Functional conservation in human and *Drosophila* of Metazoan ADAR2 involved in RNA editing: loss of ADAR1 in insects

Liam P. Keegan[1],*, Leeane McGurk[1], Juan Pablo Palavicini[2], James Brindle[1], Simona Paro[1], Xianghua Li[1], Joshua J. C. Rosenthal[2] and Mary A. O'Connell[1],*

[1]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK and [2]Institute of Neurobiology, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico 00901, USA

ABSTRACT

Flies with mutations in the single *Drosophila Adar* gene encoding an RNA editing enzyme involved in editing 4% of all transcripts have severe locomotion defects and develop age-dependent neurodegeneration. Vertebrates have two ADAR-editing enzymes that are catalytically active; ADAR1 and ADAR2. We show that human *ADAR2* rescues *Drosophila Adar* mutant phenotypes. Neither the short nuclear ADAR1p110 isoform nor the longer interferon-inducible cytoplasmic ADAR1p150 isoform rescue walking defects efficiently, nor do they correctly edit specific sites in *Drosophila* transcripts. Surprisingly, human ADAR1p110 does suppress age-dependent neurodegeneration in *Drosophila Adar* mutants whereas ADAR1p150 does not. The single *Drosophila Adar* gene was previously assumed to represent an evolutionary ancestor of the multiple vertebrate ADARs. The strong functional similarity of human ADAR2 and *Drosophila Adar* suggests rather that these are true orthologs. By a combination of direct cloning and searching new invertebrate genome sequences we show that distinct *ADAR1* and *ADAR2* genes were present very early in the Metazoan lineage, both occurring before the split between the Bilateria and Cnidarians. The *ADAR1* gene has been lost several times, including during the evolution of insects and crustacea. These data complement our rescue results, supporting the idea that ADAR1 and ADAR2 have evolved highly conserved, distinct functions.

INTRODUCTION

The conversion of adenosine (A) to inosine (I) by RNA editing occurs in CNS transcripts in both *Drosophila* and humans, diversifying ion channels and many other proteins [for reviews see (1,2)]. The ADAR RNA editing enzymes recognize specific adenosines within RNA duplexes that form, typically by base pairing between edited exons and sequences in adjacent introns, in edited transcripts. ADARs have two or more double-stranded (ds) RNA binding domains that bind dsRNA (3), and a catalytic deaminase domain that also contributes to recognition of bases adjacent to the edited site (Figure 1A). Although the ADAR RNA editing enzymes are conserved, the editing events in particular transcripts are not; edited transcripts differ substantially between fly and human and no clear example of a conserved editing site has been found. In *Drosophila* editing is extensive. A recent study identified 972 edited positions within transcripts of 597 genes, 630 of which are predicted to alter protein-coding sequences (4) It is not known which editing events are responsible for the *Adar* phenotype (5,6). Other invertebrates such as the squid, a member of the Phylum Mollusca, also show extensive RNA editing of CNS transcripts (7–10). Vertebrates have far fewer editing events that result in recoding of transcripts and only one editing event is essential (11). One recent study identified 239 edited sites in 207 human transcripts, but only 38 are predicted to change codons (12).

Mutations to both *Drosophila* and vertebrate *ADAR* genes have catastrophic effects on the CNS. *Drosophila* has a single *Adar* gene and mutations cause a loss of locomotion in adult flies from birth and drastic age-dependent neurodegeneration (13,14). Vertebrates have two catalytically active *ADAR* genes and mutations in one of

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them, the CNS-expressed *Adar2* gene, leads to seizures and early postnatal death with localized hippocampal neurodegeneration in mice (11). The mouse *Adar2* mutant is rescued by genomically encoding a single residue change in a key AMPA class glutamate receptor subunit transcript that is normally introduced by editing. By replacing a glutamine (Q) codon with an arginine (R) codon within the region of *GluR2* transcripts that encodes the ion channel pore, *Adar2* mutant mice survive to adulthood. Editing at this site has the key functions of both restraining the assembly of AMPA receptors to synapses and blocking calcium entry through the resulting channels (15,16). Reductions in RNA editing efficiency at this site leads to production of calcium-permeable AMPA receptors and may be involved in disease symptoms such as motor neuron death through glutamate excitotoxicity in ALS (17), and selective neuron death following ischaemia in stroke (18).

Vertebrates have two other *ADAR* genes; *ADAR1* is widely expressed within the CNS as well as in mesoderm and haematopoietic lineages. Mutations in *Adar1* result in death of mouse embryos by embryonic day 12.5 with failure of haematopoiesis in the liver and overproduction of interferon (19–21), preventing the role of *Adar1* in the CNS from being assessed. ADAR1 has an intrinsic RNA editing site specificity that is distinct from that of ADAR2, however to date no site-specific editing event catalysed by ADAR1 has been found to be essential. This enzymatic substrate specificity is surprising considering the overall homology between the two proteins and also that the major groove in the A structure of dsRNA is inaccessible, rendering it difficult for proteins to read the actual base sequence of dsRNA substrates (22). Selection of particular adenosines for editing at different RNA editing sites is likely to be determined by the location of the edited base within the duplex and by its proximity to imperfect pairings between base pairs in each duplex structure (3). In addition both ADAR1 and ADAR2 have distinct yet overlapping preferences for particular nucleotides 5′ and 3′ of the editing sites when editing long dsRNA (23,24). There is some evidence of competition between ADAR1 and ADAR2 in editing: in neurons cultured from *Adar1*−/− ES cells loss of ADAR1 leads to increases in RNA editing by ADAR2 at some sites in transcripts encoding 5-HT$_{2C}$ receptor (19,20).

Until recently the single *Drosophila Adar* gene appeared to be an invertebrate ancestor of both human ADARs and we wondered if it had similar or distinct substrate specificity to the human ADARs. As the edited sites in target transcripts are not conserved, the ADARs may also have diverged in their substrate specificities. We investigated this with RNA editing assays *in vitro* and by expressing the human ADARs in *Drosophila,* to determine if they can edit *Drosophila* transcripts, rescue locomotion defects and suppress neurodegeneration. It is advantageous to perform this analysis in *Drosophila* as there are a large number of editing sites in the fly to compare the editing site specificities of the different ADARs.

Surprisingly, we find that the editing specificity of an ADAR2-type protein is conserved from fly to human, allowing effective rescue of site-specific RNA editing

events, locomotion defects and suppression of neuro-degenerative phenotypes in *Adar* mutant flies by human ADAR2. ADAR1 does not efficiently edit most sites in *Drosophila* transcripts nor does it rescue the locomotion phenotype. However the different ADAR1 isoforms behave differently with regard to the neurodegeneration phenotype; ADARp110 suppress neurodegeneration whereas ADAR1p150 does not.

We conclude that *Drosophila Adar* is an orthologue of vertebrate *ADAR2*. By cloning *ADAR* genes from invertebrates and by examining data from genome sequencing projects, particularly that of the starlet sea anemone *Nematostella vectensis* (25), we show that *ADAR1* and *ADAR2* have evolved independently since early in Metazoan evolution. Both *ADAR1* and *ADAR2* genes are present in molluscs, annelids, echinoderms and even cnidarians. *ADAR1* appears to have been lost in some Arthropods, including insects, as well as in some other taxa.

MATERIALS AND METHODS

Comparison of RNA editing site specificities of *Drosophila* and vertebrate ADARs *in vitro*

All recombinant ADAR proteins were expressed and purified from *Pichia pastoris* as previously described (26). Poisoned primer extension assays in the presence of dideoxythymidine were performed with equivalent concentrations of ADAR proteins as described in (27).

Rescue of *Adar* mutant phenotypes in *Drosophila* by human *ADAR1* and *ADAR2*

cDNAs encoding full length human ADARs were cloned into the vector *pUAST* and multiple balanced transgenic *Drosophila* lines were generated with constructs inserted randomly at different locations on Chromosomes II or III. These construct lines were crossed to lines expressing GAL4 ubiquitously and strongly in all cells [*actin 5C-GAL4 25FO1* driver (28)], or strongly in cholinergic neurons [*Cha-GAL4 19B, UAS-GFP S65T* driver (29) also expressing an enhanced GFP from Chr. II]. To express ADARs in an *Adar*^{5G1} mutant background under the control of the *Cha-GAL4* driver, for example, we crossed the *UAS-ADAR* lines to females of a strain that had the first and second chromosome genotypes *y, Adar*^{5G1}*, w/w, FM6 Bar; Cha-GAL4 / SM5 Cy* and picked male *y, Adar*^{5G1}*, w; Cha-GAL4, UAS-ADAR* progeny to measure rescue of mutant phenotypes.

We also constructed a strain that had the first and second chromosome genotypes *y, Adar*^{5G1}*, w /w, FM6 Bar; UAS-dADAR S / SM5 Cy*. This strain has no GAL4 driver but it allows the rescue effectiveness of drivers expressing GAL4 in different cell types to be tested. Crossing males of some GAL4 driver lines to females of this strain gives male *y, Adar*^{5G1}*, w; GAL4 driver; UAS-Adar S* progeny in which phenotypes are rescued by expression of the *UAS-dAdar S* construct in particular cell types.

Open field locomotion assay

We measured phenotypic rescue of *Adar^1F4* and *Adar^5G1* locomotion defects with an open field locomotion assay on flies expressing the human *UAS-ADAR* constructs 2–4 days after eclosion (30). Flies were collected using CO_2 and left for 1 day to recover before performing this assay. They were placed in a 30-mm petri dish divided into seven equal areas. The dishes were tapped and the number of times a fly walked over a line separating the zones was recorded for a 2-min period. This was then repeated a further two times for each individual fly. For each *UAS-ADAR* construct multiple different transgenic lines with random insertions were generated to control for variations in expression levels due to insertion sites. Locomotion rescue was measured for 10 or more flies from each of three different transgenic lines for each construct. RNA editing *in vivo* and protein expression levels were determined for the line of each construct that rescued locomotion best or that showed the darkest red eye colour, another correlate of expression levels at different sites of chromosomal insertion.

Other *Drosophila* GAL4 driver lines used in this study

w^1118; Ddc-Gal4 L 4.3D on Chr. II expresses GAL4 in the pattern of dopa decarboxylase which is involved in synthesis of the excitatory neurotransmitter dopamine in dopaminergic neurons. *Tdc2-GAL4 C 2* on Chr. III expresses GAL4 in the pattern of tyrosine decarboxylase which is involved in synthesis of the excitatory neurotransmitter octopamine in octopaminergic neurons. Expression of two of the three motor neurone driver lines have been examined in detail elsewhere (31). The OK6 line has a GAL4 enhancer trap insertion in the *Rapgap1* gene on Chr. II and is the driver line most highly specific for motor neurons. The D42 line is a GAL4 enhancer trap insertion in the *toll6* gene on Chr. III (31). It is expressed in a very small number of brain cells and in peripheral nervous system in addition to motor neurones. *w^1118; VGlut^OK371* has a GAL4 enhancer trap insertion on Chr. II in the gene encoding the vesicular glutamate vesicular uptake receptor (32), broadly expressed in all glutamatergic neurons including motor neurons. *w^1118; OK307* is a GAL4 enhancer trap insertion on Chr. II that is expressed specifically in the giant fibre descending jump escape neuron.

Haematoxylin and eosin staining

To characterize neurodegeneration 6-μm sections of paraffin wax-embedded *Adar^5G1* mutant heads were cut and stained with haematoxylin and eosin. To remove the wax the slides were taken through three 5-min incubations in Xylene. To re-hydrate, the slides were incubated twice in 100% ethanol for 2 min, 90% ethanol for 2 min, 80% ethanol for 2 min, 50% ethanol for 2 min, 30% ethanol for 2 min and finally in H_2O for 2 min. The slides were incubated in freshly filtered haematoxylin for 4 min and then in running tap water. Once the haematoxylin had washed out the slides were dipped twice into acid alcohol and again washed in running tap water. The slides were incubated in lithium carbonate for 3 min and then in water for 3 min. The slides were incubated in 1% eosin for 4 min and quickly washed in running tap water. The slides were dipped in 100% ethanol and then incubated three times in 100% ethanol each for 2 min. Before mounting the slides were incubated in Xylene three times, each for 5 min. The slides were mounted with D.P.X. and eyes were photographed at 40× and mushroom bodies at 63× with Zeiss Plan Neofluor objectives on a Zeiss Axiophot compound microscope with Coolsnap HQ CCD camera (Photometrics Ltd. Tuscon, AZ, USA) and images processed using IPLab Spectrum (Scanalytics Corp, Fairfax VA, USA) with all alterations of brightness and contrast covering the entire image.

Oligos, RT–PCR and sequencing

The oligos used in this study to perform RT–PCR and for sequencing the edited positions are listed in Supplementary Table S1.

Quantitating RNA editing activity *in vivo*

RNA was extracted from rescue and control male flies with Trizol reagent (Invitrogen) as described by the manufacturer and sequential RT–PCR was performed on the isolated RNA. To ensure that each RT–PCR product sequenced represents a distinct initial first strand cDNA, two separate RT reactions were performed. The majority of the editing sites were analysed by sequencing the RT–PCR reaction product pools and not by sequencing individual clones. We measured the relative heights of A and G peaks in electropherograms of RT–PCR product pools covering edited sites. Editing at each site was determined using multiple sequence chromatograms in each direction. To indicate the variability in this data: for percentage editing in adult male flies at *Eag* 2107 Y/C in Table 1 the standard error is ±2% flies and for editing at *Eag* 2159 V/V the standard error is ±2.9%. If editing appeared to be zero at a position but there was a low background in the electropherogram then we inserted an asterisk in the tables to represent this.

Phylogenetic analysis of invertebrate ADAR1 and ADAR2

Putative ADAR sequences were identified using blast searches (tblastn or blastp) against invertebrate genome sequences available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm .nih.gov/sutils/genom_table.cgi?organism=euk) and the Joint Genome Institute (JGI- (http://genome.jgi-psf. org/). Initially human ADAR1 and ADAR2 were used as query sequences. As we identified invertebrate homologues, they were used as queries as well. Cephalopod ADAR deaminase domains were cloned directly using cDNA samples and PCR primers based on other invertebrate ADAR sequences. Putative ADAR hits were defined as ADAR1 or ADAR2 using several criteria. First, the core deaminase domains were aligned with vertebrate

Table 1. Percentage RNA editing at specific sites in transcripts isolated from whole wildtype Canton S male or female flies, embryos and third instar larvae

	Male	*n*	Female	*n*	Embryo	*n*	Larva	*n*
Caα1D								
2061 L/L	**36**	4	**38**	4	**0**	5	0	2
2083 N/D	**97**	4	**95**	4	**22**	6	20	3
2097 L/L	**96**	4	**89**	4	[a]	4	**0**	3
2098 R/G	**96**	4	**92**	4	[a]	4	**0**	3
2140 I/M	**100**	2	**100**	4	**14**	6	18	3
Eag								
1864 K/R	**58**	11	**66**	4	**76**	2	89	3
2107 Y/C	**89**	11	**92**	5	**46**	3	70	5
2159 V/V	**16**	7	[a]	5	**0**	3	[a]	4
2163 N/D	**88**	7	**86**	5	**52**	3	66	4
2560 K/R	**78**	3	**60**	2	[a]	2	**0**	3
Nic 34E								
1872 L/L	**100**	6	**76**	4	**85**	4	100	4
1873 I/V	**100**	6	**78**	4	**85**	4	100	4
2020 T/A	**100**	7	**97**	6	**100**	4	100	3
2023 I/V	**38**	5	**30**	5	**16**	4	17	3
2028 L/L	**35**	5	**28**	3	**15**	2	15	3
2037 I/M	**67**	5	**60**	3	**41**	2	48	3
2049 L/L	**16**	4	**17**	3	**0**	2	[a]	3
2052 S/S	**71**	4	**63**	1	**40**	1	40	3
2062 I/V	**100**	4	**100**	2	**100**	2	100	3
2065 I/V	**53**	4	**41**	1	**15**	2	11	3
Rdl								
728 L/L	**23**	8	**23**	4	**0**	2	**0**	2
735 R/G	**65**	8	**68**	4	**0**	2	[a]	2
1218 I/V	**100**	8	**87**	8	**78**	2	100	2
1251 N/D	**22**	4	**14**	8	**0**	1	**0**	2
1448 Q/Q	**8**	4	**12**	7	**0**	2	**0**	2
1449 M/V	**22**	4	**20**	7	**0**	2	**0**	2

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the different samples. The total number of RT–PCR reactions sequenced is represented by *n*.
[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.

ADAR1 and ADAR2 using T-COFFEE (http://tcoffee .vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) to assess general homology with residues previously defined as ADAR1 or ADAR2 consensus. Second, phylogenetic trees were generated using the entire deaminase domain. Alignments for ADAR1 and ADAR2 were generated using M-COFFEE (http://tcoffee.vital-it.ch/cgi-bin/ Tcoffee/tcoffee_cgi/index.cgi). Both alignment files were joined by ClustalX2 (profile mode). Gap-rich columns were removed from each alignment. The tree was generated using Phylip Package (Protdist, Neighbor, Consense) (http://bioweb.pasteur.fr/phylogeny/intro-en .html). In the following cases only partial sequences were available; *Varroa destructor* (ADAR1 and ADAR2), *Helobdella robusta* (ADAR1), *Acropora millepora* (ADAR1) See Supplementary Table S2 for the names of species in different evolutionary groups and for sequence accession numbers. For these, separate phylogenetic trees were generated using the homologous regions from both human ADAR1 and ADAR2. Based on these trees the partial sequences were classified as either ADAR1 or ADAR2. All the accession numbers for ADAR1 and ADAR2 that were used in the alignment are in Supplementary Table S2.

RESULTS

Human ADAR1 and ADAR2 proteins show greater selectivity than *Drosophila* ADAR for specific sites *in vitro*

Human ADAR1 and ADAR2 proteins (Figure 1A), have been shown to have distinct editing site specificities for vertebrate transcripts. Using an *in vitro* poisoned primer extension assay in the presence of dideoxythymidine we compared the specific RNA editing activities of dADAR 3/4, human ADAR1p110 and human ADAR2 proteins on the *Adar exon 7* substrate from *Drosophila* which dADAR edits very efficiently *in vitro* (30) (Figure 1B) and on the *GluR2 B13* minigene substrate (Figure 1C). Fly and human ADAR proteins expressed in the yeast *Pichia pastoris* were purified and cross-species editing was tested using equivalent amounts of the different proteins sufficient for maximal editing of their specific substrates.

The vertebrate proteins are much less active on the *Drosophila Adar exon 7* substrate than dADAR 3/4 is. Human ADAR2 edits the *Adar exon7* site slightly more efficiently than human ADAR1p110, but the activity is significantly lower than that of *Drosophila* ADAR (Figure 1B). This data is in agreement with what was previously observed when all three enzymes were assayed on long dsRNA for promiscuous RNA editing and dADAR edited more sites than the two human proteins (24).

The dADAR 3/4 protein edits sites in the vertebrate substrate efficiently (Figure 1C). The *GluR2 B13* minigene substrate contains an exonic Q/R editing site that is preferentially edited by human ADAR2 and an intronic hotspot site that is preferentially edited by human ADAR1 (27,33). *Drosophila* ADAR is less selective than the human ADARs on the *GluR2 B13* minigene substrate, efficiently editing both the Q/R (ADAR2-preferred) site and the hotspot (ADAR1-preferred) site.

Because relatively few of the dsRNA structures that are required for editing have been fully defined in *Drosophila*, only a limited number of site-specific RNA editing events can be assayed *in vitro*. Since *Drosophila* has so many edited transcripts, a much larger number of edited sites can be studied *in vivo* in transgenic flies. By expressing human ADAR proteins we can elucidate if some *Drosophila* editing sites respond to human ADARs differently than the *dAdar exon7* site.

Human ADAR2 rescues locomotion defects in *Adar* mutant *Drosophila*

Constructs designed to express human *ADAR* cDNAs under UAS/GAL4 control were injected into *Drosophila* and transgenic lines were generated and balanced. To measure phenotypic rescues, human and *Drosophila* ADAR proteins were expressed in two different deletion strains of *Adar* in a range of tissue-specific expression patterns by means of the *GAL4-UAS* binary system. Both *Adar^1F4* and *Adar^5G1* mutants are equally grossly defective in open-field locomotion and totally lack RNA editing in all ion channel transcripts tested (Figure 2) (14). The *Adar^1F4* deletion removes promoters of *Adar* but leaves the coding sequence intact and its expression is at
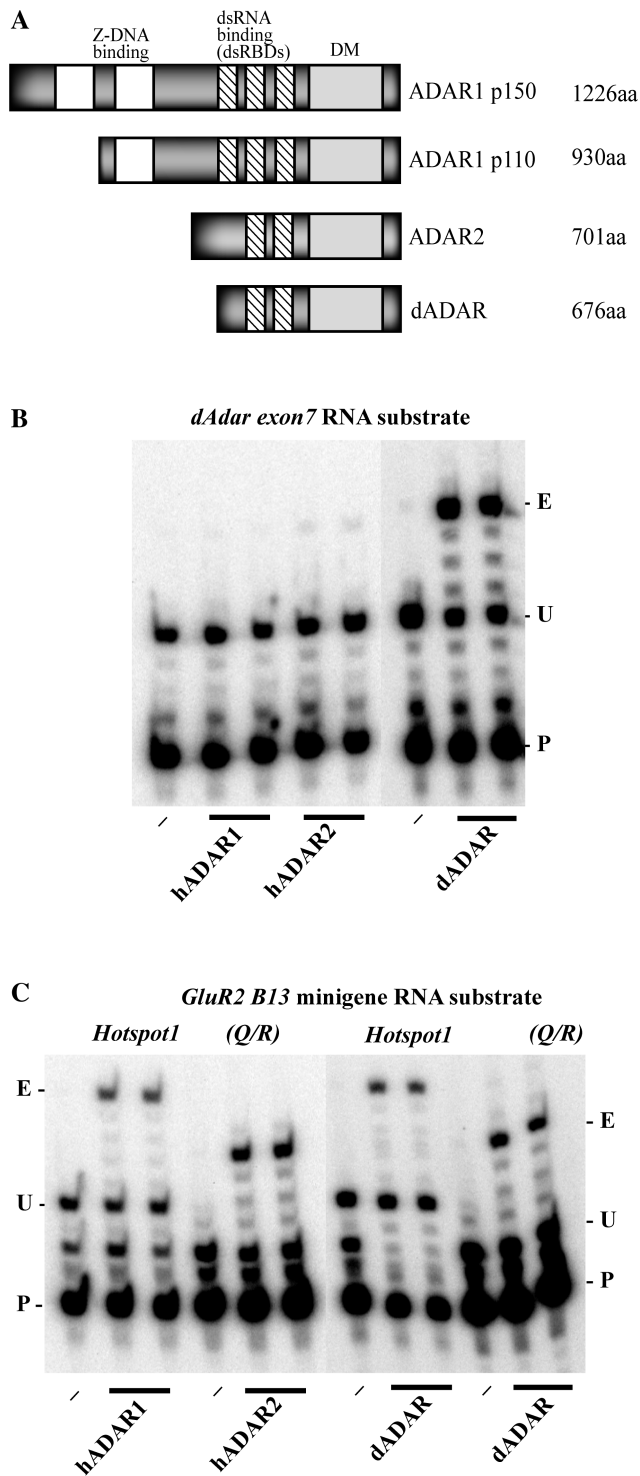
Figure 1. Comparison of human and *Drosophila* ADAR structures and activities on RNA substrates *in vitro*. (**A**) Domain structures of human and *Drosophila* ADARs. (**B**) *In vitro* RNA editing of a single site in the *Drosophila Adar exon 7* substrate by duplicate samples of *Drosophila* and human ADARs analysed by poisoned primer extension with dideoxythymidine. Dash indicates substrate RNA incubated without ADAR. For each primer extension reaction P (primer) indicates the end-labelled primer, U, (unedited) indicates the position of the next A after the primer in the template. On unedited templates primer extension terminates at the first A but if this is edited then primer extension continues to the next A, which is indicated with E, (edited). (**C**) *In vitro* RNA editing of two sites in the mammalian *GluR-2 miniB 13*

least 10- to 20-fold lower (14). This strain shows residual RNA editing at only one identified site—the *Adar exon7* site. In later stages of this study we concentrate on the *Adar*[5G1] null mutant, as it completely removes the coding sequence and expresses no ADAR protein. In addition we found age-dependent neurodegeneration proceeds more rapidly in the *Adar*[5G1] null mutant.

Strong and widespread expression of ADAR proteins in both the *Adar*[5G1] and *Adar*[1F4] mutant brains was obtained using the *Cha-GAL4* driver: choline acetyl transferase encoded by the *Cha* gene is involved in the biosynthesis of acetylcholine, the major excitatory neurotransmitter in insect neurons. Because the *Drosophila Adar* gene is on the X chromosome, rescue phenotypes were measured in male flies that had the *Adar* mutation and that also had the *Cha-GAL4* driver construct and *UAS-ADAR* constructs.

Each of the two vertebrate ADARs yield viable flies when expressed under the control of the *Cha-GAL4* driver. Figure 2 shows a comparison of open field locomotion tests on *Adar*[1F4] (Figure 2A) or *Adar*[5G1] (Figure 2B) mutant flies that have *Drosophila* ADAR protein or different vertebrate ADARs expressed under the control of the *Cha-GAL4* driver. The *Adar* mutants are both grossly defective in locomotion and this defect is efficiently rescued by either the *Drosophila* ADAR 3/4 protein or human ADAR2 in either *Adar*[1F4] or *Adar*[5G1] mutant flies (Figure 2A and B) whereas the rescue with human ADAR1p110 or ADAR1p150 is barely above background and movement is not well coordinated. For each ADAR expressed the locomotion data represents an average of results obtained with three independent insertions of the relevant *UAS-ADAR* transgene and the results obtained with different insertion lines for each ADAR are consistent with each other. The wild-type control strain is *w*[1118]; *Cha-GAL4*. This is an appropriate control because strong expression of GAL4 in neurons negatively affects locomotion in flies, (*w*[1118] flies cross 57 lines in 2 min in this test.) Expression of ADAR 3/4 restores locomotion above the level seen in *w*[1118]; *Cha-GAL4* but not quite to the level seen in *w*[1118]. Locomotion rescue by *ADAR2* is not as strong as expected since it edits most *Drosophila* sites more efficiently than dADAR 3/4.

ADAR1 is expressed as either a cytoplasmic 150-kDa protein that shuttles in and out of the nucleus but accumulates in cytoplasm or as a shorter 110-kDa protein that is primarily localized to the nucleus (34). Neither isoform efficiently rescues the locomotion defects in either *Adar*[1F4] or *Adar*[5G1] mutant *Drosophila* (Figure 2A and B). There is a small effect of ADAR1 in improving the locomotion but a similar slight effect is seen with a catalytically inactive mutant form of *Drosophila* ADAR in which an essential

substrate by *Drosophila* and human ADARs analysed with poisoned primer extension with dideoxythymidine. The *GluR-2 miniB 13* transcript contains an exonic Q/R editing site (unextended primer and unedited and edited extension product sizes indicated on the right) that is preferentially edited by human ADAR2 and an intronic hotspot site (primer and extension product sizes on the left) that is preferentially edited by human ADAR1.
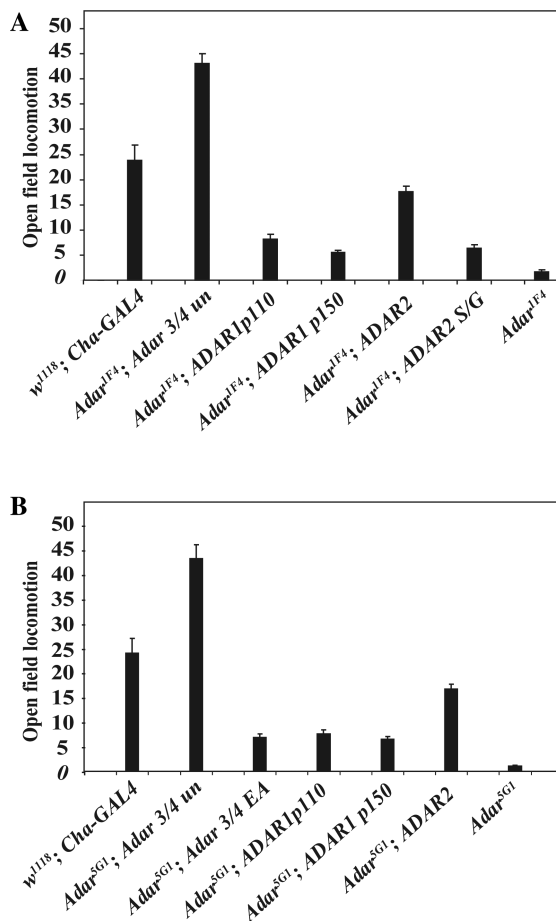
Figure 2. Human *ADAR2* rescues *Drosophila Adar* mutant locomotion defects. (**A**) Rescue by human ADAR2 of hypomorphic *Adar^{1F4}* mutant open field locomotion defects with the strong neuron-specific *Cha-GAL4* driver. Neither the long nucleocytoplasmic shuttling human ADAR1p150 isoform nor the shorter human ADAR1p110 nuclear isoform rescue locomotion defects. (**B**) Rescue of locomotion in the *Adar^{5G1}* null mutant.

glutamate residue at the catalytic site has been mutated to alanine (dADAR 3/4 EA, Figure 2B). Catalytic RNA editing activity at appropriate target sites is necessary for full locomotion rescue.

The equivalence of function between human ADAR2 and *Drosophila Adar* is further supported by the fact that ubiquitous expression of *UAS-ADAR2* with the *actin 5C-GAL4* driver is lethal to *Drosophila*; similar lethality was previously observed with the very active genome-encoded isoform of dADAR that has a serine residue as found in ADAR2 at the S/G RNA editing site in the deaminase domain (30). The lethality was attributed to premature editing of target transcripts during embryonic development, particularly in muscle tissue or heart which normally have lower ADAR expression than CNS. There is a very much weaker rescue of locomotion when the serine corresponding to the *Drosophila* self-editing site is mutated to glycine in ADAR2 (Figure 2A). Editing of the *GluR2 B13* minigene substrate at the Q/R site is reduced 8-fold by the serine to glycine mutation in poisoned primer

extension assays (Supplementary Figure S1). Widespread ADAR1 expression under *actin 5C-GAL4* driver control is not fully lethal in *Drosophila* though viability is low and only small numbers of flies are obtained.

Human ADAR2 edits many *Drosophila* editing sites similarly to dADAR but ADAR1 edits only a subset of these sites

We do not know which individual RNA editing events or which combination of editing events in the known edited transcripts in *Drosophila* are the most essential. Therefore we chose to measure RNA editing levels in a subset of the known *Drosophila* transcripts that contain sites that are highly edited at functionally important amino acids (5). These sites were originally identified by comparative genomics due to strong evolutionary conservation among fly species of exonic sequences flanking some of the highly edited positions due to conservation of RNA duplex formation. We analysed 26 RNA editing sites in four transcripts in embryos, larvae and adult male and female flies to examine developmental RNA editing levels in these transcripts and to determine if there were sex-specific effects (Table 1). Editing levels were calculated using peak height measurements of A and G peaks in sequencing electropherograms of RT–PCR products covering each the edited sites. The analysis shows that amongst this set of transcripts some sites are fully edited such as the 1218 I/V site in the *Rdl (Resistance to Dieldrin)* transcript which encodes a pore-forming alpha subunit of a member of the inhibitory GABA-gated chloride channel family. Another transcript with fully edited sites, *Nic34E*, encodes a pore-forming subunit of acetylcholine receptors. Acetylcholine has widespread significance as an excitatory neurotransmitter in insect brain similar to that of glutamate in vertebrate brain.

As previously observed, editing at most sites is low in embryos and increases during development (13,30). There was a dramatic increase in editing of the *Caα1D* transcript encoding a muscle-type voltage-gated calcium channel that is expressed in both muscle and CNS at metamorphosis. The *Nic34E* transcript encoding a pore-forming subunit of a nicotinic acetylcholine receptor is always highly edited with two sites being edited to 100% even in early developmental stages. We decided that these sites would be informative to analyse rescue of RNA editing by human ADARs since they include sites constitutively edited by dADAR as well as sites with editing levels ranging from 0 to 100%. The constitutive editing of some of these sites throughout development (Table 1), is reminiscent of the human *GluR2 Q/R* site (35) and also suggests that these editing sites might be physiologically important. Editing of these transcripts was slightly higher in males than females.

We measured RNA editing levels in these transcripts in flies expressing either human ADAR proteins or *Drosophila* ADAR and compared these to editing levels seen in wild-type *Canton S* and *Adar* mutant flies (Tables 2 and 3). Expressing *Drosophila* ADAR 3/4 under the control of the *Cha-GAL4* driver in the *Adar^{5G1}* background rescues RNA editing in these sites, substantially

Table 2. Percentage RNA editing at specific sites in transcripts from rescued *Adar^5G1* flies expressing either dADAR, hADAR1p110, hADARp150 or hADAR2 under the control of the *Cha-GAL4* driver

	WT	n	5G1	n	dAdar	n	ADAR2	n	ADAR1 P110	n	ADAR1 P150	n
Caα1D												
2061 L/L	**36**	1	**0**	4	**0**	4	**18**	5	**0**	4	**0**	3
2083 N/D	**97**	2	**0**	4	**20**	4	**56**	4	**0**	4	**0**	3
2097 L/L	**96**	2	**0**	4	**0**	4	**20**	4	**0**	4	**0**	3
2098 R/G	**96**	2	**0**	4	**11**	4	**24**	4	**0**	4	a	2
2140 I/M	**100**	2	a	2	**0**	4	**25**	3	**0**	2	**0**	2
Eag												
1864 K/R	**58**	3	**0**	11	**14**	5	**10**	2	a	4	**0**	6
2107 Y/C	**89**	5	**0**	11	**21**	5	**36**	9	**0**	7	**0**	13
2159 V/V	**16**	5	**0**	7	**0**	3	**23**	7	**0**	7	**0**	13
2163 N/D	**88**	5	**0**	7	**52**	3	**30**	7	**0**	7	**10**	13
2560 K/R	**78**	2	a	3	a	1	**31**	6	**0**	3	a	11
Nic 34E												
1872 L/L	**100**	4	**0**	6	**16**	2	**54**	6	**0**	4	**0**	3
1873 I/V	**100**	4	**0**	6	**14**	2	**56**	6	**0**	4	**0**	3
2020 T/A	**100**	3	**0**	7	**55**	2	**79**	5	**0**	3	a	3
2023 I/V	**38**	1	**0**	5	**0**	2	**10**	5	**0**	3	**0**	3
2028 L/L	**35**	1	a	5	**0**	2	**19**	5	**0**	3	**0**	2
2037 I/M	**67**	1	a	5	**6**	2	**49**	5	**0**	2	**21**	7
2049 L/L	**16**	1	**0**	4	**0**	2	**0**	4	**0**	3	**0**	6
2052 S/S	**71**	1	**0**	4	**18**	2	**10**	3	**0**	3	**0**	6
2062 I/V	**100**	3	**0**	4	**46**	2	**31**	3	**0**	2	*	6
2065 I/V	**53**	1	**0**	4	**14**	2	**11**	3	**0**	2	**11**	4
Rdl												
728 L/L	**23**	2	a	8	a	6	**12**	4	**10**	11	a	8
735 R/G	**65**	2	a	8	**12**	6	**39**	4	**16**	11	a	8
1218 I/V	**100**	3	a	8	**43**	3	**81**	5	**0**	4	**0**	4
1251 N/D	**22**	3	**0**	4	**0**	3	**0**	12	**0**	4	**0**	4
1448 Q/Q	**8**	3	**0**	4	**0**	4	**0**	7	**0**	4	**0**	5
1449 M/V	**22**	3	**0**	4	**0**	4	**0**	7	**0**	4	**0**	4

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the different samples. The total number of RT–PCR reactions sequenced is represented by *n*.
[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.

though not completely (Table 2). Editing is completely dependent on dADAR as it is eliminated in the *Adar^5G1* mutant and not restored by expression of a catalytically inactive dADAR 3/4 EA protein (data not shown). Human ADAR2 edits 22/26 sites analysed in *Drosophila* when expressed using the *Cha-GAL4* driver in *Adar^5G1* (Table 2). The levels of editing at specific sites are generally similar to, and generally higher than, levels obtained for rescue by dADAR expressed under the control of the *Cha-GAL4* driver. We have repeated this with different drivers and the pattern of editing with ADAR2 is always similar to that with dADAR. Human ADAR1p110 and p150 display low levels of editing activity, 2/26 and 3/26 sites respectively were edited.

When the *Adar^1F4* hypomorphic mutant background is used in rescue experiments with the *Cha-GAL4* driver the pattern of locomotion rescue is unchanged from that obtained in the *Adar^5G1* null background, i.e. ADAR2 rescues and ADAR1 isoforms do not (Figure 2). Levels of RNA editing at most sites are higher in *Adar^1F4* rescues with *UAS-dAdar* and *UAS-hADAR2* than in the *Adar^5G1*rescues with the same UAS-ADAR transgenic lines (Table 3), presumably due to some assistance from the low level of residual dADAR in the *Adar^1F4* strain. Also RNA editing by ADAR1 is observed at more sites in ion channel transcripts in the *Adar^1F4* rescue but the

pattern of sites with high and low levels of editing is very different from that seen in wild-type flies or in rescues by *Drosophila* ADAR protein or human ADAR2 (Table 3). This is exemplified by editing of the *Nic 34E* transcript where sites that are normally edited to 100% are edited slightly or not all by ADAR1 yet other sites within in the same transcript are highly edited by ADAR1p110 (*Nic 34E* I/M site, 84%) Editing activity is due to ADAR1 itself and not to endogenous *Drosophila* ADAR protein because no editing is observed at any site in transgenic flies expressing catalytically inactive ADAR1 EA (not shown). We conclude that human ADAR1, even when it succeeds in editing ion channel transcripts in *Drosophila*, does not restore the wild-type pattern of editing.

The ADAR proteins are expressed at low levels and cannot be detected on immunoblots of total protein extracts from embryos, whole flies or fly heads. In the case of ADAR2 low level expression in mammalian cells is due to the activity of a specific E3 ubiquitin ligase (R. Marcucci, manuscript in preparation). To express ADARs strongly in embryos male flies of *UAS-ADAR* lines were crossed to *actin 5C-GAL4 / SM5 Cy* and soluble protein extracts were made from 48-h embryo collections. The FLAG-tagged ADAR proteins were immunoprecipitated from extracts with anti-FLAG antibodies and the proteins were detected on immunoblots with anti-FLAG or

Table 3. Percentage RNA editing at specific sites in transcripts from rescued *Adar[1F4]* flies expressing either dADAR, hADAR1p110, hADARp150 or hADAR2 under the control of the *Cha-GAL4* driver

	WT	n	1F4	n	dAdar	n	ADAR2	n	ADAR1 P110	n	ADAR1 P150	n
Caα1D												
2061 L/L	**36**	1	[a]	4	**13**	5	**24**	4	**25**	2	**29**	1
2083 N/D	**97**	2	**0**	3	**31**	5	**2**	4	**0**	2	**0**	1
2097 L/L	**96**	2	**0**	3	**24**	5	**0**	2	**0**	1	**0**	1
2098 R/G	**96**	2	[a]	3	**29**	5	**32**	2	**0**	1	**0**	1
2140 I/M	**100**	2	**0**	4	**26**	5	**20**	4	**0**	1	**0**	1
Eag												
1864 K/R	**58**	3	**0**	2	**50**	2	**58**	2	**0**	7	**0**	4
2107 Y/C	**89**	5	**0**	2	**55**	3	**56**	5	**10**	7	**0**	4
2159 V/V	**16**	5	**0**	2	**10**	5	**24**	6	**0**	7	**0**	4
2163 N/D	**88**	5	**0**	2	**62**	5	**46**	6	**40**	7	**12**	4
2177 A/A	**0**	5	**0**	2	[a]	5	**0**	6	**0**	7	**0**	4
2560 K/R	**78**	3	**0**	1	**37**	2	**56**	2	**0**	3	**0**	2
Nic 34E												
1872 L/L	**100**	4	**0**	5	[b]		**76**	2	**0**	1	**0**	2
1873 I/V	**100**	4	**0**	5	[b]		**74**	2	**0**	1	**0**	2
2020 T/A	**100**	3	**0**	5	**82**	3	**80**	3	**26**	3	**0**	2
2023 I/V	**38**	1	**0**	5	**35**	3	[a]	3	**0**	3	**0**	2
2028 L/L	**35**	1	**0**	5	**29**	3	**15**	3	**0**	3	**0**	2
2037 I/M	**67**	1	**0**	4	**63**	3	**75**	3	**84**	3	**37**	1
2052 S/S	**71**	1	**0**	4	**60**	3	**8**	3	**27**	3	**0**	2
2062 I/V	**100**	3	**0**	5	**84**	3	**38**	2	**32**	3	**10**	1
2065 I/V	**53**	1	**0**	5	**42**	3	**13**	2	**54**	2	**17**	1
Rdl												
728 L/L	**23**	2	**0**	2	**29**	2	**34**	4	[a]	2	**0**	2
735 R/G	**65**	2	**0**	2	**52**	2	**64**	4	**15**	2	**0**	2
1218 I/V	**100**	3	[a]	2	**88**	2	**81**	4	**31**	2	**16**	2
1251 N/D	**22**	3	**0**	3	**0**	2	**0**	5	**0**	2	**0**	2
1448 Q/Q	**8**	3	**0**	3	**10**	2	**0**	3	**0**	2	**0**	2
1449 M/V	**22**	3	**0**	3	**12**	2	[a]	3	**0**	2	**0**	2

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the different samples. The total number of RT–PCR reactions sequenced is represented by *n*.
[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.
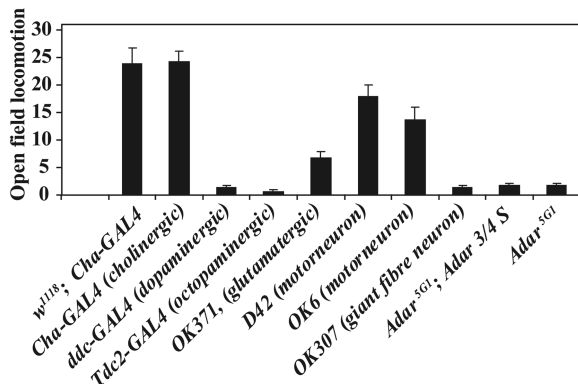[b]Sites that we were unable to obtain sequence for.

Figure 3. *Adar* expression in cholinergic or motor neurons is sufficient to rescue *Adar[5G1]* mutant locomotion defects. The chart shows open field locomotion in *Adar[5G1]* flies, *Adar[5G1]; UAS-Adar 3/4 S* flies having this UAS construct in the absence of any GAL4 driver to induce expression or lines in which the *UAS-Adar 3/4 S* construct is expressed in the *Adar[5G1]* background under the control of different GAL4 drivers. The wild-type control is *w[1118]*, *Adar* wild-type having a *Cha-GAL4* driver to control for locomotion effects of widespread and strong GAL4 expression. Drivers expressing GAL4 in motor neurons, giant fibre escape neurons and different chemical classes of neurons are indicated. Drivers expressing GAL4 specifically in motor neurons (OK6, D42 and OK371) and *Cha-GAL4* which expresses GAL4 in cholinergic neurons and some motor neurons direct efficient rescue.

anti-His antibodies. This allowed confirmation that proteins of the expected sizes are expressed at similar though not identical levels. The ADAR1p150 protein was not detected in this way but other evidence indicates that this protein is expressed and that it behaves differently than ADAR1p110 (36).

To ascertain if the fly and human proteins have similar levels of RNA editing activity in transgenic flies and therefore similar protein expression, we analysed non-specific RNA editing of the *Rnp-4F* transcript. This transcript is overlapped at the 3′-end by a convergently transcribed antisense transcript generated by read-through at the transcription terminator of the convergently transcribed gene (37). The resulting dsRNA is promiscuously edited by ADARs. Non-specific editing in the *Rnp-4F* transcript is rescued to the same level as in wild-type (approximately 14%) in *Adar* mutant flies rescued by expression of dADAR 3/4, ADAR1 p110 and p150 and human ADAR2 under *engrailed-GAL4* control.

Locomotion defects in *Adar* mutant flies are rescued by expression of ADAR specifically in motor neurons

We have tested rescue of the locomotion defect by ADARs using a wide range of GAL4 drivers in addition to *Cha-GAL4*. We constructed a strain that had *Adar[5G1]*

on the X chromosome and a *UAS-dAdar 3/4 S* construct on the second chromosome and crossed a number of different GAL4 drivers to this strain (Figure 3). Surprisingly the enhancer trap GAL4 driver lines D42 and OK6 that drive GAL4 and UAS construct expression specifically in motor neurons, give efficient rescue of the *Adar* locomotion defect (Figure 3). In *Drosophila* neuromuscular junctions are primarily glutamatergic. The GAL4 enhancer trap line OK371 has a GAL4 insert in the promoter region of the gene encoding the vesicular glutamate transporter and this line directs expression in motor neurons as well as widely in a range of other glutamatergic neurons in the brain. None of the driver lines tested has expression that is absolutely restricted to motor neurons although OK6 has very little expression elsewhere in the CNS (31). Also the locomotion rescue by all three GAL4 driver lines is consistent with motor neurons being the main focus of the locomotion defect. Among all GAL4 drivers we have tested those whose expression patterns are known to include motor neurons consistently give efficient locomotion rescue.

Drivers expressing in neurons of other pharmacological types implicated in the central control of movement such as *ddc-GAL4* (dopamine decarboxylase in dopaminergic neurons) or *Tdc2-GAL4*, (tyrosine decarboylase 2 in octopaminergic neurons) are not sufficient to direct locomotion rescue. Expression of ADARs in muscles, (*How(Held-out wings)-GAL4*) or in glia, (*nrv(nervana)-GAL4*) do not give rescue of walking defects (data not shown).

Human ADAR2 suppresses age-dependent neurodegeneration in *Adar* mutant *Drosophila*

*Adar^1F4^* flies undergo progressive vacuolization of the synaptic neuropile from 30 to 50 days (14). As the *Adar^5G1^* deletion mutant is less viable than the *Adar^1F4^* mutant strain it was hypothesized that the neurodegeneration in *Adar^5G1^* would be more aggressive. To characterize the neurodegeneration pattern of the *Adar^5G1^* mutant strain, *Adar^5G1^* mutant males were aged, and heads were sectioned at 30 days and stained with haematoxylin and eosin (Figure 4). This revealed that vacuolization occurred in the *Adar^5G1^* mutant as it did in the *Adar^1F4^* mutant. However the neurodegeneration was more aggressive in the *Adar^5G1^* mutant, not only affecting the retina (Figure 4D, compare to wild-type in B), but also the paired mushroom body (MB) calyces on the dorsal brain (Figure 4C, compare to wild-type in A). The mushroom body calyces are neuropil which is comprised of the dendrites of mushroom body Kenyon cells whose haematoxylin-stained nuclei lie above the calyces, and the axonal collaterals of projection neurons extending to them from the paired olfactory glomeruli on the ventral brain above the antennae.

To confirm that the neurodegeneration that had been observed in aged *Adar^5G1^* is due to the *Adar* deletion, the *UAS-Adar 3/4* transgenic line was crossed into *Adar^5G1^; Cha-GAL4*. The *Adar^5G1^*mutant male rescued by expression of *dAdar 3/4* in the cholinergic nervous system was aged to 30 days and the MB calyces and retina were analysed by haematoxylin and eosin staining of head sections. The vacuolization of the neuropil of the MB calyces and retina of the *Adar^5G1^; Cha-GAL4* male rescued with *Adar 3/4* is significantly reduced compared to the *Adar^5G1^*mutant strain at 30 days (Figure 4).

As neurodegeneration in the *Adar^5G1^* mutant strain is successfully suppressed by *Cha-GAL4*-driven expression of *dAdar*, it was therefore possible to compare suppression of this phenotype by human ADARs. We aged the transgenic flies to 30 days to visualize neurodegeneration (Figure 5). Human ADAR2 suppresses neurodegeneration of both the calyces of the mushroom body (Figure 5E) and in the retina (Figure 5F) as effectively as *Drosophila* ADAR in the *Adar^5G1^* mutant background in flies aged to thirty days. The suppression of neurodegeneration at thirty days is weaker with the nuclear p110 form of human ADAR1 (Figure 5A, ADAR1p110 calyx, Figure 5B, ADAR1p110 retina) but is lacking entirely with the cytoplasmically accumulating p150 isoform of ADAR1 (Figure 5C, ADAR1p150 calyx, Figure 5D, ADAR1p150 retina), suggesting that suppression of neurodegeneration is associated with nuclear localization of the ADAR proteins. It appears that suppression of neurodegeneration by ADAR proteins is easier to obtain than rescue of the locomotion defect.

Insects have lost the *ADAR1* gene

Human ADAR2 expressed in *Drosophila* matches the target site specificity of dADAR and rescues mutant phenotypes surprisingly well while human ADAR1 does not. These data suggest that *Drosophila Adar* may be a true orthologue of human *ADAR2* rather than an invertebrate gene ancestral to both vertebrate *ADAR*s. Because the *Drosophila* genome harbours a single *Adar* gene, this idea would imply that flies have lost an *ADAR1* orthologue. Sequence data from recent invertebrate genome projects supports this idea. Many genes that were previously assumed to have first appeared only at the separation of Chordates from invertebrates have now been found in some of the simplest invertebrates like cnidarians (25). Both the *ADAR1* and *ADAR2* genes are in this category.

Figure 6 shows results of our searches for invertebrate *ADAR*s mapped onto the phylogeny of all Metazoans that extend a previous report (38) (Supplementary Table S2). For all putative *ADAR* sequences, the deaminase domain was aligned with those from human *ADAR1* and *ADAR2*. In most cases each *ADAR* could be classified as an orthologue of *ADAR1* or *ADAR2* with a high degree of confidence (Supplementary Figures S2 and S3). Surprisingly, having discrete *ADAR1* and *ADAR2* genes is an ancient characteristic, present throughout the Eumetazoa lineage, including its oldest phylum, the Cnidaria. In a few cases, however, *ADAR1* appears to have been lost. For example, an *ADAR1* orthologue was not found in multiple insect and crustacean genomes. It was found in some arachnids, indicating that it was not lost in all arthropods. Among the cnidarians, hydrozoans also seem to have lost ADAR1, although it was present in anemones (its presence or absence in corals cannot be
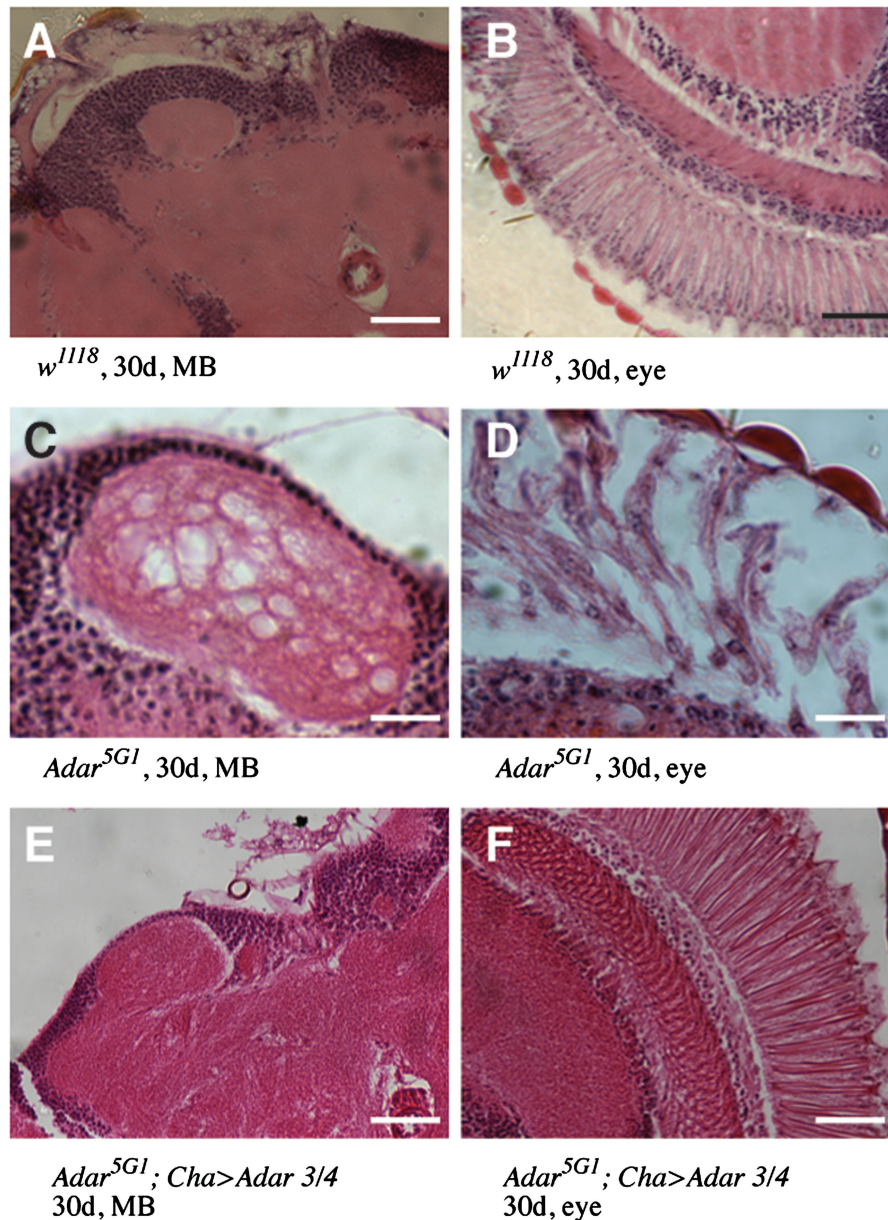
Figure 4. Suppression of neurodegeneration in *Adar*[5G1] mutant flies by *Drosophila* ADAR. (**A** and **B**). Haematoxylin and eosin stained frontal sections of 30-day-old wild-type (*w*[1118]) heads show no neurodegeneration in the mushroom body calyces or in the eye. Scale bars: 20 μM. (**C** and **D**) Frontal sections of 30 day-old *Adar*[5G1] heads show vacuolization and loss of Mushroom Body calyx neuropil (C) and large vacuoles in the retina of the eye (D) of Scale bars: 5 μM. (**E** and **F**) Frontal sections of 30-day-old *Adar*[5G1]; *Cha GAL4, UAS-Adar 3/4* heads show rescue of vacuolization in the MB calyx and in the eye. Scale bars: 20 μM.

clearly inferred because no genome is available, only a partial EST library). *ADAR2* appears to be more ubiquitous. In fact, the only genome that possibly lacks an *ADAR2* orthologue, but contains one for *ADAR1*, is *Aplysia*. However, the apparent absence of an *Aplysia ADAR2* could be due to incomplete coverage of the *Aplysia* genome. Interestingly, nematodes and flatworms have neither a true *ADAR1* nor *ADAR2* orthologue. The two *Adr* genes from *Caenorhabditis elegans* cannot be classified into either group (39).

Together the findings of an ancient Metazoan *ADAR2* conserved between fly and human, and loss of an ancient Metazoan *ADAR1* in insects explain the results of the

rescue tests with human *ADAR*s in fly and account for the surprising similarity in target site preferences between human ADAR2 and *Drosophila* ADAR.

DISCUSSION

We find that the target specificity of an ADAR2-type protein is conserved from fly to human allowing effective rescue of *in vivo* RNA editing, locomotion and neurodegenerative phenotypes in flies by human ADAR2. Neither ADAR1p110 nor ADAR1p150 efficiently edit critical sites in *Drosophila* transcripts nor rescue the *Adar* mutant locomotion phenotype. This data was
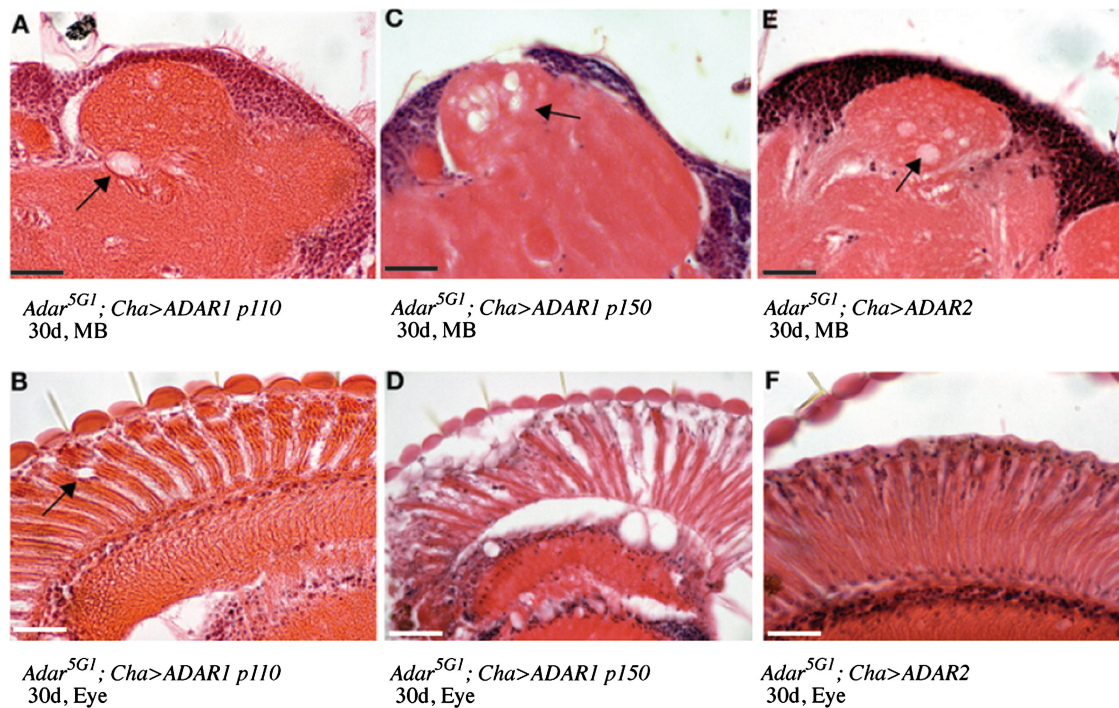
Figure 5. Suppression of neurodegeneration at 30 days in *Adar^{5G1}* mutant flies by human ADAR2. (**A** and **B**): Haematoxylin and eosin stained frontal sections of 30-day-old *Adar^{5G1}*; *Cha-GAL4, UAS-ADAR1p110* heads show rescue of neurodegeneration in the mushroom body (MB) calyces of the *Adar^{5G1}* mutant. (A). Some small vacuoles remain in the retina (B). Arrows indicate vacuolization. (**C** and **D**): Frontal sections of 30-day-old *Adar^{5G1}*; *Cha-GAL4, UAS-ADAR1p150* heads show lack of neurodegeneration rescue in the MB calyces of *Adar^{5G1}* (C) The retina degenerated rapidly (D). (**E** and **F**): Frontal sections of 30-day-old *Adar^{5G1}*; *Cha-GAL4, UAS-ADAR2* heads show rescue of vacuolization of the MB calyces (E) and the eye (F). Scale bars: 20 μM.

obtained before the recent increase in vertebrate genome sequences and is well explained by the identification of ancient Metazoan *ADAR1* and *ADAR2* genes in invertebrate genomes. Previously these *ADAR* genes had been identified only in Chordate genomes and not in *Drosophila* and other insects. We find that *ADAR2* is conserved in *Drosophila* and that *ADAR1* has been lost from insects and crustaceans but is present in Arachnid genomes. The data also show that the *Drosophila Adar* mutant represents a very useful genetic model for *ADAR2* loss of function effects in human disease even though different transcripts are edited in vertebrates and flies. Restoration of ADAR activity in motor neurons, a fundamental neuron type present in even the simplest metazoans, is sufficient to rescue locomotion defects in *Adar* mutant flies.

The lack of RNA substrates from *Drosophila* with defined ECS elements made it impossible to analyse the activities of ADAR1 and ADAR2 at many *Drosophila* editing sites *in vitro*. We find that RNA structures at specific editing sites in *Drosophila* are often difficult to predict from the genome sequence. Although vertebrate editing sites show easily recognized pairings between edited exons and editing site complementary sequence (ECS) elements that exist as contiguous stretches of sequence in nearby introns, some fly sites may have shorter fragmented ECSs, as shown for the *Drosophila synaptotagmin1 (Syt1)* transcript (40). To analyse rescue at more editing sites we expressed the human ADAR

proteins in *Drosophila* and measured editing by these proteins in *Adar* mutant flies. We focused on 26 edited positions in four transcripts that were either constitutively highly edited at all developmental stages or edited only or predominantly in adult flies. We have also analysed other edited positions in many other transcripts, though not in such depth, and the overall pattern of editing at these other positions with different ADARs did not vary from our core set. Our data showed that the set of edited sites in *Drosophila* match the specificity of an ADAR2 enzyme but not an ADAR1 enzyme to a surprising extent, i.e. the fly ADAR does not appear to represent an evolutionary precursor that might combine features of two descendant vertebrate ADARs. This is consistent with greater sequence conservation between *Drosophila* ADAR and vertebrate ADAR2.

Human *ADAR2* expressed in *Drosophila* mirrors the function of the fly gene in many respects. We found that *actin 5C-GAL4* and other drivers that direct ubiquitous, high level expression of *ADAR2* in embryos and larvae or *Mef 2-GAL4* that directs similarly premature high level expression in muscles and heart cause embryonic and larval lethality. We have previously observed similar lethality with the edited *dAdar S* isoform that is the most active *Drosophila* ADAR isoform (30). This is presumably due to some transcripts being edited inappropriately early in development. Expressing either an edited-equivalent *Drosophila UAS-ADAR 3/4 G* isoform or *UAS-ADAR2 G* do not cause this lethality. Human ADAR2 also
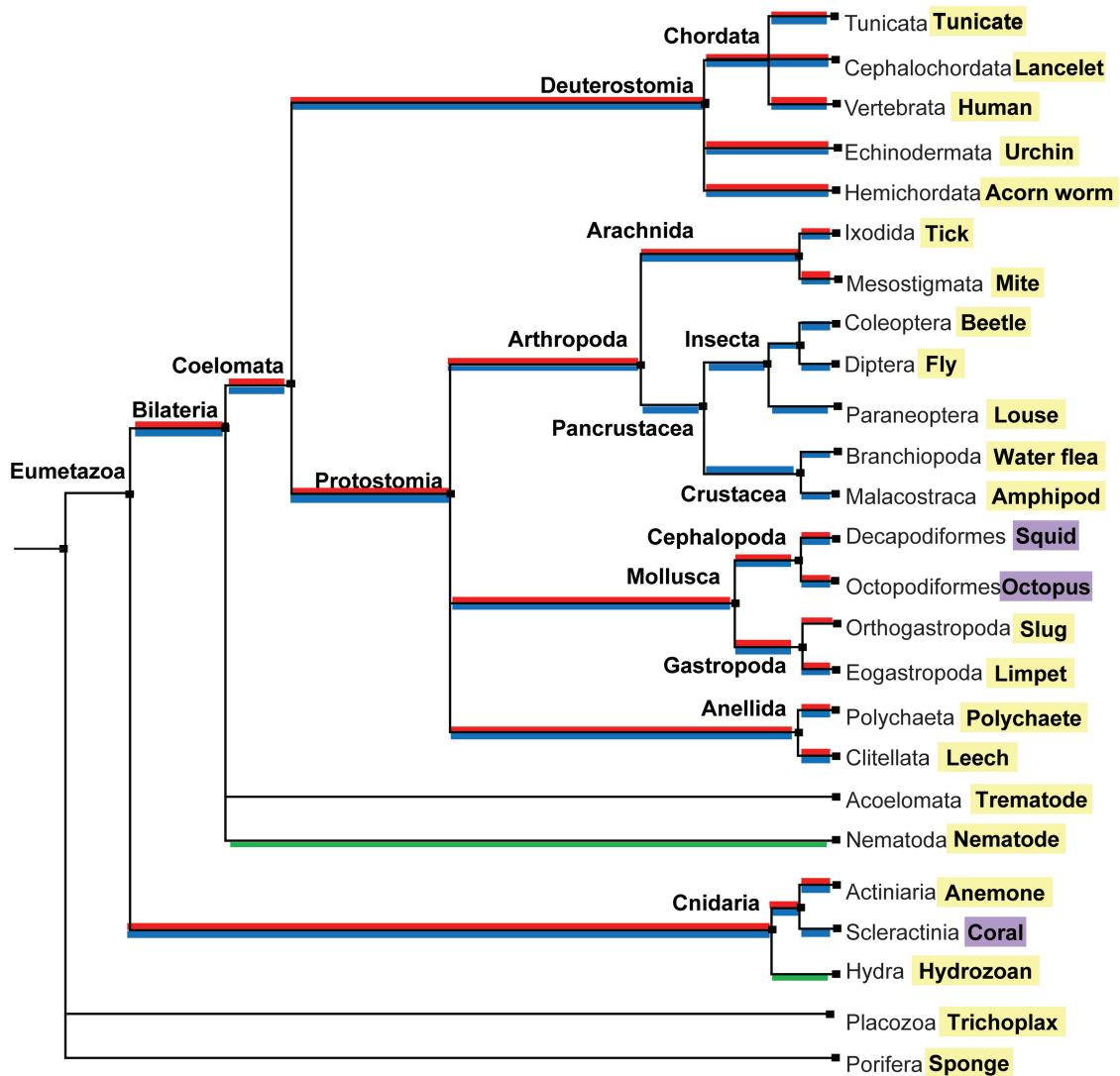
Figure 6. Occurrence of *ADAR1* and *ADAR2* genes in the Metazoa. The phylogenetic tree of species was obtained from Taxonomy Common Tree NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). Species names at the ends of branches highlighted in yellow represent available genomes that were searched for *ADAR1* or *ADAR2* orthologues. Species names highlighted in purple were cases where *ADAR*s were identified by direct cloning (cephalopods) or searching EST resources (coral). Positive identification of *ADAR1* or *ADAR2* is coloured in red and blue, respectively. *ADAR*s that cannot be classified as either *ADAR1* or *ADAR2* are coloured in green.

rescues neurodegeneration in *Adar* mutant flies as does dADAR 3/4.

Human ADAR2 does not rescue locomotion defects in the *Adar* mutants as well as expected since in the best-rescuing *UAS-ADAR2* line sites in *Drosophila* transcripts are edited more effectively than in the best-rescuing dADAR 3/4 line (Tables 2 and 3). We do not know why this is. Since ADAR2 is less active than dADAR 3/4 in editing the *dAdar exon 7* site *in vitro* (Figure 1B) it might be expected that for ADAR2 to edit sites *in vivo* in *Drosophila* more efficiently than dADAR 3/4 would require a higher level of ADAR2 expression. We cannot rule out that ADAR2 is more highly expressed than dADAR 3/4 and has also some deleterious effect due to a higher expression level that interferes with locomotion rescue.

We do not understand why ADAR1p110 also rescues neurodegeneration but the finding suggests that rescue of neurodegeneration may not be dependent on rescue of site-specific RNA editing. ADAR proteins may have dosage-sensitive effects independent of their RNA editing specificities since ADAR1p110 is able to rescue neurodegeneration even though it does not edit correctly. The ability to rescue neurodegeneration correlates with predominant localization to the nucleus. It does not appear likely that rescued RNA editing of a subset of the *Drosophila* sites is the reason that ADAR1p110 rescues neurodegeneration, since ADAR1p150 edits most of the same sites to some extent, but we cannot rule out this possibility. Editing independent effects of ADARs expressed in motor neurons might also account for the small improvements in locomotion seen when ADAR1 isoforms

or inactive dADAR 3/4 EA are expressed in *Adar* mutant flies and might also contribute to the toxicity of high level ADAR1 isoform expression.

Ironically, even though the target specificity of ADAR2-like proteins is well conserved and *Drosophila* has many edited transcripts, there is no evidence that any editing sites are conserved between *Drosophila* and vertebrates. There is no evidence for editing of transcripts encoding ionotropic glutamate receptor subunits in *Drosophila* even though this family of genes is conserved with vertebrates; vertebrate glutamate receptor editing appears to have first evolved in fish. None of the many editing sites in *Drosophila* transcripts can be related to known editing sites in vertebrate homologues and the one known case where a fly and vertebrate transcript are edited at the equivalent codon appears to have arisen by convergent evolution rather than by conservation of the underlying dsRNA target structure (41). This makes more impressive the finding that human ADAR2 has retained specificity and rescues the *Drosophila Adar* mutant.

As *Drosophila* has lost ADAR1, the possibility existed that certain sites would remain ADAR1-preferred sites since dADAR may have a higher specific activity or a slightly broader specificity than the vertebrate ADARs (Figure 1B and C). However this has not occurred and the tested editing sites in *Drosophila* are all preferentially edited by ADAR2. RNA editing sites at sites once edited by ADAR1 may have adjusted to conform better with the ADAR2-like target specificity after ADAR1 was lost in insects and crustaceans. Now that so many RNA editing events have been detected in *Drosophila* (4), evolutionary comparisons across invertebrates may be able to establish whether some RNA editing events are conserved since the insects diverged from crustaceans or arachnids or more distant groups and perhaps also determine which ADARs edited these sites in more primitive invertebrates. We cannot exclude the possibility that human ADAR1 edits some completely unknown sites in RNA duplexes in *Drosophila* transcripts that might represent relics of ancient ADAR1 editing events. This could provide one explanation for the reduced viability associated with highly expressing ADAR1 isoforms but we did not see any evidence for new human ADAR1 RNA editing events close to the *Drosophila* editing sites examined in rescue lines *in vivo*. ADAR1-type sites retained in *Drosophila* might not be edited by *Drosophila* ADAR and it would require a genome-wide search by RNA Sequencing in ADAR1-expressing flies to detect them, if they are still present. It is not clear however that ADAR1 editing sites would be conserved since the beginning of modern insects. Whole genome sequences are available for only a limited number of insect and crustacean species so there could be some insects and crustaceans that do still have *ADAR1*. With the full extent of editing in humans still to be determined, 4% of *Drosophila* transcripts affected and indications that RNA editing may be even more widespread in squid studies on the evolutionary origins of RNA editing sites and the selective forces maintaining them will expand our understanding of the role of RNA in gene expression.

What is most surprising is that *ADAR1*, an essential gene in mammals, has been lost in some invertebrates. Is there a biological role of ADAR1 other than site-specific editing that became dispensable?

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