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Chronological requirements of TDP-43 function in synaptic organization and locomotive control

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ABSTRACT

Alterations in TDP-43 are commonly found in patients suffering from amyotrophic lateral sclerosis (ALS) and the genetic suppression of the conserved homologue in *Drosophila* (TBPH) provokes alterations in the functional organization of motoneuron synaptic terminals, resulting in locomotive defects and reduced life span. To gain more insight into this pathological process, it is of fundamental importance to establish when during the fly life cycle the lack of TBPH affects motoneuron activity and whether this is a reversible phenomenon. To achieve this, we conditionally expressed the endogenous protein in TBPH minus *Drosophila* neurons and found that TBPH is a short lived protein permanently required for *Drosophila* motility and synaptic assembly through the direct modulation of vesicular proteins, such as Syntaxin 1A, indicating that synaptic transmission defects are early pathological consequences of TBPH dysfunction in vivo. Importantly, TBPH late induction is able to recover synaptogenesis and locomotion in adult flies revealing an unexpected late-stage functional and structural neuronal plasticity. These observations suggest that late therapeutic approaches based on TDP-43 functionality may also be successful for the human pathology.

Introduction

The RNA-binding protein TDP-43 is the primary component of the insoluble protein aggregates present in the majority of patients affected by amyotrophic lateral sclerosis (ALS) and fronto-temporal lobar degeneration (FTLD) (Neumann et al., 2006). More recent studies have shown that dominantly inherited genetic mutations within the coding region of TDP-43 were associated with the neurological symptoms present in some ALS and FTLD patients (Sreedharan et al., 2008). Since then, a large number of successive publications illustrating different aspects of TDP-43 biology and its role in ALS and FTLD have helped to elaborate the rising consensus that TDP-43 protein is causally linked to neurodegeneration.

TDP-43 is a 43 kDa protein of the hnRNP family, its structure and function are conserved through evolution from insects to men. It possesses two RNA recognition motifs (RRM1 and RRM2) that are required for its molecular function in vivo (Buratti and Baralle, 2001, 2012; Godena et al., 2011). In normal tissues, TDP-43 localizes in the cell nucleus together with other RNA processing proteins. On the contrary, in FTLD and ALS affected brains TDP-43 suffers a series of pathological modifications that include abnormal ubiquitylation, phosphorylation, cytoplasmic and nuclear aggregation in sarkosyl-insoluble TDP-43 clusters, proteolytic cleavages yielding TDP-43 C-terminal peptides, and altered intracellular distribution with loss of its typical nuclear localization (Arai et al., 2006; Geser et al., 2009; Neumann et al., 2006). There is some evidence that neurons with TDP 43 cytoplasmic aggregates display a depletion of this protein in the nucleus and hence a probable loss of function. On the contrary, increased levels of TDP-43 were described in the spinal cord and in the cerebrospinal fluid (CSF) of ALS patients (Kasai et al., 2009; Swarup et al., 2011), indicating that variations in the basal levels of this protein are critical for the neuronal function. Evidences in model systems reinforce but by no means prove this hypothesis. However, the interpretation of each of these observations deserves a careful analysis since it is not clear which of these findings could be the direct cause of the disease and which might be secondary to the neurodegenerative process. Thus, despite the efforts towards describing TDP-43 pathology, the original question regarding whether alterations in TDP-43 mediate neurodegeneration through a toxic gain of function or a loss of normal protein activity remains unsolved.

To test these hypotheses and determine the physiological function of TDP-43 in vivo, we have previously generated the loss of function alleles in the *Drosophila* TDP-43 homologue gene TBPH and found that flies without TBPH activity presented uncoordinated movements and

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progressive paralysis (Diaper et al., 2013; Feiguin et al., 2009; Hazelett et al., 2012; Lin et al., 2011). At the molecular level, these functional alterations were accompanied by modifications in the organization of the synaptic microtubules inside the terminals buttons. Moreover, we described that these defects were due to the down-regulation of the microtubule-associated protein MAP1B/*futsch*. This regulation seems to be achieved through a direct interaction of TBPH with *futsch* mRNA, implying that a complete loss or a reduction in TDP-43 function within the cell could lead to ALS or FTLD symptoms through defects in the processing of TDP-43 target mRNAs that codify for specific synaptic or cytoskeleton-associated proteins, essential for neuronal development and/or endurance (Arnold et al., 2013; Godena et al., 2011; Hazelett et al., 2012).

In correspondence with this view, a fundamental question to understand the physiological role of TDP-43 in vivo as well as the mechanisms behind the pathogenesis of ALS and FTLD, is to know whether the alterations described in TBPH minus flies were originated from defects in the protein function during neuronal differentiation, suggesting a temporary role for TBPH in neurodevelopment, or to determine whether TBPH is permanently required in mature neurons to prevent neurodegeneration.

To answer these fundamental questions, we have now used different genetic methodologies like the Gene-Switch and the TARGET technology (McGuire et al., 2004; Nicholson et al., 2008; Poirier et al., 2008) to manipulate TBPH expression in the nervous system at discrete temporal phases and analyze the capacity of the protein to induce or prevent the neurological phenotypes described in TBPH null flies either during development or in the adult life. This approach allowed us to define the critical periods of TBPH function as well as to characterize the early events that lead to neuronal dysfunction in the absence of TBPH.

Materials and methods

Fly strains

All flies were kept on standard cornmeal medium and the genotypes of the used animals are listed below:

W^{1118} (wild type), w; *tbph*$^{\Delta 23}$/CyOGFP, w;;*elav*-GS GAL4, w;*elav*-GAL4, w;;*elav*-GAL4, w;;D42-GAL4, w;GMR-GAL4, w;*tubulin*-GAL80^{TS}, w;;*tubulin*-GAL4, UAS-*D*cr-2, w;UAS-TBPH; w;;UAS-TBPH$^{F/L}$, w;;UAS-mCD8::GFP, w;;UAS-TBPH RNAi (ID38379), w;;UAS-Syx, w;;UAS-TDP-43, w;;UAS-TDP-43$_{A328T}$, w;;UAS-TDP-43$_{M337V}$, and w;;UAS-TDP-43$_{G348C}$. All TDP-43 constructs were Flag tagged and *EcoRI-XbaI* cloned in the pUASTattB plasmid (5xUAS, GenBank: EF362409) and transgenic flies were generated by standard embryo injection (Best Gene Inc.) through PhiC31 integrase-mediated transgenic system. The same estimated cytosite, 86Fa, on the third chromosome has been selected for all constructs (Bischof et al., 2007).

Larval movement

Wandering third instar larvae were selected individually, washed and gently transferred to the center of a Petri dish (94 × 16) with a layer of 0.7% agarose in distilled water. After a period of adaptation (30 s), larvae were analyzed measuring the number of peristaltic waves during a period of 2 min. A fresh Petri dish was used for each genotype (Feiguin et al., 2009).

Climbing assay

Flies were assessed measuring their negative geotaxis movement. Aged synchronized adult flies were transferred in a 50 ml cylinder without anesthesia. After a period of adaptation of 30 s, climbing ability has been scored counting the flies that reach the top of the cylinder (10 cm) in 15 s. Flies were assessed in batches of 20, three trials were performed on each test (Feiguin et al., 2009).

Walking assay

3 day-old flies were placed in a 145 mm Petri dish marked with 1 cm square grid. After a period of adaptation of 30 s, locomotion was quantified as the number of squares crossed during a period of 30 s (Feiguin et al., 2009).

RU486-induction protocols

Feeding larvae

A stock solution of 10 mM RU486 in 95% ethanol was added during fly food preparation. Control groups have been fed in a standard cornmeal medium containing vehicle only (ethanol). Parental flies have been left for 6 h in tubes, during which an age-synchronized embryo population has been collected. Embryos were grown at 25 °C on standard cornmeal medium containing RU486 (0.1 μM). The larvae were kept in drug food for a period in accordance to the needs of the experiment, then were transferred into normal food.

Feeding adults

RU486 was diluted to the final concentration in 2% sucrose and the solution has been added on the surface of a standard cornmeal medium to feed adults. Newborn flies were collected for 6 h and then transferred in tubes containing the drug (Osterwalder et al., 2001; Roman et al., 2001).

Target system

Larvae

We have generated, by standard genetic crosses, flies carrying a single copy of the tubP-GAL80^{TS} construct, a GAL4 driver and a UAS-transgene. We have grown a synchronized population of larvae kept in an incubator at 18 °C to repress GAL4-mediated transcriptional activation of UAS transgene. In mature larvae (L3) stage we induced the RNAi against TBPH performing a temperature shift from 18 °C to 29 °C. For each experiment a group of control (expressing an unrelated transgene) has been shifted at 29 °C and another control group (expressing UAS-target transgene) has been maintained at 18 °C (McGuire et al., 2004).

Adults

Embryonic, larval and pupal stages were maintained at 18 °C, newborn flies were collected for 1 day and then transferred at 29 °C or 31 °C. The same controls used in larvae experiments were applied in adult experiments.

Immunohistochemistry

NMJ larvae were dissected in HL-3 (Feng et al., 2004), fixed for 20 min in 4% formaldehyde (5 min in methanol for anti-GluRIIA) and labeled with antibodies. Images were captured on a Zeiss 510 Meta confocal microscope with a 63× 1.4NA oil lens and labeling intensities normalized to anti-hrp labeling were determined with ImageJ. Numbers of accumulations per synaptic area were manually quantified. The resulting area was normalized to the vesicle marker area. Dilutions of antibodies are reported below: anti-Flag M5 (Sigma 1:200); anti-TBPH (in house 1:200); anti-GFP (Invitrogen 1:200); anti-Syn 3C11c (DSHB 1:15); anti-Syx 8C3s (DSHB 1:15); anti-CSP2c (DSHB 1:50); anti-BRPs (DSHB 1.50); anti-Futsch 22C10s (DSHB 1:50); anti-Dlg 4F3c (DSHB 1:250); anti-Elav (DSHB 1:250); and anti-HRP (Jackson 1:150). Secondary antibodies: Alexa Fluor® 488 (mouse, rabbit 1:500) and Alexa Fluor® 555 (mouse, rabbit, rat 1:500).

Pre- and postsynaptic marker quantification

Larvae used for these analyses were processed simultaneously for immunohistochemistry and confocal images were acquired under

identical condition. Synaptic boutons from muscles 6 and 7 (segment A2) were used for quantitative analysis. Samples were double labeled with anti-HRP and the marker. The mean intensity of both the marker and the hrp has been quantified, and a ratio has been calculated. To quantify the cluster volume of Bruchpilot and Glutamate Receptor IIA a ratio has been calculated between the volume occupied by the marker inside a single bouton and the total volume of the analyzed bouton (adapted from Diaper et al., 2013; Thomas et al., 1997). Images were processed with ImageJ, and then statistically analyzed using Prism.

Western blot

Proteins were extracted from adult heads or larval brains squeezed in a lysis buffer 1×. The material (8–10 μl/head or brain) was processed with a lysis buffer 1× (1.5× Lysis buffer solution: 225 mM NaCl, 15 mM Tris, 7.5 mM EDTA, 15% glycerol, 7.5 mM EGTA, 75 mM NaF, 6 M urea, 7.5 mM DTT and protease inhibitors). Lysates were clarified by a centrifugation step at 6 °C. The quantification of the proteins was performed with Quant-iT™ Protein Assay Kit (INVITROGEN), following the recommended procedure. Extracted proteins were separated in SDS polyacrylamide gels and blotted on 0.2 μm nitrocellulose membrane (Sigma-Aldrich). Membranes were overnight blocked in 5% non-fat dried milk in TBS-T and subsequently incubated with primary antibodies properly diluted: anti-Flag M5 (Sigma 1:1000); anti-TBPH (in house 1:2000); anti-Syn 3C11c (DSHB 1:4000); anti-Syx 8C3s (DSHB 1:2500); anti-CSP2c (DSHB 1:9000); anti-BRPs (DSHB 1:2000); anti-Dlg 4F3c (DSHB 1:15,000); anti-Tubulin (Calbiochem 1:4000); and anti-Actin (Sigma 1:1000). Goat anti-rabbit and anti-mouse IgG HRP conjugated were used as secondary antibodies (Pierce 1:50,000). Detection was done with Femto Super Signal substrate (Pierce). Protein bands were quantified using NIH ImageJ. The intensity of the protein bands was calculated with regard to the equivalent control band. In every lane the mean percentage obtained out of three experiments has been shown normalized to control.

Immunoprecipitation and RNA identification by RT-PCR

The same procedure described in (Godena et al., 2011) has been followed. Briefly, protein G magnetic beads (Invitrogen) were coated with anti-Flag monoclonal antibody (Sigma). The prepared beads and head extracts were mixed and incubated for 30 min at 4 °C. After the washes, bounded RNA transcripts were extracted with DynaMagTM-Spin (Invitrogen). Trizol extraction of RNA has been performed, followed by DNase treatment (Promega RQ1#M610A) and oligo-dT retro-transcription with Superscript III First-Strand Synthesis for RT-PCR (Invitrogen #1808-051). Gene specific primers were designed for amplification:

Synapsin	5′ACCAGACCATCGTACTCAC3′ and 5′CCGAAAATCATATCGGCATCC3′
Syntaxin	5′TGTTCACGCAGGGCATCATC3′ and 5′GCCGTCTGCACATAGTCCATAG3′
csp	5′CCGATAAGAACCCGGACAATG3′ and 5′TCACGGCACAGCAGATAAC3′
rpl-52	5′GAAAATAACAAAGATCTGCTTGGCC3′ and 5′AAGTGGCCCTTGGGCTTC AG3′
rpl11	5′CCATCGGTATCTATGGTCTGGA3′ and 5′CATCGTATTTCTGCTGGAAC CA3′

In order to calculate the enrichment fold, initially, all data were normalized to the respective inputs. The signal was represented by how many more fold increase was measured compared to the control signal. The enrichment was calculated according to the following equation: $\Delta C_T = C_{T(\text{target})} - C_{T(\text{normalized})}$.

The results were derived from three independent immunoprecipitation experiments.

Quantitative real-time PCR analysis

Total RNA was extracted from adult heads of wild type and TBPH minus alleles by using the KIT QIAGEN RNeasy Microarray tissue mini kit (code 73304). cDNA was synthesized using random primer and oligo-dT with Superscript III First-Strand Synthesis for RT-PCR (Invitrogen #1808-051), using the same protocol described above.

Results

Temporal analysis of TBPH requirements in the Drosophila nervous system

To determine the temporal requirements of TBPH function in the nervous system we first of all utilized the Gene-Switch (GS) RU486-inducible GAL4 system to restore transgenic expression of the *Drosophila* TDP-43 homologue in fly CNS at physiological levels in temporally controlled manner (McGuire et al., 2004; Nicholson et al., 2008; Poirier et al., 2008).

For these experiments we generated flies carrying the neuronal specific driver *elav*-GS GAL4 in TBPH minus background (GS-TB: $tbph^{\Delta 23/-}$; *elav*-GS GAL4/UAS-TBPH) and treated GS-TB and wild type flies, with different concentrations of RU486 (0.05 to 0.25 μM) dissolved in the fly food immediately after embryogenesis and throughout the subsequent larval stages. We found that 0.1 μM of RU486 induced transgenic expression of TBPH at comparable levels with the endogenous protein (Fig. 1A) in third instar larval brains (L3). This dose was sufficient to rescue larval locomotion (Fig. 1B), NMJ growth (Figs. 1C–E), MAP1B-futsch protein levels and cytoskeleton organization inside synaptic buttons compared with flies treated with different doses of RU486, ethanol (not induction of transgenic TBPH protein) or flies expressing the RNA-binding defective version of TBPH ($TBPH^{F/L}$) which presented little or no differences compared to TBPH mutants alone (Figs. 1F–J).

Having confirmed the reliability of the Gene-Switch system to rescue TBPH minus phenotypes we used this methodology to define the temporal requirements of TBPH in *Drosophila* locomotion and NMJ formation in vivo.

For these experiments, we induced transgenic TBPH expression in the nervous system of TBPH mutant flies through precise periods of time during development (Fig. 2A) and analyzed the levels of protein expression at the end of the drug treatments as well as at the end of larval period or L3 stages (96–100 h after egg laying [AEL]) (Figs. 2B,C). The genetic rescue capacity obtained by TBPH expression was also evaluated at the end of larval development (Fig. 2D). We found that transient expression of the endogenous protein in TBPH null mutant backgrounds for 48 or 72 h AEL throughout the first and second instar larval stages (L1 and L2 respectively, when axonal growth and synaptogenesis takes place) failed to rescue larval locomotion (Fig. 2D) and NMJ assembly at the end of larvae development (L3) (Fig. 2Fiii,iv), compared with RU486 constitutively treated animals (Figs. 2D,Fvi). Interestingly, western blot analysis on larval brains revealed that transgenic TBPH protein was effectively induced immediately after RU486 treatments but completely disappeared from L3 brains at the end of the third instar larval stages (Figs. 2B,C), indicating that the half-life of the protein was lower than 24 h. In agreement with these results, longer drug treatments (92 hour AEL) showed a gradual reduction of TBPH protein levels from L3 larval brains 6 h after RU486 removal from the fly food, this fact becomes more evident 12 h after drug release to completely disappear by 24 h post-treatment (Fig. 2C). Quantitative analysis of the normalized TBPH/actin intensity values of three different experiments reported in Fig. 2E demonstrated that the half-life of the protein was approximately 12 h, implying that this is a short-lived protein and, moreover, considering the anatomical consequences of TBPH activity in the functional organization of NMJ (Figs. 2D,F) we conclude that TBPH is permanently required in the nervous system to regulate the functional organization of motoneuron synaptic connections.

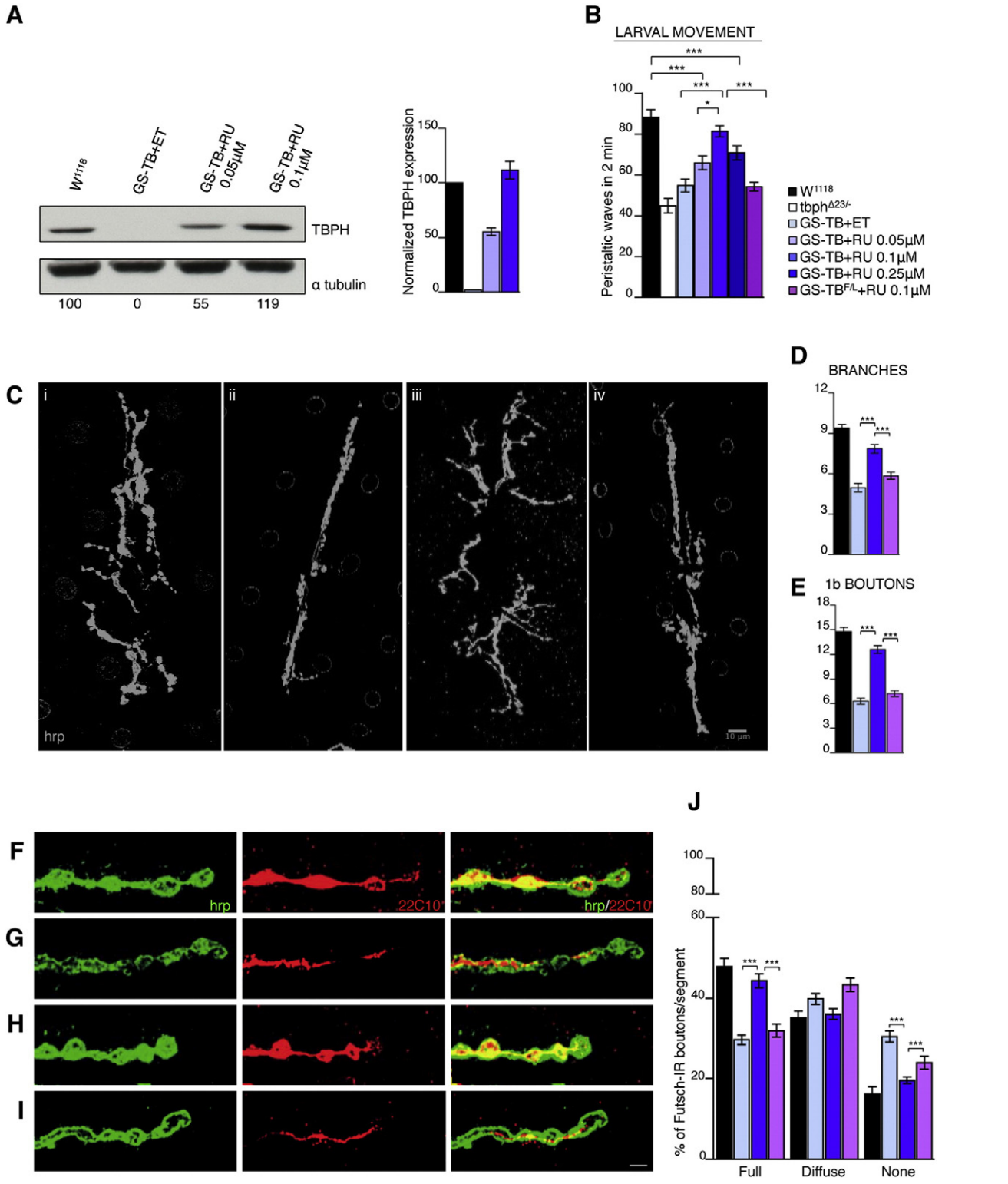


Fig. 1. Targeted TBPH neuronal expression at endogenous levels rescued larval motility, presynaptic grow and microtubule organization. (A) Western blot analysis of L3 brains probed with anti-TBPH and alpha-tubulin showing that 0.1 μM of RU486 induced TBPH transgenic protein expression at equivalent endogenous TBPH levels compared to control or 0.05 μM RU486 treated brains. Genotypes: W^{1118} (control), homozygous TBPH null allele, $tbph^{\Delta 23/-}$; *elav*-GS GAL4/UAS-TBPH (GS-TB). Treatments: GS-TB fed ethanol vehicle (GS-TB + ET) or RU486 (GS-TB + RU) at different concentrations in μM. The histogram represents the quantification of normalized values on tubulin, these quantification numbers are reported below each lane. n = 3. (B) Measurement of peristaltic waves in L3 larvae showed a complete recovery of larval motility using 0.1 μM of RU486 while a lower dose (0.05 μM) was not sufficient and 0.25 μM administration showed toxic effects. Equivalent expression of the RNA binding defective isoform of TBPH, $tbph^{\Delta 23/-}$; *elav*-GS GAL4/UAS-$TBPH^{F/L}$ (GS-$TB^{F/L}$) was unable to rescue the phenotypes. n = 30 larvae, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ calculated by one-way ANOVA. (C) Confocal images of NMJ in muscle 6/7, abdominal segment II in L3 larvae (96–100 h AEL) probed with the neuronal marker hrp, showed a significant rescue of presynaptic branches and 1b boutons in GS-TB + RU 0.1 μM larvae (iii) compared to controls (i), GS-TB + ET (ii), or (iv) GS-$TB^{F/L}$ + RU 0.1 μM. (D,E) Quantification of NMJ morphology. (F–I) Similar confocal images of L3 NMJs showing the distribution of the microtubules-associated protein *futsch* (22C10, red) and hrp (green) in synaptic terminals of (F) controls, (G) GS-TB + ET, (H) GS-TB + RU 0.1 μM and (I) GS-$TB^{F/L}$ + RU 0.1 μM L3 larvae. (J) Quantification of *futsch* staining showed the absence of this protein from distal boutons in GS-TB + ET and GS-$TB^{F/L}$ + RU 0.1 μM compared to controls where *futsch* staining fulfills the inside area of the synaptic boutons as well as in TBPH rescued flies. n = 15 larvae. $^{**}p < 0.01$ and $^{***}p < 0.001$ calculated by one-way ANOVA. Scale bars: (C) 10 μm, (F–I) 5 μm. Error bars indicate SEM.

Acute manipulation of TBPH function in adult flies produced locomotive alterations and reduced the life span

The results described before suggest that acute defects in TBPH function might be sufficient to induce neurological phenotypes in *Drosophila*. This hypothesis is particularly relevant if we consider that in the majority of the patients with ALS the neurological symptoms of the disease appear in already differentiated tissues during adulthood. Therefore, to test this hypothesis we decided to suppress TBPH expression exclusively in adult tissues. For these experiments we decided to

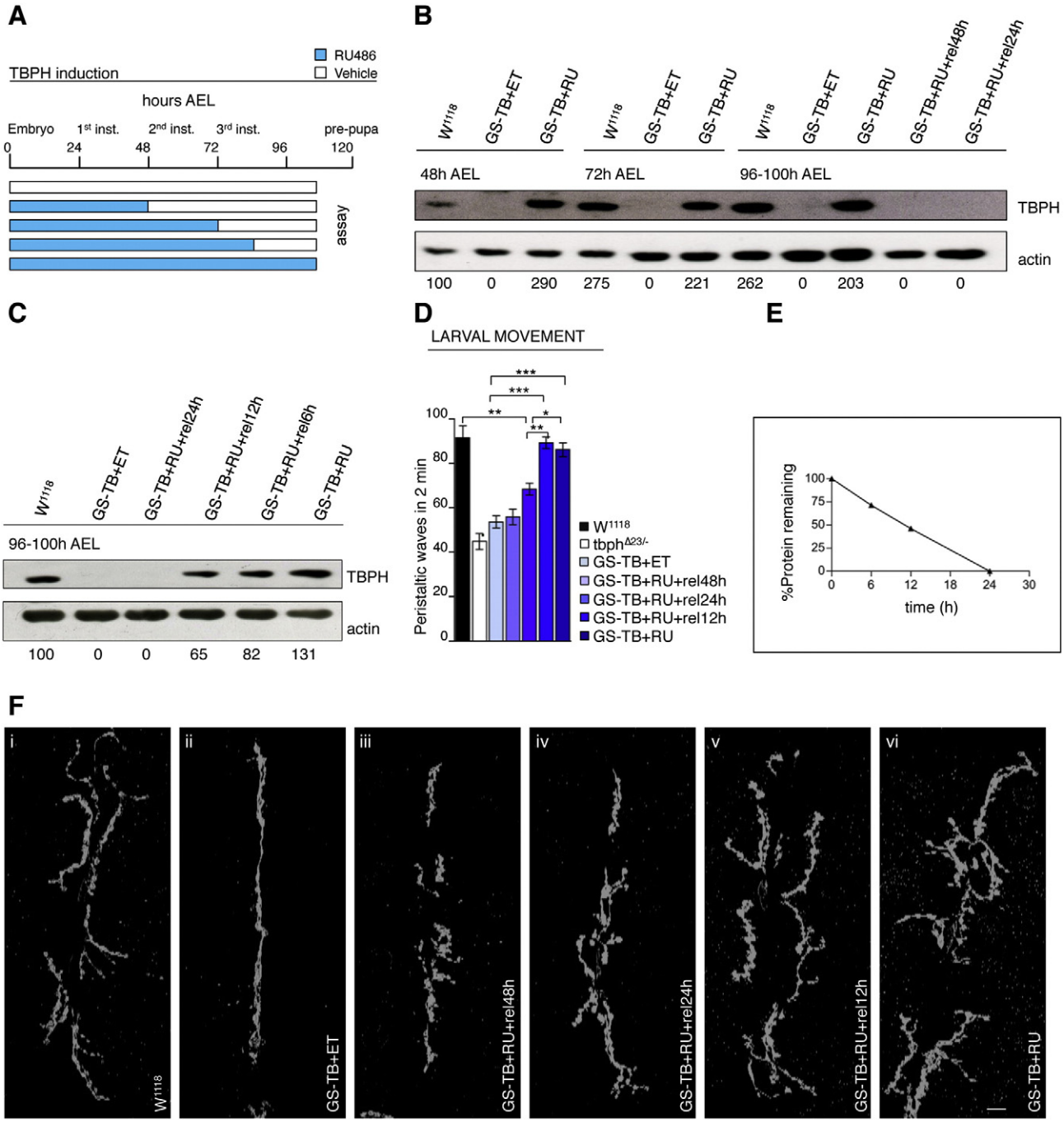


Fig. 2. Conditional TBPH expression revealed the physiological requirements and half-life of this protein in developing neurons. (A) Schematic time lines designed to indicate the early *Drosophila* larval induction of transgenic TBPH show the points of protein analysis. (B) Western blot analysis of larval brains probed with anti-TBPH and Actin. Samples were taken immediately after the 48 h, 72 h and 96/100 h of RU486 or ethanol treatments and the levels of TBPH induction in the CNS were compared with the amounts of the endogenous protein. TBPH induced expression assumed similar levels to the endogenous protein in control brains of second and third instar larvae but appeared undetectable 24 h post-treatment. Quantification of normalized protein amount was reported below each lane. (C) Western blot analysis of third instar larval brains similarly probed for TBPH (upper lane) and actin (lower lane) showed a progressive reduction in transgenic TBPH levels after 6 or 12 h post-treatment. Quantification of normalized protein amount was reported below each lane. (D) Evaluation of peristaltic waves at the end of the third instar larval stage (96–100 h AEL) in response to the temporal expression of the TBPH protein indicates that constitutive expression of the protein or a minimum of 12 hour drug release during the L3 stages guaranteed a complete recovery of larval motility. n = 30 larvae. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ calculated by one-way ANOVA. (E) TBPH protein levels normalized on tubulin at different RU486 release time points were plotted in the graph and the half-life of TBPH protein estimated in around 12 h. n = 3. (F) Analysis of NMJ morphology in L3 larvae (96–102 h AEL) stained with anti-hrp antibodies showed that transgenic expression of TBPH during 48 or 72 h after AEL was not able to rescue motoneuron synaptic terminals (Fiii and iv) compared to wild type (Fi). On the contrary, flies constitutively treated with RU486 (Fvi, GS-TB + RU) or after 12 h post-treatment (Fv, GS-TB + RU + rel12h) were able to completely recover their presynaptic architecture. Scale bar 10 μm. Error bars indicate SEM.

utilize a GS analogous system called TARGET (for temporal and regional gene expression targeting system). This technique is based on the ubiquitous expression of the temperature sensitive version of the GAL80 protein ($GAL80^{TS}$), a very strong GAL4 repressor that allows activation of gene expression by a simple temperature shift (McGuire et al., 2004). In this manner, we used the TARGET system associated to *tubulin*-GAL4 to drive the expression of specific double stranded RNAi against TBPH exclusively in adult fly tissues. For these experiments, we combined TARGET *tubulin*-GAL4 flies with anti-TBPH RNAi strains and UAS-Dcr-2 to improve the RNAi efficiency (TT-TBi: UAS-Dcr-2; *tubulin*-$GAL80^{TS}$/+; *tubulin*-GAL4/TBPH RNAi). TT-TBi flies were grown at 18 °C to maintain the repressive effect of the GAL80 on *tubulin*-GAL4 and adult individuals were shifted to 31 °C to abolish the GAL80 block and induce TBPH-RNAi expression in adult tissues exclusively with *tubulin*-GAL4. As expected, we observed that TT-TBi flies eclosed at 18 °C presented similar levels of TBPH protein in head extracts as control flies expressing Dicer (UAS-Dcr-2) alone (TT-TBc: UAS-Dcr-2; *tubulin*-$GAL80^{TS}$/+; *tubulin*-GAL4/+) indicating that the expression of the RNAi against TBPH was not activated during the period of fly development (Fig. 3A). In agreement with that, flies maintained

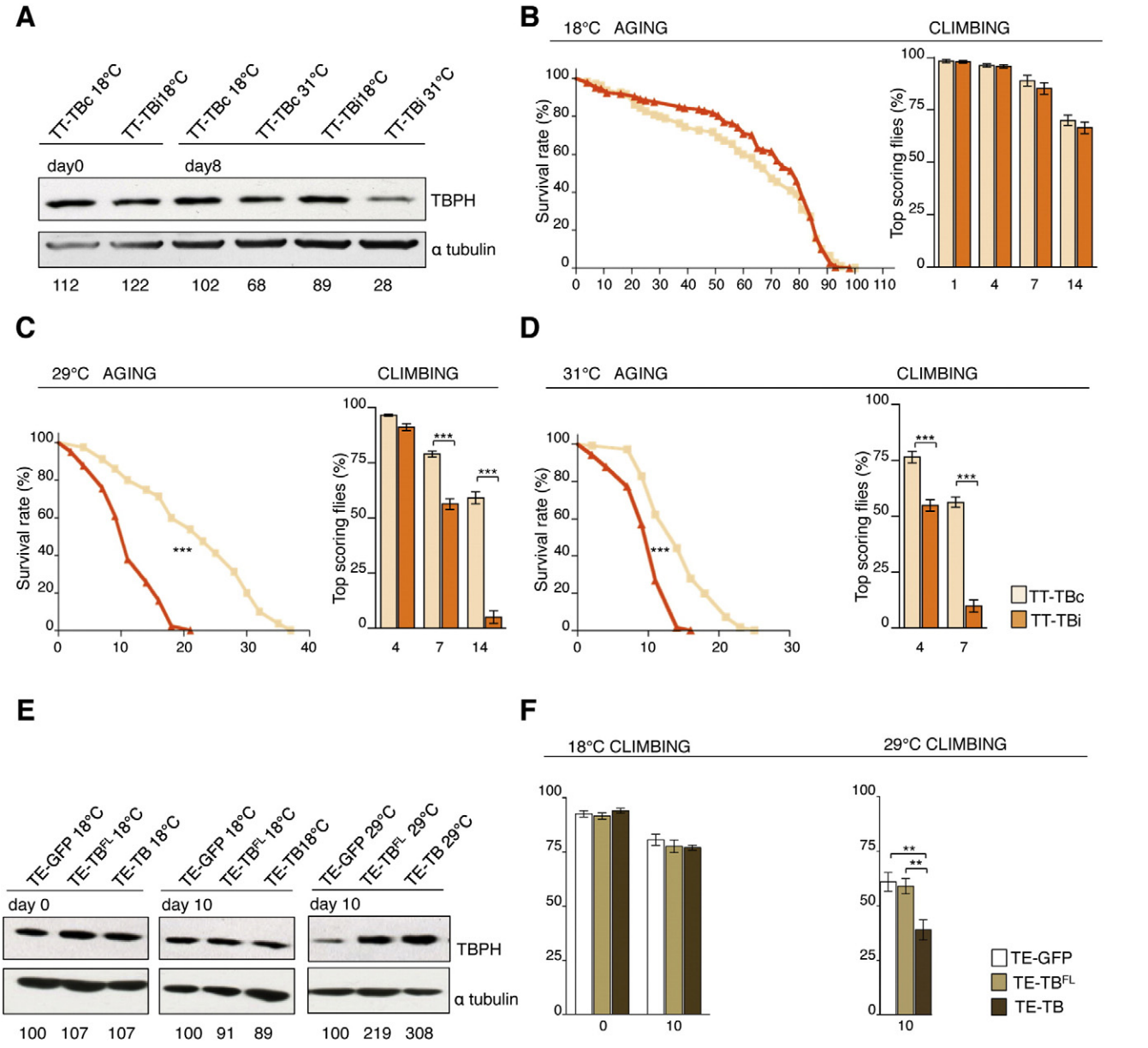


Fig. 3. Targeted suppression and overexpression of TBPH in adult flies provoked neurological defects. (A) Western blot analysis of *Drosophila* adult heads probed with anti-TBPH and alpha-tubulin showed strong reduction of TBPH protein levels after 8 days of TARGET mediated RNAi expression in adult flies at permissive 31 °C temperature compared to controls and flies maintained at restrictive 18 °C temperature. Genotypes: UAS-*Dcr-2*; *tubulin*-$GAL80^{TS}$/+; *tubulin*-GAL4/+ (TT-TBc). UAS-*Dcr-2*; *tubulin*-$GAL80^{TS}$/+; *tubulin*-GAL4/*TBPH RNAi* (TT-TBi). Quantification of normalized protein amount was reported below each lane. n = 3. (B) Life span and climbing assays performed in flies maintained at restrictive temperature (18 °C) show no differences in these traits. (C and D) Life span and climbing assays performed in flies shifted at two different 29 °C (C) and 31 °C (D) permissive temperatures immediately after adult eclosion showed serious neurological alterations in locomotive behaviors with an important reduction in their life span. n = 200, ***$p < 0.001$ calculated by one-way ANOVA, life span ***$p < 0.001$ calculated by log-rank test. Error bars indicate SEM. (E) Western blot analysis on protein extracts of *Drosophila* adult heads overexpressing TBPH in adult neurons with the TARGET system probed with antibodies against TBPH and alpha-tubulin. Do not present alterations in TBPH expression levels after being maintained at restricted temperatures during development (left panel) or for 10 days at 18 °C during adulthood (medium panel). On the contrary, TARGET flies switched during 10 days at permissive temperatures (29 °C in the right panel) show increased levels of TBPH expression. Genotypes: TE-GFP, *tubulin*-$GAL80^{TS}$/UAS-GFP; *elav*-GAL4/+. TE-TB^{FL}, *tubulin*-$GAL80^{TS}$/+; *elav*-GAL4/UAS-$TBPH^{FL}$. TE-TB, *tubulin*-$GAL80^{TS}$/UAS-TBPH; *elav*-GAL4/+. The quantification of normalized protein amounts is reported under each lane. n = 3. (F) Climbing assays performed in TARGET flies overexpressing TBPH. Adult flies shifted at 29 °C immediately after eclosion and maintained during 10 days presented neurological alterations in locomotive behaviors compared to GFP and $TBPH^{FL}$ expressing controls. n = 60, **$p < 0.01$ calculated by one-way ANOVA. Error bars indicate SEM.

at restricted temperatures did not present motility or life span defects compared to controls (Fig. 3B). On the contrary, TT-TBi flies switched at 31 °C immediately after adult flies eclosion showed a dramatic reduction in TBPH protein levels that became more evident after eight days of incubation at permissive temperatures (Fig. 3A) indicating that the TARGET system was able to efficiently reduce TBPH protein expression levels in differentiated adult tissues. More interestingly, we found that TT-TBi flies eclosed at 18 °C and shifted to 29 °C or 31 °C closely reproduced, after few days, the main neurological phenotypes described in TBPH minus flies like locomotive deficiencies in climbing assays and reduced life span (Feiguin et al., 2009). Thus, we observed that TT-TBi flies presented evident locomotion problems in climbing assays after 7 days at 29 °C compared to controls (Fig. 3C). These phenotypes became more evident after 14 days of uninterrupted TBPH-RNAi expression or at more permissive temperatures (Fig. 3D) compared with control TT-TBc flies or TBPH-RNAi flies maintained at restrictive temperatures (18 °C, Fig. 3B). Similar decline in the life span was observed in these assays, demonstrating that the reduction of TBPH expression in adult flies was sufficient to induce these pathological modifications and early neurodegeneration.

Next, we decided to test whether the acute overexpression of the endogenous TBPH protein in adult flies was able to affect locomotive behaviors in climbing assays. For these experiments, we utilized the TARGET system to overexpress TBPH in adult fly neurons with the *elav*-GAL4 (TE-TB flies: *tubulin*-GAL80^{TS}; *elav*-GAL4/UAS-TBPH) by incubating these flies at non-permissive temperatures (18 °C) during development and moving the adult flies to 29 °C. Thus, we observed that flies raised at 18 °C and maintained at this temperature during more than 10 days did not present differences in the levels of TBPH expression compared to GFP expressing flies (Fig. 3E). On the contrary, we observed that adult flies similarly developed at 18 °C and moved immediately after eclosion to 29 °C for about 10 days presented increased levels of TBPH expression and serious neurological defects in climbing assays compared with flies overexpressing GFP (TE-GFP: *tubulin*-GAL80^{TS}; *elav*-GAL4/UAS-GFP) or the TBPH^{FL} isoform of TBPH, which is unable to bind the RNA (Fig. 3F, TE-TB^{FL}: *tubulin*-GAL80^{TS}; *elav*-GAL4/UAS-TBPH^{FL}). These experiments indicated that an acute increase in TBPH activity in adult stages was able to affect fly locomotion by a pathological mechanism that depended on the RNA-binding capacity of the protein. In the same direction, the data strongly supports the idea that the expression levels of TBPH should be tightly regulated in order to prevent neurological alterations during the adult life.

Neurological deficits caused by lack of TBPH can be rescued through the late expression of the protein in mature neurons

A fundamental question for potential TDP-43 based therapies to target ALS/FTD is to know whether a late correction of TDP-43 function could revert or improve the neurological symptoms. Therefore, we analyzed next whether the reintroduction of this protein in mature animals could rescue the neurological and structural phenotypes observed in TBPH loss of function flies. To perform these experiments, we utilized the Gene-Switch system described above to induce the late expression of transgenic TBPH protein in TBPH minus neurons at mature third instar larval stages (Fig. 4A). Thus, GS-TB flies were treated with either RU486 or vehicle (ethanol) during 12 or 24 h at mature third instar (L3) larval stages until the end of the larval period (96–100 hour AEL, see Fig. 4A). The analysis of protein expression following late activation of the *elav*-GAL4 promoter showed a consistent recovery of the TBPH protein levels in mature larval brains treated with 1 mM of RU486 compared to ethanol treated controls (Fig. 4B). The examination of late rescued larvae revealed that 12 h of RU486 treatment was sufficient to induce a dramatic recovery of the normal locomotive behaviors in these flies compared to control insects expressing the TBPH isoform that is unable to bind RNA (GS-TB$^{F/L}$) or ethanol treated flies (Fig. 4C).

Most importantly, the NMJ organization in late rescued larvae (after 24 h of promoter activation) showed a clear re-elaboration of motoneuron terminals with the formation of new synaptic branches and the addition of new synaptic boutons (Figs. 4D–I). We also observed that TBPH minus synaptic boutons recovered their normal *futsch*-positive structural organization that included the formation of new cytoskeletal loops (Figs. 4Fiii, J). These structural modifications did not recover in ethanol treated (Figs. 4Eiii, J) or GS-TBPH$^{F/L}$ (Figs. 4Giii, J) expressing controls, demonstrating that a plastic recovery of the neuronal function and synaptic organization is possible in differentiated TBPH mutant neurons at mature larval stages.

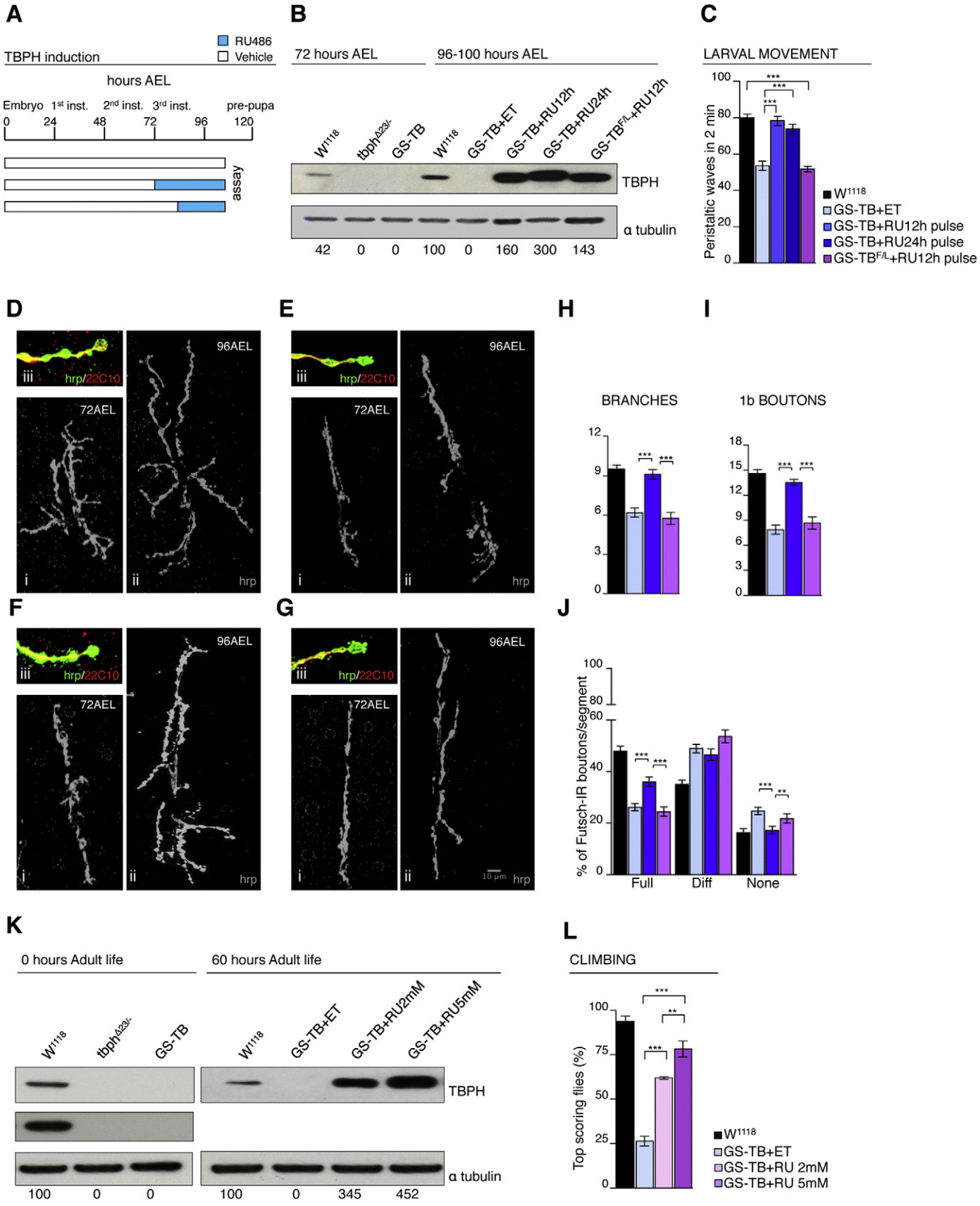
Even though the nervous system of L3 larvae is formed by highly mature and differentiated neurons, we still decided to determine whether the neurological recovery observed in late larvae was also possible in adult flies. For these experiments, we treated immediately eclosed GS-TB adult flies with two different doses of RU486 in the fly food and analyzed by Western blot the expression levels of TBPH induction in head samples exposed to 60 h of RU486 ingestion compared with untreated or ethanol treated controls (Fig. 4K). In this experiment, we observed that the expression of the TBPH protein in *tbph* mutant backgrounds induced an extraordinary and robust recovery of the climbing abilities in these animals (Fig. 4L). In particular, these experiments showed that 2 mM RU486 treated GS-TB flies recovered approximately 60% of their climbing capacities while 5 mM treated flies recovered more than 80% of their motility (Fig. 4L). These observations demonstrate that a late functional recovery of neuronal activity in adult stages was possible in *tbph* minus flies and suggest that the lack of TDP-43 function may not produce persistent defects in motoneuron function or motor circuit assemble.

Downregulation of presynaptic vesicular proteins is an early event of TBPH dysfunction in vivo

A second fundamental issue regarding the neurodegenerative diseases is to understand what are the series of events that lead to neuronal dysfunction and, in the case of TDP-43-mediated pathology, the initial modifications that may lead to ALS symptoms and neuronal death. To determine the premature alterations produced by the lack of TBPH function in *Drosophila* neurons we, therefore, decided to characterize the early alterations provoked in motoneuron synaptic terminals after the acute silencing of TBPH function in vivo.

For these experiments, we used the previously described TARGET system to induce the conditional expression of a double stranded RNAi against TBPH in mature third instar larval neurons. The anti-TBPH RNAi treatments commenced immediately after these flies reached the L3 larval stages. For these experiments TE-TBi *Drosophila* embryos (UAS-Dcr-2; *tubulin*-GAL80^{TS}, tbph$^{\Delta 23}$/+; *elav*-GAL4, TBPH-RNAi/+) were cultured at restricted temperatures (18 °C) and collected at mature third instar larval stages. Mature L3 larvae were posteriorly shifted to permissive 29 °C temperatures in order to activate the expression of the RNAi against TBPH. We observed that TE-TBi L3 flies cultured at 18 °C did not present differences in the brain levels of TBPH protein compared with TE-TBc controls (UAS-Dcr-2; *tubulin*-GAL80^{TS}, tbph$^{\Delta 23}$/+; *elav*-GAL4/+). On the other hand, we found that TE-TBi flies after 24 h of temperature switch showed a strong reduction in the neuronal levels of the endogenous TBPH compared to controls (Fig. 5A). Moreover, we observed that this reduction in protein expression was sufficient to induce locomotive defects in TE-TBi larvae compared to controls (Fig. 5B). In contrast with the phenotypes observed in TBPH null L3 larvae, these alterations in fly motility were not associated with anatomical modifications in the number of synaptic boutons or motoneuron terminal branches at NMJs except for the presence of subtle alterations in the morphology of the synaptic boutons in TE-TBi flies compared to controls (Figs. 5C–G), suggesting that functional alterations in synaptic activity or muscles innervation may precede the anatomical defects described in TBPH null flies. In order to test this hypothesis, we analyzed

whether short time suppression of TBPH in presynaptic neurons affected the expression and/or distribution of molecules involved in synaptic transmission at *Drosophila* NMJs (Keshishian et al., 1996; Majumder and Krishnan, 2010). To achieve this, TARGET TBPH-RNAi treated L3 flies and GFP expressing controls were dissected 24 h after RNAi induction and the intracellular distribution of synaptic protein levels analyzed by immunocytochemistry. We observed that essential components of the secretory synaptic machinery like syntaxin (syx), (Wu et al., 1999)



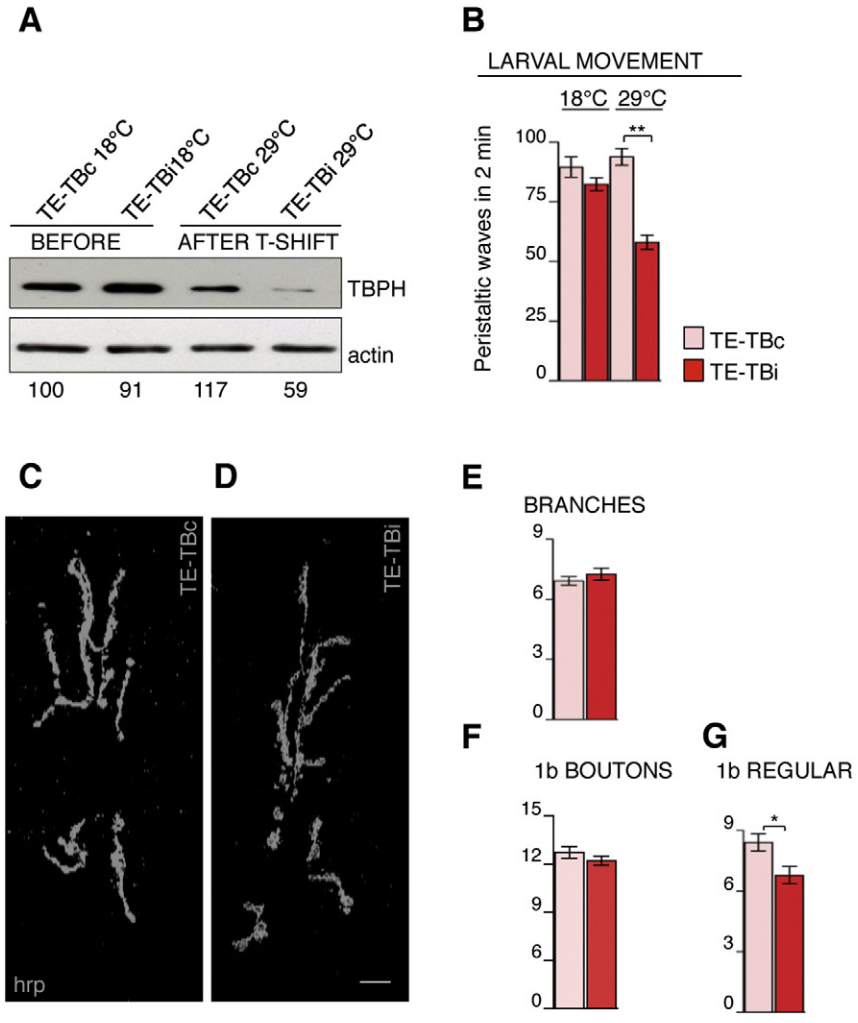


Fig. 5. Acute suppression of neuronal TBPH function in L3 larvae provokes locomotive defects. (A) Western blot analysis on larval brains probed for TBPH and actin showed strong reduction in endogenous TBPH protein after 24 h of RNAi activation in third instar larval neurons using the TARGET system (genotypes: TE-TBc = UAS-*Dcr-2*; $tbph^{\Delta 23}$, *tubulin*-GAL80^{TS}/+; *elav*-GAL4/+ and TE-TBi + 24 h = UAS-*Dcr-2*; $tbph^{\Delta 23}$, *tubulin*-GAL80^{TS}/+; *elav*-GAL4/TBPH-RNAi). Quantification of normalized protein amount was reported below each lane. n = 3. (B) Acute suppression of TBPH by RNAi expression in L3 larval neurons during 24 h induced a drastic reduction in larval motility. n = 30, $^{**}p < 0.01$ calculated by one-way ANOVA. (C and D) Analysis of NMJ morphology in L3 larvae (96–100 h AEL) stained with anti-hrp antibodies in (C) TE-TBc and (D) TE-TBi. Images showed that acute silencing of TBPH (24 h) in neurons does not alter the morphology and the number of branches and boutons, quantification shown respectively in (E) and (F). Only a mild significant alteration of bouton shape has been detected, quantification in (G). n = 15 larvae, $^{*}p < 0.05$ calculated by T-test. Scale bar 10 μm. Error bars indicate SEM.

(Fig. 6A) and synapsin (syn), (Hilfiker et al., 1999) (Fig. 6B) appeared significantly downregulated 24 h after the neuronal suppression of TBPH. Intriguingly, similar experimental conditions induced less significant modifications in the presynaptic levels of cysteine string protein (csp), (Dawson-Scully et al., 2007) (Fig. 6C). We also found that the downregulation of these synaptic markers was progressive and highly dependent of TBPH function since the constitutive expression of the TBPH-RNAi during the complete larval development exacerbated these phenotypes (Figs. S1A–D). Interestingly, we found that the acute suppression of presynaptic TBPH did not affect the expression of proteins involved in the structural organization of the synapses like Bruchpilot (brp) (Wagh et al., 2006; Wichmann et al., 2008) (Fig. 6D), a protein required in the organization of the active zones at *Drosophila* synaptic terminals. In particular, we observed that neither the distribution of brp nor the levels of protein intensity were modified in the synaptic terminals of short term TBPH suppressed flies or in constitutively TBPH-RNAi treated insects (Figs. 6D and S1D), although we cannot exclude the presence of more subtle morphological changes at the level of the T-bars. Western blot analysis performed in *Drosophila* adult heads confirmed this data showing a strong reduction in the total protein levels of the vesicular synaptic markers syx, syn and csp in TBPH minus neuronal tissues compared to wild type controls (Fig. 6E). On the contrary, we did not observe

Fig. 4. Late TBPH expression in already well differentiated neurons rescued synaptic grow and fly locomotion. (A) Schematic time line of TBPH induction. (B) Western blot of L3 brains probed with anti-TBPH and alpha-tubulin, before (72 h AEL) and after (96–100 h AEL) drug addition showed no protein detection before drug administration compared to 12 or 24 h of drug treatment. Quantification of normalized protein amount was reported below each lane. n = 3. (C) Evaluation of peristaltic waves in L3 larvae after 24 and 12 h of TBPH induction showed a significant increase in larval motility compared to similar treatment using TBPH$^{F/L}$ isoform. n = 30, $^{***}p < 0.001$ calculated by one-way ANOVA. (D–G) Confocal images of NMJ in muscle 6/7, abdominal segment II probed for hrp at 72 AEL (i), 96 AEL (ii) and hrp with *futsch* to observe synaptic bouton detail (square green and red, iii). (D) W1118 showed a robust presynaptic growth in W^{1118} L3 larvae from 72 to 96 hour AEL through the addition of newly form *futsch* positive (22C10, red in detail) synaptic boutons. (E) GS-TB + ET larvae presented reduced synaptic bouton formation and *futsch* levels. After 24 h of TBPH induction in GS-TB + RU flies, a vigorous regrowth of the synaptic structures was observed (F) compared to GS-TB$^{F/L}$ + RU treated flies (G). Scale bar 10 μm. (H) Quantification of NMJ terminal branches, (I) 1b boutons and (J) *futsch* intracellular distribution (cytoskeleton), 24 h after TBPH activation in L3 larvae from 72 to 96 hour AEL, showed that late induction of TBPH was able to recover NMJ morphology to wild type equivalent condition. n = 15 larvae, $^{**}p < 0.01$ and $^{***}p < 0.001$ calculated by one-way ANOVA. (K) Western blot of adult heads probed for anti-TBPH and alpha tubulin before RU486 induction (left panel) and after 60 h of TBPH induction (right panel). Quantification of normalized protein amount was reported below each lane. n = 3. (L) Climbing assay of late rescued adults showed a significant, dose dependent, recovery of locomotive abilities 60 h after TBPH activation. n = 200, $^{**}p < 0.01$ and $^{***}p < 0.001$ calculated by one-way ANOVA. Error bars indicate SEM.

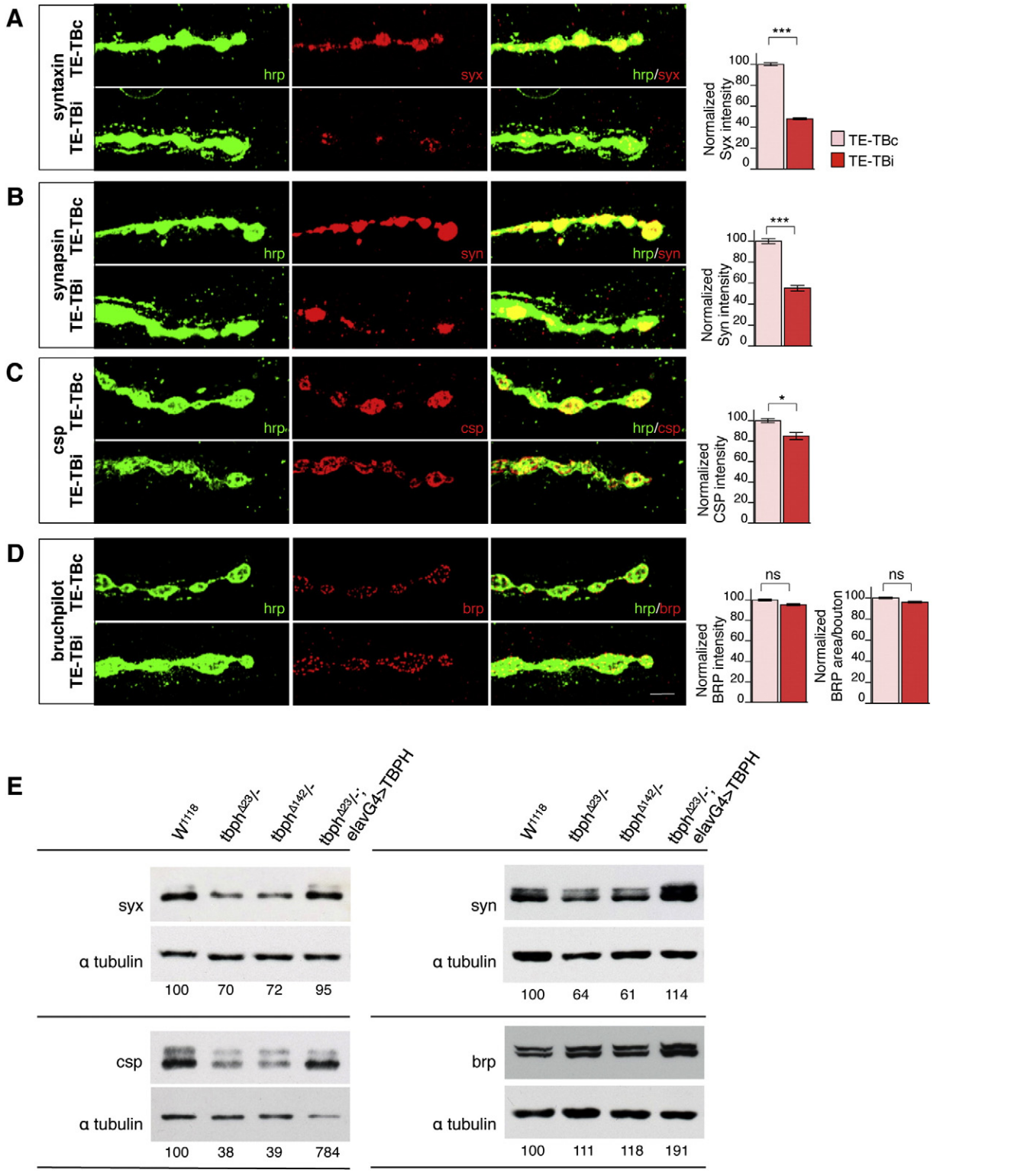


Fig. 6. Acute TBPH suppression in L3 larval neurons induced the downregulation of different presynaptic vesicular proteins. (A) Confocal images of NMJs presynaptic boutons in muscle 6/7, II segment, compare control, third instar TE-TBc larvae stained with anti-hrp (green) and anti-syntaxin (red), with acute silenced TBPH L3 larvae TE-TBi + 24 h where syx appear strongly reduced in the synaptic terminals. These differences were quantified in the graph. (B) Similarly, control TE-TBc larvae stained with anti-hrp (green) and anti-synapsin (red) presented a significant reduction in synapsin levels compared to TE-TBi + 24 h larvae and quantified in (B). (C) A mild decrease in csp staining was observed between TE-TBc controls and TE-TBi + 24 h acute silenced larvae. (D) No differences instead were found in the intracellular levels or presynaptic distribution of brp in control neurons versus acute silenced flies as reported in the quantification of brp intensity pattern (left graph) and receptor area (right graph). n = 250/300 boutons, $^{*}p < 0.05$, $^{***}p < 0.001$ calculated by T-test. Scale bar 5 μm. (E) Western blot analysis of adult heads stained with anti-syx (upper left), anti-syn (upper right), anti-csp (bottom left), anti-brp (bottom right) and anti-alpha tubulin as a loading control (lower panel) independently confirms the specific reduction of presynaptic vesicular markers in TBPH minus flies ($tbph^{\Delta 23}$ and $tbph^{\Delta 142}$, second and third lines) compared to wild type (first line) and TBPH rescued neurons ($tbph^{\Delta 23}$, UAS-TBPH/$tbph^{\Delta 23}$, *elav*-GAL4, in the fourth line). On the contrary, no changes in protein levels were observed for the structural protein brp. Quantification of normalized protein amount was reported below each lane. n = 3. Error bars indicate SEM.

modifications in brp levels between these different genotypes (Fig. 6E), indicating that these presynaptic proteins may present a differential sensitivity to TBPH function.

Finally, genetic rescue experiments confirmed this idea by showing that neuronal expression of the transgenic TBPH protein in TBPH minus flies was able to reestablish the wild type levels of the downregulated syx, syn and csp proteins in adult heads (Fig. 6E) demonstrating that TBPH posses an important role in the functional organization of the presynaptic terminals and suggesting that defects in vesicular membrane-dependent events, like exocytosis, may initiate the pathological symptoms observed in TBPH minus *Drosophila*.

The presynaptic function of TBPH is required for the differentiation and maintenance of the postsynaptic structures

During the formation of neuromuscular junctions, the activity of the presynaptic neurons is permanently required to induce the differentiation of postsynaptic structures in the surface of the subjacent muscles (Bloch and Pumplin, 1988). This neuronal function mainly involves the release of different signals, including neurotransmitters, through vesicular exocytosis, suggesting that the alterations in presynaptic motoneurons described above may lead to defects in the formation of postsynaptic structures and muscle denervation.

To address this hypothesis, we decided to analyze the distribution of different postsynaptic markers in L3 larval NMJs after 24 h of presynaptic TBPH suppression. In these experiments we used immunocytochemistry to study the distribution of Dlg, a PDZ domain scaffold molecule important for the localization of numerous postsynaptic proteins including the glutamate receptors (GluRs) (Chen and Featherstone, 2005). We observed that in wild type NMJs Dlg concentrates around the presynaptic terminals in the postsynaptic zone, surrounding the individual synaptic boutons and establishing well defined structures in the muscular surface (Koh et al., 1999) (Fig. 7A). In contrast, we found that the acute suppression of presynaptic TBPH induced dramatic modifications in the organization of Dlg which appeared strongly reduced and abnormally distributed, unable to define single synaptic structures like surrounding large groups of boutons or even absent, uncovering big areas of the presynaptic surface (Fig. 7A). We also found that these early defects in Dlg distribution became progressively worst in flies expressing anti-TBPH RNAi from the beginning of the neuronal development (Fig. S1E), indicating that these modifications are highly dependent of the presynaptic levels of TBPH expression.

Similarly, we found that the distribution of the postsynaptic receptor GluRIIA was affected 24 h after the presynaptic expression of TBPH-RNAi. In particular, we appreciated that the GluRIIA localizes in well-defined spots with a ring-shaped outline in wild type controls (Fig. 7B) while in L3 larvae treated with TBPH-RNAi for 24 h, the pattern of GluRIIA staining became more diffuse and irregular with significantly less ring-shaped spots (Fig. 7B). Identical results were found in TBPH-RNAi constitutively treated neurons (Fig. S1F), indicating that TBPH function is continuously required in presynaptic motoneurons to maintain the differentiation of the postsynaptic structures. In support of these observations, we found that transgenic expression of TBPH exclusively in neurons was sufficient to rescue the postsynaptic defects in Dlg and GluRIIA distribution observed in TBPH minus NMJs (Figs. S2A–J), confirming that early defects in motoneuron neurotransmission may lead to muscles denervation in *Drosophila*.

TBPH binds syntaxin mRNA to regulate postsynaptic differentiation and fly locomotion

What remained to be determined was whether the downregulation of these synaptic proteins was caused directly by TDP-43 or represented secondary events after TDP-43 depletion. In order to address the possibility that TBPH may interact with the mRNAs of syx, syn or csp in vivo we immunoprecipitated neuronal expressed Flag-tagged TBPH with *elav*-GAL4 from *Drosophila* heads and utilized real time quantitative PCR (qRT-PCR) to measure the mRNA enrichment in these samples compared to immunoprecipitations performed using the unrelated protein dREEP or, more representatively, the RNA-binding impaired isoform of TBPH, called TBPH$^{F/L150-152}$ (Fig. 8A) (Godena et al., 2011).

Interestingly, we observed an enrichment of syx mRNA in TBPH immunoprecipitated samples (2.5 folds) compared to syn, csp or the housekeeping genes rpl-52 and rpl-11 (Fig. 8A), indicating that TBPH may preferentially interact with syx mRNA but not with the messengers of syn or csp. In agreement with this view, we observed that TBPH-syx mRNA interactions are lost when similar immunoprecipitations were performed utilizing the RNA-binding defective form of TBPH$^{F/L150-152}$ demonstrating that these physical interactions are rather specific. Moreover, we observed in both loss of function alleles, tbph$^{\Delta 23}$ and tbph$^{\Delta 142}$, a significant and consistent reduction in the amounts of syx mRNA in *Drosophila* adult heads by qtPCR, compared to wild type control flies (Fig. 8B). Regarding these results, it is interesting to note the presence of different clusters of putative TBPH binding sequences (TG)n

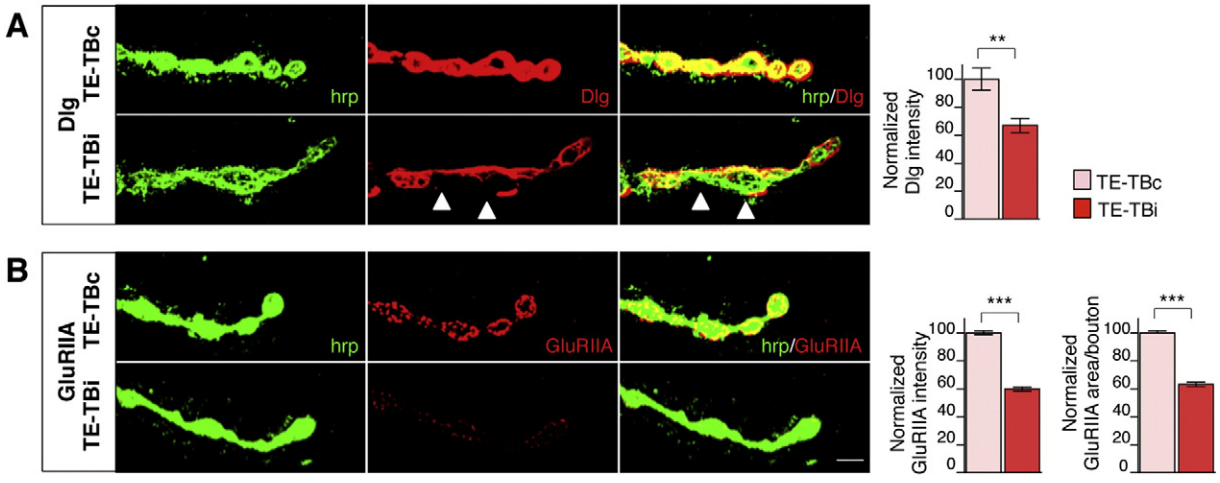


Fig. 7. Acute suppression of presynaptic TBPH in L3 larvae affects the maintenance of postsynaptic structures. (A) Confocal images of synaptic terminal boutons stained with anti-hrp (presynaptic membranes) in green and with anti-Dlg in red (postsynaptic membranes), in control (TE-TBc = UAS-*Dcr-2*; *tbph*$^{\Delta 23}$, *tubulin*-GAL80^{TS}/+; *elav*-GAL4/+) and in acute 24 h silenced TBPH (TE-TBi = UAS-*Dcr-2*; *tbph*$^{\Delta 23}$, *tubulin*-GAL80^{TS}/+; *elav*-GAL4/TBPH-RNAi) show a strong reduction in Dlg intensity with empty areas in the side of the synaptic boutons. The quantification is reported in the graph. (B) Equivalent alterations were also found in the distribution of the glutamate receptor IIA between controls and acute silenced TBPH, where GluRIIA appears diffuse or even disappears from the postsynaptic zone, quantification of intensity (left panel) and area (right panel) differences are showed in graphs. n = 250/300 boutons, $^{**}p < 0.01$, $^{***}p < 0.001$ calculated by T-test. Scale bar 5 μm (A). Error bars indicate SEM.

both within the 5′UTR and the coding sequence of the syx gene (Colombrita et al., 2012). These indications together with the co-IP results plus the observed reduction of syx mRNA and protein levels observed in TBPH null flies (Fig. 6E) would strongly suggest that the function of TBPH may contribute to stabilize the subcellular levels of syx mRNA and/or to possibly promote its translation.

Considering the role of syx in membrane exocytosis these data strongly suggest that the synaptic defective phenotypes observed in TBPH mutant flies might be influenced by the downregulation of syx levels in vivo.

In order to test this possibility, we analyzed whether transgenic expression of syx in the TBPH minus backgrounds was able to rescue the neurological problems induced by the lack of TBPH function in vivo. Consequently, we observed that neuronal expression of syx with *elav*-GAL4 in TBPH null flies ($tbph^{\Delta 23/-}$, *elav*-GAL4; UAS-Syx) was able to efficiently recover the locomotive defects observed in TBPH minus L3 larvae compared to control flies rescued with GFP (Fig. 8C). Moreover, we observed that syx-rescued motoneurons were able to recover the structural defects observed in the distribution and localization of the postsynaptic protein Dlg (Figs. 8D–G) as well as the formation of the postsynaptic clusters of GluRIIA (Figs. 8H–K), absent in TBPH minus terminals. Taken together, these experiments demonstrate that the regulation of syx levels through the direct interaction with TBPH is fundamental to promote the formation and differentiation of neuromuscular synapses.

Intriguingly, we observed that the rescue of syx levels with *elav*-GAL4 was not sufficient to recover the pupal lethality associated with the loss of TBPH function (Feiguin et al., 2009). Nevertheless, we observed that syx expression in adult TBPH hypomorphic alleles, generated by the induction of anti-TBPH RNAi expression in neurons ($tbph^{\Delta 23}$, *elav*-GAL4/+; *UAS-Dcr-2, TBPH RNAi*/UAS-Syx), was able to significantly recuperate the locomotive capacity of these flies in climbing assays compared to GFP expressing controls ($tbph^{\Delta 23}$, *elav*-GAL4/+; *UAS-Dcr-2, TBPH RNAi*/UAS-GFP) (Fig. S3A) confirming the relevance of these regulatory interactions in adult flies.

Discussion

Although a common characteristic of many neurodegenerative diseases is the late appearance of the neurological symptoms during the adult life, it is largely unknown whether these alterations originate from pathological defects that occurred during neuronal development or later on, in the already differentiated nervous system. In particular, in the case of ALS and FTD, the temporal requirements for TDP-43 activity in axon growth, synaptic formation, transmission and maintenance are still unknown. Clarifying these issues might be essential to understand ALS pathogenesis, prognosis and evolution, essential aspects required to design diagnostic tools and effective therapeutic interventions. Our work, has addressed these two issues using inducible fly systems that can either shut-off or recover TBPH expression at different time points during the developmental cycle. In this respect, therefore, our study mainly focused on the potential consequences of the loss-of-function of this protein in disease. As discussed previously, however, another possible way through which TDP-43 could play a role in disease is represented by its eventual overexpression and subsequent pathological consequences due to gain-of-function mechanisms. Regarding this, many *Drosophila* models described up to now and recently reviewed by us (Romano et al., 2012) have tried to address this issue by overexpressing human TDP-43 either wild-type or expressing disease-associated mutants in flies. In all these cases, the overexpression of wild type or mutated TDP-43 had severe consequences, including the loss of neurons and an age-dependent reduction in motility. How these two mechanisms act in human disease and whether they act through the same mechanisms/pathways are not currently known. Nonetheless, the observation that both can co-exist suggests that in *Drosophila* and humans the levels of TDP-43 must be tightly regulated to ensure that at all times its expression does not fall or increase over a critical level.

Chronological requirements of TBPH function and neurological plasticity

To study the chronological requirements of TBPH function we utilized the very well characterized Gene-Switch and TARGET systems in *Drosophila* to induce the conditional expression of the TBPH protein in TBPH null background.

First of all, we observed that constitutive expression of TBPH in neurons during *Drosophila* development could completely rescue motoneuron presynaptic grow and cytoskeleton organization with the complete recovering of normal locomotive behaviors and synapse differentiation. On the contrary, we found that restricted expression of TBPH immediately after embryogenesis during very well defined developmental larval stages (24, 48 and 72 h after AEL) was not sufficient to restore neither fly motility nor synaptic pattern at the end of the larval period (96–100 hour AEL). Western Blot analysis demonstrated that TBPH was effectively induced in these flies but the protein was not able to persist till the end of the larval period, suggesting that TBPH may represent a short lived protein. In agreement with this idea, we found that longer pulses of TBPH induction (84–90 h after AEL) allowed us to recover the structural functionality of the NMJs and to detect the presence of the protein at the end of the larval period, indicating that TBPH may present a half-life of approximately 12 h in neurons and demonstrating that its presence was permanently required during neuronal development for the functional assemble of the neuromuscular synapses in vivo. Interestingly, it should be noted that this half-life of TBPH is extremely similar to the one recently determined for human TDP-43 wild type (12.6 h) in differentiated neuronal cells using a pulse chase approach (Watanabe et al., 2013). This further highlights the similarities between the fly and human homologues.

These results, however, did not clarify whether similar requirements for TBPH function could also be necessary to guarantee the proper regulation of locomotive behaviors in adult flies. To test this hypothesis we decided to reduce the activity of TBPH exclusively in adult tissues by expressing an RNAi against TBPH using the TARGET system. Unlike Gene-Switch, the TARGET system allows gene expression by modifying the environmental temperature and we reasoned that this situation would favor the stable expression of the anti-TBPH RNAi in adult flies independently of their feeding or locomotive behaviors. In this way, we observed that the acute expression of the RNAi in adult tissues induced a marked decrease of the endogenous levels of TBPH in adult heads compared with similar samples maintained at restricted temperatures. As expected, we found that RNAi expressing flies presented strong alterations in climbing assays and in the expected life span compared to controls. These observations indicated that the abrupt reduction of TBPH activity in adult flies was sufficient to produce a severe neurological phenotypes similar to the neurological defects observed in ALS patients and suggested that incidental defects in TBPH activity during any stage of the adult life could decisively contribute to the appearance of the main symptoms of the disease.

Secondly, a fundamental question in the ALS field is to know whether it is possible to recover the neurological phenotypes and/or prevent the development of the disease by the late reintroduction of TDP-43 activity. For these experiments, we used Gene-Switch to induce TBPH expression at the end of the larval development adding the activator RU486 in the food in adult flies. Using these approaches, we found that the expression of TBPH protein in differentiated L3 neurons was able to rescue the synaptic defects detected throughout the larval development, promoting the regrowth and reassemble of motoneuron functional synapses together with the functional recovery of the alterations detected in larval motility.

We have also found that similar induction of TBPH expression in adult neurons was able to dramatically reactivate the locomotive

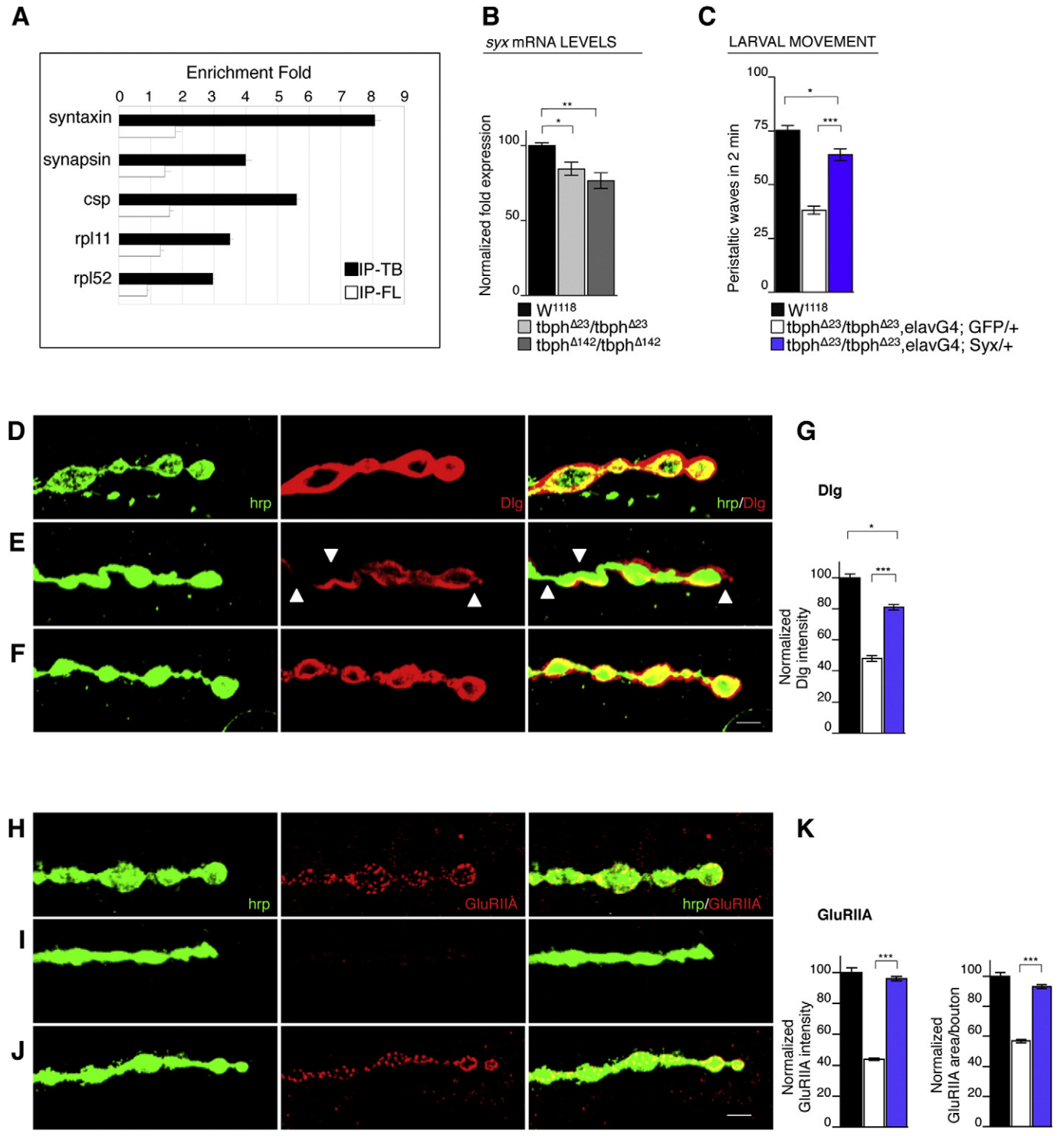


Fig. 8. Neuronal expression of syntaxin rescued larval motility and synaptic defects in TBPH minus flies. (A) qRT-PCR analysis of mRNAs immunoprecipitated by Flag-tagged TBPH (IP-TB) and its mutant variants TBPH $^{F/L}$ (IP-FL). The enrichment-folds of the two housekeeping genes *rpl-52* and *rpl-11* are 2.96 and 3.50 times respectively for IP-TB with an average of 3.23 times of enrichment. Considering this value as the background values for IP-TB, we can conclude that there is no enrichment for *synapsin* (enrichment level 3.99, 1.2 folds of the background level), a slight enrichment for csp (enrichment level 5.60, 1.7 folds of the background level), and a consistent enrichment for syntaxin, whose enrichment level is 8.07 2.5 folds of the background level. Consistently with the inability to bind RNA of the mutant variant TBPH $^{F/L}$ there is no enrichment at all for none of the IP-FL. n = 3. Error bars indicate SD. (B) Real-time PCR quantifications of the *syntaxin* transcript levels in the heads from wild type and the two TBPH minus alleles. Three independent experiments were quantified and the average is plotted in the graph. $^{*}p < 0.05$, $^{**}p < 0.01$ calculated by one-way ANOVA. (C) Quantification of peristaltic waves of third instar larvae expressing syx protein in neurons of TBPH mutant background rescued larval motility compared to GFP rescued controls (genotypes: $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-Syx/+ and $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-GFP respectively). n = 30. $^{*}p < 0.05$, $^{**}p < 0.01$ calculated by one-way ANOVA. (D–F) Confocal images of synaptic boutons stained with anti-hrp (green) and with anti-Dlg (red), (D) wild type, (E) $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-GFP/+, (F) $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-Syx/+, show recovery of the postsynaptic organization and intensity levels of Dlg compared with GFP rescued flies that conserve areas devoid of Dlg staining (arrowheads), the quantification of these events is showed in (G). (H–J) Confocal images of synaptic terminals stained with anti-hrp (green) and with anti-GluRIIA (red). (H) Wild type, (I) $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-GFP/+, (J) $tbph^{\Delta 23}$, *elav*-GAL4/$tbph^{\Delta 23}$; UAS-Syx/+, show rescue of glutamate receptor levels, postsynaptic cluster distribution and organization in syx expressing flies compared to GFP rescued controls. (K) Shows the quantification of GluRIIA intensity pattern (left graph) and receptor area (right graph). n = 300/350 boutons, $^{**}p < 0.01$, $^{***}p < 0.001$ calculated by one-way ANOVA. Scale bar 5 μm. Error bars indicate SEM.

behaviors in the otherwise paralyzed insects, demonstrating that a long lasting neuronal plasticity remained in TBPH depleted flies. Additionally, we observed that late rescue experiments of TBPH minus phenotypes required higher levels of TBPH protein expression compared to the endogenous TBPH amounts present in wild type control brains. The origin of these differences is not completely clear but they may indicate that neurons depleted of TBPH during long periods may have lost their sensitivity to the protein function and longer intervals are required to reactivate the activity of these neurons.

Our results clearly show that late corrections of TBPH activity in neurons are possible and sufficient to recover the neurological defects detected in already well differentiated mature flies. These observations indicate that therapeutic strategies aimed to re-establish or compensate TDP-43 function could successfully correct neurological defects providing a pathway to reversibility that should be explored in the therapy of human ALS patients.

TBPH function is essential for pre and postsynaptic differentiation

Taking advantage from the experimental design that allowed us to manipulate the levels of TBPH expression in a well defined temporal manner, we decided to analyze the initial events that lead to neurodegeneration immediately after TBPH downregulation. Our experiments showed that the acute reduction of TBPH levels induced an immediate drop in L3 larval locomotion that correlated with a strong decrease in the synaptic levels of the vesicular proteins syx, syn and csp that was also confirmed in flies constitutively expressing the anti-TBPH RNAi during the complete period of larval development or in adult flies carrying loss-of-function mutations in TBPH (Figs. S1A–D and 6A–D).

These results strongly suggest that vesicular exocytosis might be affected in presynaptic neurons treated with anti-TBPH RNAi and predict that pathological modifications in synaptic transmission may occur at the nerve-muscle contacts. To test this hypothesis, we therefore analyzed whether the molecular organization of the post-synaptic membranes was affected in the RNAi treated larvae. We found that the subcellular distribution of both dlg and GluRIIA receptors was highly disorganized after 24 h of anti-TBPH RNAi treatments in L3 larvae, confirming that defects in NMJ maintenance and/or complications in muscle innervation constitute early events in the degenerative process that take place after TBPH loss.

In summary, this work shows that TBPH function is permanently required in presynaptic motoneurons to induce the differentiation and maintenance of the NMJs and prevent locomotive defects. This is a situation that is probably not unique to the fly system, because recent analyses in adult mice have shown that TDP-43 can be found at the presynaptic membrane of axon terminals in the neuromuscular junction (Narayanan et al., 2013). In addition, just like in flies, the analyses of mice transcripts following RIP-Chip have shown that in mice TDP-43 binds to RNAs that codes for genes related to synaptic function. This, together with the localization of TDP-43 protein at axon terminals, suggests a role for TDP-43 in the transport of synaptic mRNAs into distal processes (Narayanan et al., 2013). Considering the extremely high functional similarity between human TDP-43 and *Drosophila* TBPH, these results suggest that similar alterations could be expected in individual suffering of TDP-43 dysfunction and indicate that *Drosophila melanogaster* may represent a suitable system to directly test the functional consequences of the ALS-associated modifications observed in this protein.

For the reasons described above, we consider that therapeutic designs aimed to potentiate synaptic transmission or maintain the physiological activity of TDP-43 function may predictably ameliorate the symptoms of the disease.

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