Adenosine signaling and its downstream target mod(mdg4) modify the pathogenic effects of polyglutamine in a Drosophila model of Huntington’s disease

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Abstract

Dysregulation of adenosine (Ado) homeostasis has been observed in both rodent models and human patients of Huntington’s disease (HD). However, the underlying mechanisms of Ado signaling in HD pathogenesis are still unclear. In the present study, we used a Drosophila HD model to examine the concentration of extracellular Ado (e-Ado) as well as the transcription of genes involved in Ado homeostasis and found similar alterations. Through candidate RNAi screening, we demonstrated that silencing the expression of adenosine receptor (adoR) and equilibrative nucleoside transporter 2 (ent2) not only significantly increases the survival of HD flies but also suppresses both retinal pigment cell degeneration and the formation of mutant Huntingtin (mHTT) aggregates in the brain. We compared the transcription profiles of adoR and ent2 mutants by microarray analysis and identified a downstream target of AdoR signaling, mod(mdg4), which mediates the effects of AdoR on HD pathology in Drosophila. Our findings have important implications for the crosstalk between Ado signaling and the pathogenic effects of HD, as well as other human diseases associated with polyglutamine aggregation.
Introduction

Adenosine (Ado) is one of the most common neuromodulators in the nervous system of vertebrates as well as invertebrates and modulates synaptic transmission\(^1\). Under normal conditions, the extracellular Ado (e-Ado) concentration is in the nanomolar range, which is sufficient to modulate the appropriate adenosine receptors (AdoRs) in the brain cells tonically\(^3\). However, under pathological circumstances the e-Ado level may increase up to 100-fold. In these conditions, Ado functions as an imperfect neuroprotector; in some cases it may be beneficial and in others may worsen tissue damage\(^4\). Recent experiments with knockout mice for all four adoRs demonstrated that Ado signaling is less involved in baseline physiology and likely more crucial for its roles as a signal of stress, damage, and/or danger\(^5\). It has also been suggested that Ado signaling is mainly engaged when an allostatic response is needed\(^6\).

Due to its impact on important physiological functions in the brain, e-Ado signaling has attracted attention as a possible therapeutic agent in Huntington's disease (HD)\(^7\), a dominant hereditary neurodegenerative disorder caused by a mutation in the Huntingtin gene (\textit{htt}). Mutated HTT protein (mHTT) contains an expanded polyglutamine (polyQ) tract encoded by 40 to more than 150 repeats of CAG trinucleotide\(^8\). Although mHTT is ubiquitously expressed in the central nervous system (CNS) and peripheral cells in HD patients, it predominantly affects striatal neurons that contain a higher density of adenosine receptors A2A (A\(_{2A}\)R) and A1 (A\(_{1}\)R)\(^9\). Several studies have demonstrated that the abnormality of AdoRs activity, especially A\(_{2A}\)R in the striatum, contributes to HD pathogenesis\(^10,11\). In addition, the alteration of adenosine tone and the upregulation of striatal equilibrative nucleoside transporters (ENTS), facilitating Ado transport across the cytoplasmic membrane, suggest that e-Ado concentration could serve as a HD biomarker for assessing the initial stages of neurodegeneration\(^12,13\). However, the complexity of the system modulating Ado metabolism and the crosstalk between individual AdoRs, as well as their interactions with purinergic (P2) or dopamine receptors, impedes the characterization of HD pathophysiology and downstream mechanisms of e-Ado signaling\(^14,15\).
Drosophila expressing human mHTT has previously been demonstrated as a suitable model system for studying gene interactions in polyQ pathology, and has been used to elicit a number of modifiers for symptoms of HD\textsuperscript{16,17}. Drosophila e-Ado signaling is a relatively simple system compared to mammals; it contains a single AdoR isoform (cAMP simulation) and lacks P2X receptors\textsuperscript{18,19}. Human homologs of the Drosophila genes involved in the regulation of Ado homeostasis and AdoR are shown in Fig. S1. The lack of adenosine deaminase 1 (ADA1) in Drosophila indicates that adenosine deaminase-related growth factors (ADGFs, related to ADA2), together with adenosine kinase (AdenoK), are the major metabolic enzymes converting extra- and intra-cellular adenosine to inosine and AMP, respectively\textsuperscript{20-22}. e-Ado signaling in Drosophila is involved in regulating various physiological and pathological processes, including modulation of synaptic plasticity, JNK-mediated stress response, hematopoiesis, and metabolic switching upon immune challenges\textsuperscript{23-25}.

In the present study, we performed a candidate RNAi screen examining the role of Ado signaling in a Drosophila HD model. We co-expressed exon 1 with a polyglutamine tract of normal human \textit{htt} Q20 or pathogenic \textit{mhtt} Q93\textsuperscript{17} together with \textit{UAS-RNAi} or \textit{UAS-overexpression constructs specific for adoR}, Ado transporters, and Ado metabolic enzymes in \textit{Drosophila}. We demonstrated that the downregulation of \textit{adoR} and \textit{ent2} expression reduces cell death, mortality and the formation of mHTT aggregates. In addition, we identified a number of differentially-expressed genes in response to Ado signaling and showed that \textit{mod(mdg4)} is a downstream target of AdoR that mediates its effect in HD pathogenesis.

Results

**Phenotypes of Drosophila expressing mHTT**

To verify the effect of mHTT expression on \textit{D. melanogaster}, we used a \textit{UAS/GAL4} system for targeted gene expression. Flies overexpressing normal exon 1 from human huntingtin (Q20 HTT), or its mutant pathogenic form (Q93 mHTT), were driven by the pan-neuronal driver, \textit{elav-GAL4}. The results showed that expression of mHTT under the \textit{elav-GAL4}
driver in the *Drosophila* brain is not lethal during the larval stage (Fig. S2A) but reduces both the adult eclosion rate (Fig. S2B) and adult lifespan (Fig. S2C). These results are consistent with previous observations\(^\text{26}\).

**Disturbance of extracellular adenosine (e-Ado) homeostasis in HD larvae**

A recent study of human HD patients reported a reduced concentration of e-Ado in the cerebrospinal fluid \(^\text{27}\). To determine whether e-Ado levels are also altered in HD *Drosophila*, we compared e-Ado levels in the hemolymph of last-instar larvae ubiquitously expressing Q20 HTT and Q93 mHTT driven by the *daughterless-Gal4* driver (*da-GAL4*). The results showed that the e-Ado concentration in the hemolymph of Q93-expressing larvae was significantly lower compared to larvae expressing Q20 or control *da-GAL4* (Fig. 1A).

Since e-Ado concentration may be associated with the level of extracellular ATP (e-ATP), we also examined its titer in the hemolymph of larvae with the same genotypes as the above experiment. As shown in Fig. 1B, there was no significant difference in e-ATP levels between Q20, Q93, and control *da-GAL4* larvae. We thus postulated that the lower level of e-Ado in Q93 larvae might be affected by changes in proteins involved in Ado metabolism or transportation.

**Altered transcriptions of genes involved in Ado homeostasis in HD *Drosophila***

Earlier reports have shown that the expression of several genes involved in Ado homeostasis, including Ado receptor, transporters, and genes involved in Ado metabolism, are abnormal in human HD patients as well as in HD mice \(^\text{28}-\text{30}\). Since homologous proteins have also been shown to control Ado homeostasis in flies (Fig. S1), we compared the expression of three *Drosophila adgf* genes (*adgf-a*, *adgf-c*, *adgf-d*), adenosine kinase (*adenoK*), adenosine transporters (*ent1*, *ent2*, *ent3*, *cnt2*), and adenosine receptor (*adoR*) in the brains of Q93- and Q20-expressing larvae. The results showed that the expression of *adgf-a* and *adgf-d*, as well as transporters *ent1*, *ent2*, and *ent3* in the brain of Q93 larvae were significantly lower than in Q20 larvae (Fig. 1D). The expression of *cnt2* and *adoR* showed no difference between Q93 and Q20 larvae.
In order to assess progressive changes in transcription profiles associated with HD pathogenesis, we further examined the expression of genes involved in Ado homeostasis in the heads of 5- and 15-day-old adults, roughly corresponding to early- and late-stage HD (Fig. S2C). Unlike in the larval stage, the expression of metabolic genes adgf-c, adgf-d, and adenoK, and transporter ent1, in five-day-old adults was found to be higher in Q93 flies than Q20 flies (Fig. 1E). In addition, 15-day-old Q93 flies showed higher expression of adgf-d and adenoK (Fig. 1F). Previous studies in Drosophila have shown that the downregulation of the transporter ents decreases e-Ado concentration \(^{23,24}\); hence, the reduced expression of three ent genes could explain why the e-Ado level is lower in Q93 larvae. Moreover, it has also been shown that the expression of adgfs as well as adenoK follows the levels of e-Ado upon stress conditions \(^{31,32}\), suggesting that the lower expression of adgfs in Q93 larvae and the higher expression in Q93 adults might be a consequence of elevated e-Ado concentrations resulting from HD pathogenesis.

**Functional characterization of Ado homeostasis and signaling in HD flies**

To understand the effects of alterations in Ado homeostasis on polyQ pathology, we used the pan-neuronal driver, elav-GAL4, for RNAi-mediated silencing of the genes involved in Ado transport, metabolism, and adoR in Q93-expressing flies and assessed their survival and formation of mHTT aggregates. In addition, we also co-expressed Q93 with RNAi transgenes in the eyes by using the gmr-GAL4\(^{33,34}\) driver and compared levels of retinal pigment cell degeneration.

Silencing the transcriptions of Ado metabolic enzymes showed that only the RNAi of adgf-D increased the number of eclosion rate (Fig. 2A). Silencing adgf-A and adenoK, but not adgf-D or adgf-C RNAi, extended the adult lifespan of Q93-expressing flies (Fig. 2B). To ensure that the mortality of the HD flies was mainly caused by Q93 expression and not by RNAi constructs, we recorded the survival of flies co-expressing normal htt Q20 together with RNAi transgenes until all corresponding experimental flies (expressing Q93 together with RNAi constructs) died (Fig. S3A). However, silencing adgfs or adenoK only affected survival and did not significantly influence mHTT aggregation (Fig. 2C&D) or retinal pigment cell degeneration (Fig. 2E).
Next, we examined the RNAi silencing of adoR and Ado transporters in Q93 and control Q20 flies. The results showed that knocking down the expression of adoR as well as two transporters, ent1 and ent2, significantly increased the eclosion rate (Fig. 3A) and adult lifespan (Fig. 3B). The RNAi silencing of ent2 and adoR extended the lifespan of HD flies to 30 and 40 days, respectively, which is about 1.5~2 times longer than that of HD flies. In contrast, knocking down cnt2 expression did not change the viability of HD flies, and knocking down ent3 did not influence the eclosion rate, although it increased mortality and shortened the lifespan of adult HD flies. The survival of control flies expressing Q20 with individual RNAi constructs are shown in Fig. S3B. mHTT aggregation was significantly reduced (to 50%) in adoR RNAi flies (Fig. 3C&D), and a similar suppression of mHTT aggregate formation was also observed in 20-day-old HD flies (Fig. S4). An examination of eye phenotypes in ent2 RNAi flies showed a significant reduction in retinal pigment cell death (Fig. 3E), but surprisingly we did not observe a significant rescue of cell death by silencing adoR (Fig S5). We therefore postulated that it might be due to insufficient RNAi efficiency for suppressing AdoR signaling in the eye. To test this, we combined Q93 flies with the adoR RNAi transgene under a adoR heterozygote mutant background (AdoR<sup>1</sup>+/+) or with AdoR<sup>1</sup> homozygote mutant, and both showed significantly decreased retinal pigment cell degeneration similar to ent2–RNAi flies (Fig. 3E).

To further validate the RNAi results, we studied flies simultaneously expressing Q93 and overexpressing ent2, adoR, adgf-A, and adenoK in the brain and assessed the adult lifespans. Since silencing these genes extended the lifespan of HD flies (Figs. 2B&3B), we expected the opposite effect upon overexpression. As shown in Fig. S6A, ent2 overexpression significantly increased the mortality of early-stage HD flies; the survival of 5-day-old flies dropped to 60% for HD flies in contrast to 90% for Q93 control flies, and the lifespan of HD flies was significantly shorter than control flies expressing either Q93 alone or together with gfp RNAi. Consistently, we co-expressed strong and weak adoR overexpressing transgenes with Q93 and both significantly increased the mortality and shortened the lifespan of Q93 flies. The effects of shortening the lifespan were more severe than with ent2 overexpression. Nevertheless, the increase in mortality by adgf-A and adenoK overexpression was not as strong as that caused by ent2 and adoR overexpression, although both still showed a significant difference to either Q93 control or Q93/gfp RNAi.
control by weighted log-rank test (Fig. S6B). Hence, we concluded that overexpressing the examined genes enhances the effect of mHTT, resulting in the increased mortality of HD flies. Our results demonstrate that the overexpression and silencing of ent2 or adoR has a stronger influence over HD pathology than genes involved in Ado metabolism.

**Interactions of AdoR with ENT1 and ENT2**

In order to investigate whether there is a synergy between the effects of AdoR and ENTs, we co-expressed adoR RNAi constructs with ent1 RNAi or ent2 RNAi in Q93-expressing flies. As shown in Fig. 4A, the silencing of both ent2 and adoR has the same effect as silencing only adoR, indicating that ENT2 and AdoR are in the same pathway. Interestingly, the double knockdown of ent1 and adoR shows a sum of individual effects on lifespan which is longer than the knockdown of adoR alone. There seems to be a synergy between ENT1 and AdoR suggesting that ENT1 may have its own effect, which is partially independent from AdoR signaling.

Next, we investigated our hypothesis that the source of e-Ado, which contributes to AdoR activation in Q93 flies, is mainly intracellular and released out of the cells by ENTs. We conducted an epistasis analysis by combining mHTT with adoR overexpression and ent1 or ent2 RNAi. The results showed that adoR overexpression increased the mortality of Q93 flies while the combination of adoR overexpression with either ent1 or ent2 RNAi minimized the increased mortality caused by adoR overexpression (Fig. 4B). Notably, Q93 flies expressing ent2 RNAi and overexpressing adoR had the longest lifespan in comparison to Q93 control or ent1 RNAi flies. These results suggest that AdoR signaling needs functional Ado transportation to carry out its effect and thus the Ado efflux from these cells is needed for AdoR activity (Fig. 4C&D). The source of e-Ado, which contributes to AdoR activation causing HD pathogenesis, seems to be intracellular and it is mainly released out of the cells through ENT2.

**AMPK is not involved in Drosophila HD pathogenesis**

AMP-activated protein kinase (AMPK) is one of the key enzymes maintaining energy balance within a cell by adjusting anabolic and catabolic pathways; both Ado receptors and transporters have been implicated in its activation. Activation of AMPK is
beneficial at early stages in mammalian HD models; however, in the late stage of the disease it may worsen neuropathological and behavioral phenotypes.

To find out whether the above-described effects of e-Ado signaling and transport on HD flies are mediated by AMPK, we co-expressed Q93 mHTT with three different recombinant forms of AMPK α subunit, including wild-type AMPKα [M], a phosphomimetic-activated form of AMPKα [T184D], and dominant negative AMPK [DN], and assessed the survival of HD flies. The results showed that neither the activation nor the inhibition of AMPK signaling influenced the eclosion rate (Fig. S7A) or lifespan (Fig. S7B).

To further confirm the genetic data related to AMPK activation or inhibition, we pharmaceutically inhibited AMPK signaling by feeding the larvae with AMPK inhibitor, dorsomorphin (Compound C). The results showed that although dorsomorphin had an effect on the development of larvae expressing normal Q20 HTT, it did not influence the eclosion of Q93-expressing larvae (Fig. S7C). Overall, our results show that, unlike in mammalian HD models, AMPK signaling does not play a significant role in the pathological manifestations of mHTT in Drosophila.

Identification of potential downstream targets of the AdoR/ENT2 pathway by microarray analysis

Our above results indicate that ENT2 and AdoR contribute to mHTT pathogenesis in HD Drosophila and work in the same pathway. To identify their downstream target genes, we compared the expression profiles of larvae carrying mutations in adoR or ent2 as well as adult adoR mutants using microarrays (Affymetrix), shown as a Venn diagram in Fig. 5A and B. The intersection between each mutant contains differentially expressed transcripts in all three data sets, including six upregulated (Fig. 6A) and seven downregulated mRNAs (Fig. 5B). Interestingly, according to Flybase (http://flybase.org), four of these genes were expressed in the nervous system (ptp99A was upregulated, while CG6184, cindr, and mod(mdg4) were downregulated) (Fig. 5C).

To validate the microarray data, we knocked down adoR expression in the brain and examined the transcription of the four candidate genes expressed in the nervous system by qPCR. The results revealed that ptp99A and mod(mdg4) had the same expression trends as
observed in the microarrays (Fig. 5D). We further examined whether the expression of ptp99A and mod(mdg4) are influenced by an increase of e-Ado level. As shown in Fig. 6E, Ado microinjection significantly increased mod(mdg4) expression and decreased ptp99A expression, confirming that mod(mdg4) is positively regulated and ptp99A is negatively regulated by the AdoR/ENT2 pathway.

Suppression of mod(mdg4) decreased mHTT aggregation and increased survival of HD flies

In order to examine the potential roles of ptp99A, CG6184, cindr, and mod(mdg4) genes in HD pathogenesis, we used RNAi to silence them in HD flies. The results showed that only the RNAi silencing of mod(mdg4) extended their lifespan. As shown in Figure 6A, the survival curve of HD flies with a silenced mod(mdg4) gene was almost identical to the curve specific for adoR RNAi HD flies; this effect was stronger than in ent2 RNAi HD flies. In addition, mod(mdg4) RNAi significantly decreased the formation of mHTT inclusions (Fig. 6B&C) and suppressed retinal pigment cell degeneration (Fig. 7D). In contrast to mod(mdg4), RNAi silencing of the other three genes did not show any significant effect.

To further confirm that mod(mdg4) is downstream target of the AdoR pathway and regulated by e-Ado signaling, we first checked the expression of mod(mdg4) in larval brains and adult heads of HD flies using qPCR. In Q93 larvae, we found that both the expression level of mod(mdg4) (Fig. 7A) and the e-Ado level was lower than in Q20-expressing controls (Fig. 1A). For the 15-day-old (roughly corresponding to late-stage HD) Q93 adults, there was no difference in mod(mdg4) expression compared to Q20 control adults (Fig. 7A). We next examined the epistasis relationship between ent2, adoR, and mod(mdg4) by combining overexpression of ent2 or adoR mod(mdg4) RNAi in Q93-expressing flies. The results showed that mod(mdg4) RNAi suppressed the lethal effects caused by the overexpression of ent2 and adoR (Fig. 8B). These results indicate that mod(mdg4) serves as a downstream target of AdoR signaling involved in the process of mHTT inclusion formation and other pathogenic effects (Fig. 7C).

The mod(mdg4) locus of Drosophila contains several transcription units encoded on both DNA strands producing 31 splicing isoforms. As shown in Fig. 5B, two of the
mod(mdg4)-specific microarray probes which target 11 mod(mdg4) splicing isoforms (Tab. S2) were downregulated in all three datasets. We performed splice form-specific qPCR analysis and found that adoR RNAi silencing leads to the downregulation of multiple mod(mdg4) isoforms (Fig. 7D), suggesting that AdoR signaling regulates multiple isoforms.

**Discussion**

Considerable dysregulation of Ado homeostasis has been observed in HD human patients and mice, but the mechanisms of such changes related to HD pathogenesis still need to be characterized. The present study examined the e-Ado titer in the hemolymph of HD *Drosophila* larvae and found that it is lower in Q93-expressing larvae (Fig. 1). Although we did not measure the e-Ado titer in adult flies (due to a problem in acquiring a sufficient amount of hemolymph), the dynamic changes in expression levels of genes involved in Ado homeostasis (Fig. 1D-E), as well as the AdoR-regulated gene, *mod(mdg4)* (Fig. 8A), indicated that e-Ado titer and AdoR activity are variable in different stages of HD. Such dynamic changes of e-Ado homeostasis have also been observed in rodent HD models, whereby striatal adenosine tone is lower during the early stage of the disease and increased during the later stages.

Both the activation and inhibition of A2AR by pharmacological treatments have shown benefits in mammalian HD models. In R6/2 mice, the beneficial effect of activating A2AR is thought to occur via the inhibition of AMPK nuclear translocation (which contributes to HD pathogenesis including brain atrophy, neuron death, and increased mHTT aggregates formation). Beneficial effects by antagonizing A2AR with SCH58261 in R6/2 mice include reduced striatal glutamate and adenosine outflow as well as restoring emotional behavior and susceptibility to NMDA toxicity. A1R activation has also been shown to have neuroprotective effects; however, the chronic administration of A1R agonists (leading to a desensitisation of A1 receptors) increases neuronal loss whereas the chronic administration of A1R antagonists (inducing an upregulation of A1 receptors) improves survival and neuronal preservation in the same model. Our results show that the genetic depletion of AdoR has beneficial effects on HD flies, while the activation of AdoR contributes to mHTT pathogenesis and aggregates formation.
We observed a non-additive interaction between AdoR and ENT2 characteristic for epistasis relationship (Fig. 4B), indicating that ENT2 is required for the transportation of Ado from the intra- to extracellular environment which activates AdoR and, in turn, enhances the effects of mHTT. Our previous report showed that both ENT2 and AdoR participate in modulating synaptic transmission, and that both adoR and ent2 mutations cause defects in associative learning in Drosophila\textsuperscript{25}. Consistently, both the inhibition of Ado release by the knockdown of ent2 in hemocytes and the mutation of adoR suppress metabolic reprogramming and hemocyte differentiation upon immune challenges\textsuperscript{23}. Furthermore, another report showed that the disruption of epithelial integrity by Scribbled (Scrib) RNAi stimulates Ado release through ENT2, subsequently activating AdoR that, in turn, upregulates tumor necrosis factor (TNF) production which activates JNK signaling\textsuperscript{24}. Interestingly, while the effects of ent2 and adoR RNAi in HD flies were found to completely overlap, ent1 RNAi showed a synergistic effect, suggesting potential AdoR-independent mechanisms (Fig. 4A). These results correspond to our previous report showing that Drosophila ENT1 has lower specificity for Ado transportation in comparison to ENT2\textsuperscript{50}. The altered expression of ent1, as well as the RNAi effect in HD flies, might be associated with the disturbance of nucleotide homeostasis, similar to that observed in R6/2 and Hdh\textsuperscript{Q150} mice\textsuperscript{51}.

We identified a downstream target of the AdoR pathway, mod(mdg4), which contributes to the effects of mHTT in the Drosophila HD model. The mod(mdg4) gene has previously been implicated in the regulation of position effect variegation, chromatin structure, and neurodevelopment\textsuperscript{52}. The altered expression of mod(mdg4) has also been observed in flies expressing untranslated RNA containing CAG and CUG repeats\textsuperscript{53,54}. In addition, mod(mdg4) has complex splicing, including trans-splicing, producing at least 31 isoforms\textsuperscript{55}. All isoforms contain a common N-terminal BTB/POZ domain which mediates the formation of homomeric, heteromeric, and oligomeric protein complexes\textsuperscript{56-58}. Among these isoforms, only two [including mod(mdg4)-56.3 (isoform H) and mod(mdg4)-67.2 (isoform T)] have been functionally characterized. Mod(mdg4)-56.3 is required during meiosis for maintaining the chromosome pairing and segregation in males\textsuperscript{59,60}. Mod(mdg4)-67.2 interacts with Suppressor of hairy wing [Su(Hw)] and Centrosomal protein 190 kD (CP190) forming a chromatin insulator complex which inhibits the action...
of the enhancer on the promoter, and is important for early embryo development and oogenesis\textsuperscript{61-63}. Although our results showed that silencing all \textit{mod(mdg4)} isoforms decreases the effects of mHTT (Fig. 6), we could not clarify which of the isoforms is specifically involved in HD pathogenesis because AdoR signaling regulates multiple isoforms (Fig. 7D). Interestingly, an earlier report on protein two-hybrid screening indicated that Mod(mdg4) interacts with six Hsp70 family proteins\textsuperscript{64,65}, and Hsp70 proteins are known for their contribution to the suppression of polyQ aggregates formation and neurodegeneration \textsuperscript{66,67}. Further study will be needed to identify the specific \textit{mod(mdg4)} isoform involved in HD pathogenesis, and whether a decrease in mHTT aggregates by \textit{mod(mdg4)} RNAi is connected to Hsp70 interaction.

In summary, we observed an alteration in the e-Ado concentration and expression of genes involved in Ado homeostasis in a \textit{Drosophila} HD model. By candidate RNAi screening, we demonstrated that the silencing of \textit{ent2} and \textit{adoR} increases the survival of HD flies in addition to suppressing retinal cell degeneration and mHTT aggregate formation. We also showed that the activation of e-Ado signaling enhances the effects of mHTT. Furthermore, we found that \textit{mod(mdg4)} is a downstream target of the AdoR pathway and plays a major role in the pathogenesis of HD flies. Our work enhances our understanding of e-Ado signaling in HD pathogenesis and may open up new opportunities for HD pharmacological intervention.

**Materials and methods**

**Fly stocks**

Flies were reared at 25 °C on standard cornmeal medium. The following RNAi lines were acquired from the TRiP collection (Transgenic RNAi project) at Harvard Medical School: adgfA-Ri (BL67233), adgfC-Ri (BL42915), adgfD-Ri (BL56980), adenoK-Ri (BL64491), ent1-Ri (BL51055), ador-Ri (BL27536), gfp-Ri (BL41552), mod(mdg4)-Ri (BL32995), cindr-Ri (BL38976), and ptp99A-Ri (BL57299). The following RNAi lines were acquired from the Vienna Drosophila RNAi Center (VDRC): ent2-Ri (ID100464), ent3-Ri (ID47536), cnt2-Ri (ID37161), and cg6184-Ri (ID107150). The following lines were
provided by the Bloomington Drosophila Stock Center: UAS-AMPKα\textsuperscript{T184D} (BL32110), UAS-AMPKα\textsuperscript{M} (BL32108), UAS-AMPKα\textsuperscript{DN} (AMPKα\textsuperscript{K57A}, BL32112), and elav\textsuperscript{C155-}

GAL4 (BL458).

Flies overexpressing human normal huntingtin (HTT) exon 1, Q20Htt\textsuperscript{exon1111F1L} or mutant pathogenic fragments (mHTT), Q93Htt\textsuperscript{exon14F132} were obtained from Prof. Lawrence Marsh (UC Irvine, USA)\textsuperscript{17}. The UAS-overexpression lines, Ox-adenoK and Ox-adoR (s), were obtained from Dr. Ingrid Poernbacher (The Francis Crick Institute, UK)\textsuperscript{24}. gmr-GAL4 was obtained from Dr. Marek Jindra (Biology Centre CAS, Czechia). da-GAL4 was obtained from Dr. Ulrich Theopold (Stockholm University). The UAS overexpression strains Ox-adgfA, Ox-ent2, and Ox-adoR (w), as well as adoR\textsuperscript{1} and ent2\textsuperscript{3} mutant flies, were generated in our previous studies\textsuperscript{25,68-70}.

**Eclosion rate and adult lifespan assay**

For assessing the eclosion rate, male flies containing the desired RNAi or overexpression transgene (RiOx) in the second chromosome with genotype w\textsuperscript{1118}/Y; RiOx /CyO; UAS-Q93/MKRS were crossed with females of elav-GAL4; +/-; +/-+. The ratio of eclosed adults between elav-GAL4/+; RiOx/++; UAS-Q93/+ and elav-GAL4/+; RiOx/++; +/MKRS was then calculated. If the desired RiOx transgene was in the third chromosome, female flies containing elav-GAL4; +/-; RiOx were crossed with male w\textsuperscript{1118}/Y; +/-; UAS-Q93/MKRS, and the ratio of eclosed adults between elav-GAL4; +/-; RiOx/UAS-Q93 and elav-GAL4; +/-; RiOx/MKRS was calculated.

For the adult lifespan assay, up to 30 newly emerged female adults were placed in each cornmeal vial and maintained at 25 °C. At least 200 flies of each genotype were tested, and the number of dead flies was counted every day. Flies co-expressing RiOx and Q20 were used for evaluating the effect of RNAi or overexpression of the desired transgenes (Fig. S3A&B).

**Extracellular adenosine and ATP level measurements**

To collect the hemolymph, six third instar larvae (96 hours post-oviposition) were torn in 150 µl of 1x PBS containing thiourea (0.1 mg/ml) to prevent melanization. The samples
were then centrifuged at 5000×g for 5 min to separate the hemocytes and the supernatant was collected for measuring the extracellular adenosine or ATP level. For measuring the adenosine titer, 10 µl of hemolymph was mixed with reagents of an adenosine assay kit (Biovision) following the manufacturer’s instructions. The fluorescent intensity was then quantified (Ex/Em = 533/587 nm) using a microplate reader (BioTek Synergy 4). For measuring the ATP level, 10 µl of hemolymph was incubated with 50 µl of CellTiter-Glo reagent (Promega) for 10 min. Then, the luminescent intensity was quantified using an Orion II microplate luminometer (Berthold). To calibrate the standard curve of ATP concentration, 25 µM ATP standard solution (Epicentre) was used for preparing a concentration gradient (0, 2, 4, 6, 8, 10 µM) of ATP solution and the luminescent intensity was measured for each concentration. The protein concentration of the hemolymph sample was determined by A280 absorbance using a NanoDrop 2000 spectrophotometer (Thermo Fisher). The adenosine and ATP concentrations were first normalized to protein concentration. Then, the values of Q20 and Q93 samples were normalized to values of the GAL4 control sample. Six independent replicates for each genotype were performed for the analysis of adenosine and ATP levels.

RNA extraction

The brains from 10 third-instar larvae (96 hours post-oviposition), heads from 30 female adults (5 days or 15 days old) or 15 whole female flies were collected. The samples were first homogenized in RiboZol (VWR) and the RNA phase was separated by chloroform. For brain or head samples, the RNA was precipitated by isopropanol, washed in 75% ethanol and dissolved in nuclease-free water. For whole fly samples, the RNA phase was purified using NucleoSpin RNA columns (Macherey-Nagel) following the manufacturer’s instructions. All purified RNA samples were treated with DNase to prevent genomic DNA contamination. cDNA was synthesized from 2 µg of total RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

Adenosine injection
Three- to five-day-old female adults were injected with 50 nl of 10 mM adenosine using a NANOJECT II (Drummond Scientific); control flies were injected with 50 nl of 1× PBS. Two hours post-injection, 15 injected flies for each replicate were collected for RNA extraction.

**Microarray analysis**

The Affymetrix GeneChip® *Drosophila* genome 2.0 array system was used for microarray analysis following the standard protocol: 100 ng of RNA was amplified with a GeneChip 3′ express kit (Affymetrix), and 10 μg of labeled cRNA was hybridized to the chip according to the manufacturer’s instructions. Statistical analysis of array data were described previously in our studies\(^{71,72}\). Storey’s q value (false discovery rate, FDR) was used to select significantly differentially transcribed genes (q < 0.05). Transcription data are shown in Table S2.

**qPCR and primers**

5× HOT FIREPol® EvaGreen® qPCR Mix Plus with ROX (Solis Biodyne) and an Eco Real-Time PCR System (Illumina) were used for qPCR. Each reaction contained 4 μl of EvaGreen qPCR mix, 0.5 μl each of forward and reverse primers (10 μM), 5 μl of diluted cDNA and ddH₂O to adjust the total volume to 20 μl. The list of primers is shown in Table S1. The expression level was calculated using the 2^−ΔΔCt method. The ct values of target genes were normalized to reference gene, ribosomal protein 49 (*rp49*).

**Imaging of retinal pigment cell degeneration**

Twenty- and thirty-day-old female adults were collected and their eye depigmentation phenotypes were recorded. At least 30 individuals for each genotype were examined under a microscope, and at least five representative individuals were chosen for imaging. Pictures were taken with an EOS 550D camera (Canon) mounted on a SteREO Discovery V8 microscope (Zeiss).

**Immunostaining**
Brains dissected from 10- or 20-day-old adult females were used for immunostaining. The brains were fixed in 4% PFA, permeabilized with PBST (0.1% Triton X-100), blocked in PAT (PBS, 0.1% Triton X-100, 1% BSA) and stained with antibodies in PBT (PBS, 0.3% Triton X-100, 0.1% BSA). Primary antibodies used in this study were mouse anti-HTT, MW8 which specifically binds to mHTT aggregates (1:40, DSHB), and rat anti-Elav (1:40, DSHB) which is a pan-neuronal antibody. Secondary antibodies were Alexa Fluor 488 anti-mouse and Alexa Fluor 647 anti-rat (1:200, Invitrogen). The samples were mounted in Fluoromount-G (Thermo Fisher) overnight, prior to image examination.

Quantification of mHTT aggregates
Images of aggregates were taken using a Flowview 100 confocal microscope (Olympus). The intensity of mHTT aggregates detected by anti-HTT antibody (MW8) or anti-Elav were quantified using ImageJ software. The level of mHTT aggregates was quantified by normalizing the mHTT aggregates intensity to Elav intensity. At least six brain images from each genotype were analyzed.

AMPK inhibitor (dorsomorphin) feeding
Thirty first instar of Q20- or Q93-exexpressing larvae were collected for each replicate 24 hours after egg laying. Larvae were transferred to fresh vials with 0.5 g instant Drosophila medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) supplemented with 2 mL distilled water containing either dorsomorphin (100 μM) or DMSO (1%). Total number of emerging adults were counted.

Statistical analysis
Error bars show standard error of the mean throughout this paper. Significance was established using Student’s t-test (N.S., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) or one-way ANOVA analysis with Tukey’s HSD post-hoc test. For the statistical analysis of survival curves, we used the online tool OASIS 2 to perform a weighted log-rank test (Wilcoxon-Breslow-Gehan test) for determining significance.73
Figure 1. Alteration of adenosine homeostasis in the Drosophila HD model. (A-B) The measurements of extracellular adenosine levels (A) and extracellular ATP levels (B) in Q93-expressing (da>Q93), Q20-expressing (da>Q20) and control da-GAL4 (da+/+) larvae. Six independent replicates were measured. Significance was analyzed by ANOVA with Tukey’s HSD post-hoc test; significant differences (P < 0.05) among treatment groups are marked with different letters. (C) Diagram showing the interaction of adenosine metabolic enzymes, transporters, and receptors in Drosophila. (D-F) Expression profiles of genes involved in adenosine metabolism (green) and adenosine transportation (blue) as well as adenosine receptors (orange) at different stages in HD Drosophila brains (larvae) or heads (adults). The expression of Q20 and Q93 were driven by the pan-neuronal driver (elav-GAL4). Three independent replicates were measured. The significances of results were examined using Student’s t-test: *P < 0.05, **P < 0.01, ***P < 0.001; N.S., not significant. All data are presented as mean ± SEM.
Figure 2. RNAi-mediated (Ri) downregulation of adenosine metabolic genes in HD

_Drosophila_. Co-expression Q93 with each RNAi transgenes were driven by the pan-
neuronal driver, _elav-GAL4_ (A-D), or eye driver, _gmr-GAL4_ (E). The adult eclosion rate
(A), adult lifespan (B), mHTT aggregate levels (C-D), and retinal pigment cell
degeneration (E) were compared. † Eye image of homozygous _adoR^1_ mutant without _htt_
expression. At least five independent replicates were measured for eclosion rate. Detailed
methodologies of the lifespan assay, eye imaging, and quantification of mHTT aggregates
are described in Materials and methods. Significance values of the eclosion rate (A) and
mHTT aggregates levels (D) were analyzed by ANOVA with Tukey’s HSD _post-hoc_ test;
significant differences (_P_ < 0.05) among treatment groups are marked with different letters.
Significance values for the adult lifespan curve (B) were analyzed by a weighted log-rank
test, and significant differences between control _gfp-Ri_ flies with each RNAi group are
labeled as follows: * _P_ < 0.05; N.S., not significant. Error bars are presented as mean ±
SEM
Figure 3. RNAi-mediated (Ri) downregulation of adenosine transporters and adenosine receptor (adoR) in HD Drosophila. Co-expression of Q93 with each RNAi transgene was driven by the pan-neuronal driver, elav-GAL4 (A-D), or eye driver, gmr-GAL4 (E). The adult eclosion rate (A), adult lifespan (B), mHTT aggregate levels (C-D), and retinal pigment cell degeneration (E) were compared. At least five independent replicates were measured for eclosion rate. Detailed methodologies of the lifespan assay, eye imaging, and quantification of mHTT aggregates are described in Materials and methods. Significance values for eclosion rate (A) and mHTT aggregates levels (D) were analyzed by ANOVA with Tukey’s HSD post-hoc test; significant differences (P < 0.05) among treatment groups are marked with different letters. Significance values for the adult lifespan curve (B) were analyzed by a weighted log-rank test; significant differences comparing control gfp-Ri with each RNAi group are labeled as follows: *P < 0.05; N.S., not significant. Error bar are presented as mean ± SEM.
Figure 4. Interactions of AdoR and ENTs in HD Drosophila. (A) Co-expression of adoR RNAi with ent1 or ent2 RNAi in HD flies. (B) Co-expression of adoR overexpressing construct (Ox-adoR) with ent1 or ent2 RNAi transgenes in HD flies. Significance values of the adult lifespan curve were analyzed by a weighted log-rank test; different letters indicate significant differences ($P < 0.05$) among treatment groups. (C-D) Diagrams showing the action of Ado in mHTT pathogenesis.
Figure 5. Identification of potential downstream targets of AdoR by microarray analysis. (A-B) Venn diagram showing the number of common genes (in intersect region) which are upregulated (A) or downregulated (B) among the *adoR* mutant larvae vs. control (*w*^1118^), *adoR* mutant adults vs. control (*w*^1118^), and *ent2* mutant larvae vs. control (*w*^1118^). The cutoff values for expression differences were set at Q < 0.05 (false discovery rate, FDR). (C) The intersection between the three datasets; tissue localization of each gene expression was obtained from Flybase (http://flybase.org/). Tissue abbreviations: midgut (MG), hindgut (HG), Malpighian tubule (MT), imaginal disc (ID), integument (I), sensory system (SS), nervous system (NS), trachea (T), testis (tes), nonspecific expression (non-spec) (D) qPCR confirmed the potential AdoR-regulated genes expressed in the nervous system. Expression of *adoR* RNAi transgenes (*adoR*-Ri) was driven by the pan-neuronal driver (elav>*adoR*-Ri), and control flies contained *elav*-GAL4 (*elav/+*) only. (E) Enhancing extracellular adenosine signaling by adenosine injection and qPCR examination demonstrated that *mod(mdg4)* is positively- and *ptp99A* is negatively-regulated by adenosine signaling. Three independent replicates were measured in qPCR experiments. The qPCR primers of *mod(mdg4)* were selected to target the common 5′ exon shared in all of the isoforms. Student’s t-test was used to examine the significance of qPCR results: *P* < 0.05, **P* < 0.01, ***P* < 0.001; N.S., not significant. Error bars are presented as averages ± SEM.
Figure 6. RNAi-mediated (Ri) downregulation of potential downstream targets of AdoR signaling in HD Drosophila. Co-expression of Q93 with each RNAi transgene, including ptp99A, CG6184, cindr, and mod(mdg4), were driven by the pan-neuronal driver, elav-GAL4 (A-B), or the eye driver, gmr-GAL4 (D). The adult lifespan (A), mHTT aggregate levels (B-C), and retinal pigment cell degeneration (D) were compared. A detailed methodology of the lifespan assay, eye imaging, and quantification of mHTT aggregates are described in Materials and methods. Significance values of the adult lifespan curve (A) were analyzed by a weighted log-rank test, and different letters indicate significant differences ($P < 0.05$) among treatment groups. Significance values of mHTT aggregate levels (C) were analyzed by ANOVA with Tukey’s HSD post-hoc test; significant differences ($P < 0.05$) among treatment groups are marked with different letters. Error bars are presented as mean ± SEM.
Figure 7. mod(mdg4) as a AdoR-regulated gene contributes to HD pathogenesis. (A) qPCR analysis of the expression of mod(mdg4) in the larval brain and 15-day-old adult heads of Q20- and Q93-expressing flies. The qPCR primers of mod(mdg4) targeted the common 5' exon shared by all isoforms. (B) Epistasis analysis showed that ent2 (Ox-ent2) and adoR overexpression (Ox-adoR) with mod(mdg4) RNAi transgenes in HD flies decreased the mortality effect caused by ent2 and adoR overexpression. This suggests that mod(mdg4) is downstream of the AdoR pathway (C). qPCR identified potential mod(mdg4) isoforms regulated by the AdoR pathway. adoR RNAi transgene (adoR-Ri) expression was driven by the pan-neuronal driver (elav>adoR-Ri); control flies contained only elav-GAL4 (elav/+). Mod_all indicates that the primers targeted all mod(mdg4) isoforms. Isoforms L and G do not have their own unique exonal region, therefore it is possible for the qPCR primers to target two isoforms simultaneously (presented as RG&RG and RL&RK). qPCR result significance was examined using Student’s t-test: *P < 0.05, **P < 0.01, ***P < 0.001; N.S., not significant. Significance values for the adult lifespan curve (A) were analyzed by weighted log-rank test, and different letters indicate significant differences (P < 0.05) among treatment groups. Error bars are presented as mean ± SEM.
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Author Contributions

Y-HL performed the experiments and prepared the manuscript. HM assisted in recording the adult lifespan and eye phenotypes as well as performed the brain dissection, immunochemistry and confocal microscopy imaging. LK performed the sample preparation and analyzed the microarray data. LR assisted in recording the adult lifespan, eye phenotype and prepared fly strains. TF established the methodologies for recording the eclosion rate, survival and prepared fly strains. MZ conceived the project and supervised manuscript preparation.

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