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A *Drosophila* adenosine receptor activates cAMP and calcium signaling

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Abstract

Adenosine receptors (AdoR) are members of the G protein-coupled receptor superfamily and mediate extracellular adenosine signaling, but the mechanism of adenosine signaling is still unclear. Here we report the first characterization of an insect AdoR, encoded by the *Drosophila* gene *CG9753*. Adenosine stimulation of Chinese hamster ovary cells carrying transiently expressed CG9753 led to a dose-dependent increase of intracellular cAMP and calcium, but untransfected controls showed no such response, showing that CG9753 encodes a functional AdoR. Endogenous *CG9753* transcripts were detected in the brain, imaginal discs, ring gland and salivary glands of third-instar *Drosophila* larvae, and *CG9753* overexpression in vivo caused lethality or severe developmental anomalies. These developmental defects were reduced by adenosine depletion, consistent with the proposed function of the *CG9753* product as an AdoR. Overexpression of the G protein subunit $G\alpha_s$ or of the catalytic subunit of protein kinase A (PKA) partially mimicked and enhanced the defects caused by ectopic expression of AdoR. Our results suggest that AdoR is an essential part of the adenosine signaling pathway and *Drosophila* offers a unique opportunity to use genetic analysis to study conserved aspects of the adenosine signaling pathway.

Keywords: Adenosine deaminase; ADGF; Adenosine deaminase growth factor; CG9753; GPCR receptor; Ca^{2+}

1. Introduction

Adenosine is an endogenous nucleoside that modulates numerous physiological processes, including oxygen and metabolic balance in tissues (Berne, 1963; Costa and Biaggioni, 1998), immune responses (Sitkovsky and Lukashev, 2005) and signaling in the nervous system (Masino and Dulla, 2005; Fredholm et al., 2005). Most of these roles in mammals are mediated by interaction of adenosine with specific G protein-coupled receptors (GPCRs). As in other GPCRs adenosine receptors (AdoR) have seven membrane-spanning α-helices with an extracellular amino terminus and an intracellular carboxy-terminal tail (Murphree and Linden, 2004).

Four mammalian subtypes of the AdoR have been identified and their genes cloned: A1, A2A, A2B, and A3. They have been shown to modulate intracellular levels of adenosine 3′, 5′-cyclic monophosphate (cAMP) in different ways: A1 and A3 inhibit adenylate cyclase, whereas A2A and A2B stimulate this enzyme (van Calker et al., 1979; Londos et al., 1980). In some cells, such as human kidney epithelial cell line HEK293 or canine mast cells, the A2B receptors are also coupled to the calcium-mobilizing G protein subunit, $G\alpha_q$ (Auchampach et al., 1997). AdoR subtypes are differentially distributed throughout the body (Murphree and Linden, 2004; Jacobson and Gao, 2006). The A1 AdoR is expressed in the brain, heart, adipose tissue, stomach, vas deferens, testis, spleen, kidney, aorta, liver, eye and bladder. The A2A receptor is highly expressed in parts of the brain (the striatum, nucleus accumbens and olfactory tubercles), in the spleen, thymus, immune cells, heart, lung and blood vessels. The A2B receptor is expressed at low levels in almost all tissues. The A3R is expressed at low levels in the thyroid gland, brain, liver, kidney, heart and intestine. The existence of four

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receptors with different functions but overlapping patterns of expression, together with the pervasiveness of adenosine-mediated physiological events, pose difficult questions in efforts to design pharmacological and biochemical interventions (Nyce, 1999). Moreover, the molecular dissection of AdoR signaling is difficult due to cross talk among various GPCR receptors (Fredholm et al., 2000; Werry et al., 2003). A better understanding of the adenosine signaling pathway would help in the elucidation of these mechanisms as well as in the development of strategies for the treatment of various human diseases, such as tachycardia, sleep disorders, immune and inflammatory disorders (for a review see Jacobson and Gao (2006)).

Understanding adenosine signaling could be advanced by the use of various genetic strategies in *Drosophila*. Accordingly, we have characterized a mutation that eliminates the major adenosine deaminase ADGF-A, and shown that this has pleiotropic effects on development associated with increased levels of adenosine and deoxyadenosine in the hemolymph (Dolezal et al., 2005). These phenotype includes larval and pupal lethality, developmental delay with block of pupariation, fat body disintegration and amplification of hemocytes. We have also generated a loss-of-function mutation in the *CG9753* gene, which encodes a putative *Drosophila* AdoR (Brody and Cravchik 2000; Vanden Broeck 2001). The *AdoR/ADGF-A* double mutants show a less extreme phenotype than the *ADGF-A* null mutant (Dolezal et al., 2005) confirming that the two genes are functionally related. Here we show that *CG9753* shares significant structural similarity with mammalian AdoRs, and that its activation leads to strong effects on intracellular cAMP and calcium signaling pathways.

2. Materials and Methods

2.1. Protein sequence alignments

Sequence alignments were produced using CLUSTAL algorithm as implemented by the Megalign program of the Lasergene package (DNASTAR Inc., Madison). The GenBank accession numbers of AdoRs used in this paper are: *Drosophila melanoster* AdoR (NP_651772); *Homo sapiens* A3R (CAA54288), A1R (NP_000665), A2AR (NP_000666), and A2BR (NP_000667).

2.2. Gene cloning and sequence analyses

The *Drosophila CG9753* sequence was predicted from the genomic database 〈www.flybase.org〉. To produce a cDNA clone for use in AdoR expression in CHO cells, reverse transcription was done with 5 μg of total adult fly RNA, oligo(dT)$_{12-18}$ and Superscript II reverse transcriptase according to the supplier's protocol (Invitrogen). The full-length *AdoR* cDNA was subcloned into the expression vector pcDNA3.1 (Invitrogen) using XbaI and KpnI restriction sites and sequenced using an ABI Prism 310 DNA analyzer. PCRs were carried out by using the following primers: AdorF- 5′ ACTCTAGACCATGTCCGCGTTTCGCTAC (incorporates XbaI site at its 5′ end); AdorR- 5′ ATGGTACCTCAACTCTCCGCGCTGGTCG (incorporates KpnI site).

To produce a *UAS-AdoR* construct, a genomic AdoR fragment was amplified using the following primers: AR-F, 5′ acaccgcccaccatgtcc (the primer also incorporates an EcoRI site at its 5′end) and AR-R, 5′ CCAGCGCGGAGAGTTGACCTTC (with a STOP codon and XbaI tail). The fragment was cloned into the EcoRI/XbaI of the pUAST vector (Brand and Perrimon, 1993).

2.3. Transient expression of AdoR in CHO cells

The full-length *AdoR* cDNA was cloned into the pcDNA3.1 vector and the construct was transfected into CHO cells for transient expression studies using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Transfections were done in 100 mm tissue culture dishes and seeded after 24 h into microtiter plates for subsequent calcium assays or 24 well plates for cAMP assays. Assays were performed 48 h after cell transfections.

2.4. Measurement of adenosine–induced cAMP increase

48 hour after transfection the cells were washed with assay buffer (OptiMEM, Invitrogen, San Diego) containing 10 μM Ro 20-1724 (Sigma). Drugs were added to a final volume of 500 μl and the cells were incubated at 37 °C for 30 min. The assay was stopped by adding ice-cold ethanol to a final volume of 60% (v/v). After freezing and thawing the cells were pelleted by centrifugation and aliquots of the supernatant were assayed for cAMP by radio-immune assay (FlashPlates, PerkinElmer, USA) according to the manufacturer's instructions.

2.5. Measurement of adenosine-induced calcium release

Calcium mobilization was assayed as described recently (Nothacker et al., 2000). Briefly, CHO cells were seeded in black microtiter plates (Costar) at 6×10^4 cells/well and grown overnight. The cells were loaded with 2 μM of the calcium indicator dye Fluo-4 (Invitrogen, San Diego) in growth medium (α MEM, 5% FBS) supplemented with 2.5 mM probenecid for 1 h at 37 °C, 5% CO_2. The cells were washed three times with Hank's balanced salt solution containing 20 mM HEPES and 2.5 mM probenecid. Adenosine-induced changes in fluorescence were measured on a FLIPR I system (Molecular Devices, Sunnyvale, CA). For calculation of dose-response curves the peak fluorescence values of the transient calcium curves were determined and analyzed by nonlinear regression using PRISM™ software (GraphPad, San Diego, CA). Triplicate values from at least two independent experiments were used for the calculations. The EC_{50} value is defined as the

adenosine concentrations generating 50% of the peak fluorescence value.

2.6. In situ hybridization

In situ hybridization to tissues of partially dissected late third-instar larvae was performed as described previously (Zurovec et al., 2002). Sense and antisense digoxigenin-labeled RNA probes were prepared from linearized cDNA clones by using T3 and T7 polymerases (MaxiScript, Ambion) and a digoxigenin labeling mixture (Roche Diagnostics). The larval tissues were fixed and hybridized with RNA probes in 50% formamide, 5 × SSC, 250 mg/ml salmon sperm DNA, 50 mg/ml heparin, 1 mg/ml tRNA, and 0.1% Tween 20, at 55 °C. Signals were detected by Sheep Anti-Digoxigenin Fab fragments Antibody, AP Conjugated (Roche Applied Science). There were no detectable signals from sense RNA probes except in the gut.

2.7. Real-time RT-PCR

PCR was carried out using the RotorGene real-time DNA amplification system (Corbett Research, Sydney, Australia), using the following cycling protocol: a 94 °C denaturation step for 5 min, followed by 45 cycles of 94 °C denaturation (20 s), 65 °C annealing (20 s), and 72 °C extension (40 s). Fluorescence was measured at the end of each cycle with the temperature held at 86 °C. Each sample was analyzed twice in triplicates. Gene expression was normalized to PCR-detected measurements of a ''housekeeping'' β-actin gene-transcript. PCR products were subjected to melting curve analysis, and the data were analyzed and quantified with the RotorGene analysis software. Relative values were standardized to β-actin and values normalized to the sample with highest expression (sample prepared from adults—Fig. 3A or male heads—Fig. 3B) arbitrarily set to 1.0. Values represent the mean of three independent experiments ± standard errors. To avoid genomic DNA amplification the primers were located in different exons (AdoR-RealFOR: 5′ CCCATCTGAACTCGGCGGTAAATC and AdoR-RealREV: 5′ GCCTCCTGCTGCTGCTGCCTCAAC).

2.8. Drosophila stocks and production of transgenic lines

Flies were raised on a cornmeal–yeast–agar–sugar diet with 0.3% Nipagin at 25 °C. Transgenic flies carrying the *UAS-AdoR* construct were produced by a modified P-element transformation method (Park and Lim, 1995). The *act-gal4* and *en-gal4* flies were kindly provided by Jessica Britton and Bruce Edgar (FHCRC, Seattle, WA); *Pros-gal4, Pnr-gal4* by Marek Jindra (Inst. Entomol., Ceske Budejovice Czech republic); *Sev-gal4* and *Elav-gal4* by Larry Marsh (UC Irvine, CA); *30A-gal4* and *UAS-PK* by J. Kiger (UC Davis, CA); *UAS-Gsα*, by M. Forte (OHSU, Portland, OR); *neuroblast-gal4* and *UAS-EGFP* were received from the Bloomington Stock Center (Indiana University, Bloomington). We always analyzed more than 100 flies unless stated otherwise.

2.9. Injection of adenosine and ADA

For injection, the pupal cases of pharate adults (grainy-G-stage or later) were pierced with forcep tips and injected with solutions (300 μM adenosine or 200 ng/μl bovine adenosine deaminase) using a glass capillary injection needle. The injections were delivered into the side of the thorax, near the wings or legs. The volume of solution injected was 0.1–0.3 μl. Injected animals were kept in moist vials (vials with water-soaked filter papers) until eclosion.

3. Results

3.1. Alignment of Drosophila AdoR with other known AdoRs

We queried the fly protein database with sequences encoding human AdoRs and found a previously identified *CG9753-AdoR* coding sequence (Brody and Cravchik, 2000; Vanden Broeck, 2001). The *CG9753* gene has four exons and is located at cytological map position 99D8. The predicted ORF encodes a protein of 774 amino acids. The N-terminal part of the molecule (around 300 amino acids) comprises the region with most conservation among species and contains the seven transmembrane helices, but unlike other known AdoRs, *CG9753* encodes a protein with a long (predicted intracellular) C-terminal extension of unknown function. Comparison with human AdoRs revealed that despite a low similarity at the amino acid level (approximately 30% identity in the N-terminal parts of the molecules) CG9753 shares most of the amino acids known to be important for binding of the ligand (Fig. 1).

3.2. AdoR expressed in Chinese hamster ovary (CHO) cells is activated by adenosine

In order to test for activation of AdoR by adenosine, the coding sequence of *CG9753* was cloned into the mammalian expression vector pcDNA3.1 in both orientations. The gene was expressed in CHO cells and the changes in secondary messenger concentrations were measured. The transfected cells responded to adenosine by an increase of intracellular cAMP in a dose-dependent manner, with an EC_{50} value (the adenosine concentration producing 50% maximum effect) of 1.3 ± 0.3 μM (Fig. 2(A)). We also observed a similar dose-dependent increase in intracellular calcium with an EC_{50} of 5.34 ± 1.2 μM (Fig. 2(B)). Adenosine did not cause these effects in untransfected cells or in cells transfected with the *CG9753* gene in antisense orientation.

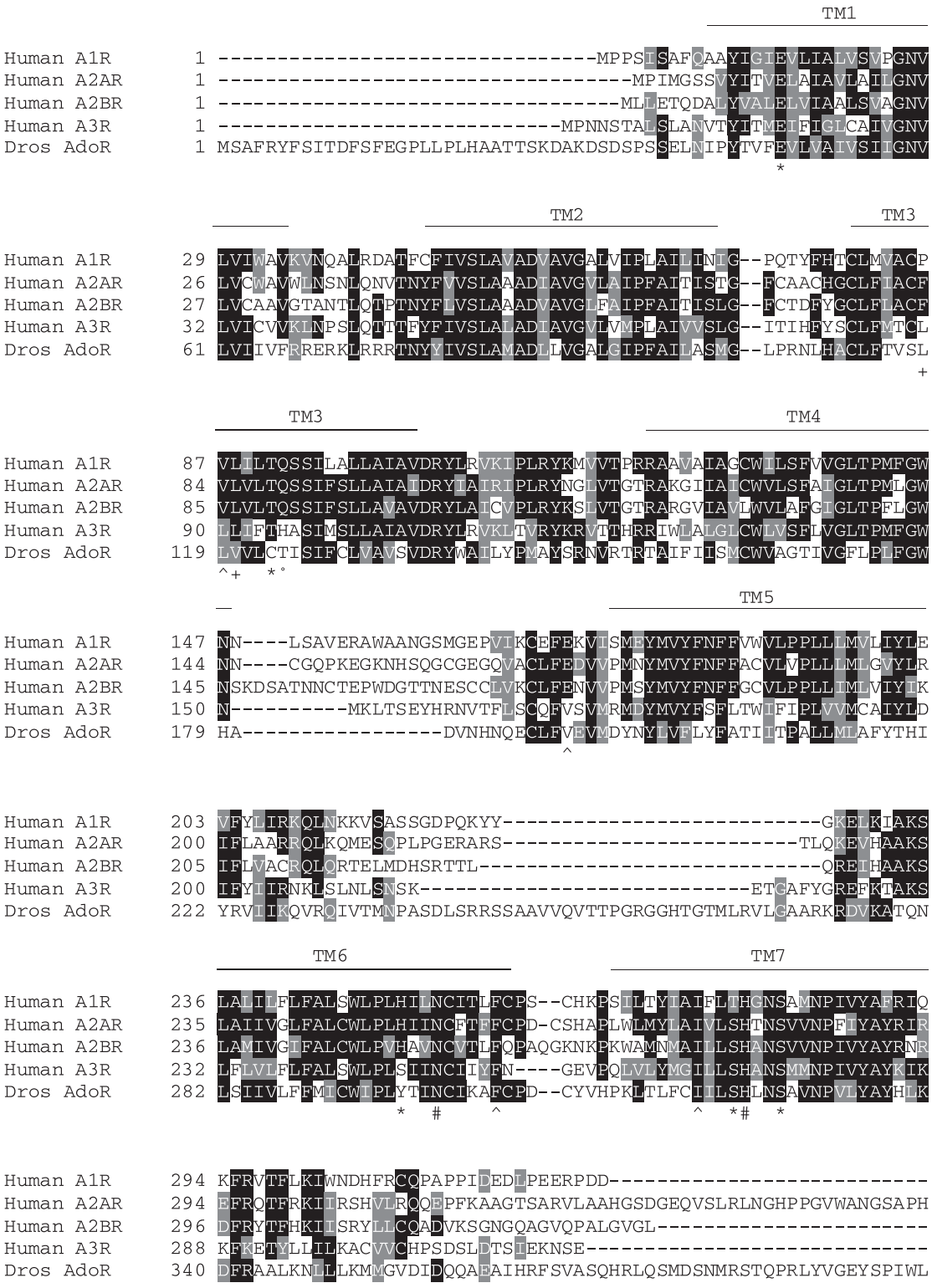

Fig. 1. Alignment of the human adenosine receptors and N-terminal part of *Drosophila* AdoR amino acid sequences. The transmembrane domains (TM) of human adenosine receptors were modeled by Tuccinardi et al. (2006) and are marked by lines above the sequences. The residues that were demonstrated important for adenosine and adenosine receptor agonist binding in point mutation experiments for the human A1, A2A and A3 receptors are indicated below the sequence alignment. "#" marks the residues important for all three receptors, "*" the residues crucial for A1 and A2A receptors, while "°" marks the residues important for A1 and A3 receptors. "+" signs mark the residues important for A1 receptor and "^" mark the A2A receptor adenosine binding residues (Kim et al., 2003, Gao et al. 2002).

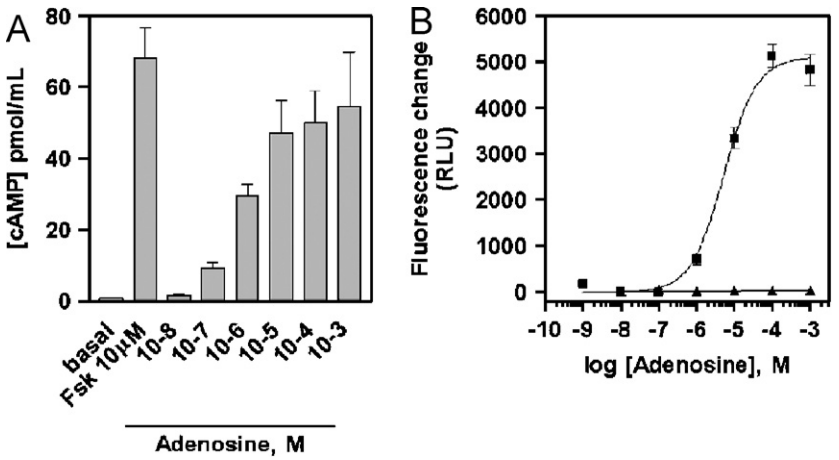

Fig. 2. Direct activation of AdoR by adenosine. AdoR was transiently expressed in CHO cells and stimulated with different doses of adenosine. (A) Adenosine stimulated dose-dependently cAMP production or (B) intracellular calcium increase. Only cells transfected with plasmids of AdoR in sense orientations (■) responded to adenosine, but not in antisense orientation (▲), basal, cAMP concentration in nonstimulated cells; FSK, cells stimulated with 10 μM forskolin. Both experiments show triplicate values of one typical experiment with error bars indicating SEM. Both experiments were done at least twice.

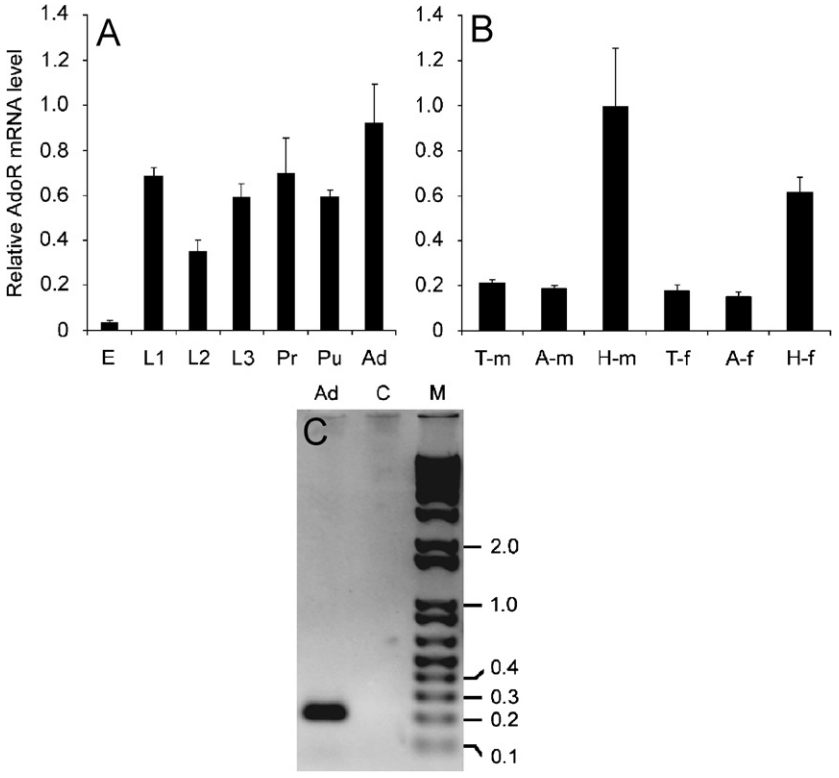

Fig. 3. The developmental and spatial expression pattern of *Drosophila AdoR* gene. Total RNA was prepared from different developmental stages and body parts of *Drosophila*, then used for quantification of AdoR gene expression by real-time PCR analysis. Bars represent the relative AdoR transcript levels ± SEM of triplicate samples normalized against β-actin transcript levels. The value 1.0 on the *Y*-axis corresponds to the highest *AdoR* expression level. (A) Relative mRNA levels of *AdoR* during developmental stages: embryos (E), larvae (L1-3), prepupae (Pr), pupae (Pu), and adults (Ad). (B) Relative *AdoR* mRNA levels in three body parts representing thorax (T), abdomen (A) and head (H) of males (m) and females (f). (C) Agarose gel electrophoresis of the 247 bp amplicon from a real-time PCR reaction. From a 20 μl reaction, 1 μl was run on an 1.5% agarose gel and stained with ethidium bromide. The lanes are as follows: an amplicon from adults (Ad), a control without cDNA (C) and a 1-kb plus DNA ladder (M).

3.3. Endogenous expression of AdoR during development

In order to assess the possible functions of the putative AdoR in *Drosophila,* we used quantitative reverse-transcription PCR (Real-time RT-PCR) to determine the temporal and spatial expression of the corresponding gene throughout development. The expected amplicon was 247 bp (Fig. 3(C)); it was gel purified and verified by

sequencing. Varying levels of *AdoR* mRNA were detected in all postembryonic stages, with the highest expression level in adults (Fig. 3(A)). The head showed higher mRNA levels per unit mass of tissue than the thorax and abdomen (Fig. 3(B)).

AdoR expression was investigated by RNA in situ hybridization to whole tissues dissected from late third-instar larvae. The *AdoR* antisense cRNA probe reproducibly detected mRNA in the optic lobes of the brain, the ring gland, all imaginal discs, and salivary glands (Fig. 4) but not in the fat body (data not shown).

3.4. Ectopic AdoR expression with some gal4 drivers causes lethality or severe developmental anomalies

To assess the physiological roles of AdoR, we expressed the gene either ubiquitously or in various patterns using the *UAS/gal4* system (Brand and Perrimon, 1993). The expression pattern of each of the combinations was monitored using the *UAS-EGFP* transgene, which was co-expressed with *AdoR*. The tissue specificity of these *gal4* drivers and the resulting phenotypes are shown in Table 1.

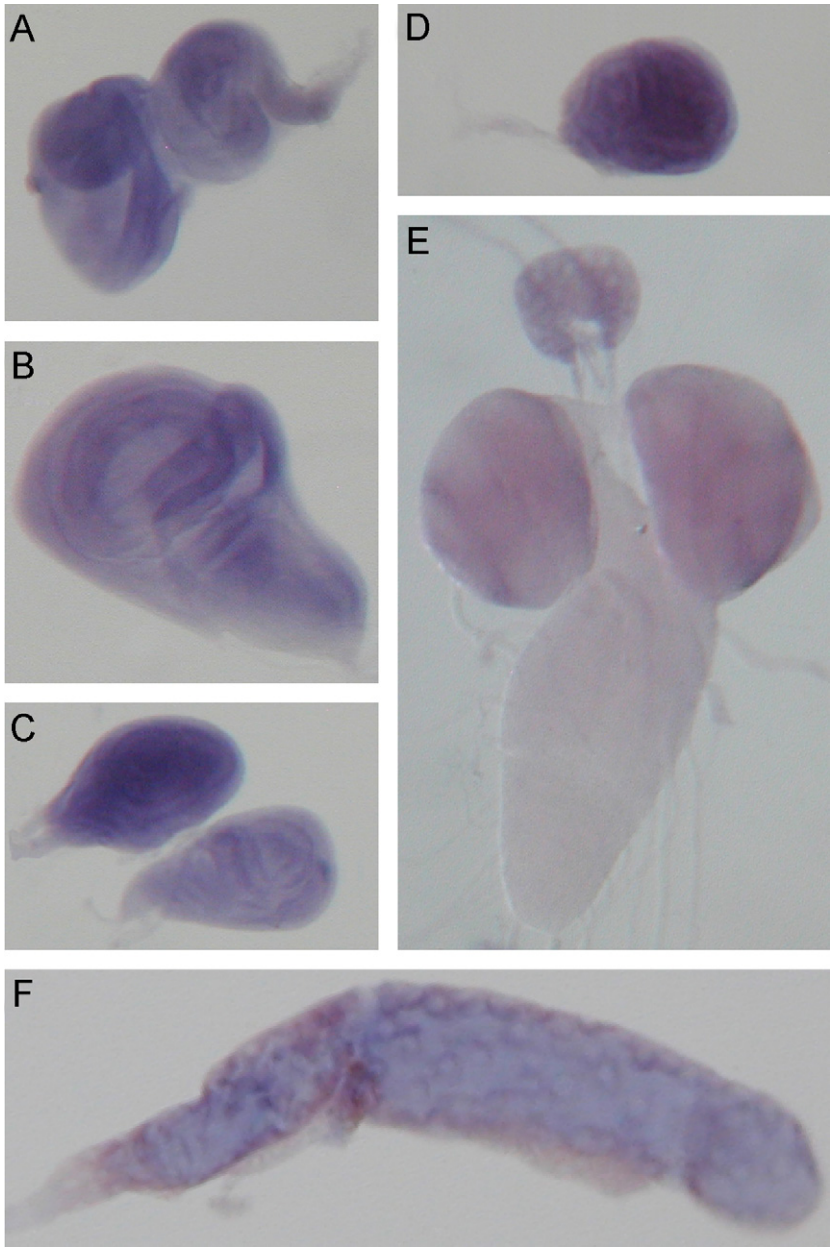

Fig. 4. Tissue-specific localization of *AdoR* RNA in third-instar larvae. In-situ hybridization to whole tissues was performed with antisense and sense *AdoR* probes. *AdoR* expression is visible in the imaginal discs, including (A) eye-antennal disc, (B) wing disc, (C) leg discs, and (D) haltere disc; *AdoR* mRNA signal also occurs in (E) the optic lobes of larval brain and the ring gland, and (F) in salivary glands.

Table 1
Effects of forced *AdoR* expression

gal4 driver	Tissues with the highest expression in L3 larva	Aberrant phenotype (% penetrance)
Actin	Ubiquitous	Lethal in L1-L2 larvae (100%)
Sevenless	CNS, eye disc, salivary glands	Lethal in late pupae (100%)
Prospero	CNS, eye disc, ring gland, salivary glands, gut imaginal islets	Lethal in late pupae (100%)
Engrailed	CNS, imaginal discs, cuticle, gut	Wing blistered, folded (100%); melanotic tumors (80%)
Pannier	Imaginal wing disc—notum, salivary glands, CNS	Reduced scutellum; defect in melanization of abdominal cuticle (100%)
30A	Salivary glands, wing imaginal discs, CNS-optical lobes	Wing blistered, folded (100%); occurrence of melanotic tumors (50%)

Ubiquitous expression of the *UAS-AdoR* transgene caused lethality during the transition from the first to second larval instar. Expression of AdoR in the central nervous system and some additional organs, under the control of either the *prospero* or the *sevenless* promoter, resulted in lethality during the final step of metamorphosis, when the adults normally emerge from the puparium. The pharate adults of these transgenic flies survived for several hours when removed from the pupal cases at the normal eclosion time, but they were unable to move or spread their wings.

Expression of the *UAS-AdoR* transgene with other tissue-specific drivers (*engrailed-gal4*, *30A-gal4*, or *pannier-gal4*) caused weaker phenotypes in which most individuals survived to adulthood, but had severe malformations of the wings or thorax (Table 1). All flies with *engrailed*- and *30A*-driven expression displayed folded and blistered wings (Fig. 5(A)–(C)), and 80% and 50% of the flies carried melanotic tumors in the head capsule, respectively (Fig. 5(D)). Folded wings were also observed in 10% of the *UAS-AdoR/neuroblasts-gal4* or *patched-gal4* flies. All *Pnr-gal4/UAS-AdoR* flies showed a reduced scutellum and crossed post-scutellar bristles (100 flies analyzed) (Fig. 5(B)). Flies homozygous for *UAS-AdoR* and heterozygous for *pnr-gal4* emerged very rarely (less than 5%), and exhibited a strong cleft in the thorax and complete loss of scutellum, as well as defects in the melanization of abdominal cuticle (Figs. 6(A) and (C)).

3.5. The AdoR overexpression phenotype can be partially rescued by decreasing the level of extracellular adenosine

To test whether the phenotypes observed in the flies overexpressing AdoR driven by *en-gal4*, *30A-gal4*, or *pnr-gal4* are caused by an over-activation of the adenosine pathway, we decreased adenosine level in the hemolymph by injecting pharate adults (smooth/grainy stage; Kimura and Truman, 1990) with commercial bovine adenosine deaminase (Roche). The injection allowed nearly 5% of the *pros-gal4/UAS-AdoR* animals ($N = 100$ pharate adults) to emerge from the puparium compared to 0% in uninjected or saline-injected controls (Table 1). The flies lived only about 24 h, had impaired locomotion and were unable to spread their wings. These results are consistent with our in vitro pharmacological data and strongly support the idea that *CG9753* encodes a functional AdoR.

3.6. The AdoR overexpression phenotype can be mimicked by injection of adenosine into the hemolymph of wild-type pharate adults

If the *CG9753* gene product activates the adenosine signaling pathway, then an increase of adenosine concentration in fly hemolymph should have an effect similar to that of AdoR overexpression. To test this hypothesis, we injected 0.1–0.3 μl of a solution of 100–500 μM adenosine (based on the concentration used previously in vitro; Zurovec et al., 2002) into the hemolymph of wild-type pharate adults. This treatment reduced the number of successfully eclosing adults (eclosion per se) from 90% (in saline-injected controls) to 70% ($N = 50$ animals). The eclosion of adults was delayed and the imagoes showed several anomalies, including folded and blistered wings, that were never seen in the controls. Melanotic tumors were present in 5–10% of emerged flies, and occurred in the same head compartment (basiproboscis) as those caused by AdoR overexpression. The similarity of these phenotypes to those produced by AdoR overexpression further support our hypothesis that *CG9753* encodes a functional AdoR.

3.7. Interaction in vivo between overexpressed AdoR and multiple members of the cAMP/PKA pathway

To test whether AdoR interacts with the cAMP/PKA pathway in vivo, we coexpressed *UAS-AdoR* with *UAS-*$G\alpha_s$ or *PKA* (Kiger et al., 2001). The results showed that overexpression of AdoR (driven by *en-gal4, 30A-gal4*, or *pnr-gal4*) has a synergistic effect with $G\alpha_s$ or PKA expression. All flies with overexpression of AdoR in combination with $G\alpha_s$ or PKA (*UAS-PKA15.3* or *UAS-*$G\alpha_s$) displayed stronger phenotypes than those seen with the overexpression of $G\alpha_s$ or PKA alone. For example, *en-gal4/UAS-AdoR/UAS-*$G\alpha_s$ flies showed 20% lethality as pharate adults, whereas neither AdoR nor $G\alpha_s$ overexpression alone caused any lethality. In addition, all emerged triple transgenic flies had melanotic tumors in the head capsule and in the wings (Fig. 7), whereas only 10% of the *en-gal4/UAS-AdoR* flies showed this phenotype.

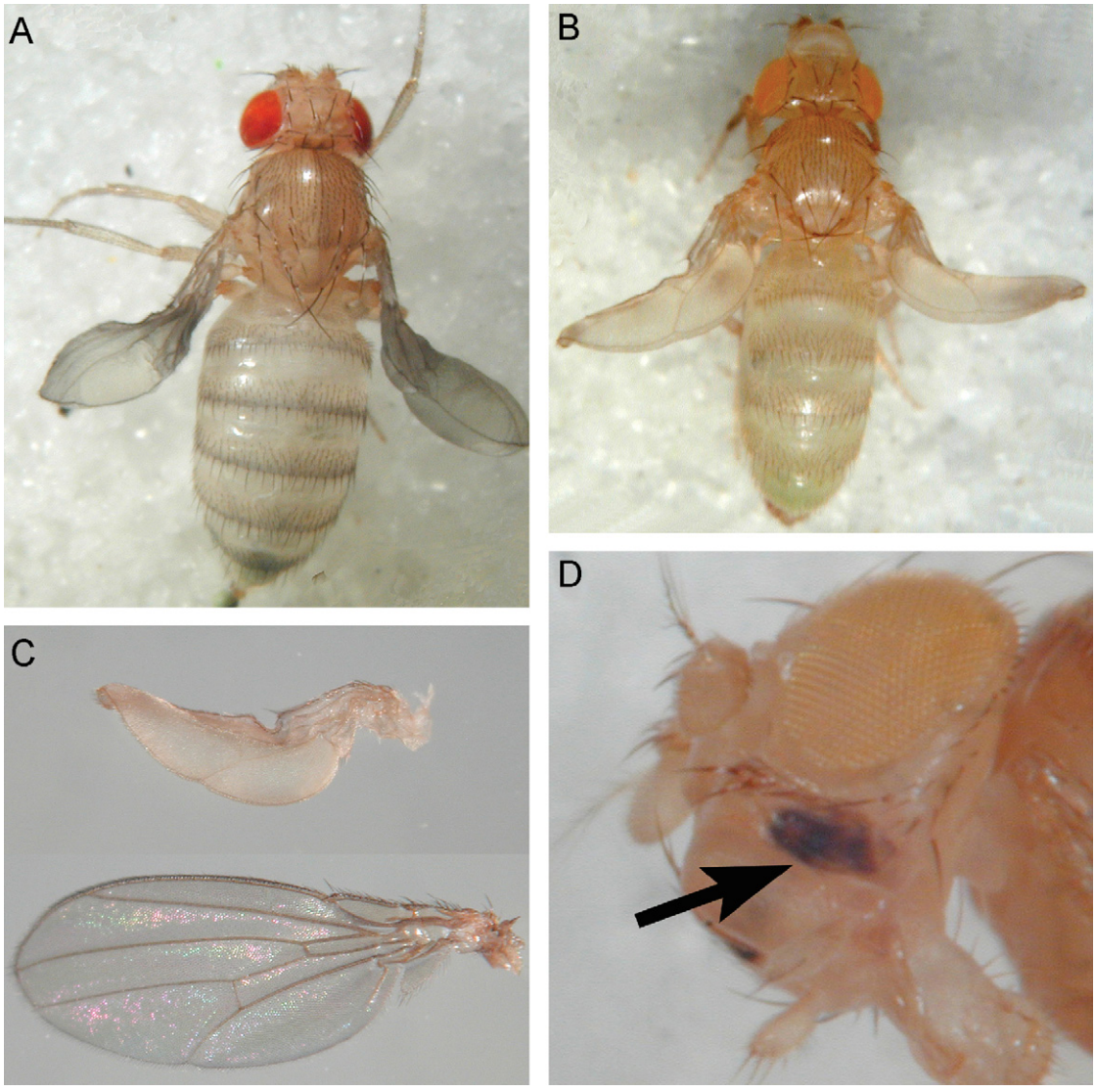

Fig. 5. Phenotypes observed in the flies overexpressing AdoR driven by *30A-gal4* and *en-gal4*. (A) *30A- gal4/UAS-AdoR* blistered wing phenotype; (B) *en-gal4/UAS-AdoR* blistered wing phenotype; (C upper) a wing of the *en-gal4/UAS-AdoR* fly; (C lower) control wing of a fly expressing the *en-gal4 only*. (D) A melanotic tumor (arrow) in the head capsule of the *en-gal4/UAS-AdoR* fly.

The synergistic effects of overexpressed AdoR and cAMP/PKA pathway members are consistent with the conclusion that AdoR mediates activation of the cAMP/PKA pathway in vivo.

4. Discussion

By conducting BLAST searches of the available *Drosophila* protein database, we have identified CG9753 as the gene encoding the *Drosophila* AdoR homolog. Closely related proteins are predicted by sequences in the databases of the malaria vector *A. gambiae* and the honeybee *A. mellifera*. The N-terminal domains in the predicted proteins from the three insect species show approximately 70% identity. While the entire *Drosophila* AdoR contains 774 amino acids, the predicted ORFs of the mosquito and honeybee AdoR encode 392 and 462 amino acids, respectively. *CG9753* differs from the known AdoRs by a long (~350 amino acids) intracellular C-terminal extension) of unknown function. The C-terminal intracellular part of AdoR is highly divergent among these species and cannot be reliably aligned (the extension is absent in the predicted AdoR from *A. gambiae* and is only about 90 amino acids long in the sequence of *A. mellifera*). Since it is likely that there is only a single ortholog of mammalian AdoRs in the three insect species with known genomic sequences, it is probable that insects in general contain only a single AdoR.

The sequence of *Drosophila* AdoR is quite divergent from the mammalian AdoRs but they still share the region

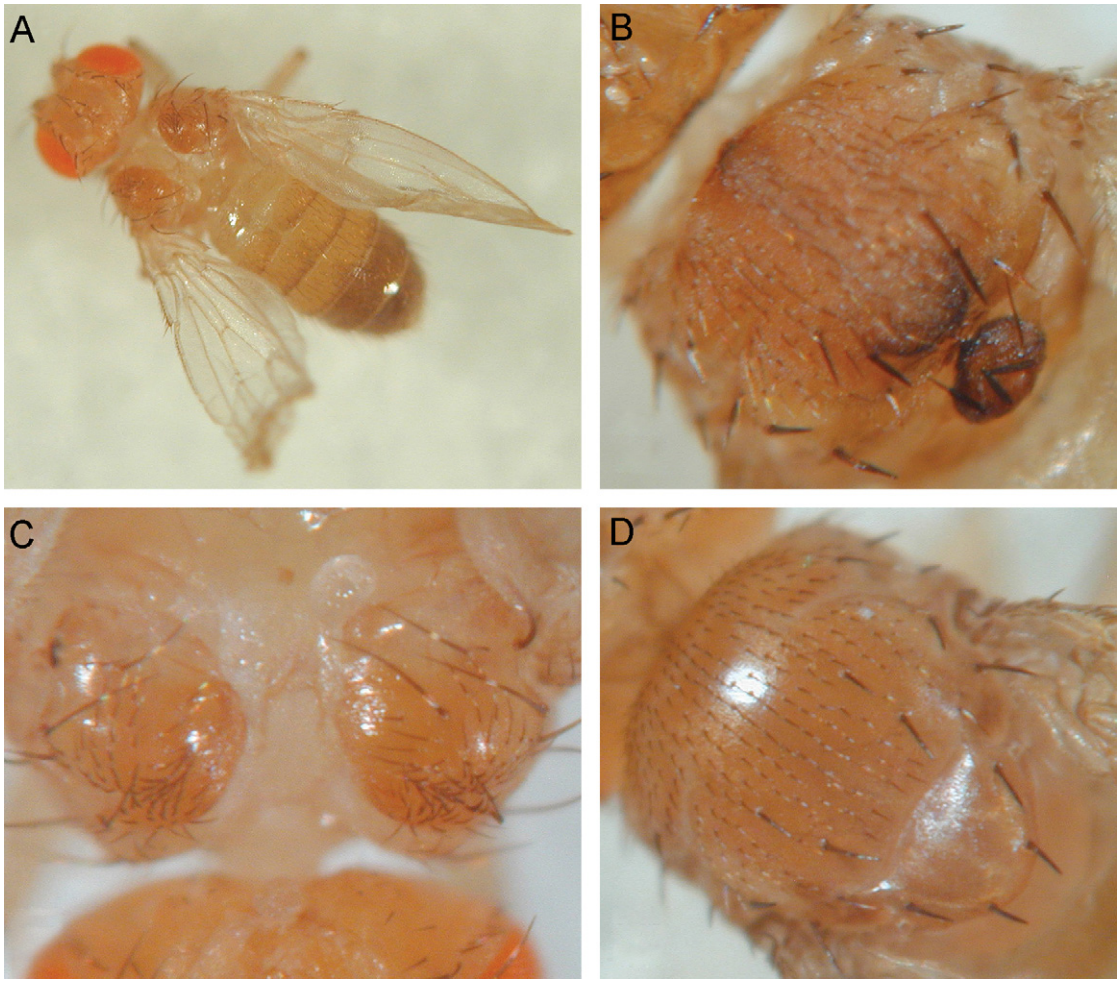

Fig. 6. Phenotypes observed in the *Pnr-gal4/UAS-AdoR* flies. (A) Thoracic and wing, abnormalities observed in the *Pnr-gal4/+*; *UAS-AdoR/UAS-AdoR* fly; (B) deformation of the scutellum observed in the *Pnr-gal4/TM3; UAS-AdoR/+* fly; (C) a strong cleft in the thorax and absence of the scutellum in the *Pnr-gal4/+*; *UAS-AdoR/UAS-AdoR* fly; and (D) the normal-appearing thorax of the *Pnr-gal4/TM3* control fly.

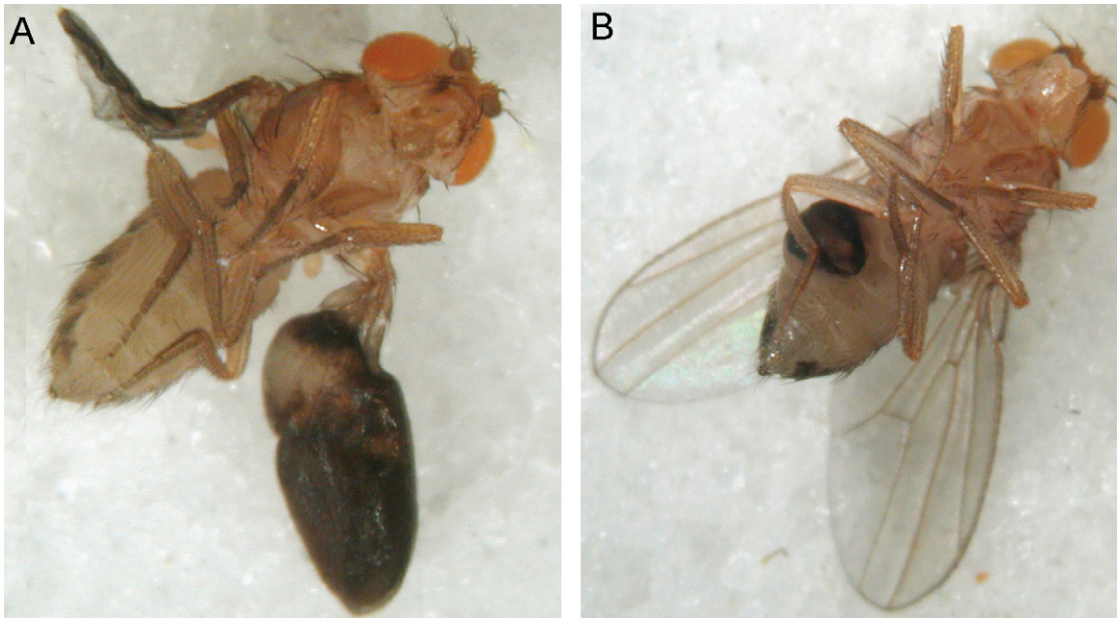

Fig. 7. The additive effects of overexpressed AdoR and cAMP/PKA pathway members. (A) Strong wing deformation and melanization observed in the *en-gal4/ UAS-AdoR/UAS-G*α_s fly. (B) A massive melanotic tumor in the abdomen of the *pros-gal4/UAS-PKA15.3c* fly.

important for adenosine binding. Furthermore, our pharmacological experiments revealed that *Drosophila* AdoR functionally responds to adenosine and is able to activate at least two second messenger pathways, involving cAMP and calcium. By assaying cAMP and Ca^{2+} levels in CHO cells transiently transfected with *Drosophila* AdoR, we detected increase in both cAMP and calcium levels after adenosine treatment. In both cases, activation occurred at physiologically relevant doses, and was only present for the AdoR plasmid in sense orientation.

The endogenous (steady state) concentration of adenosine in the hemolymph of third-instar larvae is less than 0.08 μM (Dolezal et al., 2005) although it can increase dramatically under stress. This concentration is close to the EC_{50} values established for the AdoR, which were 1.3 ± 0.3 μM for cAMP and 5.3 ± 1.2 μM for Ca^{2+}. Both of these values are therefore low enough to be physiologically relevant. The results indicate that AdoR has the potential to couple to both $G\alpha_s$ and $G\alpha_q$ proteins and to control both cAMP and calcium-mediated pathways in vivo.

In mammalian systems the local extracellular adenosine concentrations increase >100 fold after increased tissue activity, hypoxia, or ischemia (Hagberg et al., 1987; Kobayashi et al., 1998). EC_{50} values for adenosine-induced cAMP response of the human A1, A2A, A2B and A3 receptors expressed in CHO cells were estimated at 0.3, 0.7, 24 and 0.3 μM, respectively (Fredholm et al., 2001). The human AdoR A2B also displays dual coupling to the $G\alpha_s$ and $G\alpha_q$ subunits (Murphree and Linden, 2004), so it seems likely that the *Drosophila* AdoR is the counterpart of the A2B mammalian AdoR subtype.

It is of interest to compare the pharmacological properties of insect and human AdoRs. It is important to determine if the studied adenosine analogs also activate or block CG9753. A combined bioinformatic and pharmacological approach could also provide new insights into the role of specific amino acid resides in the protein, since the *Drosophila* sequence is so divergent from the usual mammalian sequences but still functions. Analyses of these interactions and such studies are under way.

Ubiquitous AdoR overexpression resulted in a larval lethal phenotype, whereas tissue-specific expression produced milder pupal lethal phenotypes. Since the expression patterns controlled by the available expression drivers show significant overlap, it is not clear from the present results which tissue is critical for lethality. It was previously suggested that adenosine signaling to the ring gland might be responsible for the lethality of the *ADGF-A* mutant (Dolezal et al., 2005). Consistent with this hypothesis, *prospero-gal4* clearly drives expression in the ring gland and produced a strong phenotype. The ring gland could therefore be involved in producing the AdoR overexpression phenotype, but the relevance of this to the tissue specificity of normal AdoR function is, of course, unclear. High levels of mouse A3AR expression also induce lethality, and it has been suggested that there is a connection between this lethality and antiproliferative effects or apoptosis induced by A3AR in various cell lines (Zhao et al., 2002). We also observed similar antiproliferative effects and the induction of apoptosis in several *Drosophila* cell lines (Zurovec et al., 2002).

We found that the phenotype caused by overexpressing AdoR could be rescued by decreasing the ligand concentration (injection of adenosine deaminase), or enhanced by adenosine injection in the pharate adult (data not shown). In addition, the dose dependency of the phenotype was apparent in the flies overexpressing AdoR driven by *pnr-gal4*. The *pnr-gal4* flies homozygous for the *UAS-AdoR* transgene had a much stronger phenotype than the flies with a single copy of *UAS-AdoR*. Consistently, the increase of signal strength might be responsible for the synergistic effect of coexpressing AdoR with $G\alpha_s$ or PKA.

The blistered wings, melanotic tumors and lethal phenotypes caused by local AdoR expression resemble those caused by mutations eliminating adenosine deaminase-related growth factors (ADGFs), which cause elevated adenosine levels in the hemolymph (Dolezal et al., 2005). For example, most of the ADGF-D and ADGF-C double-mutant flies die during adult eclosion, associated with locomotion defects (Dolezal et al., 2003). The flies overexpressing AdoR under *prospero-* or *sevenless-gal4* drivers seem to have similar phenotypes. The most severe phenotype among the *ADGF* mutations is caused by a mutation in the *ADGF-A* gene, displaying larval and pupal lethality, melanotic tumors, an accelerated differentiation and an increased number of hemocytes. Defects (developmental arrest and melanotic tumors) caused by the *ADGF-A* mutation can be partially rescued by combination with the *CG9753* null mutation (Dolezal et al., 2005), again suggesting a functional connection.

It was previously shown that blistered wings, melanotic tumors (hemocyte differentiation) and lethality can be induced by injecting cAMP into the hemolymph of *Drosophila* pharate adults (Kimura et al., 2004) or by overexpression of $G\alpha_s$ or PKA (Kiger et al., 2001; Kimura et al., 2004). The similarity of these phenotypes with those caused by AdoR overexpression is particularly striking in the case of the PKA catalytic subunit, since the expression was performed with the same *gal4* drivers as those used in the present study. These results indicate that the AdoR mediates activation of the cAMP/PKA-signaling pathway by adenosine in vivo.

Several similarities are apparent between mammalian and insect adenosine and AdoR functions: Extracellular adenosine influences immune responses in mammals, as well as in *Drosophila* (Dolezal et al., 2005). Adenosine agonists and antagonists were reported to have a modulatory role in the *Drosophila* sleep and waking cycle (Hendricks et al., 2000), which may be connected to the observed endogenous expression of AdoR in the brain (Fig. 4(E)). There is a substantial conservation of AdoRs and proteins involved in adenosine transport (Sankar et al., 2002) and metabolism (Zurovec et al., 2002). The

steady-state concentration of extracellular adenosine in fly hemolymph (below 3×10^{-7} M) is similar to the adenosine concentration in human blood (Dolezelova et al., 2005). Elevated adenosine concentrations are cytotoxic for various cells of both insects and mammals (Zurovec et al., 2002; Dolezelova et al., 2005). Adenosine agonists and antagonists were reported to have a modulatory role in the Drosophila sleep and waking cycle (Hendricks et al., 2000), which may be connected to the observed endogenous expression of AdoR in the adult heads (Fig. 3). All these connections suggest that the proposed role for adenosine as a local paracrine and autocrine homeostatic regulator (Masino and Dulla, 2005; Newby, 1984; Cunha, 2001) could apply to both mammals and *Drosophila*. The adenosine pathways in *Drosophila* and mammals may therefore represent evolutionarily well-conserved homeostatic mechanisms.

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