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Adenosine signaling in *Drosophila*

Ph.D. Thesis

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Annotation

This thesis characterizes adenosine signalization in *Drosophila* and describes response to adenosine in various cell types. Extracellular adenosine mediates most of its physiological effects through its receptors but recent data also indicate that adenosine transport also has important physiological functions. It was shown in this thesis that adenosine stimulates only cAMP second messenger system in *Drosophila* cells endogenously expressing *AdoR*. The pharmacological profile of the DmAdoR was established using the cAMP functional assay. The utility of the agonist 2-chloroadenosine and antagonist SCH58261 were examined in flies *in vivo* and compared with phenotypes of *DmAdoR* mutants. The responses of *Drosophila* cells to adenosine mediated by adenosine transport were also examined. Different cell types exhibited striking differences in adenosine uptake and adenosine recycling that were closely connected with the regulation of carbohydrate and lipid metabolism. This thesis provides an important foundation for the study of interactions between adenosine receptor and adenosine transport.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Lucie Kučerová

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Fleischmannova J, **Kucerova L**, Sandova K, Steinbauerova V, Broz V, Simek P, Zurovec M. (2012) Differential response of Drosophila cell lines to extracellular adenosine. Insect Biochemistry and Molecular Biology 42: 321-331 (IF = 3.246).
Lucie Kučerová performed isolation of RNA, cDNA synthesis and qRT-PCR from cell culture samples. She also helped with Ado incorporation assays and participated on the preparation and revision of the manuscript.

II. **Kucerova L**, Broz V, Fleischmannova J, Santruckova J, Sidorov R, Dolezal V, Zurovec M. (2012) Characterization of the Drosophila adenosine receptor: the effect of adenosine analogs on cAMP signaling in Drosophila cells and their utility for in vivo experiments. Journal of Neurochemistry 121: 383-395 (IF = 4.061).
Lucie Kučerová was responsible for isolation of RNA, cDNA synthesis, qRT-PCR, sequence alignment reconstruction, in vivo experiments with adenosine receptor analogs and statistical analysis of all experiments. She also helped with cultivation of cell cultures. She prepared the manuscript for publication in cooperation with Michal Žurovec.

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INTRODUCTION

1. Adenosine signaling

Recent progress in biology has shown that many basic mechanisms underlying development, immunity, metabolism, tumor growth and even behavior are evolutionarily conserved. Our ability to manipulate the genome of model organisms became a widely used approach to study various physiological disorders and model specific diseases. The studies of model organisms are therefore paving the way for further development in human disease research. The research on *Drosophila* played a crucial role in identifying genes controlling the development in insect and vertebrates, and now the genetic research turns its focus to other complex issues, including energy homeostasis, tumor growth and immune response (Tannahill and O'Neill, 2011) *Drosophila* provides the advantage of a lower complexity model system combined with the opportunity to use genetic approaches, which are not possible in higher organisms.

Adenosine (Ado) is an ubiquitous metabolite and its concentration in cells and tissue fluids ranges under normal conditions from 20 to 200 nM and can rise during stressful conditions into the micromolar range (Fredholm, 2010). The release and uptake of Ado requires nucleoside membrane transport, but adenosine might also be produced by apoptotic cells. The half-life of Ado in the circulation is very short, usually between 1 and 10 s (Jacobson and Gao, 2006). The steady-state extracellular concentration of Ado is maintained by the rate of its production in the extracellular and/or intracellular space, and its reutilization for ATP synthesis (salvage pathway) due to rephosphorylation or catabolism by adenosine deaminases (EC 3.5.4.4) to inosine and hypoxanthine. Elevated concentration of extracellular Ado is connected to many serious diseases including congenital immunodeficiency, diabetic complications, lung inflammation and asthma (Ponnoth and Jamal Mustafa, 2011; Sakowicz-Burkiewicz et al., 2006; Sauer et al., 2009). High concentrations of Ado have also been reported in most cancer tissues, where it was implicated as an important factor in tumor growth (Blay et al., 1997; Gessi et al., 2011; Spychala, 2000; Stagg and Smyth, 2010). Emerging evidence indicates that nearly all cancers regardless of cellular or tissue origin show impaired cellular energy homeostasis (Hanahan and Weinberg, 2011).

Depending on the cell types and conditions, extracellular Ado modulates energy metabolism, cell cycle, and apoptosis (Abbracchio et al., 1995; Apasov et al., 1995; Ceruti et

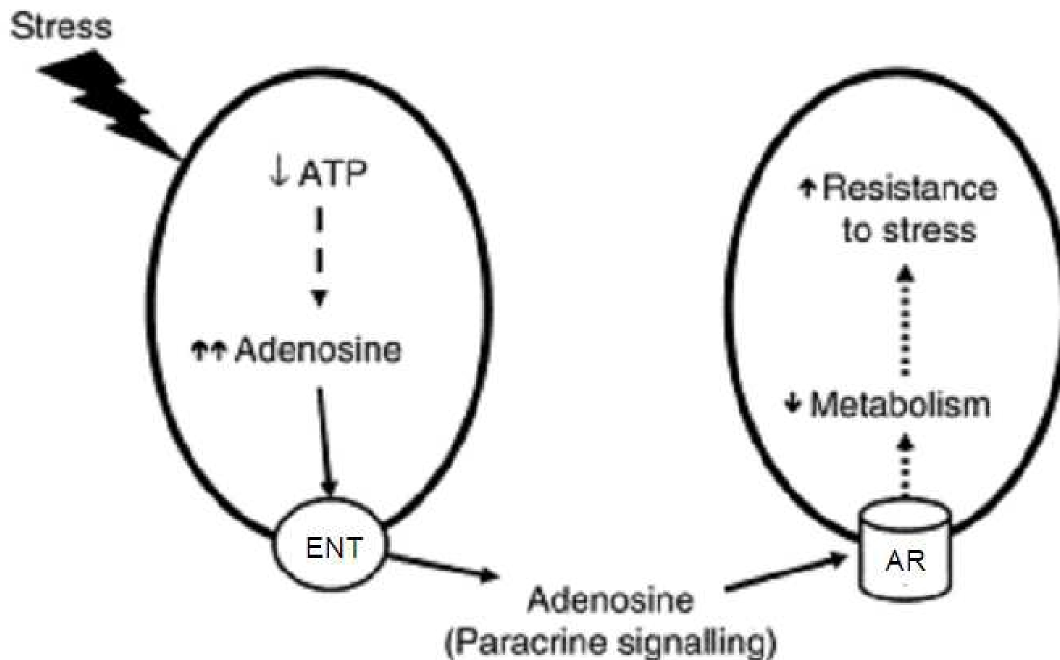

FIGURE 1. – **Ado signal as a paracrine regulator of metabolic imbalance within tissues.** The cells under stress conditions convert part of their ATP to Ado, which is then released by equilibrative transporter (ENT) to extracellular space. The signal is received by adenosine receptors (AR) in neighboring cells, which in turn decrease their metabolic activity. Adapted from Cunha, 2008.

al., 2000; Dawicki et al., 1997; Fishman et al., 2000; Ohana et al., 2001). Adenosine has a big advantage for being a homeostatic regulator because its derivatives are key molecules determining the energy charge (ATP), redox status (NADH) as well as transmethylation and cellular growth (SAM, SAH) (Gomes et al., 2011). Ado signal has a prominent role as a paracrine regulator of metabolic imbalance within tissues (Cunha, 2008). At least in some tissues Ado seems to synchronize metabolic activities of cells. The cells under stress conditions convert part of their ATP to Ado, which is then released by equilibrative transporter to extracellular space. The signal is received by adenosine receptors in neighboring cells, which in turn decrease their metabolic activity (Cunha, 2008). The cell responses result at optimizing the balance between energy consumption and production and Ado has been described as a ‘retaliatory metabolite’ (Newby et al., 1985). It took, however, 30 years to prove this concept at least for some tissues - mainly due to the local nature of adenosine signals, its short half-life in extracellular space and methodological problems of measuring adenosine concentrations.

Besides its homeostatic role Ado also plays an important role in neural tissue as a modulator of neuronal activity in mammals by regulating neurotransmitter release and

neuronal excitability (Cunha, 2008; Fredholm et al., 2005). The most evident effect is the selective depression of excitatory transmission (Dunwiddie and Masino, 2001).

2. Adenosine receptors

Four distinct Ado receptors (AR) were described in mammals, including humans, mice and rats, all well characterized by pharmacological studies and implicated in mediating most of the physiological effect of extracellular Ado (for a review see (Fredholm, 2010). Human adenosine receptors (Fig. 2) display an overall amino acid identity of 31 – 46% (Piirainen et al., 2011). They belong to the G-protein-coupled receptor family and are positively (A2A and A2B) or negatively (A1 and A3) coupled to adenylate cyclase (Fredholm et al., 1994; Linden, 1994; van Calker et al., 1979). A number of AR ligands displaying selectivity for individual receptor subtypes and much better metabolic stability than Ado were characterized. A detailed structure of the human A2AR has been solved and co-crystallization was performed with its agonists Ado, UK-432097 and 5'-N-ethylcarboxamidoadenosine (NECA) and antagonist ZM241385 (Jaakola et al., 2008; Lebon et al., 2011; Xu et al., 2011). The contact amino acid residues for binding of these Ado analogs were elucidated. The amino acid residues responsible for ligand binding differences still need to be discovered.

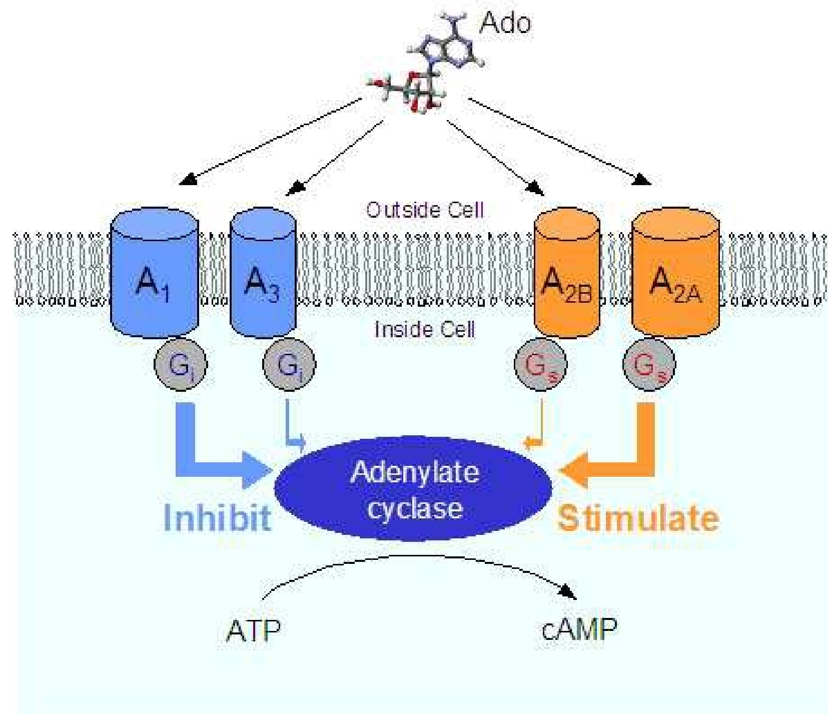

FIGURE 2. – **Human adenosine receptor (AR) subtypes and mechanism of action.** Adenosine is a naturally occurring ligand of four subtypes of G-protein-coupled cell membrane receptors, which exibit high (A_1 and A_{2A}) or low (A_{2B} and A_3) binding affinity. Human ARs either activate (A_{2A} and A_{2B}) or inhibit (A_1 and A_3) adenylate cyclase. Adapted from (Gemignani and Abbott, 2010).

It is very difficult to measure the affinity of adenosine to the receptors directly, because of its rapid metabolism and relatively high endogenous levels. Adenosine potency has

to be measured in functional assays (Fredholm, 2010). The estimated EC50 values for adenosine-induced cAMP response of the human A1, A2A, A2B and A3 receptors expressed in CHO cells are 0.3, 0.7, 24 and 0.3 µM, respectively (Fredholm et al., 2001). Another methodical problem is caused by the behavior of ARs as "spare receptors", which means that the potency of the agonist is highly influenced by the number of receptor molecules in the given cell type (Arslan et al., 1997). It is therefore not so useful to divide these receptors simply into high affinity and low affinity (Fredholm, 2010).

The ARs are widely expressed in various tissues and Ado responses are highly dependent on the receptor subtype and the level of receptor expression in a particular cell type. Mice carrying null mutations in different adenosine receptor subtypes are viable displaying only subtle phenotypic effects. The receptor knockouts show reductions or enhancements of physiological activities in cardiovascular and the nervous system specific for receptor deletion (Fredholm et al., 2005; Hasko and Pacher, 2008). Despite of the progress in the elucidation of the effects of Ado signaling, there are potential problems with compensatory mechanisms and receptor redundancy, which are poorly understood (Johansson et al., 2007). Further research of Ado signaling requires the combination of pharmacological and genetic approaches and multiple adenosine receptor subtype deficiency studies (Yaar et al., 2005).

3. Adenosine transport and metabolism

Recent reports also show the clinical importance of adenosine transporters and their interactions with receptors. Two types of specialized transporter systems mediate transport of nucleosides across cytoplasmic membranes. The first type, named equilibrative nucleoside transporters (ENTs), facilitates the diffusion of nucleosides down their concentration gradient. The second type, called concentrative nucleoside transporters (CNTs), can transport nucleosides into the cytoplasm even against concentration gradient by co-transport of Na^+ (King et al., 2006). Both equilibrative (ENT1-4 in mammals) and concentrative nucleoside transporters (CNT1-3 in mammals) are able to effectively transport Ado and thus contribute to Ado signaling regulation. Moreover, it has recently been shown that nucleoside transporters might play an important role in the cellular metabolism independent of Ado receptor signaling (Huber-Ruano et al., 2010).

The major physiological role of Ado transport was postulated as a regulator, especially a terminator of Ado signal at the receptors (Choi et al., 2004). Consistently, the pharmacological inhibition of equilibrative nucleoside transporters (ENTs) leads to increased Ado signaling through the adenosine receptors (Grenz et al., 2012). Several recent reports, however, do not support this simple model. For example, the ablation of the *ENT1* gene in the mouse, which was expected to result in the increased Ado concentration due to the decreased uptake activity of ENT1, had an opposite effect. It was found that the ablation of the *ENT1* has a physiological role in ethanol-mediated behaviors, associated with a decrease in the activation of adenosine A1 receptor connected most probably with the decrease of extracellular Ado concentration (Choi et al., 2004). Another recent study strongly suggests the involvement of human ENT3 in glucose metabolism and Pigmented hypertrichotic dermatosis with insulin-dependent diabetes (PHID) syndrome (Cliffe et al., 2009). Further progress in the research of these interactions is hampered by the complexity of the mammalian models, low metabolic stability of Ado and the use of Ado receptor analogs that interfere with Ado transport and conversion to AMP or inosine.

The major source of extracellular Ado is the catabolism of ATP released from stressed cells by 5'-nucleotidases or ecto-phosphodiesterase (Fig. 3). Alternatively, Ado is formed

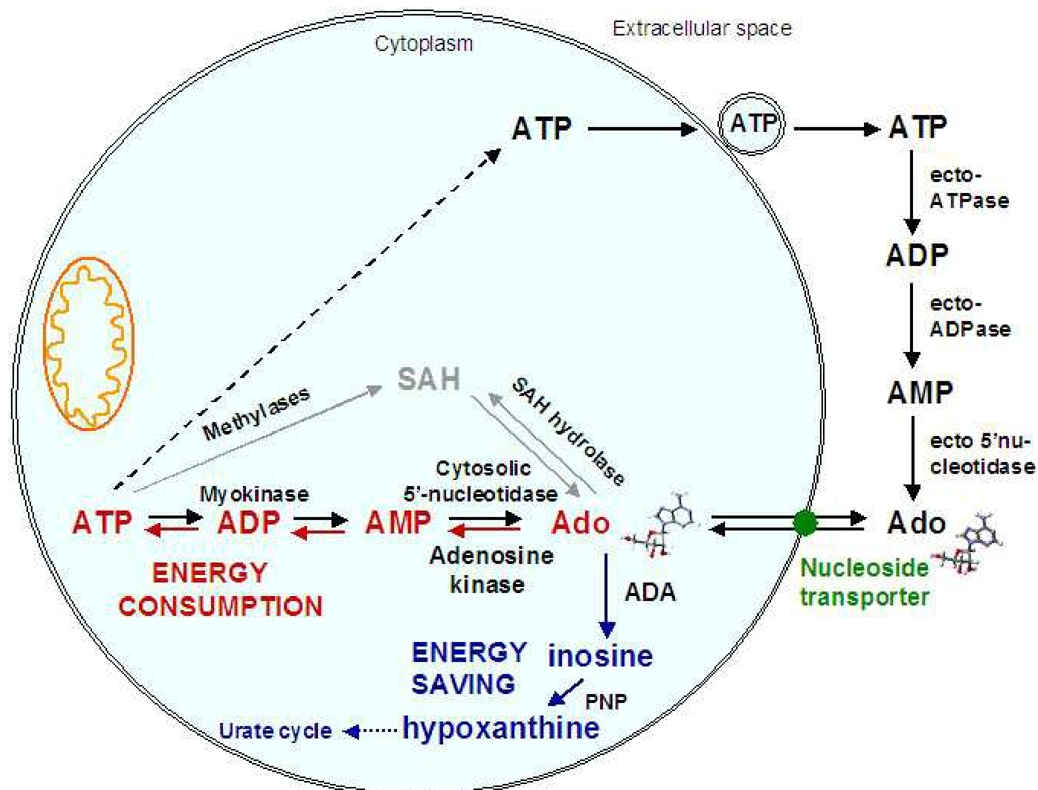

FIGURE 3. – **Schematic model of adenosine metabolism and recycling.** Red –Ado salvage pathway to ATP, blue – purine catabolic pathway, grey – less used conversion to SAH.

intracellularly by sequential dephosphorylation of Ado nucleotides and released via nucleoside transporters (Fredholm et al., 2011).

There are two major pathways for adenosine conversion in most eukaryotic cells (Fig. 3) - either the rephosphorylation to AMP or deamination to inosine; cleavage to adenine and conversion to SAH are negligible (Hershfield, 2001.). Adenosine deaminase (ADA) catalyzes the irreversible, hydrolytic deamination of Ado to inosine and ammonia and subsequently inosine can undergo reversible phosphorolysis to hypoxanthine by Purine nucleoside phosphorylase (PNP). Alternatively, Ado can be converted to adenosine monophosphate (AMP) by the adenosine kinase (AK), which has a higher affinity for adenosine than ADA. Ado kinase and nucleoside transporters are key players of Ado salvage. The recycling of Ado to AMP has a major impact for energy conservation because of the high-energy requirements of Ado *de novo* synthesis (Park and Gupta, 2008).

4. Adenosine signaling in *Drosophila*

Drosophila has only a single Ado receptor gene (*AdoR*). The closest human homolog of *Drosophila* DmAdoR is A2AR. It has about 38.3% identity in the 350 base long N-terminal part. DmAdoR is expressed mainly in the optic lobes of the brain, ring gland, imaginal discs and salivary glands of the third instar *Drosophila* larvae. In adults, the expression is higher in the head than in the rest of the body (Dolezelova et al., 2007). *Drosophila* DmAdoR null mutant flies are viable but exhibit reduced associative learning and defects in synaptic transmission (Knight et al., 2010). Ectopic expression of higher levels of DmAdoR is lethal (Dolezelova et al., 2007). So far only one AR antagonist – caffeine was tested for its interactions with DmAdoR (Wu et al., 2009). Caffeine belongs to natural xanthines. Most of its effects in mammals are mediated through the strong blocking of A1 and A2A receptors (Fredholm et al. 1999). However, the responses connected with sleep and circadian rhythms of *Drosophila AdoR* null mutants to caffeine are the same as in the wt flies, suggesting that some fundamentally different signaling mechanisms regarding adenosine and caffeine are used in mammals versus flies (Wu et al., 2009).

Other genes involved in Ado signaling and metabolism are also conserved across phyla. There are three genes encoding eqilibrative nucleoside transporters (*ENTs*) and two concentrative nucleoside transporters (*CNTs*) in flies (Machado et al., 2007). The levels of extracellular Ado in the body fluids of vertebrates and insects are similar (Dolezelova et al.,

2007). Hypomorph of *Drosophila Ent2* equilibrative transporter, have defects in synaptic transmission and reduced associative learning (Knight et al., 2010). *Drosophila* mutant in the major Ado deaminase gene *ADGF-A* is homozygous lethal (Dolezal et al., 2005). It was also found that *Ent2* and *AdoR* mutants display complex compensatory changes in the expression of the AdoR, Ado transporters and several other genes involved in Ado signaling, metabolism and transport (Knight et al., 2010).

The research regarding the relationship between Ado signaling and Ado transport in *Drosophila* offers a substantial advantage of lower complexity model system. Such research in mammalian models is hampered by the use of analogs of AR. These compounds provide a good selectivity for particular adenosine receptor isoforms; however, they also strongly interfere with the Ado transport and metabolic conversion of Ado to AMP or inosine. The relationship between Ado signaling and Ado transport seems to be crucial to our understanding of the regulation of energy homeostasis in various cell types.

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LIST OF ABBREVIATIONS

ADA – Adenosine deaminase

ADGF – Adenosine deaminase growth factor

Ado – Adenosine

AK – Adenosine kinase

AMP – Adenosine monophosphate

AR – Adenosine receptor

ATP – Adenosine-5´-triphosphate

CADO – 2-chloroadenosine

CNT – Concentrative nucleoside transporter

DmAdoR – *Drosophila* adenosine receptor

ENT – Equilibrative nucleoside transporter

NADH – Nicotinamide adenine dinucleotide

PNP – Purine nucleoside phosphorylase

SAH – S-Adenosyl homocysteine

SAM – S-Adenosyl methionine

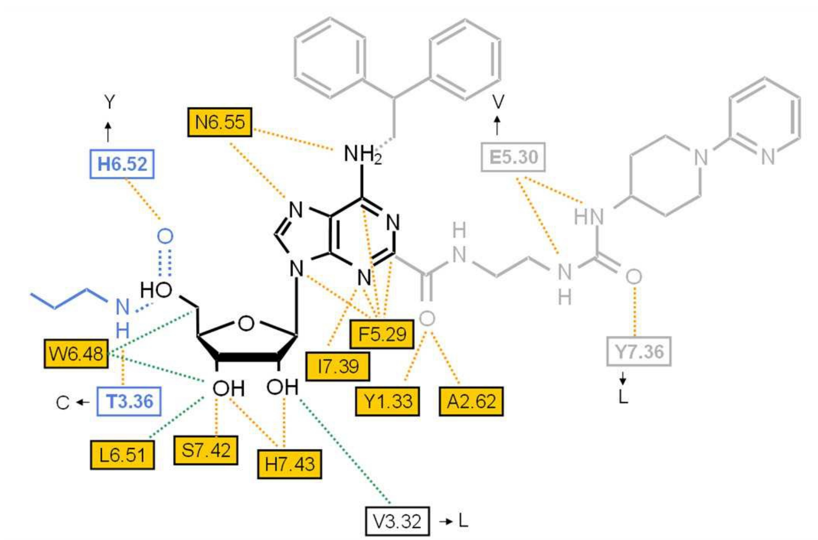
AIMS OF THE WORK

Adenosine is a key metabolite that affects a wide range of physiological processes. Deeper characterization of adenosine signaling and transport is of considerable importance for the development of treatments for diverse human disorders. Investigations on mammals are limited by the high complexity of the model system, lack of genetic tools, and also cost of experiments. The main goal of my PhD research was to investigate the physiological effects of adenosine on *Drosophila* cells. Our advantage was the availability of several *Drosophila* cell lines, which showed remarkably different responses to adenosine treatment. Our study helps to introduce *Drosophila* as an important model to study Ado function. The specific aims are as follows:

1) To establish the functional assays for the activation of *Drosophila* adenosine receptor in *Drosophila* cells *in vitro*.
2) To verify, whether AdoR activates two second messenger systems, cAMP and calcium in *Drosophila* cells.
3) To determine the pharmacological profile of DmAdoR and test utility of some adenosine receptor analogs for the *in vivo* experiments in *Drosophila*.
4) To explain toxic effects of extracellular adenosine on some *Drosophila* cells.
5) To compare the effects of extracellular adenosine on various *Drosophila* cell lines.

Part I

CHARACTERISATION OF THE *DROSOPHILA* ADENOSINE RECEPTOR: THE EFFECT OF ADENOSINE ANALOGS ON CAMP SIGNALING IN *DROSOPHILA* CELLS AND THEIR UTILITY FOR *IN VIVO* EXPERIMENTS



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ORIGINAL ARTICLE

Characterization of the *Drosophila* adenosine receptor: the effect of adenosine analogs on cAMP signaling in *Drosophila* cells and their utility for *in vivo* experiments

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Abstract

Adenosine receptors (AR) belonging to the G protein-coupled receptor family influence a wide range of physiological processes. Recent elucidation of the structure of human A2AR revealed the conserved amino acids necessary for contact with the Ado moiety. However, the selectivity of Ado analogs for AR subtypes is still not well understood. We have shown previously that the *Drosophila* adenosine receptor (DmAdoR) evokes an increase in cAMP and calcium concentration in heterologous cells. In this study, we have characterized the second-messenger stimulation by endogenous DmAdoR in a *Drosophila* neuroblast cell line and examined a number of Ado analogs for their ability to interact with DmAdoR. We show that Ado can stimulate cAMP but not calcium levels in *Drosophila* cells. We found one full and four partial DmAdoR agonists, as well as four antagonists. The employment of the full agonist, 2-chloroadenosine, in flies mimicked *in vivo* the phenotype of DmAdoR over-expression, whereas the antagonist, SCH58261, rescued the flies from the lethality caused by DmAdoR over-expression. Differences in pharmacological effect of the tested analogs between DmAdoR and human A2AR can be partially explained by the dissimilarity of specific key amino acid residues disclosed by the alignment of these receptors.

Keywords: AdoR, calcium, CG9753, cyclic AMP, GloSensor, GPCR.

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Adenosine (Ado) is a key cellular metabolite that physiologically regulates energy and oxygen homeostasis, neural functions, adipose tissue metabolism, immune reactions, and sleep (Jacobson and Gao 2009). Ado signaling is also involved in a variety of pathological conditions, including neurological, cardiovascular and inflammatory diseases as well as cancer (Trincavelli *et al.* 2010). Its concentration in cells and tissue fluids ranges under normal conditions from 20 to 200 nM and can rise during stressful events into the micromolar range (Fredholm 2010). The release and uptake of Ado requires nucleoside membrane transport. Half-life of Ado in the circulation is very short, usually between 1 and 10 s (Jacobson and Gao 2009). As Ado is a part of many metabolic pathways, it is always present both inside and outside of cells (Fredholm 2010).

Extracellular Ado mediates most of its effects through the activation of adenosine receptor (AR). Four subtypes of AR were identified in mammals displaying an overall amino acid identity of 31–46% (Piirainen *et al.* 2011). They belong to the G protein-coupled receptor family and are positively (A2A and A2B) or negatively (A1 and A3) coupled to adenylate cyclase (van Calker *et al.* 1979; Fredholm *et al.*

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Abbreviations used: AR, adenosine receptor; CADO, 2-chloroadenosine; CCPA, 2-chloro-*N*(6)-cyclopentyladenosine; CHA, *N*(6)-cyclohexyladenosine; CHO, Chinese hamster ovary; CPA, *N*(6)-cyclopentyladenosine; DmAdoR, *Drosophila* adenosine receptor; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; IB-MECA, *N*(6)-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; TM, transmembrane; XAC, xanthine amine congener.

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1994; Linden 1994). The expression of AR is widely spread in various tissues and Ado responses are highly dependent on the level of receptor expression in a particular cell type. Mice carrying null mutations in different adenosine receptor subtypes display only subtle phenotypic effects (Fredholm *et al.* 2005). The receptor knockouts pointed to clusters of activities related to the cardiovascular and the nervous systems, whose physiology is either reduced or enhanced upon specific receptor deletion (Hasko *et al.* 2008). Despite progress in the elucidation of the pathophysiology of Ado signaling, there are potential problems with compensatory mechanisms and receptor redundancy, which are not understood (Johansson *et al.* 2007). Further understanding of Ado signaling requires the combination of pharmacological and genetic approaches and multiple adenosine receptor subtype deficiency studies (Yaar *et al.* 2005).

A number of AR ligands displaying much better metabolic stability than Ado and selectivity for individual receptor subtypes were characterized. A detailed structure of the human A2AR has been solved and co-crystallization was performed with its antagonist ZM241385 and agonists UK-432097, Ado and 5′-*N*-ethylcarboxamidoadenosine (NECA; Jaakola *et al.* 2008; Lebon *et al.* 2011; Xu *et al.* 2011). Contact amino acid residues for binding of these Ado analogs were elucidated. At least three residues were crucial for binding of all mentioned analogs: F5.29 (amino acid residue 168 in A2AR primary sequence) in the extracellular loop 2, I7.39 and N6.55, which bind to the adenine core. In addition, co-crystallization of A2AR with the agonist UK-432097 suggested important roles of H7.43 and S7.42, which create hydrogen bonds with the OH groups of the ribose ring (Xu *et al.* 2011). Three more amino acid residues, including V3.32, L6.51 and W6.48, were implicated to bind ribose moiety by the recent study of Lebon *et al.* (2011). These contact amino acids are well conserved among all four human isoforms (see Fig. 1). Further information is needed to specify the role of other amino acid residues responsible for ligand binding differences of each specific receptor subtype.

A single AR was found in *Drosophila* (DmAdoR) based on its homology to the human ARs (Brody and Cravchik 2000; Broeck 2001; Dolezelova *et al.* 2007). The closest homolog of *Drosophila* DmAdoR in human is A2AR. It has about 38.3% identity in the 350 base long N-terminal part. Other ARs show following rank order of amino acid conservation: A1 having 36.2%, A2B – 35.2% and A3 – 34.5% identity with DmAdoR. DmAdoR is expressed mainly in the optic lobes of the brain, ring gland, imaginal discs and salivary glands of the third instar *Drosophila* larvae. In adults, the expression is higher in the head than in the rest of the body (Dolezelova *et al.* 2007). *Drosophila DmAdoR null* mutant flies are viable but exhibit reduced associative learning and defects in synaptic transmission (Knight *et al.* 2010). Ectopic expression of DmAdoR results in complex pleiotropic phenotypes ranging from slight wing morphology abnormalities to lethality depending on expression level and the tissue specificity of its expression.

Drosophila provides an advantage of lower complexity model system (three equilibrative nucleoside transporters and only single adenosine receptor), with the opportunity to use genetic approaches not possible in higher organisms. We previously found that a null mutant in *DmAdoR,* as well as a hypomorph in *DmEnt2*, a major *Drosophila* equilibrative nucleoside transporter (Machado *et al.* 2007), display changes in synaptic function (i.e. plasticity and presynaptic calcium influx) and altered behavioural responses (Knight *et al.* 2010). Interestingly, the phenotypes of both mutants were remarkably similar and suggest extensive adaptive responses to extracellular levels of Ado (Knight *et al.* 2010). Unraveling the relationship between the Ado transport and the receptor signaling will greatly improve our understanding of Ado function. The importance of the equilibrative nucleoside transporters for Ado receptor signaling was demonstrated earlier in mice carrying mutation in *ENT1*, which exhibited a reduced inhibitory tonus mediated by endogenous Ado (Choi *et al.* 2004). In the mammalian CNS, the inhibitory effect of Ado on cellular and synaptic activity appears to be primarily mediated by A1 receptors (Dunwiddie 1980; Greene and Haas 1991).

Our previous study showed a concentration-dependent accumulation of cAMP and calcium in Ado-treated Chinese hamster ovary (CHO) cells expressing recombinant *Drosophila* DmAdoR (Dolezelova *et al.* 2007). We re-examined the second messenger responses in native *Drosophila* cells and showed that the naturally expressed DmAdoR primarily stimulates the increase in cAMP synthesis. We also provide data on the ability of a number of the subtype selective analogs of human AR to stimulate or block DmAdoR. This study provides important information about the biological role of DmAdoR in flies *in vivo* and brings new insight into the role of specific amino acid residues in the mammalian Ado receptors.

Materials and methods

Chemicals

Ado, inosine, guanosine, deoxyadenosine, NECA, *N*(6)-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA), *N*(6)-cyclohexyladenosine (CHA), 2-chloro-*N*(6)-cyclopentyladenosine (CCPA), CGS 21680, *N*(6)-cyclopentyladenosine (CPA), 2-chloroadenosine (CADO), caffeine, theophylline, xanthine amine congener (XAC), CGS 15943, SCH58261, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and forskolin were obtained from Sigma-Aldrich (St Louis, MO, USA); CV1808, ZM241385 and pentostatin (deoxycoformycin) were purchased from Tocris Bioscience (Bristol, UK); 2-phenylaminoadenosine (ADAC) and SCH442416 were from

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Dros  AdoR     1 MSAFRYFSITDFSFEGPLLPLHAATTSKDAKDSDSPSSELNIPYTVFEVLVAIVSIIGNV
Human  A3      1 -----------------------------MPNNSTALSLANVTYITMEIFIGLCAIVGNV
Human  A1      1 --------------------------------MPPSISAFQAAYIGIEVLIALVSVPGNV
Human  A2A     1 -----------------------------------MPIMGSSVYITVELAIAVLAILGNV
Human  A2B     1 ----------------------------------MLLETQDALYVALELVIAALSVAGNV

                 ▼1.50                    ▼2.50
Dros  AdoR    61 LVIIVFRRERKLRRRTNYYIVSLAMADLLVGALGIPFAILASMGLPRNLHACLFTVSLLV
Human  A3     32 LVICVVKLNPSLQTTTFYFIVSLALADIAVGVLVMPLAIVVSLGITIHFYSCLFMTCLLL
Human  A1     29 LVIWAVKVNQALRDATFCFIVSLAVADVAVGALVIPLAILINIGPQTYFHTCLMVACPVL
Human  A2A    26 LVCWAVWLNSNLQNVTNYFVVSLAAADIAVGVLAIPFAITISTGFCAACHGCLFIACFVL
Human  A2B    27 LVCAAVGTANTLQTPTNYFLVSLAAADVAVGLFAIPFAITISLGFCTDFYGCLFLACFVL
                                                                           *

                                ▼3.50                      ▼4.50
Dros  AdoR   121 VLCTISIFCLVAVSVDRYWAILYPMAYSRNVRTRTAIFIISMCWVAGTIVGFLPLFGWHA
Human  A3     92 IFTHASIMSLLAIAVDRYLRVKLTVRYKRVTTHRRIWLALGLCWLVSFLVGLTPMFGWNM
Human  A1     89 ILTQSSILALLAIAVDRYLRVKIPLRYKMVVTPRRAAVAIAGCWILSFVVGLTPMFGWNN
Human  A2A    86 VLTQSSIFSLLAIAIDRYIAIRIPLRYNGLVTGTRAKGIIAICWVLSFAIGLTPMLGWN-
Human  A2B    87 VLTQSSIFSLLAVAVDRYLAICVPLRYKSLVTGTRARGVIAVLWVLAFGIGLTPFLGWNS

                                                                  ▼5.50
Dros  AdoR   181 DVNHNQEC--------------------LFVEVMDYNYLV-FLYFATIITPALLMLAFYT
Human  A3    152 KLTSEYH----RNVT---------FLSCQFVSVMRMDYMVYFSFLTWIFIPLVVMCAIYL
Human  A1    149 LSAVERA----WAANGSM---GEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYL
Human  A2A   145 ------NCGQPKEGKNHSQGCGEGQVACLFEDVVPMNYMVYFNFFACVLVPLLLMLGVYL
Human  A2B   147 KDSATNNCTEPWDGTTNESCC---LVKCLFENVVPMSYMVYFNFFGCVLPPLLIMLVIYI

Dros  AdoR   220 HIYRVIIKQVRQIVTMNPASDLSRRSSAAVVQVTTPGRGGHTGTMLRVLGAARKRDVKAT
Human  A3    199 DIFYIIRNKLSLNL---------------------SNSK--------ETGAFYGREFKTA
Human  A1    202 EVFYLIRKQLNKKV---------------------SASSG-------DPQKYYGKELKIA
Human  A2A   199 RIFLAARRQLKQMESQ-------------------PLPG--------RARSTLQKEVHAA
Human  A2B   204 KIFLVACRQLQRTELM------------------------------DHSRTTLQREIHAA

                ▼6.50                                 ▼7.50
Dros  AdoR   280 QNLSIIVLFFMICWIPLYTINCIKAFCP-DCYVHPK-LTLFCIILSHLNSAVNPVLYAYH
Human  A3    230 KSLFLVLFLFALSWLPLSIINCIIYF----NGEVPQLVLYMGILLSHANSMMNPIVYAYK
Human  A1    234 KSLALILFLFALSWLPLHILNCITLFCPS--CHKPSILTYIAIFLTHGNSAMNPIVYAFR
Human  A2A   233 KSLAIIVGLFALCWLPLHIINCFTFFCP-DCSHAPLWLMYLAIVLSHTNSVVNPFIYAYR
Human  A2B   234 KSLAMIVGIFALCWLPVHAVNCVTLFQPAQGKNKPKWAMNMAILLSHANSVVNPIVYAYR
                              *  *

Dros  AdoR   338 LKDFRAALKNLLLKMMGVDIDQQAEAIHRFSVASQHRLQSMDSNMRSTQPRLYVGEYSPI
Human  A3    286 IKKFKETYLLILKACVVCHPSDSLDTSIEKNSE---------------------------
Human  A1    292 IQKFRVTFLKIWNDHFRCQPAPPIDEDLPEERPDD-------------------------
Human  A2A   292 IREFRQTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVWANGSA
Human  A2B   294 NRDFRYTFHKIISRYLLCQADVKSGNGQAGVQPALGVGL---------------------
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Fig. 1 Alignment of the N-terminal parts of DmAdoR and human adenosine receptors. Amino acid residues that are identical between all receptors are highlighted in gray. Conserved positions of key amino acid residues involved in making contacts with the agonists, UK-432097, Ado and NECA are shown in boxes, using solid-line box for the amino acid residues contacting the adenosine core and dotted-line box for residues contacting the substituted side chains (Lebon *et al.* 2011; Xu *et al.* 2011). Additional amino acids described by Lebon *et al.* (2011) to be involved in binding of ribose moiety are marked with asterisks. The transmembrane (TM) alpha helices were assigned using the A2AR crystal structure information by Jaakola *et al.* (2008) and are marked by lines above the sequences. Position 50 in Ballesteros-Weinstein numbering (see Experimental procedures section) of each TM is marked by a filled triangle.

Axon Medchem (Groningen, the Netherlands). The AR analogs were dissolved to 10 mM concentration in 100% dimethylsulfoxide (DMSO). For tissue culture assays, the AR agonists were diluted to 10 and 100 μM and the AR antagonists to 1 and 10 μM final concentrations in cell culture media. DMSO was purchased from Invitrogen (Carlsbad, CA, USA). Fura-2AM and ionomycine were obtained from Sigma-Aldrich. Final DMSO concentrations in all cell culture cAMP assays were less than 10%. For *in vivo* experiments, Ado or CADO was dissolved in water and mixed into fly-food at a final concentration of 1.65 mM (500 μg/mL). SCH58261 was dissolved to a 28.57 mM concentration (10 mg/mL) in 100% DMSO and subsequently transferred to an injection mix containing Ringer’s solution, 170 μM SCH58261 (60 μg/mL) and 0.6% DMSO.

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Cell culture

Four different *Drosophila* cell lines were used for the experiments, including imaginal disc cells Cl.8+ (Peel and Milner 1990), hematopoietic cells Mbn2 (Samakovlis *et al.* 1992), embryonic cells S2 (Schneider 1971) and neuroblasts Bg2-c2 (Ui *et al.* 1994). All cells were maintained in Shields and Sang medium (Sigma, S 8398) with different supplements as described previously (Zurovec *et al.* 2002). The growth media of Cl.8+ cells were supplemented with 2% fetal bovine serum, 2.5% fly extract, and 0.125 IU/mL insulin. Embryonic S2 cells and hematopoietic Mbn2 cells were maintained in Shields and Sang medium with 10% FBS and 0.125 IU/mL insulin, whereas the medium for Bg2-c2 cells contained 10% fetal bovine serum, yeast extract (1 g/L), pepton (2.5 g/L) and 0.3 IU/mL insulin.

Measurement of Ado-induced cAMP increase

The coding sequence for a chimeric firefly luciferase carrying a cAMP-binding moiety protein was PCR amplified from the *pGloSensor-20F* plasmid (Promega, Madison, WI, USA) and subcloned into the commercial *Drosophila* expression vector *pAC5.1/V5-His* (Invitrogen), which is under a *Drosophila* actin V promoter. The following primers were used, both incorporating EcoRI site (underlined) at its 5′ ends: GsF: TTGAATTCGAA ATGCCTGGCGCAGTAGGCAAGG and GsR: GTTGAATTCT AGAGTTTAAACCCCTTCTGGAG.

The ability of the construct to emit light upon cAMP binding was first confirmed by transient transfection of the pAC-GloSensor plasmid into *Drosophila* Cl.8+ cells and treatment by forskolin (an activator of adenylyl cyclase). The cAMP responses in *Drosophila* cells were detected by luminescence readout (see the procedure below). Forskolin (20 μM) induced about three times higher level of luciferase activity than the control-uninduced cells (see Results). Ado responses were measured with or without inhibitors of adenosine deaminases (deoxycoformycin) and we did not observe any significant effect on cAMP responses.

The *Drosophila* Bg2-c2 or S2 cells were seeded into 60-mm dishes at a density of 1×10^6 cells/mL in 3 mL medium. The pAC-GloSensor plasmid (1 μg) was then transiently transfected into the cells by using the Effectene reagent (Qiagen, Hilden, Germany). After 18 h of transfection, the cells were washed with 2 mL of complete medium and 3 mL of fresh complete medium was added to the cells and incubated for an additional 24 h. A bioluminiscence-based cAMP assay was conducted using the GloSensor cAMP assay kit (Promega) following manufacturer's instructions (Fan *et al.* 2008). The cells were pre-equilibrated for 2 h with the GloSensor cAMP reagent and dispensed into 96-well plates (2×10^6 cells per mL). After loading, the cells were treated with ligands and luminescence was measured on a Berthold Orion II Microplate luminometer using the kinetic protocol (a maximum cAMP signal was reached in 8–11 min.). The results represent the average of three experiments.

Measurement of Ado-induced calcium release

The fluorescent calcium indicator Fura-2 was employed to monitor changes in intracellular calcium ion concentration (Grynkiewicz *et al.* 1985) with modifications according to (Torfs *et al.* 2000). Cells were resuspended in insect Elliot buffer (129.7 mM NaCl, 5.44 mM KCl, 1.2 mM $MgCl_2 \cdot 6H_2O$, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 20 mM HEPES, 63 mM saccharose; pH 6.2) supplemented with 1 mM $CaCl_2$, 4 mg/mL Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA), 10 mM glucose, and 1 mM probenecid and incubated in the dark for 30 min at 22°C. Then a coverslip with the attached cells was washed in fresh medium, inserted into a recording chamber and placed on a stage of an inverted fluorescence microscope (Nikon, Tokyo, Japan). The cells were alternatively illuminated at 340 nm and 380 nm and the emitted light was recorded at 510 nm using the Hammamatsu CCD camera. The cells were kept in 0.5 mL of the basal medium and were stimulated by adding 0.5 mL of the medium containing Ado. The recording chamber was kept at 25°C.

Real-time RT-PCR

Total RNA from *Drosophila* cells was isolated using the RNA Blue reagent (Top-Bio, Prague, Czech Republic). The RNA was further purified by NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany) including on-column digestion step with rDNase I. One microgram of total RNA was reversely transcribed at 42°C using oligo$(dT)_{17}$ and PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan).

The PCR reaction volume was 20 μL, containing 10 μL of diluted cDNA and reaction mix [Hot start ExTaq polymerase (Takara) 0.75 unit, ExTaq buffer, dNTPs 200 μM each, Syber green 1 : 25 000, primers 400 nM each). The amplification was carried out on a Rotor-Gene 3000 (Corbet Research, Sydney, Australia) for 50 cycles (94°C for 20 s; 62°C for 30 s; 72°C for 30 s) following an initial denaturation/Taq activation step (95°C for 2 min). Each sample was analyzed in triplicate. Primers (sequences shown in Table 1) were designed with Lasergene PrimerSelect Software (DNASTAR, Madison, WI, USA) to assure that each amplicon encompassed an exon/intron boundary. The product size was confirmed by melting analysis. Data were analyzed and quantified with the Rotor-Gene 6 analysis software. Relative values were normalized to the *Rack1* cDNA and standardized to the Cl.8+ sample. All results are presented with means and SEM from four independent biological samples.

Fly strains and survival experiments

Transgenic fly strains *w; UAS-DmAdoR* and *w; DmAdoR^1* were described previously (Dolezelova *et al.* 2007); other strains, including *yw; UAS-GFP; act-gal4:PR* (Rogulja and Irvine 2005) and w^{1118} were provided by the Bloomington Stock Center (Indiana University, Bloomington). Mutant *w; DmAdoR^1* was isogenized to w^{1118} for eight generations. Flies were kept on a cornmeal–yeast–agar–sugar diet supplemented with the mold inhibitor methylparaben at 25°C. After hatching, first instar larvae of isogenized *w, DmAdoR^1* or w^{1118} were collected and transferred to vials containing

Table 1 List of primers used in real-time RT-PCR

Primer name	Sequence
DmRack1-fw	CCC GTG ACA AGA CCC TGA T
DmRack1-rev	TAG TTG CCA TCG GAG GAG AG
DmAdoR-fw	CCC ATC TGA ACT CGG CGG TAA ATC
DmAdoR-rev	GCC TCC TGC TGC TGC CTC AAC

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3 mL of fly food with or without the addition of Ado or its analog CADO (final concentration 500 μg/mL). Numbers of pupae were counted twice a day around the 120-h time point (when the first larvae start to pupariate). 150 larvae per sample were analyzed. Late third instar larvae containing *wP*; *UAS-DmAdoR/act-gal4:PR* (or control flies without *UAS-DmAdoR*) were injected with 50 nL of Ringer's solution containing 170 μM SCH58261 and 0.6% DMSO or 0.6% DMSO alone, respectively. The injection volume was approximately 5% of the total larval volume. Injected larvae were transferred into vials with cornmeal–yeast–agar–sugar diet at 25°C (30 injected larvae per vial). Numbers of pupae were counted and compared with numbers of eclosed flies.

Sequence alignment, amino acid numbering, GenBank accession numbers

The alignment of several G protein-coupled receptors was performed using the MEGA 4 software (Tamura *et al.* 2007). To simplify the identification of corresponding residues in human and *Drosophila* AR, we used the index system established by Ballesteros and Weinstein (1995). The numbering is based on the relative position of each amino acid residue to the most conserved residue in each transmembrane (TM) segment (the most conserved residue in each TM domain is designated x.50, where x is the TM helix number). The GenBank accession numbers of ARs used in this paper are: no. NP_651772 (DmAdoR); no. NP_000665 (human A1R), no. NP_000666 (human A2AR), no. NP_000667 (human A2BR) and no. CAA54288 (human A3R).

Statistical methods

Data are presented as means ± SEM of the number of independent experiments indicated in Figure legends. In concentration–response experiments, EC_{50} (half maximal effective concentration) was determined by non-linear regression using Graphpad Prism v5.0 software (GraphPad, San Diego, CA, USA).

Results

DmAdoR -mediated stimulation of cAMP in *Drosophila* cells

We first tried to detect the Ado-induced cAMP responses in *Drosophila* imaginal disc Cl.8+ cells, but we did not obtain any response. We therefore examined the *DmAdoR* mRNA levels and found that these cells demonstrate rather low endogenous receptor mRNA expression (Fig. 2). We tried to over-express DmAdoR in these cells, however, the transfection and over-expression of recombinant DmAdoR did not improve the outcome of the Ado signaling assays. As the transfection was cytotoxic, the cells grew poorly, and the level of expressed *DmAdoR* mRNA remained very low.

To find another cell line with a higher endogenous level of DmAdoR expression, we employed real-time RT PCR and examined several *Drosophila* cell lines of different tissue origin, including embryonic S2, hematopoetic Mbn2, and neuroblast Bg2-c2 cells. We also compared the mRNA concentrations in these cell lines with the levels of *DmAdoR* mRNA in adult fly body parts. As is shown in Fig. 2, the expression of *DmAdoR* varies substantially among the cell

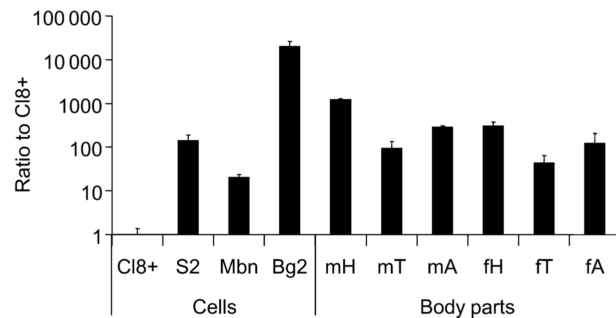

Fig. 2 Expression of *DmAdoR* mRNA in *Drosophila* cell lines and imago body parts. Real-time RT PCR was performed in order to compare the expression of *DmAdoR* in four cell types (Cl8+, S2, Mbn2, Bg2-c2) and adult male (m) and female (f) body parts including head (H), thorax (T) and abdomen (A). *Rack1* mRNA was used as an internal control. Ordinate: The level of mRNA is expressed as a log of ratio relative to Cl.8+ cells.

lines and tissues examined. Bg2-c2 cells displayed the highest level of *DmAdoR* mRNA expression, which was about 10 times higher than the natural receptor mRNA level found in heads of male flies. The level of receptor mRNA in S2 cells was about 10 times lower than that in the heads of male flies and comparable to other parts of the fly examined (female heads, thorax and abdomen of both male and female flies) but still about 100 times higher than in Cl.8+ cells. The Bg2-c2 and the S2 cells were therefore used in most of the subsequent experiments.

The examination of Ado-stimulated cAMP induction in Bg2-c2 and S2 cells transiently transfected with pGloSensor reporter showed a concentration dependent Ado-specific increase in the cAMP readout (Fig. 3a and b). This response was strong and robust in Bg2-c2 cells (about 200% induction) and, in line with the smaller *DmAdoR* mRNA expression, much lower in S2 cells (about 50% induction, Fig. 3a). The estimated EC_{50} values were 4.2 (95% confidence interval 3.7–4.8 μM) and 55 μM (95% confidence interval 13–236 μM) in Bg2-c2 and S2 cells, respectively (Fig. 3a and b).

Lack of adenosine receptor-mediated calcium responses in *Drosophila* cells

The intracellular free calcium release upon Ado stimulation of Fura-2-loaded Bg2-c2 and S2 cells was investigated by digital imaging fluorescence microscopy. Application of the calcium ionophore, ionomycin was used as a positive control to demonstrate that the calcium detection system worked properly. As shown in Fig. 4, the resting calcium concentration was very stable and the addition of ionomycin to the cells in the presence of extracellular calcium caused the expected maximal increase in the signal. However, Ado failed to stimulate a detectable increase in the concentration of calcium in both S2 and Bg2-c2 cells (Fig. 4a and b). Marginal calcium responses (just above the detection limit) were observed only in some samples of Bg2-c2 cells treated

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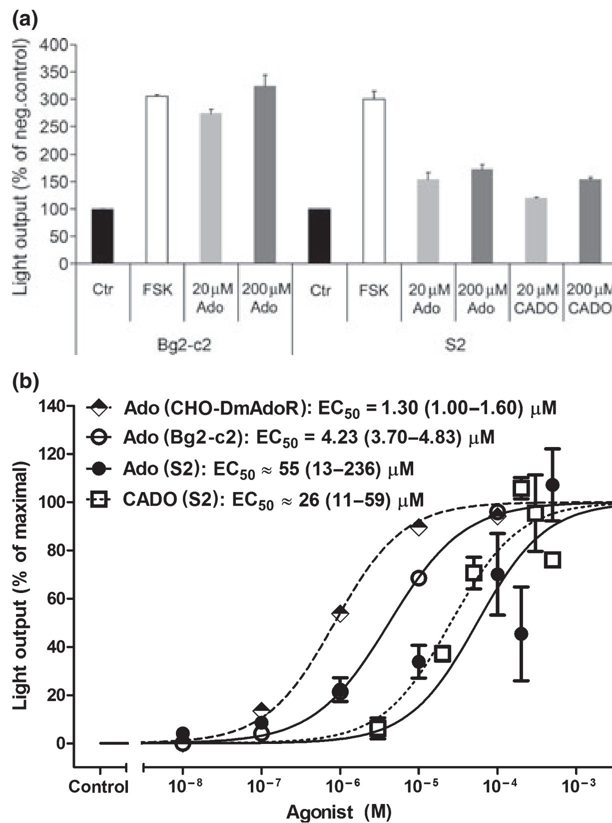

Fig. 3 The responses of Bg2-c2, and S2 cells to Ado and CADO. (a) The cells were treated with 20 or 200 µM Ado, 20 or 200 µM 2-chloroadenosine (CADO) and 20 µM forskolin (FSK). The cAMP production induced by Ado (ordinate, expressed in percent of control) was proportional to the level of adenosine receptor mRNA while forskolin-induced comparable responses in both cell types. (b) Comparison of Ado-induced cAMP accumulation in Bg2-c2 (open circles) and S2 cells (closed circles). The CADO-induced cAMP accumulation in S2 cells is also included (open square). Concentration–response curves of cAMP production in response to extracellular Ado in Bg2-c2 and S2 cells is expressed as percent of calculated maximal response (ordinate) in individual experiments. Values represent the mean ± SEM of four values obtained in one experiment for Bg2-c2 cells and 3–9 values from two independent experiments for S2 cells. Abscissa: molar concentration of analogs. The EC50 values of Ado and CADO with 95% confidence limit are shown in graph. For comparison, the dotted line with diamond symbols illustrates the concentration–response curve of DmAdoR over-expressed in Chinese hamster ovary cells, published earlier by Dolezelova *et al.* (2007). Sigmoidal concentration-response equation was fitted to normalized data.

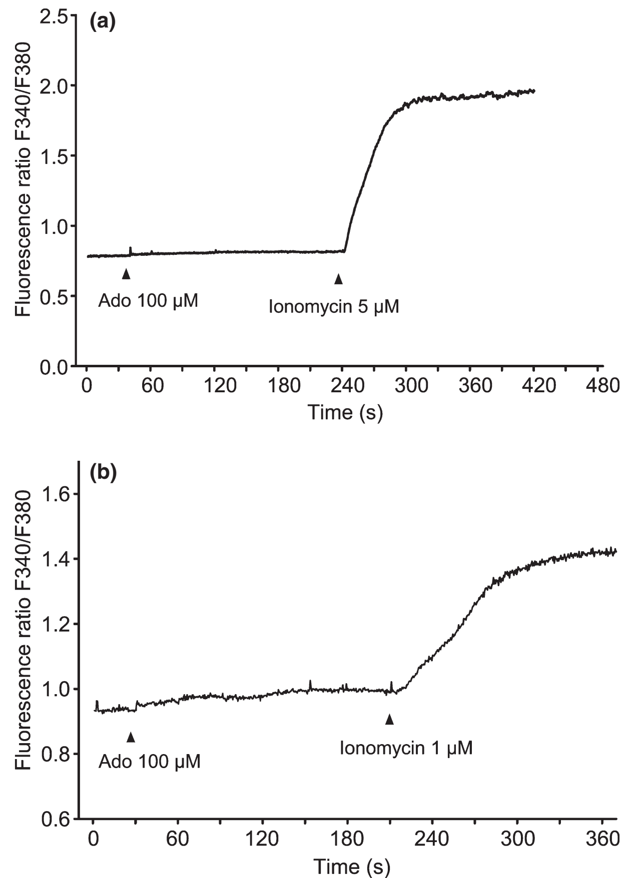

Fig. 4 Determination of intracellular calcium in response to Ado stimulation in *Drosophila* S2 and Bg2-c2 cells. The calcium level is unaffected by Ado treatment in S2 cells (a) and has at best a marginal effect in Bg2-c2 cells (b). Ionomycin in the presence of extracellular calcium elicits a maximal signal in both cell lines. Ordinate: fluorescence emission ratio recorded at 510 nm after alternating excitation at 340 and 380 nm.

with a high (100 µM) concentration of Ado. We conclude that there is no or very little calcium signal in response to Ado in the *Drosophila* cells examined.

Determining ligand specificity and pharmacological profile of DmAdoR

The ability of potential physiologically relevant natural ligands to activate DmAdoR was examined using cAMP functional assays and Bg2-c2 cells. We examined deoxyadenosine (dAdo), inosine and guanosine and found that inosine and guanosine do not activate DmAdoR and dAdo has only a marginal effect (Fig. 5a).

To further characterize the ligand sensitivity of the DmAdoR, we assayed various structural Ado analogs, including CHA, CPA, CCPA, CADO, IB-MECA, ADAC, CV1808, CGS 21680 and NECA. In the first set of experiments these ligands were tested in Bg2-c2 cells for downstream signaling (cAMP production) at two concentrations – 10 and 100 µM. The results showed that a number of analogs displayed an increase in cAMP levels, including CADO, CHA, CPA, CCPA, CV1808 and ADAC (Fig. 5b). In the next set of experiments, concentration–response relationship of ADO, CADO, CV1808, and CHA (Figs 3b and 5c, Table 2) was determined. The rank order of the tested agonist potency was CHA > ADO > CADO = CV1808 and the efficacy CADO > CV1808 = ADO > CHA. At an equi-

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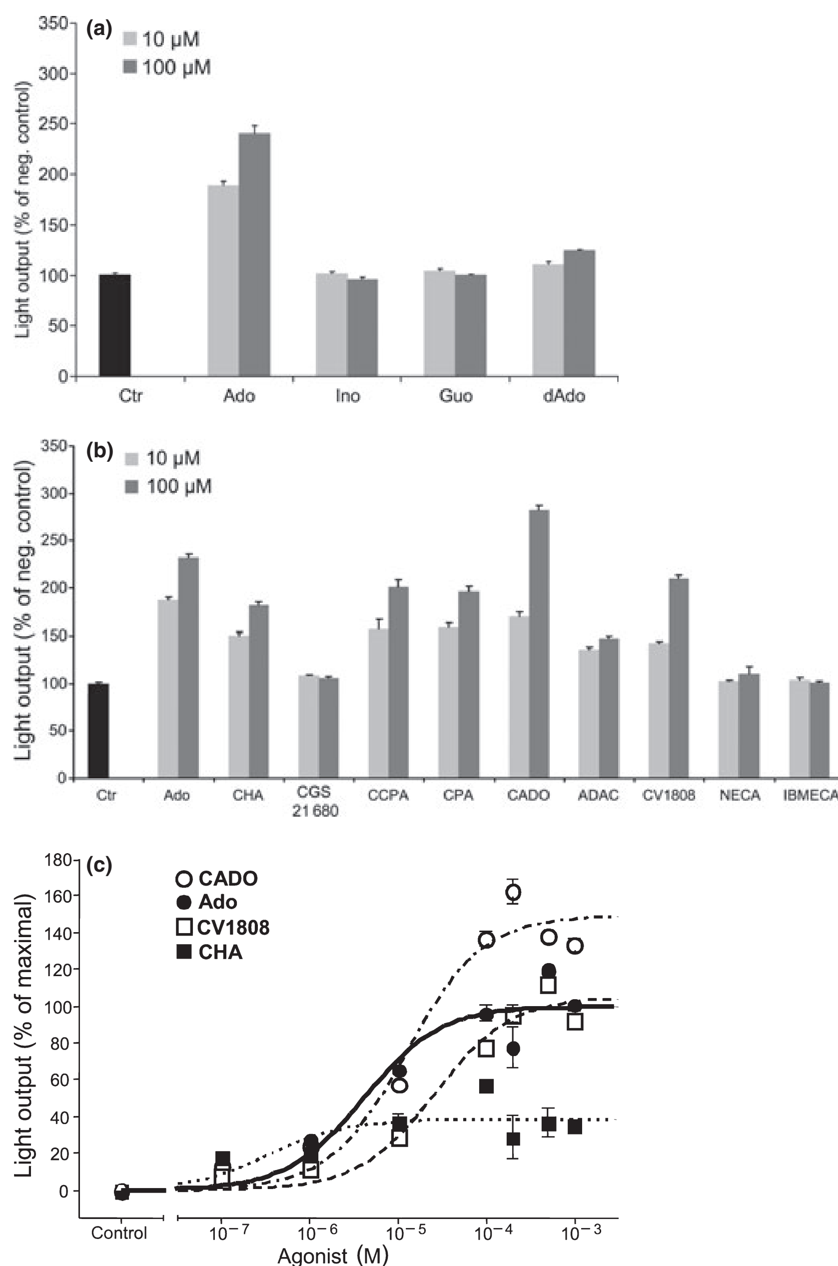

Fig. 5 Effects of AdoR agonists on cAMP accumulation in Bg2-c2 cells. (a, b) The cell responses to 10 and 100 μM concentration of Ado, or indicated potential AdoR agonists. The increase in cAMP concentration is expressed as a percentage of the basal level. (c) Concentration–response curves of cAMP accumulation in response to extracellular Ado and adenosine receptor agonist CADO, CV1808, and CHA. Individual points are expressed as a percentage of the maximal Ado response (ordinate) and represent mean ± SEM of 3–6 values. Abscissa: molar concentration of agonist. Sigmoidal concentration–response equation was fitted to normalized data. Parameters of fits are given in Table 2.

Table 2 Parameters of concentration–response curves (shown in Fig. 5C) of agonist-induced cAMP accumulation in Bg2-c2 cells

Agonist	EC_{50} (log *M*)	E_{max} (% of Ado)
Ado	−5.39 ± 0.23	100 ± 7
CADO	−4.91 ± 0,17	150 ± 8
CV1808	−4.58 ± 0.16	105 ± 6
CHA	−6.47 ± 0.47	38 ± 5

molar 100 μM concentration, the CCPA, CPA, and ADAC reached 76, 73, and 46 percent of the Ado effect, respectively, while CGS21680 and other analogs including NECA and IB-MECA had no effect (Fig. 5b). The potency of CADO-induced cAMP response was slightly higher in Bg2-c2 cells (12 μM) than in S2 cells (26 μM), as shown in Figs 3b and 5c.

We also examined activity of various antagonists on DmAdoR by assaying their ability to inhibit an Ado-stimulated cAMP response. The Bg2-c2 cells pre-loaded with GloSensor reagent were pre-incubated with 1 μM and 10 μM concentrations of antagonist for 5 minutes, followed by an addition of 10 μM concentration of Ado. As shown in Fig. 6a, the following order of potency was observed: SCH442416 ≥ ZM241385 ≥ SCH58261 > XAC > CGS15943. Pre-incubation with 1 and 10 μM SCH442416, 10 μM

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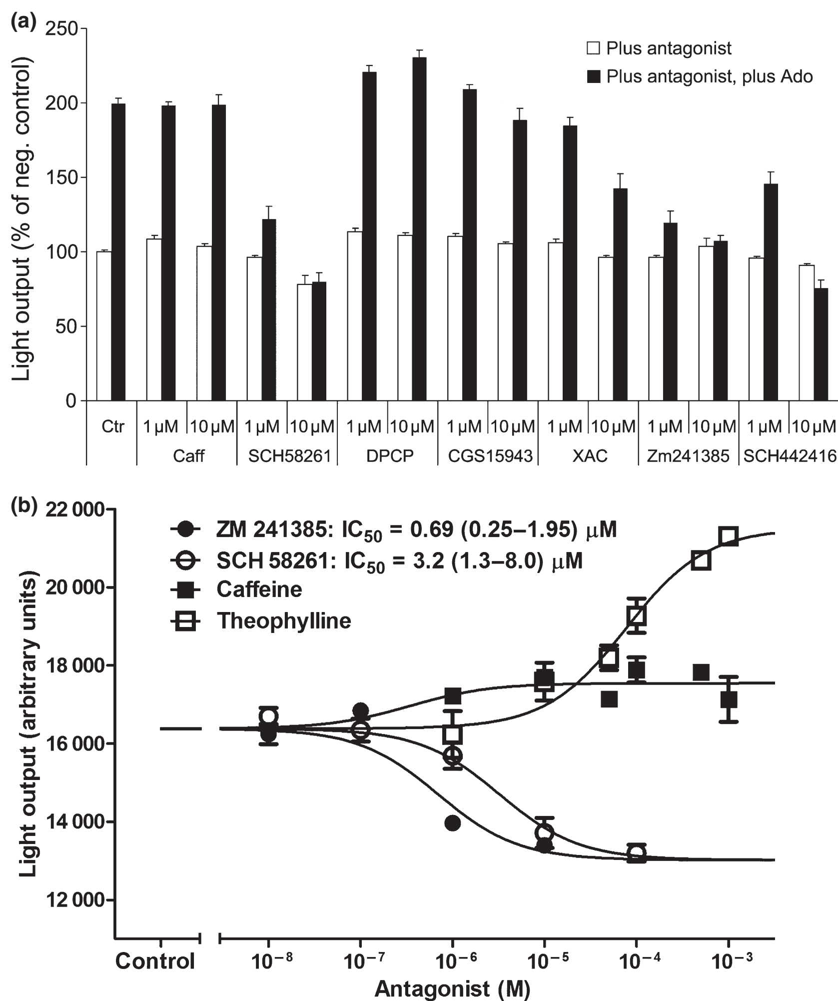

Fig. 6 Effects of AdoR antagonists on Ado-stimulated cAMP accumulation in Bg2-c2 cells. (a) The Bg2-c2 cells were pre-incubated for 5 min with 1 or 10 μM antagonist. The cells were then treated with 10 μM Ado and the cAMP accumulation was measured using the pGloSensor assay. The left chart (empty) columns are data prior to Ado treatment, the right chart (filled) columns are the records, taken after 10 μM Ado treatment. Ctr indicates control without the addition of antagonist. Columns represent mean ± SEM of 3–6 values. (b) Concentration–response curves of cAMP accumulation in response to DmAdoR antagonists. The Bg2-c2 cells were pre-incubated for 5 min with various concentrations of antagonists and then cells treated with 10 μM Ado. Individual points are expressed in arbitrary units (ordinate) and represent mean ± SEM of three values. The 50% effective concentrations of SCH 582261 and ZM 241385 (IC_{50} value with 95% confidence limits) are shown in graph.

SCH58261, and 10 μM ZM241385 completely prevented Ado-stimulated cAMP accumulation (Fig. 6a). XAC at a 10 μM concentration inhibited the increase of cAMP by about 50% but higher XAC concentrations did not cause full inhibition. Interestingly, simple xanthines like caffeine, theophylline and DPCPX failed to antagonize DmAdoR signaling (Fig. 6a and b). Slight increase in cAMP signal induced by high caffeine and theophylline concentrations (Fig. 6b), may not be receptor specific and results rather from phosphodiesterases inhibition.

To study the functional diversity of AR, we tried to find a correlation of the differences in the ligand selectivity and sequence variations among the DmAdoR and human ARs. We aligned DmAdoR with human A2AR and several AR-like sequences found in GenBank. We found that amino acids involved in contact with the Ado are perfectly conserved in *Drosophila* AdoR and these amino acid residues might be used as an important character to recognize AR orthologs in distant species. They include amino acid residues N6.55, F5.29, I.7.39, S7.42, H7.43, L6.51 and W 6.48 (Fig. 1 and Figure S1). Similarly, 6 of the 11 A2AR amino acid residues, which make contact with the antagonist ZM241385, were conserved between A2AR and DmAdoR (Figure S2).

Function of adenosine receptor analogs *in vivo*

The ectopic expression of DmAdoR in flies was shown earlier to cause gain of function phenotypes ranging from melanotic tumors to lethality, depending on the level and tissue specificity of over-expression (Dolezelova *et al.* 2007). To verify the effect of DmAdoR antagonists *in vivo*, we examined the ability of SCH58261 to rescue the lethal effect of DmAdoR over-expression. We administered the SCH58261 both by feeding and microinjection. Virtually no eclosed adults over-expressing DmAdoR under *act-gal4:PR* driver emerged in untreated (control) flies. We did not observe any influence of the Ado receptor antagonists SCH58261 in the fed flies probably because of its low water solubility. However, the microinjection at the dosage of 50 nL of 170 μM SCH58261 in Ringer's solution into the third instar larvae partially rescued the lethality and led to the emergence of approximately 20% of adult flies (Fig. 7a). The

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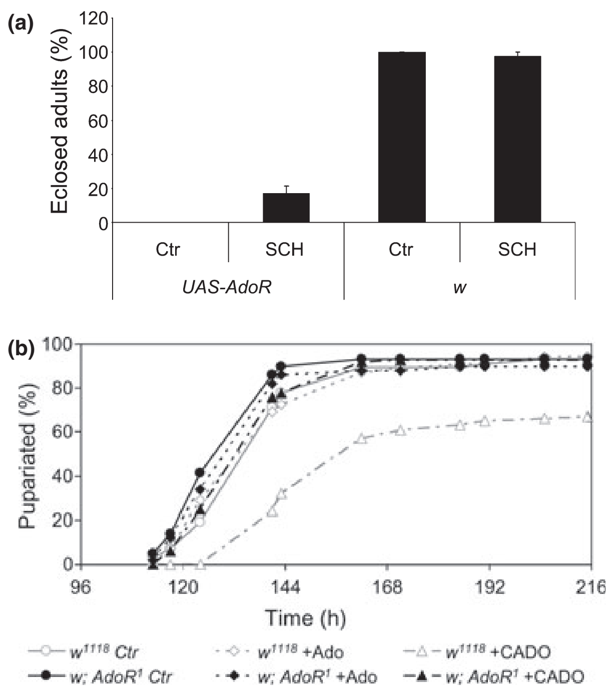

Fig. 7. *In vivo* effect of DmAdoR agonists and antagonists. (a) Antagonist SCH58261 rescues pupal lethality of DmAdoR over-expressing flies. (Ctr) Control L3 larvae injected with 50 nL of vehicle (0.6% DMSO in Ringer solution), (SCH) L3 larvae injected with 50 nL of 170 μM SCH58261. Fly genotypes: "*UAS-AdoR*" - *w*; *UAS-DmAdoR/act-gal4:PR*; "*w*" - control genotype, *w*; *+/act-gal4:PR* (b) Stable Ado agonist CADO causes larval lethality and developmental delay in *wt* flies but not in *DmAdoR null* mutants. First instar larvae were transferred to control fly food (Ctr) or fly food containing 500 μg/mL of CADO or Ado.

dosage of 55 μM SCH58261 was already too low to rescue the phenotype. Consistently, the administration of XAC, which is a less potent DmAdoR antagonist, did not show any rescue of flies (not shown). Our survival assay thus confirmed the utility of SCH58261 for *in vivo* experiments in flies.

We also tested if agonist treatment in *wt* flies results in the lethality characteristic for DmAdoR over-expression and whether *null* mutants in the *DmAdoR* gene ($DmAdoR_1$) are resistant to the treatment. Indeed, the feeding of *wt* flies with CADO led to developmental delays as well as larval and pupal lethality observed previously with DmAdoR over-expression (Dolezelova *et al.* 2007). Consistent with this result, $DmAdoR_1$ mutants were resistant to CADO treatment (Fig. 7b), confirming that the CADO effect is mediated by DmAdoR. Ado administered at the same dosage in food did not show any effect, most likely due to Ado degradation in the gut.

Discussion

Our previous results on the activation of second messenger pathways in heterologous CHO cells over-expressing DmAdoR showed a concentration-dependent increase in both intracellular cAMP and calcium (Dolezelova *et al.* 2007). Now, we confirmed that Ado activation of endogenous DmAdoRs in *Drosophila* cells induces cAMP accumulation. The Bg2-c2 and S2 cells responded to Ado by an increase in intracellular cAMP level in a concentration-dependent manner, with EC_{50} values of approximately 4 and 55 μM, respectively (Fig. 3a and b). There was an influence of the Ado solvent DMSO, which slightly interfered with the measurements, affecting especially low responses of S2 cells. The EC_{50} value received for Ado in S2 cells is therefore very approximate and had to be confirmed with metabolically more stable agonist CADO (the responses of Bg2-c2 and S2 cells to CADO showed EC_{50} values of approximately 12 and 26 μM, respectively; Figs 3b and 5c). The different potency of the same Ado receptor response can be explained by either the dissimilarity of cells in which the receptor is expressed or the level of receptor expression. Our results are consistent with the latter explanation. Ado at 200 μM concentration increased cAMP level 3 times in Bg2-c2 cells but only 1.5 times in S2 cells (Fig. 3a). This difference cannot be explained by unequal ability to produce cAMP because the direct activator of adenylyl cyclase forskolin had the same effect in Bg2-c2 and S2 cells. S2 cells also seem to have similar amount of Gαs subunits (Figure S3) and low level of adenosine deaminase activity (Zurovec *et al.* 2002) when compared with Bg2-c2 cells. However, this divergence fits well with the receptor mRNA expression level, which is about 100 times higher in Bg2-c2 cells than in S2 cells (Fig. 2). Considering also the lack of Ado-induced cAMP response in Cl.8+ cells, which demonstrate about 10 times lesser receptor gene expression level than S2 cells, we conclude that the level of the Ado-induced cAMP response is proportional to that of *DmAdoR* mRNA expression and the naturally expressed receptor in the tested cells demonstrates low or no receptor reserve.

In contrast to our previous results obtained using CHO cells over-expressing *Drosophila* DmAdoRs (Dolezelova *et al.* 2007), we observed no effect of Ado on the concentration of free intracellular calcium in either of the tested *Drosophila* cell types. This discrepancy can also be explained by the high level of receptor expression in CHO cells. The EC_{50} value for Ado-induced cAMP accumulation was about three to four times lesser in previous experiments (see dotted trace in Fig. 3b) than the one found in this study for Bg2-c2 cells. This result suggests the presence of a receptor reserve, which when activated can interact with a non-preferential signaling pathway(s). Our results are in line with reports showing that other heterologously over-expressed G protein-coupled receptors interact with multiple G-protein subunits (Robb *et al.* 1994; Reale *et al.* 1997; Jakubik *et al.* 2006; Michal *et al.* 2007), including mammalian ARs (Fredholm *et al.* 2007), and that activation of non-preferential G-protein signaling depends on receptor

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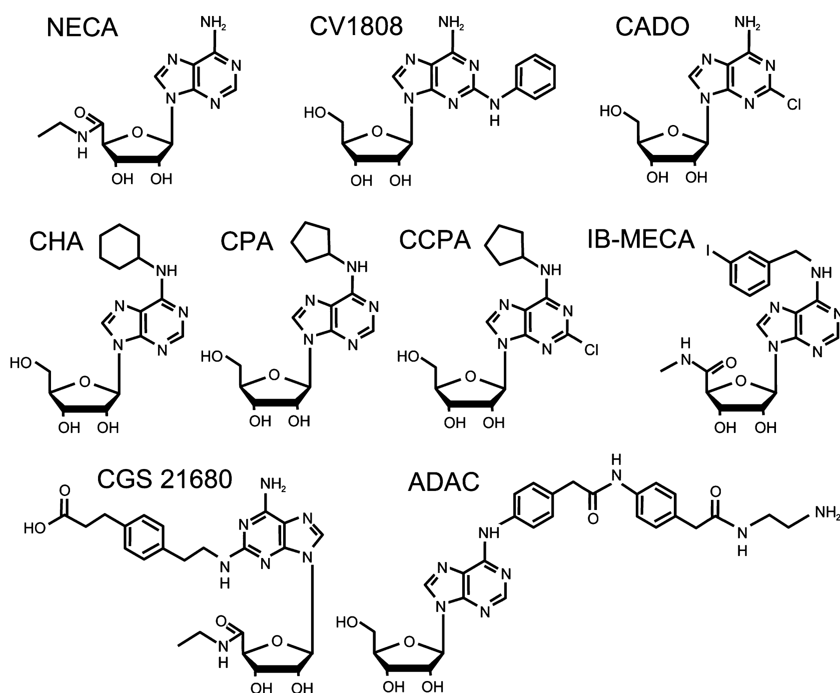

Fig. 8 Adenosine receptor agonists used in this study.

expression level (Michal *et al.* 2001). Our results thus demonstrate that in *Drosophila* cells cAMP is the preferred physiological second messenger in DmAdoR signaling.

Establishing a general pharmacological profile of the receptor is a key step towards the molecular characterization of DmAdoR signaling. From the functional point of view (stimulation of cAMP synthesis) DmAdoR resembles A2 isoforms in mammals. Recent studies on human A2A structure (Kim *et al.* 2003; Jaakola *et al.* 2008; Lebon *et al.* 2011; Xu *et al.* 2011) have described contact amino acid residues needed for ligand binding. These residues are highly conserved among human isoforms but the differences in selectivity of various Ado analogs binding and receptor activation are not yet well understood.

Most agonists of human AR carry substitutions in the C2 or N6 positions of the adenine ring or in the 5′ position of the ribose (Yan *et al.* 2003) (Fig. 8). The agonists NECA and UK-432097 used for the A2AR co-crystallization (Lebon *et al.* 2011; Xu *et al.* 2011) share the 5′ carboxyl substitutions of ribose while UK-432097 contains the two additional substitutions in the adenine ring (see Figure S1). According to these studies, the amino acid residues H6.52 and T3.36 are involved in interactions between the receptor and the 5′ carboxyl substituted Ado analog. We found that unlike human receptor subtypes DmAdoR does not tolerate the 5′ carboxyl substitutions of ribose. The difference between DmAdoR and mammalian ARs may be due to the difference in residues H6.52 and T3.36 which are conserved in mammalian receptors but replaced by Y and C, respectively, in DmAdoR. We examined three 5′-substituted Ado analogs including the non-selective human ARs agonist NECA, A3R selective agonist IB-MECA, and A2AR selective agonist CGS 21680. Regardless of the presence or absence of substitutions in the C2 or N6 positions of the adenine ring they did not activate DmAdoR. Earlier study (Kim *et al.* 1995) reported that the point mutation of H6.52 for Y in A2AR strongly reduced binding affinity of certain agonists. However, this reduction was much less pronounced for the 5′-carboxyl substituted analog NECA and CGS. As DmAdoR carries the same amino acid replacement it seems that the difference between mutated human A2AR and DmAdoR is due to the difference of the amino acid residues at position 3.36 (T is replaced by C in DmAdoR) or another alternative interaction.

The most efficacious agonists at DmAdoR were C2-substituted adenine analogs. The non-selective (with respect to mammalian AR) agonist CADO carrying a relatively small chlorine atom at the C2 position is an even more potent DmAdoR agonist than Ado itself. Our results show that DmAdoR can tolerate even larger C2 substituents including a phenylamino group of CV1808 (see Fig. 8), making its pharmacological profile more similar to human A2R. The N6 substituted analogs of Ado are selective agonists of the human A1R (Jacobson 2009). We found that all tested N6 substituted analogs CHA, CPA, and ADAC are weak agonists of DmAdoR. We searched for the difference in binding site between A2AR and A1R, which is shared with DmAdoR. Such difference is in the 7.35 position which is occupied by M in A2AR but bears a substitution M → T in both A1R and DmAdoR (Fig. 1). The M7.35 residue was previously implicated in the direct interaction of A2AR with the antagonist ZM241385 (Jaakola *et al.* 2008) (Figure S2)

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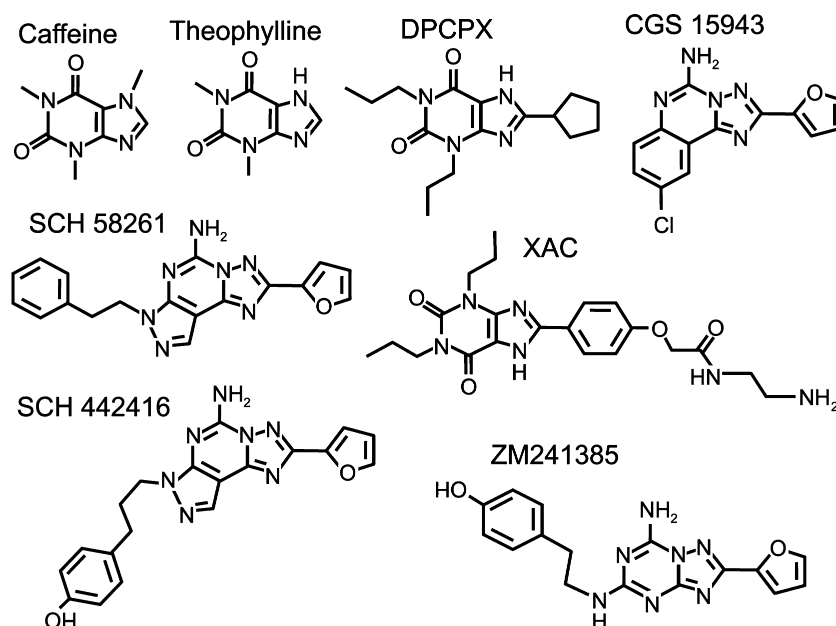

Fig. 9 Adenosine receptor antagonists used in this study.

and we suggest that T7.35 plays a role in the N6-substituted agonist selectivity of A1R and activation of DmAdoR.

The key feature that differentiates agonists from corresponding antagonists is the presence of the ribose ring (Xu *et al.* 2011) (Fig. 9). The most common weak non-selective AR antagonists are xanthines. Interestingly, simple xanthines including caffeine and theophylline, which bind to all human AR subtypes with submicromolar affinity and more potent A1R selective derivative DPCPX did not prevent Ado-induced DmAdoR activation (Fig. 6a). In addition, non-xanthine human AR antagonist CGS 15943 with affinity range 1–30 nM in human AR subtypes also showed no inhibition of DmAdoR. A marginal antagonistic activity was detected only for the non-selective antagonist XAC.

The best DmAdoR antagonists were non-xanthine poly-heterocycles, which also all work as human A2AR-selective antagonists (Muller and Jacobson 2011). They include ZM241385, SCH58261 and SCH442416. The structural analysis of the A2AR-ZM241385 complex reported earlier (Jaakola *et al.* 2008) showed that 11 amino acid residues were crucial for the antagonist-receptor contacts. Interestingly, five of the 11 human A2AR amino acid residues that make contact with the ligand are conserved between A2AR and DmAdoR (Figure S2). The substitution of V5.30 for a E5.30 in human A3R was predicted to contribute to the low affinity of ZM241385 for A3AR (Piirainen *et al.* 2011). However, the same difference in DmAdoR does not eliminate the antagonistic activity of ZM241385 in DmAdoR.

Differences in pharmacological effect of some of the tested Ado analogs between DmAdoR and human A2AR can be partially explained by replacement of specific key amino acid residues disclosed by alignment of these two receptors. Based on the pharmacological profile, sequence similarity and cAMP-linked signaling, the most closely related human subtype appears to be A2AR, while the high EC_{50} values are more reminiscent of A2B receptor.

The availability of new drugs will accelerate research of Ado signaling in *Drosophila* and other insects. Synthetic AR agonists typically persist in the body much longer than Ado. The employment of the most efficacious agonist, CADO, in flies *in vivo* mimicked the phenotype of DmAdoR over-expression, whereas the most potent antagonist, SCH58261, rescued the flies from the lethality caused by DmAdoR over-expression (Fig. 7a). CADO is more stable in the organism than Ado but does not affect $DmAdoR_1$ mutant flies (Fig. 7b). This observation proves that CADO effect is mediated by native DmAdoR. Our *in vivo* experiments show that both the CADO and SCH58261, by stimulating or inhibiting the DmAdoR-mediated cAMP response, mimic the phenotypes of the DmAdoR receptor over-expression or knockout. As Ado also influences physiology of all cells via Ado transport, the pharmacological approach will allow separating the effects of signaling through AR from the effects on Ado transport in physiological experiments.

The involvement of Ado in many distinct physiological functions such as neuromodulation, defense responses, metabolic stress responses, and even altered regulation of lipolysis and lipogenesis in *Drosophila* has been demonstrated (Dolezal *et al.* 2005; Knight *et al.* 2010; Zuberova *et al.* 2010). These physiological functions of Ado confirm an ancient origin of Ado signaling. In addition, they show a great deal of similarity with responses in mammals where Ado effects are mediated by four AdoR subtypes (for a review, see Wei *et al.* 2011). It points to the importance of

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studies on molecular mechanisms and physiological outcomes of DmAdoR activation. The confirmation of the DmAdoR coupling with cAMP signaling and the establishment of DmAdoR pharmacological profile are important steps towards understanding the complexity of Ado responses in this genetically tractable organism and constitute a necessary background for understanding evolution of Ado signaling.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Schematic representation of interactions between A2AR and agonists – Adenosine (black lines), NECA (black and blue lines) and UK-432097 (black, blue and grey lines) according to Xu *et al.* (2011) and Lebon *et al.* (2011).

Figure S2. Schematic representation of the interactions (orange dashed lines) between A2AR and antagonist ZM241385 (according to Jaakola *et al.* 2008 and Piirainen *et al.* 2011).

Figure S3. Western blotting of cell lysates (25 μg) probed with anti-Gαs/olf antibody (C-18, cat. no. sc-383; Santa Cruz Biotechnology) including S2 cells (lines 1–4), Cl.8+ cells (line 5) and Bg2-c2 cells (lines 6–8).

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Supporting informations

Figure S1

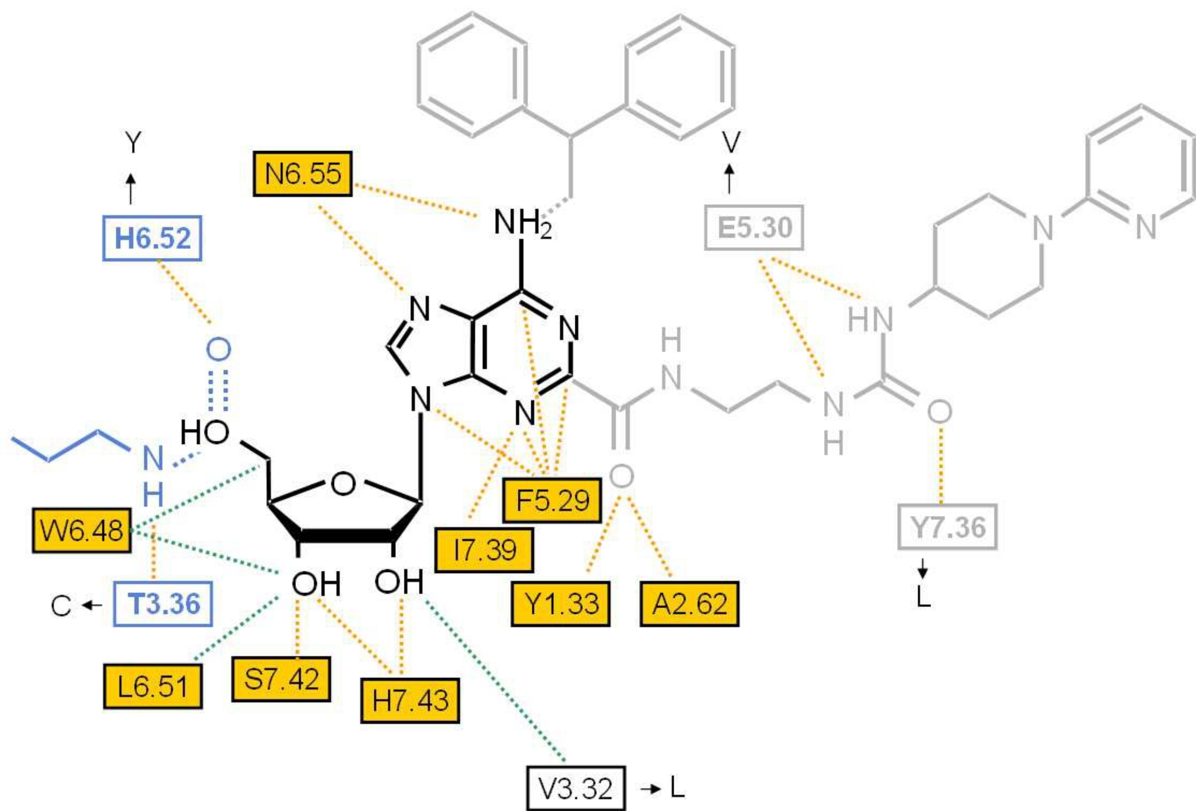


Figure S1. Schematic representation of interactions between A2AR and agonists - Adenosine (black lines), NECA (black and blue lines) and UK-432097 (black, blue and grey lines) according to Xu et al. (2011) and Lebon et al. (2011). Amino acid residues involved in contact with agonists are shown in boxes. The interactions described first by Xu et al. (2011) are shown as orange dashed lines, additional interactions of W6.48, L6.51 and V3.32 described by Lebon et al. (2011) are shown as green dashed lines. Residues conserved between A2AR and DmAdoR are highlighted by yellow boxes. Amino acid replacements in nonconserved positions are indicated by arrows and uppercase letters.

Figure S2

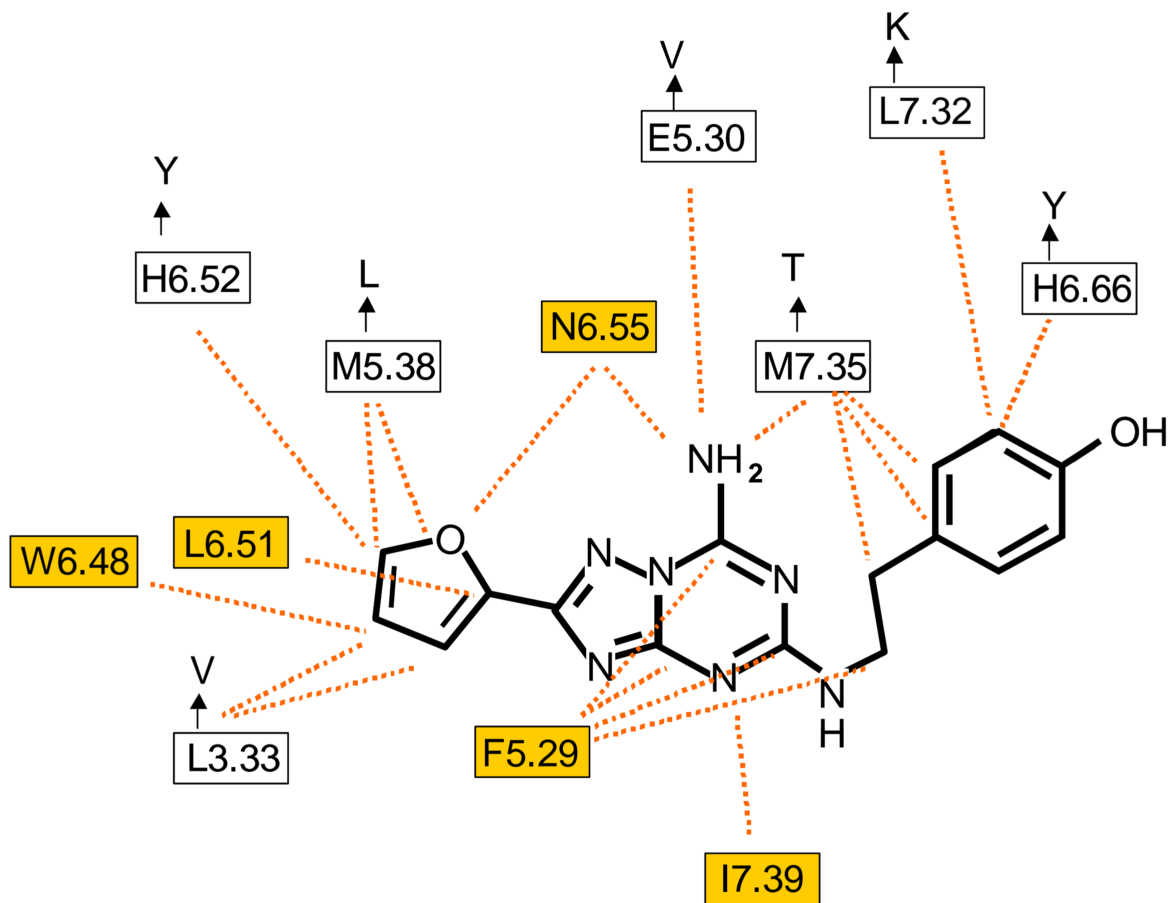


Figure S2: Schematic representation of the interactions (orange dashed lines) between A2AR and antagonist ZM241385 (according to Yaakola et al. 2008 and Piirainen et al. 2011). Amino acid residues involved in contact with ZM241385 are shown in boxes. Amino acid residues conserved between A2AR and DmAdoR are highlighted by yellow boxes. Amino acid replacements in nonconserved positions are indicated by arrows and uppercase letters.

Figure S3

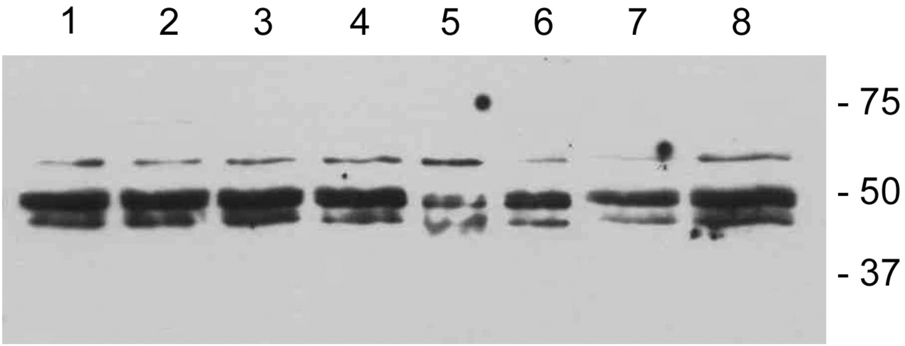
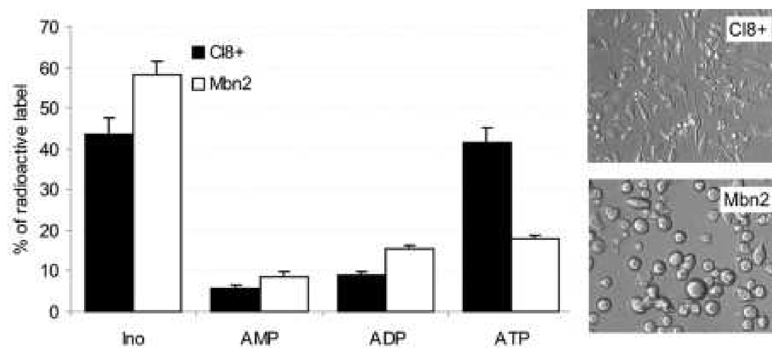


Figure S3: Western blotting of cell lysates (25 µg) probed with anti-Gαs/olf antibody (C-18, cat. No. sc-383, Santa Cruz Biotechnology) including S2 cells (lines 1-4), Cl.8+ cells (line 5) and Bg2-c2 cells (line 6-8). Position of molecular weight markers is shown on the right side of the figure.

Part II

DIFFERENTIAL RESPONSE OF *DROSOPHILA* CELL LINES TO EXTRACELLULAR ADENOSINE



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Differential response of *Drosophila* cell lines to extracellular adenosine

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ABSTRACT

Adenosine (Ado) is a crucial metabolite that affects a wide range of physiological processes. Key proteins regulating Ado signaling, transport and metabolism are conserved among vertebrates and invertebrates. It is well known that Ado influences proliferation of several vertebrate and invertebrate cells. Here we show that Ado negatively influences viability, changes morphology and mitochondrial polarity of the *Drosophila* imaginal disc cell line (Cl.8+) via a mechanism exclusively dependent on cellular Ado uptake. High transport of Ado is followed by phosphorylation and ATP production as a part of Ado salvation, which at higher concentrations may interfere with cellular homeostasis. In contrast, hematopoietic cell line Mbn2, which grows well in high Ado concentration, preferentially uses adenosine deaminase as a part of the purine catabolic pathway. Our results show that different types of *Drosophila* cell lines use different pathways for Ado conversion and suggest that such differences may be an important part of complex mechanisms maintaining energy homeostasis in the body.

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1. Introduction

Adenosine (Ado) affects a remarkable variety of key physiological processes in mammals, such as the rate of blood flow in the heart, skeletal muscle and brain, rate of lipolysis in adipose tissue and neurotransmission in the brain (Fredholm et al., 2011). Ado is capable of protecting tissues from ischemia-reperfusion injury, thrombosis and atherosclerosis and plays an important role in promoting wound healing and tissue repair (Burnstock, 2011; Feoktistov et al., 2009). A number of reports also indicated its general immunosuppressive and anti-inflammatory function (Ohta and Sitkovsky, 2009). Extracellular adenosine (Ado) also influences energy homeostasis and cell growth (Aymerich et al., 2006; Porkka-Heiskanen and Kalinchuk, 2011); however, the mechanism of Ado action on cell physiology has not been studied in detail despite its importance for understanding the cell type specific mechanisms underlying maintenance of metabolic homeostasis.

Physiological concentrations of Ado are relatively low (0.06–0.3 μM in *Drosophila* and 0.05–0.4 μM in human), however its concentration may locally increase to micromolar range under stressful conditions (Dolezelova et al., 2005; Matherne et al., 1990; Van Belle et al., 1987). Elevated concentration of extracellular Ado is connected to many serious diseases including congenital immunodeficiency, diabetic complications, lung inflammation and asthma (Ponnoth and Jamal Mustafa, 2011; Sakowicz-Burkiewicz et al., 2006; Sauer and Aiuti, 2009). *In vitro*, excessive extracellular Ado has been reported to negatively influence cell growth and cause cell death in several vertebrate as well as invertebrate cell lines (Ohana et al., 2001; Zurovec et al., 2002).

Ado physiological and pathophysiological roles are mediated mainly by the activation of specific G protein-coupled receptors. Four Ado receptors from the G protein-coupled receptor superfamily (A1, A2A, A2B, A3) are present in mammals recruiting different G protein subunits (G_α, $G_{i/o}$, G_q) (Hasko and Cronstein, 2004). A1 and A2A high-affinity receptor isoforms seem to be physiologically relevant, whereas low-affinity receptor isoforms

Abbreviations: Ado, adenosine; CHA, cyclohexyladenosine; Dipy, dipyridamole; Itu, 5-iodotubercidine; CM, complete medium; MM, minimal medium; H3-Ado, tritium labeled adenosine; C14-Uro, radioactive labeled uridine; Ino, inosine; Uro, uridine; Guo, guanosine; ADGF, adenosine deaminase growth factor; Adk, adenosine kinase; MSI, Male-specific insect derived growth factor.

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A2B and A3 could play a crucial role under pathological (e.g. inflammatory) conditions (Nyce, 1999).

As mentioned above, under stressful conditions, such as hypoxia or hypercapnia, extracellular Ado levels dramatically raise up to μM concentrations (Linden, 2001) by the action of 5′-nucleotidases (EC 3.1.3.5) or ecto-phosphodiesterase (EC 3.1.4.1) that convert adenine nucleotides (ATP, cAMP) released from cells to Ado (Fredholm et al., 2011; Jackson et al., 2007). Alternatively, Ado is formed intracellularly by sequential dephosphorylation of Ado nucleotides. The steady-state extracellular concentration of Ado is maintained by the rate of its production in the extracellular and/or intracellular space, and its reutilization for ATP synthesis (salvage pathway) due to rephosphorylation or catabolism by Ado deaminases (EC 3.5.4.4) to inosine and hypoxanthine.

Ado salvage pathway has a major impact for energy conservation because of the high energy requirements of Ado *de novo* synthesis. Key players of extracellular Ado recycling are nucleoside membrane transporters and Ado kinase (EC 2.7.1.20) rephosphorylating Ado to AMP (Park and Gupta, 2008). In most tissues and animals investigated, the Km values of Ado kinase for Ado are between one and two orders of magnitude lower than those for the deaminase allowing effective Ado recycling under conditions of low extracellular Ado concentration (Arch and Newsholme, 1978).

Nucleoside membrane transport is mediated by two types of specialized transporter systems. Equilibrative nucleoside transporters facilitate the diffusion of nucleosides down their concentration gradient. High affinity concentrative nucleoside transporters can transport nucleosides into cytoplasm even against concentration gradient by co-transport of Na^+ (King et al., 2006). All equilibrative (Ent1-4 in mammals) and concentrative nucleoside transporters (Cnt1-3 in mammals) are able to effectively transport Ado and thus contribute to Ado signaling regulation. Moreover, it has recently been shown that nucleoside transporters might play important role in cellular metabolism independent of Ado receptor signaling (Huber-Ruano et al., 2010).

Ado functions seemed to have been highly conserved during evolution; genes encoding Ado receptor (*DmAdoR*), ecto-5-nucleotidases, Ado deaminases (*ADGF A-E, DmADA, MSI*), three equilibrative (*DmEnt1-3* - CG11907, CG11045, CG11010) and two concentrative nucleoside transporters (*DmCnt1, 2* - CG11778, CG8083) and three putative Ado kinases (*DmAdk1-3* - CG3809, CG11255, CG1851) have been found in the fruit fly genome (Dolezal et al., 2003; Dolezelova et al., 2007; Fenckova et al., 2011; Sankar et al., 2002). Characterization of mutants in genes encoding *DmAdoR*, *DmEnt2* and *Drosophila* Ado deaminases revealed surprising conservation of Ado roles in mammals and the fruit fly, including the regulation of neuromuscular junction, memory, energy homeostasis and cell growth (Dolezal et al., 2005; Knight et al., 2010; Zuberova et al., 2010). Phenotypes of both *ADGF-A* and *DmEnt2* mutants indicate involvement of Ado in regulation of energy homeostasis via DmAdoR-dependent as well as independent mechanisms (mutant phenotypes can be only partially rescued by *DmAdoR* mutation) (Dolezal et al., 2005; Knight et al., 2010). The presence of alternative Ado metabolic mechanisms and tissue specificity of Ado action make the understanding of Ado roles *in vivo* difficult even in *Drosophila melanogaster*, a model organism with lower complexity of Ado regulation than mammals. In this study, we show that different types of *Drosophila* cell lines may significantly differ by their ability to reutilize Ado which has a dramatic effect on their energy homeostasis. We show that Cl.8+ cells are very effective in Ado recycling, however when the extracellular Ado concentration exceeds the physiological range it influences their viability, morphology, and mitochondrial polarity.

2. Material and methods

2.1. Reagents

Cell culture chemicals including cell culture media, insulin and antibiotics, as well as nucleosides, nucleotides, cyclohexyladenosine (CHA), dipyridamole (Dipy), and iodotubercidine (Itu) were purchased from Sigma Aldrich CO (St. Louis, USA). Fetal bovine serum (FBS, cat. number 16140071) was purchased from Invitrogen (San Diego, CA, USA). [2,8-^{3}H-]-Ado (12.8 Ci/mmol) (H3-Ado) and [2-^{14}C]-Uridine (50 mCi/mmol) (C14-Uro) were purchased from Moravek Biochemicals (Brea, USA).

2.2. Cell culture

Drosophila imaginal disc cells (Cl.8+) and hematopoietic Mbn2 cells (Samakovlis et al., 1992) were used for the experiments. Cl.8+ cells were maintained in complete medium (CM), which was Shields and Sang medium (Shields and Sang, 1970), (Sigma Aldrich CO, catalog no. S 8398) supplemented with 2% FBS, 2.5% fly extract (Cullen and Milner, 1991), 125 U/l insulin, 1% penicillin/streptomycin (10000 U/10 mg). Cells were passaged every 2 days (without trypsinization) by transferring one quarter of the suspended cells to fresh medium (at approx. cell density 4×10^5/ml). Confluent cells at 90–110 passages after cell line initiation were used for all experiments.

Mbn2 cells were maintained in Shields and Sang medium supplemented with 10% FBS, 125 U/l insulin, 1% penicillin/streptomycin (10000 U/10 mg). Cells were passaged every 3 days.

Minimal medium (MM) used for experiments was Shields and Sang medium (prepared from Sigma cell culture tested grade chemicals) supplemented with 1% penicillin/streptomycin (1000 U/mg) and 125 U/l insulin, without FBS, fly extracts and yeasts extracts. The supplements were omitted in order to keep the cells in chemically defined conditions (yeast extracts are rich source of Ado and dAdo, fly extracts and serum are sources of adenosine deaminases).

2.3. Proliferation rate analysis

Cells were plated at 4×10^5/ml in MM/CM and treated as appropriate with nucleosides or other drugs. Cell proliferation was measured by directly counting the cells on digital photographs of identical areas taken every 24 h during a period of 3 days. Three replica plates, one field per plate, were evaluated.

2.4. TUNEL assay

The cells were exposed for 16 h to various Ado concentrations. After the treatment, Fluorescein-dUTP TUNEL assays were performed according to the manufacturer's directions (In Situ Cell Death detection kit, Fluorescein, Roche, Basel, Switzerland). The cells were fixed in 4% paraformaldehyde for 1 h at room temperature, permeabilized in 0.1% Triton X-100, and then incubated with TUNEL reaction mix 1 h at 37 °C in the dark.

2.5. TMRE mitochondrial staining

After the cells were treated with 30–100 μM Ado, Cl.8+ cells were incubated with 600 nM tetramethyl rhodamine ethyl ester (TMRE, Molecular Probes, Eugene, USA) for 30 min at 37 °C in culture medium. The dye was taken up by mitochondria and the mitochondrial staining was analyzed by flow cytometry. At least 10000 events were used for analysis.

2.6. Real-time RT-PCR

Total RNA from Cl.8+ and Mbn2 *Drosophila* cells was isolated using RNA Blue (Top-Bio, Prague, Czech Republic) and subsequently cleaned with NucleoSpin RNA II kit (Macherey–Nagel, Duren, Germany) including on-column digestion step with rDNase I. 1,000 ng of total RNA was applied for reverse transcription using PrimeScript Reverse Transcriptase (Takara) and oligo(dT) (17-mer). The obtained cDNA was diluted and used for the Syber Green PCR reaction in triplets. Each 20 μl PCR reaction contained: Hot start ExTaq polymerase (Takara) 0.75 unit, ExTaq buffer 1x, dNTPs 200 μM each, Syber green 1:25,000, primers 400 nM each. The amplification was carried out on a Rotor-Gene 3000 (Corbet Research, Sydney, Australia) for 40 or 50 cycles (94 °C for 20 s; 60 °C [62 °C for *DmAdoR*] for 30 s; 72 °C for 30 s) following an initial denaturation/Taq activation step (95 °C for 2 min). Primers (Table S1) were designed with Lasergene Primer Select Software (DNASTAR, Madison, USA) to assure that all amplicons are situated in a comparative distance from the end of transcription (1 kb maximum) so the effect of reverse transcription efficiency can be excluded from our results. The amplicons encompassed exon/intron boundary with the exception of *DmEnt3*, whose introns are too distant from the transcription end/polyA tail. Amplified product specificity was confirmed by melting analysis. Data were analyzed and quantified with the Rotor-Gene 6 analysis software. Relative values were either standardized to *DmRack1* gene or standardized to *DmRack1* and normalized to the *DmAdoR* expression level in Cl.8+ cells (Pfaffl, 2006). All results are presented with means and SEM from 2 independent biological samples.

2.7. Ado and Uro uptake assay

Cells were seeded at 5 × 10^5/ml and cultured overnight. Fresh MM containing 2 μCi H3-Ado with unlabeled Ado at appropriate concentration was added to the cells. After incubation, cells were extensively washed in fresh MM and lysed in 1 M NaOH. Samples were neutralized using 5 M HCl, mixed with Triton/toluene/POPOP scintillation cocktail and the radioactivity was measured with a Packard 1500 TriCarb β-Counter.

To determine equilibrative Ado transport sodium-free MM was used with glutamic acid K^+ salt and K_2HPO_4 instead of their corresponding Na^+ salts. For competition experiments unlabeled nucleosides (Ino, Uro, Guo) at 10, 50, and 200 μM concentration were added to 10 μM labeled Ado. For inhibition experiments, 20 min pre-treatment with Dipy (1–20 μM) was applied before addition of 10 μM Ado.

For Uro transport measurements, different concentrations of Uro (labeled with 2.5 μCi of C14-Uro) were assayed for 1 h as described above for Ado transport. To see the effect of Ado on Uro transport, various Uro concentrations were assayed together with 50 μM Ado.

2.8. RNA interference

RNA interference (RNAi) approach was applied to silence expression of selected Ado signaling and metabolism genes. PCR primers were designed and about 400 nt fragments were amplified from a single exon using genomic DNA template or from a cDNA of genes involved in Ado metabolism and signaling (for primers see Table S2). The fragments were subcloned into pGemTeasy plasmids (Promega, Madison, USA). The clones were used as templates for synthesis of plus and minus RNA strands *in vitro* by using MEGAscript® T7 Kit (Ambion, Leicestershire, UK). After annealing and checking the quality of dsRNA, these molecules were transfected into the Cl.8+ cells by using transfection protocol of Lum et al. (2003). The cells were incubated in CM overnight and then treated with 40 μM Ado for 16 h. The percentage of cells with characteristic elongated cell shape and pseudopodia was calculated from the digital photographs of identical areas (0.8 × 0.8 mm). *DmCht5* gene that is not connected with Ado signaling or metabolism and mock transfected cells without Ado treatment were used as positive and negative controls, respectively.

2.9. Ado incorporation assay

5 × 10^5 cells were incubated overnight in MM, supplemented with 100 μM Ado labeled with 10 μCi H3-Ado. After 2 h, cells were washed two times in PBS, lysed in liquid nitrogen and nucleosides and nucleotides were extracted with ice-cold acetonitrile 4:1, (v/v) and separated by HPLC coupled with a UV (254 nm) and electrospray mass spectrometric detection as described earlier (Zuberova et al., 2010). Elution time of each compound was estimated by means of a standard solution mixture containing Ado, Ino, AMP, ADP, and ATP. From each cell lysate, particular fractions of the Ino (3.17 min), AMP (8.81 min), ADP (11.36 min), and ATP (12.96 min) peaks were collected manually after their elution from HPLC and their radioactivity was measured as described in the Ado uptake experiments (see 2.7).

2.10. ATP assay

ATP was measured by The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA) according to manufacturer's directions. Cells (100 μl aliquots at a concentration of 5 × 10^5/ml) were seeded on 96-well plates and incubated overnight in the presence of different concentrations of Ado in CM. After cultivation, equal volume of CellTiter-Glo reagent was added and luminescence in cell lysates was detected by Luminometer Orion II (Berthold Technologies, Bad Wildbad, Germany).

2.11. Statistical analysis

All data are presented as means ± SDs of 3 independent experiments (excerpt for RT-PCR analysis, which was performed with two independent biological samples and the results are presented as means ± SEM). *T*-test or one-way analysis of variance (ANOVA) followed by a post hoc test were used for statistical evaluation of the data.

3. Results

3.1. Effect of Ado on Cl.8+ cell proliferation and survival

We have reported earlier that high concentration of Ado in growth media may be toxic for some *Drosophila* cells, including imaginal disc cell line - Cl.8+ (Zurovec et al., 2002). In this study, we further characterized mechanism of Ado toxicity in Cl.8+ cells. First we re-evaluated the effect of Ado on cell proliferation and apoptosis under different growth conditions. Ado dose proportionally decreased growth rate of the Cl.8+ cells (Fig. 1a,b), changed their typical elongated fibroblast-like cell shape to round cells (Fig. 2a, upper row) and at higher concentrations induced cell death. In MM, 10 μM Ado substantially decreased cell growth rate (Fig. 1a) while concentrations higher than 100 μM caused cell death. No significant increase in the number of apoptotic cells was detected after treatment with 30 μM Ado (Fig. 2a, lower row), whereas the cells treated overnight with 100 μM Ado showed reduced mitochondrial polarity (visualized by marked decrease of mitochondrial TMRE staining; Fig. 2b), typical apoptotic DNA fragmentation and the

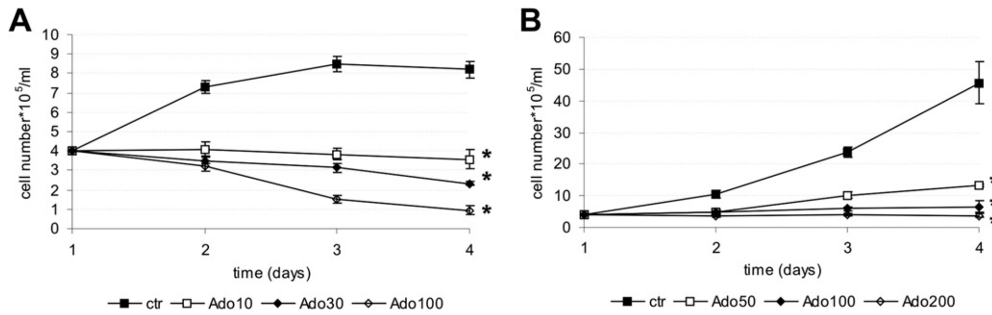

Fig. 1. Ado concentration-dependent decrease in proliferation rate of Cl.8+ cells grown in MM (A) and CM (B). Cells were incubated without Ado or with various Ado concentrations for 3 days. Cell numbers in defined areas were counted every 24 h. Values represent means ± SD from three independent experiments. *$P < 0.05$ for whole data series compared with Ado untreated cells by Tukey's test.

Fig. 2. Ado effect on cell morphology and apoptosis in Cl.8+ cells. Cells were treated with 30 or 100 μM Ado in MM overnight. (A) Ado changed the typical fibroblast like morphology of Cl.8+ cells to round shape, decreased proliferation rate (upper row) and increased numbers of TUNEL positive cells (lower row) as documented on the micrographs. (B) Ado effect on mitochondrial membrane polarity visualized by decreased mitochondrial TMRE staining on micrographs (upper row) and flow cytometry plots (lower row). The channel number is shown on the x axis and the number of events on the y axis. ctr – control cells without Ado treatment, Ado30 – cells treated with 30 μM Ado, Ado100 – cells cultured with 100 μM Ado, bar 100 μm.

partitioning of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) within 24–48 h. In CM (containing FBS, yeast extract, fly extract, and insulin), higher Ado doses were needed to inhibit cell proliferation (Fig. 1b) and the onset of cell death was substantially delayed. Only a minor decrease in TMRE staining was observed even after overnight cultivation with 200 μM probably because of presence of Ado deaminases in the media supplements.

3.2. Activation of DmAdoR in Cl.8+ cells is not responsible for Ado growth inhibitory effect

Growth inhibitory effect of Ado may result from the activation of specific receptors or its uptake into the cytoplasm via nucleoside transporters respectively (Ohkubo et al., 2007). Specific pharmacological agents were exploited to distinguish between the receptor- and uptake- dependent Ado effect. A potent agonist of DmAdoR - cyclohexyladenosine (CHA) - that has been proven previously to activate cAMP response in *Drosophila* cells (data not shown) - was applied to the cells to see if it can mimic the Ado effect. The cells treated with 100 μM CHA showed no difference in their growth rate compared to untreated control cells (Fig. 3a).

To further confirm that the effect of Ado is not mediated by DmAdoR, we reduced its expression using RNA interference and evaluated the effect of *DmAdoR* knock-down on the sensitivity of Cl.8+ cells to Ado. Silencing of *DmCht5* and mock transfected cells without Ado treatment were used as positive and negative control, respectively. Proportion of cells lacking typical Ado-dependent morphological change ("healthy cells") after 16 h cultivation with 40 μM Ado was determined. In positive control cells with silenced *DmCht5* expression, Ado treatment decreased percentage of "healthy" cells to 16% of the non-treated mock transfected control cells. In agreement with CHA treatment, *DmAdoR* gene knock-down did not increase the percentage of "healthy" cells after Ado treatment (Fig. 3b).

3.3. Characterization of Ado transport in Cl.8+ cells

The negative results of DmAdoR function elimination on Ado toxicity suggested that the effect of Ado might be uptake dependent. Cl.8+ cells showed relatively high expression of *DmEnt2* and *DmEnt1* (Fig. 4a) and were able to transport significant amounts of Ado from the extracellular space to the cytoplasm. The uptake of H3-Ado (5–50 μM) increased in time- (0–60 min interval) and concentration-dependent manner (Fig. 4b,c).

We investigated whether both concentrative and equilibrative transporters contribute to the total Ado uptake. The uptake of 10 μM Ado was inhibited in direct proportion to the concentration of Dipy, a known inhibitor of equilibrative Ado transport in mammals. Maximum 70% inhibition was observed after treatment with 10 μM Dipy (Fig. 4d). Involvement of concentrative Ado transport was tested in medium where Na^+ containing chemical components, mainly Na^+ glutamate and Na^+ phosphate, were replaced by their K^+ counterparts (see Material and Methods) to inhibit sodium-dependent concentrative nucleoside transporters. The level of Ado uptake in sodium-free medium (containing 90 mM K^+) ranged from 76 to 46% depending on the applied Ado concentration (Fig. 4e).

Since mammalian nucleoside transporters are known to transport broad spectrum of purine as well as pyrimidine nucleosides or nucleobases, we tested if common metabolites Ino, Uro, and Guo compete with Ado transport. Interestingly, even 20 times higher concentrations of these nucleosides did not inhibit transport of 10 μM Ado (Fig. 4f). This indicates that Ado transport into Cl.8+ cells is mediated via equilibrative and concentrative transporters not affected by other nucleosides.

3.4. Inhibition of Ado uptake rescues the Ado toxicity

To test if blocking of equilibrative Ado transport would be able to rescue the Ado-mediated growth arrest, Dipy was applied to the Ado treated cells in a concentration that maximally inhibited Ado uptake into cells. After 24 h, 10 μM Dipy completely rescued growth inhibitory effect of 100 μM Ado (Fig. 5a).

A presumed involvement of concentrative Ado transporters in Ado toxicity was tested in a medium with reduced Na^+ concentration. With respect to proliferation, Cl.8+ cells tolerated a decrease in Na^+ concentration up to approximately 50% (approx. 60 mM K^+, low sodium medium). When we applied Ado to the cells in the low sodium medium for 24 h, we observed partial rescue of the cell morphology (50 μM Ado) without any beneficial effect on the cell growth rate (Fig. 5b,c).

RNA interference approach (as described in Section 3.2) was applied to further investigate the role of particular nucleoside transporters. RNA silencing of *DmEnt2*, and both *DmCnts* caused a significant increase in the number of healthy cells after an overnight 40 μM Ado treatment compared to cells with silenced *DmCht5* gene. The silencing of Na^+ cotransporter *DmCnt1* (CG11778) showed almost complete rescue (98% cells with healthy morphology compared to negative control) followed by transporters *DmCnt2* CG8083 (75%) and *DmEnt2* (60%) (Fig. 5d).

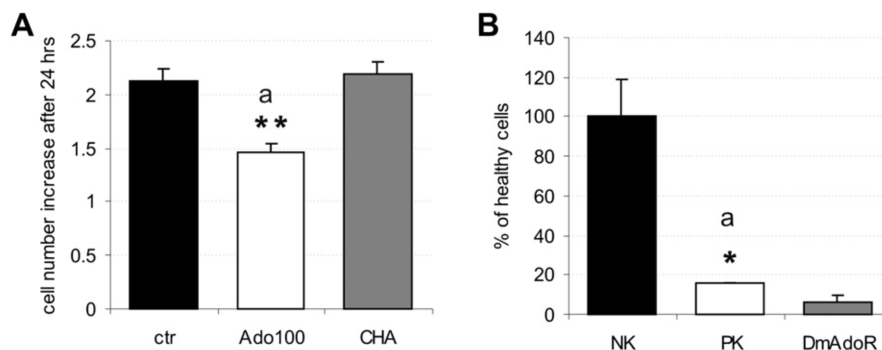

Fig. 3. Effect of pharmacological and genetic ablation of DmAdoR on Ado toxicity in Cl.8+ cells. (A) Increase in cell number after 24 h after treatment with 100 μM Ado and a previously characterized potent DmAdoR agonist CHA (100 μM). (B) Rescue of cell morphology by RNAi. Cells were transfected with dsRNA fragments designed to silence *DmAdoR* expression. Data are expressed as a percentage of cells with Ado unaffected morphology ("healthy cells") compared to mock transfected control. NK - mock transfected control without Ado treatment, PK - DmCht5 transfected cells treated with Ado. $^*P < 0.05$, $^{**}P < 0.01$, a – compared to NK.

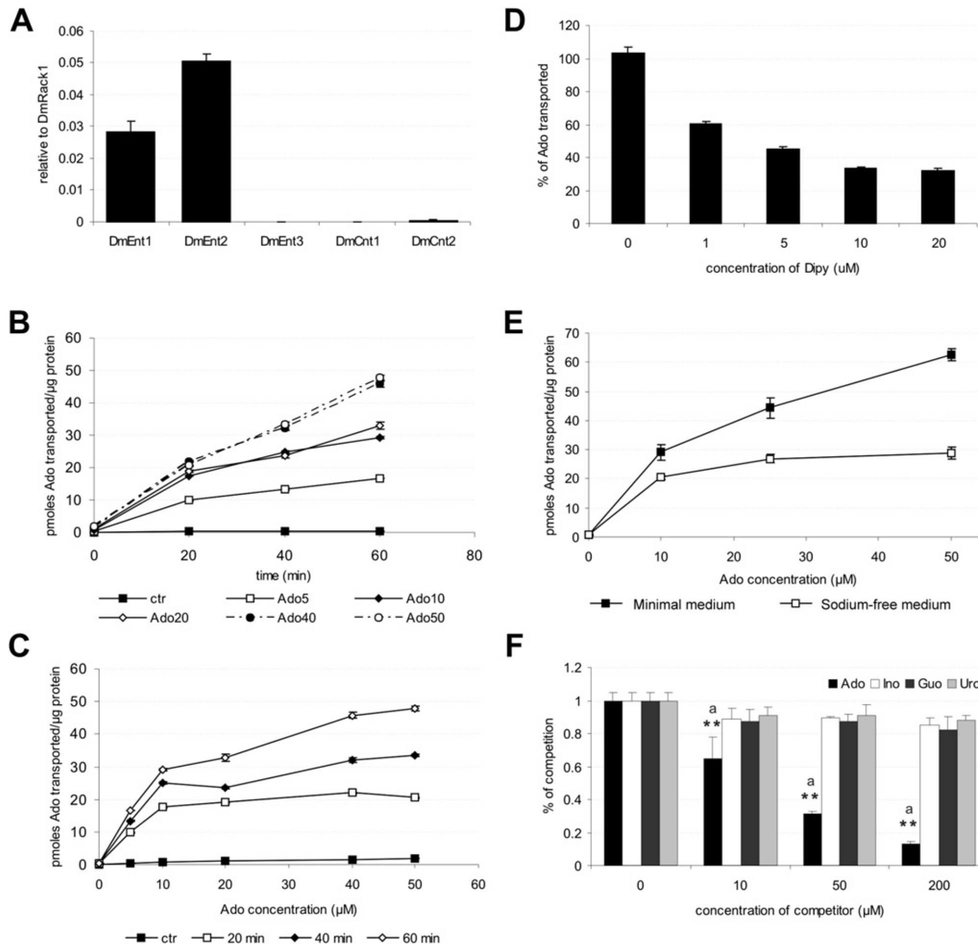

Fig. 4. Characterization of Ado uptake in Cl.8+ cells. (A) Expression of nucleoside transporters in Cl.8+ cells quantified by real time RT-PCR, relative expression values were standardized to *DmRack1* gene expression. (B) Time and (C) concentration dependency of Ado uptake into Cl.8+ cells. Cells were incubated with different concentrations of Ado (5–50 μM) for 20, 40 and 60 min. (D) Ado transport was inhibited by Dipy depending on the dose. The cells were treated with different Dipy concentrations and the uptake of 10 μM H3-Ado was measured as described in Materials and Methods. The data represent the relative uptake expressed in percent of the control 10 μM Ado transport with 0.05% DMSO. (E) Involvement of concentrative nucleoside transport in Ado uptake. Corresponding Ado concentrations were added to the cells in minimal and sodium-free medium, respectively and H3-Ado uptake was measured after 1 h. (F) Competition of Ado uptake by other nucleosides. Cells were incubated with 10 μM H3-Ado in the presence of different concentrations of other nucleosides (10–200 μM) with Ado used as control. Data are present as a decrease in Ado uptake compared to the control 10 μM Ado. Unless otherwise specified, all data are present as the amount of pmoles transported to the cytoplasm per μg of protein. Values are presented as means ± SD of three independent experiments. $^{**}P < 0.01$, a – compared to control.

3.5. Ado uptake correlates with the increase of cellular ATP pool

In order to follow the metabolic fate of the H^3-labeled Ado taken up into the Cl.8+ cells, we examined the distribution of the radioactive label in fractions corresponding to Ino, AMP, ADP, and ATP separated by HPLC. Radioactively labeled Ado (10 μCi, 100 μM) was added into the growth medium and the cells were incubated for 2 h and lysates than separated with HPLC. Our data showed that radioactivity predominantly accumulated in the fractions corresponding to Ino and ATP, both representing about the same radioactive label portion (44 and 42%, respectively). The amount of radioactivity in the AMP and ADP fractions was below 10% (Fig. 6a).

Detailed kinetics of Ado-dependent ATP production was measured by bioluminiscent ATP kit that allowed simple and rapid non-radioactive quantification of ATP amount in cells compared to HPLC separation. Cells were cultured with different Ado concentrations (50, 100, 200 μM) for 1–12 h. Both dose and time dependent massive accumulation of ATP was observed in the initial 3–6 h interval resulting in up to 9x increase of ATP level in the case of 200 μM Ado treatment. Elevated level of ATP was maintained for at least 12 h (Fig. 6b). The concentration of ATP was directly proportional to the dose of Ado over a wide range of concentrations suggesting the importance of Ado salvage in this cell type.

3.6. The Ado effect on Cl.8+ cells is rescued by Ado kinase inhibition but not by high Uro concentration

To further confirm the role of intracellular Ado phosphorylation in its toxicity, we applied a known inhibitor of Ado kinase, Itu, to the Ado treated cells. After 24 h, the number of cells cotreated with Itu and Ado was significantly higher than the number of Ado treated cells, and was similar to the control (Fig. 7b). RNAi experiments performed with constructs designed to silence all three *Drosophila* genes coding for proteins with Ado kinase activity (CG3809, CG11255, CG1851) revealed that the most abundant Ado kinase CG11255 was responsible for the Ado toxicity in Cl.8+ cells (Fig. 7a,c).

Excessive phosphorylation of Ado has been reported previously to interfere with *de novo* synthesis of pyrimidines that may result in growth arrest or even cell death (Ohkubo et al., 2007). Ado-induced pyrimidine starvation is reportedly possible to overcome by media supplementation with an alternative source of pyrimidines, such as

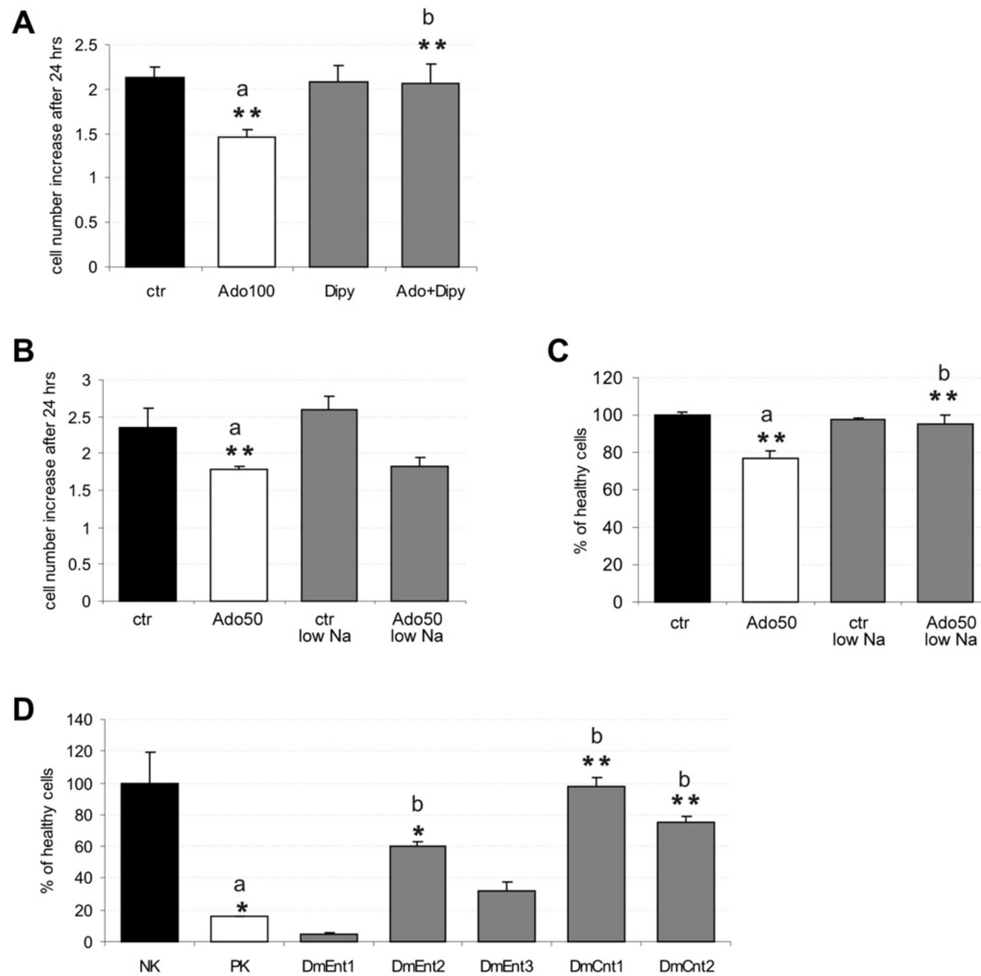

Fig. 5. Effect of pharmacological and genetic ablation of Ado uptake on Ado toxicity in Cl.8+ cells. (A) Effect of equilibrative transport inhibitor Dipy (10 μM) on Ado mediated inhibition of cell growth in Cl.8+ cells, cell number increase after 24 h was quantified. (B) Effect of low sodium medium on cell proliferation and morphology (C) after Ado treatment. Cells were treated with 50 μM Ado for 24 h in CM or low sodium medium respectively. The number and morphology of cells were assessed. (D) Genetic ablation of particular nucleoside transporters using RNAi approach. Data are expressed as a percentage of cells with Ado unaffected morphology ("healthy cells") compared to mock transfected control. NK - mock transfected control without Ado treatment, PK - *DmCht5* transfected cells treated with Ado. Values are presented as means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, a – compared to untreated control, b – compared to control Ado treatment.

Uro. We have shown that Cl.8+ cells can efficiently transport Uro even in the presence of 50 or 100 μM Ado (Fig. S1). However, the co-treatment of Cl.8+ cells with Uro, even in 4x higher doses, did not rescue cell proliferation after Ado treatment (Fig. 7d). It suggests that Ado toxicity is directly connected to the massive ATP production independent of pyrimidine starvation.

3.7. Ado-tolerant Mbn2 cells do not accumulate such high concentration of ATP upon Ado treatment

To see if such a high level of ATP accumulation is specific for Ado-sensitive cell lines, we compared Ado uptake and ATP production in Cl.8+ cells with Mbn2 cells that seem to be tolerant to high concentrations of Ado (Fig. 8a). Interestingly, real-time RT PCR analysis of Mbn2 cells showed similar pattern of nucleoside transporter genes expression as did the Cl.8+ cells (Fig. 9). However, the rate of Ado uptake in Mbn2 cells ranged from 40 to 50% of that observed in Cl.8+ cells for all time points and concentrations tested (5–50 μM ado, 20–60 min) (Fig. 8b). It probably resulted from higher activity of extracellular deamination by ADGF-A, which is more expressed in Mbn2 compared to Cl.8+ cells (Fig. 9).

ATP accumulation in Mbn2 cells was much lower than in Cl.8+ cells and maximum ATP content was reached after a shorter time period. In Mbn2 cells, maximum ATP level was approximately 3–4.5x lower than in Cl.8+ cells depending on the applied Ado concentration (50, 100, 200 μM) reached within the initial 1–4 h of Ado cultivation in contrast to the 3-6 h-long linear increase of ATP level observed in Cl.8+ cells (Fig. 8c,d). Lower incorporation of Ado into ATP was also confirmed also by radioactive labeling (18% radioactivity in the fraction corresponding to ATP in Mbn2 vs. 42% in Cl.8+ cells) (Fig. 8e). Lower radioactivity in the ATP fraction corresponded to higher level of labeled Ino in Mbn2 cell lysates (58 vs. 43% in Cl.8+ cells) corresponding to higher expression of DmADA and MSI genes in Mbn2 cells coding proteins with cytosolic Ado deaminase activity (Fig. 9). Interestingly, in addition to higher radioactivity in the Ino fraction, Mbn2 cells showed slightly, but still significantly higher levels of labeled AMP (8.5 vs. 6%) and ADP (15 vs. 9%) (Fig. 8e).

4. Discussion

Here we report that the adverse Ado effect on cell morphology, growth and viability in *Drosophila* Cl.8+ cells results from an excessive Ado uptake leading to massive ATP accumulation, whereas other cell lines including Mbn2 cells, Bg2-c2 neuroblasts and embryonic S2 cell line seem to be more Ado-tolerant.

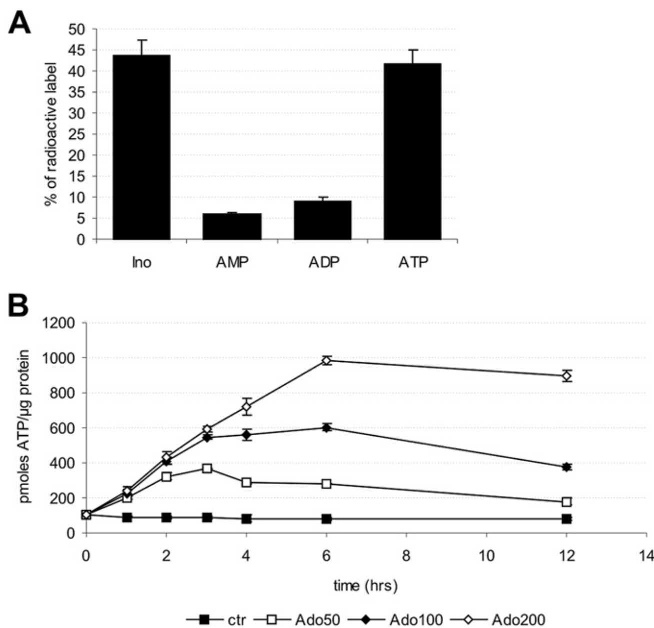

Fig. 6. ATP accumulation after Ado treatment in Cl.8+ cells. (A) Cells were incubated in 100 µM H3-Ado for 2 h and lysed, the lysates were separated by HPLC and the percentage of radioactivity in each fraction corresponding to Ino, AMP, ADP, and ATP was determined using scintillation counting. (B) Time-course study of Ado-induced increase in ATP amount in Cl.8+ cells. The cells were incubated in CM containing indicated Ado concentrations (50 µM, 100 µM, 200 µM). The amount of ATP was monitored in regular intervals for 12 h. Each data point represents mean ± SD of three independent experiments.

Comparison of *Drosophila* Cl.8+ and Mbn2 cell lines showed that Ado-tolerant Mbn2 cells utilize Ado deamination as the preferred metabolic pathway to cope with increased level of extracellular Ado.

There is a large body of literature reporting Ado-induced growth arrest or cell death (Henderson and Scott, 1980; Merighi et al., 2002; Ohkubo et al., 2007; Peyot et al., 2000; Schrier et al., 2001). Mammalian receptor subtypes were shown to have anti-proliferative and, apoptotic, as well as prosurvival function depending on the cell type and applied Ado concentration (Ohana et al., 2001). Activation of AdoR may negatively influence cell survival via activation of cAMP, Ca^{2+}, or MAP kinase cascades (Kizaki et al., 1990; Merighi et al., 2005; Szondy, 1994). On the other hand, anti-proliferative role of Ado is, in many cases, independent of its receptor activation since it can be blocked by nucleoside transport inhibitors such as Dipy (Ohkubo et al., 2007; Wu et al., 2006). Moreover, synergistic effect of both Ado uptake and signaling in apoptosis induction has also been reported in some cell lines (Di Iorio et al., 2002).

Our results demonstrate that Ado signaling through the DmAdoR plays negligible role in Ado toxicity in Cl.8+ cells. Involvement of *DmAdoR* in this phenomenon may be excluded based on both RNAi knock-down and pharmacological experiments. The expression of DmAdoR in Cl.8+ cells was very low and its agonist CHA did not mimic Ado effect on cell proliferation. In agreement with these results, RNAi of DmAdoR did not rescue Ado toxicity in Cl.8+ cells. Recently, we have tested DmAdoR signaling in various *Drosophila* cell lines and showed that Ado is not able to recruit detectable levels of cAMP or Ca^{2+} second messengers in Cl.8+ cells (data not shown).

We have several lines of evidence that Ado anti-proliferative role in Cl.8+ cell line is caused exclusively by intracellular Ado uptake. We have shown that Ado is efficiently taken up into Cl.8+ cells, in a way that is not inhibited by other nucleosides and involves both equilibrative and concentrative transporters, since Ado toxicity can be rescued by Dipy treatment or Na^{+} depletion as well as knocking down the expression of *DmEnt2*, and both *DmCnts*. Previously, functional characteristics of DmENTs were studied by Machado et al. (2007) who expressed particular DmEnts in *Xenopus* oocytes and measured their capability to transport Uro, which is believed to be universal permeant of mammalian Ents. In agreement with our findings, they showed that only *DmEnt2* was capable of transporting Uro and broad scale of other nucelosides and nucleobases - including Ado - from the extracellular space, however they were not able to block Uro transport by classical mammalian equilibrative transport inhibitors (Dipy, Diazep, NBTI). These discrepancies might be caused by separate transporters for Ado and Uro in flies (as suggested by Fig. S1) or by methodological differences between the two studies.

Once taken up into cells, Ado is rapidly metabolized. It was shown in some mammalian cells that accompanying increase in AMP and S-adenosylhomocysteine concentration may contribute to Ado cytotoxicity causing pyrimidine starvation and hypomethylation, respectively (Green and Chan, 1973; Skinner et al., 1986). In accordance we found that the inhibitor of adenosine kinase Itu prevented Ado-induced growth retardation of Cl.8+ cells pointing to the involvement of Ado phosphorylation in its toxic effect. Despite the fact that Adk inhibitor Itu was able to block Adk enzymatic activity in cell lysates (data not shown), we can not exclude the fact that the Itu effect on living cells was caused by blockade of NTs, which was also reported to be an Itu target (Sinclair et al., 2001). However, further support for the involvement of Ado phosphorylation was received from RNAi silencing of DmAdoK2 (CG11255), which was able to rescue cells from Ado toxicity.

In Cl.8+ cells, Ado phosphorylation is connected to the massive accumulation of ATP. The contribution of extracellular Ado to the elevation of adenine nucleotide pool was earlier observed both *in vitro* and *ex vivo* in mammalian muscle cells, in which virtually all transported Ado was rapidly converted to ATP (95% after 30 min) (Barron and Gu, 2000; Hellsten and Frandsen, 1997). We found that elevated level of Ado-induced multiple increment of cellular ATP level (see Fig. 6). Such massive ATP pool enhancement is believed to cause pyrimidine starvation. Excessive Ado phosphorylation to AMP causes negative feedback inhibition of PRPP (5-phosphoribosyl-1-pyrophosphate) synthase, which produces an intermediate PRPP necessary for both pyrimidine and purine *de novo* biosynthesis (Seetulsingh-Goorah, 2006). Resulting depletion of the pyrimidine nucleotides pool interferes with DNA synthesis and ultimately causes growth arrest and cell death (Green and Chan, 1973). Inhibition of pyrimidine *de novo* synthesis can be compensated by the addition of Uro, a substrate of pyrimidine salvage pathway. However, in our conditions Uro was not able to rescue the Ado toxicity in the same way as described for some other cell types (Seetulsingh-Goorah, 2006). This indicates that a mechanism other than pyrimidine starvation is involved in Ado toxicity in Cl.8+ cells.

It has been reported that Ado can specifically increase the nuclear compartment pools of ATP and nuclear ATP/ADP ratio, such increase inhibits DNA synthesis in mouse fibroblasts (Rapaport et al., 1979). Alternatively, activation of AMP kinase has been reported to cause cell death after Ado treatment in intestinal rat epithelial cells, HUVEC and human gastric cancer cells (Aymerich et al., 2006; da Silva et al., 2006; Saitoh et al., 2004). However, we were not able to detect any increase in AMPK phosphorylation after Ado treatment (data not shown) probably because of the increased ATP/AMP ratio that is not favorable for AMP kinase activation (Hardie, 2004).

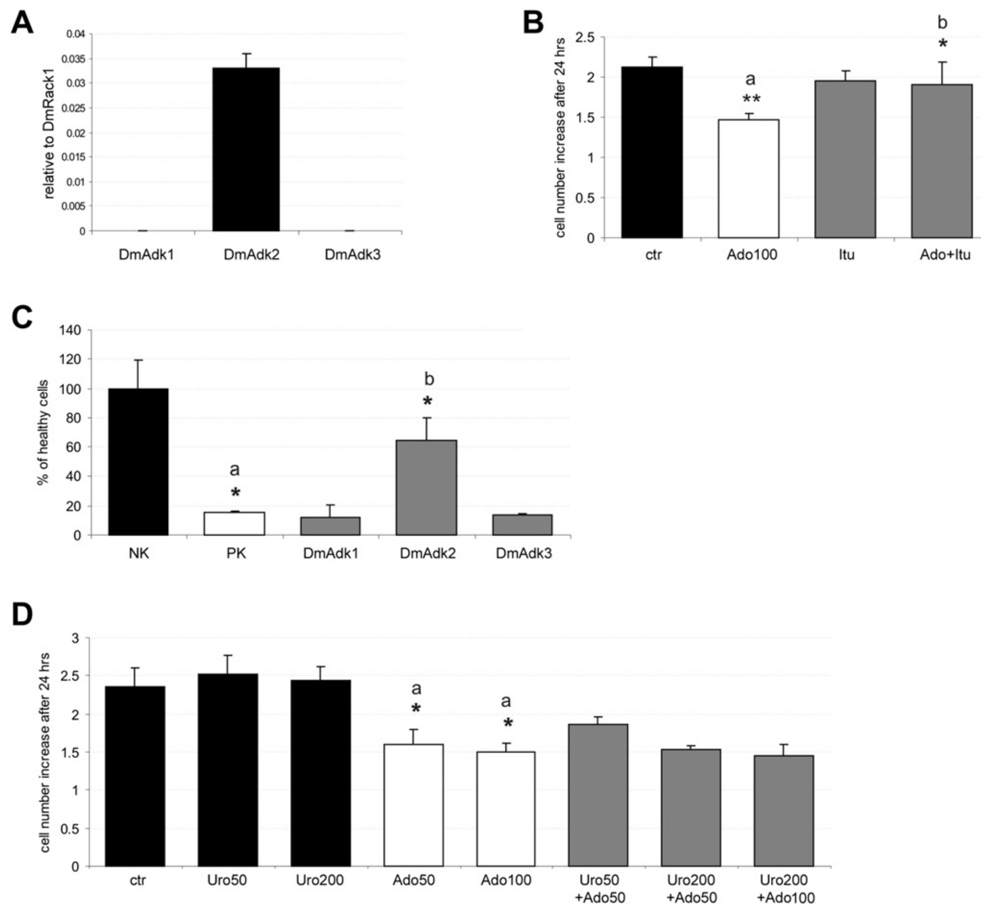

Fig. 7. Involvement of Ado phosphorylation and pyrimidine starvation in Ado toxicity. (A) Expression of three known *Drosophila* Ado kinases in Cl.8+ cells quantified by real time RT-PCR, relative expression values were standardized to *DmRack1* gene expression. (B) Effect of Adk inhibitor Itu (200 nM) on Ado mediated inhibition of cell growth in Cl.8+ cells, cell number increase after 24 h was quantified. (C) Genetic ablation of particular Adks using RNAi approach. Data are expressed as a percentage of cells with Ado unaffected morphology ("healthy cells") compared to mock transfected control. NK - mock transfected control without Ado treatment, PK - DmCht5 transfected cells treated with Ado. (D) Lack of Uro effect on Ado toxicity. Cells were treated with 50 μM and 100 μM Ado in the presence of various Uro concentrations. The number of cells per well after 24 h incubation is shown in the graph. Each data point represents mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA and Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, a – compared to untreated control, b – compared to Ado treatment.

Crucial role of ATP production in Ado toxicity is supported by the observation that Ado resistant *Drosophila* cell lines, including Mbn2 cells, do not show such massive accumulation of ATP. Ado taken up into the cytoplasm is in these cells converted to ATP to a much lower extent than in Cl.8+ cells (see Fig. 8). Ado resistance in insect cells has mainly been connected to extracellular Ado deaminases (Zurovec et al., 2002). This was supported by our results indicating that both extra and intracellular adenosine deaminations are major pathways of Ado metabolisation in Mbn2 cells. However, lower uptake of Ado and not as high levels of AMP and ADP in Mbn2 cells compared to Cl.8+ cells indicate that in addition to the higher Ado deaminase activity, less effective transport of Ado, and its phosphorylation to AMP and ADP contribute to Mbn2 resistance to Ado. In this context it is worth noting that the inhibition of AMP deaminase induces Ado toxicity in mammalian cell lines (Barry and Lind, 2000).

The differences in Ado metabolism, among different cell types at the level of its deamination, uptake and phosphorylation suggest elaborate regulation of Ado homeostasis in the *Drosophila* body that might be important especially during the period of tissue remodeling. Critical importance of Ado homeostasis during metamorphosis is supported by the fact that both null mutants in *DmENT2* as well as *ADGF-A* are lethal during late larval/early pupal stages (Dolezal et al., 2003; Knight et al., 2010). Mutants in several *de novo* synthesis pathway genes including *Prat*, *Ade2, Ade3*, and *Ade5* display pupal lethality (Ji and Clark, 2006). The key enzyme in the *de novo* purine synthesis - PRAT is not expressed in a large excess over the level necessary for normal development (Clark and MacAfee, 2000). *Drosophila* depends on purines derived from food (Burnet and Sang, 1963) supporting the important role of Ado recycling and redistribution (Henikoff et al., 1986).

For both vertebrates and invertebrates it has been previously shown that the Km value of Ado kinase for Ado was one to two orders of magnitude lower than that for Ado deaminase (Arch and Newsholme, 1978). Based on these findings we presume that Ado uptake and its effective phoshorylation even in relatively low Ado concentrations allows effective Ado redistribution and reutilization in newly formed tissues and organs, while high level of extracellular Ado deaminase enzymes in the hemolymph prevent major local Ado level fluctuations and keep the extracellular Ado below dangerous levels. Thus the maximum possible amount of Ado would be recycled in newly formed tissues (e.g. imaginal discs) while the tissues with slower growth/lower metabolic activity would have their Ado-recycling capacity limited due to high expression of both extracellular and intracellular deaminases.

We conclude that unlike the ATP production in Mbn2 cells (and other cell types, unpublished results), the amount of ATP produced in Cl.8+ cells is directly proportional to the level of extracellular Ado and is limited only by the metabolic capacity of the cells. In the

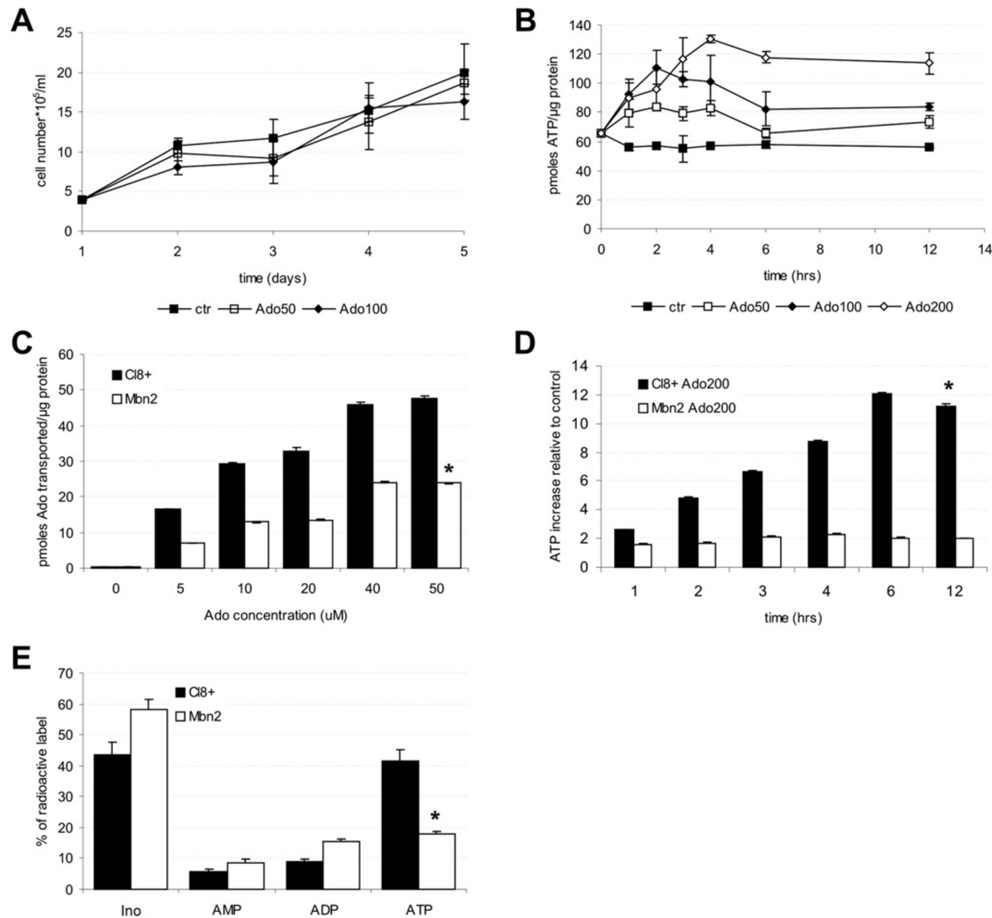

Fig. 8. Comparison of Ado response in Cl.8+ and Mbn2 cells. (A) Effect of Ado on Mbn2 cells proliferation. Cells were incubated in CM without Ado or with indicated Ado concentrations for 3 days. Cell numbers were counted every 24 h using images of the same area of Petri dish. (B) Comparison of Ado uptake in Mbn2 and Cl.8+ cells. Mbn2 cells were incubated with different concentrations of H3-Ado (5–50 μM) for an hour and the data were compared with Cl.8+ cells. Data are expressed as the amount of pmoles transported to the cytoplasm per μg of protein. (C) ATP production in Mbn2 cells treated by Ado. The cells were incubated in CM medium containing various Ado concentrations (50 μM, 100 μM, 200 μM). The amount of ATP (pmoles ATP per μg protein) was monitored in regular intervals for 12 h. (D) Comparison of ATP increase after Ado treatment of Mbn2 and Cl.8+ cells. Cells were treated with 200 μM Ado for 12 h. Data are present as relative increase in ATP level compared to control. (E) Comparison of Ado incorporation into cellular Ado metabolites pool. Cells were incubated in 100 μM H3-Ado for 2 h and lysed, the lysates were separated by HPLC and the percentage of radioactivity in each fraction corresponding to Ino, AMP, ADP, and ATP was determined using scintillation counting. Each point represents mean ± SD of three independent experiments. *$P < 0.05$ for whole data series compared with corresponding value for Cl.8+ cells by Tukey's test.

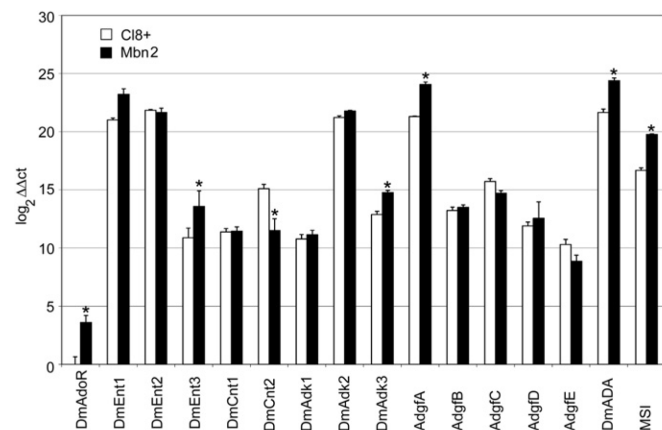

Fig. 9. RT-PCR analysis of expression profile of genes involved in Ado signaling and metabolism in Cl.8+ and Mbn2 cell lines. The relative gene expression is presented as $\log_2$ ratios to the least abundant transcript ($\log_2\Delta\Delta ct$). Data were normalized to internal control gene *Rack1*. Values represent averages from two independent biological samples. Bars indicate SEM. *$P < 0.05$ compared with corresponding value for Cl.8+ cells by Tukey's test.

other words, these cells may lack an endogenous feedback mechanism that would protect them from the excessive ATP production that at higher doses of Ado results in the depletion of cell supplies, growth arrest, and eventually cell death. The exact nature of metabolic changes causing Ado-mediated growth arrest and cell death, however, will require further studies.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2012.01.002.

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Supplementary materials

Figure S1. Uridine transport in the Cl.8+ cells. Concentration dependency of Uro transport in presence or absence of 50 μM Ado. Data are present as the amount of pmoles transported to the cytoplasm per μg of protein. Values are presented as means ± SD of three independent experiments.

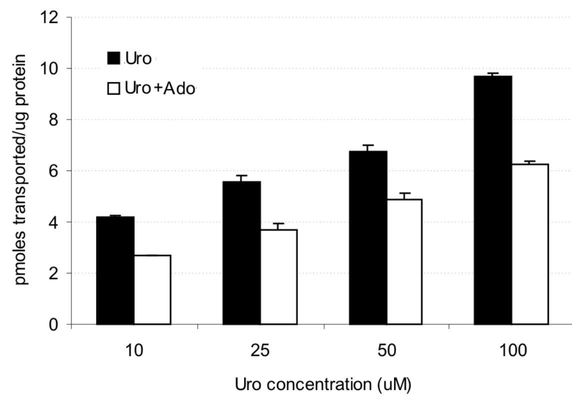

Table S1: List of primers used for the real time RT-PCR amplification of Ado signalling and metabolism genes.

Gene	Sequence
DmEnt1-fw	TCCCTGCGCACCAAGAT
DmEnt1-rev	ATAAACTCGGAGGGAAATAGACC
DmEnt2-fw	CGTGTGCAACGGCATCTAC
DmEnt2-rev	GTTGGAGCCCAGGACGAC
DmEnt3-fw	CATCGCTCTGGGCATCAC
DmEnt3-rev	CCACCGTCAGACCAACATTAT
DmCnt1-fw	GGGGGCTTATGTGTCCTTC
DmCnt1-rev	GGCCGCATCCAAAATAGA
DmCnt2-fw	CTTTGCCAATCCCAGTTCC
DmCnt2-rev	TAGTTCGCCCGCTCGTC
DmAdoR-fw	CCCATCTGAACTCGGCGGTAAATC
DmAdoR-rev	GCCTCCTGCTGCTGCCTCAAC
DmAdk1-fw	GGTCAGTGGGCAAGGATAAG
DmAdk1-rev	AGAAAGAAGCCCGTAAAGTAGAA
DmAdk2-fw	CAACAAGGCTCTGGTGGATAA
DmAdk2-rev	AATGGTGCGCTGAGGTTC
DmAdk3-fw	GGGCTCCAAGGGTGAACT
DmAdk3-rev	ACGGCGCCAAAGAAGAG
AdgfA-fw	AGGCTCATCCAGATTTCATTG
AdgfA-rev	CGGGTACTTTTCCTTTATTTGTT
AdgfB-fw	GCTGACCATTCGGGACAC

AdgfB-rev	CAACTAGATCAAAGCCAACCAT
AdgfC-fw	TGTACACAGAGATTCGGACCAG
AdgfC-rev	TAGACGGCCATAATGACTTTGA
AdgfD-fw	CTGACCACCACCAATAATCTGTA
AdgfD-rev	AGCGCTCCCAAATCTTCTT
AdgfE-fw	ATCGCGCTGGAGGTGTG
AdgfE-rev	CAGAAAGGCCATGTAGAAGTCA
DrADA-fw	GACAGTGGCGTTTTTGATACC
DrADA-rev	CGCTGGCGAATGAATGAT
MSI-fw	CGCCCGATCTGGAGTGAA
MSI-rev	ATATTTGGCAGCGAGGAACG
DmRack1-fw	CCCGTGACAAGACCCTGAT
DmRack1-rev	TAGTTGCCATCGGAGGAGAG

Table S2: List of primers used in RNAi silencing of Ado signalling and metabolism genes.

Gene	Sequence
DmCht5-fw	ACGCAGTGTGGTACGCTTTATGAA
DmCht5-rev	CGCCGCCTGCCTCCTTATT
DmAdoR-fw	GCTAGTGGGCGCATTGGGTATTC
DmAdoR-rev	CGGAGGCGGGGTTCATCGTA
DmEnt1-fw	ATCTCGAGCAGATGTCTCTTGAAAAGC
DmEnt1-rev	ATTCTAGAATGACTGCTCCTCGAACG
DmEnt2-fw	ATCTCGAGTAATCCCGAAATCAGAGTGC
DmEnt2-rev	ATCTCGAGTAATCCCGAAATCAGAGTGC
DmEnt3-fw	ATCTCGAGTGTGAATAATCCGCCAGAGG
DmEnt3-rev	ATTCTAGATGTAGTCCCAGAAATAGTGG
DmCnt1-fw	GAGAAGCCAGTGCCAGAGGTTA
DmCnt1-rev	AAGGGGAGGCGCAGGCAGAGTAT
DmCnt2-fw	GCGCATAGTCATCACGGGAATC
DmCnt2-rev	GCGGGAGCTGCCATCACC
DmAdk2-fw	GCCGCCGGACTGGATGTTCACTAC
DmAdk2-rev	CGCGTCGGGTTTTTCTTCTCCA
DmAdk3-fw	GCGGGTTTTTGGCCTCAGTAT
DmAdk3-rev	CATCGGATTTGGCCCTCTTTTC
DmAdk1-fw	CGGTGGAGCGCAAGCAAATC
DmAdk1-rev	GAATCCGGCCACAAAAGCATCTC

CONCLUSIONS

1) We have established the functional cAMP assay for the activation of *Drosophila* adenosine receptor in *Drosophila* cells *in vitro*. We used *Drosophila* neuroblast cell line with high endogenous *AdoR* expression and the cAMP responses in *Drosophila* cells were detected by the luminescence readout of expressed GloSensor reporter protein.
2) We show that cAMP is the preferred physiological second messenger of adenosine signaling in *D. melanogaster* cells.
3) We found and characterized several DmAdoR agonists and antagonists and also examined their effects *in vivo*. The most efficacious agonists at DmAdoR were C2-substituted adenine analogs, while the best DmAdoR antagonists were non-xanthine polyheterocycles. Simple xanthines like caffeine, theophylline and DPCPX failed to antagonize DmAdoR signaling. We show that adenosine analogs are metabolically more stable than adenosine and mimic DmAdoR overexpression and knockout, respectively. We also combined bioinformatic and pharmacological approach and provided new insights into the role of specific amino acid residues in the DmAdoR, since the *Drosophila* sequence is quite divergent from the mammalian receptors.
4) We detected that *Drosophila* imaginal disc cells (Cl8+) have extremely low AdoR expression, hence its adenosine response is exclusively controlled by Ado uptake via both equilibrative and concentrative transporters. We show that the adverse Ado effect on cell morphology, growth and viability in *Drosophila* Cl.8+ cells results from an excessive Ado uptake leading to massive ATP accumulation and depletion of supplies.
5) We show that different types of *Drosophila* cell lines significantly differ by their ability to reutilize Ado, which has in turn a dramatic effect on their energy homeostasis. Different response of cells to Ado is connected with the variations in expression profiles of genes connected with Ado metabolism, transport and signalisation.

Our findings will help to explain complexity and tissue specificity of disease phenotypes connected with changes in adenosine signaling and metabolism.

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