INSTITUTE OF EXPERIMENTAL ENDOCRINOROLOGY

BIOMEDICAL RESEARCH CENTER
Slovak Academy of Sciences

October 19, 2022

Review

of the Houda Ouns Maaroufi's PhD thesis

"Role of adenosine pathway on cell growth and stress response in *Drosophila*"

Thesis of PhD candidate, Houda OunsMaaroufi's, is widely concepted work and deals with (1) the effects of mutation in concentrative nucleoside transporter 1 that alters spermatid maturation and mating behavior, (2) how adenosine signaling and its downstream target *mdg4* can modify the pathogenic effects of polyglutamine in a *Drosophila* model of Huntington's disease, and (3) how expression of human mutant huntingtin protein in hemocytes impairs immune responses in *Drosophila*.

These 3 different topics find common features by acting on or via adenosine signaling pathway. In the first and I would say main topic, author described the role of Ado metabolic pathway in the regulation of cell growth in the testis. Specifically author investigated the physiological role of one of the major transmembrane proteins, *concentrative nucleoside transporter 1* (*cnt1*), in *Drosophila melanogaster*, and found that Cnt1 is highly expressed particularly in the head and tail of spermatids. To check whether Cnt1 plays a role in modulating *Drosophila* fertility, author used the CRISPR/Cas9 system to create a mutation in the *cnt1* gene. These mutants exhibited partial sterility associated with a reduced duration of copulation compared to controls, and impaired spermatid maturation. Males with defect in mating behavior have high numbers of mature spermatids released in the TE region, but a lower number in the SV, indicating impaired movement of mature spermatids. The tails of the mature sperm were not properly coiled and became tangled in the TE region, which correlates with the lack of sperm migration toward SV. Using electron microscopy authors observed that the tails of spermatids had an abnormal number of mitochondrial subunits. This defect explains that the mature spermatids have a mechanical problem with the mitochondrial defect caused by the *cnt1* mutation. Analysis of the RNAseq data revealed impaired expression of a *sperm-specific dynein intermediate chain 4* (*sdic4*), which is essential for sperm tail motorization, and also impaired ATP synthesis. All this having synergic effect on sperm dynein motorization. Overall, these results demonstrated the importance of *cnt1* gene in male fertility and suggested that the observed defects in mating behavior and spermatogenesis are due to alterations in nucleoside transport and related metabolic pathways.

The second topic deals with *Drosophila* model of Huntington's disease (Q93) and show how adenosine signaling and its downstream target *mdg4* modify the pathogenic effects of polyglutamine expansion. Houda demonstrated that HTT mutation (mHTT) acts as a stressor on the Ado pathway. mHTT, once expressed throughout the organism, causes larval lethality associated with a decrease in Ado titer in hemolymph. Thus, she hypothesized that the decrease in Ado levels is due to alterations in Ado metabolism or transport genes. Described results also show that the level of expression of *adgf-A*, *adgf-D*, *ent1*, *ent2*, and *ent3* is

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decreased in the brain of Q93 larvae. Therefore, by overexpressing *adgf-A* and *adenoK* in Q93 flies she increased the metabolism of intracellular and extracellular Ado in flies which extended their lifespan. Applicant also enhanced Ado signaling in Q93 flies by overexpressing *adoR* and caused a reduction of the lifespan of mHTT-expressing flies, in contrast to flies in which *adoR* was silenced. Furthermore, silencing *ent2* in Q93 flies reduced the lifespan of *Drosophila*, in contrast to overexpression of *ent2*. Her results suggest that *adoR* and *ent2* are key regulators of mHTT effects and that the reduction of Ado in Q93 flies is a natural response to cytotoxic stress. In addition to *mgd4*, she also identified new downstream target of AdoR pathway, the heat-shock protein 70 (Hsp70). Knock-down of *mod(mdg4)* and *adoR* suppressed the increased production of Hsp70 in Q93 flies involved in the response to various stress conditions. In conclusion, this set of author's data suggest that *ent2*, *adoR*, *mod(mdg4)*, and Hsp70 represent an important Ado pathway acting against cytotoxic stress.

The third subject of the thesis is dedicated to expression of human mutant huntingtin protein in *Drosophila* blood cells (hemocytes) where author observed an impairment of immune responses. Houda found that the number of circulating hemocytes is decreased in Q93 flies. Therefore, she investigated whether the immune response to infection was impaired in Q93 larvae. To do so, she infected *Drosophila* larvae with the nematodes *Heterorhabditis bacteriophora* or *Steinernema carpocapsae* and found that Q93 larvae had higher mortality than the control group. Author also observed a decrease in the clotting activity of the hemolymph in Q93 larvae, suggesting that the susceptibility to infection was due to the poor quality of the blood clot. Similarly, infection of Q93 larvae with a parasitic wasp *Leptopilina boulardi* showed a high number of emerging wasps compared to the control, indicating that the wasps overwhelmed the larval immune system by defecting wasp eggs melanization. PhD applicant also further investigated whether mHTT expression alters hemocyte function by using S2 cell lines expressing mHTT. The S2 cells were infected with *E. coli* particles conjugated with a pH-sensitive dye (pHrodo), and subsequently decreased phagocytic activity in mHTT-expressing cells was observed. We also checked whether the phagocytic activity of S2 cells was affected by mitochondrial dysfunction and impaired energy metabolism. In mHTT-expressing cells, ATP levels were significantly decreased, which could limit the cellular immune responses against pathogenic infections. The phenotype was associated with increased expression of cytokines, with *upd3* strongly upregulated in mHTT-expressing cells compared to controls. In addition, expression of *dome* receptor and several downstream targets of the JAK/STAT pathway were also increased. The phenotype was associated with a decrease in antimicrobial peptide production upon bacterial infection. This study provides a novel system to study tissue-specific effects of mHTT in *Drosophila* immune cells.

I am glad to summarize that these are very nice and original results that document another good piece of scientific work from the laboratory of the mentor, Michal Žurovec, in this case significantly contributed by Houda Ouns Maaroufi. Nevertheless, I have few questions to the author and her thesis, specifically quoting topic of concentrative nucleoside transporter:

1. It is my pleasure to see that data on *cnt1* mutation and phenotype are the first functional data on this gene related to sterility, and they were completely missing in FlyBase. Did author

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try to compare data on *cnt1*^{FD} mutation with those from RNAi *e.g.* using *P{TKO.GS04932}attP40* construct from TRiP project ? If yes, phenotypes were similar or different ?

2. Regarding fertility test, I have simple question: how the appearance of offspring was examined/counted: by number of deposited eggs or number of hatched larvae ?

3. On one side you stated that caspase signaling remained active in the individualized portion of the spermatids, but the apoptotic-like process required for the elimination of excess organelles were impaired in *cnt1*^{FD} mutation. If caspase signaling was not affected, can you indicate which part of the apoptotic pathway required for elimination of the excess organelles is involved and how it is related to disorganization of the spermatid's tail ?

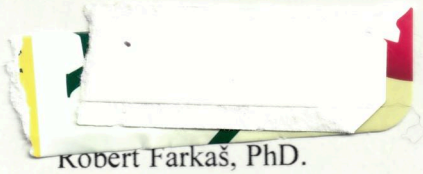
4. Do you have an explanation or model to explain how *cnt1* affect numbers of major and minor mitochondria as well as axonemes in the spermatids ?

5. What kind of steps can be implicated in the signal transduction from transmembrane Cnt1 protein to affect expression of so many ribosomal protein genes ?

6. You mentioned extracellular space factors among top overrepresented categories of genes. Could you mention at least some of them, *e.g.* those which were most affected ?

7. Can you list at least some representative immune-related proteins (genes) that have been differentially expressed in *cnt1* mutants, and how you explain this specific phenotype ?

Based on above mentioned facts, contribution of the research data with obtained evidence, experimental methods used, and number of published papers, the achievements of Houda Ouns Maaroufi meets alls acceptable scholarly criteria for PhD dissertation. Therefore, I can conclude that she fulfilled all major requirements, and I can gladly recommend the thesis for successful defense in the front of a PhD committee, and warding PhD degree.



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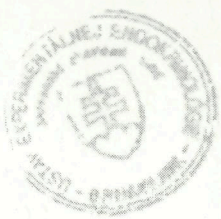

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Review of Ph.D. thesis written by MSc. Houda Ouns Maaroufi: Role of adenosine pathway on cell growth and stress response in *Drosophila*

Ph.D. thesis of MSc. Houda Ouns Maaroufi describes the role of adenosine signalling in *Drosophila melanogaster* as insect model with possible genetic modifications of metabolism. The effect on cell growth was studied mostly on concentrative nucleoside transporter 1 and 2 (*cnt1*, *cnt2*) during male spermatogenesis and also its effect on mating behaviour was clearly demonstrated. In following studies, a mutation in the huntingtin protein (mHTT) essential for neuronal development was used as a tool to promote cytotoxic stress in *Drosophila*. Results show the importance of adenosine pathway during stress conditions using genetically enhanced intracellular and extracellular metabolism and also new downstream targets of adenosine pathway were described.

The thesis (106 pages) consists of introduction part, three published articles, concluding remarks and references. The „Introduction" provides background about adenosine in *Drosophila* metabolic and signalling pathways and its role in cell growth and stress. Aims are clearly described and correspond with obtained results which are briefly summarized after reprints of scientific papers. The thesis is written in English, the text is understandable, and results are clearly documented and discussed mostly in already published scientific papers.

MSc. Houda Ouns Maaroufi published the results as first author of scientific paper in an international, peer-reviewed journal Frontiers in Cell and Developmental Biology (IF = 6.081). The thesis contains also other two papers published in Frontiers in Cell and Developmental Biology (IF = 6.081) and Frontiers in Immunology (IF = 8.786) where is MSc. Houda Ouns Maaroufi as a second-author; her individual contribution to team work is stated. She is also co-author of another two scientific papers not included in the thesis. The peer-review process in all journal redactions is also a guarantee of results quality.

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Comments and questions

- In the first study authors shows the importance of concentrative nucleoside transporters (*cnt*1 and *cnt2*) for male testis development. Is there any effect on their gene expression during male aging? What are other members of concentrative nucleoside transporter family present both in males as well as females? What about the situation in other insect males, can we estimate similar situation as in *Drosophila*?
- Adenosine signalling and metabolism in flies involves lower number of genes than in mammals, e.g. *Drosophila* contains only one adenosine receptor (AdoR) instead of four as in mammals. How it looks like from evolutionary point of view and is there also connection of AdoR with olfactory functions?
- Stress reaction can be initiated by different stressors, is there any difference in adenosine pathway according the type of stressor? At the beginning is usually stress reaction stimulatory and during long-lasting stress it moves to inhibition of physiological and immune reactions, is similar trend also in *Drosophila*?
- How cooperates adenosine signalling with hormonal control of the stress?
- Figure legends must contain the source reference when it is not created by the author, reference in the text together with link to figure is not enough. Also, the statement if the figures changed from the original should be mentioned. Reference in the text or figure legends must be a part of the sentences, sometimes are located separately after coma.

Evaluation:

To produce highly valuable results suitable for publication in reputed scientific journals MSc. Houda Ouns Maaroufi needed large experimental up to date background in molecular biology, genetics and *Drosophila* techniques. Except published articles, she attended six international conferences as main author of the talk or poster and was also awarded by several prizes. Ph.D. thesis is well written and meets the requirements, thus **I suggest accepting the thesis for defence as one part of Ph.D. degree**.

Yours sincerely,

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