A-to-I Pre-mRNA Editing in Drosophila Is Primarily Involved in Adult Nervous System Function and Integrity

Michael J. Palladino,* Liam P. Keegan,†
Mary A. O’Connell,‡ and Robert A. Reenan*‡

*Department of Genetics and Developmental Biology
University of Connecticut Health Center
263 Farmington Avenue
Farmington, Connecticut 06030
† MRC Human Genetics Unit
Western General Hospital
Edinburgh EH4 2XU
United Kingdom

Summary

Specific A-to-I RNA editing, like that seen in mammals, has been reported for several Drosophila ion channel genes. Drosophila possesses a candidate editing enzyme, dADAR. Here, we describe dADAR deletion mutants that lack ADAR activity in extracts. Correspondingly, all known Drosophila site-specific RNA editing (25 sites in three ion channel transcripts) is abolished. Adults lacking dADAR are morphologically wild-type but exhibit extreme behavioral deficits including temperature-sensitive paralysis, locomotor uncoordination, and tremors which increase in severity with age. Neurodegeneration accompanies the increase in phenotypic severity. Surprisingly, dADAR mutants are not short-lived. Thus, A-to-I editing of pre-mRNAs in Drosophila acts predominantly through nervous system targets to affect adult nervous system function, integrity, and behavior.

Introduction

The phenomenon of RNA editing was first reported in trypanosomes 14 years ago as the insertion of four ribonucleotides into a framenished mitochondrial transcript (Benne et al., 1986). The insertion restored an open reading frame that was not encoded by the trypanosome mitochondrial genome. Since then, several distinct classes of RNA editing have been found in both plants and animals (reviewed by Simpson, 1999). Despite advances in our understanding of the mechanisms that underlie RNA editing, we still are faced with the intriguing question; what is the biological function of this process? To address this question, we chose to generate null mutations of an entire class of RNA editing in an organism, Drosophila melanogaster, which possesses a single ADAR (adenosine deaminase acting on RNA) gene. ADAR enzymes convert adenosine (A) to inosine (I) in double-strand (ds)RNA and were first discovered in Xenopus oocytes (Bass and Weintraub, 1988). ADARs have been purified and cloned from many diverse sources and act via hydrolytic deamination of A-to-I (reviewed by Rueter and Emeson, 1998). In mammals, there are three known ADAR enzymes; ADAR1, ADAR2, and RED2. They contain two or three dsRNA binding domains and a catalytic deaminase domain. The deaminase domain is distantly related to cytidine deaminases, and in particular to another editing enzyme, APOBEC-1, that catalyzes the conversion of C to U in transcripts of mammalian apolipoprotein B (reviewed by Chan et al., 1997; Maas et al., 1997). The deaminase domain is also related to the ADATs (adenosine deaminases acting on tRNA) that convert A-to-I in tRNA (reviewed by Keller et al., 1999).

ADARs are defined by their ability to convert A-to-I nonselectively in extended perfect dsRNA duplexes. Such activities have been detected in extracts from lower invertebrates through vertebrates, and hence, the enzymes for converting A-to-I in mRNA have an ancient origin. The exact biological role for this promiscuous activity is not known but the activity has been proposed to play roles in viral defense, viral life cycles, and gene regulation (Bass et al., 1989; Bass, 1997; Kumar and Carmichael, 1997). Recently, edited mRNAs were identified in C. elegans on the basis that they contain inosine (Morse and Bass, 1999). These mRNAs were shown to possess extensive modification of 3′ untranslated regions within extended hairpin structures, and candidate ADARs have been identified in C. elegans (Hough et al., 1999). Likewise, a Drosophila adenosine deaminase acting on RNA, dADAR, has been identified and shown to possess promiscuous A-to-I activity (Palladino et al., 2000).

ADARs are also capable of the site-specific conversion of A-to-I in precursor messenger RNAs (pre-mRNAs). The examples of site-specific editing are limited to transcripts found in the nervous system of vertebrate and invertebrate animals. Most of these transcripts encode ligand- or voltage-gated ion channels and G protein-coupled receptors (reviewed in O’Connell, 1997; Rueter and Emeson, 1998; Keller et al., 1999). Because inosine has basepairing properties like that of guanosine (G), the translation machinery interprets I as G (Basilio et al., 1997). Thus, A-to-I conversion in mRNA has the potential to recode genomic information and alter protein function. Despite the paucity of examples of pre-mRNA editing, inosine has been detected in polyA−mRNA from a variety of tissues in mammals, reaching a maximum level in mRNAs from nervous tissue (Paul and Bass, 1998). Thus, additional pre-mRNA targets of ADARs probably exist.

The best characterized examples of A-to-I RNA editing are found in the glutamate receptor subunit genes (GluRs) of the mammalian nervous system (reviewed by Seeburg et al., 1998). One site in GluR-B transcripts, the glutamine (Q)/arginine (R) site, undergoes editing which changes a genomically encoded Q codon (CAG) to an R codon (CCR) (Sommer et al., 1991). The functional consequences of editing at this site are striking: Ca2+ permeability of GluR channels containing GluR-B subunits is determined largely by the editing status of this site (Verdoorn et al., 1991; Kohler et al., 1993). Mechanistically, RNA editing of the Q/R site is dependent upon cis-acting sequences in the intron downstream of and complementary to the editing site (Higuchi et al., 1993). This editing site complementary sequence (ECS) was shown to base-pair with the region surrounding the Q/R site and form a double-stranded (ds) RNA secondary
structure. Transgenic mice missing the ECS failed to edit the Q/R site. Mice expressing such GluR-B Q/R editing-incompetent alleles in various heterozygous combinations were shown to exhibit seizures and early death (Brusa et al., 1995; Feldmeyer et al., 1999). Thus, site-specific A-to-I RNA editing profoundly affects protein function in vivo.

Further examples of specific A-to-I editing in mammals include other GluR editing sites, several sites in the serotonin receptor (5HT2;R) (Burns et al., 1997; Niswender et al., 1999), and the ADAR2 gene (Rueter et al., 1999). Although the functional significance has been investigated for some of these sites, their in vivo significance remains largely unknown. Interestingly, while the best studied example of mammalian A-to-I editing, the GluR-B Q/R site, is edited at a very high frequency in vivo (>99% of transcripts are edited), all of the remaining examples of editing occur at lower frequencies. Such intermediate levels of editing suggest a mixture of edited and unedited proteins in neurons or tissue specificity of editing. This is particularly intriguing considering a recent proposal that RNA editing in the nervous system evolved to fine-tune the function of the nervous system through subtle functional effects on ion channels (Seeburg, 2000).

In support of such a proposal, A-to-I RNA editing as a posttranscriptional regulatory mechanism for generating protein diversity in the nervous system appears to have ancient origins, as evidenced by several invertebrate examples. For instance, the squid voltage-gated potassium channel sqKV2 has been shown to undergo RNA editing at numerous positions (Patton et al., 1997). Transcripts of the major action-potential Na+ channel in Drosophila, the product of the paralytic (para) locus, have been shown to undergo RNA editing proceeding through a mechanism similar to that of the mammalian GluR Q/R sites (Hannah et al., 2000; Reenan et al., 2000). Other reported substrates for editing in Drosophila also encode nervous system signaling components, namely, the cacophony (cac) voltage-gated Ca2+ channel (Peixoto et al., 1997; Smith et al., 1998b) and the glutamate-gated Cl- channel, DrosGluCl-α (Semenov and Pak, 1999).

Here, we report null mutations for all ADAR activity in a metazoan. dADAR null mutants in Drosophila lack nonspecific dsRNA-dependent adenosine deamination activity, a measure of ADAR activity.Surprisingly, dADAR mutants develop into morphologically normal adults. We show that site-selective A-to-I editing of all known pre-mRNA targets in Drosophila, comprising 25 specific A-to-I editing sites in three different ion channel transcripts, is abolished in dADAR mutants. Despite the absence of any obvious developmental defects, the loss of dADAR activity confers profound adult behavioral deficits that include defects in motor control, mating, and flight. These behavioral defects increase in severity with age and are associated with neurodegeneration. Surprisingly, these defects do not shorten the adult life span of mutant animals. We propose that the principal function of pre-mRNA editing in Drosophila is the modification of adult behavior by altering the functional properties of signaling components in the nervous system.

**Results**

**Targeted Mutagenesis of the dADAR Locus**

A search for available mutations near the dADAR locus revealed no breakpoints or useful transposon insertions. We obtained insertions in the dADAR locus, on the X chromosome, through site-selected P element mutagenesis. In a screen of 20,000 lines, three insertions into the 5′ regulatory region of the dADAR gene, near the two predicted promoters of dADAR, were generated (Figure 1A). All insertions were aphenotypic and resulted in no detectable change in dADAR transcription or RNA editing of transcripts of the para locus (data not shown). Starting with one of these insertions, we then proceeded to target deletions to the dADAR locus via transposase-mediated P element excision. Our screen involved recovering lethal mutations linked to the dADAR locus. In a screen of 16,000 mutagenized X chromosomes, 13 lethal mutations mapping to the dADAR locus were recovered.

Molecular analysis revealed that all 13 mutations harbored deletions of the dADAR locus and comprised several classes (Figure 1A). One class deleted DNA unidirectionally in the 5′ direction removing exon −4a and upstream 5′ regulatory regions (alleles 2B3 and 5A1). Another, the largest class, were bi-directional deletions which remove all of exons −4a and −4b and the predicted promoter regions (alleles 1F1, 1F4, 1G3, 2J3, 3A1, 4F1, 5I2, 6A2, and 6K1). Lastly, two deletions were recovered that were unidirectional in the 3′ direction; allele 6C1 deletes from the P element insertion site to a region between alternative exons −1 and 0, and allele 5G1 completely deletes the dADAR locus including all coding sequences.

**Mutations in dADAR Are Conditionally Lethal and Confer Behavioral Deficits**

We were surprised to discover that all of the dADAR mutants obtained from our screen were conditionally lethal. That is, under ideal growth conditions dADAR mutants developed into morphologically normal adults that displayed profound behavioral deficits and these phenotypes were recessive. All dADAR alleles were phenotypically indistinguishable, including the 5G1 allele that deleted the entire dADAR locus. We describe the mutant phenotypes in more detail below.

Two alleles were chosen for genetic mapping analysis, 1F1 and 1F4. Using markers defining a 1.5 centimorgan (cM) interval flanking the dADAR locus, we screened 40,000 chromosomes for recombination and generated 235 recombinant chromosomes. The presence of the mutant phenotype in recombinants correlated with the associated molecular lesion at the dADAR locus and, thus, we were unable to separate the mutant phenotypes from molecular lesions at the dADAR locus. The minimum genetic distance between dADAR lesions and the mutant phenotype determined from these experiments corresponded to 0.006 cM (~4 kb in terms of the Drosophila physical map). The region of the dADAR locus has been sequenced and no other predicted genes are present within at least 40 kb surrounding the dADAR locus. Thus, we conclude that the mutant phenotypes observed in dADAR mutants result from extremely hypomorphic or null alleles of dADAR.

**Duplication Rescue of dADAR Mutants and Lack of Maternal Effect**

All phenotypes of dADAR mutant males were rescued by a Y chromosome bearing a translocation of the tip of the X chromosome (Dup(X;Y)901) containing the complete dADAR locus. One behavioral defect observed in
**Figure 1.** Targeted Mutagenesis of the dADAR Locus and Transcriptional Analysis

(A) The restriction enzyme map of the Berkeley Drosophila Genome Project (BDGP) P1-bacteriophage clone DS04654 is shown with the region of the dADAR transcription unit expanded below. S = ScaI, X = XbaI, B = BamHI, R = EcoRI. T(1,2)dor var7 marks the site of a known translocation breakpoint. White boxes indicate exons, black or gray boxes indicate alternative exons. Arrows at exons 2a and 2b indicate the alternative transcriptional start sites. DRBM indicates the position of a double-stranded RNA binding motif. Black triangle marks the insertion site in dADARHD57. Imprecise excision of HD57 generated the deletions of dADAR indicated below the transcription unit. Parentheses indicate that the deletion endpoints have been cloned and characterized by direct sequence. Allele designations are given to the right of each deletion. Deletions whose exact endpoints are unknown are indicated (//).

(B) dADAR transcripts were undetectable in the 5I2 or 5G1 alleles of dADAR. Semiquantitative RT-PCR was performed on Canton-S (C-S) males or dADAR mutant males (alleles 5I2 and 5G1). Arrows indicate the sizes of dADAR specific products or products of the internal control, ribosomal protein 49 (RP49). Cycle numbers increase 2 cycles per lane from left to right (22–36). dADAR transcripts were undetectable in the 5I2 or 5G1 alleles of dADAR. PCR primers used to generate products are located in exons 1 and 3.

dADAR mutant males was an extreme defect in mating. dADAR males rarely successfully mate with wild-type (WT) females. Nevertheless, a rare successful mating between females heterozygous for dADAR and dADAR males was able to produce viable progeny amongst which were females homozygous for dADAR and dADAR males. Mutant females displayed behavioral defects similar to hemizygous males. We efficiently generated females homozygous for all alleles of dADAR displaying mutant behavioral phenotypes similar to hemizygous dADAR males.

In contrast, homozygous dADAR females could be mated by WT males, were fertile, and gave rise to morphologically normal dADAR male progeny which exhibit the dADAR adult behavioral defects. Since dADAR male progeny of mothers lacking dADAR are indistinguishable from those generated by mothers heterozygous for dADAR, we conclude that there is no significant requirement for a maternal contribution of
dADAR activity. Thus, dADAR would seem not to be required during early development. In addition, Dup(X;Y)901 undergoes nondisjunction at a moderate frequency. This allowed females with two doses of mutant dADAR and one copy of Dup(X;Y)901 to be generated. Dup(X;Y)901 also rescued all behavioral defects in mutant females.

dADAR Mutants Are Transcriptionally Compromised and Lack Nonspecific ADAR Activity

Semiquantitative RT-PCR revealed that two alleles with large deletions, 5I2 and 5G1, produce no detectable dADAR transcripts resulting in at least a 1000-fold reduction in message levels (Figure 1B). This is consistent with the observation that the dADAR5G1 lesion results in the deletion of the entire dADAR locus. Three other alleles tested, 1F1, 1F4, and 6C1 exhibit at least a 10- to 20-fold reduction in dADAR transcripts (data not shown). This is consistent with the deletion of promoter regions in these alleles. In addition, these transcripts lack the normal 5’ untranslated regions and numerous alternative exons and would be unlikely to support translation.

The mammalian ADARs (ADAR1 and ADAR2), which are capable of specifically editing transcripts of the glutamate receptor subunit genes and the 5-HT\sub{1C} receptor gene, also possess additional nonspecific A-to-I conversion activity on synthetic duplex RNA. Such a nonspecific ADAR activity has been directly detected in embryonic extracts of Drosophila and dADAR has been shown to possess A-to-I activity (Casey and Gerin, 1995; Palladino et al., 2000). In order to address the contribution of dADAR to total ADAR activity in flies, we assayed nonspecific A-to-I conversion activity present in crude protein extracts from WT and dADAR\sup{1F4} adults (Figure 2A). In short, [\alpha\sup{32}P]ATP labeled synthetic dsRNA was transcribed and incubated with crude head extract. Modified dsRNA was digested with P1 ribonuclease and the resulting mononucleotide mixtures separated by thin-layer chromatography to resolve adenosine from inosine. While dsRNA-dependent adenosine deaminase activity was detectable in WT male head extracts, no A-to-I conversion activity was detected in extracts from the heads of dADAR\sup{1F4}. Quantitation of the amount of inosine converted in extracts revealed an average overall decrease in promiscuous A-to-I conversion activity of at least 12-fold in dADAR\sup{1F4} (n = 4). This is a conservative estimate of the decrease in activity as the level of ADAR activity was frequently lower in the mutant flies than the negative control (no extract). Extracts from WT versus dADAR\sup{1F4} whole animals gave similar results.

Drosophila has been shown to possess at least one adenosine deaminase acting on tRNA, dADAT1 (Keegan et al., 1999). To ensure that the mutant and WT were equivalent with regard to a related enzymatic activity, the same extracts were assayed for the conversion of A-to-I at positions 34 and 37 of Bombyx mori tRNA\sup{Ala}. The mutant extract was indistinguishable from WT extract as regards specific conversion of A-to-I in the tRNA substrate (Figure 2B). The adenosine deaminases acting on tRNAs (dADATs) have adenosine deaminase catalytic domains homologous to those of ADARs but lack dsRNA binding domains and are active on tRNAs (Gerber et al., 1998; Gerber and Keller, 1999; Keegan et al., 1999). Thus, dADAR mutants were shown to abolish nonspecific A-to-I editing activity without affecting tRNA-specific editing activity. From this and our transcriptional analysis of dADAR mutants, we conclude that dADAR encodes the major dsRNA-specific adenosine deaminase in Drosophila.

Specific Editing of Ion Channel Pre-mRNAs Is Abolished in dADAR Mutants

To address the status of specific A-to-I RNA editing in dADAR mutants, we chose to analyze an RNA editing site in cac voltage-gated Ca\sup{2+} channel transcripts. We generated cac cDNAs via RT-PCR from WT and dADAR\sup{1F4} flies. In the case of the cac N/S-1 site (Table 1), editing abolishes cleavage by a restriction enzyme. RT-PCR products from behaviorally normal animals heterozygous for dADAR mutations had WT levels of RNA editing while editing in two different dADAR mutants (1F1 and 1F4) was undetectable (Figure 3A). We then tested all of the mutants in complementation tests by dADAR\sup{1F1} or dADAR\sup{1F4}. Females doubly heterozygous for either of these alleles and any of the other 11 dADAR mutations displayed behavioral phenotypes which were indistinguishable from females homozygous for dADAR\sup{1F1} or dADAR\sup{1F4}. Correspondingly, all double heterozygote dADAR combinations showed failure of complementation at the molecular level revealing no detectable editing at the cac N/S-1 site (Figure 3B). Since dADAR5G1 removes the entire dADAR transcription unit and is indistinguishable in behavioral and molecular tests from all

Figure 2. dADAR\sup{1F4} Mutant Extracts Contain No Detectable ADAR Activity

(A) Nonspecific ADAR activity from head extracts of dADAR\sup{1F4} mutants and WT C-S adult males were assayed for A-to-I conversion activity on [\alpha\sup{32}P]ATP labeled synthetic dsRNA. Control lane (–) contains no extract. Lanes 1-4 show the results with dADAR\sup{1F4} head extracts and lanes 5-8 with C-S extracts. Assays were performed with the following amounts of total protein from extracts: lanes 1 and 5 contain 4.69 \mu g, lanes 2 and 6 contain 14.07 \mu g, lanes 3 and 7 contain 29.31 \mu g, and lanes 4 and 8 contain 58.62 \mu g. The reaction products were separated on a TLC plate, the origin (Ori), AMP, and IMP are indicated.

(B) Conversion of A-to-I in tRNA\sup{Ala} from Bombyx mori. The same extracts from (A) were used to assay for the conversion of A-to-I in tRNA\sup{Ala} labeled with [\alpha\sup{32}P] ATP. The amounts of extract used per lane and (–) are the same as in (A).
of the other dADAR alleles, we conclude that all 13 dADAR mutations generated in this study are null. Extending our analysis of specific editing, we determined the editing status of all reported editing sites in transcripts of the cac, para, and DrosGluCl-α genes. Where editing events generate or abolish restriction enzyme cleavage sites, RT-PCR products were obtained from whole fly RNA and digested with the appropriate restriction enzyme (Figure 4A). Otherwise, RT-PCR products were subjected to direct sequence analysis and analyzed for the presence of a mixed A/G signal in the chromatographic data (Figures 4B and 4C). Editing was undetectable at all 25 sites analyzed (Table 1). Control experiments demonstrated that the level of detection of editing using the direct sequence method was about 5% (data not shown).

Although no editing was seen at any site using restriction enzyme assays or sequence data in dADAR mutants, we were interested in determining a lower limit to the detection of specific RNA editing in dADAR mutants. To this end, we isolated more than 100 partial cDNAs from both WT and dADAR mutants and assessed their editing status (Table 2). In wild type, where 112 cDNAs were analyzed, 116 out of a potential 336 adenosine residues were modified. In dADAR mutants, our analysis of 103 partial cDNAs from para failed to detect a single modified adenosine residue in 309 potential sites. Thus, this method for detection of site-specific editing would be sufficient to detect less than 1% residual activity remaining in dADAR mutants. From this, we again conclude that all dADAR mutant alleles described here are null mutations and that dADAR is the site-specific A-to-I RNA editing enzyme of all reported pre-mRNA editing sites in Drosophila.

**Developmental and Phenotypic Analyses of dADAR Mutants**

Since we showed that maternal dADAR is dispensable, we were interested in determining whether dADAR animals display normal development in the absence of a zygotic contribution of dADAR. We analyzed the time course of development from egg-laying to pupariation of WT and dADAR mutants (Figure 5A). No significant difference was observed. As well, we saw no significant difference between WT and mutant animals in the time course from onset of pupariation to eclosion into morphologically normal adults (Figure 5B). dADAR mutant animals showed a slight reduction in viability versus WT (71 ± 6 and 92 ± 3, respectively).

Mutant dADAR larvae appeared normal for locomotion, response to stimuli, and were also normal in a specific behavioral assay. We determined the phototactic behavior of dADAR versus WT larvae and observed no measurable difference (response indices of 0.81 ± 0.09 and 0.75 ± 0.09, respectively).
dADAR Mutants, though Not Short-Lived, Are at an Extreme Selective Disadvantage

Although dADAR mutants are severely compromised for many aspects of adult behavior, they perform the behaviors and functions necessary to sustain life (eating, respiration, metabolism). We were interested to determine whether dADAR mutants were short-lived as a result of their neurobehavioral enfeeblement. We performed life span analysis on dADAR


dADAR F1 males populations under optimal environmental conditions (low population density). As controls, we compared the life spans of populations of dADAR


B) Double heterozygotes were generated between dADAR


C) resulting in bouts of paralysis and extreme motor inactivity resting on their backs. Many animals beyond the leg-shaking characteristic of mutations which in-duced leg shaking. While


As can be seen, even under relatively low population density, dADAR mutants exhibit a high mortality rate with respect to WT animals (Figure 6B). Thus, dADAR mutants are at an extreme selective disadvantage even early in their life span, and this disadvantage is rescued by a translocation that provides dADAR + function and behavioral rescue.

Brain Degeneration Is Seen in Aging dADAR Mutants

The behavioral phenotypes of dADAR− animals become more severe with age and some new phenotypes appear. Tremors increase dramatically such that locomotion is severely compromised in animals beyond day 50. Animals fall over and become increasingly inefficient at righting themselves; sometimes leading to periods of inactivity resting on their backs. Many animals beyond day 30 exhibit circling behavior that varies from wide circling to circling while standing in place. A majority of animals beyond day 50 exhibit a persistent upheld wing phenotype. Marked asymmetries appear in animals, manifesting as one upheld wing or leg, extension of one or both back legs, and more severe asymmetries in posture. The progressive nature of these defects prompted us to determine the gross nervous system morphology of dADAR mutants. Frontal sections through adult heads of male dADAR F1 and dADAR F4 were stained with hematoxylin and eosin. As controls, we used Dup(X;Y)901 rescued males or males harboring a dADAR− recombinant chromosome derived from the original dADAR F1.

Figure 3. Specific Editing of a cac Editing Site Is Abolished in all dADAR Mutants

(A) Partial cDNAs of the cac locus were generated from dADAR mutants and WT controls by RT-PCR. These partial cDNAs spanned the cac asparagine-serine (N/S)-1 editing site. Bulk RT-PCR product was then digested with a diagnostic restriction enzyme (SspI). RNA editing at this site destroys a restriction enzyme recognition site. An arrow indicates the position of product indicative of editing. As can be seen, dADAR mutant alleles 1F1 and 1F4 produce no detectable edited product while a heterozygote for dADAR F4 (HF4) produces edited product. (B) Double heterozygotes were generated between dADAR F1 or dADAR F4 and all remaining alleles described in this study. RNA editing of the cac N/S-1 site was undetectable in all allelic combinations tested demonstrating failure of complementation between all alleles of dADAR at the level of specific editing activity.

dADAR mutant adults, however, exhibit severe neurobehavioral phenotypes. Motor deficits, manifesting as slow uncoordinated locomotion, occasional tremors and varying degrees of abnormal body posture are observed immediately upon eclosion. Also apparent soon after eclosion, dADAR− animals spend an inordinate amount of time grooming (5 ± 0.5% for WT, 27 ± 6% for dADAR F2). This obsessive cleaning is apparent throughout the lifetime of mutant animals. dADAR mutants are capable of flying and jumping but do so only when repeatedly provoked and then only rarely. Not unexpectedly, flight in dADAR mutants is erratic. dADAR− males exhibit an extreme mating defect. When presented with WT or dADAR− virgin females, males were not observed to initiate any displays of courtship.

Since we have shown that the targets of dADAR activity are ion channel transcripts of the nervous system, we tested dADAR mutants using two paradigms that have been extremely successful in detecting ion channel mutations, temperature-sensitive (ts) paralysis and ether-induced leg shaking. While dADAR mutants recover more slowly from ether anesthesia, they do not exhibit the leg-shaking characteristic of mutations which increase membrane excitability. However, dADAR mutant flies exhibit a strong temperature-dependent enhancement of behavioral defects at the restrictive temperature (37.5 °C) resulting in bouts of paralysis and extreme motor uncoordination. Mutations in a number of genes affecting ion channels or the process of neurotransmission confer similar phenotypes (Wu and Ganetzky, 1992; Littleton et al., 1999). The lack of a significant developmental phenotype, outwardly normal adult morphology, varied behavioral deficits, and ts phenotype are all consistent with the primary role of dADAR being the editing of transcripts encoding signaling components of the adult nervous system.
Figure 4. All Specific Editing Sites Are Abolished for Three Ion Channel Genes

(A) Partial cDNA were generated spanning all reported RNA editing sites in para and cac from dADAR<sup>£1</sup> and dADAR<sup>£4</sup> animals and control animals heterozygous for the dADAR 1F1 allele (HF1) or the 1F4 allele (HF4). Where RNA editing either creates or destroys diagnostic restriction enzyme sites this assay was used. Arrows indicate the position of bands indicative of RNA editing. RNA editing was not detectable in either dADAR mutant tested for the sites indicated.

(B) Direct sequence analysis of cac cDNA generated by RT-PCR. For the sites indicated, editing is seen as a mixed sequence signal (A and G) in the chromatogram.

(C) Direct sequence analysis of DrosGluCl-α cDNA generated by RT-PCR. For the sites indicated, editing is seen as a mixed sequence signal in the chromatogram.

mutant chromosome. One gross difference between dADAR mutants and controls is the organization of the retina. The photoreceptors in dADAR<sup>−/−</sup> animals appear disorganized and extend longitudinally projecting further to reach the laminar layer in mutants versus the more compact, organized retinal structure seen in WT controls (Figure 7). This retinal abnormality is seen in all sections through mutant heads of all ages tested.

Other than structural abnormalities in the retina, young dADAR mutant brains (1-3 day) appear grossly normal. However, by day 30, lesions appear in the brains of dADAR mutants and are distributed randomly in the central brain, optic lobes, and retina (Figures 7A-7C). The lesions appear as vacuolated regions, are distributed throughout the sectioned brains, and are most prevalent in the retina, lamina, and optic lobes. Vacuoles appear to increase in size and number with age and, by day 50, animals can be found with extensive brain degeneration (Figure 7E, compare to 7D). Control animals never demonstrated brain degeneration and seldom had even one small vacuole per head.

**Discussion**

ADAR activity and ADAR homologs have been identified in a wide range of metazoaons demonstrating that the conversion of A-to-I in RNA substrates is an ancient process. Still, there are relatively few identified pre-mRNA targets of ADARs and even less data on the in vivo significance of A-to-I RNA editing. Thus, an important question remains. What are the global roles of ADARs in gene regulation and organismal function? A hint at the answer may be the prevalence of nervous system targets of ADARs in both invertebrates and mammals. From data presented here, we conclude that the process of A-to-I RNA editing in Drosophila is, primarily, a process subserving the execution of adult behaviors through effects on numerous and varied nervous system targets.

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<td>0.0% (n = 103)</td>
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<tr>
<td>N/S</td>
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Table 2. Editing at Three Sites in para Transcripts
targets of ADAR activity. Moreover, we propose that the process of A-to-I editing is, by its very nature, ideally suited for modifying activity in the nervous system.

dADAR Encodes the Pre-mRNA Editing Enzyme for Every Reported Editing Site in Drosophila

We report here null mutations of the Drosophila ADAR, dADAR. Using a targeted mutagenesis scheme to obtain lethal mutations mapping to dADAR, we generated deletions of the dADAR locus. Due to the nature of the screen, we were surprised to discover that these mutations were conditionally lethal. Under ideal conditions, morphologically normal adult mutants were obtained which exhibited profound behavioral deficits. All dADAR alleles were recessive and genetic analyses were unable to separate the dADAR mutant phenotype from the molecular lesions at the dADAR locus. In addition, a translocation containing the dADAR locus was able to rescue all phenotypes of dADAR mutants.

Transcriptional analysis revealed that all mutants tested were compromised in transcription of the dADAR locus and that some alleles produced no detectable transcripts. In addition, ADAR activity was undetectable in crude extracts from a dADAR mutant while assays of a related enzymatic activity, which specifically modifies A-to-I in tRNAs, was unaffected. Thus, we have shown that dADAR encodes the major dsRNA-specific adenosine deaminase in Drosophila.

In order to address the function of dADAR in vivo, we tested the specific pre-mRNA editing of all reported targets of A-to-I RNA editing in Drosophila (Table 1). These are transcripts of the voltage-gated Na⁺ channel para, the voltage-gated Ca²⁺ channel cac and the glutamate-gated Cl⁻ channel DrosGluClα. Editing was abolished at all 25 of these sites in dADAR mutants. Thus, we have shown that dADAR encodes the site-specific adenosine deaminase acting on all reported pre-mRNA targets in Drosophila. Taken together with the result that
dADAR mutants abolish ADAR activity in extracts, we show that dADAR encodes the major pre-mRNA editing enzyme in Drosophila. Analysis of the completed Drosophila genome sequence supports our data as dADAR and dADAT1 (a tRNA adenosine deaminase) are the only predicted genes in the Drosophila genomic sequence that have ADAR-type deaminase motifs (unpublished observation).

The Primary Role of Pre-mRNA Editing in Drosophila Is in Adult Nervous System Function

The developmental time course of dADAR− animals was essentially the same as that of WT controls. We also showed that there is no requirement for maternal contribution of dADAR−, strongly suggesting that dADAR activity is not necessary maternally for oocyte formation nor for early development. Our data do not rule out a subtle zygotic role for dADAR during development.

Morphologically normal adult dADAR− mutants display profound behavioral defects including locomotor defects, jump and flight defects, tremors, and mating defects. In addition, mutations in dADAR confer ts uncoordination and paralysis. Most of these phenotypes worsen with age and new phenotypes appear including upheld wings, severe debilitating tremors, falling, difficulty righting, and circling behavior. Despite the extreme neurological dysfunction of mutants and age dependence of dADAR− phenotypes, these animals are not short-lived. However, their behavioral deficits place dADAR mutants at an extreme selective disadvantage even early in their lives when most mutant phenotypes are much less severe. Gross analysis of brain structure in dADAR mutants revealed anatomical defects in the retina as well as brain lesions appearing in all areas of the brain with increasing age. Further studies using transmission electron microscopy will be necessary in order to elucidate the exact nature of the ultrastructural brain defects seen in dADAR mutants.

The presence of ADAR activity in many mammalian tissues along with the observation that inosine-containing mRNAs are present in all mammalian tissues make our results surprising (Wagner et al., 1990; Paul and Bass, 1998). dADAR activity does not appear to be in Drosophila Is in Adult Nervous System Function. In fact, mutations have been generated in some of the ion channel targets of dADAR (described in this paper) which confer behavioral phenotypes. For instance, cac mutants exhibit specific defects in male courtship song, visual defects, and temperature-dependent convulsions and uncoordination (Peixoto and Hall, 1998; Smith et al., 1998a). Mutations of para also confer a wide range of behavioral phenotypes including recessive and dominant ts-paralysis, defects in learning and olfaction, cold-sensitive lethality, genetic suppression of certain mutations in the Shaker K+[Cl] channel gene, and resistance to insecticides (Loughney et al., 1989; Stern et al., 1990; Lilly et al., 1994; Pittendrigh et al., 1997). Glutamate-gated Cl− channels, like DrosGluCl-α (Cully et al., 1996), have only been reported in invertebrates. They are a
target of the avermectin class of insecticides and are found in several neuronal and muscle preparations (Delgado et al., 1989; Cleland, 1996). Though no mutations in DrosGluCl-5 have been reported, the presence of glutamate-gated Cl- channels on the soma of neurons and their similarity to the mammalian inhibitory glycine receptor, mutations in which cause inherited startle disorders to unexpected stimuli (Rajendra and Schofield, 1995), are also consistent with the idea that RNA editing of DrosGluCl-5 modulates neuronal membrane excitability.

The extensive and varied behavioral defects in dADAR mutants are consistent with effects on target genes such as para, cac, and DrosGluCl-5; ion channels of the nervous system. Like all other examples of site-specific A-to-I editing though, these 25 sites in three Drosophila genes were discovered serendipitously and other unidentified targets of dADAR activity surely exist. Thus, it appears unlikely that the nervous system impairment seen in dADAR mutants is due solely to any one particular dADAR target site. A more likely scenario, given the potential for hundreds of dADAR editing sites in scores of nervous system genes, is that the dADAR phenotype is complex and results from effects on the posttranscriptional processing of many transcripts in the nervous system.

Implications of RNA Editing for the Evolution of Animal Behavior
Since most A-to-I editing sites have been discovered in signaling components of the nervous system, one role of editing that has been proposed is in the fine-tuning of neurophysiological processes (Seeburg, 2000). Site-specific mRNA editing can have profound biological significance for individuals. The only example, and an extreme one, is the in vivo consequences of failure to edit the GluR-B Q/R site in mice. Editing at this site occurs at high frequency (~99%) and mutant mice expressing an editing-incompetent allele of GluR-B die postnatally from neurological dysfunction (Brusa et al., 1995). However, most editing sites are not edited at such high levels and must confer altered signaling properties in a cell-specific manner or alter neuronal signaling properties through dominant effects on protein function.

The singular role of dADAR in Drosophila pre-mRNA editing along with the adult neurological phenotypes of dADAR null mutants suggest a novel evolutionary role for dADAR in supplying genetic diversity. Genetic variation in large, diploid, randomly mating populations appears through mutation and is discrete in nature. That is, an individual animal can have zero, one or two alleles for a given nucleotide position and stochastic or selective processes act to determine the final frequency of that allele in a population over time. Cells can usually express, at most, two alleles differing at a single amino acid in a 1:1 ratio.

An important example of selection acting on variation in nature exists for the para gene. In natural insect pest populations, such as Heliothis (tobacco horn worm) or Blatella (cockroach), resistance to pyrethroid insecticides is prevalent and confers a strong selective advantage on animals carrying knock-down resistant (Kdr) alleles in para orthologs (Feyereisen, 1995). In the laboratory as well, certain Drosophila para mutations which are recessive ts-paralytic mutations confer dominant resistance to pyrethroid insecticides and DDT in heterozygotes (Pittendrigh et al., 1997). In fact, certain doubly heterozygous mutant combinations confer even higher levels of resistance. Thus, the advantage of modifying certain amino acid positions in ion channels is not without precedence and demonstrates the selective value of possessing two different alleles of a particular locus.

A-to-I RNA editing via ADARs is different. Changes introduced by editing are not discrete, such as naturally occurring genetic variation, but appear as a continuum. Within the 25 examples given in this report, the level of editing at particular sites ranges from ~5% to 90%. Though little is known about what determines the efficiency of deamination of pre-mRNAs in vivo, the substrate requirements of ADARs must play a large role. Specific pre-mRNA editing via ADARs has been shown, in mammals, to proceed through dsRNA intermediates in which intronic editing-site complementary sequences (ECSs) base-pair with regions around the edited adenosine (Higuchi et al., 1993; Herb et al., 1996). This mechanism has been conserved in Drosophila para editing (Reenan et al., 2000). Thus, the evolution of such RNA editing sites likely proceeds through the mutation of noncoding sequences. Many mutational changes to the genome may be required in intronic sequences to generate an initial, weakly complementary ECS. This nascent ECS would then direct modification of a particular nucleotide position at a low efficiency in nearby coding sequences resulting in an amino acid change in a small percent of messages. Certain de novo editing sites, though edited at low levels, would be selectively advantageous through dominant effects on protein function and resultant changes in behavior. Such a model provides an ideal substrate for evolution, the intron, for which mutation can provide a large sequence space of variants most of which would be selectively neutral. Larger introns would perform generate more sequence variants through time. Editing introduced at a low level at a particular site within a protein would introduce subtle variants within a population which could benefit from a selective advantage in a niche-specific manner altering behavior to suit environment. Future enhancements of editing, through improvements of the ECS-editing site interaction to generate a better ADAR substrate, could titrate in the edited form of the protein to maximally advantageous levels; all via changes to noncoding sequences. In effect, RNA editing provides the possibility to make use of amino acid changes that would be selectively disadvantageous at 50% (as a mutation appearing in coding sequence) offering a continuum of expression of two residues at a given amino acid position. Further diversity could be obtained in cases where the process of RNA editing became spatially regulated.

Conclusion
A-to-I RNA editing, while providing the potential for tailored mixtures of proteins differing at a single amino acid position, seems to have supplied this diversity primarily to the nervous system; a tissue whose hierarchical function, complex structure and direct influence on organismal behavior ideally suit it to take advantage of such continuous and subtle genetic variation. The future challenge of studying dADAR mutants will be in addressing the potential multitude of unknown pre-mRNA targets, the role of different dADAR isoforms in the editing of targets, the association of specific editing sites with specific modifications of behavior, and the natural history of ADARs and their substrates in the course of evolution.
Experimental Procedures

Fly Stocks
WT flies were C-S. Transposase overexpression stocks were: y w; K' P' y t7.2-Delta2-3 Y;9B and TMS, Birmingham 2 (17 defective P elements) was used as source of P elements in the site-selected mutagenesis. Dup xpath1 Y;901 - T(1;Y)901(dADAR1) and was the stock used to rescue the dADAR mutants. Balanced chromosomes used were FM7a and FM7G (contains actin promoter driving GFP expression).

Two-Step Targeted Mutagenesis
Site-selected mutagenesis was performed essentially as described (Ballinger and Benzer, 1989). Females with a balanced, P element mutated X chromosome were pooled (45) and screened by PCR for insertions in the dADAR locus (n - 20,000). dADAR was recovered as a positive generated with the following primers: P311R (element specific) and S105 (5\' - CAAACTGACCATCAGGCT TCCAGGC-3'). For targeted deletions y dADAR w; y/TMS males were generated and crossed en masse to wT/ww virgin females. F1 non-TMS virgin females from this cross were crossed singly to wT/ww males. F2 progeny were screened for the deletion of the y w+ male class, indicating a lethal mutation on the X chromosome. Flies were tested for editing (n = 25,000). Females with a balanced, P element (n = 6) and heterozygous control animals (n = 6). To determine the presence or absence of editing one or both of the following were performed: direct sequence of the RT-PCR product with a nested primer or restriction digestion of RT-PCR products with a diagnostic enzyme. Both y dADAR w/Y and y/ dADAR y/ males were tested for editing (n = 2-6). Control animals were dADAR w/FM7a and dADAR w/FM7a (n = 2-6). Details of the primers used for RT-PCR and sequence analysis are available on request.

Genetic Characterization of Lethals
All mutants were maintained as dADAR w/FM7a stocks. dADAR w FM7a females were crossed to Dup xpath1/Y males to generate dADAR w/Dup xpath1/Y males bearing a P element to dADAR w/Dup xpath1/Y males bearing dADAR w/Dup xpath1/Y homozygous females and dADAR w/Dup xpath1/Y doubly heterozygous females for complementation tests. Genetic data shows that the dADAR locus to 0.13 cM on the genetic map. (31 recombinants on the y-dADAR interval out of 237 recombinants total on the y-w interval. Total chromosomes screened n = 39,062.)

Semiquantitative RT-PCR
Whole RNA from mutant and control males was isolated using the LiCl/aqueous method (Auffray and Rougeon, 1980). The RNA was split, 1/2 for -RT controls and 1/2 was used for standard RT (reverse transcription) and PCR reactions. 5' -CTTGAGGACGAGAAGAGC ACC GTTGG-3' (RP49) and 5' -GGTTATCATATACGGT GTCGTTGC-3' (dADAR) were used in the RT reaction at -1 mM each. PCR primers were: 5' -CCAAGGACTTACCCGACCGAC CCC -3' and 5' -GGGGTGGCGG TTGTCGATTGC-3' (RP49-specific, added at end of cycle 7); and 5' -AATGCTATAACCGAATTTGCCAC-3' and 5' -TTGTCGGTCG TCAGGGCCGG-3' (dADAR-specific). Aliquots were taken from the reactions every cycle from 22 to 36, run on a 1.7% agarose gel with ethidium bromide (n = 3-7 for each genotype reported).

dsRNA Adenosine Deaminase Activity from Drosophila
Head Extracts
Approximately 600 dADAR w males or WT C-S males were frozen in liquid nitrogen and heads were isolated. Tissue was homogenized on ice (20 strokes with a Dounce homogenizer) in 3 ml of buffer C (50 mM Tris-HCl pH 7.9, 50 mM KCl, 0.2 mM EDTA and 10% Glycerol, 0.1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.7 mg/ml pepstatin and 0.4 mg/ml leupeptin). The extracts were centrifuged for 5 min at 2000 g and concentrated to a final volume of 150 µl using a Centricon 10 centrifugal concentrator (Amicon). The protein concentration was determined using bovine serum albumin as a standard and the extracts were stored at -70 C. The crude head extracts were divided into two aliquots to assay for dsRNA adenosine deaminase activity on extended dsRNA or for conversion of A-to-I at position 34 and 37 in tRNA as from Bombyx mori. Both assays were performed in parallel. Additional KCl and EDTA were added to the extract used for the dsRNA adenosine deaminase assay so that the final concentration of KCl was 100 mM and EDTA was 5 mM. The protein concentration was normalized so that the same concentration of either mutant or WT protein extract was added to the corresponding tube. The dsRNA was transcribed and annealed as previously described (O'Connell and Keller, 1994). Bombyx mori tRNA was transcribed with T3 RNA polymerase and [-32P]ATP and also gel purified. The dsRNA adenosine deaminase assay was performed as previously described (O'Connell and Keller, 1994) except that the incubation was at 30 C for 4 hr. The lower temperature was necessary due to the high level of RNases in the extracts. MgSO4 was added to the extract used for the tRNA assay so that the final concentration was 2.5 mM and this assay was performed as previously described (Auxilien et al., 1996; Gerber et al., 1998). In all assays, cold inosine was spotted onto TLC plates to serve as a standard.

Assays of In Vivo RNA Editing
For each site analyzed independent RT-PCR products were obtained using gene-specific RT and nested PCR primers from mutants (n = 6) and heterozygous control animals (n = 6). To determine the presence or absence of editing one or both of the following were performed: direct sequence of the RT-PCR product with a nested primer or restriction digestion of RT-PCR products with a diagnostic restriction enzyme. Both y dADAR w/Y and y/ dADAR y/ mutant flies were tested for editing (n = 2-6). Control animals were dADAR w/FM7a and dADAR w/FM7a (n = 2-6). Details of the primers used for RT-PCR and sequence analysis are available on request.

To quantify the frequency of editing remaining in dADAR mutants, a more sensitive cloning-based assay was employed. para RT-PCR products were generated with 5'-gtcagtagcgcagcaaagga gattaattgtgcgtgcc3'-3' and -ccgacgcattaattgctgctgcc-3' which span three editing sites that can be assayed by restriction digestion. These fragments were directionally cloned into pBluecript SK- (Stratagene) as SacI-Xbal inserts. Twelve independent clones of groups were obtained, three each from the following genotypes: y dADAR w/Y males, y/ dADAR w/Y males, y dADAR w/Y females, and y/ dADAR w/Y females. Clones from each group were obtained and independently digested with TaqI, SspI, and SfiI to indicate the editing status of para Q/R, K/R, and N/S editing sites, respectively.

To determine possible sexual dimorphism for editing, 10 editing sites described in this study were analyzed and editing was seen in both WT males and females.

Developmental Analysis
Nonfluorescing 48 hr old larvae were collected from apple-juice agar plates containing the progeny of y dADAR w/FM7G X FM7G/Y and y/ dADAR w/FM7G X FM7G/Y. Larvae were put into 9 independent control and mutant vials with 6-30 larvae each (n = 295 total). The timing of pupariation and fly eclosion were noted daily and lengths of development were determined. Survival to pupariation and total are the ratio of pupae and adults to initial larvae, respectively. Survival to eclosion are the ratio of those that reached pupariation. Experimental errors are SEM.

Larval Phototaxis Assay
The assay and RI (response index) calculations were performed as in Lilly and Carlson (1989). Late third instar larva were collected from a y dADAR w/FM7G X FM7G/Y population and sorted by GFP fluorescence. These larvae were allowed to choose dark or light areas on an agarose plate. The dark fields contained 3% dye (McCormick) and the clear fields did not contain dye. Three independent trials were performed with mutant (n = 60) and nonmutant (n = 58) larvae (R1 = .81 .99 and .75 .99, respectively). These responses are not significantly different (t test). Experimental errors refer to SEM.

Life Span Analysis
Flies were generated that contained either 10 mutant or control newly eclosed WT males. Genotypes tested were: y dADAR w/Y; y/ dADAR w/Y; and FM7G (n = 414, 350, 581, respectively). Flies were passed onto fresh food every 24-36 hr. Survival of the flies was recorded until all flies had died. Competition life span vials contained 10 y dADAR w/Y males, 10 y/ dADAR w/Y Dup xpath1/Y males, and 20 y/ dADAR w/FM7G females, newly eclosed. 14 groups.
of 10 vials were constructed and each day all of the surviving flies in a single group were counted for 14 consecutive days. Data for two-day intervals were used to calculate survivorship. For both of the above experiments vials did not contain supplementary yeast and were kept at room temperature. Experimental errors are SEM.

Drosophila Histology

Flies were manually decapitated and fixed in Carnoy’s fixative (4 hr at room temperature). The fixed heads were processed into paraffin, then serial sections were obtained (5 μm) and stained with hematoxylin and eosiin (all standard procedures). Serial sections through ~35 heads were obtained for each of the following genotypes: dADAR<sup>−/−</sup>, dADAR<sup>−/−</sup>, and dADAR<sup>−/−</sup>. Mutant flies were always processed in parallel with age-matched control animals.

Acknowledgments

We would like to thank the following for help with fly husbandry and screening: Laurie Bonneau, Christopher Hanrahan, Steve Helfand, Barry Hoopengardner, Blanka Rogina, Alicia Scilla, and Lee Ann Smith. In addition, we would like to thank: Brenton Graveley, Stephen Helfand, Jack Lalande, and Lee Ann Smith for helpful discussions and comments on the manuscript. This work was supported by NSF grant 9728737 to R. A. R.

Received April 20, 2000; revised June 20, 2000.

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