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Extensive Recoding of the Neural Proteome in Cephalopods by RNA Editing

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Abstract

The coleoid cephalopods have the largest brains, and display the most complex behaviors, of all invertebrates. The molecular and cellular mechanisms that underlie these remarkable advancements remain largely unexplored. Early molecular cloning studies of squid ion channel transcripts uncovered an unusually large number of A→I RNA editing sites that recoded codons. Further cloning of other neural transcripts showed a similar pattern. The advent of deep-sequencing technologies and the associated bioinformatics allowed the mapping of RNA editing events across the entire neural transcriptomes of various cephalopods. The results were remarkable: They contained orders of magnitude more recoding editing sites than any other taxon. Although RNA editing sites are abundant in most multicellular metazoans, they rarely recode. In cephalopods, the majority of neural transcripts are recoded. Recent studies have focused on whether these events are adaptive, as well as other noncanonical aspects of cephalopod RNA editing.

DISCOVERY OF A→I RNA EDITING

Of the many types of RNA modifications, only a few alter the coding capacity of the RNA sequence (1). These modifications are termed RNA editing. In multicellular metazoans, the most frequently encountered form of RNA editing is the process of adenosine deamination, catalyzed by the adenosine deaminases acting on RNA (ADAR) family of enzymes (2–6). ADARs recognize higher-order structures within RNAs, bind to them, and then convert select adenosines (As) to inosines (Is) through a simple hydrolytic deamination (5, 7, 8) (**Figure 1*a*,*b***). The ribosome reads Is as guanosines (Gs) (9, 10), and thus RNA editing can recode codons in messenger RNAs (mRNAs)

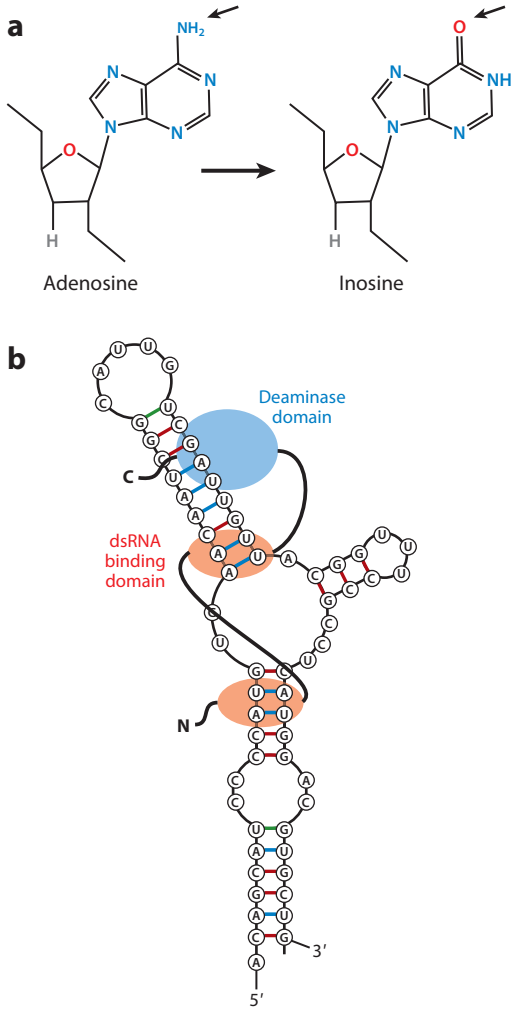

Figure 1

The biochemistry of RNA editing by adenosine deamination. (*a*) Adenosines are converted to inosines by a hydrolytic deamination of the primary amine at the base's number 6 position (*arrow*), converting it to a carbonyl oxygen. The new molecular species is called inosine. (*b*) A cartoon of an ADAR (adenosine deaminase acting on RNA) enzyme binding to an imperfect double-stranded RNA (dsRNA) structure. The dsRNA binding domains (*red*) bind to the dsRNA and position the catalytic deaminase domain (*blue*) next to the target adenosine.

prior to translation. The field of RNA editing was founded on seminal, almost parallel studies on the biochemistry of adenosine deamination and a recoding event in mRNAs encoding mammalian GluA2, a glutamate receptor of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subfamily. Studies out of the Bass lab (11, 12) demonstrated that double-stranded RNA (dsRNA), when injected into the nucleus of *Xenopus* oocytes, would unwind and become accessible to RNAse as a result of adenosines being converted to inosines, thus producing mismatches in the duplex. Subsequent biochemical purifications, and molecular cloning, identified ADARs as the enzymes that catalyze the editing reaction (13–16). Two ADARs are encoded in vertebrate genomes, ADAR1 and ADAR2. Except for the inosines found in transfer RNAs that are produced by ADAT (adenosine deaminases acting on transfer RNA), all other inosines in RNAs are thought to be produced by ADARs. Thus, these early studies showed that ADAR1 and ADAR2 are the enzymes that recode mRNAs. It stands to reason that amino acid changes produced by RNA editing could alter protein function in a precise manner.

Concurrent to the identification of ADARs as the RNA editing enzymes, Seeburg and coworkers (17, 18) pioneered the first studies on the functional consequences of individual RNA editing events. By comparing genomic DNA and complementary DNA (cDNA) sequences for an AMPA-type glutamate receptor expressed in the mouse brain, they identified a position that was an A in the genome but a G in the mRNA. They went on to show that this site was produced by RNA editing and had profound functional consequences on receptor function. The edit recodes a genomically encoded glutamine codon to an arginine at a position in the channel's ion selectivity filter, changing the channel from one that allows monovalent and divalent cations to permeate to one that allows only monovalent cations to pass and reducing the channel's ion conductance. It was then shown that this edit is critical for survival, as mice unable to edit this position in even one allele develop severe epilepsy and die shortly after birth (19). These elegant studies laid the groundwork for the next ∼15 years of investigation, which focused heavily on the ADAR enzymes and specific recoding events that they catalyze, with a particular emphasis on ones that occur in the nervous system (3, 20). Further studies on recoding events in other glutamate receptors, serotonin receptors and the resulting G protein signaling, and voltage-dependent ion channels led to the initial impression that RNA editing is relatively common in the nervous system and that its primary purpose is to recode (21–24). These assumptions were heavily influenced by the technology of the time. Few genomes were available, and the vast majority of molecular cloning was directed toward the isolation of specific mRNAs from shotgun libraries. Research focused on protein coding sequences, and not surprisingly, that is where editing sites were found. The encoded proteins were then studied, and in some instances the editing sites altered function. The advent of deep-sequencing methodologies and the associated computational tools have revealed that RNA editing is far more common in noncoding portions of mRNAs and in noncoding RNAs. However, these technologies were not available when the first RNA editing sites in squid were identified.

DISCOVERY OF RNA EDITING IN SQUID

From the 1950s to the 1980s, squid were one of the most important models for neuroscience. Specific species of loliginid squid possess a system of three synaptically connected giant nerve fibers that are used to drive jet-propelled rapid escape jetting (25). The giant synapse between the second- and third-order fibers and the giant axon of the third-order fiber that innervates the musculature of the mantle have been particularly important. The giant synapse is large enough to insert microelectrodes and microinject compounds into both the pre- and postsynaptic terminals to study the details of synaptic transmission. The giant axon is large enough to insert a wire down its core to achieve exceptionally fast voltage clamp, allowing the dissection of the ionic currents

that produce action potentials. Taken together, synaptic transmission and action potentials are the basic currencies of communication within the nervous system, and much of our understanding about how they work comes from studies on squid. For example, the fundamental concepts that synaptic transmission between neurons is mediated by a chemical signal and requires calcium influx into the presynaptic cell come from the giant synapse prep (26–28). The fact that action potentials turn on and off because of voltage-dependent Na^+ and K^+ conductances comes from Hodgkin & Huxley's (29) groundbreaking studies on the squid axon. These studies nucleated an entire generation of scientists using the squid preps to better understand the physiological principles of excitability.

Methodologies that enabled molecular cloning, and the expression of those clones in heterologous systems, were critically important advancements toward our ability to dissect the physiological mechanisms that generate action potentials. With them, the primary structures of voltage-dependent ion channels could be deduced and manipulated to study the relation between structure and function. For example, the parts of channels that are responsible for critical properties such as ion selectivity, voltage sensitivity, and transient pore formation across the plasma membrane could be identified and studied. The first Na^+ and K^+ channels were cloned and expressed in the late 1980s, the former from rat and the electric eel, and the latter from *Drosophila* (30–34). It naturally followed that researchers were interested in isolating the clones for the squid giant axon Na^+ and K^+ channels because they had been studied for approximately four decades and their functional properties in their native system were better understood than those from any other model. Sequences of the first squid giant axon Na^+ and K^+ channel clones were published in the mid-1990s via traditional cDNA library screens (35, 36). Sequencing of multiple clones from the same library revealed multiple splice variants and site-specific A-or-G variation between different clones for the same channel, and it was speculated that this variation was due to RNA editing by adenosine deamination (37). A further study showed that the variable sites affected channel function and biosynthesis (38). In all, the sequencing of 30 individual clones revealed 10 sites of A-or-G variation and no other discrepancies. The uncovering of so many sites between relatively few clones prompted further exploration.

Studies on a K_v2 subfamily K^+ channel from the squid central nervous system, as well as a K_v1 K^+ channel from the giant axon, were the first to comprehensively map sites of A-or-G variation across entire messages and showed that they were due to RNA editing (39, 40). The key to these works was that the sequences of multiple cDNA and genomic DNA clones were isolated from individual squid, removing allelic variation as a variable. Edited positions were sometimes G in cDNA but always A in genomic DNA. What stood out from these studies was the sheer number of editing sites. For the K_v2 subfamily message, individual clones were compared, and 17 editing sites were uncovered. For the K_v2 subfamily message, a direct sequencing assay for polymerase chain reaction products was developed, which enabled a more accurate estimate of editing frequencies for the 14 uncovered sites. The frequencies varied tremendously, from some at a very low level to others that were edited close to completion. In both studies, the electrophysiological properties of edited and unedited channels were examined under voltage clamp in *Xenopus* oocytes, and individual sites were found to affect channel gating and inactivation in complex ways. In addition, for the K_v1 channel, there were a cluster of editing sites in codons coding for the channel's N terminus, in a cytoplasmic domain called T1, responsible for promoting channel tetramerization. Individual editing sites had a strong impact on the binding affinity of one T1 domain for another, and this regulated channel expression. In addition, it was postulated that the T1 edits could regulate heteromultimerization between different subunits. Later studies confirmed high-level RNA editing in voltage-dependent K^+ channel messages across coleoids (41–43).

These studies have raised the question of whether RNA editing is focused just on K_v channel messages or whether it occurs more generally across different neural messages, with clear implications for potential neural plasticity. In mammals, the sequencing of individual cDNAs in early RNA editing studies had uncovered multiple sites that altered protein function, particularly in messages encoding different neurotransmitter receptors (e.g., γ-aminobutyric acid, serotonin, and glutamate receptors) and ion channels (Ca_v and K_v) (17, 18, 23, 24, 44–50). Work in squid showed a similar pattern, with an important difference: In mammals, editing sites were rarely encountered in new cDNA clones, and they were found only due to the massive efforts on mammalian models. In squid, editing sites were found in most examined neural transcripts. Four editing sites were found in the α-subunit of the Na^+/K^+ ATPase, and one site that converts an isoleucine to a valine in the seventh transmembrane span (I877V) is particularly interesting (51). By transporting three Na^+ out of the cell and two K^+ into the cell, the Na^+/K^+ ATPase generates the ionic gradients that drive excitability. Because the ion transport is asymmetric, the Na^+/K^+ ATPase generates an electric current that can be measured, providing a direct correlation with the transport rate. The I877V edit increases the velocity of ion transport at physiologically relevant voltages. A detailed analysis of the protein conformations involved with the release of Na^+ revealed that I877V shifted the equilibrium of the voltage-dependent state occupancy for bound Na^+ ions, favoring their release at negative voltages. Interestingly, the editing frequency at this position varied between nervous tissues, and even at the subcellular level between the soma and axon (51, 52). Besides the Na^+/K^+ ATPase, 16 editing sites were also identified in messages encoding squid ADAR2 (sqADAR2), an RNA editing enzyme. Although the functional significance of these sites was not explored, editing frequencies, and the presence or absence of specific sites, depended on the specific ADAR splice variant. Taken together, these early squid studies naturally led to an interest in exploring the full scope of RNA editing in cephalopods and its contribution to transcriptome plasticity.

TRANSCRIPTOME-WIDE EDITING REVEALS EXCEPTIONALLY HIGH-LEVEL RECODING IN CEPHALOPODS

The first discoveries of recoding sites in mammals were serendipitous. A or G variation was identified in individual cDNA clones at positions that were always A in the genome. This variation was due to the fact that most current RNA sequencing schemes start with the reverse transcription of RNA into cDNA. Like ribosomes, reverse-transcriptases recognize inosines as guanosines. Consequently, inosines in mRNA appear as guanosines in cDNA, and the editing events show up as A→G DNA–RNA mismatches. With the advent of deep-sequencing technologies, it was soon realized that RNA editing events could be detected systematically, across entire transcriptomes, by analyzing RNAseq data. However, a fundamental problem stems from the fact that the number of editing-induced A→G mismatches is usually dwarfed by mismatches due to technical and biological noise (e.g., sequencing errors, incorrect alignment, genomic polymorphisms, or somatic mutations) (53–58). Since 2003, several groups have developed computational approaches that scan through large-scale sequencing databases to look for these mismatches and apply various filters to the multitude of A→G mismatches observed in a given sample, or set of samples, to identify the relatively few originating from an editing event (59–62). Advances in sequencing technologies have increased the availability of high-coverage multi-sample data sets, enabling systematic mapping of the editome across multiple species (63–65).

Transcriptome-wide mapping studies revealed that A→I editing is abundant in all multicellular metazoans. Millions of sites have been detected in humans (62–64), and tens to hundreds of thousands have been identified in other multicellular metazoans screened so far (66), including

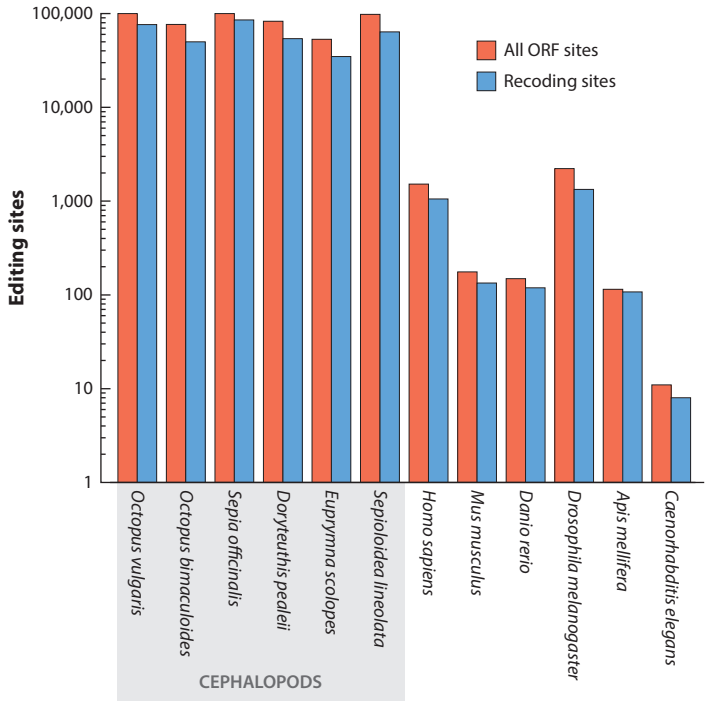

Figure 2

Editing across taxa. Numbers of all editing sites within the coding region (including synonymous editing) and recoding sites from multiple studies are plotted. For the coleoids, *Octopus vulgaris* (OL, SG), *Octopus bimaculoides* (OL, ANC, Sub, Supra), *Sepia officinalis* (OL, SG), *Doryteuthis pealeii* (OL, GFL), *Euprymna scolopes* (VL, SG), and *Sepioloidea lineolata* (OL, SG) come from Shoshan et al. (82). Abbreviations: ANC, axial nerve chord; GFL, giant fiber lobe; ORF, open reading frame; OL, optic lobes; SG, stellate ganglia; Sub, subesophogeal ganglia; Supra, supraesophogeal ganglia; VL, vertical lobe. Human data come from Gabay et al. (based on Genotype-Tissue Expression Project data; 73). Mouse data come from Licht et al. (brain; 123). Zebrafish data come from Buchumenski et al. (head; 74). *Drosophila* data come from Duan et al. (brain; 79). Honeybee data come from Duan et al. (head; 77). *Caenorhabditis elegans* data come from Zhao et al. (whole animals; 124).

corals (belonging to Cnidaria, an early-branching metazoan phylum) (67). However, virtually all A→I editing sites reside outside mRNA coding regions and have no direct effect on the protein products. The extent of noncoding editing varies considerably across organisms and depends strongly on the properties of the mobile elements present in the genome (66, 68): High numbers of repetitive elements from lowly diverged families will generate large numbers of dsRNA structures that may be potential ADAR targets. These noncoding editing events are often clustered and concentrated in well-identified repetitive elements. For instance, >99% of human editing sites (65) are located in the primate-specific *Alu* repeats (69–72). In all cases studied to date, noncoding editing substantially outweighs recoding activity. In fact, except for cephalopods, recoding is usually only an exception to the rule (**Figure 2**). For example, a recent, thorough analysis of ~10,000 human samples identified ~1,000 recoding sites, ~200 of which are known to be conserved in non-primate mammals (73), compared to millions of noncoding sites. Similarly, 100–200 sites were found in zebrafish (74), ants (75), and bees (76, 77). Recoding is somewhat more abundant in *Drosophila*, in which nearly a thousand recoding sites were shown to be conserved across the lineage (78–80), compared to a relatively low number (~38,000) of noncoding sites (66).

Early discoveries described in the preceding sections provided tantalizing hints that recoding RNA editing might be unusually common in cephalopods and called for a systematic search for recoding sites in this taxon using deep-sequencing approaches. However, the absence of a complete cephalopod reference genome (in 2013) presented a major challenge, because all computational pipelines used one as an anchor to map the short reads. In response, an alternative bootstrap approach was devised (42). Instead of mapping the RNA reads to a reference genome, they were first assembled to construct a model transcriptome, and then this transcriptome was used as the reference against which the same RNA reads were aligned, looking for systematic A→G mismatches. DNA sequencing data from the same individual specimen were mapped to filter out mismatches due to genomic polymorphisms. In addition, strongly edited sites, where the majority of RNA reads were edited, resulted in a G in the constructed transcriptome. The DNA reads were used to identify these sites as well. This method was first applied to the squid *Doryteuthis pealeii* (42). As suggested by the early cloning reports, the results revealed recoding at an unprecedented level. Despite the limited number of samples used, and the inherent difficulties associated with the bootstrap method, 57,108 recoding sites were identified in the transcriptome of the nervous system, affecting the majority of the encoded proteins (6,991 of 12,039 open reading frames identified).

Follow-up studies demonstrated that this extraordinary level of recoding is common among coleoid cephalopods, as observed in *Octopus bimaculoides*, the first cephalopod to have its genome sequenced (43, 81); *Octopus vulgaris*; the cuttlefish (*Sepia officinalis*) (43); the Hawaiian bobtail squid (*Euprymna scolopes*); and the striped pajama squid (*Sepioloidea lineolata*) (82). Interestingly, extensive recoding was not seen in *Nautilus pompilius*, suggesting that it has evolved along the coleoid lineage. This correlates with the behavioral complexity exhibited by the coleoids but not by nautilus (83). Recoding levels vary considerably across tissues and are highest for neural tissues, where recoding accounts for 11–13% of the global RNA editing activity measured (43, 84), compared to <1% in mammals (65). In nonneural tissues of *O. bimaculoides*, recoding activity is three- to sixfold lower compared to in neural tissues (43, 84). Within the neural transcriptome, recoding is enriched in genes with neuronal and cytoskeletal functions, suggesting it plays an important role in brain physiology (43, 81). In *D. pealeii*, for example, 27% of all messages in the nervous system harbored ≥3 sites that were edited at levels ≥10%, and 7.3% had ≥10 sites.

In sharp contrast with mammals, a large fraction of the recoding sites is shared between species. For example, most sites are shared between the closely related *O. bimaculoides* and *O. vulgaris*, but even *S. officinalis* and *D. pealeii*, estimated to have diverged >120 Mya (85), share more than 10,000 recoding sites. Based on the sites identified in all six coleoid species analyzed, one may estimate that at least ~5,000 recoding sites have been conserved (in some species, at least) since the divergence of the coleoid lineage, estimated to have occurred ~200–350 Mya (86). Even editing levels at the shared sites are well-conserved between evolutionarily distant species. This high degree of conservation contrasts sharply with other evolutionary lineages. Only a few dozen recoding sites have been conserved since mammalian divergence (73), and a similar number were conserved across the *Drosophila* genus (78, 79). These data suggest that recoding by RNA editing is performing an important function.

IS CEPHALOPOD RECODING ADAPTIVE?

In principle, mRNA recoding by RNA editing provides the ability to express functionally different protein isoforms to meet developmental or environmental demands. It is well poised to provide a precise system for acclimation and as such would be adaptive (87). Is this really the case? The strong conservation of recoding sites across cephalopods through millions of generations is a strong indication for an adaptive advantage. In addition, a detailed study of recoding of potassium

channel messages in octopus (41) demonstrated recoding's capacity to confer adaptability. Related octopus species can inhabit diverse temperature environments, ranging from warm tropical waters to the icy waters near the poles. As all enzymatic systems are governed by the three laws of thermodynamics, changes in temperature present formidable challenges to all organisms. These challenges are particularly acute within the nervous systems of poikilotherms like cephalopods, given the high temperature sensitivity of neuronal synaptic transmission and the need for precise integration of billions of neurons. Interestingly, RNA editing, rather than genomic mutations, enabled potassium channels of Antarctic octopuses to tune their function to the extreme cold. In particular, the editing level at a single isoleucine-to-valine recoding site correlated negatively with temperature across species. This single-amino-acid substitution accelerates the channel's closing rate to compensate for low temperature. Closing kinetics are highly temperature sensitive, and faster closing would permit faster repetitive firing rates.

This octopus study looked at different species, each living in its own environment over many generations. In principle, these species could have adapted through changes in the genomic sequence. However, several features of RNA editing may make it advantageous to genomic mutations as a means for adaptions, in some cases. Genomic mutations affect all precursor mRNAs transcribed from an allele, in all cells of the organism, and under all conditions; recoding mRNAs may be used to fine-tune the proteome, creating a tissue-dependent (88) or developmental stage–dependent (89) balance of edited and unedited transcripts. In addition, RNA editing is well-positioned to compensate for temperature changes (40, 87). Based solely on the genetic code, 18 codons can be recoded to another amino acid through A→I editing. In 13 cases, side-chain volume is reduced, increasing the activation entropy of an enzyme. Extensive recoding in cephalopods' neurons could therefore serve as a global mechanism for temperature adaptation.

Indeed, global analysis of recoding in coleoids supports its functional importance. First, the nonsynonymous-to-synonymous ratio for strongly conserved cephalopod edits (editing levels $\geq$10%) is substantially higher than expected under neutrality, strongly indicating positive selection (43). Analysis of genomic mutations at editing sites further shows that genomic guanosines (roughly equivalent to inosines in terms of protein production) are generally favored at the editing sites, supporting the notion that editing fine-tunes the protein as an alternative means for genomic evolution (90). Furthermore, editing site conservation is reflected in the conservation of the genomic DNA sequence that flanks the sites. Because editing depends on specific surrounding dsRNA structures, maintaining editing throughout evolution requires elevated sequence conservation in the vicinity of editing sites to keep these structures intact (17, 91). Indeed, a marked depletion of interspecies mutations and intraspecies genomic polymorphisms is observed up to ~100 bp on each side of a recoding site. Given the large number of sites, this effect greatly reduces the number of mutations and genomic polymorphisms in protein coding regions of the neural transcriptome. This trade-off between genome evolution and transcriptome plasticity further highlights the functional importance of RNA recoding (43).

When considering whether recoding is adaptive, it is important to distinguish between two scenarios (**Figure 3**). First, editing at a given position could provide a selective advantage because it replaces an inferior A allele with a preferred G allele, but there is no inherent advantage to having an edit over a genomically encoded G. Such edits exhibit positive selection, as they may fix the harmful effect of a previous G→A mutation (restorative editing) or, equivalently, introduce a beneficial A→G alteration to the RNA as a replacement to a similarly beneficial genomic mutation that is yet to occur, regardless of the ancestral state (92–94). Note, however, that under such a "harm-permitting" scenario (95), there may be no selective advantage associated with the A/G flexibility offered by having an editing site, and the fitness of having a recoding site is equal to or lower than that of having a guanosine encoded in the genome. On the other hand, a unique

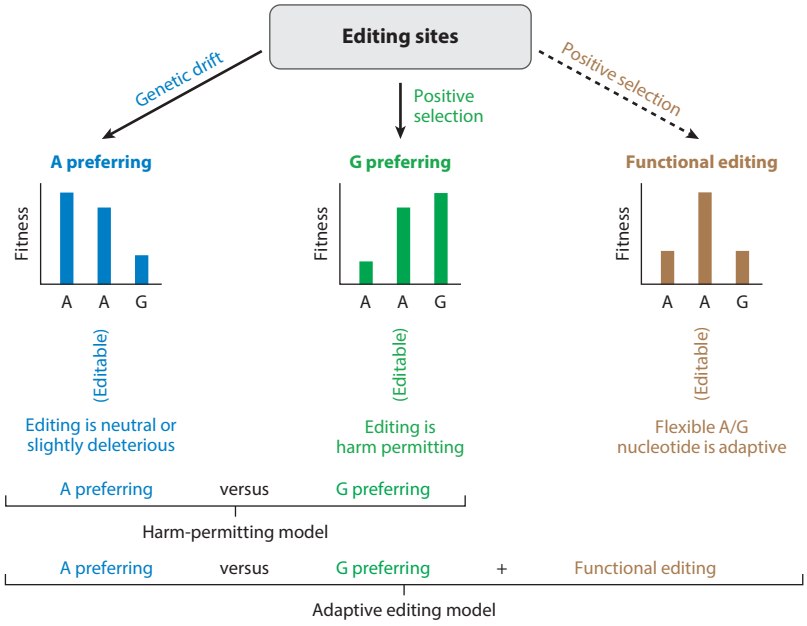

Figure 3

The harm-permitting and adaptive editing models. Recoding sites may be fixed into the genome due to random genomic drift, even though their editing does not provide any selective advantage and may even be slightly deleterious. These randomly fixed sites, where transcripts containing the genomically encoded adenosine allele are functionally superior to the edited ones (A-preferring sites), are not expected to be enriched in nonsynonymous editing (*left*). In a second class of recoding sites, editing does provide a selective advantage, as it replaces an inferior genomically encoded A allele with the preferred G allele (*middle*). For these G-preferring sites, editing does increase the fitness of the organism over an uneditable A, but having a genomically encoded G is equally beneficial or even better (*middle*). In both cases, fitness does not improve due to the protein diversity and flexibility provided by recoding. The harm-permitting model asserts that all (or almost all) recoding sites belong to these two categories. In contrast, according to the adaptive editing model, some of the recoding sites belong to a third class, where the proteomic complexity generated by having a recodable codon is functionally important, and the editable A provides a selective advantage over both an uneditable A and a genomically encoded G (*right*).

feature of editing, which allows for the expression of multiple protein isoforms at varying levels in different tissues or conditions (96), may confer a selective advantage for some edits. In these cases, the fitness of having a recodable codon is higher than having both an uneditable genomic A and a genomically encoded G.

Several lines of evidence support the harm-permitting model. Restorative sites, where there was indeed an ancestral G→A mutation before the emergence of recoding, exhibit both a higher incidence rate than expected and higher recoding levels than other sites (95). Furthermore, over time one observes an elevated level of A→G mutations at recoding sites, suggesting that recoding is an interim substitute for the preferred genomic mutation (90, 97). Thus, it was suggested that restorative editing may fully explain the positive selection of recoding edits in cephalopods, and there is no evidence for a specific adaptive advantage of recoding itself over genomically encoded messages (95). However, some of the above results are also consistent with adaptive editing. A necessary condition for adaptive editing, whereby fitness increases via the flexibility inherent in the process to produce both unedited and edited alleles, is that both alleles are tolerated, regardless of whether one of the two alleles is beneficial. Thus, it is not surprising to find recoding more

abundantly at places where a genomic G was already demonstrated to be tolerated (in an ancestral species).

More importantly, several previous works have analyzed the evolutionary dynamics at recoding sites in terms of two competing, mutually exclusive, hypotheses: Conserved recoding sites either restore a preferred G allele or provide an adaptive advantage due to flexibility (90, 95, 97). Clearly, the actual situation may be more complicated, and both of the above scenarios may be realized concomitantly. Namely, some of the conserved recoding sites may serve merely as a partial replacement for an A→G mutation, whereas others do provide a selective advantage given the inherent flexibility editing provides. Thus, the question is not which scenario is correct but rather what fraction of sites adhere to each model. Answering this question is not easy and requires a large statistical sample. In an attempt to provide a partial answer (82), A→G substitution rates for the thousands of conserved recoding sites were analyzed along the inferred evolution of select coleoid species. Adenosines undergoing harm-permitting recoding are expected to mutate to G at a rate that exceeds the neutral rate, whereas sites where editing is advantageous to both an unedditable genomic A and a genomically encoded G should mutate at a lower rate. Thus, the actual rate of A→G substitutions that mutate an editable A into a genomically encoded G provides a lower bound on the fraction of conserved recoding sites where flexible editing is adaptive. By using this approach, it was shown that the fraction of all strongly edited sites that are recoding preferring (rather than A preferring or G preferring) is higher than 11% to 41% (numbers vary for different paths along the coleoid evolutionary tree). Note that these numbers apply only to conserved sites and that they are lower bounds.

We conclude that harm-permitting editing likely accounts for a substantial amount of conserved recoding sites. An important example for this may be the first discovered example of A→I recoding, the Q/R site in mammalian GRIA2 transcripts. However, this is not the only way in which recoding is used, and the inherent flexibility provided by recoding is used in a sizable fraction of conserved cephalopod recoding sites to provide a selective advantage over both an unedited adenosine and a genomically fixed guanosine. Although these statistical analyses demonstrate the existence of sites where the flexibility inherent in recoding is adaptive, identifying the specific adaptive sites is difficult. To date, there is not a single known example of a recoding site, in cephalopods or any other taxa, where one can definitively demonstrate that there is an advantage to having both the edited and unedited version of the protein available. Therefore, revealing the adaptive role played by transcriptomic plasticity is an open challenge.

COMBINATORIAL EDITING IN CEPHALOPODS

The transcriptome-wide mapping studies discussed above revealed that many neural transcripts in cephalopods harbor multiple editing sites. Typically, in metazoans, editing at neighboring sites is correlated, but not fully so (98). Thus, the editing status at one editing site within a given copy of a transcript does not determine the editing status at a different site within the same molecule. Accordingly, for a transcript with N editing sites and a sufficiently large number of molecules, one may expect to find all 2^N possible combinations, resulting in an exponentially large number of potential isoforms. For example, the mammalian serotonin receptor 2C (5-HT_{2C}), a member of a family of serotonin receptors expressed in the central nervous system, is edited at five different sites affecting three amino acids (23). These sites are neither fully edited nor fully correlated, and thus editing could potentially lead to 32 (24) distinct transcripts, which encode 24 different protein isoforms, and these changes lead to varying effects on the receptor's response to serotonin and a cascade of downstream pathways (23, 99). Transcripts encoding at least 20 of the different protein variants were observed in human brain tissues (100, 101). Despite this diversity of sequence and function, the situation with cephalopod neural transcripts is far more complex.

The number of proteins encoded by edited transcripts grows rapidly as the number of editing sites increases. For example, 242 recoding sites were found in squid α-spectrin messages. Theoretically, these could lead to $2^{242} \approx 7 \cdot 10^{72}$ different variants of the α-spectrin protein. To put this number in perspective, suffice it to say that it dwarfs the number of all α-spectrin molecules (or, for that matter, all protein molecules) synthesized in all cells of all the squids that have ever lived on our planet since the dawn of time. Clearly, not all combinations are expressed, and the question is how many of these are expressed at biologically relevant levels. How many protein isoforms can be observed concurrently? Are all of these isoforms functional, or could some of them misfold, creating dangerous, possibly toxic, substances? What is the regulatory mechanism determining the relative expression of the multitude of various variants? Assuming that a large number of functionally distinct isoforms are produced due to a complex pattern of site–site correlations, one cannot characterize the editing status of the transcript by looking only at the set of editing levels per site. Instead, the full distribution of isoforms, reporting the relative abundance of the different isoforms, should be reported to understand the effect of editing on the protein products and its dependence on internal and external conditions.

Finally, the combinatorial nature of editing may present an additional argument against the harm-permitting model. The harm-permitting model assumes that for each edited message there is basically one advantageous version of the protein. If this version were encoded in the genome, editing would not be required. According to this model, editing is beneficial and conserved by evolution only due to the need to compensate for harmful G→A mutations in the genomically encoded genes. If this is the case, one would expect editing to produce mainly a single protein version (presumably, the fittest one). However, in the presence of multiple editing sites, we expect RNA editing of neural cephalopod messages to result in a wide distribution over a multitude of protein isoforms expressed concurrently in the cell, with no single version expressed to the level of more than a few percent. If these many isoforms are also functionally distinct, the outcome of editing is a large diversity in protein function rather than one optimized protein. Thus, it is difficult to view recoding as a restorative mechanism that merely replaces a genomic mutation in an attempt to produce the one optimized version of the protein. Whether large numbers of protein isoforms are produced concurrently by editing, and the functional implication of this diversity, remains to be seen.

CEPHALOPOD ADARS

Vertebrate genomes possess two genes encoding active ADARs, *adar*1 and *adar*2. cDNAs from these genes were cloned before the genome sequences were published (13, 102–104). In mammals, there are two versions of ADAR1, termed p110 and p150, each driven by a different promoter (105, 106). p110's expression is constitutive, whereas that of p150 is interferon induced. Besides playing a limited role in mRNA recoding, the ADAR1 isoforms are thought to be important components of the innate immune response to dsRNA viruses (107, 108). p110 edits endogenous dsRNA structures, preventing their recognition by MDA5, a cytosolic dsRNA sensor that triggers an innate immune response. p150, on the other hand, is activated, with a delay, by the interferon that is produced during an innate immune response and presumably plays a role in shutting down the response. From the N to C termini, the canonical vertebrate ADAR1 consists of two Z-DNA/RNA binding domains (Zα and Zβ), three dsRNA binding domains (dsRBDs), and a deaminase domain (DD) that catalyzes the conversion of A to I (**Figure 4**). The primary sequence of squid ADAR1 has been deduced from genome and transcriptome assemblies (42, 43, 84). It contains an N-terminal Zα, a single dsRBD, and a DD; thus, it is missing the Zβ domain and 2 dsRBDs. It is expressed predominantly in the nervous system and testes, at relatively low levels. Nothing is known currently about its function.

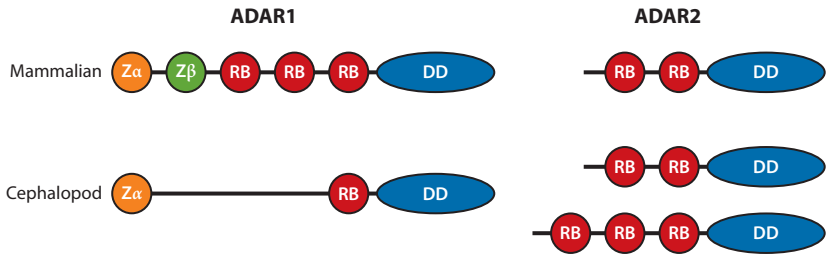

Figure 4

Cephalopod versus mammalian ADAR structures (ADAR1 and ADAR2). Abbreviations: ADAR, adenosine deaminase acting on RNA; DD, deaminase domain; RB, double-stranded RNA binding domain; Zα, Z-DNA binding domain α; Zβ, Z-DNA binding domain β.

In addition to ADAR1, cephalopods also possess a gene for ADAR2, and its function has been studied in greater detail than SqADAR1. SqADAR2 cDNAs were originally cloned from the nervous system of *D. pealeii*, and the corresponding genomic loci have been identified in both squid and octopus (81, 84, 109). Canonical vertebrate ADAR2s contain two dsRBDs and a DD. Squid express two splice variants of ADAR2, one that shares the same design as that of vertebrates (SqADAR2B) and another with an extra dsRBD (SqADAR2A). The added dsRBD of SqADAR2A makes this variant bind more tightly to dsRNA, conferring higher editing activity (110). Because inorganic anions like Cl^- tend to impede ADAR activity, and marine invertebrates are osmoconformers, being isotonic with seawater, it was proposed that the extra dsRBD in SqADAR2A helps to compensate for the elevated ionic environment. Besides the splice variants, messages encoding SqADAR2A and -B are themselves edited at 16 sites, but the functional consequences of these editing events have not been explored (109). The catalytic activities of the SqADAR2 variants are not unusually high when compared to human and *Drosophila* ADAR2 in vitro and are unlikely to explain high-level recoding in cephalopods (109, 110). Thus, coleoid cephalopods, like vertebrates, express a single gene for both ADAR1 and ADAR2. Each has some novel structural features. The function of ADAR1 in particular merits further investigation.

SPATIALLY REGULATED RECODING IN SQUID

In principle, RNA editing allows the regulation of protein function across many spatial scales. Clearly, editing levels can be regulated between tissues, and between cell types within those tissues. This is the case for numerous mammalian mRNA targets (88, 111, 112). Editing could also vary between different regions in a cell, and this idea is particularly compelling for neurons because of their complex morphologies and large size. Regions like dendrites, axons, and synapses are often distant from the nucleus, and thus transcriptional-level regulation would be necessarily slow. The ability to edit messages and translate them on-site would be a source of rapid plasticity. In most organisms, including mammals, this appears unlikely. First of all, both mammalian ADAR1 p110 and ADAR2 are localized to the nucleus, predominantly within the nucleolus (113–115). Although the interferon-inducible ADAR1 p150 shuttles between the nucleus and the cytoplasm (106, 113, 116, 117), no recoding events have been identified for this enzyme (118, 119). Second, many of the RNA structures that ADARs recognize are made up of both intronic and exonic sequences in premRNAs (7, 8, 120, 121). Thus, neither the RNA editing enzymes nor most of the structures that they recognize exist outside of the nucleus.

The spatial regulation of RNA editing in coleoids appears to be complex. Like in mammals, editing varies between tissues and different regions of the nervous system (42, 43, 84). In general,

editing is most abundant in the nervous system, and most mRNA recoding happens in nervous tissue. As with mammals, editing outside of the nervous system tends to be concentrated in repetitive elements in the noncoding portions of RNAs (43, 84). Unlike with mammals, however, editing in squid appears to vary within neurons. Vallecillo-Viejo et al. (52) recently showed that SqADAR2 protein is expressed abundantly outside of the nucleus in neurons. This study took advantage of the giant axon system where axons and cell bodies can be separated manually via dissection and pure axoplasm extruded from the axon. Western blots revealed strong SqADAR2 signals in the axoplasm. Immuno-stained sections from other regions of the nervous system revealed strong extranuclear SqADAR2 signals. Interestingly, there was robust staining in the plexiform layer of the optic lobe, a region of synaptic connections. In addition to the presence of SqADAR2, general A→I editing activity was also demonstrated in axoplasm, as well as the ability to edit known editing sites within the K_v1.1 channel message. Finally, transcriptome-wide editing was mapped and compared between messages isolated from axons and cell bodies from the giant axon system. In general, editing was more extensive in the axons for the same message. Taken together, these data strongly suggest that squid, and probably other coleoids, can regulate RNA editing between different regions of a neuron.

FUTURE DIRECTIONS

In general, there are several major questions relating to cephalopod RNA editing. First, on a molecular level, what mechanism underlies the high-level mRNA recoding? On this point it is important to emphasize that overall editing levels are not unusually high in cephalopods; it is their propensity to use it to recode mRNAs that is unusual. As discussed, cephalopod ADARs have some novel structural features. Do these contribute to the high-level editing, is it due to association with other proteins, or is there something different about cephalopod mRNAs that makes their coding regions more likely to fold into editable structures? Second, what are cephalopods using the recoding for? Editing may be used simply as a mechanism to generate protein diversity. However, it is tempting to speculate that it is used for plasticity because as a process it is well-positioned for this role. By turning editing on or off in an editing site–specific manner, it could be used to acclimate to changing environmental conditions, such as temperature, oxygen availability, light, or pH. Besides physical environmental variables, differences in the social environment may also influence editing. The messages encoding proteins known to be involved in learning and memory, like neurotransmitter receptors and the machinery for synaptic vesicle release and reuptake, are recoded extensively. Because of the sheer number of editing sites in the coleoids, a wide variety of physiological processes could be regulated.

To be edited, editing sites require large surrounding RNA structures. Thus, to maintain the ability to edit them over evolutionary timescales, comparatively large swaths of sequence space must remain invariant. This relationship presents an interesting evolutionary trade-off. Very little is known about how editing sites evolve. This is largely because editing has not been closely examined between closely related species or different populations of the same species. Because so much structure is required to generate an editing site, one might think that new sites are slow to come into being. However, the introduction of various ADAR1 and ADAR2 enzymes into yeast, an organism that lacks ADARs and RNA editing, resulted in editing at thousands of sites (S. Ben-Aroya, unpublished data). Thus, it appears that a neutral genome contains editable structures in the open reading frames and that the lack of recoding editing in most organisms with active ADARs has occurred through evolutionary purification. Do coleoids contain a common complement of structures that require few mutations to become editable? The vast majority of coleoid editing sites are edited at very low frequencies. Maybe these are the set of possible sites that can be recruited into action as conditions change? How quickly can new sites evolve in the

face of selective pressures? A more systematic search for adaptive recoding sites may permit us to better correlate proteome flexibility with selective advantage.

RNA editing in the coleoid cephalopods displays many novel features that warrant further investigation. Chief among these is the propensity to recode. How recoding sites are selected is unclear. A lack of genetically tractable models has been a major obstacle to our understanding of cephalopod editing. Without genetics, it is difficult to better understand ADAR activity, proteins that interact with ADAR, and the functional significance of RNA editing events on the physiology of the whole organism. In addition, a lack of cellular and molecular tools for this taxon, such as cultured cells, viral delivery systems, and identified promoters and enhancers to drive gene expression, further complicates experimental approaches. However, the situation is starting to improve. A recent gene knockout study using CRISPR-Cas9 on squid opens many doors to better understanding RNA editing in cephalopods (122). As a new generation of marine model organisms are generated, progress should advance more quickly.

SUMMARY POINTS

1. A→I editing, rarely used to recode codons in mammals and other species, is used extensively for protein recoding in the nervous system of coleoid cephalopods.
2. The majority of messages in coleoid neural tissues are subject to recoding, and many of them are multiply recoded, potentially leading to an extremely large number of protein isoforms.
3. Recoding levels vary across tissues, depend on external conditions, and vary even between different regions within the same neuron.
4. Recoding sites, and even recoding levels, are conserved across coleoid species that diverged ~200–350 Mya.
5. In some cases, recoding may serve to substitute a genomic mutation. However, evidence shows that thousands of recoding sites are adaptive, in the sense that the proteomic flexibility conferred by editing is superior to a genomically encoded sequence.
6. Cephalopods, like vertebrates, have orthologs for the editing enzymes ADAR1 and ADAR2. Their versions differ from the vertebrate ones, though whether these differences are relevant for the exceptionally high recoding levels is unclear.
7. Cephalopod ADAR2, unlike orthologs from vertebrates and *Drosophila*, can be found in both the cytoplasm and nucleus of neurons.

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