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AN AGE-RELATED REDUCTION OF BRAIN TBPH/TDP-43 LEVELS PRECEDES THE ONSET OF LOCOMOTION DEFECTS IN A *DROSOPHILA* ALS MODEL

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Abstract—Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. The average age of onset of both sporadic and familial cases is 50–60 years of age. The presence of cytoplasmic inclusions of the RNA-binding protein TAR DNA-binding protein-43 (TDP-43) in the affected neurons is seen in 95% of the ALS cases, which results in TDP-43 nuclear clearance and loss of function. The *Drosophila melanogaster* ortholog of TDP-43 (TBPH) shares many characteristics with the human protein. Using a TDP-43 aggregation inducer previously developed in human cells, we created a transgenic fly that shows an adult locomotive defect. Phenotype onset correlates with a physiologically age-related drop of TDP-43/TBPH mRNA and protein levels, seen both in mice and flies. Artificial reduction of mRNA levels, *in vivo*, anticipates the locomotion defect to the larval stage. Our study links, for the first time, aggregation and the age-related, evolutionary conserved reduction of TDP-43/TBPH levels with the onset of an ALS-like locomotion defect in a *Drosophila* model. A similar process might trigger the human disease.

Key words: TDP-43/TBPH levels, aggregation, *Drosophila*, ALS.

INTRODUCTION

The major histopathological feature, seen in around 95% of amyotrophic lateral sclerosis (ALS) patients, is the presence of cytoplasmic TAR DNA-binding protein-43 (TDP-43) inclusions in the affected neurons (Ling et al., 2013; Gomez-Deza et al., 2015), resulting in TDP-43 nuclear clearance and loss of TDP-43 nuclear function, which in turn may lead to cellular dysfunctions (Neumann et al., 2006; Arai et al., 2006).

TDP-43 is a protein that has a marked tendency to unfold and become insoluble both *in vitro* and *in vivo*. In particular, its C-terminal end has a so-called "prion-like domain" between amino acids 331–369 (Udan and Baloh 2011; Nonaka et al., 2013). This sequence is rich in Glutamine (Q) and Asparagine (N), and is involved both in the interactions with other proteins and the self-aggregation process (D'Ambrogio et al., 2009; Igaz et al., 2009; Budini et al., 2012; Budini et al., 2014). We have previously developed a cellular model of aggregation using the TDP-43 Q/N rich amino acid sequence repeated 12 times linked to the EGFP sequence. These cellular inclusions were shown to be capable of sequestering the endogenous TDP-43 both in non-neuronal and neuronal cells (Budini et al., 2012).

The link between aggregate formation and loss of function, to date is still not fully understood. It has been suggested that the late onset of ALS in humans may be due to mitochondrial dysfunction, ineffective unfolded protein response and defects in the autophagy or proteasome systems (Ling et al., 2013; Cozzolino and Carrì, 2012). Indeed, if the clearance system is defective there will be persistence of aggregates in the cell. However, this does not provide an explanation for why the aggregates may persist for many years with no ALS-related symptoms. In fact, 29% of normal individuals over 65 years old present TDP-43 inclusions in the brain (Geser et al., 2010). Furthermore, there is still no answer to the question of why mutations in TDP-43 or other TDP-43-ALS related genes do not anticipate the onset of the symptoms in human patients.

In this work, we have used the EGFP-12xQ/N aggregation model to explore the connection between the aggregate formation and the onset of the disease. We present here data indicating that the combination of physiological age-related reduction of TAR DNA-binding protein-43 *Drosophila melanogaster* ortholog (TBPH)/TDP-43 levels with the presence of aggregates, that sequester this protein in the cytoplasm, leads to a locomotion defect due to the depletion of functional TDP-43 in a *Drosophila* ALS model.

EXPERIMENTAL PROCEDURES

Fly stocks

EGFP-12xQ/N (12 repetitions of the TDP-43 amino acids stretch 331–369 sequence tagged with EGFP) and EGFP constructs were cloned in the pUASTattB vector. All the

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Abbreviations: 12xQ/N, repeated TDP-43 amino acid sequence 331–369; ALS, amyotrophic lateral sclerosis; CNS, central nervous system; TDP-43, TAR DNA-binding protein-43; TBPH, TAR DNA-binding protein-43 *Drosophila melanogaster* ortholog.

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constructs have been sequenced and subsequently used to create transgenic flies by standard embryo injections (Best Gene Inc.). Specific insertion of the transgene was performed using strain 24486. Transgenic flies have been subsequently balanced on the required chromosome.

RNA interference flies for TBPH (ID 38377) were obtained from VDRC Vienna. W1118 and ELAV-Gal4 were obtained from Bloomington *Drosophila* Stock Center at Indiana University. Flies were fed on standard cornmeal (2.9%), sugar (4.2%), yeast (6.3%) fly food, maintained and crossed in a humidified incubator at 25 °C (unless stated otherwise) with a 12 h–12 h light–dark cycle.

Climbing assay

Flies were transferred without anesthesia to a 50 ml glass-cylinder, taped to the bottom of the tube, and their subsequent climbing activity was quantified as the number of flies that reach the top of the tube in 15 s. At least 100 flies of each genotype were tested. In each set of experiments 20 flies (1:1 male:female ratio) were introduced in the cylinder and tested in triplicate. The number of top climbing flies was converted into % value, and the mean % value (±SEM) was calculated for at least five experiments.

Life span

Adult flies were collected for 2 days and transferred to tubes with food. Each tube contained 20 flies in a proportion 1:1 male:female ratio. Every third day, flies were transferred to a fresh tube and deaths were scored. Survival rate was plotted as percentage of survival flies against day. Approximately 100 flies were tested of each genotype.

Larval movement

Wandering third instar larvae were selected, washed and transferred to a Petri dish with a layer of 0.7% agarose. After a period of adaptation, the peristaltic waves were counted within 2 min. At least 20 larvae from each genotype were counted, and the mean was calculated.

Drosophila immunoblotting

Drosophila heads were homogenized in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% Glycerol, 50 mM NaF, 5 mM DTT, 4 M Urea, and protease inhibitors (Roche Diagnostic # 11836170001, Mannheim, Germany)).

Larval brains were dissected in Phosphate Buffer with 0.1% Tween 20 (PBT) and collected in Loading Buffer (30 mM Tris, pH 6.8, 9% Glycerol, 2.4% SDS, 3% 2-mercaptoethanol, 0.03% bromophenol blue) and homogenized.

Proteins were separated by 8% SDS–PAGE, transferred to nitrocellulose membranes (Whatman # NBA083C, Clifton, USA) and probed with primary antibodies: rabbit anti-TBPH (1:1500, home-made), mouse anti-synapsin (1:4000, Hybridoma Bank # 3C11, Iowa, USA) and mouse anti-tubulin (1:4000, Calbiochem # CP06, Billerica, USA). The membranes were incubated with the secondary antibodies: HRP-labeled anti-mouse (1:1000, Thermo Scientific # 32430, Rockford, USA) or HRP-labeled anti-rabbit (1:1000, Thermo Scientific # 32460). Finally, protein detection was assessed with Femto Super Signal substrate (Thermo Scientific # 34095).

Protein bands were quantified using NIH ImageJ. The intensity of the band of interest was normalized with tubulin. The respective histogram for each western blot shows the relative expression of at least two independent experiments.

Mouse immunoblotting

Whole brains were homogenized with mechanical agitator (ForLab, Bergamo, Italy) in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris HCl, pH8, 2× protease inhibitors (Roche Diagnostic # 11836170001)). Total protein extracts were separated by 10% SDS–PAGE, transferred to nitrocellulose membranes (Whatman # NBA083C) and probed with primary antibodies: rabbit anti-TDP43 (1:1000, Proteintech # 10782-2-AP), rabbit anti-synapsin I (1:1000, abcam # ab18814), rabbit anti GAPDH (1:1000, Santa Cruz # sc-25778). The membranes were incubated with the secondary antibodies: HRP-labeled anti-rabbit (1:1000, Thermo Scientific # 32460). Finally, protein detection was assessed with ECL Western Blotting Substrate (Thermo Scientific # 32106). Protein bands were quantified using NIH ImageJ. The intensity of the band of interest was normalized with GAPDH. The histogram for the western blot shows the relative expression of at least three independent experiments.

RNA extraction and real-time PCR

Total RNA was extracted from *Drosophila* heads (25 °C) with TRIzol reagent (Invitrogen # 15596-026) according to manufacturer's instructions. Regarding mouse brain total RNA, the same protocol was performed with the exception that the whole brain was homogenized using a mechanical agitator (ForLab, Bergamo, Italy) in TRIzol prior to RNA extraction. cDNA was synthesized starting from 1 μg of total RNA by using Superscript III (Invitrogen # 18080-044) reverse transcriptase and OligodT primers. Specific primers were designed to amplify TBPH (forward: 5′-CGGCAAGCCGAGCACGAT GAG-3′, reverse: 5′-CGCGGAGTTCGCTCCAACGAG-3′) and mouse TDP-43 (forward: 5′-GCAGTCCA GAAAACA-3′, reverse: 5′-CACCATCGCCCATCTA-3′). The expression levels were assessed by real-time PCR using Rpl-11 as fly housekeeping gene (forward: 5′-CCA TCGGTATCTATGGTCTGGA-3′, reverse: 5′-CATCG TATTTCTGCTGGAACCA-3′) and GAPDH as mouse housekeeping gene (forward: 5′-AGGTCGGTGT GAACGG-3′, reverse: 5′-AGTCATACTGGAACAT-3′). All amplifications were done on CFX96 real-time PCR detection system (Biorad) using SYBR Green technology (Biorad # 720000601). The relative expression levels were calculated according to the Livak

method (Schmitten and Livak 2008), using the equation $\Delta C_T = C_{T(target)} - C_{T(normalizer)}$ for Ct normalization; and the difference between $\Delta C_{T(test)}$ and $\Delta C_{T(calibrator)}$ to calculate the expression ratio and compare the expression levels. Statistical significance was calculated using student's *t*-test (indicated as * for $P \leqslant 0.05$ and ns for not statistically significant).

Mouse brain RNA Northern Blotting

Total RNA from mouse brains was isolated as already described in the real-time PCR section. RNA samples (20 μg) were loaded on 1% formaldehyde agarose gels, transferred to Hybond N^+ nylon membranes (Amersham Biosciences # RPN119B) and probed with internally ^{32}P-labeled sequences following pre-hybridization in ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion # AM8670). Pre-hybridization and hybridization were carried out at 40 °C. The probes were generated by PCR using primers Mus TDP-43 ex2 Fw 5′-atgggacggtgttgctgt-3′ and Mus TDP-43 ex3 Rv 5′-agtctttcagatcctgct-3′ and labeled with Rediprime II DNA Labeling System (GE Healthcare # RPN1633). The probe to detect GAPDH was generated using the same primers as for the mouse brain real-time PCR. Visualization of transcripts was carried out with a Cyclone Plus Storage Phosphor Scanner and the included OptiQuant Software (Perkin Elmer).

Statistics

Statistics were performed using GraphPad Prism. One-way ANOVA followed by Bonferroni's multiple comparison was used to compare measures among three groups. Unpaired *t*-test analysis was used to compare measures between two groups. Long rank test was performed to compare survival distribution between genotypes. The significance between the variables was shown based on the *p*-value obtained (ns indicates $p > 0.05$,* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$). Values are presented as a mean and error bars indicate standard error means (SEM), unless otherwise stated.

RESULTS AND DISCUSSION

The expression of the TBPH aggregate inducer in *Drosophila* CNS does not hamper normal development

In this study we have exploited our previous work on *Drosophila* models for TDP-ALS (Feiguin et al., 2009; Romano et al., 2014; Cragnaz et al., 2014), and created a fly TDP-43/TBPH aggregation model centered on the cellular inclusions of EGFP-12xQ/N. This aggregation model was previously shown (Budini et al., 2012; Budini et al., 2014) to be capable of sequestering TDP-43 in cell lines. To construct the transgenic fly expressing EGFP-12xQ/N, the construct coding for the protein was placed under the control of the upstream activating sequence (UAS). After crossing the transgenic line EGFP-12xQ/N with the pan-neuronal ELAV-Gal4 (Brand and Perrimon, 1993), brain neurons showed co-localization of *Drosophila* TDP-43 (TBPH) with EGFP-12xQ/N aggregates, as previously reported in the eye tissue (Cragnaz et al., 2014). The flies developed normally, and no early neurological toxicity caused by these aggregates was observed. This is shown by the identical larval peristaltic waves count both at 25 °C and 29 °C (the higher temperature accelerates aging in the flies), in larvae expressing EGFP-12xQ/N or EGFP alone (Fig. 1).

EGFP-12xQ/N expression triggers an age-related locomotion defect in *Drosophila*

After the pupae stage, the adult flies were kept either at 25 °C or 29 °C. Also in the adult fly brain, TBPH co-localized with EGFP-12xQ/N aggregates (data not shown). The locomotion phenotype was measured by climbing assay at regular intervals. As shown in Fig. 2, the climbing ability at 25 °C of the transgenic flies constitutively producing aggregates starts to be significantly lower than the control at 14 days. The flies kept at 29 °C display a climbing deficit already at day 3. The survival curves follow a similar tendency, with the 25 °C and 29 °C flies having, respectively, a half-life 20% and 40% shorter than the EGFP control.

The locomotion defect follows an age-related decrease in TBPH levels

A peculiarity of the flies was that notwithstanding the constitutive production of EGFP-12xQ/N, the resulting aggregates do not result in a phenotypic consequence until adulthood. Previous reports have shown that TDP-43 levels decrease with age in different regions of the mouse brain such as cerebellum, forebrain and brainstem (Huang et al., 2010; Liu et al., 2015). It was then decided to investigate whether this programed reduction of TDP-43 levels arose early in evolution and can be observed also in non-mammalian species, specifically in *Drosophila* TBPH expression levels. As it can be seen in Fig. 3a, the relative TBPH levels decrease during aging in wild-type fly heads at 25 °C and 29 °C. It should

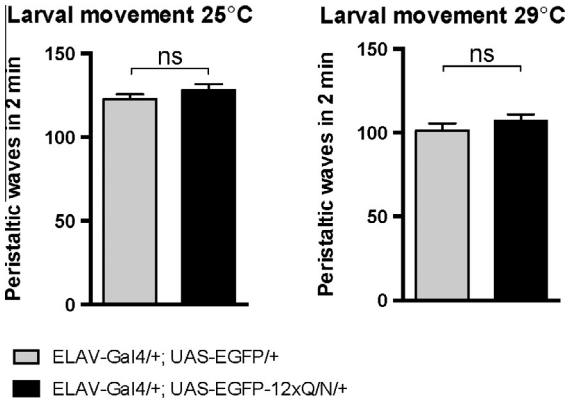

Fig. 1. EGFP-12xQ/N expression generates no early neurological toxicity. Quantification of peristaltic waves of third instar larvae, expressing EGFP-12xQ/N or EGFP alone under ELAV-Gal4 driver. More than 20 larvae from each genotype were counted, and the mean was calculated. ns indicates $p > 0.05$ (not significant) calculated by unpaired *t*-test. Error bars indicate SEM.

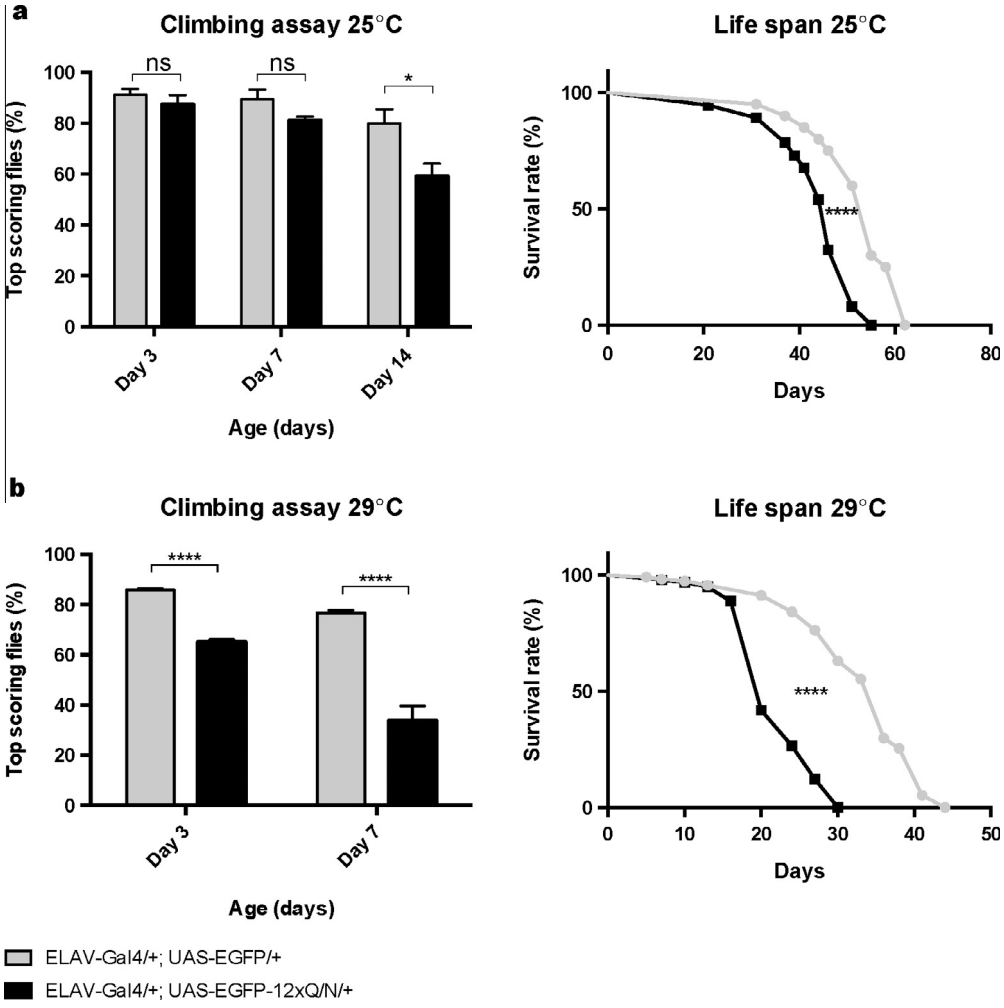

Fig. 2. The climbing ability and percentage of survival of EGFP-12xQ/N flies are compromised during aging. (a) Climbing assay and life span analyses at 25 °C (b) and 29 °C. Unpaired t-test analysis was used to compare measures between two groups. Long rank test was performed to compare survival distribution between genotypes. ns indicates $p > 0.05$ (not significant), * indicates $p < 0.05$, **** indicates $p < 0.0001$. Error bars indicate SEM.

be noted that the TBPH levels decrease faster in the case of accelerated aging (29 °C). Interestingly, the final drop (4-fold lower than in the immediate after pupa stage) coincides with the time point where the locomotive defect starts to be visible in the transgenic EGFP-12xQ/N fly (indicated by an arrow). Furthermore, the same trend in the levels of endogenous TBPH during aging was also confirmed in EGFP-12xQ/N- and EGFP-expressing flies (Fig. 3b). Fig. 3c confirms previous reports of an age-related decrease of TDP-43 protein levels in mouse brain samples following a pattern similar to the fly.

To discard an age-related neuronal loss we compared in fly and mouse the variations of TBPH/TDP-43 levels during aging with the fluctuation in levels of synapsin, a strictly neuronal protein (Figs. 3a, c). Data clearly indicate that the reduction of brain TBPH/TDP-43 levels is not due to significant neuronal loss as in that case a similar decrease in synapsin would have been observed. Furthermore, synapsin levels were previously shown to increase during mouse development between days 5 and 20 (Bogen et al., 2009). In addition, we are analyzing relatively young age groups not expected to have any significant neuronal loss. This observation is also consistent with what has been reported to occur in humans (Wickelgren 1996).

The lower protein levels correspond to lower mRNA levels measured by real-time PCR both in flies (Fig. 3d) and mouse (Fig. 3e, left panel), indicating that there is a pre-translational mechanism for age-programed reduction of TDP-43/TBPH levels. The drop in TDP-43 levels in mouse brain was further confirmed by Northern Blot analyses (Fig. 3e, right panel).

Our data strengthen the hypothesis that the decrease of TBPH/TDP-43 levels represents an evolutionary conserved feature characteristic of aging of the organisms.

Constitutive reduction of TBPH levels from birth anticipates the locomotive defect in EGFP-12xQ/N expressing flies

If low TBPH levels seen with aging predispose to the locomotion defects observed upon expression of EGFP-12xQ/N, it follows that the sensitivity to the presence of

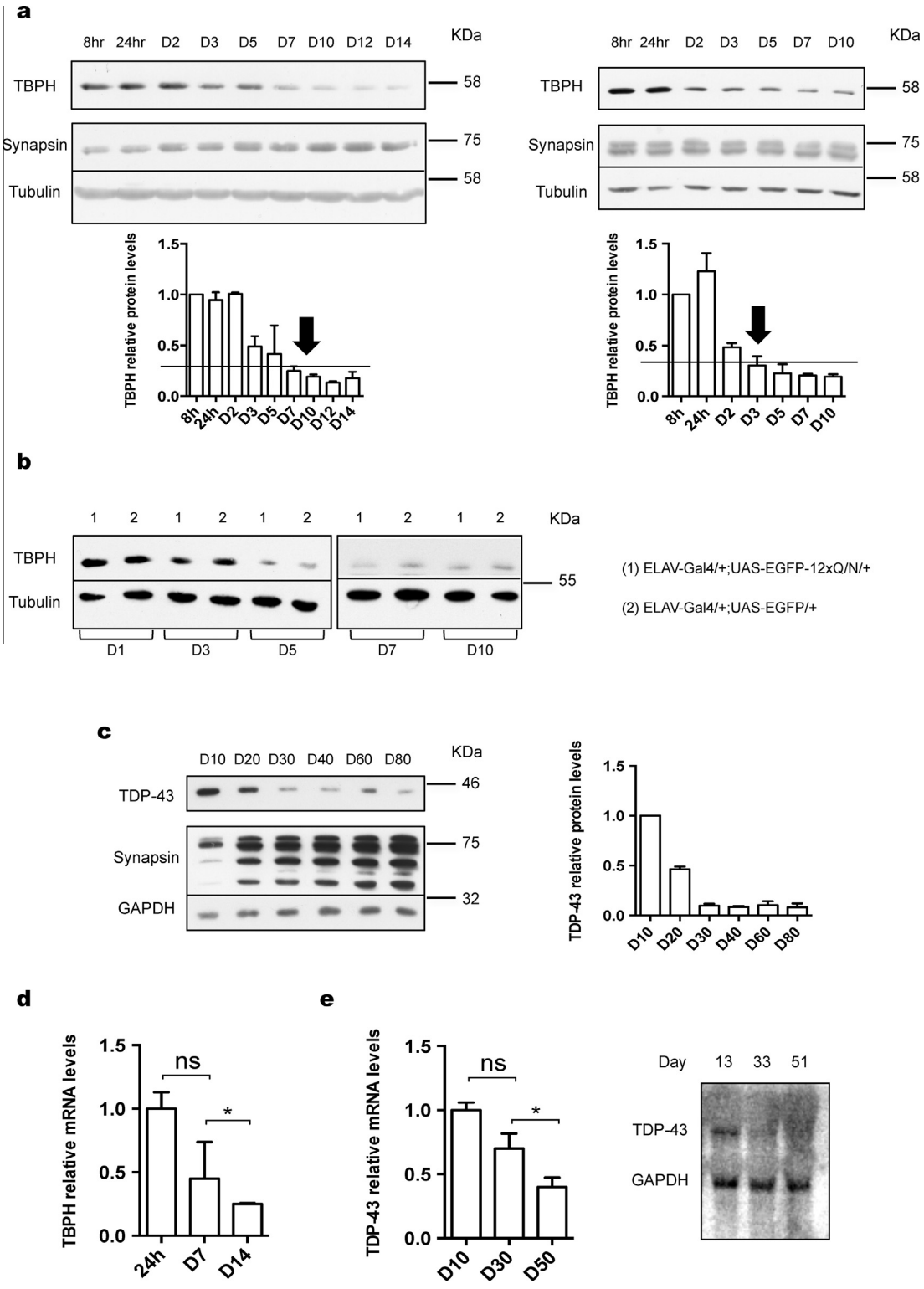

Fig. 3. *Drosophila* and mouse TDP-43 levels drop during normal aging. (a) Western blot analyses showing endogenous TBPH and synapsin levels from adult wild-type fly heads aged at 25 °C (left panel) and 29 °C (right panel). The histograms show the TBPH expression levels normalized with tubulin. D stands for day. The arrow indicates the point where the climbing ability of the flies is significantly reduced (see Fig. 2). Error bars indicate SD. (b) Western blot analyses showing endogenous TBPH levels from adult (1) ELAV-Gal4/+; UAS-EGFP-12xQ/N/+ and (2) ELAV-Gal4/+; UAS-EGFP/+ fly heads aged at 25 °C. Tubulin was used as loading control. (c) Western blot analyses showing endogenous TDP-43 and synapsin levels from wild-type mice during aging. The corresponding histogram shows the relative expression levels of TDP-43 normalized with GADPH. Error bars indicate SD. (d) Real-time PCR quantification of fly TBPH at different time points. The RNA levels were normalized with Rpl-11. ns indicates $p > 0.05$ (not significant) and * indicates $p < 0.05$. Error bars indicate SD. (e) Real-time PCR quantification of mouse TDP-43 at different time points (left panel). RNA levels were normalized with GADPH. ns indicates $p > 0.05$ (not significant) and * indicates $p < 0.05$. Error bars indicate SD. Northern Blot of mouse TDP-43 at different time points (right panel). GADPH was used as loading control.

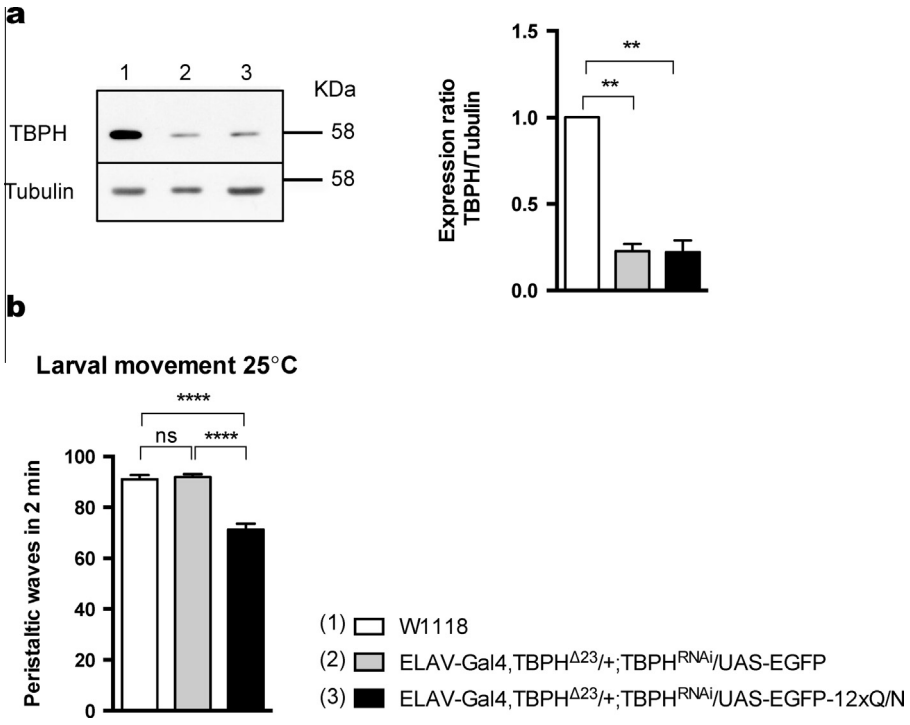

Fig. 4. EGFP-12xQ/N expression induces a defect in larval motility when TBPH levels are down regulated by silencing. (a) Quantification of TBPH level in wild-type (W1118) larval brains and larvae expressing TBPH siRNA. The different genotypes are indicated and defined by the color and number code at the bottom of the figure. The histogram shows the TBPH expression levels normalized with tubulin. One-way ANOVA followed by Bonferroni's multiple comparison was used to compare measures among three groups. ** indicates $p < 0.01$. Error bars indicate SD. (b) Quantification of peristaltic waves of third instar larvae. A one-way ANOVA followed by Bonferroni's multiple comparison was used to compare measures among three groups. ns indicates $p > 0.05$ (not significant) and **** indicates $p < 0.0001$. Error bars indicate SEM.

aggregates will be higher in a fly with low TBPH levels from birth and hence the phenotype should arise earlier than day 14 of the adult life. To test this hypothesis we created a fly expressing constitutively a siRNA against TBPH in neurons. Fig. 4a shows that this fly has from birth reduced TBPH levels, comparable to 14 day-old flies, but that this level is sufficient for their development. However, when EGFP-12xQ/N aggregates are present, the locomotion phenotype can be already observed at the third instar larval stage at 25 °C (Fig. 4b), indicating that the phenotypic consequence of the EGFP-12xQ/N aggregates is also dependent on endogenous TDP-43 levels.

Our data demonstrate the existence of a physiological decrease of TDP-43/TBPH levels with aging in brain tissue both in wild-type mice and flies, showing that it is an evolutionary conserved phenomenon. The maintenance of most functions sustained by TBPH/TDP-43 depends on the cell capacity of producing this protein and its availability in a functional form and in the proper cellular location. Available TDP-43 levels lower than a certain threshold would lead to abnormal function of TDP-43 on targets critical for neuronal structure and function, which might be responsible for the ALS pathologies.

CONCLUSIONS

Our observations suggest that sequestering of TBPH by the aggregates in young animals can be overcome by the higher production of this protein. However, during aging the lower cellular capacity for TDP-43 production becomes critical, and the continuous capture of endogenous TDP-43 by the aggregates will likely produce a loss of its nuclear function, impossible to be compensated. Furthermore, the profile of the decline suggests an evolutionary conserved, programed reduction during the life span of the organism.

Based on this evidence, we suggest that a critical event for the onset of the human disease could be an age-related, physiological drop in TDP-43 expression. At this point, the continuous capture of endogenous TBPH/TDP-43 by the aggregates will likely produce a loss of its nuclear function. We are currently following up these observations to definitively prove this point. It should be noted that, according to this model, aggregates could exist from a very early stage without causing symptoms.

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