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Review of Ph.D. thesis of Yu-Hsien Lin "Pathological mechanisms of polyglutamine disorder in Drosophila model of Huntington disease"

Yu-Hsien Lin's thesis consists of a general introduction and two first-author publications, one already published in journal Frontiers in Immunology and one deposited in bioRxiv preprint server. From this point of view, I can conclude that the thesis presents high-quality Ph.D.-level work with everything formally required, the thesis is quite clear and easily readable.

The introduction is clear, succinct and contains everything important to introduce the reader into the topics addressed in the thesis. On one side, I appreciate rather short and focused introduction with important facts about adenosine and Huntington disease (it is a good review of the known facts) but on the other side, I still lack a little broader context with raising interesting points of possible connections. For example, Part 3. on page 11, describing a crosstalk between HD and Ado signaling, just summarizes individual particular findings but does not connect them, does not speculate how and why these connections could happen.

What I am also missing is the final conclusion part, the thesis ends with the publication II but there is no additional part that would summarize the whole thesis, connect the findings (for example Ado signaling, immunity and HD), put them again into a broader perspective, offer new directions in research based on the findings and newly established models. This is pity, the overall high-quality thesis really lacks this part, which would make the thesis really strong. There is not much space in each publication for such broader connections and speculations and the Ph.D. thesis offers an excellent opportunity for it, but such potential was not used.

I have some particular critical notes and questions below but in summary I can conclude that even without the above mentioned parts, Yu-Hsien Lin clearly showed his ability as Ph.D. candidate by finishing two very good publications (I truly believe that the manuscript deposited in bioRxiv preprint server is publishable, and I hope that also my notes may help with that) and **without hesitation I recommend the presented thesis for successful defense.**

In České Budějovice, February 17, 2020

Tomáš Doležal

Particular critical notes and questions:

- Publication I - using letters above graph columns to mark the significant differences is often confusing, for example Fig. 1A, what is marked by B above the third column? It is not explained in any legend.
- Publication I, page 30 – Tab. S2 with splicing isoforms of *mod(mdg4)* is missing.

Introduction:

- Page 4 – "Hence SAHH also plays the role, but a minor one, in adjusting the Ado level." Can you explain why do you think that it plays a minor role? Can you put it into the context of the role of methylation cycle, can that play an important role, at least in some situations?

Publication I:

- Page 24 – "... the lower expression of *adgfs* in Q93 larvae and the higher expression in Q93 adults might be a consequence of elevated e-Ado concentrations resulting from HD pathogenesis." First, in larvae there was actually a lower, not elevated e-Ado concentration. Second, how would lower and higher expression of *adgfs* lead to the same effect? In addition, overexpression and RNAi of several enzymes, transporters and receptor in Ado cascade were used to analyze Ado effects in HD model. Looking at Fig.2, 3 and S6, it seems that similar effects were expected from manipulation of all these players in Ado cascade. For example, overexpressions of *ent2, adoR, adgf-a and adenoK* (page 26) were all expected to increase mortality and while *ent2* and *adoR* indeed convincingly did, the effect of *adgf-a* and *adenoK* were quite weak.
  However, I would not expect the same effects of *ent2* and *adoR* on one side and *adgf-a* and *adenoK* on the other. Suppressing *adgf-a* and *adenoK* should rather increase Ado while *ent2* and *adoR* should decrease its effect. **It think it is quite confusing throughout the manuscript and this should be clarified. Can you address this during the defense, what effects do you actually expect from these manipulations?**
- Fig. 1A and 1D are compared together (lowered expression of ENTs – 1D - explain the lowered Ado levels – 1A - in larvae) – I would be very careful with such comparison, while the effect in 1A is achieved by expressing Q93 by ubiquitous Da-Gal4, the effect on expression is achieved only in brain. In addition, completely opposite effects on expression in adults compared to larvae (1D vs 1E) are not really discussed. Can you offer some explanation?
- Two genes, which were found in microarrays, were verified by q-PCR – *mod(mdg4)* and *ptp99A*. While increased Ado resulted in increased *mod(mdg4)* expression, it decreased expression of *ptp99A.* Is then the silencing of *ptp99A* by RNAi the right way to examine its role in the presented model? What is known about *ptp99A* and how it could influence the studied pathology?
- Page 24 – "Silencing the transcription of Ado metabolic enzymes showed ..." Were the effects of RNAi verified for all the studied genes by qPCR?
- Page 58 – Imaging of retinal pigment cell degeneration – "at least five representative individuals were chosen for imaging" – can you explain how were they chosen to prevent any bias?
- I think that the presented manuscript convincingly show that HD pathology may somehow increase intracellular Ado, which is released and consequently increases AdoR signaling,

worsening thus the pathology. **Can author speculate (1) how the HD pathology may increase the intracellular Ado and (2) what cells/tissue receive the Ado signal via AdoR and how would this worsen the HD pathology in the presented fly model?**

Publication II

- Fig. 2 shows that mHTT decreased hemocyte numbers. However, in fact only GFP positive cells were counted. Is it possible that mHTT suppresses the GFP expression but cells are still present? Did you check that? This is in fact known for phagoless flies that the Hml>GFP expression is suppressed in these flies but they still contain non-GFP phagocytic-active cells. **Do you know what is in fact happening with the mHTT-expressed hemocytes?** Do they die, do they just lose the GFP expression?
- Fig. 8B shows that Q93 expression in hemocytes decreases AMPs expression in larvae infected with bacteria compared to uninfected larvae. The AMPs might be expressed both in hemocytes (affected by Q93 expression) and in the fat body (expression was analyzed in whole larvae). Since Q93 somewhat activates immune cells (making them pro-inflammatory, for example leading to expression of Upd3 – Fig.7), I wonder is it possible that Q93 activates AMPs expression in fat body of uninfected larvae? This is not possible to see in Fig. 8B since the relative values of infected to non-infected larvae are present. Can you compare the results of AMPs expression in non-infected larvae, Q20 vs. Q93, and separately in infected larvae?
- As a follow-up on previous question, in humans, mHTT leads to increased inflammation and some results with Drosophila also suggest that the Q93-expressing hemocytes might actually become pro-inflammatory on one side but eventually less immunocompetent. But the potential increased pro-inflammatory state of Drosophila hemocytes is not really addressed and discussed in the presented publication. **Can you address this more, are there any other results supporting this idea, did you check any other signs of overall immune activation in larvae expressing Q93 in hemocytes?**

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ASSESSMENT OF PhD DISSERTATION

The PhD thesis of Yu-Hsien Lin entitled '*Pathological mechanisms of polyglutamine disorder in Drosophila model of Huntington disease*' presents novel and relevant findings on the role of adenosine in the control of histological and molecular modifications present in flies expressing a mutant huntingtin, modeling Huntington's disease.

Thus, by choosing a simple experimental model (Drosophila), a robust strategy to induce alterations pertinent to Huntington's disease, genetic manipulations of different elements of the adenosine system and some selected endpoint classically measured to assess neurodegeneration in Drosophila, the candidate was able to obtain clear observations sustaining some pivotal conclusions that make the core of this thesis. Thus the conclusions obtained are based on a solid group of results, sustained by a robust methodological approach that was based on clear and logical scientific questions.

Therefore, **I have no reservations in recommending that this PhD dissertation should be publically discussed**, under the anticipation that the degree should be awarded to the candidate if his performance matches the quality of the thesis presented.

In spite of its quality, there are still several aspects that should be discussed in more detail. In fact, the Introduction is small and is prone to raising some questions on the properties and function of the purinergic system in Drosophila. Also the methodology used is presented in a rather synthetic manner, prompting the need of some clarifications. Additionally, the strategy used to tackle the role of the adenosine system was based on tinkering with its removal whereas its producing was not experimentally tackled and some of the mechanistic insights, certainly of great interest, may be further detailed to consolidate the evidence.

In summary, I anticipate a vivid and challenging discussion of this thesis, which formal and scientific quality, heralds my recommend for its acceptance for a public discussion.

Miami, 17th February 2020

Rodrigo Cunha, Full Professor

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Review

of the Yu-Hsien Lin's PhD thesis

"Pathological mechanisms of polyglutamine disorder in *Drosophila* model of Huntington disease"

Thesis of PhD candidate, Yu-Hsien Lin, deals with Huntington disease (HD), an inherited neurodegenerative disorder caused by an abnormal expansion of CAG trinucleotide in DNA sequence of *Huntingtin* (*htt*) gene, and with associated dysregulation of adenosine (Ado) homeostasis in *Drosophila* HD model. In humans, mutant HTT protein (mHTT) contains an extended polyglutamine (polyQ) tract encoded by 40 to over 150 CAG repeats, which causes cytotoxicity and leads to neurodegeneration; this results in involuntary movements, cognitive impairment and psychiatric abnormalities. The PhD thesis, of total length of 115 pages contains good overview and exhaustive introduction to the field of adenosine metabolism and signaling as well as pathophysiology of Huntington's disease, and it is divided into two main portions. In the first portion Yu-Hsien addresses adenosine signaling and the pathogenic effects of polyglutamine in a *Drosophila* model of HD. Because underlying mechanisms of Ado signaling in HD pathogenesis are still unclear, author in his study used a *Drosophila* HD model to examine the concentration of extracellular Ado (e-Ado) as well as the transcription of genes involved in Ado homeostasis. Through candidate RNAi screening, he demonstrated that silencing the expression of adenosine receptor (*adoR*) and equilibrative nucleoside transporter 2 (*ent2*) not only significantly increases the survival of HD flies but also suppresses both retinal pigment cell degeneration and the formation of mutant Huntingtin (mHTT) aggregates in the brain. Author compared the transcription profiles of *adoR* and *ent2* mutants by microarray analysis and identified a downstream target of AdoR signaling, *mod(mdg4)*, which mediates the effects of AdoR on HD pathology in *Drosophila*. These findings have important implications for the crosstalk between Ado signaling and the pathogenic effects of HD, as well as other human diseases associated with polyglutamine aggregation.

Thesis contains also several other very interesting results. Since homologous proteins have been shown to control Ado homeostasis in flies, Yu-Hsien compared the expression of three *Drosophila adgf* genes (*adgf-a*, *adgf-c*, *adgf-d*), adenosine kinase (*adenoK*), adenosine transporters (*ent1*, *ent2*, *ent3*, *cnt2*), and adenosine receptor (*adoR*) in the brains of Q93- and Q20-expressing larvae. The results showed that the expression of *adgf-a* and *adgf-d*, as well as transporters *ent1*, *ent2*, and *ent3* in the brain of Q93 larvae were significantly lower than in Q20 larvae. The expression of *cnt2* and *adoR* showed no difference between Q93 and Q20 larvae. Moreover, it has also been shown that the expression of *adgfs* as well as *adenoK* follows the levels of e-Ado upon stress conditions, suggesting that the lower expression of

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adgfs in Q93 larvae and the higher expression in Q93 adults might be a consequence of elevated e-Ado concentrations resulting from HD pathogenesis.

Author observed that the RNAi silencing of *ent2* and *adoR* extended the lifespan of HD flies to 30 and 40 days, respectively, which is about 1.5 to 2 times longer than that of HD flies. In contrast, knocking down *cnt2* expression did not change the viability of HD flies, and knocking down *ent3* did not influence the eclosion rate, although it increased mortality and shortened the lifespan of adult HD flies. An examination of eye phenotypes in *ent2* RNAi flies showed a significant reduction in retinal pigment cell death, but surprisingly he did not observe a significant rescue of cell death by silencing *adoR*. The effects of shortening the lifespan were more severe than with *ent2* overexpression. Nevertheless, the increase in mortality by *adgf-A* and *adenoK* overexpression was not as strong as that caused by *ent2* and *adoR* overexpression, although both still showed a significant difference to either Q93 control or Q93/*gfp* RNAi control by weighted log-rank test. Hence, author concluded that overexpressing the examined genes enhances the effect of mHTT, resulting in the increased mortality of HD flies. The results nicely demonstrate that the overexpression and silencing of *ent2* or *adoR* has a stronger influence over HD pathology than genes involved in Ado metabolism.

Reults indicated that ENT2 and AdoR contribute to mHTT pathogenesis in HD *Drosophila* and may act in the same pathway. To identify their potential downstream target genes, author compared the expression profiles of larvae carrying mutations in *adoR* or *ent2* as well as adult *adoR* mutants using Affymetrix microarrays. The intersection between each mutant (presentred as Venn diagrams) contains differentially expressed transcripts in all data sets, including six upregulated and seven downregulated mRNAs. Interestingly, according to Flybase, four of these genes are expressed in the nervous system (*ptp99A* was upregulated, while *CG6184*, *cindr*, and *mod(mdg4)* were downregulated). Moreover, to validate the microarray data, PhD candidate knocked down *adoR* expression in the fly brain and examined the transcription of the four candidate genes expressed in the nervous system by qPCR. The results revealed that *ptp99A* and *mod(mdg4)* had the same expression trends as observed in the microarrays. Thus, he further examined whether the expression of *ptp99A* and *mod(mdg4)* are influenced by an increase of e-Ado level. As it has been shown, Ado microinjection significantly increased *mod(mdg4)* expression and decreased *ptp99A* expression, confirming that *mod(mdg4)* is positively regulated and *ptp99A* is negatively regulated by the AdoR/ENT2 pathway.

Second part of the thesis is dedicated to HD and abnormalities related to the immune system that, in fact, were observed in a number of studies of HD patients. The expression of mHTT in both brain and peripheral immune cells (microglial and myeloid cells) is known to induce the NF-κB signaling pathway which elevates levels of pro-inflammatory cytokines and chemokines, leading to systemic inflammation. In addition, macrophages isolated from HD model mice exhibited migration deficits, and microglia showed a delayed response to laser-induced injury in the brain. Although several studies proposed that the mammalian immune cell response is impaired in HD, this phenomenon is still poorly characterized in relation to host responses to pathogens. Since *Drosophila melanogaster* has been long-term established as a HD model, *in vivo* experiments have revealed that the ectopic overexpression of mutant human *htt* (exon 1 with expanded CAG repeats) in the neural tissue of transgenic flies causes

neurodegeneration. The mechanisms of cellular pathology observed in the HD flies seems to be similar to those in human patients, including the suppression of mitochondrial function, transcriptional dysregulation and neuronal apoptosis. Genetic screening for disease modifiers in HD model flies led to the identification of the effects of sumoylation and HSP70 chaperone machinery on neurodegeneration. The subsequent confirmation that these pathways are involved in the pathology of human patients validates the *Drosophila* model for investigating HD.

In this context, Yu-Hsien found out that expression of mHTT in hemocytes did not affect larval viability but decreased the number of circulating and sessile hemocytes, and it also impaired the immune response to parasites, such as entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*, and the parasitoid wasp infections.

In addition, he performed series of *in vitro* experiments with S2 cell line, which consists of macrophage-like cells with phagocytic activity with the ability to produce antimicrobial peptides (AMPs). He transfected the cells with four different recombinant constructs encoding green fluorescent protein (GFP) fused to HTT repeats under an inducible metallothionein promoter. He confirmed that the S2 cells expressed HTT-fusion proteins by observing the GFP, and most of the cells in all cell lineages were positive for the fluorophore. Furthermore, the cells containing the mHTT Q46, Q72, and Q97 constructs (all except wild-type Q25) showed formation of mHTT aggregates. Yu-Hsien also observed the upregulation of cytokines expression and downstream JAK/STAT signaling in mHTT expression cells, and importantly also reduced antimicrobial peptide (AMP) production in response to bacteria.

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, Prof. Michal Žurovec, in this case significantly contributed by Yu-Hsien Lin. Nevertheless, I have several questions and comments to the author and his thesis:

1. Why human *htt* gene constructs (Q20 and Q93) and not endogenous *htt* gene constructs of *Drosophila* were used in the experiments ? Is it known what null allele or RNAi knockdown of the *htt* will do in flies ?

2. Did you run any experiments along these lines with different numbers of polyQ repeats than Q20 and Q93 ? By other words is there anything known how the number of expanded Q-repeats contribute to HD pathogenesis and to the fly phenotype ?

3. What is known about HSP and other chaperon interaction with HTT pathway and HD pathology progression ?

4. How mutant HTT protein affects functioning of the A2A and A1 receptors in flies ?

5. Although *elav-Gal4* driven overexpression of mHTT in the flies is not lethal during larval stages, did you observe any partial phenotype that could contribute to the systemic HD pathology in an animal ?

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6. Values of eAde in the hemolymph in the Figure 1A of the first portion of the thesis are expressed as relative abundance. What are real values of the eAde that could be measured (*e.g.* nmol, pmol or ng/g or pg/g of body weight or the protein) ?

7. Is the reduced eclosion rate in Q93 expressing flies due to neuroedegenaretaive effects in the brain that affect behavioural pattern required for eclosion ? Did you do observations of posteclosion behaviour and or locomotor activity in those cca 20-25% of escaper flies that succesfully emerged ?

8. As for the second part of the thesis: in addition to *hml-Gal4* and *he-Gal4* drivers did you do observations also in ubiquitously expressed constructs and made comparison to the phenotype caused by these hemocyte-specific drivers ?

9. In contrast to *in vivo* studies where Q20 and Q93 constructs were expressed, in the S2 cell cultures (pages 73 to 76) you used Q25, Q46, Q72 and Q97 constructs. Why, and what comparison could be made to Q20 and Q93 data ?

10. Also can you explain why pahocytic rate of S2 cells was more affected after epxression of Q72 than Q93 form of the HTT ?

11. Becuase Affymetrix microarrays were run also from total RNA extracted from whole animals, did author found any significant changes to the expression of immune response components in whole organism ?

12 On pages 71 and 82 author used term „circular" hemocytes - does author mean a circulating or round hemocytes (by shape/morphology) ?

Based on above mentioned facts, contribution of the research data with obtained evidence, experimental methods used, the achievements of Mr. Yu-Hsien Lin meets alls acceptable scholarly standards for PhD dissertation. Therefore, I can conclude that he fulfilled all major requirements, and I can gladly recommend the thesis for successful defense in the front of a PhD committee.

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