed by sequencing. The *UAS-TBPH^{A765}* line was used for all behavioural, electrophysiology and immunohistochemistry experiments. To specifically knockdown TBPH, the target region was chosen by E-RNAi web service (59). TBPH RNAi construction was carried out as previously described (33). PCR primers with EcoRI (forward) and XbaI (reverse) sites were designed to subclone TBPH RNAi construct into pMF3 vector (33).

**TBPH-RNAi**

forward primer: 5'-GGGAATTCCACTCATACCACCCACAGGG-3';
reverse primer: 5'-GGTCTAGAGTTATGCGGCTGGTTCATTC-3'.
Insertion of RNAi construct was confirmed by restriction enzyme digestion and sequencing. Plasmid injection and generation of transgenic flies were performed by BestGene Inc (CA, USA).

To generate a TBPH genomic rescue construct, we used TBPH genomic DNA from pacman bac library (CH322-116J04, bacpac.chori.org) covering position 19751906-19744713 on the second chromosome, which encodes the entire TBPH coding sequence. TBPH\textsuperscript{genomic} was integrated into the pUC 3GLA plasmid (gift from Matthias Soller) by recombination. TBPH\textsuperscript{genomic} in pUC 3GLA was used to generate transgenic flies by site-specific integration into the attp86Fb fly strain (Bischof et al., PNAS 2007) and flies harboring TBPH\textsuperscript{genomic} pUC 3GLA were selected by compound eye-specific GFP expression.

**P-element mediated mutagenesis.** The \textit{TBPH}\textsuperscript{DD96} and \textit{TBPH}\textsuperscript{DD100} null alleles were generated by imprecise excision of the EY10530 P-element, which is inserted in the promoter region of the \textit{TBPH} locus. It was mobilized using HoPl \textDelta2-3 transposase (gift from Iris Salecker). 450 excision lines were established from individual events and genomic deletions within the TBPH locus were identified by PCR and confirmed as protein null by Western blot. \textit{TBPH}\textsuperscript{DD96} and \textit{TBPH}\textsuperscript{DD100} breakpoints are 19750093…19747817 and 19750093…19747925 respectively. The primers use to confirm presence of neighbouring gene CG4585 were:

- forward primer: 5’-CTGAGCATCTTCTGCGAC-3’;
- reverse primer: 5’- GAAATTAGCTCGCCATGG-3’.

Breakpoints and P-element insertion sites were sequenced using the following primers;

- \textit{TBPH}\textsuperscript{DD100} forward primer: 5’-TTCCATGGCGAGCTAATT-3’;
- reverse primer: 5’- GTGTCCAGGTTGCGGTACTT-3’;

- \textit{TBPH}\textsuperscript{DD96} forward primer: 5’-CGAGTTGCCGCCGTATAAT-3’;
reverse primer: 5’-CCTTTCACTCGACTTTAT-3’.

Sequencing was carried out at MWG Eurofins.

**Generation of TBPH antibody.** The anti-TBPH antibody was generated by injecting two rabbits with peptide sequences PQGNHMNPGRNGHHR, corresponding to residues 291-305 and QSSSQNAAEKSNFL, residues 517-531. Immunization was carried out by Eurogentec.

**Immunohistochemistry.** Adult and larval CNS dissection was carried out as previously described (23). Larval NMJ dissections were carried out according to established protocol (60). Primary antibodies used were: mouse anti-Repo (1:20); rat anti-Elav (1:30); mouse anti-Futsch (1:50); mouse 3C11 (1:100); mouse anti-synaptotagmin (1:25); mouse anti-FasII (1:10); mouse NC82 (anti-bruchpilot) (1:100), and mouse anti-Neuroglian (1:4) all obtained from the Developmental Studies Hybridoma Bank (DSHB) under the auspices of the NICHD and maintained by The University of Iowa; rabbit anti-TBPH antibody (1:3000); rabbit anti-Poxn (1:100; Adachi, Ludlow & Hirth, unpublished); mouse anti-polyubiquitin (FK2, 1:200; ENZO); rabbit anti-FLAG (1:500; Cell Signaling Tech); mouse anti-HA (1:40; Roche); goat anti-HRPCy3 (1:100; Stratech), 488-Phalloidin (1:1000; Invitrogen). Secondary antibodies were Alexa fluor 488, 568 and 647 (each 1:150; Invitrogen).

**Image acquisition and analysis.** Images were obtained either with Motic BA400 or Leica TCS SP5 confocal microscope with Leica Application Suite Advanced Fluorescence (LAS AF) version 2.0.2 software. For confocal images, channels were scanned sequentially. For comparative images, the same microscope settings were used for the control and experimental genotypes. For Figures 6 and 7, confocal z-stacks were rendered as 3-D projections. Signal quantification of digital images were carried out using Fiji (FIJI). Significance of GFP and
FasII signal intensity was calculated using unpaired student’s t-test. Equal variance assumptions were based on Levene’s test for equality of variance.

**NMJ bouton count.** Early L1 larvae were picked from 4hr egg collection plates (fruit agar) and placed on cornmeal food. At wandering L3, larvae were dissected according to established protocol (60). All preparations were dissected within a 1.5 hr window to avoid differences in circadian rhythm effect on bouton number. Z-stacks were taken of the MN6/7-Ib motor neurons innervating muscle group 6/7 in segment A3. Boutons were counted by hand and the number of NC82 puncta were analyzed using the ITCN plug-in for ImageJ.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** Tissues of interest were fixed in 4% formaldehyde and subsequently permeabilized in 100 mM Citrate/0.1% Triton X-100 for 30 min at 65°C. Tissues were briefly washed with 0.5% Triton-X 100 in PBS and rinsed twice with TUNEL assay buffer (In Situ Cell Death Detection Kit; Roche). After incubating for 90min at 37°C, TdT enzyme was added and further incubated for 3hrs at 37°C. For positive control, freshly dissected tissue was soaked in 2N HCl for 30min to induce apoptosis.

**Western blots.** Fly tissue was lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sodium, deoxycholate and 0.1% SDS, 50 mM Tris, pH8.0) containing complete proteinase inhibitor (Roche) and phosstop phosphatase inhibitor (Roche). Samples were centrifuged to take the RIPA-soluble fraction. SDS-PAGE was run in 8% SDS gel. Rabbit anti-TBPH antibody was used at 1:2000-3000 and anti-β-Tub (DSHB) was used at 1:300. Secondary antibodies were IRDye 800 conjugated goat anti-rabbit (1:10000, Rockland
Immunochemicals) and Alexa Fluor 680 goat anti-mouse (1:10000, Invitrogen). Membrane images were acquired using Odyssey.

**Reverse transcriptase PCR (RT-PCR).** RNA was extracted by homogenizing whole flies in Trizol, adding chloroform, incubating on ice for 15 minutes, followed by centrifugation at 16,000g at 4°C. The upper phase was added to isopropanol and incubated and spun as before. The pellet was washed in 70% ethanol and dissolved in nuclease-free H2O. RNA purity was measured using a Nanodrop. DNase treatment was carried out according to manufacturer’s instructions (Ambion). cDNA was generated by mixing 1ug RNA and random hexamers, heating to 70°C and adding 5 x M-MLV reaction buffer, M-MLV-RT, RNAsin and dNTPs according to manufacturer’s instructions (Ambion). Samples were incubated at 37°C for 1 hour and 70°C for 15 minutes. PCR products were run on 1% agarose gel. The primers used were as follows;

**TBPH**
forward primer: 5’- ATCTTGGATGGCTCAGAACG-3’
reverse primer: 5’-GTCGGTCTTTATTCCGTTGG-3’;

**RPL32**
forward primer: 5’-CGCCGCTTCAAGGGACAGTATC-3’
reverse primer: 5’-CGACAATCTCCTTGCCTTCTT-3’,

**Footprint/gait analysis.** Flies were briefly anaesthetized with CO2 and one third of the wings were removed to prevent escape. Flies were left to recover in a petri dish for 10-15 minutes before being tapped onto a soot-covered glass microscope slide (slides were held over a candle flame until covered in an even coating of soot). Footprints were visualized by lighting the slide from below and images were captured with a Q-capture camera.
**Startle-induced negative geotaxis.** Flies’ climbing ability was assayed as described earlier (23). Equal variance assumptions were based on Levene’s test for equality of variance.

**Eclosion analysis.** Embryos were collected on fruit agar plates in 5 hours batches and left in a 25°C incubator until larvae had hatched. Fifty first instar larvae were placed in a vial and a tally made of all fully eclosed adult flies, mean values were calculated and plotted as a percentage of total number of larvae picked. Significance was calculated using unpaired t-test. This was carried out in triplicate for each genotype.

**Larval motility.** Thirty wandering third instar larvae were individually placed on a fruit agar plate and allowed to recover for 30 seconds. The number of peristaltic waves, travelling in either direction, was scored over 1 minute. Significance was calculated using unpaired t-test (two-tailed). Equal variance assumptions were based on Levene’s test for equality of variance.

**Video-assisted motion tracking.** Tracking arenas were modified 6-well tissue culture plates (35mm diameter wells) filled with silicon rubber (Sylgard) to leave a 3mm space so that flies could walk freely but not hop or fly. 18-24 flies were briefly anaesthetized with CO₂, placed in separate arenas and left to recover at 25°C for 45 minutes before being placed above an array of white LEDs within a temperature-controlled incubator. Tracking was carried out at 25°C. A black and white CCD camera (Hitachi, MP-M1A) positioned above the arenas was connected to a PC via an analogue capture card (Integral Technologies, Flashbus MV Lite). Recordings were carried out during the same time slot. Recorded videos were converted to fly movie format using the motmot package (61) and loaded into Ctrax software (62) to analyse the positions of the flies throughout the video. Position data for the 30-minute file was exported as a Matlab-compatible (Mathworks) matrix file. Errors in the tracking were fixed using Matlab.
(Mathworks) as well as FixErrors GUI (62), which is described in further detail at http://ctrax.sourceforge.net/fixerrors.html. Fixed trajectories were analysed in Matlab using custom scripts (written by DMH). This analysis determined the mean velocity, mean activity, activity over time and mean cumulative distance travelled by the population of flies in the arena. Activity was defined as movement per frame above a velocity of 2 mm/second. Average activity was the percentage of frames where the fly was active (>2 mm/second velocity). Mean velocity was the average of velocities in each frame of the recording only when the fly was active. Box-plots were generated in Matlab where the boxes show median, upper and lower quartiles; whiskers contain data 1.5x the interquartile range; + indicates a data point within 3x the interquartile range. Significance was calculated using the Mann-Whitney U-test with a Bonferroni correction to account for multiple comparisons.

**Escape response.** The amplitude of the escape response was carried out as previously described (63). In summary, anesthetized flies were fixed to a tungsten pin on the end of a cocktail stick using glue gum. The pin was placed so that it covered the thorax. Flies were left to recover for 20 minutes before being mounted over the flexible ergometer and lowered gently until the flies reached out and place their legs on the jump platform, bringing it up to a resting position. A stimulating electrode was placed in the eye and one in the cervical connective to stimulate the Giant Fiber System. A supra-threshold 0.8mV was applied and as the flies jumped, the amount of beam displacement was measured.

**Electrophysiology.** Wandering third instar larvae were dissected in HL3 buffer (64) containing 1.8 mM CaCl₂. Nerves innervating the body muscle walls were cut near the ventral ganglion and stimulated using a suction electrode and isolated pulse stimulator Digitimer DS2A (constant current modification), with a current double that needed to initiate a compound response. All recordings for TBPH LOF/GOF and appropriate controls were made
intracellularly in muscle 6, abdominal segment 3, at ambient room temperature using
microelectrodes filled with 3M KCl that had tip resistances of 5 to 10 MΩ. In 1.8 mM
extracellular Ca\(^{2+}\) resting membrane potentials were -61.1 ± 1.0 mV (n = 10) and -55.8 ± 2.5
mV (n = 9, P < 0.05) and input resistances were 10.1 ± 0.7 MΩ (n = 10) and 9.6 ± 0.4 MΩ (n =
9, P > 0.05) for w\(^{1118}\) and TBPH/- larva, respectively. Resting membrane potentials were -
54.1 ± 1.4 mV (n = 7) and -52.0 ± 1.5 mV (n = 8, P = 0.0018) and input resistances were 5.9 ±
0.2 MΩ (n=7) and 6.4 ± 0.3 MΩ (n = 8, P < 0.05) for ELAV/+ and ELAV>TBPH respectively.
Data, filtered at 1 kHz and digitized at 10 kHz, were acquired using an Axopatch 200B
amplifier and a Digidata 1320A data acquisition board (Molecular Devices Data) were acquired
using pClamp8.02 (Molecular Devices) and analyzed with Clampfit8.02 (Molecular Devices)
or MiniAnalysis (Synaptosoft). EJP amplitude histograms were constructed by averaging 50
separate events stimulated at 0.2 Hz from an individual muscle cell and then calculating the
mean response from at least 6 larvae per line. Spontaneous release events were recorded for
120 seconds without any electrical stimulation. All events were analyzed and mean mEJP
amplitudes from individual larvae were averaged to generate the histograms. To obtain the
frequency of spontaneous release events the number of events for each recording was divided
by 120 and then averaged to generate histograms. Significance was calculated using unpaired
students t-test (two-tailed).

**Electroretinograms.** Female flies were aspirated into a pooter, and then gently blown into a
truncated pipette tip. They were restrained with nail varnish (Creative Nail Design). No
anesthesia was used. Blunt glass pipettes, filled with simple *Drosophila* saline (130 mM NaCl,
4.7 mM KCl, 1.9 mM CaCl\(_2\) (65) were used as recording electrodes, placing one tip centrally
on the surface of the eye and the other in the mouthparts. Flies were adapted to the dark for two
minutes and then presented with a standard blue light stimulus from three Kingbright, KAF-
5060PBESEEVGC light-emitting diodes (maximum emission wavelength 465 nm) situated 6
cm away from the eye. Light pulses were monitored with a BPX65 photodiode (Centronics) next to the head of the fly. In the dark, the photodiode current was 0.5 nA; during the stimuli 400 nA. Each fly was tested with at least 3 blue light stimuli, >10 s apart. Their responses were captured using Dasylab software (measX, Mönchengladbach, Stuttgart) and the average waveform calculated. At least 9 flies of each genotype were tested at each timepoint. Significance was calculated using Bonferroni post hoc analysis.

**Statistical analysis.** Statistical analysis was carried out as previously described (24); for details of the statistical tests used, see Supplementary Table 1.

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_The authors declare no competing financial interests._


FIGURE LEGENDS

Figure 1. TBPH is expressed in neurons, glia and muscle cells. (A-E) Endogenous TBPH expression was examined at the neuromuscular junction of muscle group 6/7 of abdominal segment II in w^{1118} wandering third instar larvae. TBPH is expressed in the nucleus of muscle cells (C-E). Images are z-projections of a 10μm area scanned in 2μm steps. (F-J) Endogenous TBPH expression was examined in the adult brain of flies expressing membrane-bound GFP in ellipsoid body neurons of the central brain that are considered to be upper motor neurons (EB1>mCD8::GFP; CNS hemisphere shown in J, the dotted white box corresponds to F-I). TBPH is expressed in neurons (F and G) and glia (H) throughout the adult brain, including ellipsoid body neurons. Images are single z-slices taken in 1μm steps. (K-O) TBPH expression is also seen in perinuclear regions of ellipsoid body neurons as shown by its co-localisation with membrane bound mCD8::GFP (white arrowheads). Images are single z-sections taken in 1.5μm steps. Scale bars; 50μm (A-E); 10μm (F-I, K-O).

Figure 2. Loss and gain of function alleles of the Drosophila TDP-43 homolog TBPH. (A-C) TBPH null mutant alleles and (D-F) UAS-TBPH overexpression alleles. (A) TBPH null mutant alleles (TBPH^{DD100} and TBPH^{DD96}) were generated by imprecise P-element excision. Two types of deletion mutant were generated lacking distinct sections of the TBPH genomic region. The TBPH^{DD100} allele is lacking the promoter region of TBPH, including the start codon. The TBPH^{DD96} allele is lacking a larger amount of gDNA including the promoter region, the start codon and bases encoding the RNA recognition motifs (RRMs). (B) RT-PCR revealed lack of TBPH mRNA in homozygous TBPH mutants. Ribosomal protein L32 (RPL32) was used as loading control. Control genotype: w^{1118}. (C) TBPH^{DD100} and TBPH^{DD96} mutant alleles are protein null, as revealed by absence of the TBPH-specific 58kDa protein band present in w^{1118}. 

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flies (control); shown is a Western blot using protein extracts from six fly heads blotted with anti-TBPH antibody. (D) Western blot analysis of GMR-Gal4 driven UAS-TBPH shows excess TBPH protein expression in adult heads. Two different UAS-TBPH lines with insertion on different chromosomes (#763 on II, #765 on III) show comparable expression levels. (E) Quantification of GMR>TBP shows a dramatic increase in TBPH protein levels. Western blot TBPH signal intensities were normalized against β-Tubulin. Mean and SEM are shown.

Figure 3. Both loss and gain of TBPH affect survival and motor behavior. (A) Survival analysis quantified the number of larvae that survived to adulthood. The number of fully eclosed adults was significantly reduced in loss-of-function (LOF) TBPH null mutants and gain-of-function (GOF) ELAV>TBP flies, compared to respective control (w^{1118}/+, control A; Elav/+, control B). (B) Both TBPH LOF and GOF show impaired larval locomotion with a reduction in the number of peristaltic waves per minute compared to controls (w^{1118}/+, control A; Elav/+, control B). (C) TBPH LOF and GOF adults also show poor climbing performance in a startle-induced climbing assay (n=15) compared to controls (w^{1118}/+, control A; Elav/+, control B). (D-H) Video-assisted motion tracking of TBPH^{DD96/-} LOF and cell type-specific EBl>TBP GOF targeted to upper motor neurons. (D) Representative walking tracks recorded over 3 minutes for TBPH^{DD96/-} and EBl>TBP flies, together with their respective controls (w^{1118}/+, control A; EBl/+, control C). (E-G) TBPH^{DD96/-} LOF and EBl>TBP GOF flies show a reduction in walking activity over time, activity and total distance travelled compared to the respective controls (w^{1118}/+, control A; EBl/+, control C). (H) TBPH^{DD96/-} null mutants show reduced walking velocity compared to controls, whereas EB1-specific overexpression of TBPH does not affect the mean velocity (w^{1118}/+, control A; EBl/+, control C). (I) TBPH^{DD96/-} LOF, Tub>TBP-RNAi downregulation and ELAV>TBP GOF flies display disturbed tripod gait. Left cartoon shows tripod gait with left foreleg (1), right middle leg (2), and left hind leg (3). Control flies (w^{1118}) walk in a stereotyped alternating tripod gait pattern. This pattern is
disrupted in \(TBPH^{DD96-/-}\) null mutants (\(TBPH^{-/-}\)), ubiquitous RNAi-mediated TBPH knockdown flies (\(Tub>TBPH-RNAi\)) and pan-neuronal Elav-Gal4 mediated TBPH overexpression flies (\(ELAV>TBPH\)). Box-plots show median, upper and lower quartiles (box); whiskers contain data 1.5x the interquartile range; + indicates a data point within 3x the interquartile range (outliers). **=P<0.01; ***=P<0.001. Mean and SEM are shown (A, E).

**Figure 4.** TBPH dysfunction affects synaptic efficacy. (A) Representative excitatory junction potential (EJP) traces are shown for LOF. EJP amplitudes for \(TBPH^{DD96-/-}\) LOF and pan-neuronal \(ELAV>TBPH\) GOF flies are not significantly different from the respective controls (control A, \(w^{1118/+}\) and control B, \(Elav/+\)), however quantal content is significantly increased in \(TBPH\) mutant larvae. (B) Representative traces of spontaneous neurotransmitter release (mEJP) shown for \(TBPH^{DD96-/-}\) LOF. Both the mEJP amplitude and frequency are significantly reduced in \(TBPH\) mutant larvae. The mEJP is unaffected in \(ELAV>TBPH\) gain-of-function flies (\(w^{1118/+}\), control A; \(Elav/+\), control B). (C) Representative ERG traces of the indicated TBPH LOF conditions. The white-eyed \(TBPH^{DD96-/-}\) mutants and \(w^{1118}\) flies show a strong, habituating response, steady maintained response, and off transient. The red-eyed OregonR, \(GMR>Shibire-IR\) and \(GMR>TBPH-IR\) show a smaller amplitude response; \(GMR>Shibire-IR\) flies lack on- and off transients. Chequered bar indicates duration of light pulse. (D) Mean peak-peak amplitude of the ERG from the indicated conditions at day 1 and day 5 time points. (E) \(TBPH^{DD96-/-}\) mutant and \(GMR>TBPH-IR\) have significantly smaller off-transients than the respective eye colour-matched controls. The negative \(GMR>Shibire-IR\) controls, in which synaptic transmission is blocked, show a severely reduced off-transient. The solid line shows the regression line between off-transient and maintained response for wild-type flies. (F) Representative ERG traces of the indicated TBPH GOF conditions. The \(UAS-TBPH/+\) and \(GMR/+\) flies show a normal amplitude response, the smaller response seen in the \(GMR/+\) flies is due to the darker red pigment. \(GMR>UAS-TBPH\) flies have a very small response, with no
off/on transients visible. (G) Mean peak-peak amplitude of the ERG from the indicated GOF conditions at day 1 is significantly decreased in GMR>TBP H flies compared to the controls (D, E, n=9; *=P<0.05, **=P<0.01, ***=P<0.001). Mean and SEM are shown (A-D, F, I).

Figure 5. Similar to TBP H mutants, cell-type specific knockdown of TBP H leads to impaired motor behavior. Gal4-mediated UAS-TBP H-RNAi knockdown targeted either ubiquitously (tub-Gal4) or to all neurons (Elav-Gal4), or targeted to upper motor neurons (EB1-Gal4). RNAi efficiency was enhanced by co-expressing UAS-Dcr2. (A-J) Motor behaviour analysis using video assisted open-field motion tracking. (A) Representative walking tracks recorded over 30 minutes for 5-day-old Tub>Dcr2, TBP H-RNAi flies and heterozygous Dcr2,TBP H-RNAi controls. (C-F) Tracking analyses reveal that, comparable to TBP H<sup-DD96/-</sup> mutants, Tub>Dcr2,TBP H-RNAi flies have a significantly reduced average activity, total distance travelled, mean speed and activity over time when compared to heterozygous Dcr2,TBP H-RNAi controls. (G-J) Dcr2, TBP H-RNAi expression was activated by either the pan-neuronal ELAV-Gal4 driver, or the EB1-Gal4 driver (ELAV>Dcr2,TBP H-RNAi or EB1>Dcr2,TBP H-RNAi respectively). Tracking analyses reveal that, comparable to TBP H<sup-DD96/-</sup> mutants, the walking activity, total distance travelled, mean speed and activity over time of 5 days old ELAV>Dcr2,TBP H-RNAi and EB1>Dcr2,TBP H-RNAi flies is significantly reduced when compared to that of the control (heterozygous Dcr2,TBP H-RNAi). Box-plots show median, upper and lower quartiles (box); whiskers contain data 1.5x the interquartile range; + indicates a data point within 3x the interquartile range (outliers). (n=24; *=P<0.05, **=p<0.01, ***=p<0.001). Mean and SEM are shown (F, J).

Figure 6. Both cell type-specific TBP H LOF and GOF cause trans-synaptic defects. (A) Expression of presynaptic marker Syt::GFP (green) and dendritic marker DenMark (red) in upper motor neurons of the adult central brain of 50-day old flies using the ellipsoid body...
neuron-specific \textit{EB1-Gal4} driver (\textit{EB1}>\textit{Syt::GFP}, DenMark). Syt::GFP (green) expression is seen in the presynaptic region, whereas \textit{DenMark} visualizes postsynaptic dendritic compartment. DenMark signal and Syt::GFP are indicated in single panels (B and C, respectively). (D-F) Driving TBPH-RNAi in EB1 neurons leads to downregulation of DenMark (E) and Syt::GFP (F). (G) DenMark signal is significantly reduced in TBPH LOF (\textit{EB1}>\textit{Syt::GFP}, DenMark, TBPH-IR). (H) TBPH knock down significantly downregulates Syt::GFP. (I-K) DenMark (J) and Syt::GFP (K) expression in EB1 neurons of 40-day old flies. (L-M) TBPH overexpression in EB1 neurons leads to downregulation of DenMark (M) and Syt::GFP (N). (O) DenMark signal is significantly reduced in TBPH GOF (\textit{EB1}>\textit{Syt::GFP}, DenMark, TBPH). (P) TBPH overexpression significantly downregulates Syt::GFP. *=P<0.05, **=P<0.01, ***=P<0.001. Mean and SEM are shown. Scale bar: 50 µm.

\textbf{Figure 7.} Upper motor neuron specific RNAi-mediated downregulation and gain of TBPH causes loss of neuronal connections and age-related neurodegeneration. (A-C) Control flies with \textit{mCD8::GFP} overexpression in upper motor neurons at day 40 (B, enlargement of dashed box in A; C, additional example). (D-F) TBPH LOF flies (\textit{EB1}>\textit{mCD8::GFP}, Dcr2, TBPH-IR) with TBPH-RNAi targeted to upper motor neurons at day 40. The GFP expression in the ellipsoid body structure does not diminish in the aged brain, however, dispersed cells with fragmented and granular GFP expression are detectable (E, enlargement of dashed box in D. F, additional example). (G) Quantification of EB1 neuron cell number (GFP positive) in control and TBPH LOF flies at day 5, 40 and 50. A significant decrease in neuron number is observed in aged TBPH LOF flies. (H-J) Control flies with mCD8::GFP overexpression in EB1 ring neurons at day 40 (I-J, enlarged example of cell bodies). (K-M) TBPH and mCD8::GFP overexpression in upper motor neurons leads to an age-related reduction in mCD8::GFP expression (L-M, enlarged example of cell bodies). (N) Quantification of EB1 cell numbers (GFP positive) in control and TBPH GOF flies at day 5, 40 and 60. A significant decrease in
neuron number is observed over time in TBPH GOF flies. **=P<0.01, ***=P<0.001. Mean and SEM are indicated. Scale bar: 50 µm.

**Figure 8.** GMR-Gal4 mediated gain of TBPH does not downregulate or mislocalize endogenous TBPH. (A) Western blot analysis of GMR-Gal4 driven untagged (58kDa) or tagged UAS-TBPH (Flag-TBPH-HA, 60kDa; RFP-TBPH, 85kDa). Levels of endogenous TBPH (arrow at 58kDa) are unaffected in day 1-3 flies overexpressing either form of TBPH. (B) Quantification of endogenous TBPH shows no alteration in TBPH protein levels. Endogenous TBPH signal intensities were normalized against β-Tubulin. Mean and SEM are indicated. (C,G,K) Third instar eye imaginal discs immunolabeled with anti-TBPH does not reveal TBPH-positive aggregates in cell bodies nor in the optic stalk (dotted area). (D-F, H-J, L-N) Third instar eye imaginal discs immunolabeled with anti-TBPH and DAPI identifies that nuclear expression of endogenous TBPH is maintained in gain of TBPH. Note that signal intensity in panels I, J, M and N is adjusted for overexpressed TBPH to avoid saturation of the signal. Scale bar: 10um.
ABBREVIATIONS

ALS, Amyotrophic lateral sclerosis
Dcr2, Dicer 2
EJP, Excitatory junction potentials
ERG, Electroretinogram
FTLD, Fronto-temporal lobar degeneration
GFP, Green fluorescent protein
GOF, Gain of function
HRP, Horse radish peroxidase
IR, inverted repeat
L3, Third instar
LOF, Loss of function
mEJP, Miniature excitatory junction potentials
NMJ, Neuromuscular junction
RFP, Red fluorescent protein
RRM, RNA recognition motif
RNAi, RNA interference
RT-PCR, Reverse transcriptase polymerase chain reaction
SOD1, Copper/zinc super oxide dismutase 1
TARDBP, Transactive response DNA binding protein
TBPH, TAR DNA binding protein homologue
TDP-43, Transactive response DNA binding protein of 43 kDa
UAS, Upstream activating sequence