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Research Report

AUTEN-67 (Autophagy Enhancer-67) Hampers the Progression of Neurodegenerative Symptoms in a *Drosophila* model of Huntington's Disease

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Abstract.
Background: Autophagy, a lysosome-mediated self-degradation process of eukaryotic cells, serves as a main route for the elimination of cellular damage [1–3]. Such damages include aggregated, oxidized or misfolded proteins whose accumulation can cause various neurodegenerative pathologies, including Huntington's disease (HD).
Objective: Here we examined whether enhanced autophagic activity can alleviate neurophatological features in a *Drosophila* model of HD (the transgenic animals express a human mutant Huntingtin protein with a long polyglutamine repeat, 128Q).
Methods: We have recently identified an autophagy-enhancing small molecule, AUTEN-67 (autophagy enhancer 67), with potent neuroprotective effects [4]. AUTEN-67 was applied to induce autophagic activity in the HD model used in this study.
Results: We showed that AUTEN-67 treatment interferes with the progressive accumulation of ubiquitinated proteins in the brain of *Drosophila* transgenic for the pathological 128Q form of human Huntingtin protein. The compound significantly improved the climbing ability and moderately extended the mean life span of these flies. Furthermore, brain tissue samples from human patients diagnosed for HD displayed increased levels of the autophagy substrate SQSTM1/p62 protein, as compared with controls.
Conclusions: These results imply that AUTEN-67 impedes the progression of neurodegenerative symptoms characterizing HD, and that autophagy is a promising therapeutic target for treating this pathology. In humans, AUTEN-67 may have the potential to delay the onset and decrease the severity of HD.

Keywords: Huntington's disease, Huntingtin, polyQ, neurodegeneration, autophagy, AUTEN-67, *Drosophila*, climbing assay, ubiquitinated proteins, Ref(2)P/SQSTM1/p62

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ABBREVIATIONS

ATG	autophagy-related protein
AUTEN-67	autophagy enhancer-67
EDTP	egg-derived tyrosine phosphatase
HD	Huntington's disease
AD	Alzheimer's disease
PD	Parkinson's disease
LC3B	MAP1LC3B (LC3B), microtubule-associated protein 1 light chain 3 beta
PtdIns	phosphatidylinositol
RFP	red fluorescent protein; Ref(2)P/SQSTM1/p62, sequestosome 1/hypothetical protein 62 kDa
Vps34	vacuolar protein sorting-associated protein 34

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative genetic disorder caused by an autosomal dominant mutation in the *Huntingtin* (*HTT*) gene [5]. The encoded protein (HTT) contains a poly-glutamine (polyQ) tract (an extended CAG trinucleotide repeat in *Htt*) that varies in length among individuals. When the repetition of the polyQ tract passes a critical threshold (typically over 39) due to a genetic alteration in *HTT*, it forms an altered, toxic form of HTT. The intracellular accumulation of mutant HTT can frequently lead to the loss of certain type of neurons in different parts of the brain, thereby causing severe changes in personality, cognition and physical skills (e.g., movement coordination) in the affected individuals. HD still represents a largely untreatable pathology.

Autophagy (cellular self-eating) functions as a major molecular mechanism to degrade superfluous and damaged (e.g., aggregated, oxidized or misfolded) proteins in eukaryotic cells [1–3]. During the autophagic process, parts of the cytoplasm are delivered into the lysosomal system containing acidic hydrolases (proteases, nucleases, lipases and glycosidases). Besides microautophagy and chaperone-mediated autophagy, macroautophagy (hereafter referred to as autophagy) represents a main form of the autophagic breakdown. It involves the generation of a double membrane-bound vesicle-like structure called autophagosome to sequester the cytoplasmic cargo destined for degradation. The autophagosome then fuses with a lysosome to form an autolysosome in which the enzymatic degradation actually occurs. The relationship between autophagy and HD is compound. For example, the mutant hHTT protein is a subject to autophagic degradation [6]. Autophagic receptor proteins, such as SQSTM1/p62, ALFY and Tollip, have crucial roles in the elimination of mutant HTT via selective autophagy [7–9], during which specific cytoplasmic constituents (molecules or organelles) are labeled for degradation. Mutant forms of HTT can interfere with the autophagic process, including inefficient cargo loading, impaired movement and maturation of autophagosomes, and perturbed post-Golgi trafficking to lysosomes (that results in decreased lysosomal activity) [10–12]. It is still unclear that during the pathogenesis of HD which defects dominate [13]. In addition, the wild-type HTT was recently found to act as a scaffold protein for selective autophagy [14, 15]. Loss of normal HTT function may lead to defective autophagy that contributes to disease pathogenesis.

Formation of the autophagosomal (also called isolation) membrane requires the class III PtdIns3K (phosphatidylinositol 3-kinase, formerly called Vps34 –vacuolar protein sorting-associated protein 34) enzyme complex that converts PtdIns (phosphatidylinositol) into PtdIns3P (phosphatidylinositol 3-phosphate) [16]. This biochemical reaction is reversible; certain myotubularin-related phosphatases (MTMRs) antagonize PtdIns3K to generate PtdIns from PtdIns3P [17]. Indeed, MTMR14, also called Jumpy in mammals, was demonstrated to inhibit autophagy in cell cultures [18]. Thus, MTMR proteins may have evolved to protect the eukaryotic cells from the injurious hyperactivation of autophagy under various cellular stress conditions, which can frequently lead to the loss of the affected cell [19, 20].

By participating in the effective elimination of cellular damage, autophagy acts a central regulator of the aging process in various animal taxa [3, 21–23]. It is also implicated in the development of various age-dependent pathologies including cancer, neurodegenerative diseases, tissue atrophy, diabetes, immune deficiency and intracellular infection caused by microbes [2, 22, 23]. These observations defined autophagy as a promising therapeutic target in treating such pathologies [24–27]. To identify novel autophagy-enhancing drug candidates, we have recently screened a small molecule library for inhibitors of human MTMR14/Jumpy [4]. From this screen, AUTEN-67 (autophagy enhancer-67) was

isolated. AUTEN-67 potently induces autophagy in human cell cultures, as well as in model organisms including *Drosophila*, zebrafish and mice, significantly extends life span in flies, increases the survival of isolated neurons under both normal and oxidative stress-induced conditions, and improves nesting behavior in mice expressing an aggregation-prone human APP (amyloid precursor protein).

In this study we examined the effects of AUTEN-67 on a *Drosophila* model of HD (the transgenic animals express the full length human mutant HTT protein with a long polyQ tract, 128Q, and display several neuropathological features observable in human Huntington pathology). We found that the molecule significantly increases autophagic activity in the treated animals, decreases mutant HTT levels, inhibits the lifelong, progressive accumulation of ubiquitinated proteins in the brain, and increases the climbing ability and life span of animals. AUTEN-67 thus impedes the progression of neurodegenerative symptoms in this HD model. We also showed that human brain tissue samples exhibit markedly increased levels of SQSTM1/p62 protein (which acts as a substrate for autophagy) in patients diagnosed for HD, as compared with unaffected, age-matched controls. We suggest that autophagy serves as a potential therapeutic target for treating HD in humans.

MATERIALS AND METHODS

Culturing and drug treatment of flies

For maintenance fly stocks were raised on standard cornmeal-sugar agar medium at 18–25°C. We obtained the stocks from Bloomington *Drosophila* Stock Center: Appl-Gal4 (BL32040), Ddc-Gal4 (BL7009), UAS-16Q-hHTT/CyO (BL33810), UAS-128Q-hHTT (BL33808), P{GawB}EDTP^{DJ694} (*pEDTP*-Gal4) (BL8176), UAS-myrGFP (BL32199). The only exception is UAS-mCherry-Atg8a, which was kindly provided by Gábor Juhász (Eötvös University, Hungary), described in [28]. During all experiments flies were kept at 29°C. Flies were placed into vials containing treated medium immediately after eclosion. AUTEN-67 dissolved in DMSO (Sigma, D8418) was added to yeast suspension (final concentration was 50 or 100 μM), and dropped 65 μl to the surface of each vial. For control we used the same volume DMSO without AUTEN-67. Flies were transferred into a fresh vial every second day.

Measurement of autophagic structures in the brain of Drosophila *adults*

Brains from the heads of 7 day-old adults was dissected. Preparation of brains was carried out in PBS (Sigma, P4417) solution. Covering was achieved in glicerine:PBS (8:2) solution containing Hoechst 33342 (Life Technologies, H-1399) at 10 μM concentration. Images were captured with Zeiss Axioimager Z1 upright microscope (with objective Plan-NeoFluar 20 × 0.3 NA) equipped with an ApoTome, and AxioVision 4.82 and ImageJ 1.45 s software were used to examine and evaluate data. Results are presented as mean ± SD. Genotype: w*; UAS-mCherry-Atg8a/+; Ddc-Gal4/+.

Climbing assay of Drosophila *expressing a human HTT (16Q-hHTT or 128Q-hHTT)*

20 female adult flies that express the transgene under the control of Appl–Gal4 driver were anesthetized, and placed in a vertical glass column (length, 25 cm; diameter, 1.5 cm). After 2 hours of recovery period from CO_2 exposure, flies were gently tapped 5 times to the bottom of the column. The number of flies that reached the line at 21.8 cm height within 20 and 40 sec was counted. Three trials of four-six parallel measurements were performed in each experiment. Scores represent the mean percentage of flies that reached the top line against the total number tested. Results are presented as mean ± SD. Genotypes: ApplGal4/+; UAS-16Q-hHTT/+and Appl-Gal4/+; UAS-128Q-hHTT/+.

Determining the average speed of flies expressing a human mutant form of HTT, 128Q

10 female adult flies expressing the UAS-128Q-hHTT transgene under the control of an Appl–Gal4 driver were anesthetized, and placed in a vertical glass vial (length, 9 cm; diameter, 2.2 cm). After a 45 min recovery period from CO_2 exposure, flies were gently tapped to the bottom of the vial. The speed of individuals that reached the line at 5 cm height within 1 min (in the case of 7 and 14-day-old animals) or 3 min (21-day-old animals) was calculated. Three parallel experiments and three trials were executed. Results are presented as mean ± SD of the average speed (cm/s). Genotypes: ApplGal4/+; UAS-16Q-hHTT/+and Appl-Gal4/+; UAS-128Q-hHTT/+.

Western blot analyses

Protein samples of flies stem from adult heads. Membranes were probed with anti-Ref(2)P/SQSTM 1/p62 (rabbit, 1:2500, [29]), alpha-Tub84B (mouse, 1:2500, Sigma, T6199), anti-Atg8a (rabbit, 1:2500), anti-Ubiquitin (mouse, 1:500, Merck, ST1200), anti-polyQ (mouse 1:1000, Merck, MAB1574), anti-hHTT (1:1000, Viva Bioscience, VB3130) anti-rabbit IgG alkaline phosphatase (1:1000, Sigma, A3687) and anti-mouse IgG alkaline phosphatase (1:1000, Sigma, A8438) and developed by NBT-BCIP solution (Sigma, 72091). ImageJ 1.45 s software was used to examine and evaluate data. Results are presented as mean ± SD. Genotypes: ApplGal4/+; UAS-16Q-hHTT/+and Appl-Gal4/+; UAS-128Q-hHTT/+.

Immunohistochemistry on Drosophila *brain samples*

The cuticles of adult heads were opened in PBS and fixed in PBS containing 4% formaldehyde for overnight at 4°C. After fixation, samples were washed in PBST (PBS containing 0.3% Triton X-100) for 20 min by three times. Then, samples were incubated in blocking solution (PBST containing 5% goat serum) for an hour. Samples were subsequently incubated overnight at 4°C in blocking solution containing the primary antibodies. After incubation with the given primary antibody, samples were washed in 5xNaCl PBST for 20 min, then in PBST for 20 min. It was followed by the blocking solution for 30 min. Samples were then incubated in blocking solution containing the given secondary antibodies for 60 min. It was followed by washing steps as had been carried out after incubation with primary antibodies. For staining nuclei, we used Hoechst solution (1 mM was dissolved in PBS, 1:200) for 6 min. Then, samples were washed in PBST for 20 min, and in PBS for another 20 min. The dissection of stained brain sample was performed in PBS, and for mounting we used ProLong Gold antifade reagent (Molecular Probes, P36934). Every step was executed at room temperature, otherwise indicated. The following primary antibodies were used: Anti-Ref(P)2/p62 (rabbit, 1:200 [29], anti-polyQ (1:100 Merck, MAB1574). For nuclear staining, Hoechst 33342 (0.1 mg/ml, Molecular Probes) dye was used. Anti-Rabbit Alexa Fluor 488 (Life Technologies, A11008) and anti-Mouse Texas Red (Life Technologies, T862) in 1:500 were used as secondary antibodies. Images were captured with a Zeiss Axioimager Z1 upright microscope (with objective Plan-NeoFluar 20 × 0.3 NA and Plan-Apochromat 63 × 1,4 NA) equipped with an ApoTome, and AxioVision 4.82 and ImageJ 1.45 s software were used to examine and evaluate data. Results are presented as mean ± SD. Genotypes: ApplGal4/+; UAS-16Q-hHTT/+and Appl-Gal4/+; UAS-128Q-hHTT/+.

Life span measurements

To analyze the life span, adult females were selected. The drug treatment was performed as described above. Flies were transferred into fresh medium containing vials every second day. The number of dead animals was counted daily. Measurements were carried out with eight parallels, with 20–30 flies in each. Mean life spans are presented as mean ± SEM. Genotypes: ApplGal4/+; UAS-16Q-hHTT/+and Appl-Gal4/+; UAS-128Q-hHTT/+.

Brain tissue samples

Paraffin-embedded human brain tissue samples from parietal and temporal cortices (or subfields of these areas) of normally aged, non-affected (control) subjects and HD-affected individuals were obtained from a brain tissue bank. Samples were collected from donors for or from whom a written informed consent for a brain autopsy, the use of tissue samples and permission for anonymous use of clinical information was obtained. Control samples were obtained from clinical patients having no central nervous system (CNS) medication before death and showed no sign of disease.

Immunohistochemistry on human brain tissue samples

Paraffin-embedded tissue sections were deparaffinized, rehydrated and used in light microscopic immunohistochemistry. For identification of neurons and demonstration of the presence of ubiquitinated proteins in the autophagy pathway, anti-NeuN and anti-p62 antibodies were used in consecutive sections. NeuN and SQSTM1/p62 immunohistochemistry was performed as described [30]. For antigen recovery, deparaffinized sections were boiled in 0.01 M citrate-buffer solution (pH 6.0) in a microwave oven for 2 min (set at 900 watts). After blocking the endogenous peroxidase in 0.05 M PBS

containing 3% H_2O_2, sections were washed for 3–5 min in 0.05 M PBS (pH 7.5) at RT. Tissue sections were next permeabilized, and the background binding of antibodies was reduced in a blocking solution (0.1 M PBS containing 5% normal goat serum, 1% BSA, 0.05% Triton X-15 100) for 30 min at 37°C. Sections were covered with the above solution containing either mouse anti-NeuN primary antibody (1:500 final dilution; Chemicon, Billerica, MA, USA) or mouse anti-p62 primary antibody (1:150 final dilution; Abcam, Cambridge, MA, USA) at 4°C for overnight. After incubation with the primary antibodies, sections were washed for 4 × 5 min in 0.05 TBS (pH 7.5) at RT. Sections were then treated with biotinylated anti-mouse IgG secondary antibody (1:200 final dilution; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) in a blocking solution (where Triton X-100 was omitted) for 5 hours at RT. After several washes (4 × 5 min), biotinylated streptavidin-peroxidase tertiary antibody (1:200 final dilution; Amersham) in a blocking solution (without Triton X-100) was applied to the sections overnight at 4°C. Sections were washed again in 0.05 M TBS (pH 7.5) for 4 × 5 min at RT, and processed for peroxidase enzyme histochemistry using Sigma Fast DAB Tablet (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Sections were washed for 3 × 5 min in 0.05 M TBS (pH 7.5) at RT, washed in distilled water for 1 min, dehydrated in a series of ethanol solutions, covered with DPX mounting medium (Fluka, 30 Buchs, Switzerland), and coverslipped. Quantitative analysis of immunohistochemically processed tissue sections was performed as described [31]. NeuN- and SQSTM1/p62-positive cells were counted with the use of the computer program ImageJ (version 1.47; developed by W. Rasband at the U.S. National Institutes of Health, and available from internet at http://rsb.info.nih.gov/ij). After calibration and setting the appropriate scale, the digital image file was opened and the background was subtracted by setting the "rolling ball radius" to 50 pixels. Overlapping objects in the resulting binary images were then separated via the menu command "Process/Binary/Watershed" [32]. For counting NeuN-positive cells, "Analyze/Analyze Particles" menus were selected, size (56–2500 m) and circularity (0.5–1.0) values were chosen, then the output was copied to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for statistical analysis. SQSTM1/p62-positive cells were counted manually.

Statistics

Lilliefors test were used to know that the distribution of samples examined is normal or not. If it was normal, F test was performed to compare two variances. If the variances were equal, two-sample Student's *t*-test was used. If the variances were unequal we used Welch-test (*t*-test for unequal variances). If the distribution of a sample is not normal, Mann-Whitney U test was performed. We used the software MatLab 7.12.0 (R2011a). For life span analyses we used SPSS 17 software to perform statistical analysis. *p* values for comparing Kaplan-Meyer survival curves between two groups were determined by log-rank (Mantel-Cox) tests, and *p* values for comparing mean life spans were determined by Mann-Whitney Test. Bonferroni corrections were carried out.

RESULTS

AUTEN-67 enhances autophagy in the brain of Drosophila *expressing 128Q hHTT*

A transgenic *Drosophila* strain containing a UAS-driven full-length human Huntingtin protein with a large poly-glutamine expansion in the N-terminus (128Q-hHTT) serves as a tractable *in vivo* model system for studying the pathology of HD [33, 34]. Flies transgenic for 16Q-hHTT (containing a relatively short poly-glutamine repeat) were used as control. In the transgenic 128Q-hHTT animals (in which *c164-Gal4* was used as a driver), the progressive accumulation of toxic proteins in certain types of neurons (motoneurons) can cause massive levels of cell death, which is associated with various neurobiological abnormalities. We aimed to assess the effects of autophagy on the progression of neurodegenerative symptoms in a similar *Drosophila* model of HD. In this particular model, the full length *128Q-hHTT* transgene is driven by *Appl-Gal4/+* that is active in all neurons. Characterization of the strain revealed a pleiotropic HD-like phenotype including a short life span and reduced climbing ability (see later in the Results section), but not lowered flight performance (Supplementary Figure S1). We treated *Appl-Gal4/+; UAS-128Q-hHTT/+* transgenic flies with AUTEN-67, a potent enhancer of autophagy [4]. As shown previously, AUTEN-67 increases autophagic flux in the *Drosophila* fat body [4]. Consistent with these data, we found a significant increase in the number of autophagic structures labeled by a mCherry-Atg8a

reporter (Atg denotes an autophagy-related protein) in brain samples of treated animals, as compared with those from untreated controls (Fig. 1A, A'). Atg8a is a ubiquitin-like key autophagy protein that is conjugated to the forming isolation membrane; the mCherry-Atg8a reporter readily marks autophagosomes and autolysosomes [28, 35]. However, an elevated number of Atg8a-specific structures is not necessarily indicative of enhanced autophagic activity. It can even indicate defects in the process (e.g., autophagosome formation is not followed by autophagosome-lysosome fusion). To confirm that AUTEN-67 indeed triggers the autophagic degradation in the transgenic flies, we measured Ref(2)P protein levels, and compared with those found in untreated animals. Ref(2)P is the *Drosophila* ortholog of human SQSTM1 (sequestosome 1)/p62 protein serving as a substrate for autophagic degradation; its intracellular level inversely correlates with autophagic activity [36, 37]. According to our Western blot (Wb) analysis, Ref(2)P/SQSTM1/p62 levels were significantly lower in AUTEN-67-treated animals than in untreated (DMSO) controls in both 16Q-hHTT and 128Q-hHTT transgenic backgrounds (Fig. 1B, B'). Moreover, Atg8a-II (the membrane-bound form of Atg8a) levels were decreased in the treated animals, further suggesting an elevated autophagic activity (flux) as a result of adding AUTEN-67 (Atg8a also becomes degraded during the autophagic process) (Fig. 1B, B'; Supplementary Figure S1). We also found that *EDTP* (egg-derived tyrosine phosphatase), the fly ortholog of human *MTMR14/Jumpy* gene, is strongly expressed in the *Drosophila* brain (Fig. 1C, C'). These data explain why AUTEN-67 could influence autophagic activity in this tissue (the molecule enhances autophagy through inhibiting MTMR14/Jumpy; [4]). Together, AUTEN-67 potently increased autophagic flux in UAS-128Q-hHTT transgenic flies (Fig. 1).

The Wb analysis also showed that relative levels of Ref(2)P/SQSTM1/p62 proteins are less in 128Q-hHTT than in 16Q-hHTT animals (Fig. 1B, B'). This suggests that basal autophagic activity is significantly higher in 128Q-hHTT flies than in those expressing non-toxic 16Q-hHTT (Fig. 1B, B'). The pathological 128Q-hHTT protein (proteotoxicity) may trigger a general stress response that involves autophagy, explaining why the autophagic process is increased in transgenic flies expressing the toxic form.

Alternatively, in 128Q-hHTT samples the majority of Ref(2)P/SQSTM1/p62 may be present in insoluble forms (e.g., associated with inclusion bodies/cytoplasmic aggregates), thereby remaining undetectable by the protein isolation method (Wb) used in this study. Indeed, in cell culture and *Drosophila* models of HD the mutant 128Q-hHTT forms protein inclusions in the cytoplasm or in the nucleus, and SQSTM1/p62 strongly associates with these structures [7, 38]. This could explain why soluble Ref(2)P/SQSTM1/p62 levels are lower in 128Q than in 16Q samples on the Wb shown in Fig. 1B.

To address this issue we performed immunohistochemical staining with anti-Ref(2)P antibody (this method is capable of detecting both soluble and insoluble Ref(2)P/SQSTM1/p62 proteins) (Fig. 2A, A'). The analysis revealed that Ref(2)P/SQSTM1/p62 levels are much higher in the brain of animals transgenic for the mutant 128Q-hHTT, as compared with 16Q-hHTT samples (rows 1 vs. 3 in Fig. 2A). Most of the Ref(2)P/SQSTM1/p62 proteins localized to inclusion-like structures (row 3 in Fig. 2A). Treatment with the autophagy-enhancing AUTEN-67 markedly reduced the amount of Ref(2)P/SQSTM1/p62 in 128Q-hHTT samples. Based on these results we conclude that the Western blotting shown in Fig. 1B demonstrates only soluble Ref(2)P/SQSTM1/p62 proteins.

AUTEN-67 treatment reduces toxic polyQ/ 128QhHTT protein levels

We next asked whether enhanced autophagic activity triggered by AUTEN-67 treatment can reduce toxic 128Q-hHTT levels in brain samples of transgenic flies. An immunohistochemical staining with anti-polyQ antibody revealed a non-significant trend towards a decrease in the amount of 128Q-hHTT proteins in the treated animals, as compared with untreated controls (Fig. 2B, B'). An intriguing aspect of these results is that 128Q-hHTT predominantly accumulated in protein inclusions (red foci on the last fluorescent image in Fig. 2B), rather than exhibiting a diffuse staining pattern. 128Q-hHTT-positive structures were surrounded by Ref(2)P/SQSTM1/p62 proteins, suggesting that the aggregates are subject to elimination by selective autophagy.

Using anti-polyQ and anti-hHTT antibodies, we also performed a Wb analysis to quantify relative 128Q-hHTT levels in brain samples (Fig. 2C, C'). A significant decrease in 128Q-hHTT accumulation was evident from this set of experiments. Taken together, AUTEN-67 administration reduces the level of toxic 128Q-hHTT proteins in this animal model of HD.

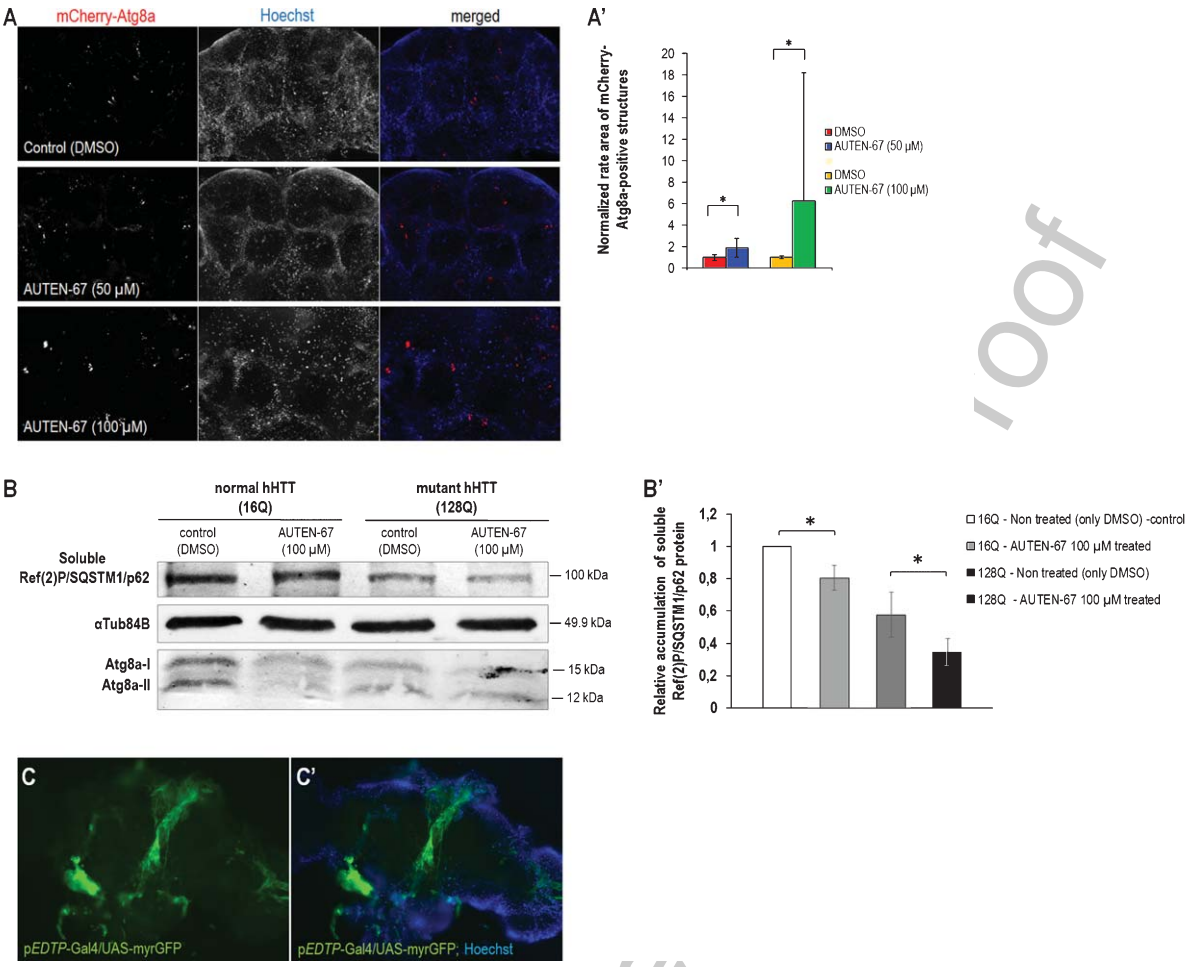
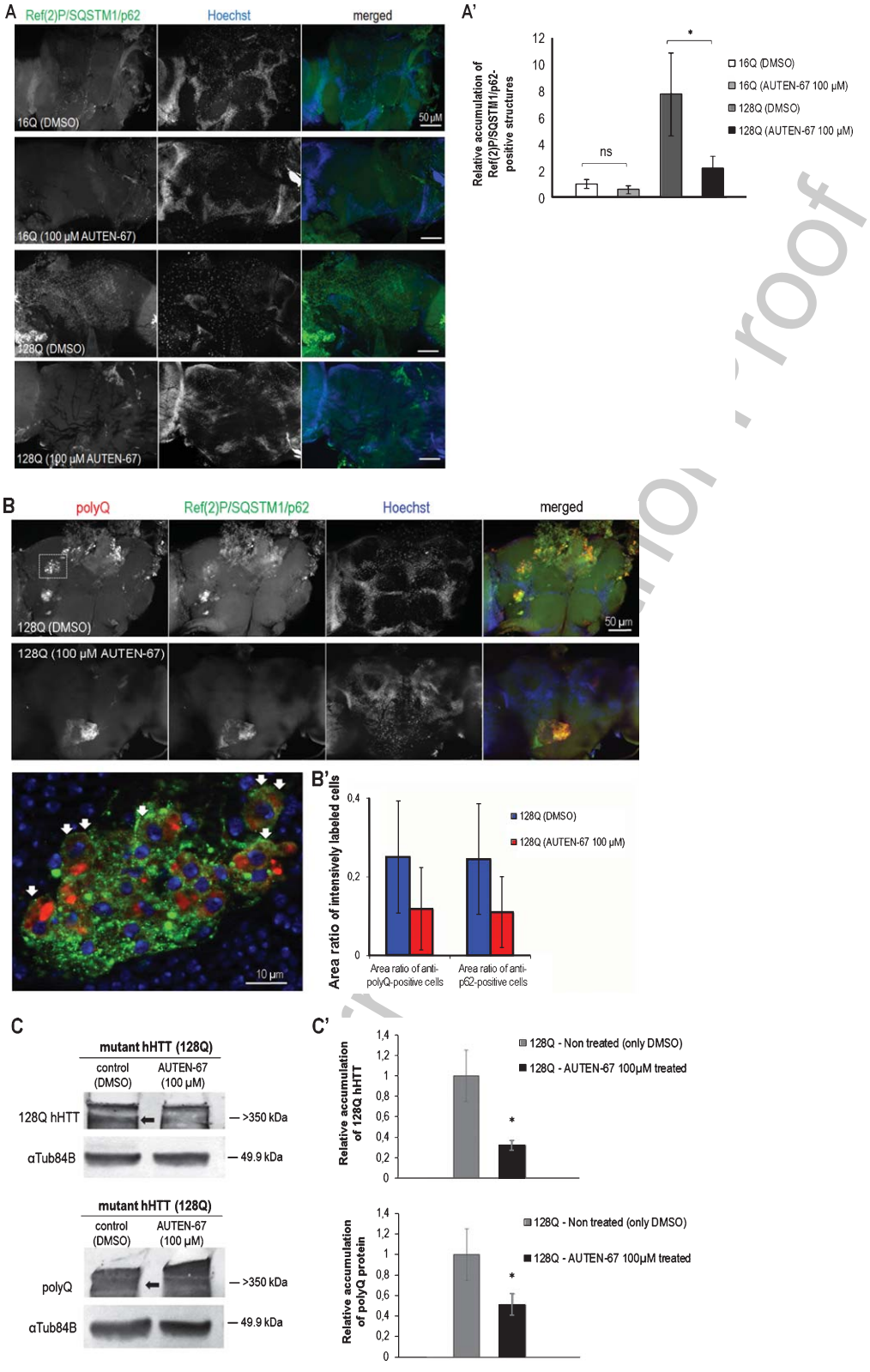


Fig. 1. AUTEN-67 enhances autophagy in the brain of a *Drosophila* model of Huntington's disease. A, AUTEN-67 elevates the number of Atg8a-positive structures (autophagosomes and autolysosomes) in brain samples in a concentration-dependent manner. Left panels: mCherry-Atg8a reporter indicates autophagic structures (red foci); middle panels: Hoechst staining indicates nuclei; right panels: Merged images. Fluorescent images. A', Quantification of mCherry-Atg8a-positive structures in brain samples from untreated (control) vs. AUTEN-67-treated animals. Bars represent mean ± S.D, $^{*}p < 0.05$; Welch-test (*t*-test for unequal variances) ($n = 7$, $p = 0.0297$) and Mann-Whitney *U*-test ($n = 13$, $p = 0.0236$). B, A representative Western blot (Wb) showing that AUTEN-67 decreases soluble Ref(2)P/SQSTM1/p62 protein levels in the head of animals expressing a human normal (16Q) or mutant (128Q) Huntingtin protein (hHTT). This suggests that the small molecule increases autophagic degradation in these transgenic flies. Alpha-Tubulin84B (αTub84B) serves as an internal control. Unconjugated (I) and conjugated (II) forms of Atg8a (a key autophagic protein) are also shown. Analyses were carried out with 7 day-old adult flies. "hHTT" denotes human Huntingtin protein. B', Quantification of soluble Ref(2)P/SQSTM1/p62 protein levels. In both 16Q and 128Q samples, AUTEN-67 treatment significantly reduced Ref(2)P/SQSTM1/p62 levels. Statistics were calculated from 4 independent Wb experiments ($n = 4$). Bars represent mean ± SD, $^{*}p < 0.05$, two-sample Student's *t*-test [16Q DMSO – 16Q AUTEN-67 (100 μM) $p = 0.01467$; 128Q DMSO – 128Q AUTEN-67 (100 μM) $p = 0.03394$]. C, EDTP/MTMR14/Jumpy promoter is highly active in the *Drosophila* brain. Animals of w^{1118}; $P\{GawB\}EDTP^{DJ694}$ (p*EDTP*-Gal4)/UAS-myrGFP were examined. C', The corresponding Hoechst staining indicates nuclei.

AUTEN-67 retards the accumulation of ubiquitinated proteins in the aging brain

Ubiquitination of damaged proteins is a general feature of all aging cells [39]. Thus, we decided to monitor ubiquitinated protein accumulation in the brain of AUTEN-67-treated versus untreated control animals. Staining of brain samples with a Ubiquitin-specific antibody revealed that AUTEN-67 significantly reduces the relative amount of damaged proteins at the age of 21-day-old adulthood (Fig. 3). This phenomenon was evident not only in animals expressing the mutant 128Q-hHTT protein, but also in those expressing its non-toxic version (16Q-hHTT). It is intriguing that relative levels of ubiquitinated proteins were lower in animals that express the pathological 128Q-hHTT protein (Fig. 3). This is in good accordance with our previous results


A
Ref(2)P/SQSTM1/p62
Hoechst
merged
16Q (DMSO)
16Q (100 μM AUTEN-67)
128Q (DMSO)
128Q (100 μM AUTEN-67)
50 μM
A'
Relative accumulation of Ref(2)P/SQSTM1/p62-positive structures
ns
*
16Q (DMSO)
16Q (AUTEN-67 100 μM)
128Q (DMSO)
128Q (AUTEN-67 100 μM)
B
polyQ
Ref(2)P/SQSTM1/p62
Hoechst
merged
128Q (DMSO)
128Q (100 μM AUTEN-67)
50 μm
10 μm
B'
Area ratio of intensively labeled cells
128Q (DMSO)
128Q (AUTEN-67 100 μM)
Area ratio of anti-polyQ-positive cells
Area ratio of anti-p62-positive cells
C
mutant hHTT (128Q)
control (DMSO)
AUTEN-67 (100 μM)
128Q hHTT
αTub84B
>350 kDa
49.9 kDa
polyQ
C'
Relative accumulation of 128Q hHTT
Relative accumulation of polyQ protein
128Q - Non treated (only DMSO)
128Q - AUTEN-67 100μM treated

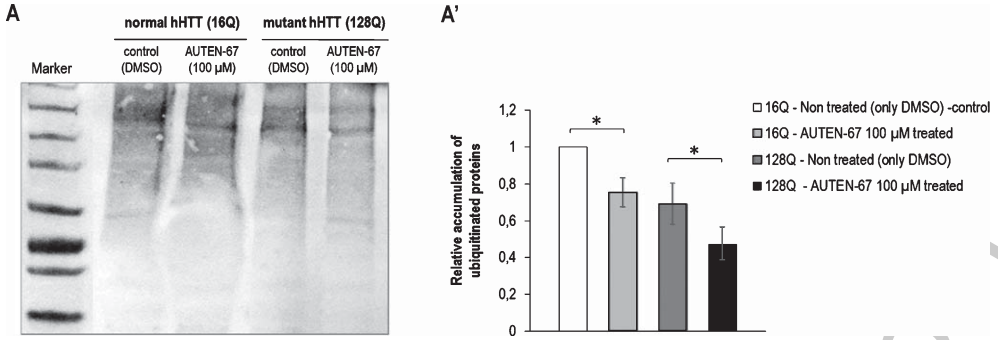


Fig. 3. AUTEN-67 retards the accumulation of soluble ubiquitinated proteins in the head of a *Drosophila* model of HD. A, A representative Western blot (Wb) showing the accumulation of (damaged) ubiquitinated proteins in head samples of animals at adult age of 18–21 days. Proteins were visualized by anti-Ubiquitin antibody staining. The compound significantly lowered relative levels of soluble ubiqitinated proteins in animals expressing normal (16Q) and mutant (128Q) hHTT. "hHTT" denotes human Huntingtin protein. A', Quantification of relative soluble ubiquitinated protein levels from three independent Wb experiments ($n = 3$). Bars indicate mean ± SD, $^*p < 0.05$, two-sample Student's *t*-test [16Q DMSO – 16Q AUTEN-67 (100 μM), $p = 0.03178$; 128Q DMSO – 128Q AUTEN-67 (100 μM), $p = 0.04472$]. Similar to Ref(2)P/SQSTM1/p62 protein localization (see Figs. 1B and 2A), a significant portion of ubiquitinated proteins may accumulate in insoluble forms in 128Q samples, and thereby is not detectable by Wb analysis as performed in this study.

showing decreased Ref(2)P/SQSTM1/p62 protein levels in animals transgenic for 128Q-hHTT, relative to those expressing 16Q-hHTT (Fig. 1B, B'). Ubiquitinated (damaged) proteins can also associate with protein inclusions in aged or HD-affected brain tissues [37, 40]. So, only the soluble fraction of ubiquitinated proteins may be detectable by Western blotting performed in this study. Nevertheless, AUTEN-67 hampered the age-dependent accumulation of ubiquitinated proteins in neurons of this HD model.

AUTEN-67 treatment improves climbing ability in flies expressing 128QhHTT

HD is a neurodegenerative disorder that affects among others muscle coordination [5]. This knowledge prompted us to investigate the capacity of HD model animals to climb up on the wall of glass vials in the presence or absence of AUTEN-67. This climbing assay actually exploits the negative geotaxis behavior of *Drosophila*. When animals transgenic for the non-pathological (16Q) hHTT protein were collected

Fig. 2. Immunohistochemical detection of Ref(2)P/SQSTM1/p62 and polyQ (mutant hHTT) proteins in flies expressing normal (Q16) and mutant (128Q) hHTT. A, Anti-Ref(2)P/SQSTM1/p62 antibody staining (green) indicates both soluble and insoluble protein levels in *Drosophila* brain samples. Total amounts of Ref(2)P/SQSTM1/p62 are much higher in samples expressing the mutant (128Q) hHTT protein (1 vs. 3 row). However, in 128Q samples the majority of Ref(2)P/SQSTM1/p62 localizes to large foci that are likely to correspond to inclusion bodies/cytoplasmic aggregates (i.e., exists in insoluble forms). AUTEN-67 treatment significantly lowered Ref(2)P/SQSTM1/p62 levels in 128Q samples (3-4 rows). In 16Q samples, Ref(2)P/SQSTM1/p62 levels are relatively low (only a faint staining is detectable, occasionally small foci can also be found; 1-2 rows). Hoechst staining (blue) indicates nuclei. Experiments were performed on 18–21 day-old adult flies. "hHTT" denotes human Huntingtin protein. A', Quantification of (total) Ref(2)P/SQSTM1/p62 levels in immunohistochemically stained samples. Bars represent mean ± SD, $^*p < 0.05$, two-sample Student's *t*-test or Welch-test. [16Q – DMSO vs. 16Q – AUTEN-67 (100 μM), $p = 0.1019$ (two-sample Student's *t*-test); 128Q – DMSO vs. 128Q – AUTEN-67 (100 μM), $p = 0.015$ (Welch-test)]. "ns" denotes not significant. Analyses were carried out with 18–21 day-old adult flies. B, Anti-polyQ (red) and anti-Ref(2)P/SQSTM1/p62 (green) antibody staining on 128Q brain samples. AUTEN-67 treatment reduced both toxic 128Q hHTT and autophagy substrate Ref(2)P/SQSTM1/p62 protein levels. The large figure (at the bottom) is an enlargement of the area indicated by a white dashed line in the first panel. Ref(2)P/SQSTM1/p62 proteins (green) localize around hHTT aggregates (red). White arrows point to the periphery of such aggregates which is positive for green labeling. Hoechst staining (blue) indicates nuclei. B', PolyQ (which corresponds to mutant hHTT) and Ref(2)P/SQSTM1/p62 proteins largely colocalize, and their levels are reduced upon AUTEN-67 treatment. Area ratio of polyQ- and Ref(2)P/SQSTM1/p62-positive cells are not significantly different between treated or untreated samples. Bars represent mean ± SD, *p* values indicate tendentious differences (for polyQ: $p = 0.2045$; for Ref(2)P/SQSTM1/p62: $p = 0.1798$), two-sample Student's *t*-test. C, Representative Western blots (Wb) showing polyQ/mutant hHTT levels in *Drosophila* head samples (performed by anti-polyQ antibody staining and anti-hHTT antibody staining). AUTEN-67 reduced polyQ/128Q hHTT levels (the corresponding lanes are indicated by black arrows). αTub84B was used as a control. C', Quantification of mutant hHTT/polyQ protein levels were calculated from 4-4 independent assays ($n = 4$). Bars represent mean ± SD, $^*p < 0.05$, two-sample Student's *t*-test (for mutant hHTT, $p = 0.03841$; for polyQ, $p = 0.01109$).

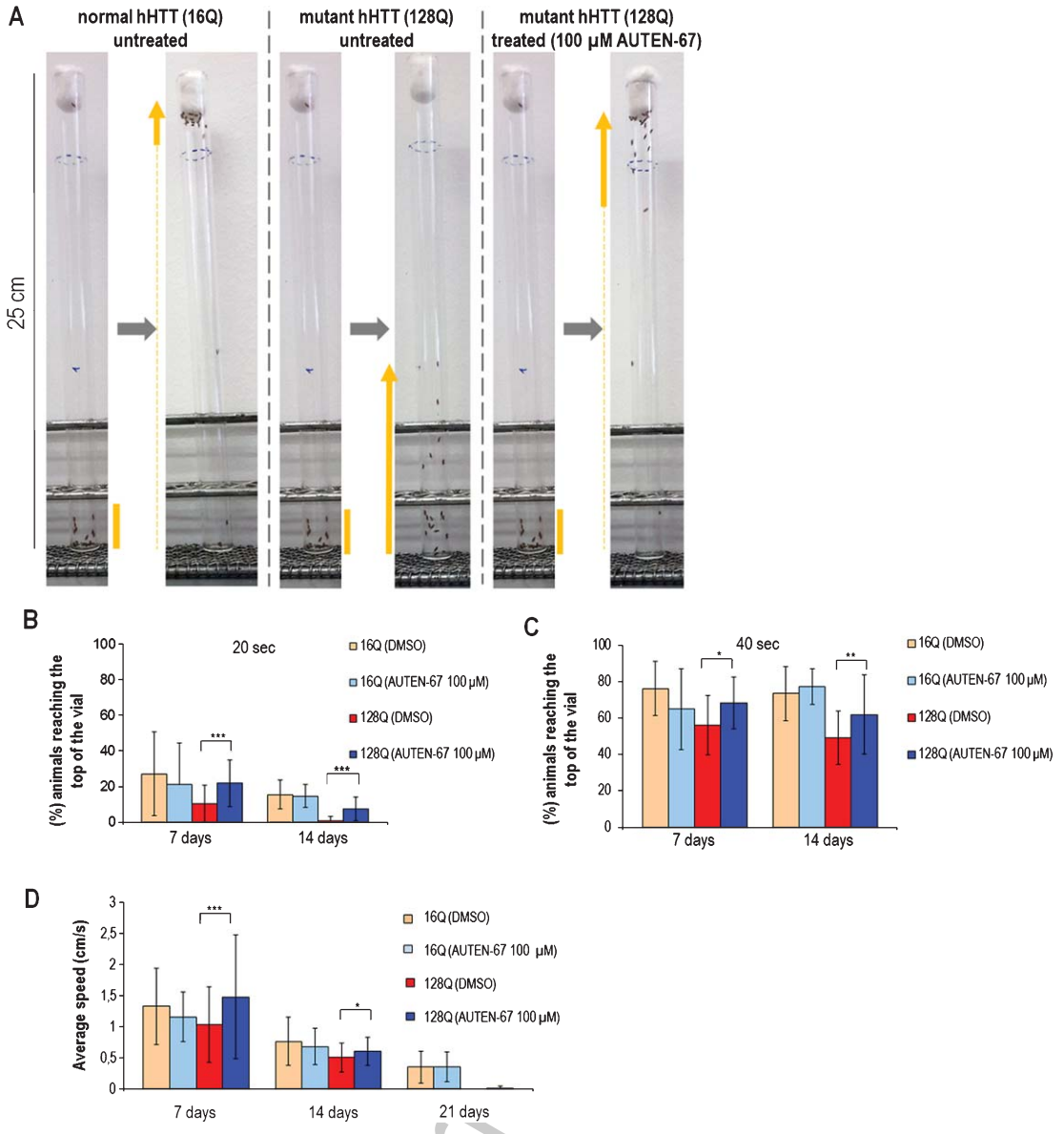


Fig. 4. AUTEN-67 improves climbing ability in flies expressing a human mutant Huntingtin protein (128Q). A, Climbing assay used in this study. 25 cm long glass vials were used to monitor the ability of animals to climb on the wall of test tubes. Left: At the starting point (0 sec) animals were shaken down at the bottom of the glass vial. After 20 sec, the vast majority of the animals climbed up to the top of the vial (negative geotaxis). Middle: Animals expressing the mutant hHTT (128Q) are unable to climb up on the wall of the glass vial. Right: AUTEN-67 markedly improved the ability of animals transgenic for 128Q-hHTT to climb up to the top of the tube. Orange bars indicate the regions where the animals accumulated. "hHTT" denotes human Huntingtin protein. B, AUTEN-67 significantly restored climbing ability in flies expressing 128Q hHTT. Testing was performed at 20 sec after shaking down. C, AUTEN-67 also restores climbing ability when animals were tested at 40 sec after shaking down. D, AUTEN-67 increased the speed at which the animals climb up to the top of vials. Adults at different ages (7, 14 and 21 days) were assayed. In panels B to D, bars indicate mean ± SD, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, Mann-Whitney *U*-test.

at the bottom of test tubes (25 cm in length) by tapping, they could climb up to the top within around 40 seconds (Fig. 4A-C). In contrast, animals expressing the mutant 128Q-hHTT protein were largely unable to climb up on the wall within the same timeframe. The addition of AUTEN-67 to the growth medium restored the climbing ability of affected (128Q-hHTT) flies to nearly normal levels

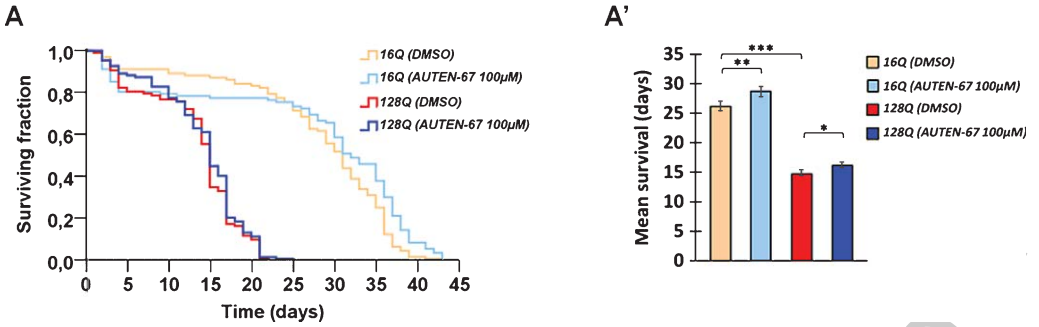


Fig. 5. AUTEN-67 extends the life span of *Drosophila* expressing the human normal (16Q) and a mutant (128Q) Huntingtin protein. A, Difference in life span between untreated (DMSO) and treated (100 μM AUTEN-67) animals expressing the human normal (16Q) and mutant (128Q) Huntingtin protein is significant: $p < 0.001$, long-rank (Mantel-Cox) test. A', Mean life span data from panel A. Bars indicate mean ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, Mann-Whitney *U*-test.

(Fig. 4A-C). In the presence of AUTEN-67, animals transgenic for 128Q-hHTT could reach the top of the vials when they were at the age of 7- and 14-day-old adulthood. This positive effect of AUTEN-67 on the climbing capacity of HD model animals was more obvious when the assay was performed for 20 seconds only (Fig. 4B, C). We also tested the speed at which 128Q-hHTT animals climb up on the glass wall at different (7-, 14- and 21-day-old) adult ages. The results demonstrated that animals climb up faster on the wall when AUTEN-67 is added to the medium (Fig. 4D). Thus, elevated autophagic activity significantly increases the climbing capacity of flies representing a HD model.

AUTEN-67 extends life span in Drosophila *expressing 16Q or 128Q hHTT proteins*

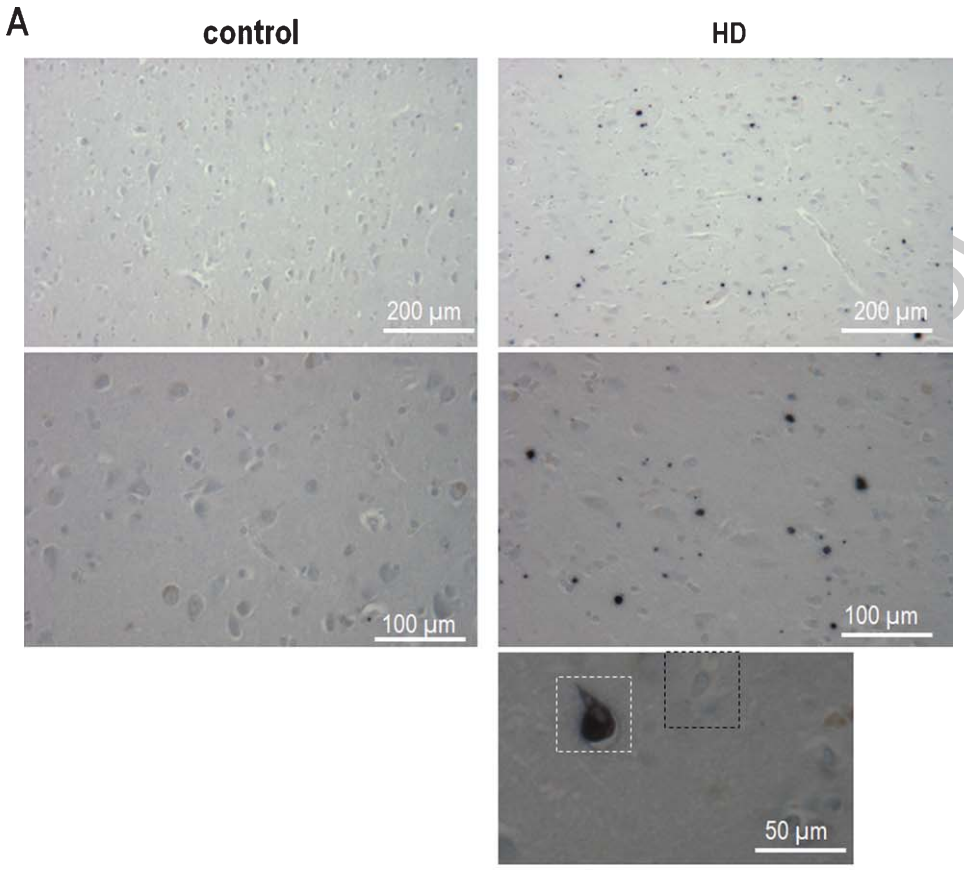
If the severity of neurodegenerative symptoms in this *Drosophila* HD model is attenuated by the administration of AUTEN-67, one can ask whether the compound delays the deterioration of affected animals. To this end we measured the life span of untreated versus AUTEN-67-treated animals transgenic for 128Q-hHTT. We found that in the absence of AUTEN-67, 128Q-hHTT animals live significantly shorter than 16Q-hHTT ones (i.e., 128Q-hHTT-mediated proteotoxicity markedly decreased the ability of animals to survive), and that AUTEN-67 increases, although only moderately, longevity in 128Q-hHTT animals (Fig. 5 and Supplementary Table S1). Based on these data we conclude that pharmacologically-enhanced autophagy can extend the life span of animals expressing the pathological 128Q-hHTT protein.

SQSTM1/p62 accumulates in postmortem human brain samples affected with HD

Finally, we also examined the accumulation of SQSTM1/p62 proteins in postmortem human brain tissue samples obtained from patients that were diagnosed for HD or as non-affected, age-matched controls. Frontal and parietal lobe brain tissue samples were immunohistochemically stained with a SQSTM1/p62-specific antibody (see the Materials and Methods). Intracellular localization of SQSTM1/p62 appeared to be both cytoplasmic and nuclear. Similar to data we obtained from *Drosophila* (Fig. 2A, A'), the number of SQSTM1/p62-positive cells was significantly higher in the disease-affected samples than in control ones (Fig. 6A, A'). It is worthwhile to note that the average number of neurons was not significantly different between the control and affected samples (Fig. 6A). The cytoplasmic diffuse staining was also elevated in the disease-affected samples. This finding is indicative of decreased autophagy in the brain of HD patients. Thus, these results suggest that defects in autophagy contribute to the development of HD.

DISCUSSION

Autophagy functions as a major cellular process to degrade aggregation-prone proteins in the cytoplasm [2, 3, 24]. Defects in autophagic degradation have indeed been widely implicated in the development of various neurodegenerative conditions [13, 24, 25]. In the present study we examined the effects of enhanced autophagic activity on the pathogenesis of HD in a *Drosophila* model expressing the full length human mutant hHTT protein (128Q) in all neurons.



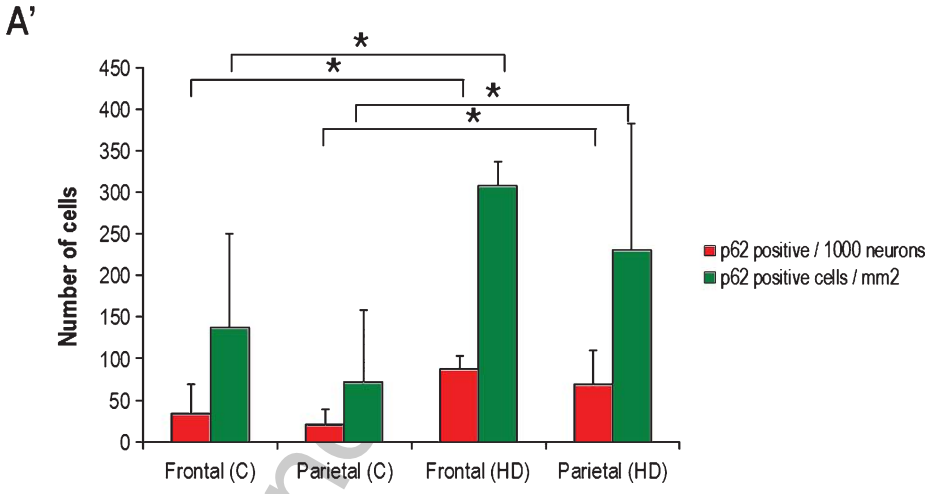


Fig. 6. Postmortem brain samples from patients diagnosed for HD display increased levels of SQSTM1/p62. A, Light microscopy shows SQSTM1/p62 accumulation in neurons from frontal parts of human brain tissues (SQSTM1/p62 serves as an autophagy substrate, so its presence may be indicative for lowered or absent autophagic activity). Postmortem samples were prepared by using anti-SQSTM1/p62 antibody staining. Age-matched, unaffected controls (left panels) and affected (diagnosed for HD; right panels) samples are compared. White scale bars are indicated. At the bottom panel, dashed white and black framings show a SQSTM1/p62-positive and a SQSTM1/p62-negative neuron, respectively. A', Quantification of neurons positive for SQSTM1/p62 antibody staining in control vs. HD samples. Bars indicate mean ± SD. For statistics, $^{*}p < 0.05$; paired Student *t*-test. Note that there was no statistically significant difference in the number of neurons found in control versus disease-affected samples. Average number of neurons/mm^2: 11157.7 ± 25.5 control frontal, 9782.2 ± 11.6 control parietal; 11122.6 ± 10.9 HD frontal, 9435.05 ± 26.8 HD parietal. In panels A and A': C indicates control, "HD" denotes Huntington's disease.

To modulate autophagy, we used a recently identified autophagy-enhancing small molecule, AUTEN-67 [4]. We revealed here that AUTEN-67 increases autophagic flux and reduces (128Q) hHTT protein levels in the brain of this *Drosophila* HD model (Fig. 1A-B'), attenuates the age-dependent accumulation of damaged (polyubiquitinated) proteins in this HD model (Fig. 3), significantly improves the climbing capacity (i.e., motor activity) of the treated animals (Fig. 4), and moderately extends the survival of flies transgenic for 128QhHTT (Fig. 5 and Supplementary Table S1). Although the latter effect is relatively small, but appears to be remarkable in light of the fact that the autophagy-inducing rapamycin-ester CCI-779 has no significant effect on the survival of a mouse HD model [41]. These data imply that activation of autophagy by pharmacological means hampers the progression of neurodegenerative symptoms triggered by the expression of the pathological full length 128Q-hHTT protein. Thus, AUTEN-67, which has potent neuroprotective effects, but no known undesired side-effect [4], serves as a promising drug candidate for treating HD.

AUTEN-67 appears to be capable of penetrating through the blood-brain barrier, and to specifically inhibit the phosphatase activity of hMTMR14/Jumpy [4], a negative regulator of autophagic membrane formation [18]. We observed that EDTP/hMTMR14/Jumpy is expressed in the *Drosophila* brain (Fig. 1C, C') and thus, AUTEN-67 may modulate autophagy induction in neurons. Although mutations in certain *MTMR* genes can lead to human disorders such as myotubular myopathy and Charcot-Marie-Tooth peripheral neuropathy [17, 42], until now there was no study reporting the involvement of hMTMR14/Jumpy deficiency in any neuropathological condition. In addition, hMTMR14/Jumpy is also readily detectable in mammalian brain tissues (see at the Expression Atlas; https://www.ebi.ac.uk/gxa/experiments/E-MTAB-2798?_specific=on&queryFactorType=ORGANISM_PART&queryFactorValues=&geneQuery=mtmr14&exactMatch=true). Based on these results, we propose that AUTEN-67 has the potential to be used to enhance autophagic activity via inhibiting hMTMR14/Jumpy in the human central nervous system.

An intriguing result we obtained in this study is that Ref(2)P/SQSTM1/p62 protein levels in the brain of *Drosophila* expressing the pathological 128Q-hHTT protein are significantly lower than in animals transgenic for non-toxic 16Q-hHTT (Figs. 1B, B', 3A, A'). In good accordance with this observation, relative levels of ubiquitinated proteins in the brain were also decreased in 128Q-hHTT transgenic animals, as compared with controls (16Q-hHTT) (Fig. 3). To explain these results, we argue that a significant amount of Ref(2)P/SQSTM1/p62 and ubiquitinated proteins may localize to protein aggregates. Thus, only a fraction of these proteins should present in a soluble form that is detectable by Western blotting as performed here. To support experimentally this assumption, a Ref(2)P/SQSTM1/p62-specific antibody staining was performed on brain tissue samples (Fig. 2A, A'). This immunohistochemical labeling could detect both soluble and insoluble (inclusion-associated) Ref(2)P/SQSTM1/p62, and showed much higher levels of the protein in 128Q-hHTT than in 16Q-hHTT brain samples. In addition, it may explain why the level of ubiquitinated proteins is lower in 128Q-hHTT than in 16Q-hHTT samples, because protein inclusions in aged or HD-affected brains also contain ubiquitin [37, 40]. Nevertheless, AUTEN-67 treatment markedly enhanced autophagic activity and reduced the accumulation of damaged proteins in brain samples prepared from both transgenic strains (Figs. 1B, B', 2A-B' and 3A, A'). Postmortem human brain tissue samples from patients diagnosed for HD also displayed highly increased levels of Ref(2)P/SQSTM1/p62, relative to age-matched, unaffected controls (Fig. 6A, A'). As Ref(2)P/SQSTM1/p62 acts as a substrate in selective autophagy, one can speculate that in humans either mechanistic defects in autophagy and/or altered *Atg* gene expression are involved in HD pathogenesis, and accumulation of pathological hHTT proteins interferes with the autophagic process [10–12]. Therefore, our results presented here point to autophagy as a promising therapeutic target in treating HD. Pharmacological agents that enhance autophagic flux in neurons may be used effectively in treating HD-related symptoms, and AUTEN-67 represents such a drug candidate.

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CONFLICT OF INTEREST

Velgene Ltd. is an inventor of patents relating to the use of autophagy induction for treating neurodegenerative and other age-dependent diseases, as well of autophagy markers for detecting early stages of neurodegenerative diseases. The company is grateful for funding from the European Union (grant GOP-1.1.1-11-2012-0405). This sponsor has not reviewed the manuscript.

SUPPLEMENTARY MATERIAL

The supplementary table and figure are available in the electronic version of this article: http://dx.doi.org/10.3233/JHD-150180.

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