The examination of two autophagy-enhancing small molecule, AUTEN-67 and AUTEN-99, in a *Drosophila melanogaster* Huntington's Disease model

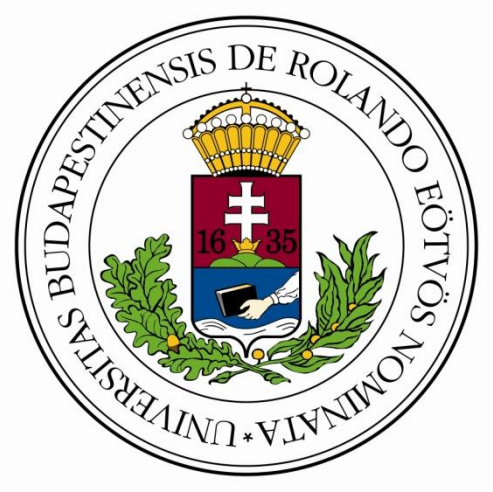
Main points of the PhD thesis

Viktor András Billes

Biological Doctoral School, Head of Doctoral School: Prof. Anna Erdei

Classical and Molecular Genetics Doctoral Program, Head of Program: Prof. Tibor Vellai

Eötvös Loránd University, Faculty of Sciences



Supervisor: Prof. Tibor Vellai

Eötvös Loránd University, Faculty of Sciences

Department of Genetics

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Introduction

Autophagy (cellular "self-eating") is a highly conserved, lysosome-mediated self-degradative pathway of essentially all eukaryotic cells. Its most significant type is called macroautophagy, in which a double membrane forms, sequestering parts of the cytoplasm. When the membrane growing is completed, an autophagosomal structure is formed. The autophagosome fuses with a lysosome (or before with a late endosome), thereby forming an autolysosome in which the degradation takes place by lysosomal hydrolases (Feng et al. 2014). Hereafter, macroautophagy is referred to as autophagy. Autophagy is executed by a set of proteins, encoded by *Autophagy-related* (*Atg*) genes, which are evolutionarily conserved from yeast to mammals (Meijer et al. 2007). ATG proteins form different complexes to carry out autophagy. Environmental cues are able to induce autophagy. The best known such factor is starvation. During starvation, autophagy contributes to the survival of cells by supplying energy and metabolites via the degradation (Rabinowitz and White, 2010; Mizushima and Komatsu, 2011). On the other hand, autophagy can operate in a selective manner, removing the long-lived and misfolded proteins, as well as damaged or superfluous organelles (Anding and Baehrecke, 2017). Autophagic receptors have a key role in the process of selective autophagy. These proteins generate molecular bridges between the structures destined for degradation and the forming autophagic membrane (Johansen and Lamark, 2011). The most ubiquitous autophagic receptor is p62 (in humans, SQSTM1 - *Sequestosome-1*, in *Drosophila*, Ref(2)P). p62 is also degraded by autophagy, hence it is widely used as a marker for the autophagic degradation (Shvets et al. 2008; Nagy et al. 2014). The quality control of cellular constituents is particularly important in non-dividing, terminally differentiated cells, including neurons (Menzies et al. 2017). Several human diseases associated with defective autophagy, and defects in autophagic activity especially in neurons are involved in the phatology of neurodegenerative conditions such as Alzheimer's, Parkinson's or Huntington's Disease (Mizushima et al. 2008; Nixon, 2013). Therefore, autophagy becomes a target of therapeutic strategies in treating these disorders (Martinez-Vicente, 2015; Towers and Thorburn, 2016).

Huntington's Disease (HD) is an autosomal dominant, fatal disorder with complex motor, cognitive and psychiatric disturbances. The underlying genetic mutation affects the *Huntingtin* (*HTT*) gene. Abnormal expansion of the polymorphic polyglutamine (polyQ) stretch, encoded by CAG repeats, leads to the manifestation of the disease, the symptoms definitely appear over 39 repeats (Rüb et al. 2016). The wild-type HTT protein has various cellular functions; it participates in the transport of numerous kinds of vesicles, in the primary cilia for-

mation, and in the regulation of transcription. In addition, it promotes selective autophagy by acting at several steps in the process (Saudou and Humbert, 2016). The mutant HTT leads to the damage of various cellular functions. However, it is a substrate for selective autophagy (Sarkar and Rubinsztein, 2008; Saudou and Humbert, 2016). Numerous autophagy-enhancing approaches and pharmacological agents are proved to be neuroprotective in various HD models (Sarkar et al. 2009; Martinez-Vicente, 2015). However, the effort of these approaches mainly focuses on mTORC1, the foremost negative regulator of autophagy, or AMPK, the main energy-sensor kinase of eukaryotic cell. By influencing these central regulators also affects many other cellular processes, in addition to autophagy (Laplante and Sabatini, 2009; Hardie, 2014; Martinez-Vicente, 2015). The situation is quite similar in case of the other group of autophagy enhancers, which act independently of mTORC1 and AMPK, but more upstream of autophagy (Williams et al. 2008; Sarkar et al. 2009). Thus, identification and examination of such autophagy enhancers, which targets autophagy in a much more direct way, is definitely needed.

A key step in the initiation of autophagy is the conversion of phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P), which is catalyzed by Vps34 kinase complex (Feng et al. 2014). The opposite reaction, the dephosphorilation of PI3P to PI, can be catalyzed by phosphatases belonging the Myotubularin protein family (Robinson and Dixon, 2006). This family of lipidphosphatses consists of 16 members in vertebrates, and some of the members are able to dephosphorilate another lipid signalling molecule, $PI3,5P_2$, which regulates several pathway and cellular processes (Alonso et al. 2004; Robinson and Dixon, 2006; Hnia et al. 2012). MTMR14 (Myotubularin-related 14, also known as Jumpy or MIP) acts on the pool of PI3P at the isolation membrane, but does not affect the other most important pool of PI3P at the sorting endosome, unlike most members of the Vps34 complex (Vergne et al. 2009). Searching for MTMR14 inhibitor small molecules, we identified AUTEN-67 (autophagy enhancer-67) and AUTEN-99 (Papp et al. 2016; Kovács et al. 2017). AUTEN-67 and AUTEN-99 inhibit the phosphatase activity of MTMR14 *in vitro*, and enhance autophagic activity in cell cultures and *in vivo* models; hence these molecules are found to be suitable to analyze them in *Drosophila* neurodegenerative disease models, including HD. The fruit fly *Drosophila melanogaster* is a widely used genetic model organism, the research of which has a more than hundred-year-old history. A quite large range of genetic tools and strain collections have been established so far for this organism (Duffy 2002; Venken and Bellen 2014). The autophagy described in mammals and *Drosophila* shows a high level of similarity. Thus, *Drosophila* appears to be applicable for autophagy research (Mulakkal et al. 2014; Nagy et al. 2014). It is

also widely used for modelling various neurodegenerative disorders. The *Drosophila* HD models recapitulate the vast majority of the pathological features of human HD. Moreover, it is suitable to get a better knowledge of the patomechanism of HD, and to identify relevant drug candidates (Xu et al., 2015). During the work presented in my PhD thesis, I investigated the effects of AUTEN-67 and AUTEN-99 in a *Drosophila* HD model, which transgenically expresses a pathogenic form of human HTT protein (128Q-HTT).

Aims of the study

Our main goal was to examine the effects of two MTMR14 inhibitor autophagy enhancers, AUTEN-67 and AUTEN-99, in a *Drosophila* HD model.

1. First, we wanted to clarify whether the intake of the drug candidates with food is able to enhance autophagic activity in the brain of adult flies, as it did in case of larval fat body.
2. In case of positive results, we wanted to investigate the effects of AUTEN-67 and AUTEN-99 on i) autophagy by examining the levels of the autophagic receptor p62/Ref(2)P and ubiquitinated proteins, ii) motor functions by analyzing the climbing and flying ability of transgenic animals, iii) the amount of mutant HTT proteins, and iv) the life span of transgenic animals expressing the pathogenic (mutant) 128Q-HTT and non-pathogenic (control) 16Q-HTT proteins.

Materials and Methods

Fly work

Drosophila strains were maintained on standard cornmeal-sugar-yeast-agar medium. Animals were kept at 29°C for the experiments. Stocks were obtained from the Bloomington Drosophila Stock Center (referred as BL), with genotype *UAS-128Q-HTT* (BL33808) and *UAS-16Q-HTT/CyO* (BL33810). The expression of both transgenes was driven by the panneural driver *Appl-Gal4* (BL32040).

Treatment with drug candidates

AUTEN-67 and AUTEN-99 dissolved in DMSO were added to yeast suspension (as final concentration would be the desired one, e.g. 50, 100 or 200 μM), and dropped 65 μl solution to the surface of each agar medium. For control, we used the same volume DMSO-containing yeast suspension without AUTEN-67 or AUTEN-99. Flies ate these yeast suspensions for two days, as the animal were transferred into a fresh vial every second day.

Climbing assay and determining the average speed of movement

1) We placed 20 females into a narrow glass column, and examined how many of them can climb up on the wall of the column, reaching the height 21.8 cm in 20, 40 and 60 sec after we had tapped the bottom of the column gently to induce movement (negative geotaxis).
2) We placed 10 females into a glass vial, and examined how many of them can climb up on the wall of the vial, reaching the height 21.8 cm in 10 and 20 sec after we had tapped the bottom of the vial gently. We also determined the average speed of the climbing flies by using video recording.
Three trials of 3-6 parallel measurements were performed in both cases, testing 7, 14 or 21 day-old animals.

Life span measurements

To analyze the life span of animals, adult females were selected. Flies were transferred into fresh medium containing vials every second day. The number of dead animals was counted daily. Measurements were carried out with 5-11 parallels, with 20-30 flies in each.

Western blot and immunohistochemistry experiments

To analyze the amount and localization of given proteins, Ref(2)P/p62, ubiquitin, mutant HTT, we applied immunolabelling methods. We carried out western blots to reveal the amount of soluble proteins, while we performed immunohistochemistry to detect insoluble protein aggregates.

Microscopy

Fluorescent images were captured with a Zeiss Axioimager Z1 upright microscope equipped with semi-confocal ApoTome, using AxioVision 4.82 software.

Quantification of the data and statistical analysis

Quantifications of fluorescent and western blot images were carried out by AxioVision 4.82 and/or ImageJ 1.45s softwares. We applied MatLab 7.12.0.635 (R2011a), RStudio 0.98.1102 or a SPSS 17.0 programmes for statistical analyses.

Results and theses

1. AUTEN-67 (50 μM) and AUTEN-99 (100 μM) are able to enhance the amount of mCherry-Atg8a-positive dot-like structures (mainly autolysomes) in the brain of adult flies.

2. AUTEN-67 (50 μM) and AUTEN-99 (100 μM) do not increase the ratio of animals with improved moving ability, expressing (control) 16Q-HTT or (mutant) 128Q-HTT proteins in

climbing assays at any ages examined or experimental settings. However, treatment with AUTEN-67 (50 μM) increases the average speed of 7-day-old animals.

3. AUTEN-67 (100 μM) improves the climbing ability of HD model flies expressing 128Q-HTT. It increases the ratio of animals reached the goal in 20 and 40 sec at the age of 7 and 14 days, but does not affect the climbing performance of control animals expressing 16Q-HTT at any given time.

4. AUTEN-99 (200 μM) improves the climbing ability of HD model flies expressing 128Q-HTT. It increases the ratio of animals reached the top of the vial within 20 sec at the age of 14-day-old adulthood, while it does not affect the climbing performance of animals expressing 16Q-HTT at any given time.

5. AUTEN-67 (100 μM) improves the flying ability of HD model flies expressing 128Q-HTT at the age of 14-day-old adulthood, while the performance of control animals expressing 16Q-HTT is not affected.

6. AUTEN-67 (100 μM), and presumably AUTEN-99 (200 μM), reduces the level of Ref(2)P/p62 and ubiquitinated proteins at the adult age of 21 days in flies expressing 16Q-HTT or 128Q-HTT proteins, suggesting elevated levels of autophagic activity.

7. AUTEN-99 (200 μM) inhibits the age-dependent accumulation of Ref(2)P/p62 in HD model animals expressing 128Q-HTT.

8. The levels of soluble Ref(2)P/p62 and ubiquitinated proteins are lower in 128Q-HTT-expressing flies than in those expressing 16Q-HTT. The reason behind this phenomenon is that 128Q-HTT proteins form large protein aggregates and inclusion bodies, which captures these proteins together with mutant HTT.

9. AUTEN-67 (100 μM) decreases the amounts of insoluble protein aggregates and mutant HTT proteins in the brain of flies expressing 128Q-HTT.

10. AUTEN-67 (100 μM) and AUTEN-99 (200 μM) decrease the level of toxic soluble mutant HTT proteins.

11. AUTEN-67 (100 μM) and AUTEN-99 (200 μM) increase the life span of control animals expressing non-toxic 16Q-HTT proteins.

12. AUTEN-67 (100 μM) increases the life span of HD model animals expressing the pathogenic 128Q-HTT protein, but not of those treated with AUTEN-99 at concentration 200 μM.

Discussion

The cellular degradative pathways, including autophagy and ubiquitin proteasome system (UPS), are crucial in maintaining cellular homeostasis via the removal of damaged proteins and other cellular constituents. This function is particular important in non-dividing, terminally differentiated cells, where the damaged components cannot be diluted by cell division (Rabinowitz and White, 2010; Mizushima and Komatsu, 2011; Menzies et al. 2017). Proteins, which tend to form aggregates are degraded easier by autophagy than the UPS. The appearance of misfolded mutant proteins is a common feature of numerous neurodegenerative diseases, including Alzheimer and Parkinson's Disease, amyotrophic lateral sclerosis (ALS), prion and polyglutamine diseases, the latter includes forms of spinocerebellar ataxia and Huntington's Disease (HD) (Ravikumar et al. 2002; Sarkar et al. 2009; Menzies et al. 2011; Sweeney et al. 2017). Autophagy is able to degrade selectively the mutant HTT protein, the factor responsible for HD, and this became more significant in the onset of disease, due to the progressive damage of UPS (Ravikumar et al. 2002; Bennett et al. 2005; Bennett et al. 2007; Sarkar and Rubinsztein, 2008). In line with that, genetic or pharmacological enhancement of autophagic activity has certainly beneficial effects in various HD models and in case of other neurodegenerative disorders as well. One reason of that is autophagy becomes also damaged in the pathogenesis of HD and in other neurodegenerative disorders (Nixon, 2013; Martin et al. 2015; Martinez-Vicente, 2015; Menzies et al. 2017). The autophagy inducing/enhancing agents studied so far target such regulators of autophagy, which function rather far from the core machinery of autophagy, and/or affect many other cellular processes (Sarkar et al. 2009; Martinez-Vicente 2015).

We searched for inhibitors of MTMR14, a potent negative regulator of the autophagic process, by screening a small molecule library. The candidates potentially act as autophagy inducers. We characterized two of the candidates, AUTEN-67 and AUTEN-99, which enhance

autophagic activity in HeLa cells, primary neurons, and in *Drosophila* and vertebrate models as well (Papp et al. 2016; Kovács et al. 2017). According to the *Drosophila* results, both AUTEN-67 and AUTEN-99 act through the *Drosophila* orthologue of MTMR14, called EDTP, as these small molecules lost their ability to enhance autophagic activity in *EDTP* mutant genetic background (Papp et al. 2016; Kovács et al. 2017). During my thesis work we ascertained that intake with the food both small molecules leads to enhanced autophagic activity in the brain of adult animals. AUTEN-67 and AUTEN-99 decrease the levels of autophagic receptor Ref(2)P/p62 and ubiquitinated proteins in animals expressing the pathogenic 128Q-HTT. These results suggest that AUTEN-67 and AUTEN-99 enhance autophagic activity in HD model animals as well. 128Q-HTT forms cytoplasmic protein aggregates in the brain, in contrast with earlier experiments using the full-length mutant HTT where the protein did not form aggregates either in larval motoneurons or interneurons of ventral nerve cord at the age of 20 days adulthood (Romero et al. 2008). Ref(2)P/p62 is also found in large cytoplasmic inclusions in the brain of flies expressing 128Q-HTT. In addition, it forms a shell around mutant HTT aggregates, as described in a HeLa HD model (Bjørkøy et al. 2005). The major fraction of insoluble p62 and ubiquitinated proteins colocalize, indicating that the cytoplasmic inclusions containing mutant HTT may also consist ubiquitin, like previous observations (Bjørkøy et al. 2005; Iwata et al. 2005; Heng et al. 2010). AUTEN-67 also reduced the amount of protein aggregates (we did not test the effect of AUTEN-99 in this paradigm), and both AUTEN-67 and AUITEN-99 decreased the amount of toxic soluble 128Q-HTT proteins. These data are consistent with previous result reporting that mutant HTT is a substrate of selective autophagy. Hence, the enhanced autophagic activity is neuroprotective through lowering the level of mutant HTT (Ravikumar et al. 2002; Sarkar and Rubinsztein, 2008; Sarkar et al. 2009). The life span of animals expressing non-pathogenic 16Q-HTT was increased by the administration of AUTEN-67 and AUTEN-99. This is consistent with findings that both small molecules are able to increase the life span of w^{1118} animals, in which *HTT* transgenes are not expressed (Papp et al. 2016; Kovács et al. 2017). It is also in good accordance with the role of autophagy in determining of life span (Vellai 2009; Rubinsztein et al. 2011; Pyo et al. 2013). In addition, AUTEN-67 also increases the life span of HD model animals expressing 128Q-HTT. AUTEN-67, and in a lesser extent AUTEN-99, improves the impaired motor functions of 128Q-HTT animals. It is a quite rare phenomenon that a single drug candidate molecule can positively affect life span and motor functions in a HD model. Metformin, for example, which activates AMPK, has a similar beneficial effect in male mice (Ma et al. 2007). Administration of cystamine, an organic disulfide that inhibits transglutaminase, is able to ameliorate

impaired motor functions and to increase life span HD model mice, where aggregates are also formed in the periphery (Dedeoglu et al. 2002). On the other hand, if the expression of mutant HTT is driven by its own promoter, cystamine has no effect on motor functions, as well as it is also ineffective in *Drosophila* model of HD (Agrawal et al. 2005; Van Raamsdonk et al. 2005).

AUTEN-67 and AUTEN-99 are able to enhance the autophagic activity in a *Drosophila* HD model, and to reduce the amount of mutant (128Q) HTT proteins, and toxicity they causes. Presumably because of these, AUTEN-67, and partly AUTEN-99, improves motor functions and increase life span. Therefore, further examination of these molecules is well-founded. We hope that AUTEN-67 and AUTEN-99 will be investigated in both mammalian cell cultures and mice HD models. If so, these small molecules would also be combined with other drugs or drug candidates. This procedure may reduce the undesired side effects of AUTEN molecules. Either with these approaches or via other methods, HD becomes treatable disease in the near future.

Publications related to the doctoral thesis

Billes V, Kovács T, Hotzi B, Manzéger A, Tagscherer K, Komlós M, Tarnóci A, Pádár Z, Erdős A, Bjelik A, Légrádi A, Gulya K, Gulyás B, Vellai T. 2016. AUTEN-67 (Autophagy Enhancer-67) Hampers the Progression of Neurodegenerative Symptoms in a Drosophila model of Huntington's Disease. *Journal of Huntington's disease* **5**: 133-147.
IF: 1.58; independent citations: 1

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IF: 4.259; independent citations: 1

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