Sex-specific regulation of aging and neuronal functions in genetic model organisms

Main points of the PhD thesis

Bernadette Hotzi

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

Classical and Molecular Genetics Doctoral Program,
Head of Program: Prof. Tibor Vellai DSc, professor and head of department

Eötvös Loránd University, Faculty of Sciences

Supervisor:
Prof. Tibor Vellai DSc
professor and head of department
Department of Genetics

Eötvös Loránd University, Faculty of Sciences
Department of Genetics
Budapest, 2019

Introduction

During my PhD work I studied the regulation of aging and various neuronal functions in the nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster*. Aging is a complex, multifactorial biological process, which can be characterized by a gradual decrease in the functional integrity of cells and organs over time, and, as a result, the organism becomes more sensitive to acquire degenerative pathologies and to various environmental stress factors. The risk of several diseases and the death increase with age. Aging is caused by the lifelong, progressive accumulation of cellular damage. As life expectancy increases worldwide in developed societies, aging research has significant medical, social and economic implications. Therefore, understanding the regulation and mechanism of the aging process represents currently one of the most important and intense research areas in biology[1,2].

An interesting aspect of aging is that the lifespan of the two sexes differs in many sexually dimorphic animal species[3], including humans. For example, women lives longer than men by approximately 5-7 years[4]. In addition, there are sex-specific differences in both the frequency and process of age-related pathologies and responses to therapy as well[5]. Examining the nervous system is a prior area for exploring aging control and sex differences in various physiological parameters. *C. elegans* and *Drosophila* are model organisms being popular for studying aging and neurobiology because their lifespan is relatively short, and their nervous system is relatively simple, making the analysis of molecular changes behind longevity and neuronal functions to be easy.

The two sexes of *C. elegans*, hermaphrodites (which are actually females with a short period of sperm production) and males, show significant differences in anatomy, physiology, behavior and lifespan. In this organism, the ratio of chromosome X to the set of autosomes is the primary determinant of sex. This ratio determines the activity pattern of the so-called sex-determination genetic pathway that ensures each somatic cell to develop according to the proper sex identity. The terminal (effector) transcription factor of the pathway is TRA-1, whose mammalian orthologs are the GLI proteins, the effector of Hedgehog signaling. TRA-1 is active in hermaphrodites, while inactive in males. The protein determines hermaphrodite fates by repressing male-specific genes. Changes in its activity are sufficient to alter the sex-specific phenotypes of the animal regardless of karyotype. Although sexual identity of somatic cells is under TRA-1 function, relatively few direct target genes of TRA-1 have been

identified so far[6]. Our group previously identified 42 potential target genes of TRA-1 by using *in silico* methods[7].

In worms, *daf-16/FoXO* gene encodes the effector transcription factor of the insulin/IGF-1 signaling pathway. DAF-16 regulates development, aging, metabolism, immunity and various stress responses[8,9]. *daf-16* has a number of isoforms. Only three of these isoform groups, *a1/a2*, *b* and *d/f(/h)*, appear to be functional[10]. These proteins regulate their target genes in tissue and process-specific ways[8]. We found two conserved TRA-1 binding sites in the *daf-16* genomic region. One of them is located in the 5' region of the *d/f* isoform and the other does in the first exon of isoform *b*[10]. *d/f* isoform is implicated in lifespan control, while *b* isoform has a role in regulation of dauer larval development (an alternative developmental stage)[11]. Sex-specific differences have been observed in both lifespan and dauer development[12,13]. In my thesis I focus on the results mainly related to *d/f* isoforms and aging.

goa-1 is the sole nematode ortholog of mammalian Gαi/o proteins, which form one type of the α subunit of heterotrimer G proteins. It plays important roles in the regulation of numerous neuronal functions and behaviors, some of them display marked sex-specific characteristics[14,15]. We first demonstrated that TRA-1 directly represses *goa-1* transcription through a conserved binding site in exon 5 we identified previously, and this interaction is established throughout adulthood[16]. Accordingly, *goa-1* levels were significantly lower in hermaphrodites than in males[16]. This difference may influence sex-specific behavioral patterns[16]. Further results we obtained suggest that the TRA-1 - *goa-1* regulatory axis may be even more significant at embryonic stages, and may play a role in the development of the sex-specific pattern of the nervous system. The risk of acquiring neurodegenerative diseases increases with age. Such diseases are basically characterized by an increased amount of aberrant proteins, and their toxic aggregates, which can lead to the loss of the affected neurons[17]. One of the most effective ways to delay brain aging and treat the progression of neurodegenerative pathologies could be the increase of autophagic activity, which reduces the amount of abnormal protein aggregates by degrading these factors enzymatically[18,19]. Numerous current therapeutic methods are focusing on autophagy activation. However, proteins targeted by these efforts influence many other cellular processes, hence their modulation can result in undesired side-effects[19]. A more specific therapeutic target could be a myotubularin-like lipid phosphatase, MTMR14 which normally inhibits autophagy-specific membrane formation by reducing phosphatidylinositol 3-phosphate (PI3P) levels[20]. Our

research group has previously shown that two small molecules, AUTEN-67 and -99 (*autophagy enhancer*) derived from a screen for autophagy inducers are capable of potently inhibiting MTMR14[21,22]. *Drosophila* is an excellent model organism to examine brain aging[23]. We tested the effect of both AUTENs in a *Drosophila* model of Huntington's disease (HD). Based on our results, treating flies with these AUTENs increase the autophagic flux in the adult (aging) brain, reduce the amount of toxic mutant proteins and protein aggregates, and improve neuronal (*e.g.* motor) functions[21,24]. AUTEN-67 also increases lifespan[21,24]. In my thesis I have discussed which steps of autophagy become changed upon treatments.

Aims of the study

- My goal was to determine whether TRA-1 controls the transcription of *daf-16d/f,* and this does through the identified conserved TRA-1 binding site. I also wandered to know whether this regulatory relationship is responsible for establishing the longer lifespan of hermaphrodites by which they outlives males.
- My further goal was to examine the effect of TRA-1 on the transcription of *goa-1* at embryonic stages, and whether this regulatory effect occurs through the identified TRA-1 binding site in the 5th exon. I also asked whether the regulatory relationship (TRA-1 – *goa-1*) has a role in the development of nervous system of the two sexes.
- My goal was also to examine whether two small molecules, AUTEN-67 and AUTEN-99, have a protective effect on the autophagic process in a *Drosophila* model of HD, and if so, which step/steps of autophagy is/are affected.

Methods

Generation of *C. elegans* males

As the rate of males is very low in natural *C. elegans* populations (<0.2%), I increased the number of males by heat shock and maintained them using crossing[25].

Lifespan assays

Lifespan assays were carried out at 25°C. Animals were propagated on NGM plates containing FUdR (5-fluoro-2'-deoxyuridine). In the assays for determining sex-specific differences, approximately 50–60 hermaphrodites and 10-15 males were kept on each plate. In other assays, 60-70 animals were kept on each plate. I counted and removed animals that died due to aging daily.

RNA interference

We applied the so-called "feeding" RNAi treatment method, using L4440 vector[26]. The RNAi constructs silenced all isoforms of target genes.

Cloning of transgenic constructs, and generating transgenic strains and analyzing their expression pattern

Recombinant DNA techniques were used to produce transcriptional and translational fusion GFP reporter constructs (for *daf-16* and *goa-1*), which contained the identified conserved TRA-1 binding sites. Using *in vitro* mutagenesis, altered variants were also produced which contained no conserved TRA-1 binding site (control). Transgenic animals were generated by biolistic cotransformation. Quantifications of fluorescent images were carried out by ImageJ software. Whole animals were involved in the analysis in case of *daf-16* reporter constructs, while whole embryos were examined in case of *goa-1* constructs. Head and tail regions were analyzed in animals carrying *pkd-2* reporter.

Quantitative (q) and semi- quantitative (sq) PCR

Total RNA was isolated from 30 L4/young or aged (6 days old) adult animals (for *daf-16* qPCR measurements) or from multiple plates covered by embryos (for *goa-1* sq-PCR measurements). Then, cDNA was synthesized with reverse transcription. Adult animals were selected to achieve the appropriate genotype/sex, while in case of embryos only a certain percentage of the embryos were appropriate. Melting curve analysis was performed to confirm correct PCR product size and the absence of nonspecific products. Evaluating qPCR results, relative gene expression values were determined using the comparative CT method (2-ΔΔCT formula)[27]. sq-PCR results were evaluated by ImageJ with densitometric analysis.

Chromatin-immunoprecipitation (ChIP)

Chip analysis was essentially performed as described previously by Ratajewski et al. (2012)[28], with some modifications. The amount of specific DNA was quantified using qPCR and locus-specific primers designed by the BiSearch software[29]. The TRA-1 target gene *xol-1* was used as a positive control. *daf-11*, which does not contain TRA-1 binding site, was used as an internal negative control to normalize quantification.

AUTEN-67 and AUTEN-99 treatments

Both small molecules were dissolved in DMSO and then added to the yeast suspension serving as nutrition for *Drosophila*. In control, only DMSO was mixed with yeast suspension. Animals were transferred every two days into new, freshly made 'treated vials'. The experiment was performed at 29°C.

Western blot analysis

Proteins were isolated from 200 young adult *C. elegans* or 7 adult *Drosophila* head. Samples were run on SDS-PAGE and transferred to nitrocellulose membrane. After incubation with the appropriate antibodies, detection was carried out using NBT-BCIP solution. Evaluation was performed by ImageJ program with densitometric analysis.

Results and theses

Sex-specific regulation of aging in *C. elegans*

- According to our view, mixed populations with a high rate of hermaphrodites provide the best opportunity to study how they behave and age ("natural populations"). Under these conditions, wild-type hermaphrodites live longer than males.
- For testing DAF-16 activity, the *daf-2/IGFR* loss-of-function mutant genetic background was used, in which DAF-16 is hypercativated. In the insulin/IGF-1 signaling-defective background, hermaphrodites have a longer lifespan, and this phenotype is manifested in a DAF-16-dependent manner.
- I also found that the nematode sex-determination pathway regulates lifespan, and the longer lifespan of hermaphrodites is TRA-1-dependent.
- TRA-1 and DAF-16 act in the same genetic pathway to control aging, and *daf-16* loss-of-function mutations suppress the effect of sex-determination pathway on lifespan.
- I have demonstrated that TRA-1 directly promotes the transcription of *daf-16d/f* isoform, via the identified binding site.
    - Using a ChIP analysis (by applying two different TRA-1-specific antibodies) we showed that TRA-1 is capable of binding the identified conserved binding site in the regulatory region of *daf-16 in vivo*.
        - I demonstrated by a Western blot analysis that the generated TRA-1 antibody labels TRA-1 protein.
    - Using quantitative-PCR, TRA-1 was proved to directly stimulate the expression of *daf-16d/f* isoform in hermaphrodites.
- With the expression analysis of reporter constructs generated by us (*lifeIs300, lifeIs302*), I showed that TRA-1 promotes the expression of *daf-16d/f* isoform in hermaphrodites through the identified conserved binding site.

- The expression level of the functional translational reporter *daf-16d/f (lpIs14*) is higher in hermaphrodites than in males, and the construct increases lifespan more significantly in hermaphrodites.
- An *in silico* analysis identified a conserved GLI binding site in the human *FoXO3* gene, suggesting an evolutionarily conservation for this molecular regulatory system.

Sex-specific regulation of neural functions in *C. elegans*

- By analyzing the expression pattern of reporter constructs generated by us (*lifeIs306, lifeIs307*), TRA-1 was shown to inhibit the expression of *goa-1* through the identified binding site at embryonic stages
- Expression analysis of a male-specific marker, *pkd-2::gfp* (*bxIS14*), suggests that GOA-1 activity may function in the development of sexually dimorphic patterns in the nervous system.
    - The expression level of *pkd-2::gfp* is changed in *goa-1* mutant males in the head and in the tail region as well.
    - In *goa-1* gain-of-function mutant hermaphrodites, which show male-specific behavioral patterns, ectopic PKD-2::GFP expression is appeared in the head with a small penetrance.

Examination of autophagy-activating small molecules in a *Drosophila* model of HD

- The level of the autophagic substrate p62/Ref(2)P protein is reduced by treatment with AUTEN-67 and AUTEN-99 in a HD *Drosophila* model, suggesting that both molecules enhance the autophagic flux.
- AUTEN treatment-induced changes in Atg8a-I and Atg8a-II protein levels suggest that both small molecules promote autophagosome formation, and AUTEN-67 also triggers autophagosome maturation.

Conclusions

I showed that in *C. elegans*, the sex-determination pathway directly regulates the expression of *daf-16* (actually its *d/f* isoforms), the terminal effector of the insulin/IGF1 signaling system, which plays a major role in aging control. This regulatory axis (TRA-1 – *daf-16*) may establish a sex bias in lifespan determination. Sex-specific differences in aging control thus are determined genetically in nematodes. The insulin/IGF1 signaling pathway influences various biological processes. Some of them show sex-specific differences and it is possible that the TRA-1 – *daf-16* regulatory axis underlies these differences. Until now TRA-1 has

been largely identified (except from one example) as a transcriptional repressor. Data generated by my PhD work however identify *daf-16* as a TRA-1 target that is upregulated by this transcription factor. This has revealed a more complex regulatory system by which TRA-1 (and the sex-determination machinery) controls sexual cell fate determination.

We provided evidence that the *C. elegans* sex-determination pathway also directly regulates, in fact represses, the transcription of *goa-1*[16], which encodes the α subunit of the heterotrimeric G protein. GOA-1 controls various behavioral patterns and neuronal functions. As TRA-1 inhibits the activity of *goa-1*, this inhibitory interaction may be responsible for some sex-specific behavioral and neural differences. Indeed, we showed that this regulatory axis plays a role in male mating behavior[16]. According to our results, this regulatory interaction between TRA-1 and *goa-1* is more significant during embryogenesis than in adulthood. It may also influence the development of the nervous system in a sex-specific manner.

Based on our *in silico* analysis, these molecular interactions (TRA-1 – *daf-16* and TRA-1–*goa*-1) may be evolutionarily conserved, and thus also exist in human (between GLI - *FoXo3* and GLI – *Gi/o*)[10,16]. These can be a role in gender specific differences in lifespan and many behavioral, neuronal and physiological traits.

I also dealt with potential therapeutic treatment of neurodegenerative diseases. I examined the influence of two small molecules (AUTEN-67 and -99) with autophagy-enhancing effects on the pathogenesis of Huntington's disease, using a *Drosophila* model. Both agents enhanced the autophagic flux, and delayed and weakened pathological symptoms[21,24]. These AUTENs induce autophagosome formation, and, in addition, AUTEN-67 promotes the maturation of autophagosomes. Thus, these drug candidates may serve as potent tools for delaying the incidence of HD and other neurodegenerative disorders.

Publications related to the doctoral thesis

Hotzi B*, Kosztelnik M, Hargitai B, Takács-Vellai K, Barna J, Bördén K, Málnási-Csizmadia A, Lippai M, Ortutay Cs, Bacquet C, Pasparaki A, Arányi T, Tavernarakis N, Vellai T (2018): Sex-specific regulation of aging in Caenorhabditis elegans. *Aging Cell* 17(3): e12724. ISSN: 1474-9726

IF: 7.627; independent citations: 2

Billes V*, Kovacs T, **Hotzi B**, Manzeger A, Tagscherer K, Komlos M, Tarnocia A, Padar Zs, Erdos A, Bjelik A, Legradi A, Gulya K, Gulyas B, Vellai T (2016): AUTEN-67 (Autophagy Enhancer-67) Hampers the Progression of Neurodegenerative Symptoms in a Drosophila model of Huntington's Disease. *J Huntington's Disease* 5: 133–147.
IF: 1,58; independent citations: 4

Kovács T*, Billes V*, Komlós M*, **Hotzi B**, Manzéger A, Veszelka Sz, Walter F, Deli M, Hackler L, Alfoldi R, Borsy A, Welker E, Kovács A, Pádár Zs, Erdős A, Legradi A, Bjelik A, Gulya K, Gulyás B, Vellai T (2017): The small molecule AUTEN-99 prevents the progression of neurodegenerative symptoms. *Sci Rep.* **7**: 42014.
IF: 4,122; independent citations: 8

*: first author(s)

References

1. Kenyon, C. J. The genetics of ageing. *Nature* **464**(7288), 504-12 (2010).
2. Kirkwood, T. B. L. Understanding the odd science of aging. *Cell* **120**, 437–447 (2005).
3. Regan, J. C. & Partridge, L. Gender and longevity: Why do men die earlier than women? Comparative and experimental evidence. *Best Pract. Res. Clin. Endocrinol. Metab.* **27**, 467–479 (2013).
4. Austad, S. N. & Fischer, K. E. Sex Differences in Lifespan. *Cell Metab.* **23**, 1022–1033 (2016).
5. Tower, J. Sex-Specific Gene Expression and Life Span Regulation. *Trends Endocrinol. Metab.* **28**, 735–747 (2017).
6. Zarkower. Somatic sex determination. *WormBook* 1–12 (2006).
7. Hargitai, B. *et al.* xol-1, the master sex-switch gene in C. elegans, is a transcriptional target of the terminal sex-determining factor TRA-1. *Development* **136**, 3881–7 (2009).
8. Murphy, C. T. & Hu, P. J. Insulin/insulin-like growth factor signaling in C. elegans. *WormBook* 1–43 (2013).
9. Martins, R., Lithgow, G. J. & Link, W. Long live FOXO: Unraveling the role of FOXO proteins in aging and longevity. *Aging Cell* **15**, 196–207 (2016).
10. Hotzi, B. *et al.* Sex-specific regulation of aging in Caenorhabditis elegans. *Aging Cell* **17**, e12724 (2018).
11. Kwon, E.-S., Narasimhan, S. D., Yen, K. & Tissenbaum, H. a. A new DAF-16 isoform regulates longevity. *Nature* **466**, 498–502 (2010).
12. Ailion, M. & Thomas, J. H. Dauer formation induced by high temperatures in Caenorhabditis elegans. *Genetics* **156**, 1047–67 (2000).
13. Gems, D. & Riddle, D. L. Longevity in Caenorhabditis elegans reduced by mating but

not gamete production. *Nature* **379**, 723–725 (1996).
14. Koelle, M. R. Neurotransmitter signaling through heterotrimeric G proteins: insights from studies in C. elegans. *WormBook* 1–78 (2016).
15. Bastiani, C. Heterotrimeric G proteins in C. elegans. *WormBook* 1–25 (2006).
16. Kutnyánszky, V. *et al.* Sex-specific regulation of neuronal functions in Caenorhabditis elegans. *Mol. Genet. genomics* **under rev.**, (2019).
17. Mulakkal, N. C. *et al.* Autophagy in drosophila: From historical studies to current knowledge. *Biomed Res. Int.* **2014**, 273473 (2014).
18. Menzies, F. M. *et al.* Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. *Neuron* **93**, 1015–1034 (2017).
19. Tan, C. C. *et al.* Autophagy in aging and neurodegenerative diseases: Implications for pathogenesis and therapy. *Neurobiol. Aging* **35**, 941–957 (2014).
20. Vergne, I., Roberts, E., Elmaoued, R. A., Laporte, J. & Deretic, V. Control of autophagy initiation by phosphoinositide 3-phosphatase jumpy. *EMBO J.* **28**, 2244–2258 (2009).
21. Kovács, T. *et al.* The small molecule AUTEN-99 prevents the progression of neurodegenerative symptoms. *Scientific reports* **7**, 42014 (2017).
22. Papp, D. *et al.* AUTEN-67, an autophagy-enhancing drug candidate with potent antiaging and neuroprotective effects. *Autophagy* **12**, 273–286 (2016).
23. Marsh & Thompson, L. M. Drosophila in the Study of Neurodegenerative Disease. *Neuron* **52**, 169–178 (2006).
24. Billes, V. *et al.* AUTEN-67 (Autophagy Enhancer-67) Hampers the Progression of Neurodegenerative Symptoms in a Drosophila model of Huntington's Disease. *J. Huntingtons. Dis.* **5**, 133–147 (2016).
25. Sulston, J. & Hodgkin, J. Methods. In The nematode C. elegans *Cold Spring Harbor Laboratory Press* pp v 587-606. (1988)
26. Ahringer, J. Reverse genetics *. *WormBook* April 6, (2006).
27. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
28. Ratajewski, M. *et al.* ABCC6 Expression Is Regulated by CCAAT/Enhancer-Binding Protein Activating a Primate-Specific Sequence Located in the First Intron of the Gene. *J. Invest. Dermatol.* **132**, 2709–2717 (2012).
29. Arányi, T., Váradi, A., Simon, I. & Tusnády, G. E. The BiSearch web server. *BMC Bioinformatics* **7**, 431 (2006).