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ORIGINAL ARTICLE

Reduction of RNA A-to-I editing in *Drosophila* acclimated to heat shock

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Abstract Although an increasing number of RNA adenosine-to-inosine (A-to-I) editing sites are being discovered, how the editing frequencies of these sites are modulated to fine-tune protein function in adaptive responses is not well understood. A previous study screening for heat tolerance in *Drosophila* mutants discovered a *hypnos-2* mutant strain that was later found to be defective in *dADAR*, the *Drosophila* gene encoding the A-to-I editing enzyme. This supports the hypothesis that cells and organisms respond to stressful environments by ADAR (adenosine deaminase acting on RNA)-mediated RNA editing. Here, we investigated changes in the RNA A-to-I editing frequencies of 30 *Drosophila* nervous system targets in response to heat shock, a stress acclimatization that requires the *dADAR* function. To our surprise, most of these nervous system editing targets showed reduced editing. Our results suggest that a change in RNA editing pattern is a mechanism by which organisms acclimate to drastic environmental

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change. However, how RNA editing confers heat resistance is more complicated and requires further investigation.

Introduction

Drosophila premRNA adenosine deaminase (dADAR), which mediates RNA adenosine-to-inosine (A-to-I) editing, is highly expressed in the adult central nervous system [1]. Various genes involved in electrical and chemical transmission in the nervous system are subject to RNA A-to-I editing [2, 3]. Since inosine has base-pairing properties similar to those of guanine, A-to-I editing has effects equivalent to those of C-to-G mutation in DNA. Disruption of the *dADAR* gene results in a lack of editing of ion channels, prolonged recovery from anoxic stupor, vulnerability to heat shock, neuronal degeneration in aged flies, and severe neurologic dysfunction [4–8].

The activities of certain voltage-gated channels are highly controlled by adenosine deaminase. Electrophysiologic recordings show that a lack of editing of ion channels can lead to an increase in ion influx into neurons [9–11] and therefore plays a pivotal role in nerve cell injury caused by oxygen deprivation [9,12,13]. In human sporadic amyotrophic lateral sclerosis patients, editing of *GluR2* is reduced in spinal motor neurons [14]. Reduced adenosine-to-inosine

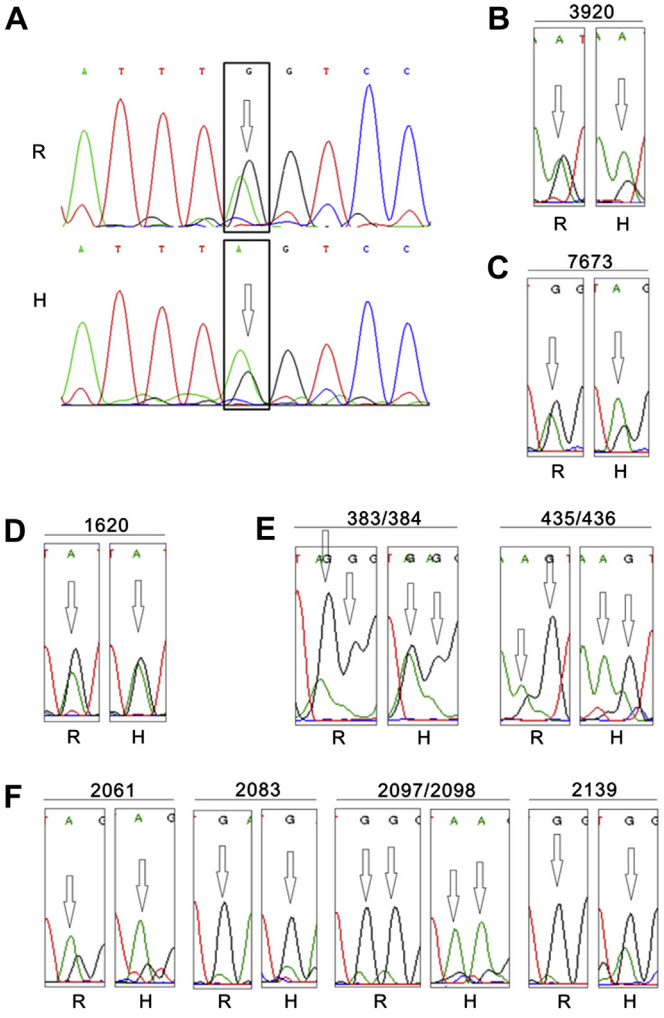

Figure 1. RNA A-to-I editing sites showing reduced editing in flies adapting to heat shock. Representative electropherograms covering editing sites of (A) *dADAR* exon 7, (B) *DSC* (nt 3920), (C) *unc-13* (nt 7673), (D) *Shaker* (nt 1620), (E) *ARD* (nt 383/384 and 435/436), and (F) *DmCa1D* (nt 2061, 2083, 2097/2098, 2139). The traces of A, G, T, and C are indicated by green, black, red, and blue, respectively. Peaks representing editing sites are indicated by arrows. Representative results of experiments in triplicate are shown. H = heat shock (37°C); R = room temperature (25°C).

(A-to-I) editing activity has been reported in human brain, prostate, kidney, and testis tumors [15]. Reductions in human ADAR2 activity are correlated with the malignancy of glioblastoma multiforme and pediatric astrocytomas [16]. These findings indicate that dysregulation of RNA A-to-I editing is an underlying pathogenic mechanism.

RNA A-to-I editing also helps the nervous system adjust to dramatic temperature changes by editing and fine-tuning the activity of ion channels. Temperature change impacts the function of the nervous system by affecting synaptic gain, as well as synaptic and conduction delays [17]. In their pioneering study of the electrical activity of the squid giant axon, Hodgkin and Katz found that the falling phase of the action potential was particularly sensitive to temperature [18], which was later shown to be caused by the temperature sensitivity of the gating property and closing kinetics of the potassium channel [19]. In cold temperatures, in the absence of functional adjustment through RNA editing, the delayed closing of the potassium channel would broaden the action potential, thereby limiting the firing frequency [20]. It is not surprising that the delayed rectifier potassium channels of squid and octopuses are found to be extensively edited [21–23]. Their editing patterns change in concert with the temperature of their living environment [23–25].

In this study, we sought to investigate how RNA A-to-I editing changes to compensate for temperature stress using *Drosophila* as a model. A previous study of *hypnos-2*^{P} mutant flies found that their reduced resistance to heat shock was due to a defective *dADAR* gene [4]. These findings, together with the aforementioned reports indicating that *dADAR* is required in the stress response of flies [5–8], led us to hypothesize that a general increase in editing frequency would be found in animals acclimating to heat shock.

Materials and methods

Drosophila stock

All reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were performed with Canton-S (C-S) flies. The stock was maintained on standard cornmeal/molasses/yeast agar medium at 25°C.

Heat shock

Adult flies (age 3–4 days, 50 flies per bottle) were placed in a 37°C incubator overnight in milk bottles containing culture medium.

RNA editing analysis

Whole-body RNA extraction from *Drosophila* (15–20 per sample) was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were amplified via RT-PCR using target-specific primers (see Supplementary Table 1 for details). Since I is reverse-transcribed to G, levels of editing were determined by measuring the height of the A and G peaks in individual electropherogram traces using ImageJ software (http://rsb.info.nih.gov/ij/). The percentage of editing was expressed as $G/(A + G) \times 100$.

Results

To evaluate the effect of heat shock on RNA A-to-I editing frequencies, we subjected *Drosophila* to 37°C heat shock overnight. In previous studies in which flies had been kept inside empty vials while receiving heat shock, the mortality rate reached 80% in 6 hours [4]. We noticed that by keeping flies in medium-containing vials, they could survive overnight incubation at 37°C. These flies had undergone physiologic changes to acclimatize to a high temperature, as we noticed that they required a significantly longer time to be anesthetized by ether compared to the room temperature control flies. Therefore, they were used to investigate how

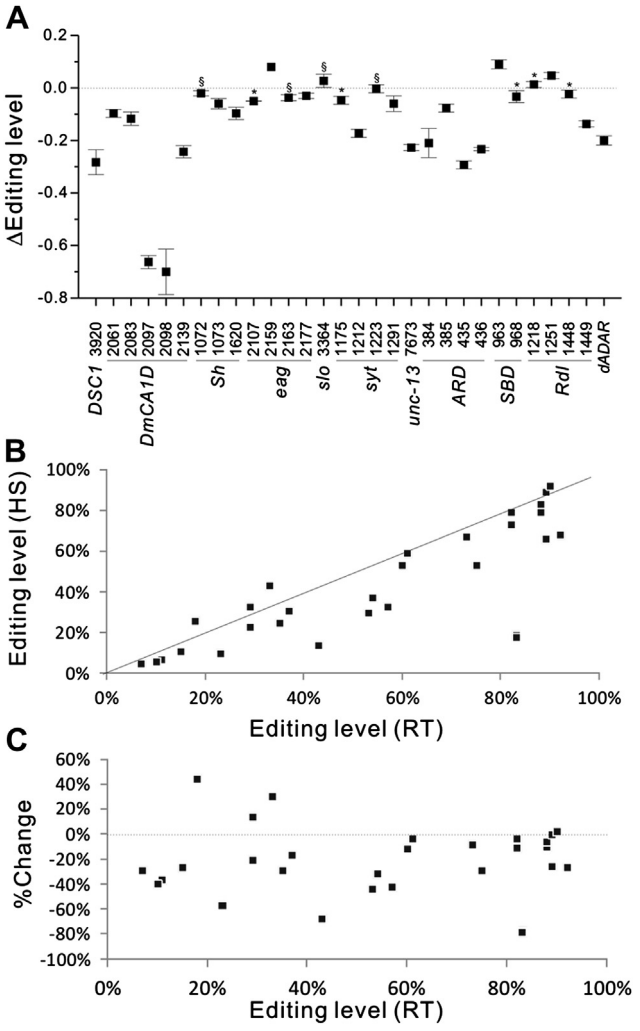

Figure 2. Lack of significant correlation of heat shock-induced editing level change to editing levels at 25°C. (A) Change of editing levels by heat shock. $^{\S}p > 0.05$; $^{*}0.5 > p > 0.1$; unmarked data points, $p < 0.01$. (B) Editing levels of 29 nervous system editing sites at room temperature (RT), were plotted against those seen after heat shock (HS). The slope of the solid line is equal to 1. (C) Percent change in editing levels in relation to baseline editing levels at RT. %Change = change in editing level normalized to the editing level at RT.

the A-to-I editing system is altered to counteract the environmental stress.

Total RNA was extracted from two groups of 25 flies, one exposed and one not exposed to heat shock. The editing patterns of the 29 nervous system editing sites identified by Hoopengardner et al. [2] and the *dADAR* exon 7 self-editing sites [26] were determined by RT-PCR and automatic sequencing as described in the Materials and methods section (see Supplementary Table 2 for a complete list of editing sites surveyed in this study). Most of the editing sites examined showed reduced editing (Fig. 1; note the lowered G peaks in the heat shock samples). Four sites showed a minor but significant increase in editing frequency (<10%; $p < 0.5$). Four sites showed no significant change, and the other 22 sites showed a significant increase in editing frequency after heat shock (Fig. 2A). All the reductions in editing frequency were within 30%, except for the 2097/2098 sites of *DmCa1D*. There was no clear correlation between the reduction in editing frequencies and the basal editing frequencies at room temperature (Fig. 2B and C).

RT-PCR was used to detect the expression of the various isoforms of *dADAR* that can be produced by using alternative promoters (−4a/b), alternative splicing of −1 and 3a exons, and self-editing at the single site within exon 7 (Fig. 3A). Isoforms expressed by both promoter −4a and −4b showed different expression patterns after heat shock (Fig. 3B and C), whereas the expression levels of the 3a and 3/4 isoforms were about the same (Fig. 3D). Exon 7 was also detected by RT-PCR as an indicator of overall *dADAR* expression levels, which showed a slight increase after heat shock (Fig. 3E). The constitutive ribosomal gene *RP49* showed no difference (Fig. 3F). Overall, there was a slight increase in *dADAR* expression and a reduction in the expression of −1 exon-containing isoforms (Fig. 3G).

Discussion

Previous studies have established that RNA A-to-I editing is reduced in nerve cells injured by oxygen deprivation

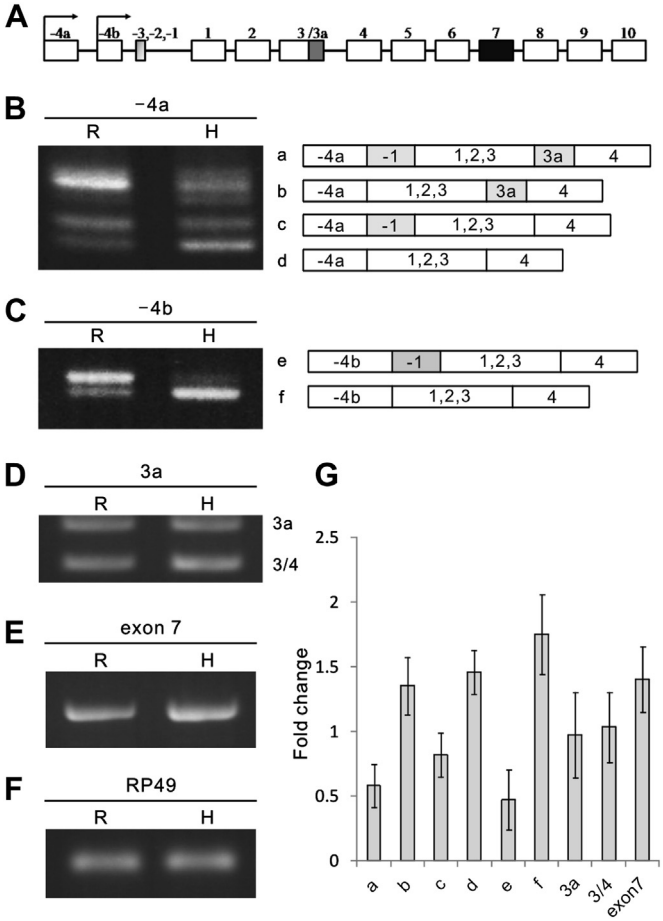

Figure 3. *dADAR* isoform expression. (A) Exon structure of *dADAR*. (B) Reverse-transcriptase polymerase chain reaction (RT-PCR) detection of isoforms expressed by −4a promoter using primers anchored in exon −4a and exon 4 (left panel). A total of four isoforms, a−d, are generated by alternative splicing of −1 and 3a exons (right panel). (C) RT-PCR detection of isoforms e and f expressed by −4b promoter using primers anchored in exon −4b and exon 4 (left panel). They differ by the inclusion of the −1 exon (right panel). (D) RT-PCR detection of 3a-containing (3a) and 3a-lacking (3/4) isoforms. (E, F) RT-PCR detection of *dADAR* exon 7 and *RP49*, respectively. The constitutive ribosomal gene *RP49* served as an internal control. (G) Heat shock-induced fold change in isoform expression. The RT-PCR results were quantified by densitometric analysis and normalized to the expression levels of *RP49*. Data are mean ± standard deviation, $n = 3$.

[9,12,13], and that flies with reduced A-to-I editing activity are vulnerable to hypoxia or heat shock [4]. One would assume that increased editing is required for cells encountering environmental stress, and, moreover, that an inability to increase editing frequency would lead to their demise. Investigating the editing patterns of nerve system target genes in flies surviving heat shock, we found that editing frequencies in the genes we examined in fact, in general, decreased in animals that survived heat shock. These unexpected results raise a number of interesting questions.

First, contrary to the results of previous studies [11,27], our findings indicate that reduced editing is not limited only to cells or animals that become vulnerable to stress. In prior studies, neurons showed reduced editing efficiency, higher ion permeability, excitotoxicity, and, subsequently, expiration due to drastic stress such as hypoxia [27]. In our system, flies that were able to withstand 37°C also showed a general reduction of editing efficiency.

Second, if *dADAR* is required for the stress response, why is editing reduced in flies acclimatized to stress? It could be that editing, although reduced, is still critical for the proper functioning of proteins in stressful conditions. The remaining edited forms, although expressed at lower levels, may still be required. Another, and more likely, explanation is that a new set of editing sites is edited by *dADAR* to enable flies to overcome stress.

Supporting evidence comes from recent research comparing the editing patterns of the delayed rectifier potassium channel in Antarctic and tropical octopuses that live in cold and warm waters, respectively [23]. The transcript sequences of the potassium channels are virtually identical in these two species, and together there are 12 non-silent editing sites. Eight of the nine sites that are edited in the Antarctic form show reduced editing in the tropical form. More importantly, three other sites are edited at high frequencies only in the tropical form. Therefore, it is likely that editing patterns in regular and stressful conditions may share largely overlapping editing sites, and, at the same time, there are some editing sites that are unique to, and only edited under, stressful conditions. Since the current lists of editing sites have been obtained using flies under regular conditions, sites only edited in heat shock conditions have not been documented and cannot be analyzed in this study.

Third, considering that the fly has only one *dADAR*, how can the editing frequencies in different genes, or even in different sites within the same gene, be altered differentially? In *dADAR* hypomorph mutants, reduction of *dADAR* activity affects editing sites differentially, with sites of low editing efficiency showing a greater reduction in editing [28]. One explanation is that highly edited sites possess better secondary structures that can still be recognized and edited in spite of reduced *dADAR*, and therefore they are less affected [28].

However, we do not believe that a change in *dADAR* expression level alone accounts for the editing pattern change observed in this study. We found that heat shock drastically reduced the editing frequencies of two edited sites of *DmCa1D*, which are in fact edited to almost 90% at room temperature (Fig. 1F). In addition, there is no clear correlation between the reduction of editing and the basal editing frequency (Fig. 2B and C). We believe that a better explanation is that differential editing site preferences are displayed by the expression of different *dADAR* isoforms [29]. This interpretation is supported by our results, which show that heat shock changed *dADAR* isoform expression by alternative splicing, such as the exclusion of exon-1 (Fig. 3).

Fourth, there is a possibility that sites that are edited under regular physiologic conditions may revert to unedited forms to help animals deal with environmental stress, for instance the 2097/2098 sites of *DmCa1D*, whose editing frequencies dropped from 84% to 14% after heat shock. *DmCa1D* encodes an essential L-type calcium channel [30,31]. In motor neurons, it is localized to somatodendritic processes where it may adjust the intrinsic excitability of the neuron by amplifying the postsynaptic current, regulating the activity of Ca^{2+}-activated potassium channels, and inducing gene expression through the calcium signaling pathway [32]. Editing of the 2097/2098 sites of *DmCa1D* alters the encoded amino acid from arginine to glycine. How such an editing event affects the functional properties of *DmCa1D* is still unknown, but one can envision that fine-tuning the activity of *DmCa1D* can effectively modulate neuronal activity in response to environmental stress.

In conclusion, the present study suggests that *dADAR* mediates resistance to stress without globally increasing editing activities. Furthermore, we propose that novel editing sites are edited in response to environmental stress. Our findings also highlight the need for methods that can conveniently survey dynamic shifts in editing pattern at the transcriptome level.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.kjms.2013.01.001.

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