University of South Bohemia
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Characterization of *Drosophila melanogaster* adenosine receptor

Master thesis

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Annotation

Adenosine receptors belonging to G protein-coupled receptor family are involved in the regulation of wide spectrum of physiological properties. In order to characterize *Drosophila* adenosine receptor, I compared its properties with its closest human receptor isoform A2A in cell cultures and in *Drosophila in vivo*. My work involved the construction of several transgenic fly strains, testing new antibody, FlyFos *in vivo* protein tagging, cAMP assays, confocal microscopy etc. I show that human A2A has higher affinity to adenosine.

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

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Contents:

1. Introduction

1.1 Adenosine receptors

Adenosine receptors (ARs) belong to G protein-coupled receptor (GPCR) family. GPCRs are a widely studied class of signalling proteins. All GPCRs have a similar structure. They have seven transmembrane α-helices, extracellular N-terminus and short intracellular C-terminus. A signalling cascade starts, when an extracellular signal molecule binds to a receptor. The receptor undergoes a conformational change and activates a heterotrimeric guanine nucleotide-binding protein (G protein), which relay signals to intracellular target proteins of the signalling cascade.

Adenosine signalization is involved in the number of key physiological processes including energy homeostasis, cell division or cell death (Fredholm et al., 2011; Wang and Ren, 2006; Ohkubo et al., 2007; Aymerich et al., 2006). Adenosine signalization is also important in many human diseases. Therefore, ARs are important targets for new drug development and treatment strategies (Jacobson, 2009).

1.2 Human adenosine receptors

Four subtypes of ARs were identified in mammals: A1, A2A, A2B and A3. The amino acid identity is 31-46% among human adenosine receptors. Couples A2A and A2B and also A1 and A3 have 46% of identity (Piirainen et al., 2011). It was previously shown that all human adenosine receptors affect intracellular level of cyclic adenosine monophosphate (cAMP). They are coupled to adenylate cyclase. The A1 and A3 receptor subtypes inhibit this enzyme, whereas A2A and A2B stimulate it (van Calker et al., 1979, Fredholm et al., 1994).

All four receptor subtypes are expressed in various tissues and organs. A1 AR is expressed for example in spleen, kidney, liver, heart or aorta. A2A receptor is expressed in some parts of the brain (striatum, olfactory tubercle and nucleus accumbens). A2B receptor has low expression level in almost all tissues. A3 receptor has low expression level in the thyroid gland, brain, liver, heart, kidney and intestines. (Doleželová et al., 2007). Type of expressed AR and its expression level influences the adenosine response level.

A2A receptor is one of few GPCRs with known crystal structure. Co-crystallization was performed with several ligands - A2A receptor antagonist ZM241385 and agonists UK-432097, adenosine and synthetic agonist NECA (Jaakola et al., 2008; Xu et al., 2011; Lebon et al, 2011).

Those structures enable to study different conformations of the receptor molecule. Moreover, the crystal stuctures revealed amino acids residues crucial for binding all of the analogs mentioned previously, including Phe168$^{5.29}$ (part of extracellular loop 2) and residues, which bind to adenine core - Ile274$^{7.39}$ and Asn253$^{6.55}$ (see Fig.1). Some other amino acid residues are important for binding of ribose part of adenosine molecule. Despite of the identity of all mentioned contact amino acids, there are significant differences in the affinity of ligand to various human adenosine receptor isoforms (Fredholm, 2001).

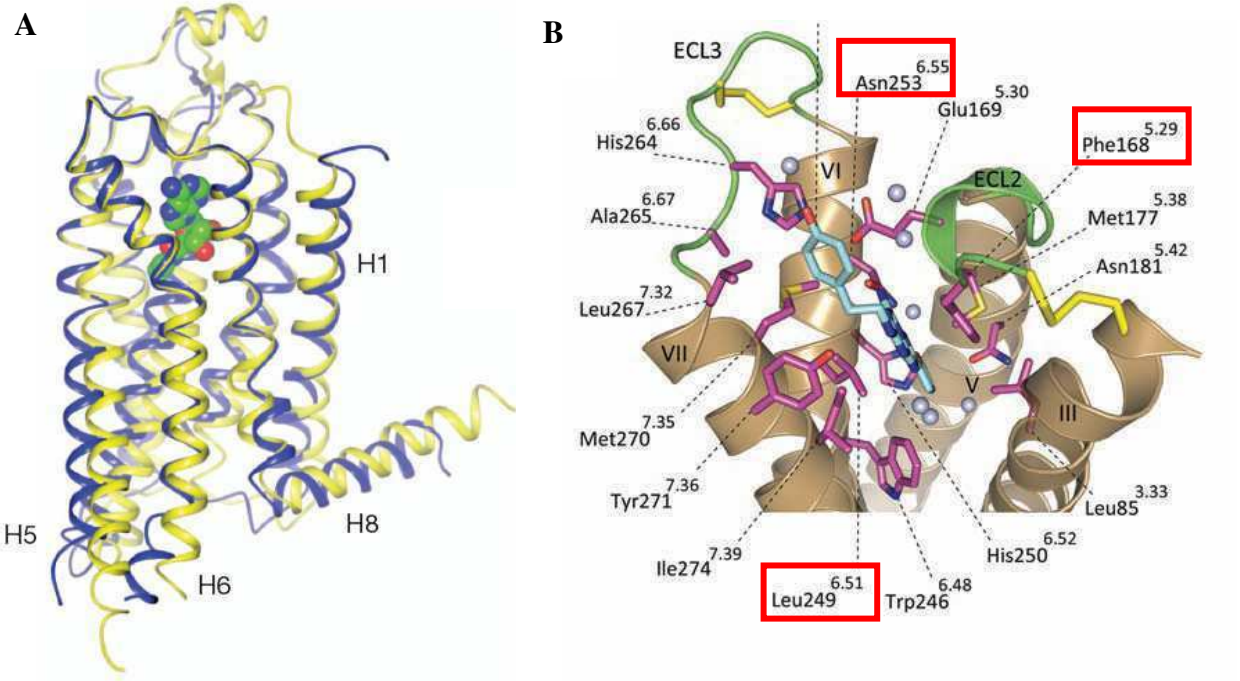

Figure 1. (A) The structure of A2AR bound to its agonist NECA (yellow) in comparison to A2AR structure with bound antagonist ZM241385 (blue). From Lebon et al., 2011. **(B)** A2A receptor binding cavity with bound antagonist ZM241385. Amino acids residues, which bind to adenine core, are highlighted in red rectangle. Nitrogen atoms are blue labeled, sulphur atoms are yellow and oxygen atoms are red. There are shown only interacting helices, the interacting part of extracellular loop 2 (ECL2) and extracellular loop 3 (ECL3). (From Jaakola et al., 2008).

It is also interesting, that A2A receptor has longer intracellular C-terminus compared to the most of GPCRs and other ARs: A2A C-terminus has 122 amino acids, A2B, A1 and A3 have 40, 38 and 34 amino acids, respectively. Therefore, it is possible that A2A receptor C-terminus can have more functions than the C-termini of other ARs, it can engage different proteins, for example proteins necessary for receptor folding (Keuerleber et al., 2011). Multiple binding sites of A2A C-terminus can enable A2A receptor to integrate more different signals and switch among different signalling pathways (Zezula and Freissmuth, 2008).

Even though a lot of information is known about human ARs, there are still many question to be solved about them. For example, it is not clear, which amino acid residues make the difference among ARs affinity to adenosine. Moreover, there is an evidence that at least some GPCRs can dimerize (Pin et al., 2005) and it is possible that they can even heterodimerize (Milligan, 2009) and it makes the research of GPCRs even more complicated. Almost nothing is known about the crosstalk between AR and adenosine transport and adenosine metabolism.

1.3 *Drosophila* adenosine receptor

A single human adenosine receptor homolog was found in *Drosophila* (Brody and Cravchik, 2000; Broeck, 2001; Doleželová et al., 2007). It has the highest identity with A2A human AR – 38% (350 bases of N-terminal part were compared). The identity with other receptors is as follows: 36,2% with A1, 35,2% with A2B and 34,5% with A3 receptor (Kučerová et al., 2012). The *Drosophila* adenosine receptor (DmAdoR) differs from other AR by its very long C-terminus (about 350 amino acids) of unknown function.

It was previously shown that DmAdoR expressed in heterologous Chinese hamster ovary cells (CHO) induced the changes of secondary messenger concentrations. The changes of intracellular calcium and cAMP level were dependent on dose of DmAdoR expression (Doleželová et al, 2007). In contrast, the adenosine treatment of *Drosophila* cells with endogenously expressed DmAdoR stimulates only the increase of cAMP, not calcium level (Kučerová et al., 2012).

The DmAdoR has relatively low expression throughout the body (www.flybase.org) with the highest expression level in adult *Drosophila* head compared to the rest of the body (Doleželová et al., 2007; Kučerová et al., 2012). Endogenous DmAdoR expression was also measured in several *Drosophila* cell lines derived from different tissues - including Cl8+ cells derived from imaginal discs, embryonic S2 cells, neuroblasts Bg2-c2 and hematopoetic Mbn2 cells. The Cl8+ cells were shown to have very low endogenous level of DmAdoR, whereas the highest expression level among the cells examined was found in neuroblasts (Kučerová et al., 2012).

The low DmAdoR endogenous expression makes the study of DmAdoR *in vivo* difficult. Moreover, due to the cytotoxicity it is difficult to overexpress DmAdoR in *Drosophila*.

The ectopic overexpression of DmAdoR causes severe phenotypic changes – wing deformations, anatomy abnormalities and lethality, dependent on tissue and DmAdoR expression level (Doleželová et al., 2007). The flies carrying overexpressed DmAdoR can be partially rescued from lethality by feeding with SCH58261, the DmAdoR antagonist (Kučerová et al., 2012).

1.4 *Drosophila* adenosine receptor model

Future research of adenosine signalling will require to elucidate the crosstalk between ARs, adenosine transport and adenosine metabolism. Since there are four different receptor isoforms expressed in various tissues it is even more difficult to study adenosine receptors in human. Drosophila contains just one adenosine receptor similar to A2A receptor. Therefore, *Drosophila* is a model organism for the research of adenosine receptor signalling with much lower complexity. Another advantage is the opportunity to use all power of *Drosophila* genetics. But if we want to use this model, it is necessary to characterize differences between DmAdoR and A2A receptors.

Several problems were addressed in this work: I tested a quality of DmAdoR primary antibody and FlyFos labeling and evaluated both methods for the detection of DmAdoR *in vivo.*

Since the overexpression of DmAdoR is toxic for the cells, I tested a possibility of use of novel cell culture inducible expression system.

The effects of ectopic expression of human A2A in *Drosophila* were compared and the necessity of DmAdoR C-terminus for its function was tested.

2. Specific Aims

- To test the quality of DmAdoR primary antibody and FlyFos labeling and evaluated the utility of both methods for the detection of DmAdoR *in vivo*.
- To modify one of the commercially available inducible systems for the expression in *Drosophila* cell cultures.
- To compare the function of DmAdoR and human A2A receptors in cell culture and in *Drosophila in vivo* .

Následující pasáž o rozsahu 24 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále diplomové práce uloženém na Přírodovědecké fakultě JU.

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8. Appendix

List of abbreviations

A1	human adenosine receptor subtype A1
A2A(R)	human adenosine receptor subtype A2A
A2B	human adenosine receptor subtype A2B
A3	human adenosine receptor subtype A3
ADA	adenosine deaminase
AR	adenosine receptor
Bg2-c2 cells	neuroblast cell line
CADO	2-chloradenosine
cAMP	cyclic adenosine monophosphate
Cl8+ cells	cell line derived from imaginal discs
dH_20	distilled water
DmAdoR	*Drosophila* adenosine receptor
Dm/A2R	chimeric gene consisting of DmAdoR N-terminus and A2A C-terminus
ER	estrogen receptor
FlyFos	genomic fosmid library, which allows to label the reporter genes by fluorescent tag and subsequently express these transgenes *in vivo*
GAL4	yeast transcription factor
GAL4/ER	chimeric protein consisting of GAL4 DNA binding domain and estrogen receptor ligand binding domain
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
Mbn2 cells	hemapoetic cell line
qRT-PCR	quantitative real-time RT-PCR
PCR	polymerase chain reaction
Rack1	receptor of activated protein kinase C1
Rp49	ribozomal protein 49
S2 cells	embryonic cell line