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REVIEW

The emerging role of RNA editing in plasticity

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ABSTRACT

All true metazoans modify their RNAs by converting specific adenosine residues to inosine. Because inosine binds to cytosine, it is a biological mimic for guanosine. This subtle change, termed RNA editing, can have diverse effects on various RNA-mediated cellular pathways, including RNA interference, innate immunity, retrotransposon defense and messenger RNA recoding. Because RNA editing can be regulated, it is an ideal tool for increasing genetic diversity, adaptation and environmental acclimation. This review will cover the following themes related to RNA editing: (1) how it is used to modify different cellular RNAs, (2) how frequently it is used by different organisms to recode mRNA, (3) how specific recoding events regulate protein function, (4) how it is used in adaptation and (5) emerging evidence that it can be used for acclimation. Organismal biologists with an interest in adaptation and acclimation, but with little knowledge of RNA editing, are the intended audience.

KEY WORDS: ADAR, Acclimation, RNA modifications, RNA processing, Adaptation

Introduction

RNA is a well-positioned target for acclimation because it is transient. In contrast to the case with DNA, alterations to genetic information at the RNA level can be reworked when environmental conditions change. In addition to their inherent flexibility, changes to RNA are relatively safe because they are not permanent. Because of these incentives, organisms use diverse mechanisms to regulate genetic information as it passes through RNA. In terms of quantity, it is precisely controlled over orders of magnitude by regulating the amount of mRNA that is transcribed from a gene, and the rate at which the message decays. Organisms even choose to use RNA, in the form of micro RNAs (miRNAs), to regulate coding RNAs. In terms of genetic complexity, alternative RNA splicing is used to create proteins with an almost endless combination of domains; splicing also offers organisms control over where and when they wish to deploy specific isoforms. When it comes to mutations in codons, however, we invariably focus on DNA: mutations arise within genes. Sometimes they provide a selective advantage, become fixed, and ultimately become the currency for evolution. Mutations also can be introduced within RNA. Various enzymatic processes, collectively known as RNA editing, generate precise point mutations within RNA. The most widespread of these catalyzes the deamination of adenosine residues.

In the strictest sense, RNA editing implies that protein coding information is altered. Using this stringent definition we must focus on mRNAs. There are many ways to biochemically modify a mRNA, but only a subset of these will recode the genetic blueprint. To accomplish this, an RNA edit must change the way a codon is interpreted by the ribosome; or, more specifically, the tRNA anticodon with which it base-pairs. Biochemically, adenosine deamination is exceptionally simple; it exerts an outsize effect because of where it happens (Fig. 1). This form of editing is catalyzed by the adenosine deaminase that acts on RNA (ADAR) family of enzymes (Bass and Weintraub, 1988; Kim et al., 1994a,b; Melcher et al., 1996; Wagner et al., 1989). ADARs are composed of two types of domains: a variable number of N-terminal double-stranded RNA binding motifs (dsRBMs) followed by a single C-terminal catalytic domain. The dsRBMs bind to higher order structures in RNA and position the catalytic domain adjacent to the adenosine to be edited. The adenosine is then thought to be 'flipped' out of the structure into a catalytic pocket where it undergoes a hydrolytic deamination of the primary amine at the base's number 6 position, a position that directly contributes to Watson–Crick base pairing. This reaction creates inosine, a relatively rare base. Inosine, like guanosine, has a carbonyl oxygen at position 6; both base pair with cytosine. During translation, inosine is interpreted as guanosine (Basilio et al., 1962), so the net effect of editing is an A→G change. Several important points relate to this mechanism. First, editing does not occur randomly. On the contrary, it is quite specific and, to be edited, an adenosine requires a complex surrounding structure. Second, editing need not be complete; at any given time, only a portion of a gene's transcripts may be edited. This allows for regulation. Finally, multiple editing sites within a transcript create exponential diversity. All true metazoans express ADARs so they probably also edit their RNAs (Keegan et al., 2011). Vertebrates have two functional isoforms (ADAR1 and ADAR2), *Drosophila* has one (an ADAR2 ortholog), and most other invertebrates have at least two. In this review, I will discuss why adenosine deamination is an ideal tool for plasticity, how it is used to regulate protein function, and emerging evidence that it is used for both adaptation and acclimation.

ADARs do much more than alter codons

If we adopt a looser definition of RNA editing which includes adenosine to inosine (A→I) changes in all RNA molecules, we see that ADARs regulate diverse cellular systems (Fig. 2). Besides tRNAs and rRNAs, virtually all types of cellular RNAs, and some extracellular ones, are regulated by editing. Perhaps the best-studied examples are miRNAs. Genetic studies in *Caenorhabditis elegans* first linked ADARs with the RNA interference (RNAi) pathway (Knight and Bass, 2002; Tonkin and Bass, 2003). ADAR knockouts in these organisms caused chemotaxis defects. Interestingly, the wild-type phenotypes could be restored by further knockouts of elements in the RNAi pathway. These results led to a host of studies in mammals that focused on miRNAs. miRNAs undergo complex processing to reach their final form. Within the nucleus, the primary miRNA transcripts (pri-miRNAs) are cleaved by the endonuclease Drosha into a precursor miRNA transcript (pre-miRNA), and then exported into the cytoplasm by Exportin. Once outside the nucleus, they are further processed by the endonuclease Dicer before being loaded onto Argonaute within the RNA-induced silencing

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List of abbreviations

ADAR	Adenosine Deaminase that Acts on RNA enzyme
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
dsRBMs	double-stranded RNA binding motifs
miRNA	micro RNA
pre-miRNA	precursor miRNA transcript
pri-miRNA	primary miRNA transcript
RISC	RNA-induced silencing complex
RNAi	RNA interference

complex (RISC) complex. Messages are selected for silencing, or translational inhibition, via Watson–Crick base-pairing with the 'seed' region of a mature miRNA. As one might imagine, an A→I RNA edit could affect different steps related to miRNA biogenesis, nuclear export or targeting. First, miRNAs that contain multiple inosines can themselves be targeted for degradation by specific ribonucleases (Garcia-Lopez et al., 2013). Editing sites in miRNA precursors can interfere with the efficiency of processing by Drosha (Yang et al., 2006b) or Dicer (Kawahara et al., 2007a), thus regulating the abundance of the mature miRNA. ADAR can also be beneficial to processing. A recent study in mice suggested that direct interactions between ADAR and Dicer augment the efficiency of processing (Ota et al., 2013). Editing sites within the seed region can influence target recognition, and in some cases even redirect the RISC complex to an entirely different target mRNA (Kawahara et al., 2008, 2007b). ADAR also regulates the expression of elements within the miRNA processing machinery (Nemlich et al., 2013). Clearly, ADAR, through its interactions with RNAi, affects global gene expression. Although unstudied, its potential as a switch for acclimation is obvious.

Besides miRNAs, editing also plays a key role in the defense against viral RNAs and in innate immunity. Any virus whose chromosome exists as a dsRNA during infection can potentially be edited. Recent evidence shows that many viruses, upon entering a cell, do in fact undergo RNA editing (see Tomaselli et al., 2014 for an excellent review). The list includes some notorious members such as HIV-1, the Epstein–Barr virus, herpes virus 8, dengue

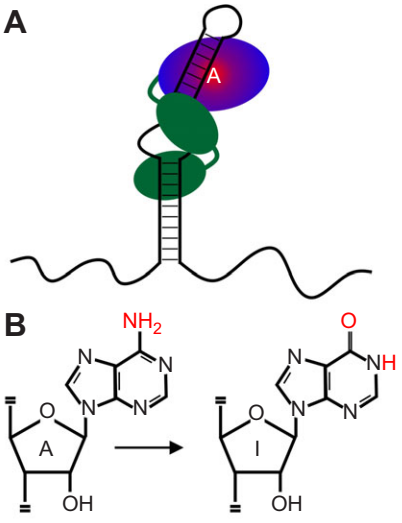

Fig. 1. The biochemistry of adenosine deamination. (A) A schematic diagram of Adenosine Deaminase that Acts on RNA enzyme (ADAR) binding to an RNA substrate. dsRBMs are shown in green and the catalytic domain in blue. A, adenosine. (B) Adenosine is converted to inosine. Groups in red are affected by the deamination reaction.

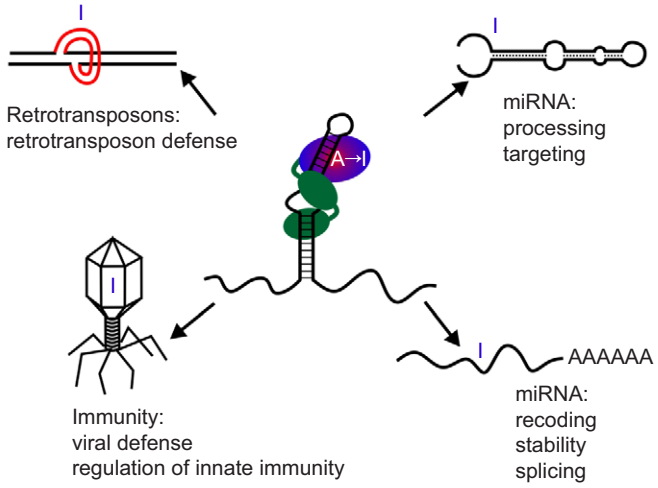

Fig. 2. RNA editing affects different classes of RNA and associated cellular pathways. Examples of different RNAs, and cellular functions, that can be affected by the enzymatic conversion of adenosine (A) to inosine (I).

virus, hepatitis C virus, influenza A virus and the measles virus (Mehedi et al., 2013). Interestingly, the relationship between RNA editing and infectivity is not always clear-cut: in some cases, editing appears to combat it and in others it promotes it (Pfaller et al., 2011; Samuel, 2011). When anti-viral, editing is thought to interfere with the virus's ability to replicate. When pro-viral, it is thought to down-regulate players in the innate immune response, like the interferon-inducible, dsRNA-activated protein kinase R. An intriguing relationship between RNA editing and innate immunity has been further explored in a recent study (Mannion et al., 2014). The interferon-induced innate immune response is often triggered by viral dsRNA entering the cell. Although effective in combatting viruses, it also causes inflammation and stress; thus, it is important for innate immunity to be carefully regulated. The authors suggest that inosine within RNA acts as an off-switch for innate immunity. They also posit that this is an ancient, well- conserved pathway. The interactions between editing and immunity should prove interesting for invertebrates as well.

RNA editing may serve as a defense against more than viruses. Recent advances in high-throughput DNA sequencing technologies have enabled transcriptome-wide tabulations of RNA editing events. Current estimates suggest that there may be more than a million editing sites in the human transcriptome, but almost all of them are in transcribed repetitive elements (Bazak et al., 2014; Carmi et al., 2011a,b; Eisenberg et al., 2005; Li et al., 2009; Paz-Yaacov et al., 2010; Porath et al., 2014; Ramaswami et al., 2012; St Laurent et al., 2013). The genomes of humans and other primates are full of repeats. Most are Alu element repeats, but there are some others. When two DNA repeats with highly similar sequences are close to one another, and one is inverted, they will create an RNA duplex when they are transcribed because one is essentially the antisense of the other. ADARs bind and edit such duplexes with high efficiency. This happens very frequently. Accordingly, it has been proposed that one of ADAR's primary functions may be to combat retrotransposon insertion within the genome (Levanon and Eisenberg, 2015). In addition, when inverted Alu repeats occur in the 3′ untranslated region of a mRNA, the message is silenced in an ADAR-dependent mechanism (DeCerbo and Carmichael, 2005).

Other frequent locations for editing sites, across all phyla that have been studied, are untranslated regions and introns (Park et al., 2012; Ramaswami and Li, 2014; Rodriguez et al., 2012; Zhao et al.,

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2014). As one might predict, editing has been shown to influence splicing (Agrawal and Stormo, 2005; Bratt and Ohman, 2003; Jin et al., 2007; Laurencikiene et al., 2006; Lev-Maor et al., 2008; Rueter et al., 1999; Ryman et al., 2007; Solomon et al., 2013) and mRNA stability (Chen and Carmichael, 2012; DeCerbo and Carmichael, 2005; Wang et al., 2013). It is reasonable to assume, therefore, that editing affects most RNA processing pathways. For the rest of this review, I will focus on changes to protein coding brought about by RNA editing. It is important to bear in mind, however, that there are many essential roles for editing outside of protein recoding. Proper ADAR regulation is vital, as small perturbations will affect many cellular functions.

Until recently, recoding RNA editing was thought to be rare

In general, when considering RNA, biologists are myopic, focusing almost exclusively on its role in genetic information transfer. Likely this is because the central dogma is one of our first lessons in introductory biology. At its inception, the field of RNA editing was no different. Early studies focused on edited codons and how they affect protein function. The first editing sites were discovered through serendipity: an astute investigator would notice a G in a cDNA sequence at a position occupied by an A in the corresponding genomic DNA and chalk it up to something other than a sequencing artifact. Only a handful of sites were uncovered by this means, but that was not surprising considering the vast number of messages, and the few people working on editing. Overall, the field was very interested in finding out whether RNA editing is widespread, but lacked the proper tools to tackle the issue. The generation of whole-genome sequences, and the advent of high-throughput DNA sequencing technologies, enabled direct answers to this question. They also funneled research into humans and the standard model organisms.

In mammals, insects and nematodes, recoding RNA editing is rare (Fig. 3). Most studies that have tabulated transcriptome-wide RNA editing have taken a similar approach: using a genome sequence as an anchor, RNAseq and DNAseq reads, generated from an individual, are aligned. Positions that contain a G in the RNA and an A in the DNA are candidates for editing sites. Then, various algorithms are used to filter out errors caused by single nucleotide polymorphisms (SNPs), allelic variation, multi-locus genes, sequencing artifacts and other sources. Finally, a subset of editing sites are further validated by standard Sanger DNA sequencing.

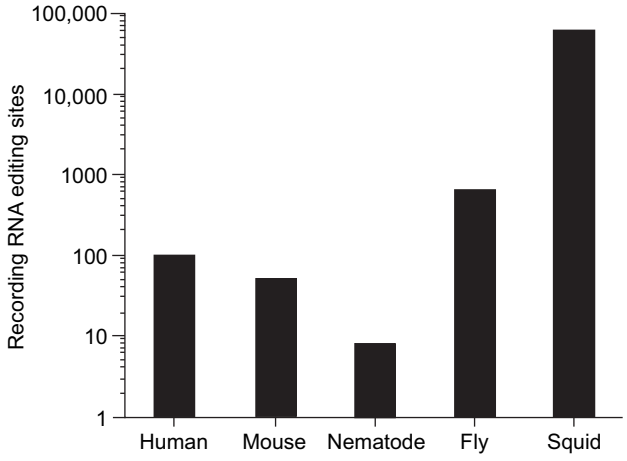

Fig. 3. The extent of recoding RNA editing across taxa. Nematode refers to *Caenorhabditis elegans*, fly refers to *Drosophila melanogaster*, and squid refers to *Doryteuthis pealei*. Data sets for graphed values are cited in the text.

A recent compilation of human RNA editing sites, generated from multiple studies, estimated that there were ∼1200 in total (Xu and Zhang, 2014). Most of the sites, however, only occurred in a single sample and at very low frequency. Individual studies put the number at ∼100 (Pinto et al., 2014; Ramaswami et al., 2013). In mice, there are even fewer sites. A recent estimate using multiple strains uncovered ∼50 (Danecek et al., 2012), 34 of which are conserved across mammals (Pinto et al., 2014). In *C. elegans*, only eight recoding sites were identified (Zhao et al., 2014). In leaf-cutter ants there are thousands of sites, many of which are caste-specific; however, only 57 sites recode (Li et al., 2014) and in the diamond-back moth there were 152 recoding sites (He et al., 2014). In *Drosophila melanogaster*, comparatively more recoding sites have been identified but they are still rare among messages. An Encode consortium effort (Graveley et al., 2011) and a subsequent study using a novel single-molecule sequencing pipeline (St Laurent et al., 2013) discovered 630 and 645 recoding sites, respectively. In both studies, recoding sites are enriched in mRNAs encoding proteins with neuronal functions. In particular, ion channels, ion transporters and proteins involved in synaptic vesicle release are frequent targets; however, mRNAs encoding other proteins are edited as well. Although there are about an order of magnitude more recoding sites in *D. melanogaster*, they only occur in ∼3% of all transcripts. In other organisms, less than 1% of transcripts harbor a recoding event.

Editing precisely regulates protein function

As might be expected for a natural system of site-directed mutagenesis, RNA editing is well equipped to make precise changes to protein function (Fig. 4). About half of all codons can be reprogrammed by an adenosine to guanosine (A→G) substitution, and this subset of changes is the theoretical limit for RNA editing functionality. Not only can it create missense 'mutations' but also it can also create and abolish start codons, and abolish termination codons. Without a doubt, the best-studied RNA editing site occurs within the GluA2 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit in the mammalian brain. The AMPA subclass of glutamate receptors underlies the bulk of fast, excitatory synaptic signaling in the brain. As with other ionotropic glutamate receptors, they are tetramers, normally composed of two different subunits. For mRNAs encoding the GluA2 subunit, editing occurs at codon 586, changing a genomically encoded glutamine to an arginine (Sommer et al., 1991). This edit is colloquially referred to as the Q/R site. Codon 586 encodes an amino acid at the narrowest aperture of the channel's ion conducting pore. Under physiological conditions, receptors with a glutamine at this position allow both sodium and calcium to permeate. An edit to an arginine renders the receptor impermeable to calcium and also decreases its conductance ∼10-fold (Burnashev et al., 1992a,b; Monyer et al., 1992; Sommer et al., 1991). Excessive calcium permeation through a major neurotransmitter receptor would be expected to wreak havoc on intracellular signaling. Not surprisingly, editing at the Q/R site occurs at close to 100% efficiency; editing at this site is required for survival. In mice, the Q/R site is edited by ADAR2; mice with their ADAR2 gene knocked out die shortly after birth (Higuchi et al., 2000). In fact, mice that contain a single allele of Gria2 (the gene encoding GluA2) that encodes RNAs that cannot be edited at the Q/R site also die shortly after birth (Brusa et al., 1995). In contrast, mice that are homozygous for a *Gria2* gene that contains the Q586R mutation can tolerate the ADAR2 knockout (Higuchi et al., 2000). These results underscore the importance of a single RNA editing event for mammalian neurophysiology.

There are other editing sites in mammalian glutamate receptors. AMPA receptor mRNAs encoding GluA2–4 can all be edited at

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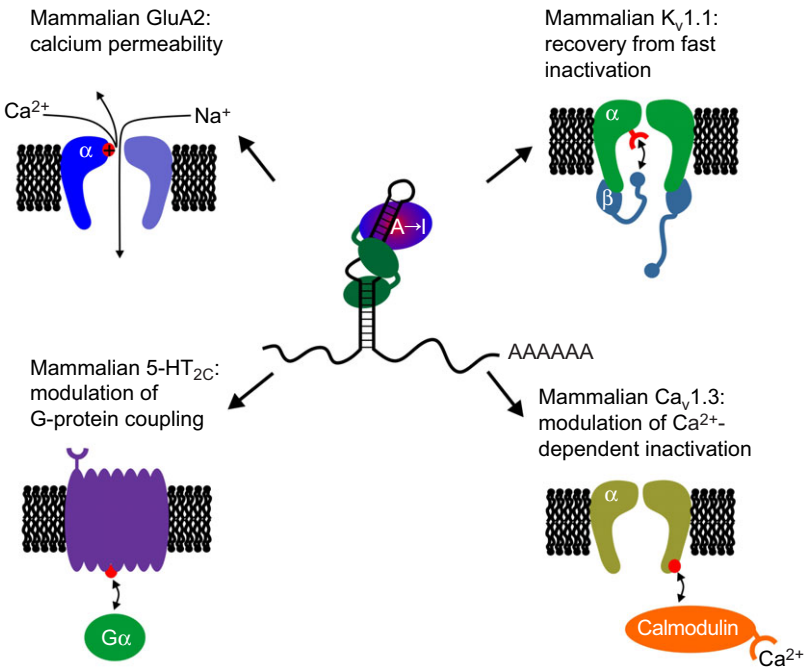

Fig. 4. Functional regulation by RNA editing in the mammalian nervous system. Select examples of mammalian ion channels and receptors that are regulated by RNA editing.

codon 764, a change that creates a glycine from a genomically encoded arginine (Lomeli et al., 1994). This position is within an extracellular loop involved in neurotransmitter binding. A glycine speeds recovery from desensitization and could therefore increase the magnitude of excitatory post-synaptic potentials during bouts of repetitive firing. The kainite receptors GluK1 and GluK2 can also be edited at a position orthologous to the Q/R site of GluA2 (Herb et al., 1996; Maas et al., 1996). Interestingly, although this edit has similar functional effects on the channel (Egebjerg and Heinemann, 1993; Köhler et al., 1993), in this case editing is far less efficient (Bernard et al., 1999; Egebjerg and Heinemann, 1993; Paschen et al., 1997). GluK2 mRNAs are also edited at two additional sites, one being an isoleucine to valine change, and the other a tyrosine to cysteine change (Köhler et al., 1993). Little is known about the functional effects of these edits, if there are any at all. Glutamate receptors are the most important players for excitatory synaptic signaling in the mammalian brain. It is noteworthy that RNA editing has converged on these targets. Because glutamate receptors are located at the post-synaptic membrane, editing regulates electrical signaling as it enters a neuron.

Other neuronal targets that are affected by editing include voltage-dependent channels and a G-protein-coupled neurotransmitter receptor. Voltage-dependent potassium channels are responsible for repolarizing the action potential and setting the resting potential. When it comes to shaping excitability, they have outsized effects. On a basic level, the job of a voltage-dependent potassium channel is simple: it opens when the transmembrane potential is sufficiently positive in order to allow potassium ions to flow through its central pore. The channel will then close when the membrane potential returns to rest. Sometimes, however, even at depolarized potentials, the channel will spontaneously close through the process of inactivation. Inactivation occurs in some potassium channels when an inactivation particle, which is part of the channel protein, enters the open pore and physically occludes the ion conduction pathway (Armstrong, 1969, 1971; Hoshi et al., 1990). Once inside, the inactivation 'ball' is stabilized via interactions with the surrounding residues that line the pore (del Camino et al., 2000; Holmgren et al., 1996, 1998, 1997). Potassium channels are multimers of pore-forming α-subunits and regulatory β-subunits.

For mammalian $K_v1.1$, the inactivation ball is located at the N-terminus of a β-subunit (Rettig et al., 1994). mRNAs encoding the mammalian $K_v1.1$ α-subunit can be edited at codon 400, changing a genomically encoded isoleucine to a valine (Bhalla et al., 2004). Position 400 is at the apex of a region called the inner vestibule, immediately before the internal entrance of the ion conducting pore. The subtle change in hydrophobicity caused by editing hastens the dissociation of the inactivation particle from the inner vestibule (Gonzalez et al., 2011). Physiologically, this translates into a faster recovery from inactivation, an effect that would presumably enable higher action potential firing frequencies. Thus, through editing, mammals can regulate the action potential, the principal component of electrical signaling within a neuron.

Once an action potential reaches the presynaptic membrane, it causes a transient depolarization that opens calcium channels. Calcium entry then triggers synaptic vesicle release. Recent reports show that mRNAs encoding mammalian $Ca_v1.3$, a neuronal calcium channel isoform, can be edited at three codons whose amino acids reside in the channel's cytoplasmic C-terminus (Huang et al., 2012). Much like potassium channels, calcium channels can also spontaneously inactivate after opening. Unlike potassium channels, however, inactivation may depend on the local cytoplasmic calcium concentration. In this case, calcium is sensed by calmodulin, which can bind directly to the channel (Shen et al., 2006; Yang et al., 2006a). The edited codons are within the calmodulin binding domain and the edited versions decrease the extent of calcium-dependent inactivation. The degree of $Ca_v1.3$ editing varies between different brain regions and the fact that edited codons do indeed get translated was shown via proteomics.

Inhibitory synaptic signaling can also be regulated in mammals. mRNAs encoding the gabra-3 subunit of the GABA-A receptor, a glutamate-gated chloride channel, can be edited at an isoleucine codon, changing it to a methionine (Ohlson et al., 2007). This change greatly reduced the efficacy of inhibitory signaling by slowing activation kinetics, speeding deactivation kinetics (Rula

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et al., 2008) and reducing the amount of receptor that makes it to the cell's surface (Daniel et al., 2011). Coupled with the previous examples, these data show that RNA editing can tune diverse aspects of electrical signaling, both within a neuron and between them. However, cells use signals besides electrical currents.

G-protein-coupled receptors are the most diverse class of signaling molecules. When bound to one of a seemingly endless array of ligands, they activate specific G-proteins, which, in turn, can affect the global protein phosphorylation state, gene expression and excitability, among other things. In mammals, messages encoding 5-HT_{2C}, a metabotropic (G-protein-coupled) serotonin receptor, undergo RNA editing in the brain (Burns et al., 1997). Three codons in the pseudoprotein's second intracellular loop can be recoded; this region is directly involved in G-protein coupling and the edited versions of the receptor bind less effectively and dampen signaling (Berg et al., 2001; Burns et al., 1997; Marion et al., 2004). Interestingly, altered RNA editing in this target has been associated with neuropsychiatric disorders such as anxiety, depression and suicide (Gurevich et al., 2002; Martin et al., 2013; Niswender et al., 2001). Because the 5-HT_{2C} receptor is involved in many aspects of behavior, it is likely that the behavioral outcomes associated with editing this molecule are exceedingly complex and we are just beginning to understand them.

The above examples of how RNA editing can affect protein function are just a subset of what is known. Indeed, RNA editing does occur outside of the nervous system. For example, mRNAs encoding filamin A are edited in smooth muscle cells (Stulić and Jantsch, 2013) and this opens the possibility that the actin cytoskeleton is regulated by this process. In addition, mRNAs encoding the DNA repair enzyme NEIL1 can be edited at a lysine codon to produce an arginine (Li et al., 2009). This edit redirects NEIL1 from removing thymine glycol in dsDNA to the repair of guanidinohydantoin lesions (Onizuka et al., 2012; Yeo et al., 2010). It is intriguing to think that editing might influence DNA repair in a manner that is consistent with environmental insults. In spite of these examples, to date most studies have focused on nervous system targets for editing. Transcriptome-wide efforts to identify new editing sites seem to support the idea that most (but by no means all) editing sites occur in neurons.

Cephalopod RNA editing breaks the rules

The idea that protein-recoding RNA editing is rare is based on few organisms. In fact, of the nine animal phyla, most of what we know is based on the vertebrata subphylum of chordates, and the Insecta class of arthropods. Needless to say, this is not a representative sampling. Early work by my group and others hinted that recoding RNA editing might be particularly robust in cephalopods. For example, two messages encoding voltage-dependent potassium channels in *Doryteuthis pealeii* and *Doryteuthis opalescens* (formerly known as *Loligo pealeii* and *Loligo opalescens*) exhibited over 30 individual recoding sites (Patton et al., 1997; Rosenthal and Bezanilla, 2002). Subsequent studies on mRNAs encoding other ion channels, ion pumps and ADARs in squid revealed as many recoding editing sites in these few messages as had been identified in the entire human transcriptome (Colina et al., 2010; Liu et al., 2001; Palavicini et al., 2009; Pinto et al., 2014; Ramaswami and Li, 2014; Ramaswami et al., 2013; Rosenthal et al., 1997). It seemed unlikely that the squid studies happened upon highly edited messages by chance. If so few messages contain so many sites, what then is the true extent of proteome recoding by RNA editing in squid?

In a recent study, transcriptome-wide recoding RNA editing in squid was tabulated and the results were unlike those seen for any other organism (Alon et al., 2015). As mentioned, previous studies have relied on a sequenced genome to anchor editing site discovery. Although A→G discrepancies between RNA and DNA can be identified by sequence data alone, the genome tells you their genomic location, in which transcripts they reside, and whether they occur in coding sequence. To date, no genome sequence is available for a cephalopod. To resolve this issue, Alon and colleagues used a *de novo* transcriptome as an anchor. This switch required an entirely new analysis routine that would get around problems associated with the use of a transcriptome. For example, in the genome, an editing site is always an A. Thus, one looks for sites in the RNA that can sometimes be a G. A transcriptome, in contrast, is a consensus of RNA sequences. Accordingly, an editing site can appear as a G if it is edited at >50% efficiency or an A when edited at <50% efficiency. Because of this, low efficiency and high efficiency sites required separate analysis pipelines. Using appropriate methods, the authors uncovered a remarkable 57,111 recoding RNA editing sites. This number withstood stringent validation. In fact, the estimate was quite conservative by design. Because only sites within annotated transcripts were scored (many transcripts from the transcriptome were clearly open reading frames but did not bear sufficient similarity to known transcripts for annotation), the true number of recoding sites is probably more than twice that reported. In any case, the number of recoding sites in the squid nervous system is orders of magnitude larger than that for other organisms. In fact, the majority of squid mRNAs harbor one or more editing sites. Obviously, squid editing entails some mechanistic differences.

To better understand the underpinnings of high-level editing in squid, studies focused on their ADARs. The first squid ADAR cloned was a vertebrate ADAR2 ortholog (sqADAR2.1; Palavicini et al., 2009). This message had some distinguishing features. Vertebrate ADAR2s invariably have two N-terminal dsRBMs. There were two versions of the sqADAR2 message created by alternative splicing: one encoded an ADAR2 with three dsRBMS, the other encoded an ADAR2 with two dsRBMS. Recombinant forms of both versions of the enzyme were then made in yeast. When mixed with squid potassium channel mRNAs *in vitro*, the version with three dsRBMs edited far more sites than the version with two. A subsequent study showed that the 'extra' dsRBM drastically increased the enzyme's affinity for dsRNA (Palavicini et al., 2012). However, even with the added binding domain, many sites that are edited *in vivo* went unedited *in vitro*. Thus the 'extra' binding domain can only account for a portion of high-level editing at best.

Another novel feature of sqADAR2 is that its messages are themselves edited at many sites, leading to further protein diversity. The role that these sites play in substrate recognition is unknown. Recent unpublished work in my lab shows that squid also express a vertebrate ADAR1 ortholog that has novel features. For example, at its N-terminus it possesses a serine-rich, basic domain that has scores of phosphorylation motifs. The activity of sqADAR1 is currently under investigation. Taken together, the two squid ADARs do indeed have novel structural elements. The extent to which they underlie high-level editing is presently unknown.

With many thousands of editing sites affecting over half of all transcripts, it is reasonable to speculate on the extent to which these events alter protein function. Functional studies on a handful of transcripts show that in many cases they do affect protein function. For example, editing sites in mRNAs encoding squid potassium channels alter kinetics, subunit tetramerization and surface expression levels (Patton et al., 1997; Rosenthal and Bezanilla,

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2002). A site in a mRNA encoding a Na^+/K^+-ATPase α-subunit increases the rate of ion pumping using a novel mechanism (Colina et al., 2010). At negative membrane potentials like the resting potential, the Na^+/K^+-ATPase's ability to pump Na^+ ions out of the cell is inhibited by both voltage and the high extracellular Na^+ concentration. An isoleucine to valine edit in the protein's seventh transmembrane span reduces the voltage-dependent inhibition, allowing the pump to transport more rapidly. The Na^+/K^+-ATPase releases three Na^+ ions in succession. Interestingly, the I→V edit exerts its effect almost exclusively on the last Na^+ to leave.

The above studies purposefully selected editing sites that made easily observed differences in protein function. Many editing sites will undoubtedly exert subtle effects, if any at all. However, with at least 57,111 RNA editing sites in squid, it is safe to assume that many will affect function. Pathway analysis suggests that certain cellular functions are affected more than others (Alon et al., 2015). These include electrical excitability, synaptic vesicle release and recycling, and the actin cytoskeleton architecture, among others. Data from squid raise some interesting and obvious questions. Why is recoding RNA editing so common in squid, while being excluded in other organisms? How prevalent is recoding by RNA editing in other taxa?

The role of RNA editing in adaptation

As a tool for adaptation, RNA editing has much to offer. As with alternative splicing, it serves to increase protein diversity. In addition, it enables the potential fitness of new mutations to be sampled gradually. In a heterozygote, a new mutation is present in 50% of mRNAs and in a homozygote, 100%. A new RNA editing site can be present in a small fraction of mRNAs to begin with, and then gradually increase its penetrance through selection. Although potentially important, these attributes are hypothetical. Whether RNA editing is adaptive or not, at least in its role in recoding, is presently a point of debate. The answer, it seems, depends on which organism one chooses to examine.

Before addressing the specifics of RNA editing's role in adaptation in different organisms, it is useful to consider how an adenosine gets edited in the first place. The mechanisms underlying editing site selection are well conserved. ADARs recognize tertiary and quaternary structures in RNAs that must be largely double stranded (Bass and Weintraub, 1988; Maas et al., 1996; Melcher et al., 1995; Stefl et al., 2010, 2006). The dsRBMS that are present at the N-terminus of all known ADARs bind these structures and position the catalytic domain adjacent to the target adenosine. The RNA structures vary in size, but are often quite large, requiring a hundred or more base-pairing interactions. In most cases, they form between exonic and intronic sequences within pre-mRNAs, and sometimes these elements are separated by kilobases of intervening sequence (Daniel et al., 2012; Herb et al., 1996). The requirement for intronic sequence, and the fact that ADARs are confined to the nucleus, dictates that editing also occurs within the nucleus.

Target site recognition, however, is not exclusively determined by the dsRBMs and the structures that they recognize. The portion of the RNA structure that immediately surrounds an editing site is thought to lie against an external binding surface of the catalytic domain, a region that has a strongly positive electrostatic potential (Macbeth et al., 2005). The adenosine is then 'flipped out' of its structure, into a catalytic pocket that is located at the center of the external binding surface. The precise topology of the binding surface probably influences the relative efficiency of editing for adenosines with different neighboring residues (Eggington et al., 2011; Polson and Bass, 1994). It also governs that fact that certain codons (e.g. glutamate GAT) will be edited less efficiently than others (e.g. cysteine UAC). Thus, the residues that immediately surround an editing site, and the overall higher order structure to which the dsRBMs bind, influence the efficiency with which an adenosine is edited. These features should then be critical for adaptation. Alternatively, changes to ADAR itself could be involved.

In an elegant study, Robert Reenan first highlighted how changes to RNA structure drive the evolution of an editing site between species (Reenan, 2005). As a first step, he tracked five editing sites in messages encoding synaptotagmin 1 through over 250 million years of insect evolution. This protein is the presynaptic calcium sensor that triggers neurotransmitter release. Bees, beetles and roaches do not edit synaptotagmin 1; however, fruit flies, mosquitos and butterflies do, and each has shared and species-specific sites. In fact, the acquisition of the editing sites faithfully overlapped the phylogeny of the taxa. Next, Reenan examined the RNA structures that drive editing. Editing commenced with the acquisition of a basal structure. Small subsequent changes to this structure drive species-specific editing patterns. Intriguingly, to create certain species-specific sites, the structure requires a pseudoknot that utilizes distant intronic sequence. Although the functional effects of the editing events were not tested, it was noted that they occurred at highly conserved positions and were therefore likely to alter activity. In addition, other editing sites in *Drosophila* have clear physiological effects (Horn and Reenan, 2010; Ingleby et al., 2009; Jones et al., 2009; Ryan et al., 2012, 2008). The idea that silent sites at the wobble positions of codons or in introns can influence protein coding is an important tenet of editing. In fact, bioinformatics screens for editing site discovery have been designed around this premise (Hoopengardner et al., 2003). In insects, therefore, there is abundant evidence that recoding RNA editing is adaptive. What about other animals?

As previously mentioned, recoding RNA editing is quite rare in mammals, and possibly other vertebrates. Guixia Xu and Jianzhi Zhang create a compelling argument that RNA editing is generally not used for adaptation in mammals (Xu and Zhang, 2014). In their study, the authors tabulated all the human recoding events in coding regions and found that, after normalizing for the abundance of adenosine targets, non-synonymous edits were less frequent than expected and synonymous changes more frequent. In addition, the level of editing at non-synonymous sites was lower than it was at synonymous sites, indicating that, if anything, the average site is deleterious. They also found that the more conserved a protein, or functionally constrained a position, the less likely it is to be recoded. Of course, these results do not speak for every editing site. As pointed out in previous sections, some human recoding sites are functionally critical. It is interesting to note, however, that these results contrast sharply with those in invertebrates. In *Drosophila*, non-synonymous sites in coding regions are in fact over-represented (Graveley et al., 2011; St Laurent et al., 2013). In squid, the same is true (Alon et al., 2015). In addition, for all squid sites edited at low frequencies, the proportion of non-synonymous and synonymous sites is pretty much as expected by chance. However, as one selects sites with higher editing frequencies, the proportion of non-synonymous changes increases. Sites that are edited to near completion are nearly all non-synonymous. These data suggest that editing in invertebrates is in fact used for protein adaptation. This conjecture is supported by a functional study on octopus potassium channels (Garrett and Rosenthal, 2012a). Octopus mRNAs encoding $K_v1.1$ are edited at several sites. One site

recodes an isoleucine to a valine change in the channel's fifth transmembrane span, a region close to the gate. Editing this position makes the channel close faster. Interestingly, tropical octopuses edit this position at low levels, but as species were sampled from progressively colder environments, the frequency of editing increased. Both arctic and Antarctic species edited it to near completion, presumably to offset the effect of temperature on channel closing kinetics.

Is RNA editing a tool for acclimation?

Based on its ability to direct precise changes in protein function, and its mechanistic underpinning, RNA editing would be an ideal tool for environmental acclimation. Surprisingly, this is an under-studied aspect of the process, but recent reports would suggest that it can be used for this purpose. How might editing be regulated in response to the environment? For most environmental factors, an organism might regulate ADAR expression levels, or its access to an RNA substrate. In the case of temperature, one of the most important environmental variables, the stability of the RNA structures that surround editing sites might themselves be directly affected. It is well established that RNA editing levels at specific editing sites can change acutely within an organism's lifetime. For example, changes in editing frequency for the mammalian GluA2 and 5-HT_{2C} receptors have been associated with disease (Gurevich et al., 2002; Hideyama et al., 2011, 2010; Kawahara et al., 2004; Niswender et al., 2001; Yang et al., 2004). Perhaps a better example comes from *Drosophila* ADAR itself. Messages for this protein are edited at a single recoding site (Keegan et al., 2005); the unedited form is more active than the edited form. As *Drosophila* has but a single ADAR, this process of auto-editing is thought to be a negative feedback circuit. In a follow-up study, auto-regulation was genetically hardwired or abolished (Savva et al., 2012). These manipulations had large effects on the extent to which many other messages were edited, and on the ability of the flies to carry out complex, adaptive behaviors. In addition, the extent of ADAR auto-editing depended on temperature, establishing an intriguing link between the physical environment and the global editing landscape.

At this point it is too early to make firm conclusions regarding the relationship between RNA editing and temperature; however, recent results provide fertile ground for speculation. Reenan and co-workers examined the temperature dependence of editing at 54 sites across several species of *Drosophila* that span about 15 million years of evolution (Rieder et al., 2015). Editing levels at most sites decreased with temperature. This effect was mostly attributed to a decrease in ADAR expression; however, in a few cases the RNA structures that drive editing appeared to be thermally labile. At a few sites, RNA editing efficiency went up with temperature. The mechanism underlying the increase was not clear.

In principle, RNA editing could respond to any environmental condition at any given site. At I321V in octopus $K_v1.1$, and at most *Drosophila* sites, editing increases as temperature decreases. It has been proposed that this makes sense (Garrett and Rosenthal, 2012a, b; Rosenthal and Bezanilla, 2002). Based on the genetic code, an A→G change most frequently replaces a relatively large –R group with a relatively small one. Because of the neighboring residue preferences for ADARs, this bias is even stronger. In both cephalopods and *Drosophila*, RNA editing creates many glycines, valines and alanines (St Laurent et al., 2013; Alon et al., 2015). Many years ago, George Somero and colleagues, while comparing metabolic enzymes from organisms that inhabit different thermal environments, hypothesized a good reason for these changes (Fields et al., 2002; Fields and Somero, 1998; Hochachka and Somero, 1968; Low et al., 1973; Low and Somero, 1974; Somero, 1975): the substitution of small amino acids at hinge regions within proteins tends to reduce activation energy barriers for enzymatic reactions. It also has the negative effect of increasing entropy, but on balance the reductions in enthalpy outweigh the increases in entropy, and the net effect helps offset the effect of low temperature on catalysis. It is entirely possible that RNA editing is predisposed to be a 'Someric tool' for temperature adaptation or acclimation.

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Competing interests

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