Review of the Ph.D. Thesis by Mgr. Michaela Fenckova:

Role of extracellular adenosine in *Drosophila*

The Ph.D. Thesis by Michaela Fenckova focuses on the role of adenosine signaling in *Drosophila*. Ph.D. Thesis consists of two papers published in impacted journals (one with Michaela Fenckova as the first author) and two chapters presenting unpublished data. Specific contribution of Michaela Fenckova in the two published papers is clearly stated. Overall, the presented Ph.D. Thesis is very well readable and comprehensively written. Thesis contains all what the Ph.D. is about: high quality published data significantly advancing the field, exciting new technologies but also confusing and largely frustrating (meaning unsuccesful) attempts to knockdown AdoR.

Minor comments to formal quality.

1) Despite the fact that Thesis is written in a fairly good English several mistakes or mispellings have escaped during the final reading.

p.2, in brain when (*correctly perhaps* where) they...

p.3, histidin (histidine)...

p. 5, 1.2.2....A2 AR (A2A AR), A2B (A2B AR)

p.7, neuropotection (neuroprotection)...

p. 10, are high (highly) expressed...

p.10, posses (possesses)

p. 11, adenylate cyclase (kinase)

p.46, ADGF-A>GFP (ADGF-A:GFP)

2) In part III, Fig.7 is missing (Fig.8 follows Fig.6). It would look more consistent if Fig.8A keeps the same layout/colors as Figs.4-6 presenting the same kind of data.

3) In part IV, Fig.6 is missing (Fig.7 follows Fig.5).

4) In the Introduction, a reference Guieu, 1998 is used but not cited in the list of references

5) It would make sense to have a pre-formed list of abbreviations. This helps to avoid using Ado (not even defined first time used) and adenosine in the same paragraph (p.8) or using ADGF before the full name Adenosine Deaminase-related Growth Factors (p.10-11).

6) Table 1, Introduction should contain a formal reference where all the data comes from.

7) Supplementary part of paper 1 (Zuberova et al.) is unfortunately not included in the Thesis.

8) In the absence of statistical data, it is better to avoid the word significant(e.g. Part III, Fig.4, Fig.8).

Scientific comments and questions.

1. Zuberova et al.: What was the rationale for screening chromosomes II and X for suppressors of adgf-a phenotype ? Where is AdoR located? Any follow-up study is planned on the other recovered suppressor alleles?

2. Fenckova et al.: What sequences is the phylogenetic tree based on (full-lenght with indels, domains)?

3. Fenckova et al.: CG30103 shows high degree of homology but no enzymatic activity likely explained by the large number of missense mutations in key positions. However, looking at the data in Figs.5 and 6 is it possible that CG30103 rather functions as a dominant negative lowering the basal level of E-Ado (as compared to pAc transfection)? Any speculation what the function of CG30103 could be?

4. Part III: It does not appear correctly interpreted (Fig.4, p.28), that there is no effect of non-induced RNAi on adult survival. What is the explanation for differential adult survival of various Gal4 drivers (without RNAi alleles) in adgf-a mutant background (Fig.5A,C,A,G)? What is the reason for an apparent difference in adult survival of adgf-a mutants in 5% sugar (Fig.4A 30%, Fig.8A 10%)?

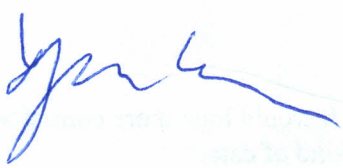
5. Part III: It is surprizing that independent knockdown alleles (VDRC and shmiR-1) are lethal in WT background. Was the same region of AdoR used for dsRNA in VDRC alleles and in the Thesis presented here? Are there any data (sequence homology search, microarray, qRT-PCR) for the existence of off-targets?

6. Part III: Can a dominant-negative (interfering)AdoR be constructed? Was a strategy for tissue specific interference with AdoR using a dominant-negative AdoR cDNA ever considered?

7. Part III: Effectivity of shmiR knockdown was directly quantitated by qRT-PCR. Was there a similar attempt to measure effectivity of dsRNA-mediated knockdown?
8. Part IV: Was it considered that the N-terminal fusion of some genes (NT5E-1, NT5E-2) would make more sense? Protein fusion makes sense if protein localization/metabolism is a primary interest, but may cause problems such as those seen in ADGF-A:GFP. In case of expression studies (questions where? When?) a simple EGFP knock-in into ATG of the gene of interest by recombineering might be more beneficial.
9. Part IV: What are there data about the sufficiency of fosmid clones to recapitulate expression of a given gene in *Drosophila*? Before the construction of conditional AdoR allele it would make sense to establish that the construct is able to complement AdoR defficiency. How?

Conclusion: Ph.D. Thesis by Michaela Fenckova has brought valuable and high quality data and experimental tools in the field of adenosine signaling. The employed methods and approaches document a large spectrum of theoretical and practical knowledge achieved during realization of the Ph.D. project. In conclusion, I highly recommend this Ph.D. Thesis for its defense.

RNDr. Zbynek Kozmik CSc.
Prague, January 4, 2012.



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Evaluation of PhD thesis by Michaela Fenckova titled "Role of extracellular adenosine in Drosophila".

Michaela Fenckova presents a PhD thesis describing various experimental approaches towards establishing Drosophila as a model for systematic studies of the role of adenosine signaling in animal physiology. This is a primarily a cumulative thesis; its foundation are two published reports where Michaela is the primary and supporting author. The papers are supplemented by general introduction and detailed descriptions of two on-going projects that originate from the published research, but are still work in progress. I focus in my evaluation particularly on the previously unpublished parts of the thesis.

The introduction begins with a detailed compilation of the current knowledge about adenosine biogenesis, trafficking and metabolism while distinguishing between its role inside the cell and in the extracellular environment. Special attention is given to the adenosine receptors particularly in the context of the mammalian genome, the various roles of adenosine signaling in different tissues particularly the nerve cells and the interplay between adenosine signaling, energy metabolism and immune response. This overview is scholarly, well-referenced, using up-to-date literature and represents a good introduction to the topic. What is missing is a schematic overview of the adenosine biochemistry, with some basic chemical fomulas and the placement of the various enzymes in the complex metabolic sub-network. This would be useful to enhance clarity of the text for an uninitiated reader like myself. Nonetheless there is a strong emphasis on placing adenosine signaling in the context of the common human disease, which highlights the general importance of the subject. The second part of the introduction deals with the comparably less worked out adenosine biology in Drosophila. Michaela argues that the insect model system promises to contribute significantly to the progress in understanding the role of adenosine signaling in general, using the powerful genetic and reverse genetic toolkit of Drosophila augmented by reduced genetic redundancy. In several places an argument is put forward stating that while adenosine is thought to act locally. the haemolymph of insects and the relatively small 'footprint' of the animal opens up the possibility that in Drosophila it may elicit more systemic, hormone like, response. I would be interested to hear more specific elaboration on this theme.

The first paper, where Michaela is a second co-author, proposes extracellular adenosine as an anti-insulin hormone that promotes release of energy stores during immune response. This conclusion is based on detailed phenotypic analysis of adenosine deaminase mutant and further corroborated by strong genetic interaction with adenosine receptor mutation. It represents an elegant demonstration of the power of Drosophila genetics to uncover new biological phenomena relevant for human pathologies.

The second paper, authored primarily by Michaela, presents a comprehensive characterization of Drosophila homologs of enzymes involved in production of extracellular adenosine. Although the study is descriptive, it provides a wealth of information about expression and function of nucleotidases involved in adenosine production. I believe that in combination with the ongoing in vivo expression studies presented later in the thesis, this research will provide valuable insights into tissues specific activity of adenosine signaling which would be hard to achieve in mammalian systems.

The third section of the thesis presents a comprehensive and honest description of largely unsuccessful attempts to interfere with the expression of adenosine receptor in a tissues specific manner. Despite engaging the entire repertoire of modern Drosophila reverse genetics, including the GAL4-UAS system, RNAi knockdown by hairpins and synthetic microRNAs, Michaela was unable to reconstitute the same strong genetic interaction between adenosine deaminase and adenosine receptor mutation when interfering with the receptor only in some tissues of the larva. She discusses extensively the possible reason for the failure of these experiments, invoking particularly the cursed 'of target' effect which seems to make the RNAi lines particularly sickly, possibly masking the adenosine-receptor-specific suppression. It takes some patience to quantify the phenotype of a large panel of genetic backgrounds with no sign of the desired effect and I applaud the effort. It will be interesting to discuss the possible reasons for this lack of interaction. In the discussion, Michaela proposes two alternative strategies designed to overcome the technical difficulties associated with RNAi. One relies on proven and tested mosaic analysis applied to a non-traditional tissue (the fat body). I wonder what molecular markers will be used to compare the glycogen breakdown phenotype of the wild-type and mutant clones. The second strategy is a novel approach based entirely on reverse genetics manipulation of third copy allele reporter in the background of a genetic null. The details of this strategy are not completely worked out in the thesis with respect to the two rounds of recombineering and the required genetics. It is something to discuss during the defense.

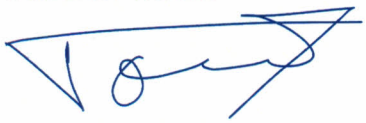
Finally, Michaela, employed the FlyFos system to generate in vivo fluorescent reporters for many components of adenosine signaling and biogenesis pathways. The production of transgenics is an ongoing process, however the initial results are very promising. The characterization of three GFP tagged transgenic lines is presented in the final chapter of the thesis. This is clearly work in progress, but it is encouraging to see that a) the expression of the GFP from the fosmids is detectable in the living larva, b) the GFP fluorescence remains detectable after fixation and c) the fosmids recapitulate the patterns described in the literature. It would be useful to provide a control panel in the Figures to show that the observed fluorescence is clearly distinct from the auto-fluorescence of the tissue. Why is the dsRed visible in the GFP channel? Was antibody staining with anti-GFP antibody attempted and if so did this provide additional information on the sub-cellular localization of the proteins? Finally, the Figures must have error bars….

Overall, this is a very well structured thesis. The writing is very clear and although the Czech language sometimes does shine through (as in my report undoubtedly) it does not impair the

understanding of the text. Michaela has in the course of her PhD employed impressive array of experimental techniques, some old and tested, some cutting-edge, collected vast amounts of data that were quantitatively analyzed and used to test hypothesis. The conclusions drawn from the experiments significantly contribute to the progress in the field of adenosine signaling, which is best documented by the publications. Finally, the work clearly shows how modern Drosophila model system technology can boost the mammalian research field and provide new hypothesis and insights.

I believe that the thesis represents a strong foundation for a PhD degree.

With Best Wishes



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