AUTOREGULATION OF ADAR2 FUNCTION BY RNA EDITING

By

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LIST OF ABBREVIATIONS

5-HT_{2C}R – the 2C subtype serotonin receptor

A – adenosine

ACF – apobec-1 complementation factor

ADAR – Adenosine deaminase that act on RNA

AMPA – -amino-3-hydroxy-5methyl-4isoxazolepropionate

apoB – apoplipoprotein B

Apobec-1ApoB editing catalytic subunit 1

A-to-I – Adenosine-to-Inosine

BLCAP – bladder cancer associated protein

C – cytidine

CDA – cytidine deaminase

CNS – central nervous system

CYFIP2 – cytoplasmic FMR1 interacting protein 2

C-6 – carbon number 6 of the purine ring

C-to-U – Cytidine-to-Uridine

dADAR – drosophila ADAR

dsRNA –double-stranded RNA

dsRBDs – double-stranded RNA binding domains

ECS – editing site complimentary sequence

EDTA – ethylenediaminetetraacetic acid

FLNA – filamin A alpha subunit

G – guanosine

GluRs – glutamate gated ion channel subunits

GPCR – G-protein coupled receptor

HDV – Hepatitis delta virus

I – inosine

IFN – Interferon

IGFPB7 – insulin-like growth factor binding protein 7

kD – kilo Dalton

$K_v1.1$ – the voltage-gated potassium channel subunit

LDL – low density lipoprotein

M – molar

NLS – nuclear localization signal

NMR – Nuclear magnetic resonance

NP-40 – Nonidet P-40

PCR – polymerase chain reaction

PKR – double-stranded RNA-dependent protein kinase

PMSF – phenylmethylsulphonylfluoride

RT – reverse transcription

SDS – sodium dodecyl sulfate

SDS-page – SDS polyacrylamide gel electrophoresis

SINEs – short interspersed elements

T – Thymidin

U – Uridine

UTR – untranslated region

CHAPTER I

INTRODUCTION

Although the “central dogma of molecular biology” proposed that genetic information flows from DNA through RNA to the encoded protein (Crick, 1970; Crick, 1958); one can not always predict the exact RNA or amino acid sequence from the nucleotide sequence in the genomic DNA. The first example is RNA splicing in adenovirus (Berget et al., 1977; Chow et al., 1977), where segments of viral RNA from different regions of the viral genome are joined together to form the mature transcripts, suggesting the possibility of generating multiple gene products from the same genomic locus via RNA processing. Since then, numerous mechanistically distinct modifications have been identified in the process of eukaryotic RNA maturation, including capping, polyadenylation, alternative splicing, and most recently, RNA editing (Emeson et al., 1989; Kable et al., 1996; Keller and Minvielle-Sebastia, 1997; Varani, 1997; Wang and Manley, 1997).

RNA editing was first discovered in a mitochondria transcripts encoding the cytochrome oxidase subunit II (coxII) in *Trypanosoma brucei*. In *trypanosome* coxII transcripts, four uridine residues are inserted into the RNA sequence to generate a open reading frame that can not be deduced from the genomic sequence (Benne et al., 1986). The existence of nucleotide insertion and deletion

within RNA sequences via different mechanisms has then been described in a variety of organisms, including amoebid protozoa, myxomycetes, chytriomycete fungi, nematodes, and RNA viruses (Grosjean and Benne, 1998). Although initially coined to describe nucleotide insertion/deletion, the term “RNA editing” is now used to describe a number of mechanistically distinctive RNA modifications, including post-transcriptional insertion/deletion (Kable et al., 1996; Seiwert, 1996), co-transcriptional insertion (Visomirski-Robic and Gott, 1997), nucleotide exchange (Price and Gray, 1999) and base modification/substitution (Auxilien et al., 1996; Sommer et al., 1991).

RNA editing by base modification

While purine-to-pyrimidine and pyrimidine-to-purine alterations have been reported (Price and Gray, 1999), most of the base modification/substitutions RNA editing identified to date result from deamination (adenosine-to-inosine, A-to-I and cytidine-to-uridine, C-to-U) or trans-amination (uridine-to-cytidine, U-to-C) reactions (Backus and Smith, 1991; Burns et al., 1997; Sharma et al., 1994). In mammals, the majority of RNA editing events involve nucleotide deamination, with the best-characterized examples being C-to-U and A-to-I conversions, in which hydrolytic deamination of cytidine or adenosine lead to the production of uridine and inosine nucleosides, respectively (Figure 1). These base modifications have been observed in both messenger RNA precursor (pre-mRNA) and mature mRNA transcripts. When such conversions occur in coding regions, it can lead to alterations in the coding potential of the transcript, resulting in the

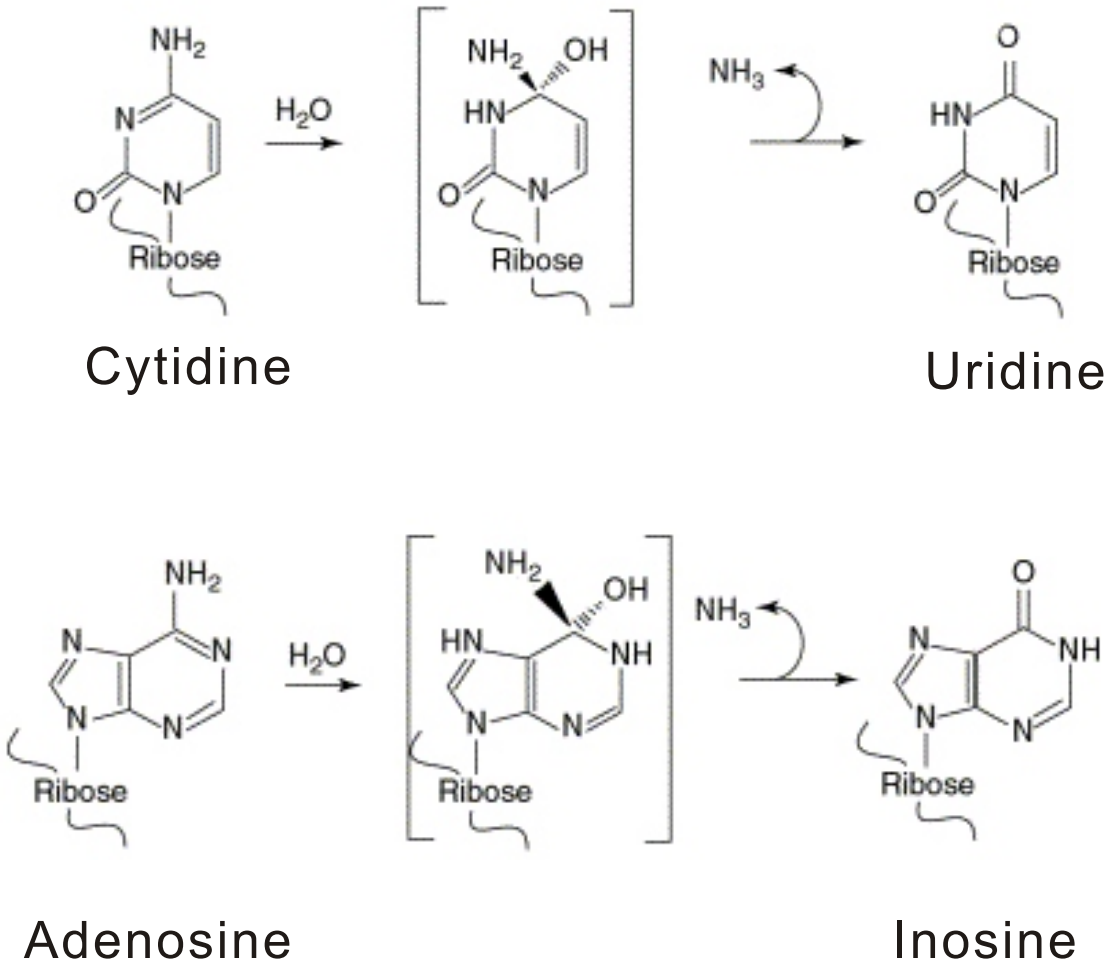


Figure 1 RNA editing by hydrolytic deamination
C-to-U and A-to-I conversions occur as results of hydrolytic deamination at C4 and C6 positions of the pyrimidine and purine rings, respectively, while the hydroxyl group (-OH) in water (H2O) serves as the nucleophile

synthesis of protein products with altered functions (Bhalla et al., 2004; Burns et al., 1997; Chester et al., 2000; Lomeli et al., 1994; Sommer et al., 1991); whereas, nucleotide deamination in the non-coding region may affect almost all aspects of cellular RNA function including structure, stability, translation efficiency and splicing patterns (Knight and Bass, 2002; Kumar and Carmichael, 1997; Prasanth et al., 2005; Rueter et al., 1999; Scadden and Smith, 2001).

C-to-U RNA editing

The first RNA editing identified in mammals was the C-to-U deamination in mRNA transcripts encoding apoplipoprotein B (apoB) (Chen et al., 1987), a key component in mammalian cholesterol metabolism. The ApoB protein exists in two functionally distinct forms, ApoB-48 and ApoB-100. Due to a single C-to-U editing event in the intestine, a glutamate codon (CAA) is converted into a stop codon (UAA), rendering an ApoB transcript to encode a protein isoform (Apo-48) lacking the low density lipoprotein (LDL) receptor binding domain at its carboxy-terminus, which facilitates the transport of dietary lipids in the intestine. In the liver, the full-length ApoB protein (ApoB-100) is secreted as a component of lipoproteins to bind the LDL receptor, delivering cholesterol to cells by receptor-mediated endocytosis (Figure 2) (Wang et al., 2003; Yao and McLeod, 1994). The C-to-U conversion in ApoB transcripts is catalyzed by a complex of proteins including a zinc-dependent cytidine deaminase, ApoB editing catalytic subunit 1 (Apobec-1), and an accessory protein, apobec-1 complementation factor (ACF), which binds to apoB transcripts at an 11nt mooring sequence immediately downstream of the editing site (Backus and Smith, 1991; Mehta et al., 1996).

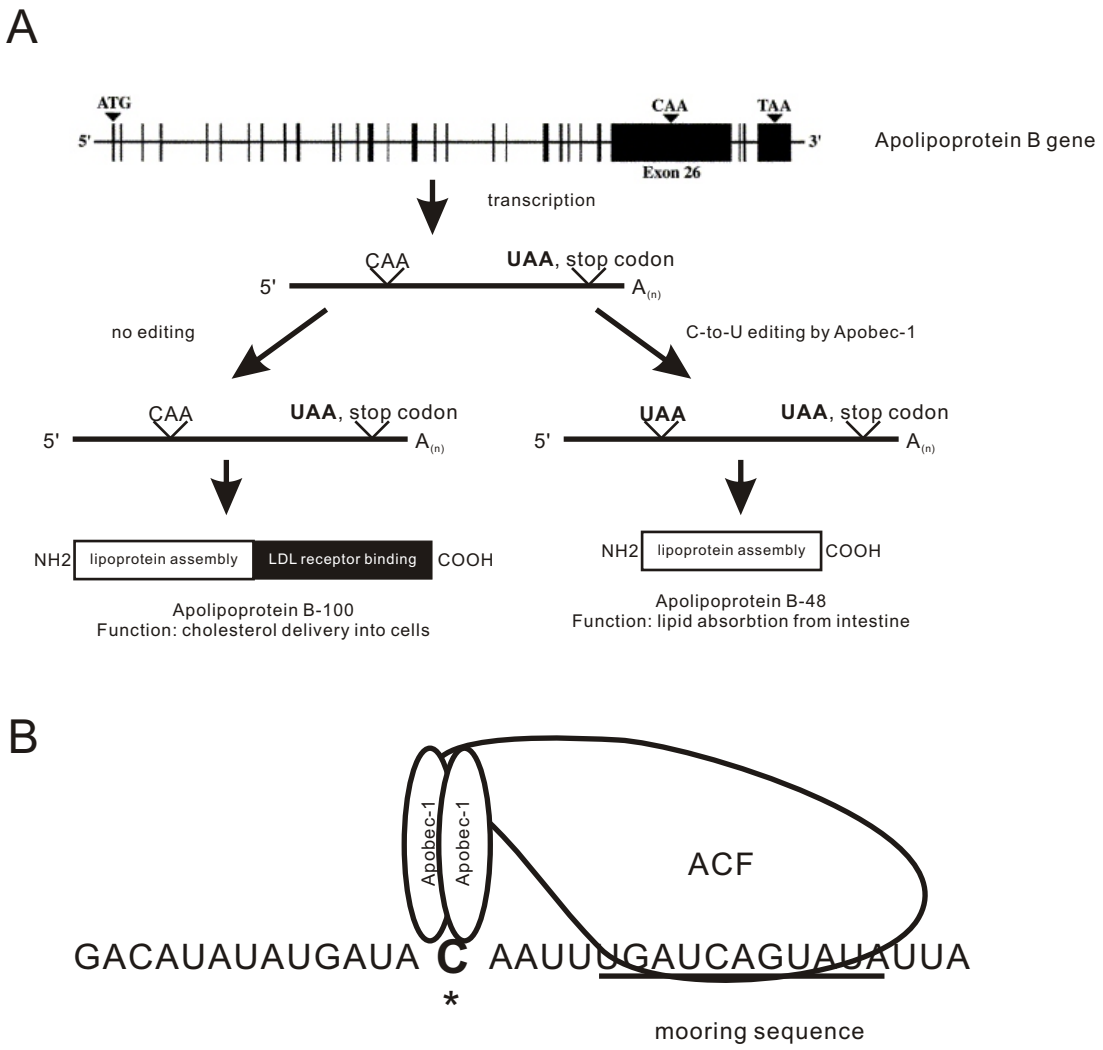


Figure 2 Apolipoprotein B editing

A. A schematic diagram of human apoplipoprotein B biosynthesis pathway is presented with the genomic organization of human ApoB gene shown at the top. Exons and intron are presented as solid bars and lines, respectively. The position of the start (ATG) and stop (TAA) codons are indicated. A C-to-U conversion in a glutamine codon (CAA) creates an in-frame stop codon (UAA) in ApoB transcripts expressed in the intestine, therefore producing a truncated protein, ApoB 48. (Adapted from Benny Hung-Junn Chang 1998)

B. A schematic representation of the minimal editing complex required for C-to-U editing in ApoB transcripts, including apobec-1 dimer and ACF which binds to the mooring sequence (underlined) near the editing site (*). (Adapted from Dr. Harold C. smith, http://www2.envmed.rochester.edu/envmed/TOX/faculty/smith.html.)

Yeast mRNA containing mooring sequence homologous motifs also can be edited by ectopically expressed Apobec-1 (Dance et al., 2001), suggesting that the mooring sequence is sufficient for Apobec-1 mediated RNA editing.

A second example of C-to-U RNA editing in mammals generates a translational termination codon at position 3916 in the neurofibromatosis type 1 (NF1) mRNA by site-specific deamination of an arginine (CGA) to a stop (UGA) codon (Mukhopadhyay et al., 2002). A truncated protein lacking a critical domain for GTPase activation is predicted to be encoded, however, the existence of such a protein has not yet been proved. This C-to-U modification has been observed in peripheral nerve sheath tumors from patients and may share elements of the same machinery responsible for apoB RNA editing (Blanc and Davidson, 2003).

In transgenic mice and rabbits, overexpression of Apobec-1 in the liver had resulted in liver dysplasia and hepatocellular carcinomas. Aberrant editing also was found in transcripts encoding a translation factor, NAT-1, which is not edited in normal tissues. These results suggest that C-to-U editing may be important for the maintenance of normal cell growth; uncontrolled C-to-U RNA editing may aberrantly modify RNA transcripts and lead to tumorigenesis (Yamanaka et al., 1995; Yamanaka et al., 1997).

A-to-I RNA editing

The most widespread type of base-modification editing is the conversion of adenosine to inosine (A-to-I), in which the C6-amino group on the adenine ring is substituted by a ketone (Auxilien et al., 1996), changing the targeted adenosine residue into inosine (Figure 1). Since the base-pairing properties of inosine are

similar to guanosine, the edited adenosine will be read as guanosine during splicing, reverse transcription and translation. Inosine was first detected at position 34 and 37 in yeast alanine tRNA ($tRNA^{Ala}$), where inosine 34 is essential for base-pairing with cytosine, uridine or adenosine at the wobble position of degenerate codons during translation (Holley et al., 1965). In addition to tRNA, A-to-I RNA editing events have also been described in pre-mRNA, mature RNA (Brusa et al., 1995; Burns et al., 1997; Higuchi et al., 2000; Hoopengardner et al., 2003; Rueter et al., 1999; Sommer et al., 1991), and RNA viruses (Polson et al., 1996), including the transcripts encoding the glutamate gated ion channel subunits (GluRs), the 2C subtype serotonin receptor ($5\text{-}HT_{2C}R$), the voltage-gated potassium channel subunit ($K_v1.1$), and Hepatitis delta virus (HDV), to modulate the coding potential of the targeted transcripts (Table 1). A-to-I conversion also has been described in non-translated RNA species and non-coding regions of RNA transcripts. Despite that an A-to-I conversion in the intron of RNA editing enzyme (ADAR2) alters the pattern of alternative splicing (Rueter et al., 1999), the functional consequences of most non-coding region RNA editing have not been examined. There have been speculations that such RNA modifications may affect various aspects of RNA function including splicing, trafficking, translation efficiency and transcript stability (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004; Morse et al., 2002).

Table 1 A-to-I RNA editing results in non-synoymous amino acid alterations

A list of known codon-altering A-to-I modifications in vertebrate, invertebrate and viral RNAs is presented. In a majority of cases, the functional consequences of amino acid changes resulting from A-to-I editing have not been examined.

RNA Substrate	protein function	Functional consequences
Vertebrate		
ADAR2	A-to-I RNA editing enzyme	Alteration in splicing pattern, proteine expression
GluR-2,3,4	Glutamate-gated cation channel subunits (AMPA subtype)	Alteration in calcium permeability and kinetics
GluR-5,6	Glutamate-gated channel subunit (Kainate subtype)	Alteration in calcium permeability
5-HT_{2C}R	G-protein coupled receptor	Reduction in G-protein coupling
Kv1.1	votage-gated potassium channel	Faster recovery from inactivation
ETB	endothelin receptor	unknown
PTPN6	phosphatase	unknown
FLNA	Filamin, alpha	unknown
BLCAP	bladder cander associated protein	unknown
CYFIP2	cytoplasmic FMR2 interacting protein	unknown
IGFBP7	insulin-like growth factor binding protein 7	unknown
Invertebrate		
4f-rnp	unknown	unknown
Para	sodium channel	unknown
Da5	nACh receptor subunit	unknown
ARD	nACh receptor subunit	unknown
SBD	nACh receptor subunit	unknown
Rdl	GABA receptor	unknown
DSCI	sodium channel	unknown
Ca-alpha1T	Calcium channel	unknown
DmCa1D	Calcium channel	unknown
$\alpha 2\delta$	Calcium channel accessory subunit	unknown
Shaker	votage-gated potassium channel	unknown
Eag	votage-gated potassium channel	unknown
Slowpoke	votage-gated potassium channel	unknown
synaptotagmin	synaptic vesicle calcium sensor	unknown
Dunc-13	SNARE protein	unknown
stnB	unknown	unknown
complexin	SNARE protein	unknown
lap	adapter protein in synaptic release	unknown
Pop-1	C elegans HMG-box protein	unknown
Laminin-γ	unknown	unknown
SSADH	aldehyde dehydrogenase	unknown
sqKv1.1	votage-gated potassium channel	Alteration in channel gating and tetramerization
sqKv2	votage-gated potassium channel	unknown
virus		
Hepatitis delta virus HDAg	virus replication/release	Switch from HDAg-S (replication) to HDAg-L (release)
measle virus hemagglutinin	hemadsorption	generate nonlytic persistant virons

Adenosine deaminases that act on RNA (ADARs)

A-to-I conversions are catalyzed by a family of enzymes known as adenosine deaminase that act on RNA (ADAR) (Bass et al., 1997). ADARs were identified initially based upon their ability to "unwind" double-stranded RNAs (dsRNA) when injected into *Xenopus* oocytes (Bass and Weintraub, 1988), resulting from the conversion of stable adenosine-uridine (A-U) to unstable inosine-uridine (I-U) base-pairs. Meanwhile, A-to-I modifications were discovered to produce functional alterations in measles viral packaging proteins that generate nonlytic, persistent virions (Cattaneo et al., 1988; Cattaneo et al., 1989) and in the transcripts encoding the second subunit of the α-amino-3-hydroxy-5methyl-4isoxazolepropionate (AMPA) glutamate receptor subunit 2 (GluR2) to alter the electrophysiological properties of the heteromeric glutamate gated ion channels (Sommer et al., 1991). Biochemical purification and molecular cloning have allowed the identification and characterization of ADAR proteins from variety of organisms. So far, three ADAR genes (ADAR1, ADAR2 and ADAR3) have been identified in mammals, (Chen et al., 2000; Kim et al., 1994; Melcher et al., 1996a; Melcher et al., 1996b), while two (ADR1 and ADR2) were found in *C. elegans* (Tonkin et al., 2002). In *Drosophila*, zebrafish and pufferfish, a single ADAR gene has been identified, respectively (Palladino et al., 2000b; Slavov et al., 2000).

ADAR functional domains

ADARs from all organisms share a common modular domain organization, including a variable amino-(N-) terminal region followed by one or several

double-stranded RNA (dsRNA) binding domains (dsRBDs) and a highly conserved catalytic domain in the carboxy- (C-) terminus (Figure 3).

The N-terminal region – In mammals, the N-terminal region of ADAR1 contains one or two Z-DNA binding motifs (Herbert et al., 1997), which bind specifically to the left-handed Z-DNA conformation with high affinity. As the formation of Z-DNA occurs during transcription, there has been speculations that the Z-DNA domains of ADAR1 recognize Z-DNA to facilitate ADAR1 targeting a nascent RNA so that editing may occur prior to splicing. For ADAR2, a nuclear localization signal (NLS) was identified in front of the first dsRBD domain of the protein (Melcher et al., 1996b); whereas in ADAR3, an arginine-rich single-stranded RNA (ssRNA) binding domain at the N-terminus is responsible for ADAR3 binding to ssRNA (Chen et al., 2000; Melcher et al., 1996b).

The dsRBD – While C-to-U editing in ApoB transcripts targets a specific cytidine moiety by recognizing *cis*-acting elements including an 11-nucleotide motif (mooring sequence) four nucleotides downstream from the editing site (Figure 2) (Anant and Davidson, 2001; Maas and Rich, 2000), no specific primary sequences in the RNA are required for site-selective A-to-I conversion. Instead, A-to-I editing requires the binding of the dsRBD to regions of duplex RNA where the targeted adenosine residues are found (Bass, 2002; Higuchi et al., 1993; Rueter et al., 1999) (Figure 4). dsRBDs were first discovered in staufen, a protein required to localize mRNA transcripts to specific cytoplasmic sites for proper anterior patterning in *Drosophila* oocytes (Ferrandon et al., 1994; St Johnston et al., 1992). To date, dsRBDs have been identified in a group of proteins with

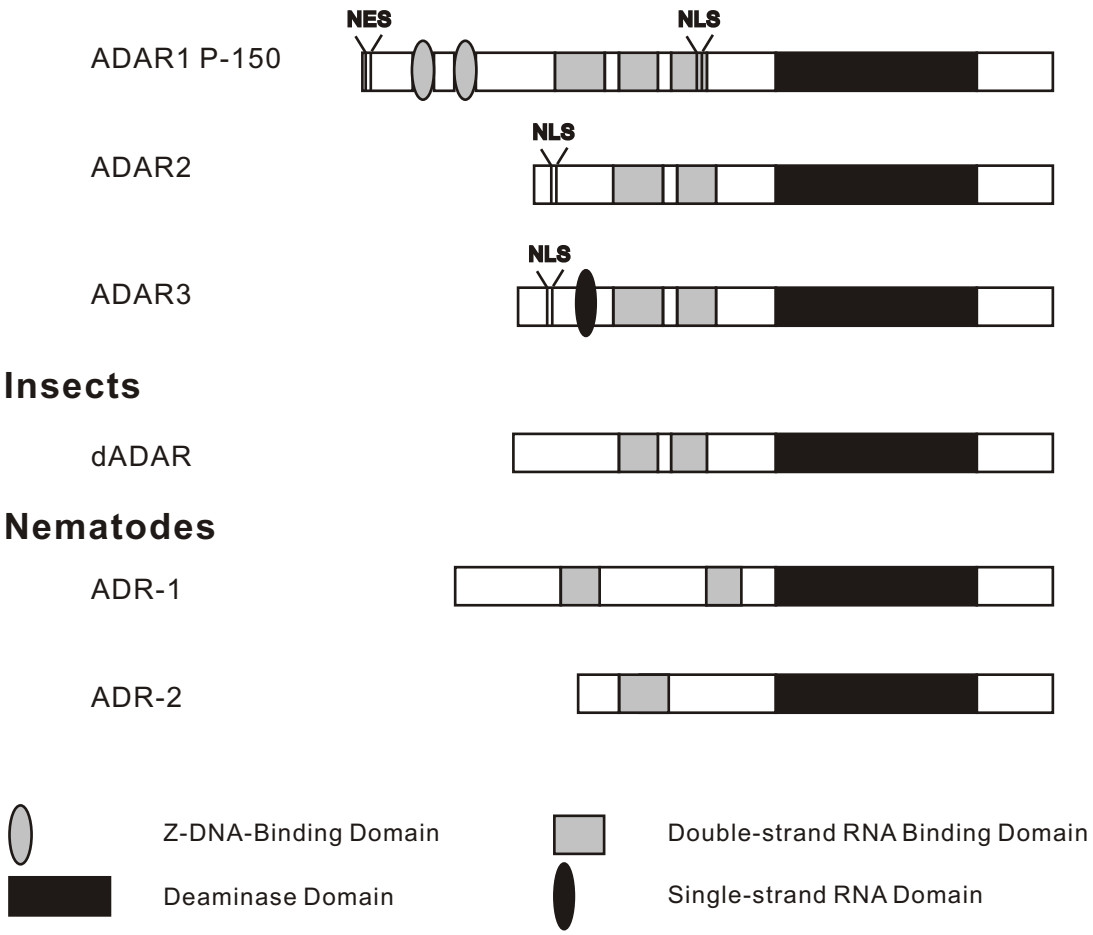
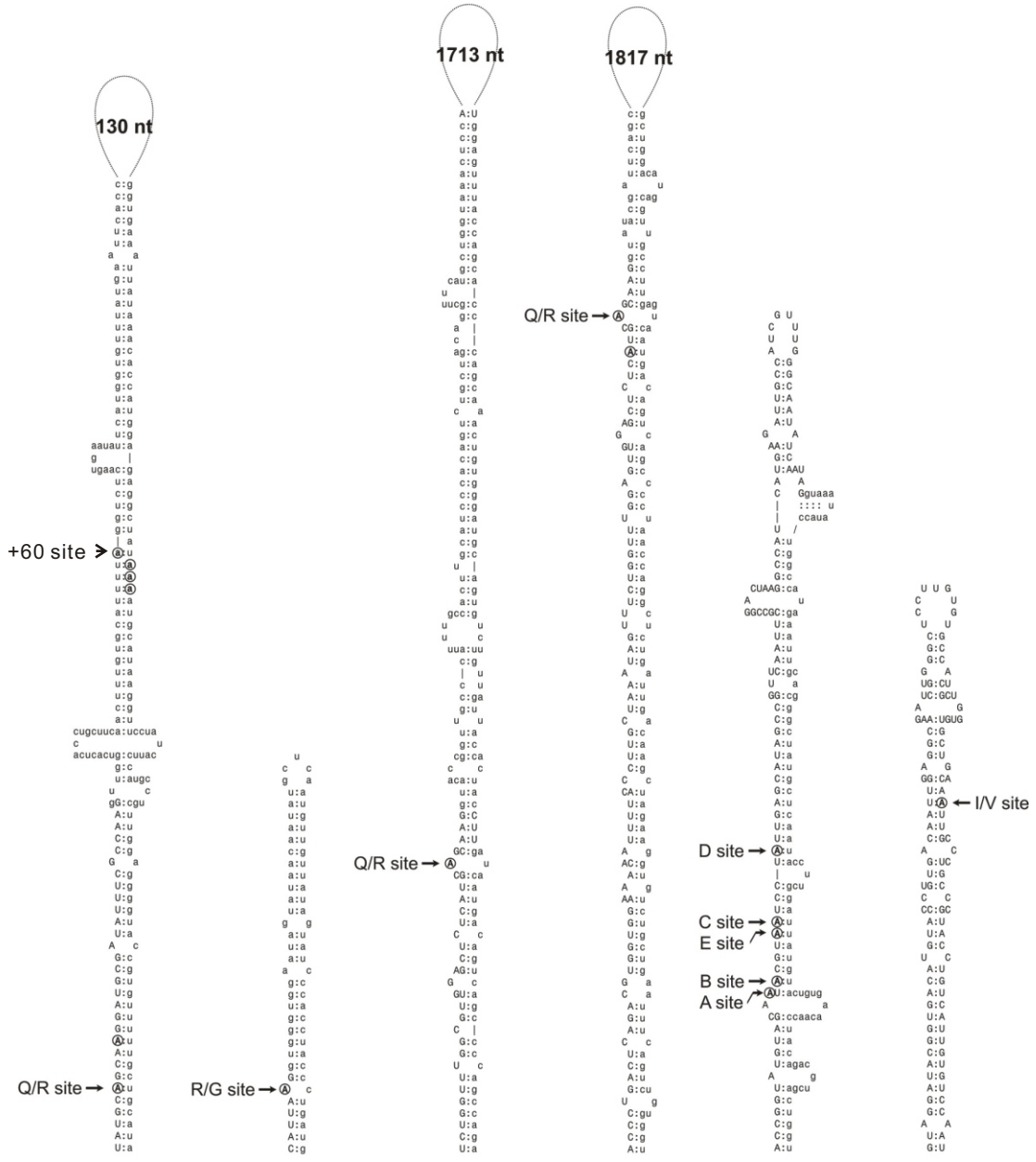


Figure 3 Functional domains of ADARs from different species. Schematic diagrams of domain structures for ADAR proteins from vertebrates, insects and nematodes are presented to depict the organization and phylogenetic conservation of functional domains in the enzymes that catalyze the A-to-I conversion in double-strand RNA substrates. The positions for nuclear export signal (NES) and nuclear localization signal (NLS) for vertebrate ADARs are indicated.

Figure 4 ADAR substrates require extended regions of duplex RNA. The predicted secondary structures of pre-mRNA transcripts encoding non-NMDA receptor subunits (GluR-2, GluR-5, and GluR-6), the 5-HT_{2C} receptor and the K_v1.1 voltage-gated potassium channel are presented in the regions of major editing modifications using RNA folding algorithms. The positions of edited adenosine residues are indicated with open circles; exon and intron sequences are represented with uppercase and lowercase lettering, respectively, and the number of nucleotides omitted from the figure is indicated in the loops.


130 nt
+60 site
Q/R site
1713 nt
Q/R site
R/G site
1817 nt
Q/R site
D site
C site
E site
B site
A site
I/V site
GluR-2 (Q/R site)
GluR-2 (R/G site)
GluR-5 (Q/R site)
GluR-6 (Q/R site)
5-HT2CR
Kv1.1 (I/V site)

diverse functions. In addition to Staufen, double-stranded RNA-dependent protein kinase (PKR) represents another well-characterized dsRNA-binding protein. Upon activation by dsRNA, PKR protects the host from viral infection by phosphorylation of eukaryotic initiation factor 2α (eIF2α), an important factor in the translation initiation machinery, thereby inhibiting protein translation in infected cells (Thomis et al., 1992). A third dsRBD containing protein, *Escherichia coli* (*E. coli*) ribonuclease III (RNase III), is part of a large family of dsRNA endonucleases that involve in the processing of mRNA, ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) and micro RNA (miRNA) (Lamontagne et al., 2001). A consensus sequence that defines a 65- to 68-amino acid of the dsRBD was first derived by comparing *Drosophila* staufen and *Xenopus laevis* RNA-binding protein A (Xlrbpa) (St Johnston et al., 1992). Among all known dsRBDs, a 42% amino acid sequence homology is revealed while the conservative amino acid changes are taken into account (St Johnston et al., 1992). For members of ADAR family, all proteins demonstrated high amino acid sequence conservation to the dsRBDs consensus sequence (Stefl *et. al.*, 2005, in press).

Nuclear magnetic resonance (NMR) studies on single dsRBD demonstrated that the dsRBD folds into a compact α–β–β–β–α structure with the alpha helices positioned on one side of an anti-parallel three-stranded beta-sheet (Bycroft et al., 1995; Kharrat et al., 1995). X-ray crystallography studies on the second dsRBD of Xlrbpa complexed with dsRNA demonstrated that the protein spanned 16 basepair (bp) of dsRNA, interacting with two successive minor grooves and

across the intervening major groove on one face of a primarily A-form RNA helix. Further x-ray crystallographic and NMR studies of dsRBDs in complex with dsRNA showed that most interactions are sequence-independent and occur with the minor groove and the phophodiester backbone (Bass, 2002). These observations supported the specificity of dsRBD for dsRNA over ssRNA or dsDNA, as well as the apparent lack of sequence specificity for dsRBD binding to dsRNA (Ryter and Schultz, 1998). Key residues making contacts with RNAs in the staufen and Xlrbpa dsRBDs also have been identified by mutational analyses and are generally conserved in the dsRBDs of ADAR1 and ADAR2. NMR solution structures of tandem dsRBDs from PKR showed that high affinity binding is achieved through cooperation between the interactions of multiple dsRBDs (Nanduri et al., 2000). A similar strategy may be used by ADARs, which have multiple dsRBDs and bind to dsRNA with nanomolar affinity (Ohman et al., 2000).

It is well accepted that the dsRBDs are important to the in A-to-I editing, but the exact roles of each dsRBD in the ADAR proteins are not clear. While the deletion of the first or the third dsRBDs from ADAR1 inhibited editing activity *in vitro*, suggesting that dsRBD is essential for targeting the ADAR substrates, the second dsRBD seemed to be dispensable. These results suggeste that the three dsRBDs are not functionally equivalent in the catalytic action of ADAR1 (Lai et al., 1995). Replacing the dsRBDs of ADAR1 with those of PKR decreased the editing efficiency of natural ADAR1 sites, while non-specific editing within a synthetic, perfect RNA duplex was not significantly altered. In addition, ADAR1 splice

variants that differ in length of the spacer region between dsRBDs or the dsRBD and the deaminase domain have different editing efficiencies on the A-site of the 5-HT_{2C} receptor transcripts (Liu et al., 1999). These results suggest that the arrangement of the dsRBDs might be a key determinant of site specificity (Liu et al., 2000).

Deaminase domain – Phylogenetic analyses have suggested that the ADAR family of enzymes evolved from a common ancestral mononucleotide cytidine deaminase (CDA) (Carter, 1995). The molecular mechanism for CDA deamination involves nucleophilic attack of the aminated carbon of pyrimidine (C-4) rings (Carter, 1995). Crystallographic studies of *E. coli* CDA have shown that a glutamate residue participates in acid-base catalysis, facilitating protonation of the N-3 nitrogen, while a histidine and two cysteines comprise a signature metalloprotein zinc-coordination domain (Ireton et al., 2002). The equivalent glutamate residue in ADARs is conserved among multiple species, including human, mouse, rat, fly and worms, and is required for editing activity (Lai et al., 1995). Substitution of this critical glutamate residue results the loss of ADAR catalytic activity, suggesting that ADARs catalyze adenosine deamination in dsRNA by a mechanism similar to that of CDA (Lai et al., 1995).

In a recent study, the crystal structure of the catalytic domain of human ADAR2 at 1.7 angstrom resolution has been reported (Macbeth et al., 2005). Detailed analysis revealed an ordered zinc ion surrounded by histidine 394 (H394), cystine 451 and 516 (C451 and C516), and glutamate 396 (E396) of human ADAR2 deaminase domain, which coordinates a water molecule that presumably

displaces ammonia during the deamination reaction. Unexpectedly, an inositol hexakisphosphate (IP_6) molecule was found buried within the enzyme core. IP_6 has been implicated in many cellular functions, including RNA export, DNA repair, endocytosis, and chromatin remodeling (Hanakahi and West, 2002; Shen et al., 2003; York et al., 1999). Although there are no reports that ADARs require a cofactor, ADAR2 expressed in a yeast strain that is deficient in IP_6 synthesis demonstrates no catalytic activity, suggesting that IP_6 is required for ADAR2 activity (Macbeth et al., 2005).

C-terminus – While the C-6 position of the adenosine ring is hidden in the narrow major groove of the dsRNA, the dsRBDs appear to interact solely with the minor and sugar-phosphate backbone (Polson et al., 1991). There has been speculation that ADARs overcome this inaccessibility through a base-flipping mechanism mediated by the extreme C-terminus of ADARs which has sequence homology to N-6-adenine methyltransferases (Hough and Bass, 1997). However, direct evidence for the existence of such a functional domain and the exact role of the C-terminus in A-to-I conversion has not been identified.

ADAR editing Specificity

In contrast to the mechanism of Apobec-1-mediated C-to-U deamination, ADAR proteins target sites of deamination through recognition of highly ordered secondary structure (Auxilien et al., 1996; Higuchi et al., 1993) (Figure 4), and to date, no evidence suggests that any accessory factor is required to mediate ADAR-RNA interaction (Chen et al., 2000; Dawson et al., 2004). These enzymes can either deaminate up to 50% of the adenosine residues in an extended,

perfect dsRNA, or precisely target one specific adenosine moiety among many adenosines in a natural substrate (Polson et al., 1996). Although ADARs recognize their targets with no specific sequence requirements, several lines of evidences have shown that ADAR1 and ADAR2 have their own preferred neighboring sequence for targeting. Previous studies have shown that both ADAR1 and ADAR2 prefer a uridine as 5' neighbor to the targeted adenosine. Although ADAR1 rarely target adenosine close to either terminus of synthetic dsRNA substrates, ADAR2 can deaminate adenosines as close as 3 nucleotides from either terminus, and it has a 3' neighbor preference as uridine (U) (Lehmann and Bass, 2000; Polson and Bass, 1994; Polson et al., 1991). In a recent study, Dawson *et. al.* compared the surrounding sequences of several adenosines by ADAR2 , and suggested a "consensus" sequence surrounding ADAR2-modified residues may exist as an important contributor to ADAR2 preference and editing efficiency (Dawson et al., 2004).

The detailed structures of RNAs may also influence the editing specificity. Adenosines in long, perfectly base-paired RNA duplex can be deaminated nonselectively (Nishikura et al., 1991; Polson and Bass, 1994); while shorter double-stranded RNA or RNAs with mismatches, bulges, or loops, undergo more selective deamination. It has been suggested that the disruptions within the duplex contribute to the selectivity of the reaction by limiting the access of ADAR binding to the short, perfect-matched RNA duplex regions (Lehmann and Bass, 1999).

ADARs in multiple species

Mammalian ADAR

Three ADAR genes have been identified in mammals. ADAR1 and ADAR2 are ubiquitously expressed, whereas ADAR3 is exclusively expressed in the brain (Chen et al., 2000). The highest expression of ADAR2 is in the brain (Melcher et al., 1996b), consistent with the observation that the brain has the highest level of inosine-containing mRNA compared with other tissues (Paul and Bass, 1998). While ADAR1 has three dsRBDs, ADAR2 and ADAR3 have two dsRBDs (Chen et al., 2000; Melcher et al., 1996a; Melcher et al., 1996b; Patterson et al., 1995). All three ADARs contain nuclear localization signals (NLS) and are localized to the nucleus, with the exception of the inducible isoform of ADAR1, which also carries an nuclear export signal (NES) in the N-terminal region of the protein and shuttles between the nuclear and cytoplasmic compartment of the cells (Strehblow et al., 2002). All mammalian ADAR proteins carry a conserved adenosine deamination domain, but only ADAR1 and ADAR2 demonstrate editing activity on extended duplex regions in synthetic RNA as well as naturally occurring pre-mRNA and viral RNA transcripts; ADAR3 is catalytically inactive on either extended RNA duplex or known editing substrates, despite the fact that it can bind both single- and double-strand RNA (Chen et al., 2000; George and Samuel, 1999; Kawakubo and Samuel, 2000; Melcher et al., 1996a; Melcher et al., 1996b). It has been suggested that ADAR3 might serve as a regulatory competitor of ADAR1 and ADAR2 within RNA substrates, thereby acting as an

inhibitor of A-to-I conversion (Chen et al., 2000), yet no evidence has been reported to support this hypothesis *in vivo*.

Drosophila ADAR

Drosophila expresses a single ADAR gene, dADAR, which encodes a protein with a deaminase domain and two dsRBDs. There is 42% amino acid homology between dADAR and human ADAR2 (Palladino et al., 2000a). Multiple dADAR mRNA isoform are generated by alternative transcription initiation and splicing (Figure 5 A). The transcripts driven by the 4a promoter are expressed during all developmental stages and undergo alternative splicing in the 5'-untranslated region and the region encoding the linker between the two dsRBDs; whereas, the expression from the 4b promoter is specific to adult flies (Palladino et al., 2000a). Interestingly, the dADAR transcript undergoes developmentally-regulated A-to-I modification, which changes a highly conserved serine (S) codon into a glycine (G) codon in the catalytic domain (Keegan et al., 2005) (Figure 5). The S/G site is close to motif II of the deaminase domain that is thought to chelate a zinc ion at the active site. Editing at the S/G site of dADAR was low in embryonic and pupal mRNAs and increased more than 40-fold from embryo to adult (Palladino et al., 2000a). DADAR isoforms with the serine to glycine substitution are less active in vitro and in vivo compared with the genome-encoded, unedited isoform. Mutant flies ubiquitously expressing an dADAR cDNA engineered to resist RNA editing demonstrated embryonic lethality, suggesting the physiological significance of S/G site editing in the embryonic development of *Drosophila* (Keegan et al., 2005).

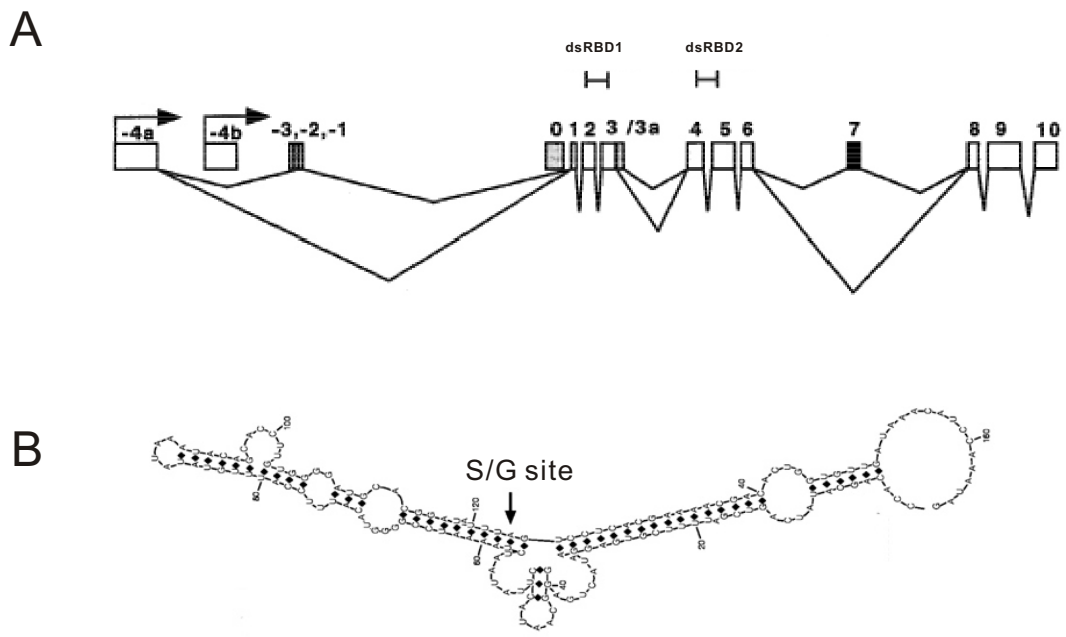


Figure 5 Alternative splicing and RNA editing of Drosophila ADAR.
A. The genomic organization of dADAR gene is presented with the constitutive exons (□) and alternatively spliced exons (▒) indicated. Exons encoding 5' UTR are labeled with negative numbers and arrows represent the alternative transcription initiation sites. RNA editing occurs within exon 7 (■). The regions encoding the two dsRBDs are indicated on top of the gene. (Adapted from Palladino et. al. 2000)
B. The predicted RNA structure for exon 7 of dADAR transcript. The edited adenosine is indicated with an arrow. (Adapted Keegan et. al. 2005)

C. elegans ADAR

The *C. elegans* genome encodes two ADAR genes, ADR-1 and ADR-2. ADR-1 encodes an enzyme with two dsRBDs and an atypical deaminase domain lacking the zinc-coordination and proton-shuttling residues (Tonkin et al., 2002). Five splice variants of ADR-1 have been identified. Expression of ADR-1 begins early in embryogenesis and continues to be expressed in all larval stages with the highest expression in the neuronal tissues. ADR-2, which encodes a protein containing a single dsRBD and a highly conserved catalytic deaminase domain, is the second gene in an operon of six genes and is expressed from early embryogenesis in most cells in the nervous system. ADR-2 mRNA are heterogeneous at their 5' termini since multiple spliced leader variants are used during processing from the polycistronic transcripts (Tonkin et al., 2002). Both ADR-1 and -2 are required for the A-to-I editing in *C. elegans* (Tonkin et al., 2002). Genetic studies indicate that while ADR-2 can function alone, the activity of ADR-1 is depend on the presence of ADR-2 (Tonkin et al., 2002).

ADAR substrate identification and characterization

Numerous RNAs have been found undergo A-to-I modifications, including those that alter the coding potential of the transcripts, such as RNA editing in transcripts encoding multiple subunits of ionotropic glutamate receptors (GluR2-6), 5-HT_{2C} receptors and $K_v1.1$ potassium channels. While most of the well-characterized ADAR targets were discovered serendipitously, recent studies

using systematic screening strategies have predicted thousands of new editing sites, most of which are located in the non-coding regions of RNA transcripts.

Substrate identification

Originally, a limited number of A-to-I editing events were identified based on adenosine-to-guanosine (A-to-G) discrepancies during comparison of genomic and cDNA sequences (Burns et al., 1997; Rueter et al., 1999; Smith et al., 1996; Sommer et al., 1991). However, the limited number of endogenous ADAR substrates identified could not explain the significant amount of inosine in the brain mRNAs (Paul and Bass, 1998), nor do they provide adequate explanations for the adverse phenotype of mice with aberrant ADAR activity (Higuchi et al., 2000; Wang et al., 2000). Therefore, several experimental approaches have been employed using either biochemical strategies or bioinformatics paradigms for systematic identification of novel A-to-I editing events.

Biochemical approach

Morse *et.al.* developed a series of biochemical strategies to specifically identify new A-to-I RNA editing substrates (Morse and Bass, 1997). The authors took the advantage of ribonuclease T1, which selectively cleave the 3' of inosine residues within glyoxal-treated RNAs. The digested fragments were then amplified by RT-PCR followed by sequence analysis. This approach allowed the successful identifications of ten and nineteen A-to-I editing events in RNA isolated from *C. elegans* and human brain, respectively (Morse et al., 2002; Morse and Bass, 1999). All the novel editing substrates are predicted to form long hairpin structures in the targeted region, and further analysis revealed that a majority of

these edited regions were contained within repetitive elements, such as IR-2, -3, -4 and -5 elements in *C. elegans* and Alu and LINE elements in human.

Comparative genomics approach

A second approach to identify ADAR substrates is developed based on the fact that the sequences surrounding the editing site as well as the sequences complimentary to the editing site region are conserved among species (Rueter et al., 1999; Slavov and Gardiner, 2002). Using the high degree of sequence identity as a potential signature of ADAR substrates, Hoopengardner et.al compared genes annotated as ion channels (*n* = 135), G protein–coupled receptors (GPCRs, *n* = 178), proteins involved in synaptic transmission (*n* = 102), and transcription factors (*n* = 499), between *Drosophila melanogaster* and *Drosophila pseudoobscura*. They identified numerous new ADAR targets and successfully verified sixteen previously unknown ADAR target genes in the fruit fly (Table 1). All of these genes are involved in rapid electrical and chemical neurotransmission and many of the edited sites alter the codons for conserved and functionally important amino acids, indicating a pivotal role for RNA editing in regulating nervous system function in *Drosophila* (Hoopengardner et al., 2003). Further investigations on one of the novel *Drosophila* targets, Shaker, revealed that the human homologue of this potassium channel undergoes A-to-I conversion in a conserved isoleucine (I) codon. Substitution of the isoleucine residue with a valine residue by A-to-I conversion alters the channel kinetic in recovery from inactivation (Bhalla et al., 2004).

Computational approach

With the recent knowledge of complete human genome sequences and the advanced technology in computational science, several groups have attempted to identify ADAR targets by comparison between cDNA and genomic sequence throughout the genome with the help of computational algorithm. Results from these studies revealed over 12,000 novel sites in more than 1600 genes in the human transcriptsome. 26 genes were experimentally validated, raising the number of known editing sites by two orders of magnitude (Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004), Strikingly, most of these newly-identified A-to-I editing events occur in inverted repetitive elements, such as *Alu* (92%) and L1 (1.3%) sequences, within the untranslated region. Search of database from other species using similar approach revealed that the frequency of A-to-I editing in humans is at least an order of magnitude higher than in the mouse, rat, chicken or fly transcriptsomes (Eisenberg et al., 2005). The extraordinary abundance of RNA editing in human can be explained by the dominance of Alu elements in the human transcriptsome, which dramatically increases the number of double-stranded RNA. In the latest computational search in human transcriptsome, researchers identified and experimentally verified four codon-altering editing events in transcripts encoding the α subunit of filamin A (FLNA), bladder cancer associated protein (BLCAP), cytoplasmic FMR1 interacting protein 2 (CYFIP2) and insulin-like growth factor binding protein 7 (IGFBP7) (Table 1). Three of the four editing sites were verified in mouse while two were validated in chicken. None of these substrates encodes a receptor protein but two of them are strongly

expressed in the CNS suggesting that some of the affected proteins might be related to the altered physiological phenotypes of ADAR mutant mice (Levanon et al., 2005).

Massive sequencing approach

Taking the advantage of high-throughput DNA sequencing technique, Blow *et. al.* have conducted a survey of RNA editing in human brain using sequence analysis on clones from a human brain cDNA library and comparing those to the reference human genome sequence and to genomic DNA from the same individual (Blow et al., 2004). In their study, approximately 12,000 nucleotides were edited out of >3 mega base-pair (Mb) surveyed. All editing events identified were A-to-I conversions and were predominantly in intronic and intergenic RNAs, especially in high-copy-number repeats, such as Alu elements. While examining the sequences surrounding the editing site, they found the A-to-I conversion is less likely to happen at an adenosine 3' to a guanosine and more likely at an adenosine 5' to a guanosine. Simulation by BLAST alignment of the double-stranded RNA molecules that underlie known editing sites indicates that there is a greater likelihood of A-->I editing at A:C mismatches than editing at other mismatches or at A: U matches. However, because A:U matches in double-stranded RNA are more common than all other mismatches, overall the likely effect of editing is to increase the number of mismatches in double-stranded RNA (Blow et al., 2004).

Functional consequences of A-to-I conversions

Once the targeted sites are modified by ADARs in the nucleus, the resultant transcripts are transported into the cytoplasm where they can be translated. Several well characterized A-to-I conversions involve non-synonymous codon changes in mRNA sequences, resulting in the production of protein products with altered functional properties. The most prominent examples are editing in transcripts encoding the GluR-2 to 6 subunits, the 5-HT_{2C} receptor, and the K_v1.1 channel subunits (Bhalla et al., 2004; Brusa et al., 1995; Burns et al., 1997; Hoopengardner et al., 2003; Niswender et al., 1999; Sommer et al., 1991). In addition, recent studies on A-to-I RNA editing events in the non-coding region demonstrated that these modifications are involved in modulation of splicing pattern and nuclear retention of the targeted transcripts (Kumar and Carmichael, 1997; Prasanth et al., 2005; Rueter et al., 1999).

RNA editing on GluR transcripts

Glutamate is the major excitatory neurotransmitter in the vertebrate CNS and plays an important role in fast excitatory neurotransmission, synaptic plasticity, and is involved in both chronic and acute neural disorders including stroke, epilepsy, amyotrophic lateral sclerosis and Parkinson's disease (Wisden and Seeburg, 1993). The ionotropic glutamate receptors (iGluRs) mediate fast neurotransmission at excitatory synapses in CNS and comprise 16 related subunits that can assemble into 3 oligomeric cation channels in postsynaptic membrane, including NMDA (N-methyl-D-asparate), AMPA and kainate receptors. They share the same tetrameric structure, in which four subunits

contribute to the inner channel lining a pore loop structure (Seeburg and Hartner, 2003). A-to-I conversions can functionally modify 5 of these 16 subunits (subunit 2-6) that are involved in AMPA and kainite receptor assembly, while no RNA editing events have been identified in transcripts encoding NMDA receptor subunits.

A-to-I RNA editing in AMPA receptor transcripts constituted the first and best characterized example of A-to-I editing in mammals. The glutamine residue (Q) positioned at the hairpin loop in GluR2 is important in forming the narrow constriction of the channel and in determining the ion permeability of the channel. The modification of the CAG (glutamine, Q) codon into a CGG (arginine, R) codon by A-to-I editing (Q/R site editing) renders the channel impermeable to calcium ions and affects channel assembly in the endoplasmic reticulum (Figure 6) (Greger et al., 2003; Sommer et al., 1991). The Q/R site is edited at almost 100% in GluR2 transcripts isolated from the whole brain, with the exception of subsets of striatal and cortical neurons where underedited (Q/R site) GluR-2 subunits are expressed. This decreased level of editing provides an potential explanation for the high vulnerability to excitotoxicity of these neurons (Kim et al., 2001). Decreased GluR-2 Q/R site editing was also observed in human brain tumors, and a link was proposed between lowered GluR-2 editing (Q/R site) and the occurrence of epileptic seizures associated with malignant gliomas (Kim et al., 2001). Genetically modified mice expressing only one non-edited (Q/R site) GluR-2 allele die of seizures at three weeks of age, demonstrating the importance of editing at this particular site for normal brain function (Brusa et al.,

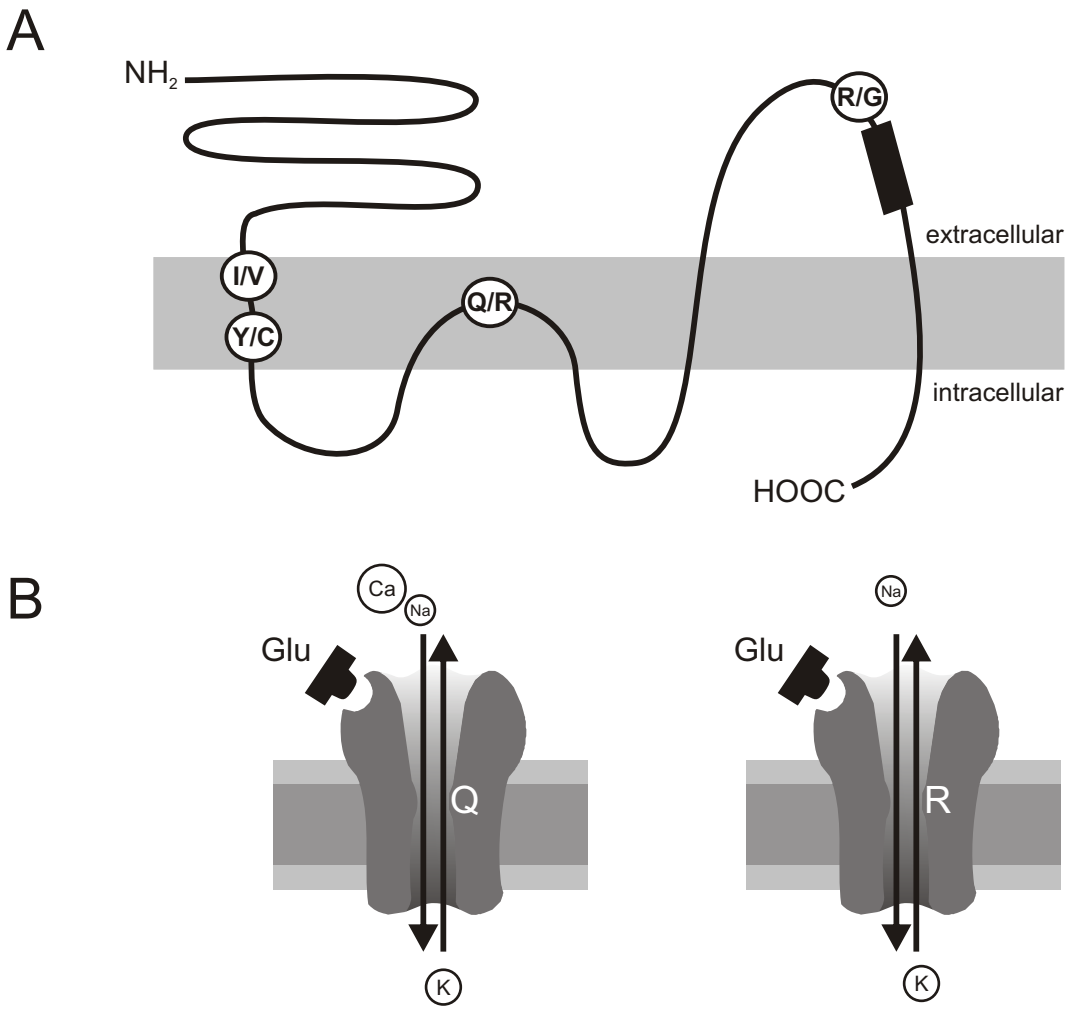


Figure 6 RNA editing of ionotropic glutamate receptor subunits.
A. A schematic diagram of the proposed topology structure of non-NMDA ionotropic glutamate receptor subunits is presented with the editing site indicated. The amino acids encoded by the nonedited/edited codon are represented in circles. The Q/R site is contained in RNAs encoding GluR-2, -5 and -6 subunits, the R/G site is in transcripts encoding GluR-2, -3 and -4, while the I/V and Y/C site are only found in GluR-6 transcripts.
B. A cartoon diagram of the ion permeation properties of heteromeric AMPA receptor containing GluR-2 subunits encoded by the nonedited (Q) and edited (R) transcripts, respectively. The cations through the channel are indicated as balls with labels and the arrows indicate the direction of the cation flow.

1995). Both *in vitro* and *in vivo* studies have indicated that ADAR2 is responsible for Q/R site editing in GluR-2 transcripts (Higuchi et al., 2000; Melcher et al., 1996b). Similar to the editing-deficient GluR-2 mutant mice, the ADAR2 null mice is prone to seizures and died shortly after birth (Higuchi et al., 2000). Interestingly, the adverse phenotype demonstrated in mice lacking the expression of ADAR2 can be rescued by introducing a point mutant at the Q/R site of GluR-2 gene to generate sole arginine-containing subunits, suggesting the seizure and lethal phenotype in the ADAR2 deficient mice is primarily due to lack of editing at the GluR-2 Q/R site (Higuchi et al., 2000). It is still puzzling why GluR-2 Q/R site occurs as a result of A-to-I editing rather than encoding the critical arginine residue within genomic DNA, as genetically modified mice expressing GluR-2 with a genomically encoded arginine at the Q/R site demonstrated no discernable phenotype (Kask et al., 1998).

In addition to Q/R site editing, ADAR binding to a separate duplex structure in the GluR-2 transcripts (Figure 4) converts an arginine (R) into a glycine (G) codon. The conversion is important in regulating channel kinetics, since the glycine-containing AMPA receptor recovers more rapidly from receptor inactivation compared with the arginine containing isoform (Lomeli et al., 1994).

Similar A-to-I modifications are also observed in the subunits of heteromeric kainate receptors, which are less abundant in the brain than the NMDA and AMPA receptors. At the position homologous to the GluR-2 Q/R site, the GluR-5 and GluR-6 kainate receptor subunits are expressed in both their edited (R) and nonedited (Q) forms. In contrast to the GluR-2 Q/R site editing, the efficiencies of

GluR-5 and -6 Q/R site editing are low at embryonic stage and rise during postnatal developmental maturation, suggesting that the channels may play different roles during development. The physiological and functional relevance of Q/R site editing in GluR-6 RNA has been revealed by genetically modified mice, in which Q/R site editing in GluR-6 transcripts is abrogated by deletion of an intron sequence that is crucial for editing (Vissel et al., 2001). The mutant mice exhibited NMDA receptor-independent long-term potentiation (LTP) at the medial perforant path-dentate gyrus synapse and both heterozygous and homozygous mutant mice were more vulnerable to kainate-induced seizures (Vissel et al., 2001). These findings indicate a clear role for GluR-6 Q/R site editing in synaptic plasticity and circuit excitability. Increased Q/R site editing of GluR-5 and GluR-6 subunits was observed in brain tissue excised from patients with temporal lobe epilepsy, and was suggested to result from an adaptive reaction to ongoing seizure activity (Kortenbruck et al., 2001); however, the mechanism underlining this proposed regulation of A-to-I editing for kainate receptors is unknown.

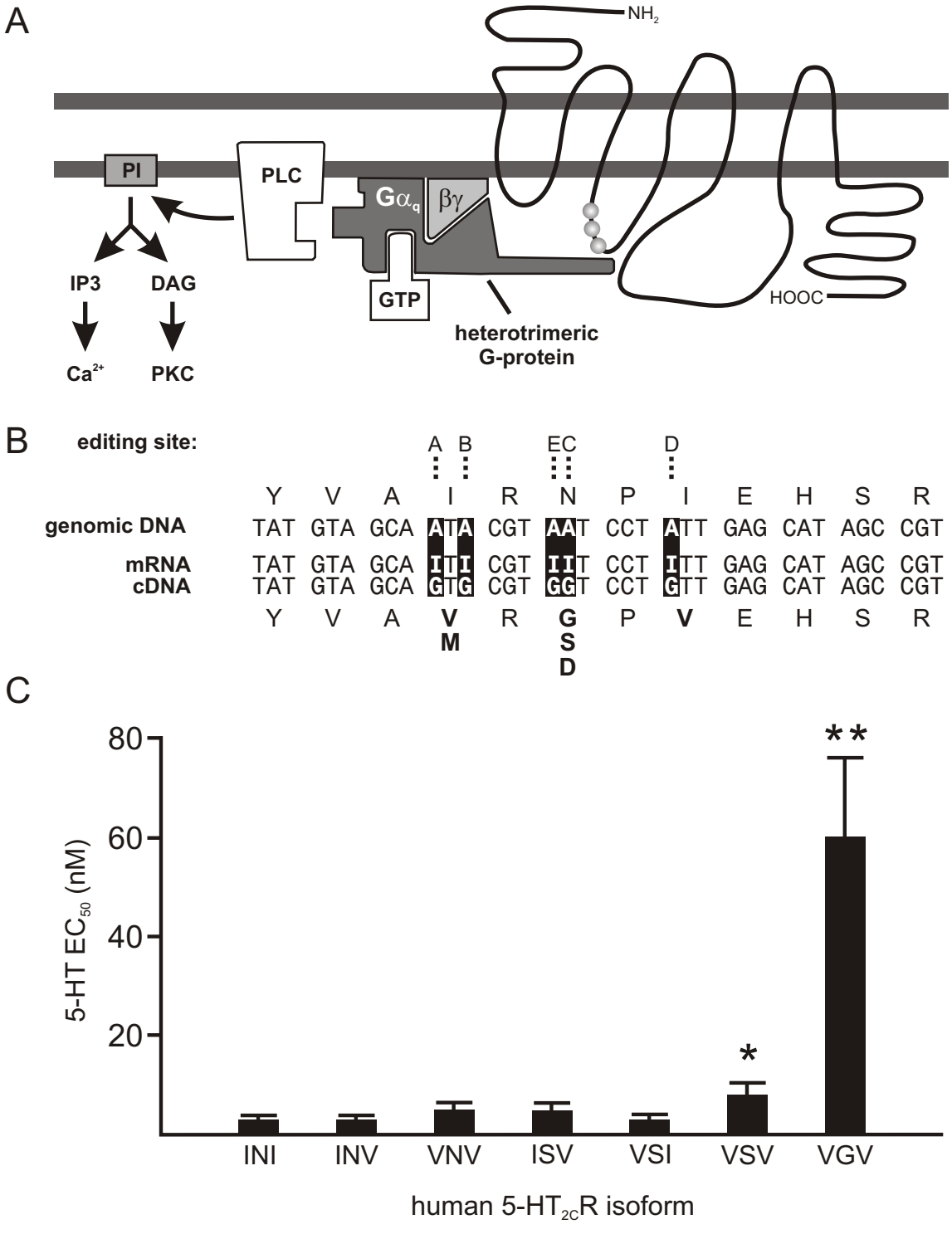
RNA editing on 5-HT$_{2C}$ receptor transcripts

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that modulates numerous sensory and motor processes as well as a wide variety of behavior including locomotion, thermoregulation, pain perception, sleep, appetite, and sexual behavior (Sanders-Bush et al., 2003). Serotonin activates at least 14 distinct receptor subtypes that differ in their tissue localization, binding affinity for 5-HT and coupling to intracellular signaling pathways (Hoyer et al., 1994). The 5-HT2 family includes three members, 2A, 2B and 2C receptors. The typical

signaling pathway for this family of serotonin receptor is Gq-coupled activation of phospholipase C (PLC), therefore increasing intracellular concentrations of inositol phosphates (IP) and diacylglycerol (DAG). In addition, both phospholipase D (PLD) and phospholipase A2 (PLA2) can also be activated by 5-HT_{2A} and 5-HT_{2C} receptors of 5-HT2 family, by interacting with additional G proteins (Sanders-Bush et al., 2003).

To date, 5-HT_{2C} receptor is the only known GPCR of whom the transcript undergoes RNA editing modification. In this post-transcriptional process, five genomically encoded adenosines can be converted into inosines in pre-mRNA, changing up to three amino acids codons in the second intracellular loop of the receptor, to generate 23 more protein isoforms for 5-HT_{2C} receptor (Figure 7). The functional consequences of RNA editing in the 5-HT_{2C}R transcripts was revealed in heterologous tissue culture system where different 5-HT_{2C} receptor isoforms were expressed and assayed for their ability to activate PLC in response to 5-HT. These results demonstrated that the fully edited isoforms (VGV) exhibit the lowest potency for agonist stimulation of PLC activation and have reduced constitutive activity compared to the non-edited (INI) isoform due to less efficiency in G-protein coupling (Niswender et.al., 1999; Price et. al., 2001; Wang et. al., 2000b) Different brain regions express unique complement of nonediting/edited 5-HT_{2C} receptor transcripts (table 2), while the non-edited INI isoform is dominant in chorus plexus in both human and rodents, the most prevailing isoform in the cortex, hypothalamus, hippocampus, olfactory bulb and striatum are VSV (human) and VNV (rat, mouse), suggesting that editing may

Figure 7 RNA editing of 5-HT2C R transcripts.
A. A schematic diagram of the predicted topology structure of 5-HT_{2C} receptor and signaling pathway involved in activation of PLC. The approximate positions of amino acid alterations within the second intracellular loop of the receptor, resulting from RNA editing, are indicated.
B. The nucleotide sequence alignment between 5-HT_{2C}R genomic, mRNA and cDNA sequences; the nucleotide discrepancies (inverse letters) and the predicted amino acid alterations (bold letter) and five editing sites are indicated.
C. 5-HT potency to activate phosphoinositide hydrolysis in NIH3T3 fibroblasts transiently transfected with different human 5-HT_{2C} receptor editing isoforms, (*, $p<0.05$; **, $p<0.01$). (Adapted from Niswender et. al. 2001)


A
NH2
PI
PLC
Gαq
βγ
IP3
DAG
GTP
HOOC
heterotrimeric
G-protein
Ca2+
PKC
B
editing site:
A B
EC
D
Y V A I R N P I E H S R
genomic DNA
TAT GTA GCA ATA CGT AAT CCT ATT GAG CAT AGC CGT
mRNA
cDNA
TAT GTA GCA ITI CGT IIT CCT ITT GAG CAT AGC CGT
TAT GTA GCA GTG CGT GGT CCT GTT GAG CAT AGC CGT
Y V A V R G P V E H S R
M
S
D
C
5-HT EC50 (nM)
80
60
40
20
**
*
INI
INV
VNV
ISV
VSI
VSV
VGV
human 5-HT2CR isoform

Amino Acid Isoform	RNA Isoforms	WB	CP	C	Hi	Hy	OB	Str
INI		8%	36%	8%	13%	7%	15%	16%
MNI	B	0%	2%	0%	0%	0%	0%	0%
ISI	C	0%	2%	0%	0%	1%	0%	0%
INV	D	10%	40%	19%	12%	9%	4%	6%
IDI	E	0%	0%	0%	2%	0%	0%	0%
ISV	CD	2%	10%	5%	2%	0%	0%	0%
IDV	ED	0%	3%	0%	0%	0%	0%	0%
VNI	AB A	8%	0%	16%	15%	10%	12%	10%
VNV	ABD AD	38%	3%	38%	38%	46%	44%	32%
VDI	ABE BE	0%	0%	0%	0%	3%	0%	0%
VSI	ABC AC	6%	0%	3%	2%	6%	6%	10%
VGI	ABCE ACE	0%	0%	0%	0%	0%	0%	2%
VDV	ABDE ADE	2%	3%	0%	2%	3%	2%	2%
VSV	ABCD ABD	27%	0%	11%	13%	15%	15%	18%
VGV	ABCDE ACDE	0%	0%	0%	0%	3%	2%	4%

Table 2 5-HT2C receptor editing isoform distribution in different brain regions of adult mice

RNA from whole mouse brain (WB), choroid plexus (CP), cortex (C), hippocampus (Hi), hypothalamus (Hy), olfactory bulb (OB) and striatum (Str) of 129Sv/Ev/Tac adult male mice were amplified by RT-PCR, subcloned into pGEM-T vector and individual cDNA isolates (>50 from each brain region) were analyzed by Pyrosequencing™ analysis. The percentage of cDNA isolates encoding each amino acid isoform for each brain region is presented.

represent a fine-tuning mechanism to modulate 5-HT_{2C} receptor function in various areas in CNS.

RNA editing of K_v1.1 transcripts

Voltage-gated potassium channels (K_v) are integral membrane proteins that enable the passage of potassium ions across cell membranes. These channels open and close in response to changes in transmembrane voltage and play a critical role in limiting neuronal excitability (Sands et al., 2005). Mice lacking the Shaker-like voltage-gated potassium channel K_v1.1 alpha-subunit developed recurrent spontaneous seizures early in postnatal development (Rho et al., 1999). Mutations in the KCNA1 locus (the human homologue of K_v1.1) also have been identified in patients affected by episodic ataxia and myokymia (Lerche et al., 2001). The voltage-gated potassium channels are comprised of four subunits that encircle a central ion conduction pathway. Each subunit consists of six alpha helices (S1- S6) (Figure 8). The first four transmembrane helices (S1-S4) form the voltage sensing domain, whereas the last two transmembrane helices (S5-S6) along with an intervening reentrant P loop, form the pore domain (Yellen, 2002). The very rapid signaling in neurons requires a fast mechanism for closing and opening the pore. The inactivation mechanism for K_v1 channel involves the N-terminus of an associated beta-subunit blocking the channel (Rettig et al., 1994).

Using comparative genomic analysis, Hoopengardner et. al. first identified multiple editing sites in three Drosophila potassium channel genes, shaker, ether-a-go-go and slowpoke. While applying the same screening strategy to mammalian shaker genes, orthologs of the Kv1.1 gene in mouse, rat and human

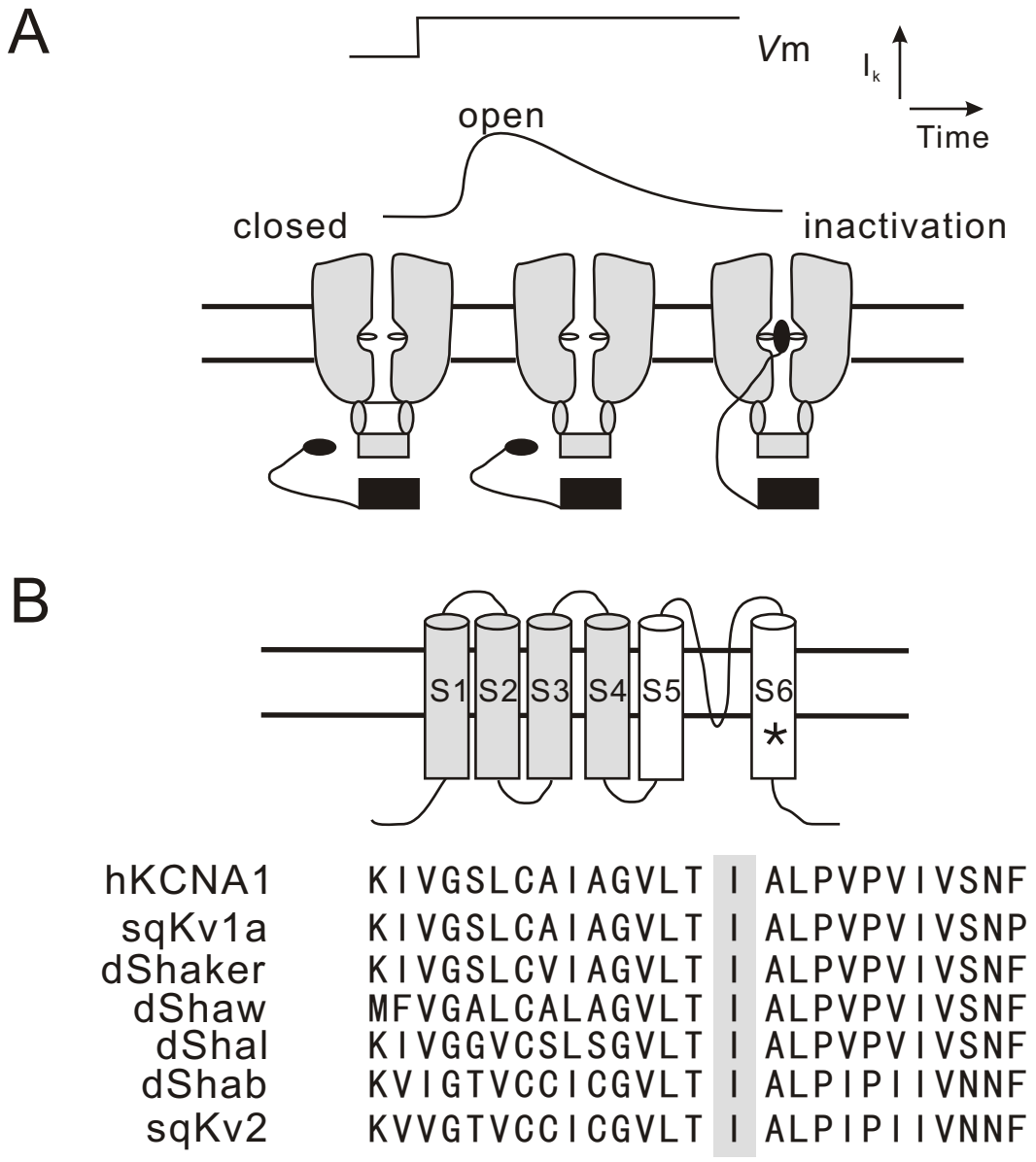


Figure 8 RNA editing of K_v1.1 transcripts

A. A schematic representation of the three states of K_v1.1 subunits (gray) associated with the β subunit (black). A K+ current (I_K) can be elicited in response to membrane depolarization (V_m) and then decreased by the inactivating particle (black oval) interacting with the isoleucine in the pore (open ovals). Substitution of the isoleucine to a valine moiety, as a result of A-to-I editing modification, is predicted to weaken this interaction. (Adapted from Bezanilla,2004) B.The predicted topology structure of K_v1.1 subunit within the membrane with membrane-spanning domains (S1−S6) and the position of the edited I/V site (*) represented on the top. The amino acid sequence alignment of the S6 domains of K_V channels from multiple species are demonstrated at the bottom with the RNA editing targeted isoleucine shaded. (Adapted from Hoopengardner et. a., 2003)

demonstrated a region of high sequence conservation and a single A-to-I conversion was found within this conserved region (Hoopengardner et al., 2003). This RNA modification occurs at an isoleucine (I) codon, conserved through fly to human, in the S6 fragment of the K_v1.1 protein. Once the isoleucine codon is converted into valine (V) codon by RNA editing, the transcripts encode channels with a 20 time faster rate of recovery from inactivation (Bhalla et al., 2004; Hoopengardner et al., 2003), suggesting a potential regulatory role of RNA editing in potassium channel kinetics.

RNA editing in UTRs

Although systematic screenings for novel ADAR substrates in human transcriptsome increased the number of editing substrates by at least an order of magnitude, only a few editing sites were identified in the coding region (Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004); the majority of the A-to-I conversions are predicted in non-translated RNA species and UTRs of RNA transcripts, especially inverted Alu repeats. Alu elements are short interspersed elements (SINEs), comprising >10% of the human genome. They are typically repeats can form extended RNA duplexes, the only known requirement for ADAR-mediated deamination, it is not surprising to find that the RNA sequences containing Alu repeats undergo extensive A-to-I modifications. It is not clear which ADAR is responsible to the Alu editing (Eisenberg et al., 2005).

Using the same strategy to identify new ADAR substrates in mouse, chicken and flies, Eisenberg *et. al.* found that the frequencies of A-to-I editing in these species are at least an order of magnitude lower than in the human, even though

the total numbers of SINEs in human and rodent genomes are similar (Eisenberg et al., 2005). This discrepancy can be explained by the fact that while rodent genome contains various SINEs, only one SINE, Alu element, dominates human genome, so it is more probable to form dsRNA from two consecutive and oppositely orientated SINEs in the human transcriptsome.

It has been speculated that UTR editing may affect RNA splicing, trafficking, translation efficiency and transcript stability. Previous studies from polyoma virus showed that early-strand transcripts isolated late in infection underwent intensive A-to-I RNA editing and accumulated within the nucleus of the infected cells, suggesting that extensive A-to-I modifications may induce nuclear retention of target transcripts (Kumar and Carmichael, 1997). Recently, an 8 kb, non-protein producing transcript transcribed from the mouse cationic amino acid transporter2 gene (CAT2), CTN-RNA has been identified to localize in paraspeckles and to be in complex with $p54^{nrb}$, an inosine-containing RNA binding protein. The 3'UTR of CTN-RNA contains sequence elements that can form an imperfect RNA for ADAR mediated adenosine deamination and undergoes intensive A-to-I conversions. A-to-I editing in the 3'UTR of CTN-RNA and its interaction with $p54^{nrb}$ suggest that A-to-I conversion in the untranslated regions may serve as a mechanism to regulate RNA translocation, therefore affecting the translational efficiency or stability of the target transcripts.

A-to-I editing in other species

A remarkable number of transcripts in invertebrates are also targeted by ADARs. The majority of known invertebrate editing sites occur in the coding

region of transcripts encoding neurotransmitter receptors, channels and proteins involved in rapid electrical and chemical neurotransmission. To date, RNA editing in invertebrates includes A-to-I conversions in transcripts encoding nicotinic acetylcholine receptors (Grauso et al., 2002), GABA receptors (Hoopengardner et al., 2003), calcium channel subunits (Palladino et al., 2000b; Smith et al., 1998), sodium channels (Hanrahan et al., 1999; Hanrahan et al., 2000; Song et al., 2004), delayed rectifying potassium channels (Bhalla et al., 2004; Patton et al., 1997; Rosenthal and Bezanilla, 2002), glutamate-gated chloride channels (Semenov and Pak, 1999) and proteins involved in synaptic release (Hoopengardner et al., 2003) (Table 1).

Preliminary analysis of the RNA editing in squid potassium channel, $SqK_v1.1$, revealed that editing-mediated amino acid alterations affect both channel gating properties and subunit tetramerization (Rosenthal and Bezanilla, 2002); in addition, two editing events in SqKv2 modulated the rates of channel closure and slowed inactivation (Patton et al., 1997). A single editing event in the German cockroach Na channel transcript, BgNav1-2, has also been found to be critical to the voltage dependence of channel activation (Song et al., 2004). However, in most cases, the functional consequences of these editing events have not yet been elucidated. Further investigations of the biological roles of the gene products encoded by these transcripts and the consequences of editing will help to provide more insight into the physiological significance of A-to-I RNA editing in the invertebrate species.

Physiological significance of ADAR activity

dADAR mutant *Drosophila*

dADAR-null flies have been generated using transposon-mediated P-element excision (Palladino et al., 2000b). Under ideal growth conditions, the dADAR deficient flies developed into morphologically normal adults, with only a structural defect in the retinas of young animals. However, adult mutants demonstrated uncoordination and paralysis with severe motion defects in jumping, fighting, tremors and mating (Palladino et al., 2000b). In addition, vacuolated lesions appeared in the brain by day 30 in mutant flies. Most of the behavioral defect and the lesions in the brain of the dADAR null files became more severe during aging (Chen et al., 2004). The phenotypes associated with loss of dADAR activity are consistent with perturbations of electrical and chemical signaling mechanisms in the nervous system and proximal effects on behavior (Palladino et al., 2000b). Mutations generated in some of the ion channel which are targets of dADAR developed similar behavioral phenotypes to the dADAR deficient flies, suggesting that it is those targets responsible for the defect observed in dADAR mutant flies. With most of the newly identified A-to-I conversions occurring in the coding regions of genes involved in rapid neurotransmission (Hoopengardner et al., 2003), it is suggested that a primary role of dADAR is to modulate neurotransmission in the CNS. Recently, it was found that dADAR deficient flies had prolonged recovery from anoxia stupor and altered gene expression for reactive oxygen species (ROS) scavengers, suggesting that dADAR may also be involved in regulating the expression of genes encoding ROS scavengers (Chen

et al., 2004; Ma et al., 2001). The ability of dADAR to edit its own mRNA has been identified at a serine codon in the conserved region of the deaminase domain; dADAR isoforms with the serine to glycine substitution close to the ADAR active site are less active *in vitro* and *in vivo* than the genomically encoded, nonedited isoform (Keegan et al., 2005). Ubiquitous expression of an non-edited dADAR transcript in embryos and larvae is lethal, suggesting dADAR autoediting is essential for normal development in *Drosophila* (Keegan et al., 2005).

ADR mutant *C. elegans*

While *C. elegans* lacking ADR-1 had severely reduced A-to-I editing activity, ablation of ADR-2 expression eliminated the editing activity entirely, suggesting that ADR-2 can function by its own but ADR-1 may require ADR-2 for activity and perhaps the two enzymes act together as a heterodimer (Tonkin et al., 2002). Although *C. elegans* strains containing homozygous deletions in either or both ADAR genes are viable, they both demonstrated chemotaxis defects (Tonkin et al., 2002). More intriguing, the ADR mutant worms can be rescued by loss-function mutations in genes involved in RNA interference (RNAi) pathways, suggesting that the phenotype of ADR deletion strains may be caused by aberrant RNAi activity and ADARs may play a role in regulating the RNAi pathway (Tonkin and Bass, 2003).

ADAR mutant mice

In contrast to ADARs in worms and flies, ADAR1 and ADAR2 expressions are absolutely essential in mammals. ADAR1 and ADAR2 knockout mice generated

by gene-targeted homologous recombination both die during development. As early as embryonic day 11.5 (E11.5), mouse embryos lacking ADAR1 expression demonstrated rapid disintegration of the liver structure, severe defects in embryonic erythropoiesis and stress-induced apoptosis (Hartner et al., 2004; Wang et al., 2000; Wang et al., 2004), suggesting that ADAR1 serves critical steps in embryonic development, especially in developing non-nervous tissue. Intercrosses between heterozygote ADAR2 null mice produced homozygote mice at the expected Mendelian frequency, indicating that ADAR2 deficiency does not result in embryonic development (Higuchi et al., 2000). However, homozygote ADAR2 null mice developed progressive seizure after birth and died between postnatal day 0 (P0) and 21 (P21) (Higuchi et al., 2000). Remarkably, the phenotype of ADAR2 null animals can be rescued by replacing both wild-type GluR-2 alleles with an arginine-containing alleles at the Q/R site, despite the fact that editing of many other sites, including the R/G site of GluR-2, the Q/R site of GluR-5 and the D site of 5-HT_{2C}R, were all significantly reduced in ADAR2 null animals (Higuchi et al., 2000). These results demonstrated that the lethality in ADAR2 null mice primarily results from the loss of Q/R site editing in GluR-2 transcripts.

Recent studies from our laboratory demonstrated that transgenic mice expressing rat ADAR2 protein under the control of Cytomegalovirus (CMV) promoter developed adult-onset obesity characterized by hyperglycemia, hyperleptinemia and increased adiposity. This phenotype predominantly resulted from hyperphagia rather than a metabolic derangement, because during a

paired-feeding analysis, mutant mice on caloric restriction had similar growth rate and body composition to wild-type littermates. Although the molecular mechanism underlining the obesity phenotype has not been identified, the editing activity of ADAR2 is not required for the hyperphagia since an enzymatically inactive rat ADAR2 transgene expression gave rise to similar outcome. Expression of inactive rADAR2 also had decreased editing at ADAR1 substrates suggesting a molecular mechanism by which the double-stranded RNA binding activity of ADAR2 could interfere with the actions of other double-stranded RNA binding proteins therefore producing phenotypic alterations in the animals (Dr. Minati Singh, unpublished data).

Regulation of ADAR activity

Unlike the static changes imparted by genetic polymorphisms, RNA editing modifications are subject to spatial and temporal regulation in response to cellular cues (Gurevich et al., 2002a; Yang et al., 2004), offering an organism great potential for complex patterns of gene expression. When functioning improperly, this essential process can give rise to pathological conditions ranging from epilepsy to embryonic lethality. Therefore, it is critical for the organisms to precisely regulate ADAR activity.

ADAR Dimerization

As previously identified for CDA, ADARs have been shown to form dimers on dsRNA (Cho et al., 2003; Gallo et al., 2003). Cho *et. al.* investigated the

formation of complexes between differentially epitope-tagged ADAR monomers by sequential affinity chromatography and size exclusion column chromatography, and demonstrated that while both ADAR1 and ADAR2 form a stable enzymatically active homodimer complex, ADAR3 remains as a monomeric, enzymatically inactive form. No heterodimer complex formation among different ADAR gene family members was detected. Interestingly, endogenous ADAR3 in brain extracts was found to form a homodimer complex, indicating the presence of a brain-specific mechanism for ADAR3 dimerization (Cho et al., 2003). In addition, dADARs form homodimers on dsRNA, and the minimum region required for dimerization is the N-terminus and the first dsRBD (dsRBD1). Single point mutations within dsRBD1 abolish RNA-binding activity and dimer formation. In addition, mutant dADAR protein with deletion of the N-terminus, although retains the ability to bind dsRNA, is monomeric and inactive, indicating that dimerization is essential for editing but not dsRNA-binding activity (Gallo et al., 2003).

Transcriptional regulation

Although in situ hybridization studies showed that ADAR2 mRNA expression is developmentally regulated (Paupard et al., 2000), studies on the transcriptional regulation of ADAR2 gene have not been reported. However, two alternative exon 1 structures in human ADAR1 gene have been identified using 5' rapid amplification of cDNA ends (RACE) assay (Figure 9). While the exon 1B-exon 2 transcripts encoding an N-terminally truncated 110-kD protein are synthesized constitutively, exon1A-exon2 transcripts encoding a 150-kD protein are interferon

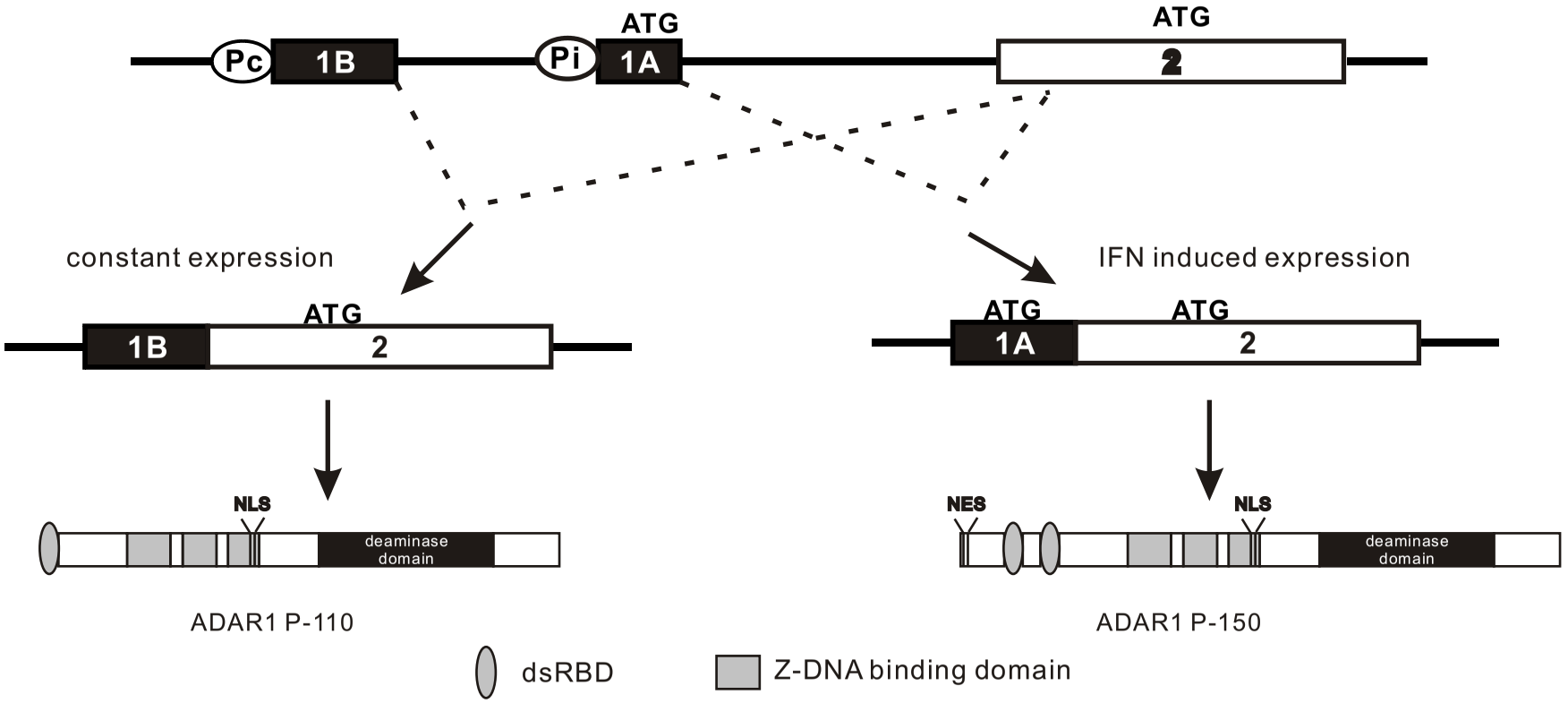


Figure 9 Transcriptional regulation of ADAR1 gene.
A schematic diagram of ADAR1 biosynthesis pathways are presented with the 5' region of human ADAR1 gene at the top. The two alternative promoters Pi and Pc and the alternative first exons, exon 1A and 1B are demonstrated. Pc driven exon 1B containing transcripts are constantly expressed, leading to the production of a smaller protein, ADAR1 p110; while upon IFN stimulation, the exon 1A containing transcripts are synthesized to produce a larger protein, ADAR1 p150. The functional domains of ADAR1 proteins are indicated and gray ovals representing Z-DNA binding domains and gray boxes for dsRBDs.

(IFN) inducible (George and Samuel, 1999). The constitutive P110 ADAR1 protein has one Z-DNA binding domain, three dsRBDs and a deaminase domain, whereas the inducible P150 ADAR1 protein has an extra Z-DNA binding domain and a nuclear export signal (NES) at the N-terminal of the protein (George and Samuel, 1999). To date, similar alternative transcription initiation has also been identified in mouse ADAR1 gene (George et al., 2005). Using transient transfection analysis with reporter constructs, two functional promoters was characterized as Pc, for constitutional expression of exon1B-exon2 transcripts, and Pi, for inducible expression of exon 1A-exon2 transcripts (George and Samuel, 1999). The IFN responsive Pi promoter of the ADAR1 gene possesses an IFN-stimulated response element (ISRE) responsible for IFN-inducibility, as well as an adjacent upstream sequence, designated kinase conserved sequence (KCS), which has been identified as an important IFN responsive element in the PKR gene promoter (Markle et al., 2003).

The IFN inducibility of ADAR1 expression is thought to represent a defensive mechanism protecting cells from viral infection. One ADAR1 target is the antigenome of Hepatitis delta virus (HDV), an RNA intermediate formed during the replication of HDV RNA genome (Polson et al., 1996). ADAR1-mediated RNA editing at the amber/W site of HDV antigenomic RNA results in the conversion of an in-frame stop codon into a tryptophan (W) codon, leading to the production of a longer form (HDAg-L), which is required for viral packaging and can also serve as a potent trans-dominant inhibitor of HDV RNA replication. Increased expression of ADAR1 strongly correlates with the inhibition of HDV RNA

replication, suggesting overexpression of ADAR1 may compromise the virus viability (Jayan and Casey, 2002). The increased expression of p150 ADAR1 also has been found in response to acute inflammation, microbial infections, and IFN treatment, resulting in an elevation of the overall inosine content in the inflammatory organs as well as increased editing at specific ADAR1 sties (Jayan and Casey, 2002; Yang et al., 2003a; Yang et al., 2003b; Yang et al., 2004). However, the exact role of ADAR1 during these inflammatory processes is under further investigation.

Subnuclear localization

Since most known RNA duplex structures targeted by ADAR include both exon and intron sequences, it is reasoned that ADARs must function before RNA splicing and translocation from the nucleus to the cytoplasm. Despite the postulated role of ADARs in the nucleoplasm near sites of transcription, several groups have demonstrated that ADAR1 and ADAR2 accumulate in the nucleolus, a subnuclear structure where ribosomal RNA (rRNA) synthesis, processing and ribosomal subunit assembly take place (Desterro et al., 2003; Sansam et al., 2003) There are multiple nucleoli in a cell, and each nucleolus is formed around many actively transcribed rDNAs within chromosomal loci called nucleolar organizing regions (NORs). Using a GFP-ADAR2 fusion protein, Sansam *et. al.* demonstrated that ADAR2 shuttles between the nucleoplasm and the nucleolus in the living cells (Sansam et al., 2003). When cells express the GluR-2 RNA duplex, endogenous ADAR1 and ADAR2 de-localized from the nucleolus and accumulated in the nucleoplasm where the substrate transcripts accumulate

(Desterro et al., 2003), suggesting a binding competition between the GluR2 duplex in the nucleoplasm and the extensive intermolecular base-pairing formed in rRNA. When NIH3T3 cells were treated with ribonuclease A (RNase A), a rapid loss of nucleolar localization of the GFP-ADAR2 was seen; and inhibition of rRNA synthesis caused translocation of ADAR2 from nucleolus to the nucleoplasm, suggesting that the ADAR2 nucleolar localization is RNA dependent. To test whether the nucleolar localization of ADAR2 affects editing activity, rRNA synthesis in rat C6 glioma cells and mouse NIH3T3 fibroblasts was inhibited to force the translocation of ADAR2 from nucleoli to the nucleoplasm. As measured by the extent of editing at endogenous ADAR2 substrates, a significant increase in ADAR2 activity was observed, indicating that the nucleolus serves as a storage depot for ADAR2, not a site of action, and alteration of ADAR subnuclear localization represents a mechanism for prompt regulation of ADAR activity in response to the abundance of ADAR substrates in the nucleoplasm.

Sumoylation

Sumoylation is a highly dynamic posttranslational modification that involves the covalent attachment of the small ubiqutin-like modifier, SUMO, to target proteins in eukaryotes. It can affect the target protein functions by altering its subcellular localization, activity or stability. The modification process involves formation of an isopeptide bond between the C-terminus of SUMO and the ε-amino group of a lysine residue of the target protein; while the removal of SUMO from proteins is carried out by specific cysteine proteases that have both hydrolase and

isopeptidase activity. In vertebrates, three SUMO proteins are identified as SUMO-1, -2 and -3; and sumoylation has been found in proteins such as RanGAP1, PCNA, IkBa, p53, c-jun, topoisomerases, promyelocytic leukemia protein, Sp100 and the mitogen-activated protein kinase kinase 1, most of which are nuclear proteins or proteins shuttling to the nucleus (Hay, 2005). In addition, since most enzymes involved in SUMO pathway are also localized in the nucleus, it is believed that sumoylation is predominantly a nuclear process (Seeler and Dejean, 2003; Zhang et al., 2002).

In recent studies (Pinto Desterro et al., 2005), subnucleolar localization of SUMO-1 was found to overlap with ADAR1 in a region that is distinct from fibrillar center, the dense fibrillar component and the granular component, raising the possibility that ADAR1 is a target for SUMO-1. Further analysis demonstrated that ADAR1 can be modified by SUMO-1 at lysine residue 418. Substitution of this lysine residue with an arginine abolished sumoylation but did not affect the nucleolar localization of ADAR1. Moreover, modification of ADAR1 by SUMO-1 reduced RNA editing activity. Although ADAR1 and ADAR2 co-localize in the nucleolus, ADAR2 does not have any SUMO-1 consensus motif, therefore it is not surprising that ADAR2 is not modified by SUMO-1 *in vitro* (Pinto Desterro, unpublished data). In summary, results from Pinto Desterro *et. al.* demonstrated a novel posttranslational modification mechanism to regulate ADAR1 activity.

Alternative Splicing

Alternative splicing is an important mechanism for generating multiple protein isoforms possessing different biological activities from a single genomic locus.

Both ADAR1 and ADAR2 genes undergo alternative splicing to diversify the function of the encoded products. For ADAR1, three naturally occurring splice variant isoforms were identified for the IFN inducible mRNA isoform. While the full-length 1226-amino acid ADAR1 protein was designated ADAR1-a, the variant designated ADAR1-b contains a deletion of 26 amino acids between the third dsRBD and the catalytic deaminase domain due to 5'-splice site selection at the exon 7-intron 7 junction. The ADAR1-c variant has an additional deletion of 19 amino acids at the intron 5-exon 6 junction which lies between the second and third dsRBDs (Liu et al., 1997). All three alternative splice variants are expressed in human kidney, while only the ADAR-a and -b forms were detected in a human placenta library (Liu et al., 1997). Since these alternative splicing events retain the dsRBDs functionally intact, all three of the ADAR1 mRNA isoforms encode active enzymes that possess comparable deaminase activity measured with a synthetic dsRNA substrate. However, site-directed mutagenesis of the dsRBDs revealed that the presence of one or both of the deletions in the spacer regions between the dsRBDs and the catalytic domain of the ADAR-b and -c variants altered the functional importance of each of the individual dsRNA binding motif relative to that observed for ADAR-a. The 26-amino acid deletion of exon 7 between the third dsRBD and the deaminase domain in ADAR-b, as well as the additional 19-amino acid deletion between the last two dsRBDs in ADAR-c, appeared to gradually reduce the functional significance of the first dsRBD (Liu et al., 1997).

Similar to ADAR1, multiple splice variants of ADAR2 mRNA have been identified in ADAR2 mRNA in rats, mice and humans (Gerber et al., 1997; Rueter et al., 1999; Slavov and Gardiner, 2002). A subset of these RNA processing events are conserved in all three species (Figure 10). One such splicing event in the region of the transcripts encoding the deaminase domain results in the production of ADAR2 protein isoforms containing an additional 40 (human) or 10 (rat, mouse) amino acids between the second and third zinc coordination residues of the deaminase domain, to generate enzymes that are approximately twice as active as those lacking the insertion (Gerber et al., 1997; Rueter et al., 1999).

A second alternative splicing event near the 5'-end of the coding region in ADAR2 transcripts was identified to insert a 47-nucleotide cassette immediately upstream of exon 5, generating a -1 frame-shift that is predicted to produce a 9kD protein lacking the dsRBDs and the deaminase domain required for protein function, due to premature translation termination (Rueter et al., 1999; Slavov and Gardiner, 2002). This alternative splicing event is dependent upon the ability of ADAR2 to edit its own pre-mRNA (ADAR2 autoediting), converting an adenosine residue within intron 4 to inosine (-1 site), thereby creating a non-canonical AI dinucleotide that serves as a novel 3'- splice acceptor (Rueter et al., 1999). As with all identified ADAR substrates, editing at the -1 site of ADAR2 requires an imperfect RNA duplex formed by the base-pairing of sequences surrounding the -1 site with an inverted repeat referred to as the editing site complimentary sequence (ECS). In the rat, the ECS is located approximately

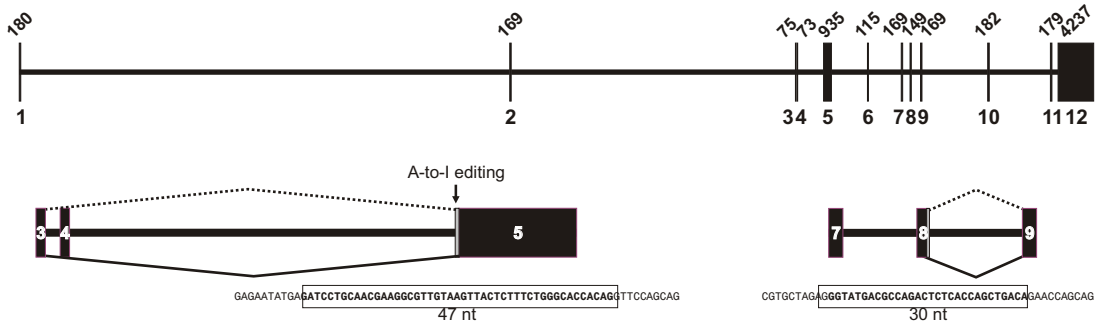


Figure 10 The conserved alternative splicing events in ADAR2 gene.

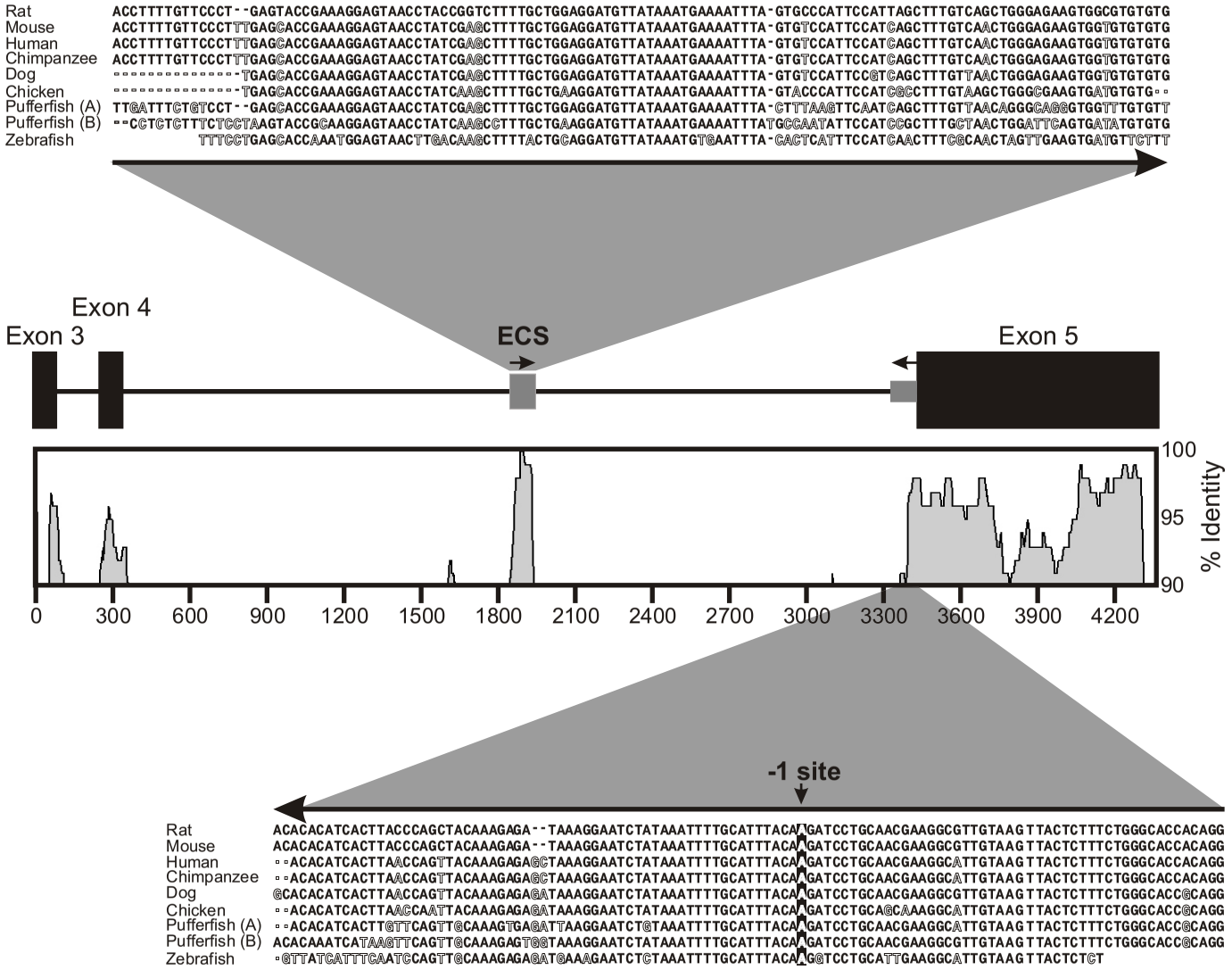
The genomic structure of mouse ADAR2 gene is presented with the sizes of the exons indicated at the top. The alternative splicing events between exon 3 and 5, and exon 8 and 9, leading to the introduction of 47 or 30 nucleotides (▭) in the open reading frame (boxed letters), respectively, are demonstrated at the bottom. The RNA editing site that creates a proximal splice acceptor site upstream of exon 5 is indicated.

1.4kb upstream of the -1 site in intron 4 (Rueter et al., 1999), and this region of the intron demonstrates a high level of sequence conservation with corresponding sequences from ADAR2 genes isolated from all characterized vertebrate species including pufferfish, zebrafish, chicken, mouse and human (Figure 11) (Rueter et al., 1999; Slavov and Gardiner, 2002). Deletion of the ECS region resulted in the ablation of editing at the -1 site in heterologous expression systems, while deletion of intronic sequences between the ECS and the region surrounding the -1 site had little effect on the extent of A-to-I conversion (Dawson et al., 2004; Rueter et al., 1999).

Specific aims

Since the ADAR2 autoediting and subsequent alternative splicing result in the generation of a premature translation termination in ADAR mRNA and are conserved among all vertebrate species, ranging from zebrafish, to human (Slavov and Gardiner, 2002), we hypothesized that ADAR2 autoediting represents a negative feedback mechanism to modulate the level of ADAR2 protein expression and activity. As ADAR2 protein expression increases, the enzyme can promote the splicing machinery to generate 47 nucleotides containing mRNA isoforms by producing more A-I dinucleotide at the proximal splice acceptor, therefore decrease the level of mRNA isoforms that encode full-length functional protein, to counteract the increase of ADAR2 protein (Figure 12). To test whether this molecular processing is essential for the ADAR2 expression and activity *in vivo*, genetically modified mice have been developed in which

Figure 11 Evolutionary conservation of regulatory elements required for ADAR2 autoediting.
A nucleotide sequence alignment and VISTA plot (Frazer et al., 2004) are presented for multiple vertebrate species highlighting regions of >90% identity for a portion of the ADAR2 gene extending from exon 3 through exon 5. Nucleotide sequences differing from the rat are indicated with open letters, the position of the edited adenosine moiety at the 3'-proximal splice acceptor (-1 site) is shown with inverted lettering and sequence gaps are indicated with dashes. The relative orientation of inverted repeats in the editing site complementary sequence (ECS) and surrounding the -1 site is indicated with arrows; coordinates are relative to the beginning of exon 3.


Rat
Mouse
Human
Chimpanzee
Dog
Chicken
Pufferfish (A)
Pufferfish (B)
Zebrafish
Exon 3
Exon 4
ECS
Exon 5
100
95
90
% Identity
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900
1200
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1800
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3600
3900
4200
-1 site

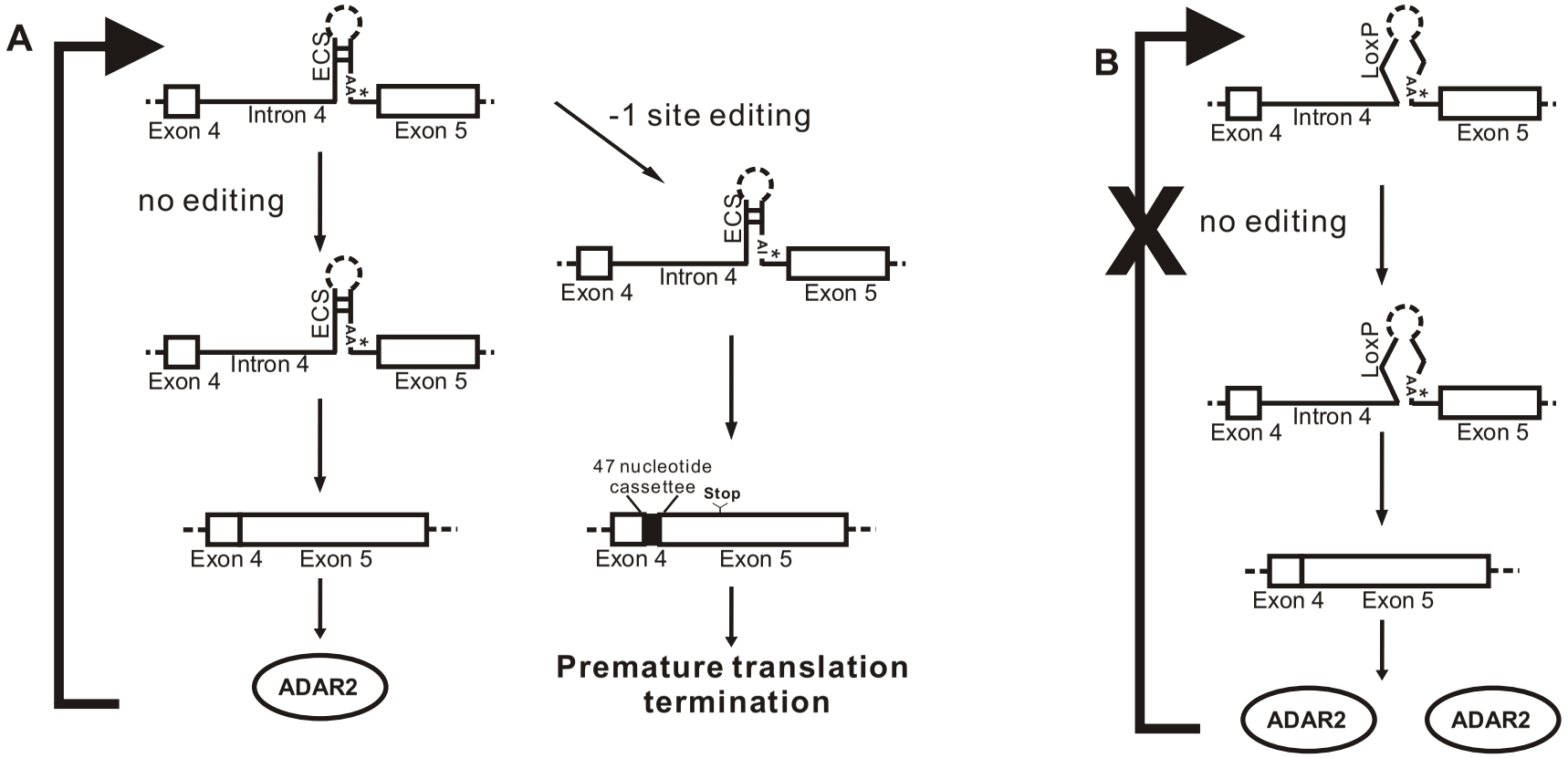


Figure 12 Regulation of ADAR2 expression by autoediting.
A. In wild-type mice, ADAR2 protein binds to an RNA duplex formed by an editing-site complementary sequence (ECS) and the region surrounding the proximal 3'-splice site (-1 site; *), converting a genomically encoded AA dinucleotide into a functional A-I splice junction. Subsequent splicing to this novel 3'-splice acceptor generates an ADAR2 mRNA containing a 47 nucleotide insertion, from which protein translation is pre-maturely terminated.
B. In ΔECS mice, the duplex structure required for A-to-I conversion is disrupted, ablating ADAR2 autoediting and use of the proximal 3'-splice site to increase ADAR2 protein levels.

ADAR2 autoediting was selectively ablated (ΔECS mice), giving rise to the following specific aims.

Specific Aim 1: to examine the consequence of ECS deletion on ADAR2 RNA processing.

Previous studies have demonstrated that the ECS element is essential for the -1 site editing in ADAR2 minigene constructs transfected in the heterologous tissue culture systems (Dawson et al., 2004; Rueter et al., 1999). In the current study, RNA from ΔECS mice will be assessed for the extent of editing at the -1 site as compared with wild-type littermates.

Since -1 site editing is a prerequisite for the 47 nucleotide inclusion in the ADAR2 mRNA, it is reasoned that ablation of ADAR2 autoediting will abrogate the generation of ADAR2 mRNA isoforms containing the 47 nucleotide cassette. The 47 nucleotide insertion will be characterized in RNA isolated from multiple tissues of ΔECS and wild-type mice.

Specific Aim 2: to examine the effect of ablation of ADAR2 autoediting on ADAR2 function.

Once the ADAR2 autoediting and consequent alternative splicing are ablated, we hypothesized that the proposed negative feedback loop by which ADAR2 control its own protein expression will be disrupted in the homozygous ΔECS mice, and therefore ADAR2 protein expression as well as ADAR2 enzymatic

activity will be altered. Total proteins from ΔECS mice will be quantified for ADAR2 expression and compared with the expression in wild-type counterparts. For analysis of ADAR2 activity, extent of editing at ADAR2 substrates will be quantified.

Specific Aim 3: to characterized the phenotypic alteration in mice lacking ADAR2 autoediting.

ADAR2 autoediting is conserved in all vertebrate species; it is an essential regulation for the normal function of the animals. In addition, many ADAR2 substrates are transcripts encoding proteins with critical CNS functions. It is then reasoned that deregulation of ADAR2 activity will result in abnormality in animal physiology and behavior. Wild-type and ΔECS homozygous mice will be subjected to physiological and neurological examinations to provide deeper insight on the physiological significance of ADAR2 activity.

CHAPTER II

REGULATION OF ADAR2 EXPRESSION BY AUTOEDIITNG

Introduction

The conversion of adenosine to inosine by RNA editing is a widespread posttranscriptional modification resulting in the hydrolytic deamination of selective adenosine residues to alter the sequence of RNA transcripts from that encoded by genomic DNA. The majority of well characterized A-to-I editing events involve non-synonymous codon changes in mRNA sequences, resulting in the production of proteins with altered functional properties. In mammals, the most prominent examples of A-to-I editing have been described for transcripts encoding ionotropic glutamate receptor subunits (GluR), a voltage-gated potassium channel subunit (K_v 1.1) and the 2C-subtype of the serotonin receptor (5-HT_{2C}R), which lead to the production of channels with altered electrophysiological and ion permeation properties (Bhalla et al., 2004; Hoopengardner et al., 2003; Kohler et al., 1993; Krampfl et al., 2002; Lomeli et al., 1994; Seeburg and Hartner, 2003) and receptors with decreased G-protein coupling efficiency (Berg et al., 2001; Burns et al., 1997; Niswender et al., 1999). A-to-I modifications have also been described in non-translated RNA species and non-coding regions of RNA transcripts, suggesting that such RNA modifications may also affect other aspects of RNA function including splicing, trafficking,

translation efficiency and transcript stability (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004; Morse et al., 2002).

A-to-I RNA editing in mammals is mediated by a family of enzymes known as ADARs, including ADAR1, ADAR2 and ADAR3 (Bass et al., 1997). While ADAR3 is exclusively expressed in the brain with no detectable deaminase activity (Chen et al., 2000), ADAR1 and ADAR2 are ubiquitously expressed and mediate A-to-I conversions in synthetic dsRNAs as well as naturally occurring substrates (Higuchi et al., 2000; Lehmann and Bass, 2000; Melcher et al., 1996a; Melcher et al., 1996b; Wang et al., 2000). ADAR2, a 80 kD nuclear protein with two dsRBDs in the N-terminal and a deaminase domain in the C-terminal is responsible for the majority of the known codon-altering A-to-I modifications (Hartner et al., 2004; Higuchi et al., 2000; Wang et al., 2004). Genetically modified mice lacking ADAR2 expression develop progressive seizures and die by day 21 due to lack of editing (Q/R site) in transcripts encoding GluR-2 subunit (Higuchi et al., 2000), demonstrating the critical role that ADAR2 plays in the normal development and function of the CNS.

Multiple splice variants of ADAR2 have been identified in rats, mice and humans, yet only a subset of these RNA processing events are conserved in all three species (Gerber et al., 1997; Rueter et al., 1999; Slavov and Gardiner, 2002). One such splicing event results in the production of ADAR2 protein isoforms containing an additional 40 (human) or 10 (rat, mouse) amino acids between the second and third zinc coordination residues of the deaminase domain, generating enzymes that are approximately twice as active as those

lacking the insertion (Gerber et al., 1997; Rueter et al., 1999). Alternative 3'-splice site selection near the 5'-end of the coding region introduces additional 47 nucleotides, creating a -1 frame-shift that is predicted to produce a 9KD protein lacking the dsRBD and the deaminase domain required for protein function (Rueter et al., 1999; Slavov and Gardiner, 2002). Previous studies have demonstrated that this alternative splicing event is dependent upon the ability of ADAR2 to edit its own pre-mRNA, converting an adenosine residue within intron 4 to inosine (-1 site), thereby creating a non-canonical AI dinucleotide that serves as a novel 3'- splice acceptor (Rueter et al., 1999). As with all identified ADAR substrates, editing at the -1 site of ADAR2 requires an imperfect RNA duplex formed by the base-pairing of sequences surrounding the -1 site with an inverted repeat referred to as the editing site complimentary sequence (ECS). In the rat, the ECS is located approximately 1.4kb upstream of the -1 site in intron 4 (Rueter et al., 1999), and this region of the intron demonstrates a high level of sequence conservation with corresponding sequences from ADAR2 genes isolated from all characterized vertebrate species including pufferfish, zebrafish, chicken, mouse and human (Figure 11) (Rueter et al., 1999; Slavov and Gardiner, 2002). *In vitro* analysis showed that deletion of the ECS region resulted in the ablation of editing at the -1 site, while deletion of intronic sequences between the ECS and the region surrounding the -1 site had little effect on the extent of A-to-I conversion (Dawson et al., 2004; Rueter et al., 1999), demonstrating that ECS is required for the editing at the -1 site.

The ability of ADAR2 to edit and direct alternative splicing of its own pre-mRNA may represent an autoregulatory mechanism by which ADAR2 can prevent its own overexpression to avoid increased levels of editing for numerous ADAR2 substrates. To determine whether autoediting can serve to prevent ADAR2 overexpression, and to characterize the subsequent consequence of lacking ADAR2 autoediting, our laboratory generated genetically modified mice in which the ability of ADAR2 to edit its own transcript has been selectively ablated.

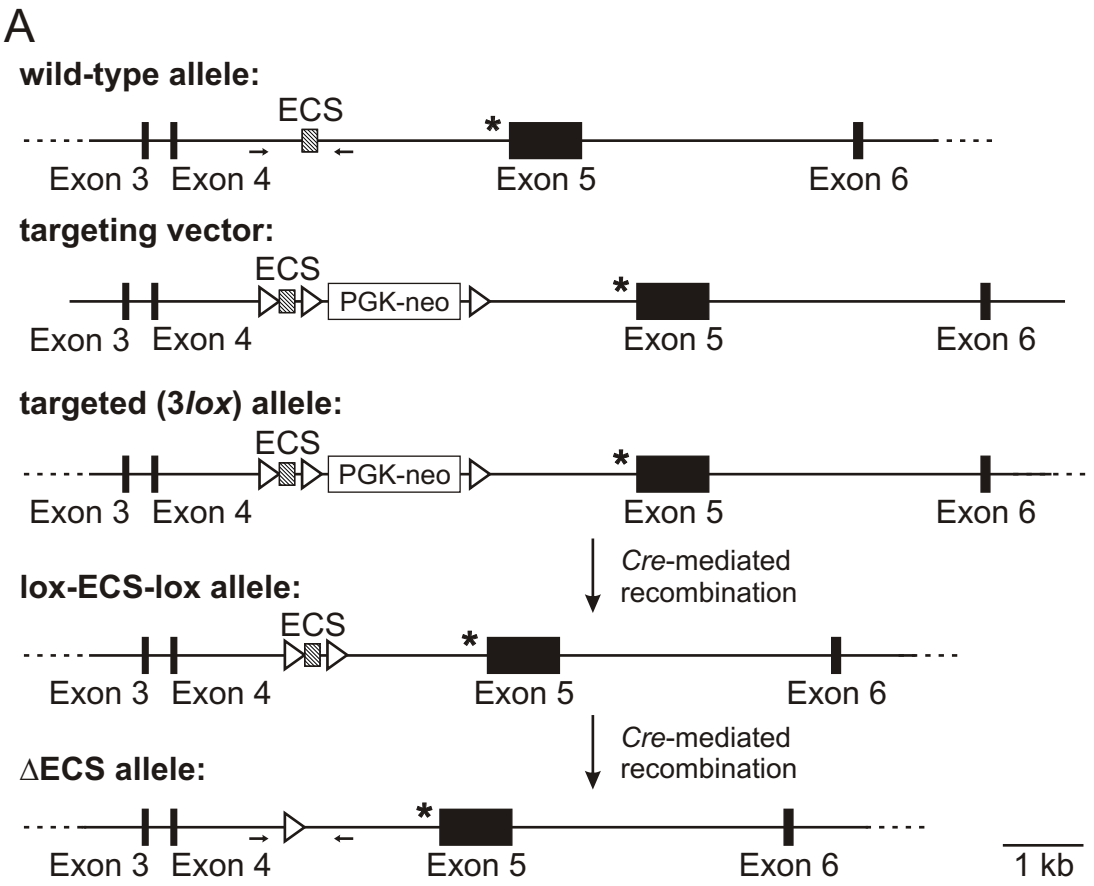
Materials and Methods

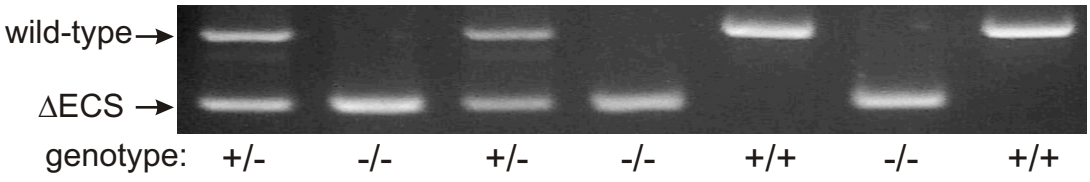
Generation of mutant mice

Detailed description of the targeting vector construction is presented in the doctoral thesis of Dr. Chris Sansam. Briefly, the targeting vector was generated from two 129/SvEvTac mouse genomic bacterial artificial chromosome (BAC) clones (Genome Systems, Inc.) containing the ADAR2 gene. A short arm fragment, extending from the 3'-end of intron 2 to a region upstream of the ECS element in intron 4 (-4053 to -1575- base pairs relative to the -1 editing site), was floxed by PCR with primers containing the loxP sequence and inserted between XhoI and BamHI sites, replacing the first LoxP site within sites in the LoxP-Neo-TK vector (a generous gift from Dr. Mark Magnuson, Vanderbilt University). A 7.4kb long arm, harboring the remainder of intron 4 downstream of ECS element through intron 6 (-1369 to +6061 relative to the -1 editing site), was inserted

between SalI and NotI sites in the LoxP-Neo-TK vector. A 195bp ECS containing intronic fragment (-1517 to -1322 bp relative to the -1 site) was inserted between the two adjacent LoxP sites using BamHI digestion. The thymidine kinase gene was excised from the targeting vector by EcoRI digestion to eliminate the previously described toxicity of thymidine kinase to the male germline (Braun et al., 1990; Ellison et al., 1995).

The ClaI-linearized targeting construct was electroporated into 129/SvEvTac derived embryonic stem (ES) cells. The integration of the targeting vector and the fidelity of homologous recombination were confirmed by Southern blotting analysis using 5'- and 3'- probes outside the region of homology and the correctly targeted ES cells were microinjected into C57BL/6 blastocysts. High percentage chimeras were mated with 129/SvEvTac animals to obtain heterozygous mice bearing the targeted allele (*3lox*; Fig 12A). The heterozygous *3lox* mice in 129/SvEvTac background were then mated with EIIA-Cre transgenic mice developed on a C57BL/6 background (a generous gift from Dr. Richard Breyer, Vanderbilt University) to delete the region between the loxP sites early in embryogenesis (Lakso et al., 1996; Xu et al., 2001). Progeny from this mating were analyzed by PCR and direct DNA sequence analysis for the loss of the entire region containing the selectable marker and a 209 bp region of ADAR2 gene containing the ECS (*ΔECS*, Figure 13). Mutant mice bearing the ΔECS alleles were mated with wild-type C57Bl/6J animals and subsequent progeny were assessed for the presence of the modified ADAR2 allele and segregation of the *Cre* transgene.


A
wild-type allele:
ECS
Exon 3
Exon 4
Exon 5
Exon 6
targeting vector:
ECS
PGK-neo
Exon 3
Exon 4
Exon 5
Exon 6
targeted (3lox) allele:
ECS
PGK-neo
Exon 3
Exon 4
Exon 5
Exon 6
Cre-mediated recombination
lox-ECS-lox allele:
ECS
Exon 3
Exon 4
Exon 5
Exon 6
Cre-mediated recombination
ΔECS allele:
Exon 3
Exon 4
Exon 5
Exon 6
1 kb


B
wild-type
ΔECS
genotype:
+/-
-/-
+/-
-/-
+/+
-/-
+/+

Genotype analysis

The wild-type and targeted (*3lox*; Figure 13A) alleles were distinguished using a polymerase chain reaction (PCR)-based strategy with sense (VU 649, 5'-TGTGAGCAGATGTAGCGTGTGAT-3') and antisense (VU 695, 5'-AATTTTCAT TTATAACATCCTC-3') primers, flanking the upstream loxP site to generate PCR amplicons of 140 and 174 bp for wild-type and targeted alleles, respectively. To distinguish between the wild-type and ΔECS alleles, PCR was performed using sense (VU 649, 5'-TGTGAGCAGATGTAGCGTGTGAT-3') and antisense (VU 650, 5'-GCGGGCAGGGTTAATGTGATGA-3') primers flanking the ECS region to generate a 343 and a 172 bp amplicons for the wild-type and the ΔECS alleles, respectively.

Tissue collection and RNA isolation

All animals were provided *ad lib* access to food and maintained on a 12hr light/dark cycle. Male ΔECS mice and wild-type litter mates were sacrificed at the age of 4 weeks according to the procedures approved by the institute of animal care and us committee (IACUC) at Vanderbilt University. Organs were quickly removed, immediately frozen in liquid nitrogen, and later stored at -80^{o}C. Total RNA was isolated from 50-100mg tissue using the Versagene RNA tissue kit (Gentra Bystems). All procedures were conducted according to the manufacturer's instructions. Genomic DNA contamination was eliminated from all

RNA samples using Versagene DNase Kit (Gentra Biosystems) followed the manufacturer's protocol. The quantity and qualify of the RNA samples were assessed using the Nano-drop ND-3300 Fluorospectrometer (NanoDrop Technologies). The 260/280 absorbance ratio of samples ranged from 1.90 to 2.10, indicating good integrity and little DNA or protein contamination of the isolated RNA.

Quantitative Fluorescent RT-PCR analysis of ADAR2 mRNA splice variants

To quantify the relative expression of ADAR2 mRNA splice variants in RNAs isolated from tissues in wild-type and ΔECS mice, first-strand cDNA was synthesized from 1 ug of total RNA by AMV RT (Promega) in a 10ul reaction containing an antisense primer (VU 459, 5'-GCGGTTTTCTTTAACATCAGTGC-3'), 1mM dNTPs (New England Biolabs) and 10U RNasin (Promega) at 42^{o}C for 1 hour followed by 95^{o}C for 5min. The cDNA was then amplified with 6-carboxyfluorescein (6-FAM) labeled sense primer (VU929, 5'-CGCTTGCTATTTTAGTGCTGCGG-3') and an antisense primer (VU 459, 5'-GCGGTTTTCTTTAACATCAGTGC-3'), using Ampli Taq (Applied Biosystems) as follows: 94^{o}C for 2min, followed by 35 cycles of 94 ^{o}C for 15 seconds, 60^{o}C 30 seconds and 72^{o}C for 2 minutes, and 1 cycle at 72 ^{o}C 7min. 5ul of the PCR reactions was then resolved by 2.5% agarose gel electrophesis at 80 volts in darkness at room temperature for 2.5 hours, and fluorescence quantified using a Molecular Dynamics phosphorimager (Amersham Biosciences).

Quantification of RNA Editing

To quantify the site-selective editing of different ADAR substrates, first-strand cDNA was synthesized from 1ug of total RNA using 10U AMV RT in a 10ul reaction containing 5uM random hexamers (Applied Biosystems, Foster City, CA), 1mM dNTP (New England Biolabs, Beverly, MA) and 10U Rnasin, under condition 25^{o}C for 10minuts, 42^{o}C for 1hr and 95^{o}C for 5 minutes, and then PCR amplified using Ampli Taq DNA polymerase (Applied Biosystems), and assessed by a modified primer-extension analysis (Rueter et al., 1999). The PCR primers, extension primer and mixtures of dideoxynucleotides used in each primer-extension assay and the sizes of expected products are listed in Table 3. The extension products were resolved by denaturing poly acrylamide gel electrophesis, and quantified using a Molecular Dynamics phosphorimager (Amersham Biosciences).

Real-time RT-PCR analysis

To quantify the mRNA expression of ADAR1 and ADAR2 in RNAs isolated from tissues in wild-type and ΔECS mice, first-strand cDNA was synthesized from 500 ng of total RNA using 10U AMV RT in a 10ul reaction containing 5uM random hexmers, 1mM dNTPs and 10U RNasin as follows: 25^{o}C for 10 minutes, 42^{o}C for 1hr and 95^{o}C for 5 minutes and subjected to Taqman real time PCR analysis (Applied Biosystems). All primers and probes used in the real-time PCR

Figure 13 Targeting strategy for the selective ablation of ADAR2 autoediting.

A. A schematic diagram of a portion of the mouse ADAR2 gene is presented, extending from exon 3 through exon 6, before and after targeted gene modification. The positions of the positive selectable marker (PGK-neo), the editing-site complementary sequence (ECS) and the alternative 3'-splice site generated by ADAR2 autoediting (*) are indicated. The positions of primer pairs used for genotype analysis (➔), introduced loxP recombination sites for selective deletion of the PGK-neo cassette and the ECS region (▷), and DNA sequences outside the region of targeting vector homology (- - -) are shown.

B. Analysis of mouse genotype by PCR amplification of mouse tail genomic DNA. The migration positions for PCR amplicons corresponding to the wild-type (+/+; 343 bp) and ΔECS (-/-; 172 bp) alleles are indicated.

Primer-extension analysis of ADAR substrates				
transcript	editing site	PCR primers	extension primer	dideoxynucleotides
ADAR2	-1 site	5'- CTAGCCCCGAGCAGTTACATCCTT -3' 5'- ACCTGCCCGTTTACCTCCACA -3'	5'- CGCCTTCGTTGCAGGAT -3'	ddTTP
5-HT_{2C}R	D	5'- ATTAGAATTCTATTTGTGCCCCGTCTGG -3' 5'- ATCAAAGCTTGACGGCGTAGGACGTAG -3'	5'- GAGCATAGCCGGTTCAATTCG -3'	ddCTP, ddGTP
GluR-2	R/G	5'- TTTGCCTACATTGGGGTCAGTG -3' 5'- AACGTTGCTCAGACTGAGGGC -3'	5'-CACACCTAAAGGATCCTCA-3'	ddGTP
K_V1.1	I/V	5'- TCATTGGGGTCATACTGTTTTCTA -3' 5'- CCTGTCTGTAATGGGCTATGCTA -3'	5'-GACAGGTACGGGCAGGGC-3'	ddCTP, ddGTP
5-HT_{2C}R	A	5'- ATTAGAATTCTATTTGTGCCCCGTCTGG -3' 5'- ATCAAAGCTTGACGGCGTAGGACGTAG -3'	5'-CGCTGGACCGGTATGTAG-3'	ddTTP, ddGTP
GluR-2	+60 site	5'- TTTGCCTACATTGGGGTCAGTG -3' 5'- GCCCATTTTCCCATATACAGGTC -3'	5'-GCTTCAACTTTGTGCAT-3'	ddATP, ddCTP

Table 3 Primer-extension strategy for quantitative analysis of editing in ADAR substrates
A series of mouse ADAR substrates is presented along with the primers used for RT-PCR amplification and primers and deoxynucleotide mixtures necessary for quantification of site-selective editing efficiency using a modified primer-extension strategy. Editing sites preferentially modified by ADAR2 or ADAR1 are indicated in the open and shaded regions, respectively.

reactions were products of Assay-On-Demand from Applied Biosystmes (ADAR1, Assay ID Mm00508001_m1; ADAR2, Assay ID Mm00504621_m1). VIC/MGB labeled Eukaryotic 18S rRNA endogenous control (product# 4319413E, Applied Biosystems) was included in each multiplex PCR. The Real-time PCR reaction and subsequent analysis were performed with the ABI Prism 7900HT Sequence Detection System (SDS v2.1, Applied Biosystems). PCR amplification conditions were as follows: 1 cycle at 50°C, 2min; 1 cycle at 95°C, 10min; 40cycles of 15 seconds at 95°C followed by 1 minunite at 60°C. Quantitation of target gene expression in all samples was normalized to 18S rRNA expression (Ct_{target}-Ct_{18S}=ΔCt). The mean ΔCt value of samples from each tissue for all wild-type animals was determined and used as a reference point for the samples from the same tissues in ΔECS mice. Differences between wild-type and ΔECS mice, including individual variation, were calculated by the equation ($\Delta Ct_{\text{individual } \Delta ECS}$ - $\Delta Ct_{\text{mean wild-type}}$ =$\Delta\Delta$Ct). Fold changes in target gene expression in each sample were calculated by $2^{-\Delta\Delta Ct}$, from which the mean and SEM were derived.

Ribonuclease Protection Assay

For quantification of ADAR2 mRNA isoforms, ribonuclease (RNase) protection analyses was performed, essentially as described (Emeson et al., 1989), using an antisense probe uniformly labeled with [α-^{32}P]-UTP corresponding to a region from -88 to +314bp, relative to the start codon of the mouse ADAR2 cDNA (Genbank accession no. AF403109). The probe generates a 408 nt protected fragment from RNA isoforms lacking of both the 47 nucleotide insertion and exon

4, and a 321 nt partially protected fragment from RNA isoforms containing 47 nucleotide insertion or exon 4 or both and the unprocessed RNA precursor (Figure 17A). A 111 nt antisense RNA probe for cyclophilin was used as an internal control for RNA loading and normalization of expression levels. Reaction products were separated on a 4% poly acrylamide/7M urea gel and the relative levels of protected fragments were quantified using a Molecular Dynamics phosphorimager (Amersham Biosciences).

Quantitative Western blotting analysis

Tissue homogenates were prepared in RIPA buffer (150mM NaCl, 1% Igepal CA630, 0.5% Na deoxycholate, 0.1% SDS, 50mM Tris pH 7.6, 1mM PMSF, 2ug/ml Leupeptin, 0.1% Aprotonin) using a polytron homogenizer. The homogenate was spun in a microcentrifuge at 13000 rpm for 15min at 4^{o}C to remove particulate matter. The supernatant was then sonicated for 5 seconds on ice to shear genomic DNA followed by centrifugation at 13000 rpm at 4^{o}C for 10 minutes and then the supernatant was stored at -80^{o}C. Expression levels of ADAR2 and β-actin in tissue homogenates from wild-type and ΔECS mice were analyzed by Western blotting analysis using an anti-ADAR2 polyclonal antibody (1:250 V/V) (Rueter et al., 1999; Sansam et al., 2003) and an anti-β-actin monoclonal antibody (1:500 V/V, sc-1616, Santa Cruz Biotechnology) respectively. An anti-goat secondary antibody labeled with Alexa Fluor 680 that excites at 650 nm and emits at 702 nm (1 to 10,000, V/V, A21084, Molecular Probes, Eugene, OR) was used to recognize both primary antibodies and the

fluorescent was directly detected using the Odyssey infrared imaging system in accordance with the manufacturer's instructions (Li-Cor Biosciences, Lincoln, NE).

Mouse growth rate analysis

To compare the growth rate between wild-type and ΔECS mice, age-matched male littermates were provided *ad lib* access to food and maintained within a facility of 12hr light/dark cycle. Body weight was monitored weekly at 3pm Thursday afternoon to minimize the variability from feeding schedule and the circadian rhythm.

Neurological examination - IRWIN screen

Age-matched male littermates were genotyped at age of week 3. 5 animals of the same genotypes (wild-type and homozygous ΔECS) were maintained ad lib in the same cage since week 3 within a facility of 12hr light/dark cycle. Age-matched male heterozygous littermates were used to make the population in each cage equal to five. The animals were undisturbed till week 8, and then transferred to the room next to the screening facility and stayed for another week. During the week before screening, one cage with 4 homozygous ΔECS mice was flooded by water leaking, so the mice were sacrificed since stressed animal can not be used in the test. 7 wild-type and 7 homozygous ΔECS mice were screen in two consecutive morning followed by the IRWIN screen protocol provided by the Vanderbilt mouse neuro-behavior laboratory (Appendix I).

Statistical analysis

For most experiments, student's t-test was performed using GraphPadPRISM (GraphPad Software, Inc.). Values are reported as mean±SEM. For growth rate analysis, two-way ANOVA was performed using GraphPadPRISM (GraphPad Software, Inc.). Values are reported as mean±SEM; $P < 0.05$ was considered statistically significant. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Results

Generation of ΔECS mice

The ΔECS mice were developed initially by Dr. Chris Sansam, a former graduate student in the laboratory. Detailed descriptions of the strategy for assembling of the targeting vector to generate ΔECS animals are presented in the Ph.D. thesis of Dr. Sansam. A brief summary of this work has been included here to provide for better understanding of the studies presented in this dissertation.

To selectively eliminate ADAR2 autoediting, the ECS region in mouse ADAR2 gene was deleted to disrupt the conserved duplex structure, similar to previous studies in which editing of the Q/R site in GluR-2 pre-mRNAs was disrupted by deletion of intronic sequences containing the ECS (Brusa et al., 1995). Since

previous studies have demonstrated that ablation of ADAR2 expression results in early-onset seizures and postnatal lethality (Higuchi et al., 2000), the P1 bacteriophage *Cre-loxP* system (Xu et al., 2001) was used to generate mutant mouse strains in which the ECS region could be conditionally deleted to circumvent the possibility of a lethal phenotype. Mutant mice that were heterozygous for the targeted ADAR2 allele (*3-lox,* Figure 13A) were mated to transgenic animals expressing *Cre* recombinase under the control of the EIIA promoter (Lakso et al., 1996) to delete the DNA sequences between the loxP sites early in embryogenesis. Progeny from this mating lost either the PGK-neomycin cassette alone (*lox-ECS-lox,* Figure 13A) or the region of ADAR2 gene containing the ECS (*lox-neo-lox*) or both (*ΔECS*, Figure 13A). The heterozygous ΔECS mice were then mated with C57Bl/6 to segregate the Cre transgene from the mutant ADAR2 allele. Heterozygous ΔECS mice without the Cre transgene were then interbred and progeny demonstrated the expected Mendelian frequency (34 wild-type (+/+), 67 heterozygous (+/-), and 44 homozygous mutant (-/-); χ^2=2.2, p>0.25), as assessed using a PCR-based assay (Figure 13B); no obvious phenotypic alterations were observed in animals homozygous for the ΔECS mutation.

Ablation of ADAR2 pre-mRNA -1 site editing and alternative splicing in ΔECS mice

To determine if the ECS element is essential for ADAR2 autoediting *in vivo*, we quantified the extent of editing at the -1 site in ADAR2 pre-mRNA transcript from

multiple tissues of wild-type and homozygous ΔECS mice. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the -1 site containing fragment from ADAR2 pre-mRNA. Since the PCR strategy amplifies both ADAR pre-mRNA and genomic DNA, an RT-PCR reaction with no reverse transcriptase was included for each sample to monitor possible genomic DNA contamination. The amplicons were then subjected to a modified primer-extension assay (Rueter et al., 1999). The extent of editing at the -1 site in ADAR2 pre-mRNAs varied widely in tissues from wild-type animals (Figure 14), with the greatest levels of editing observed in lung (32%), followed by the brain (23%), the heart (17%), the liver (16%) and the lowest in the testis (8%). By contrast, only background levels of editing were observed in tissues from the homozygous ΔECS mice. These data are consistent with previous *in vitro* studies and further confirmed the critical role of the ECS element for the A-to-I conversion at the -1 site.

Since the generation of an A-I dinucleotide to form a functional 3'-splice site in intron 4 requires ADAR2 autoediting, abrogation of this RNA processing event should result in the sole use of the distal splice acceptor immediately preceding exon 5, leading to production of ADAR2 mRNAs encoding a full-length, functional protein. Recent studies have demonstrated that in addition to alternative 3'-splice site selection, an additional exon (exon 4) was identified 174 bp downstream of exon 3 (Slavov and Gardiner, 2002). Inclusion of this 73 bp exon was found to take place at a low frequency in ADAR2 transcripts isolated from mouse brain and is predicted to cause a frameshift resulting in premature translation

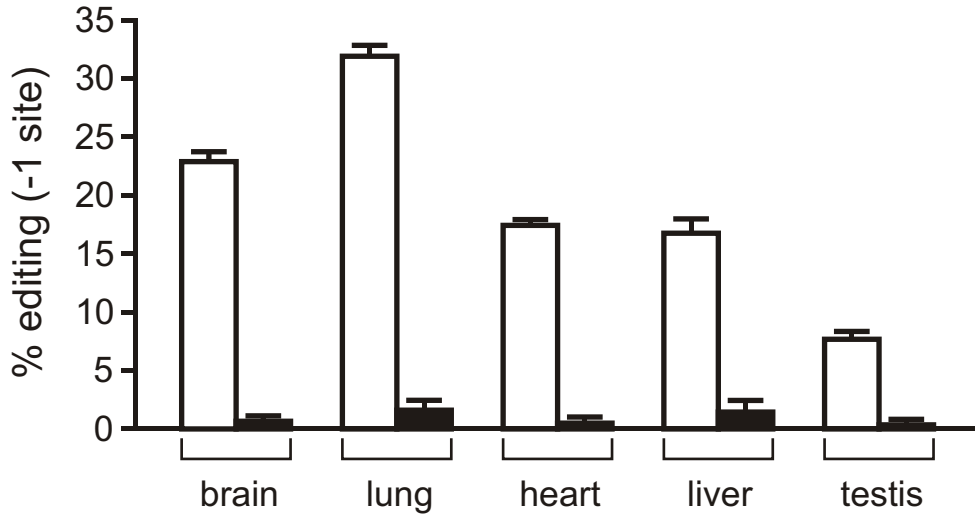


Figure 14 Ablation of ADAR2 autoediting ΔECS mice.
The extent of editing at the -1 site in ADAR2 pre-mRNA isolated from different tissues of wild-type (□) and ECS(■) mice was determined by primer-extension analysis; values were obtained from tissue isolated from 3 animals per genotype (mean±SEM)

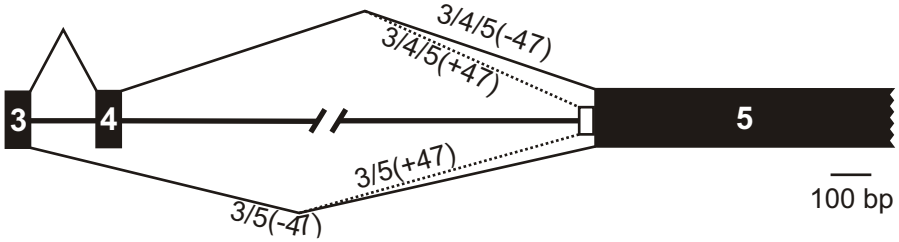
termination (Slavov and Gardiner, 2002). As a result of these alternative splicing events, four ADAR2 splice variants can be generated in the region between exon 3 and 5 (Fig 15A). Among these four mRNA isoforms, only the isoform lacking both exon 4 and the 47-nucleotide cassette (3/5 -47) is able to encode a full-length, functional ADAR2 protein. To quantify the relative abundance of ADAR2 splice variants, we developed a quantitative RT-PCR-based strategy using a 6-carboxyfluorescein (6-FAM) labeled sense PCR primer, which allowed us to directly quantify the PCR amplicon.

The quantitative accuracy of this method was tested by amplifying a series of templates with mixtures of plasmids containing or lacking the 47 nucleotide cassette at fixed ratios. The amplicons from the plasmid PCR were quantified and the observed ratio of the +47 and -47 isoform was plotted against the expected ratio (Figure 15B). The linear regression analysis demonstrated that the data fits very well with the equation Y=X, where Y stands for the observed ratio, and X for the expected, demonstrating that this method can precisely reflect the relative abundance of different ADAR2 mRNA isoforms.

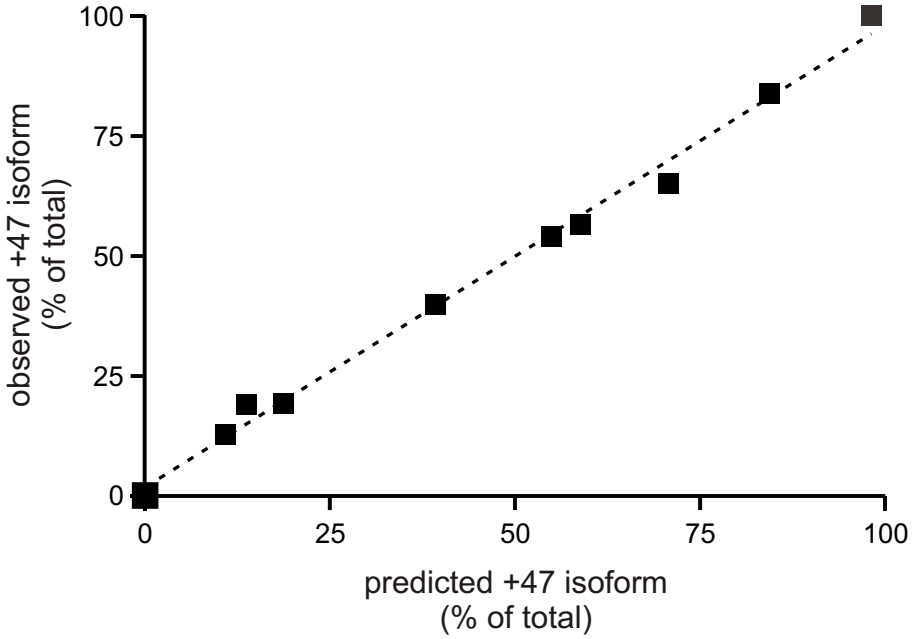
Consistent with previous reports (Slavov and Gardiner, 2002), mRNA isoforms containing exon 4 represented a small percentage of total ADAR2 transcripts in the brain of wild-type mice (Fig 15C). Our data also demonstrated that exon 4 is rarely used in all the non-neuronal tissues tested. In addition, the use of the proximal A-I splice site roughly paralleled the tissue-specific editing at the -1 site in wild-type animals (Figure 14). In RNA isolated from various tissues of ΔECS mice however, ADAR2 mRNA isoforms containing the 47 nucleotide cassette

Figure 15 Ablation of ADAR2 alternative splicing in ΔECS mice.
A. A schematic diagram of four possible ADAR2 mRNA isoforms resulting from alternative splicing of exon 4 and inclusion or exclusion of 47-nucleotide cassette (-47 and +47) at the 5'-boundary of exon 5 are indicated.
B. Standard curve for fluorescent RT-PCR analysis. Plasmids encoding ADAR2 isoforms that contained (+47) or lacked (-47) the 47-nucleotide cassette were mixed at different ratios and amplified by PCR using a fluoroscein-labeled sense and a non-labeled antisense primer. Amplicons corresponding to alternatively spliced ADAR2 variants were resolved by 2.5% agarose gel electrophoresis, and quantified by phosphorimager analysis. A correlation between the experimentally observed ratio of ADAR2 isoforms and the ratio predicted by the plasmid mixture is indicated with the best-fit line determined by linear regression ($R^2 = 0.9764$).
C. Quantitative analysis of ADAR2 alternative splicing. The positions of RT-PCR amplicons corresponding to the four alternatively spliced ADAR2 transcripts described in Fig.15A are indicated. The percentage of total ADAR2 transcripts containing the 47-nucleotide cassette but not exon 4 (3/5 +47) is shown (mean ± SEM; n=3).

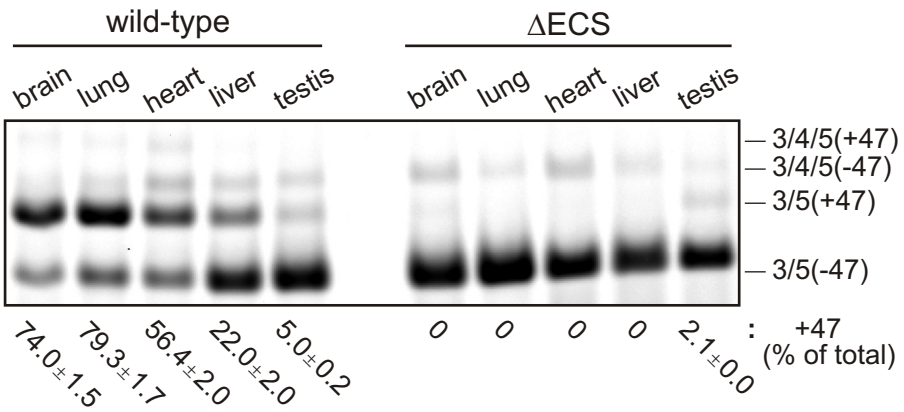
A


3/4/5(-47)
3/4/5(+47)
3
4
5
3/5(+47)
3/5(-47)
100 bp

B


100
75
50
25
0
observed +47 isoform
(% of total)
0
25
50
75
100
predicted +47 isoform
(% of total)

C


wild-type
ΔECS
brain
lung
heart
liver
testis
brain
lung
heart
liver
testis
3/4/5(+47)
3/4/5(-47)
3/5(+47)
3/5(-47)
74.0±1.5
79.3±1.7
56.4±2.0
22.0±2.0
5.0±0.2
0
0
0
0
2.1±0.0
:
+47
(% of total)

were not observed (Figure 15C), demonstrating that deletion of the ECS region concomitantly ablated ADAR2 autoediting and the ability to use the proximal splice site in intron 4 to include an additional 47 nucleotides in the ADAR2 coding region *in vivo*. The single exception to this observation was the presence of a PCR amplicon from testis RNA that migrated at the same position as the 3/5+47 isoform, representing less than 2% of the ADAR2 mRNA transcripts. Due to the low abundance of this amplicon, we were unable to identify and further characterize this molecule.

ADAR2 protein expression in the ΔECS mice

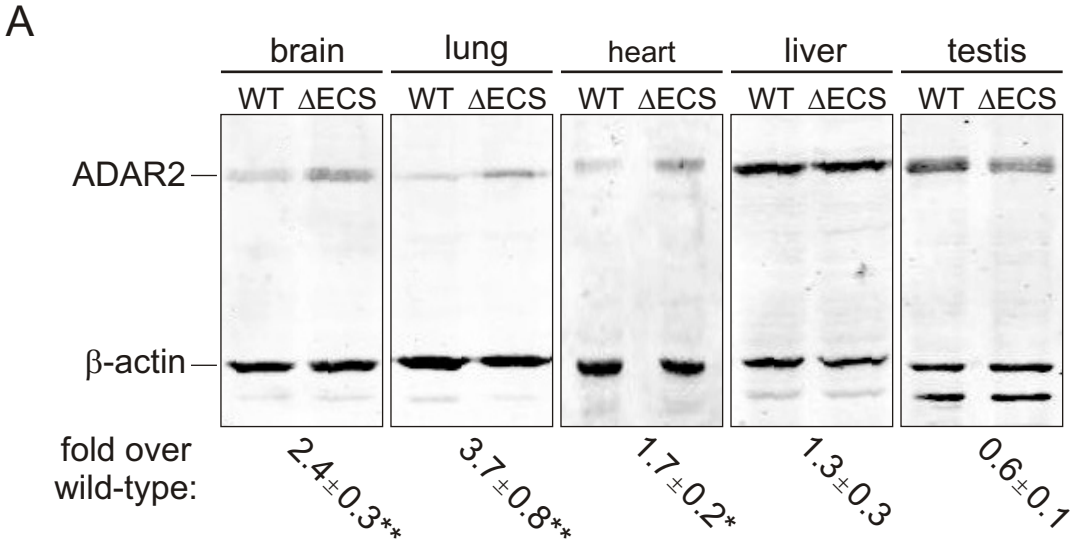
The ablation of ADAR2 autoediting and proximal splice acceptor use in ΔECS mice resulted in the predominant production of the 3/5 -47 mRNA isoform that encodes the full-length, functional ADAR2 protein. By contrast, the 3/5 +47 isoform is the predominant isoform of ADAR2 mRNA in most tissues from wild-type animals, encoding a putative truncated, inactive ADAR2 protein resulting from the premature translation termination in the altered reading frame. We therefore anticipated that the ablation of ADAR2 autoediting would lead to elevated ADAR2 protein expression in ΔECS mice in comparison to the wild-type animals.

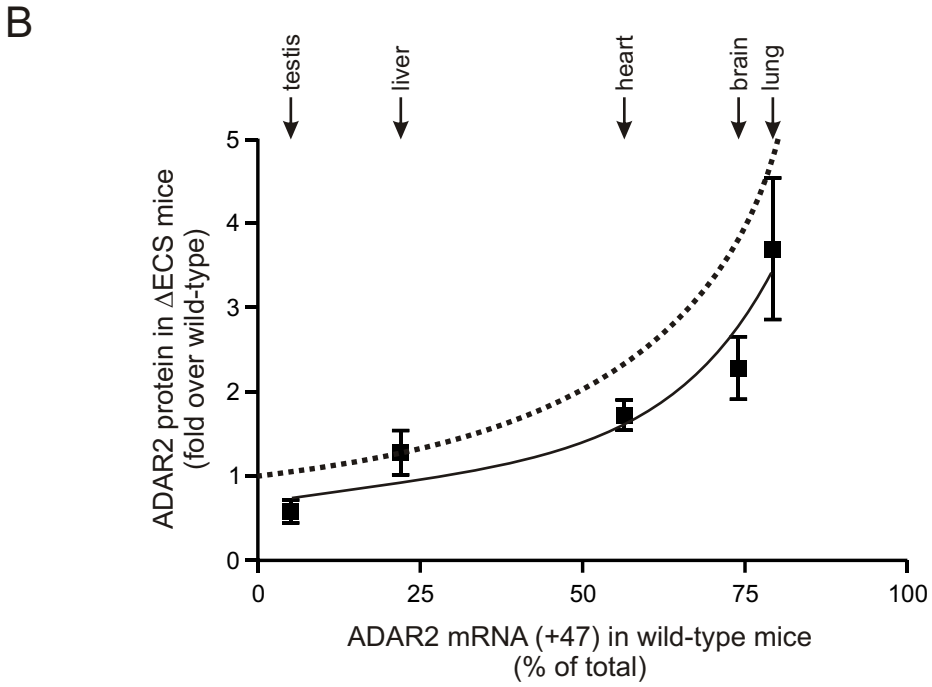
Homogenates were prepared from multiple tissues of wild-type and ΔECS mice and ADAR2 protein levels were assessed by quantitative Western blotting analysis using antisera directed against ADAR2 and a constitutively expressed

house-keeping protein, β-actin, which served as a endogenous loading control. We first performed the quantitative Western blotting analysis on a series of sample dilutions to identify the linear range of detection for both ADAR2 and β-actin. 20ug was determined to be the optimal amount for this analysis as it was in the middle of the linear range of detection. As shown in figure 16, in the lung, where the greatest level of ADAR2 autoediting and 47 nt inclusion was observed in wild-type animals (Figure 14, 15), the greatest increase (3.7 fold) in ADAR2 protein expression was identified in ΔECS mice. Similarly, in the brain and heart, where the +47 isoform represented >50% of the total ADAR2 mRNA (Figure 14, 15), a 2.4- and 1.7-fold increase in ADAR2 protein was revealed, respectively. In tissues such as the liver and testis, where ADAR2 autoediting and proximal splice site use occurred at a low frequency in wild-type mice (Figure 14, 15), ablation of this RNA modification did not affect the ADAR2 protein expression significantly (Figure 16A).

In the simplest model, where ADAR2 autoediting represents the rate-limiting step for determining alternative splice-site selection, the equation Y=100/(100-X) can define the relationship between the extent of tissue-specific proximal splice site use in wild-type tissues (X) and the expected increase in ADAR2 protein expression (Y) in the ΔECS mice. While plotting the observed increase of ADAR2 protein expression in ΔECS mice (Figure 16A) against the percentage of 47 nucleotide cassette containing mRNA isoform in wild-type tissues (Figure 15C), we obtained a curve roughly paralleled with the predicted curve (Figure 16B).

Figure 16 Quantitative Western blotting analysis of ADAR2 protein levels in tissues isolated from wild-type and ΔECS mice.
A. A representative Western blot using 20 μg of total protein from each tissue is presented; the expected migration positions for ADAR2 and -actin are indicated. The fold increase of ADAR2 protein expression in ΔECS versus wild-type tissues was quantified after normalization to the internal -actin control (mean±SEM; n=5; **, p<0.01; *, p<0.05).
B Correlation between ADAR2 autoediting levels in isolated tissues from wild-type mice (from Fig. 14) and the relative increase in ADAR2 protein expression from the corresponding tissues in ΔECS animals was determined by non-linear regression with the equation Y = A/(B-X) (A=70; B=98, r2=0.92). The best fit line defined by the observed [Y=70/(98-X)] and predicted [Y=100/(100-X)] data are indicated with solid and dashed lines, respectively.


A
brain
lung
heart
liver
testis
WT ΔECS
WT ΔECS
WT ΔECS
WT ΔECS
WT ΔECS
ADAR2
β-actin
fold over wild-type:
2.4±0.3**
3.7±0.8**
1.7±0.2*
1.3±0.3
0.6±0.1

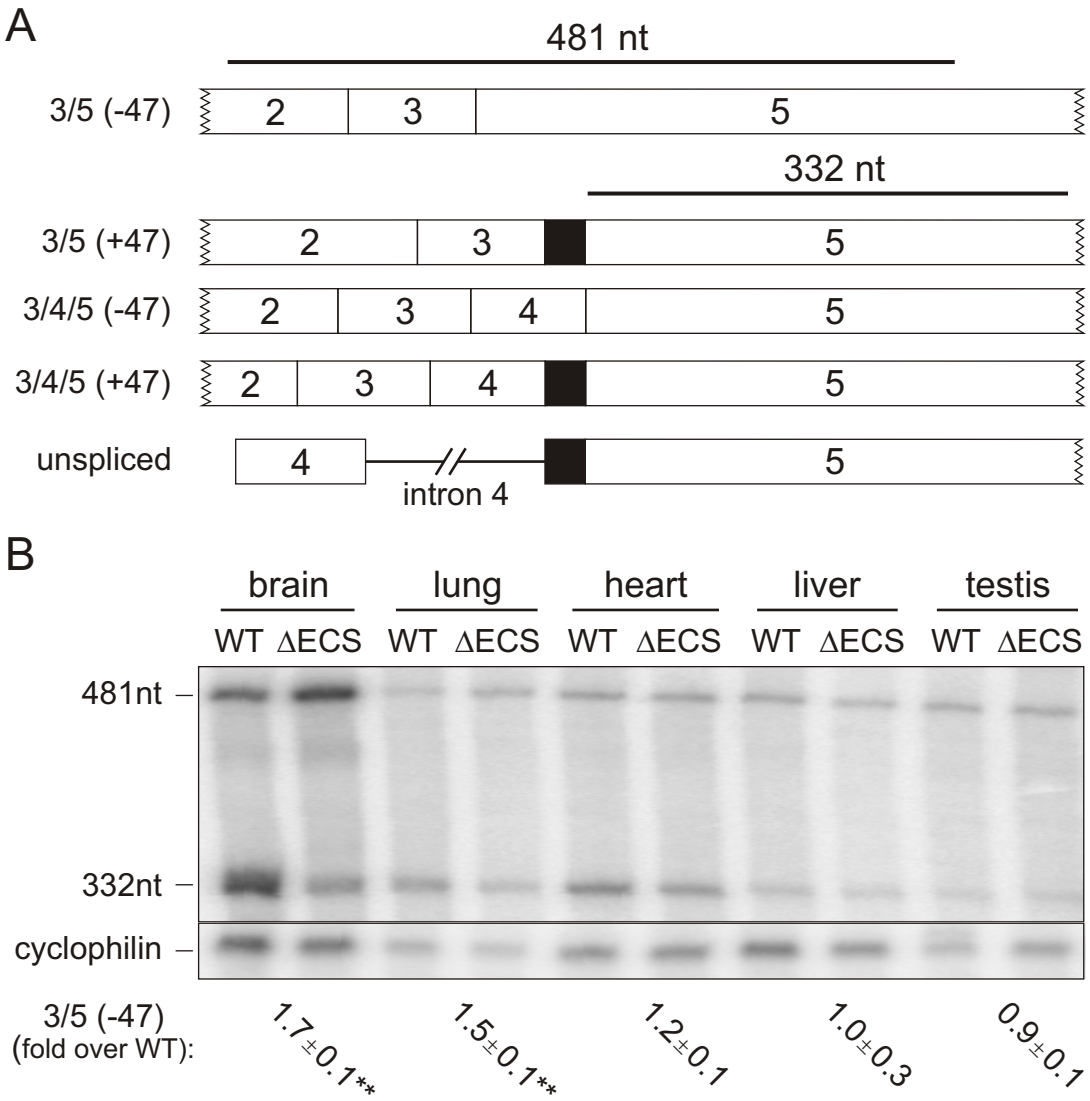

B
testis
liver
heart
brain
lung
ADAR2 protein in ΔECS mice (fold over wild-type)
0
1
2
3
4
5
0
25
50
75
100
ADAR2 mRNA (+47) in wild-type mice (% of total)

These results confirmed that ADAR2 autoediting is a key regulator for modulating the expression of ADAR2 protein *in vivo.*

Characterization of ADAR2 mRNA expression in ΔECS mice

To confirm that the elevated ADAR2 protein levels in ΔECS mice can be attributed to the alterations in 3'-splice site selection, rather than an increase in the steady state levels of ADAR2 transcripts, a ribonuclease protection assay was developed to quantify isoform-specific ADAR2 mRNA expression in tissues of wild-type and ΔECS mice. Due to the complexity of alternative splicing events between exon 3 and 5 to generate four mRNA isoforms, the ribonuclease protection probe was designed to generate a 481 nt fragment from the 3/5 -47 mRNA isoform, whereas a partial protection by all other mRNA isoforms and the unspliced ADAR2 pre-mRNA gave rise to a 332nt protected specie (Figure 17A). We were unable to develop a probe that specifically recognizes the mRNA isoform containing the 47 nucleotide cassette due to the presence of multiple editing sites at positions 10, 23 and 24 relative to the -1 site (Dawson et al., 2004). Results from this assay demonstrated significant increases in the 3/5 -47 ADAR2 mRNA isoform expression in the brain and lungs of ΔECS mice. Consistent with the lower levels of autoediting observed in wild-type animals (Figure 14), no significant increase in the 3/5 -47 isoform was seen in RNAs isolated from the liver and the testis in ΔECS mice (Figure 17).

Figure 17 Characterization of ADAR2 mRNA isoform expression.
A. A schematic diagram indicating the structures and sizes of predicted RNase protection products resulting from ADAR2 pre-mRNAs or ADAR2 mRNA isoforms containing or lacking the 47-nucleotide cassette or exon 4.
B. Ribonuclease protection analysis of total RNA (10 μg) from tissues of wild-type and ΔECS mice; the predicted migration positions for protected RNA fragments are indicated. The fold increase in ADAR2 RNA isoforms lacking both the 47-nucleotide cassette and exon 4 (3/5 -47) in ΔECS versus wild-type tissues was quantified after normalization to the internal cyclophilin control (mean ± SEM; n=6 for brain, lung and heart; n=3 for liver and testis).
C. Quantification of relative ADAR2 mRNA expression between wild-type (▭) and ΔECS (▬) using real-time RT-PCR analysis (mean ± SEM; n=6 for brain, lung and heart; n=3 for liver and testis).


A
481 nt
3/5 (-47)
2
3
5
332 nt
3/5 (+47)
3/4/5 (-47)
4
3/4/5 (+47)
unspliced
intron 4
B
brain
lung
heart
liver
testis
WT ΔECS WT ΔECS WT ΔECS WT ΔECS WT ΔECS
481nt
332nt
cyclophilin
3/5 (-47)
(fold over WT):
1.7±0.1**
1.5±0.1**
1.2±0.1
1.0±0.3
0.9±0.1
C
Relative ADAR2
mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
*
brain
lung
heart
liver
testis

To further confirm that deletion of the ECS region did not result in alterations of the steady-state level of ADAR2 transcripts, a real-time RT-PCR-based strategy using a Taqman probe spanning exon 9 and 10 was performed to quantify total ADAR2 mRNAs in multiple tissues from wild-type and ΔECS mice. Results from this analysis revealed that despite the increase in ADAR2 protein expression in ΔECS animals, there was no significant change in the total level of ADAR2 mRNAs in most tissues (Figure 17C). Interestingly, a 30% decrease in ADAR2 mRNA was observed in whole mouse brain that may have resulted from a tissue-specific instability of the modified ADAR2 pre-mRNA or a compensatory alteration to counteract elevated ADAR2 protein levels.

Selective increase in RNA editing by ADAR2 overexpression

ADAR2 has been shown to be responsible for the site-selective editing of numerous mammalian RNAs (Bhalla et al., 2004; Burns et al., 1997; Niswender et al., 1999; Rueter et al., 1999; Wang et al., 2000). To examine whether the observed increase in ADAR2 protein expression in ΔECS mice resulted in changes for ADAR2 substrates editing, we quantified the extent of editing for known ADAR2 targets, including the D- site of 5-HT_{2C} R transcripts, the R/G site of the GluR-2 pre-mRNA and the I/V site of the Kv1.1 mRNA (Bhalla et al., 2004; Hartner et al., 2004; Hoopengardner et al., 2003; Wang et al., 2000; Wang et al., 2004). Since the expression of most identified ADAR2 substrates is restricted to the nervous system (Higuchi et al., 2000), we first determined the editing efficiency of these ADAR2 targets in RNA isolated from whole brain of wild-type

and ΔECS mice using a modified primer-extension analysis (Burns et al., 1997). While the level of editing at the D-site for 5-HT_{2C} R transcripts (47.8%) isolated from wild-type mice is comparable to previous studies (Burns et al., 1997; Wang et al., 2004), a 15% increase in editing was seen for ΔECS animals. Similarly, editing at the R/G site and I/V sites of GluR-2 and Kv1.1 transcripts were increased by 11% and 10%, respectively, in ΔECS mice (Table 4).

To determine whether the changes in editing were limited to ADAR2-selective sites, we also examined the editing efficiencies of two well-characterized ADAR1 sites, the +60 site in the GluR-2 pre-mRNA and the A-site in 5-HT_{2C}R transcripts (Burns et al., 1997; Hartner et al., 2004; Higuchi et al., 2000; Wang et al., 2000). The extent of editing at the A-site in 5-HT_{2C}R transcripts isolated from whole mouse brain decreased from 74% in wild-type animals to 64% in ΔECS mice; however, editing at the +60 site was not significantly changed. Since ADAR1 mRNA expression in the brains of ΔECS mice was not different from wild-type animals as determined by real-time RT-PCR analysis (Figure 18), it is unlikely that the decrease in the A-site editing in 5-HT_{2C}R transcripts of ΔECS mice results from decrease in ADAR1 expression. As suggested by previous studies, a competition between ADAR1 and ADAR2 for binding to the 5-HT_{2C}R RNA substrate (Chen et al., 2000; Hartner et al., 2004) could explain how elevated ADAR2 protein expression in the ΔECS brains could lead to decrease in ADAR1-selective modification of the A-site.

In addition to the ADAR2 pre-mRNA transcript, the only other known ADAR2 substrates expressed in non-neuronal tissues is the RNA encoding the K_v1.1

Site-specific RNA editing of ADAR substrates				
			% editing	
transcript	editing site	tissue	wild-type	ΔECS
5-HT_{2C}R	D	brain	47.8±2.5	63.0±4.7**
GluR-2	R/G	brain	68.6±1.8	79.8±0.9**
K_V1.1	I/V	brain	37.0±3.1	46.7±2.6*
K_V1.1	I/V	lung	ND	ND
K_V1.1	I/V	heart	ND	ND
K_V1.1	I/V	liver	ND	ND
K_V1.1	I/V	testis	ND	ND
5-HT_{2C}R	A	brain	74.3±1.2	64.4±0.7***
GluR-2	+60 site	brain	61.8±1.3	60.2±1.7

Table 4 Site-specific RNA editing of ADAR substrates
The extent of editing for ADAR substrates from wild-type and ΔECS mouse tissues was determined by primer-extension analysis (see Material and Methods). Shaded are ADAR1 specific sites. Values for editing sites in brain-derived RNAs were obtained from 6 animals of each genotype, whereas values for RNAs isolated from other tissues were obtained from 3 animals of each genotype (mean ± SEM).

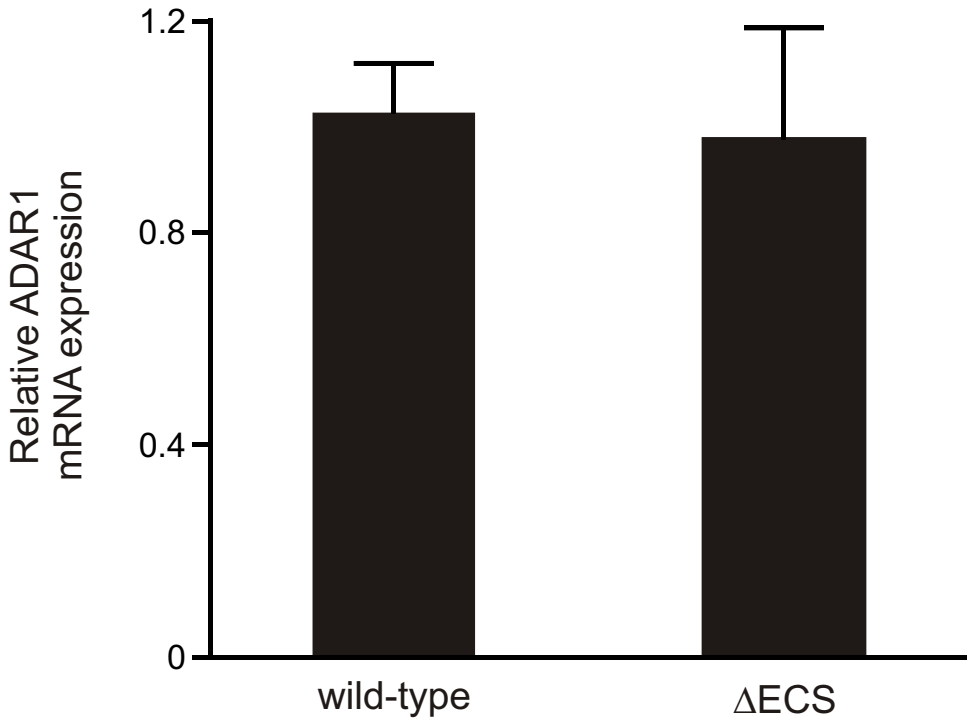


Figure 18 ADAR1 mRNA expression in wild-type and ΔECS mouse brain.
The relative level of ADAR1 mRNA expression in whole mouse brain from wild-type and ΔECS mice was quantified by real-time PCR (mean ± SEM; n=6).

channel subunit. As ADAR2 transcripts can no longer be edited due to genetic modification in ΔECS mice, the I/V site in K_v 1.1 transcripts represents the only site that can provide further information about alternation in endogenous ADAR2 activity in non-neuronal tissues due to ablation of ADAR2 autoediting. The region containing I/V site was amplified from total RNA of multiple wild-type and ΔECS peripheral tissues. Since K_v1.1 transcripts are encoded by an intronless gene, KCNA1, an RT-PCR reaction with no reverse transcriptase was included with every sample to confirm the absence of genomic DNA contamination. Surprisingly, no detectable levels of editing at the I/V site were observed in K_V 1.1 transcripts from lung, heart, liver and testis of either wild-type or ΔECS mice, suggesting A-to-I conversion to regulation K_V1.1 channel function occurs exclusively in the brain.

Phenotypic analysis of ΔECS mice

Recent study from our laboratory have demonstrated that mice expressing a rat ADAR2 transgene, under the control of cytomegalovirus (CMV) promoter, developed adult onset obesity (unpublished data from Dr. Minati Singh), yet the ΔECS mice which have elevated ADAR2 expression have no obese phenotype (Figure 19). While ΔECS mice have increased ADAR2 protein expression in the lung, brain and heart, the CMV-driven rADAR2 transgene is highly expressed in skeletal muscle and demonstrated only marginal overexpression (1.2-fold) in the brain. The growth differences between the rADAR2 transgenic mice and ΔECS

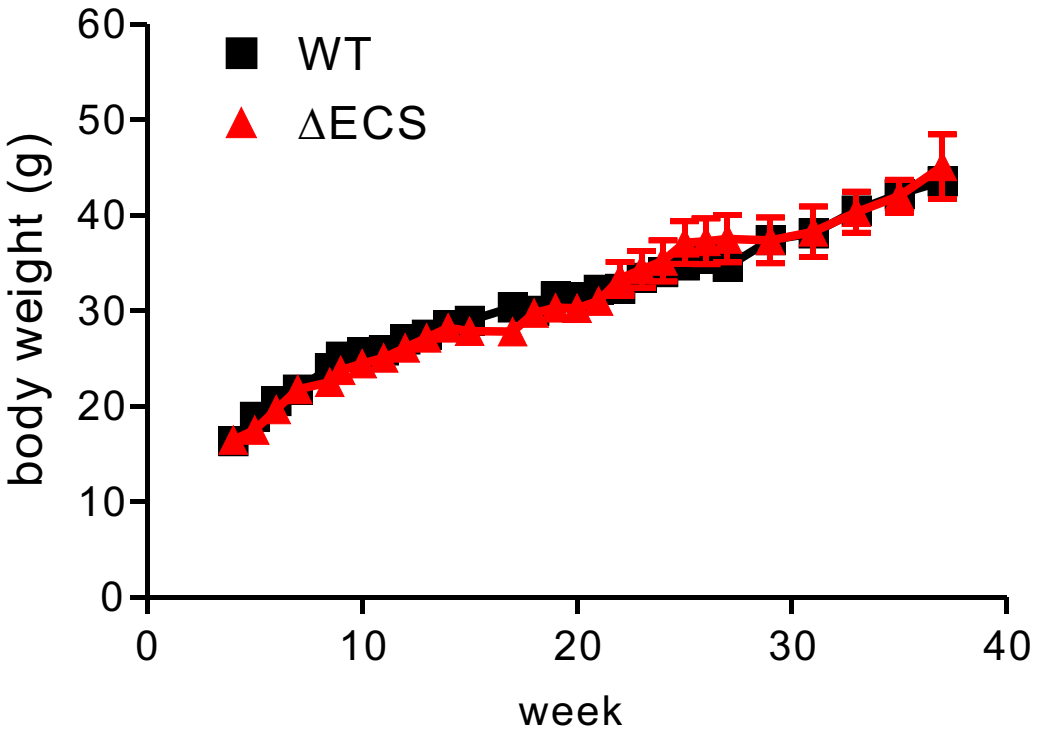


Figure 19 Body-weight analysis of wild-type and ΔECS animals. Body-weight of wild-type mice and ΔECS mice were taken once a week for 35 weeks (mean ± SD; n=6), statistic significance was determined by two-way ANOVA analysis, and no significance was detected.

mice could result from the differential expression profile of ADAR2 protein in these distinctive mouse models. The exact mechanism and the target tissue for the obesity in rADAR2 transgenic mice are still under investigation.

Despite significant alterations in the editing of ADAR substrates in ΔECS mice (Table 4) however, no overt phenotypic alterations were observed in mutant mice from birth through adulthood. Since any expected changes in ΔECS mice are dependent upon the repertoire of ADAR targets affected by changes in ADAR2 expression, and the majority of characterized editing events are limited to neurotransmitter receptors in the CNS, we assessed alterations in animal behavior using a gross examination based upon the neurological screen developed by Irwin (Irwin, 1968). This standardized method provides a behavioral and functional profile of wild-type and mutant mice by observational assessment of potential defects in gait or posture, motor control and coordination, changes in excitability and aggression, salivation, lacrimation, piloerection, defecation, and muscle tone. Results from this neurological screen identified no significant alterations in ΔECS mice from wild-type animals.

ADAR2 autoediting during brain development.

Previous studies using *in situ* hybridization have demonstrated that ADAR2 expression in the brain is developmentally regulated (Paupard et al., 2000), with the expression of ADAR2 mRNA was first detected at embryonic day 19 (E19) and reaching maximal levels by postnatal day 21 (P21). To further examine the expression of ADAR2 mRNA in the brain during development, Michelle Jacobs in

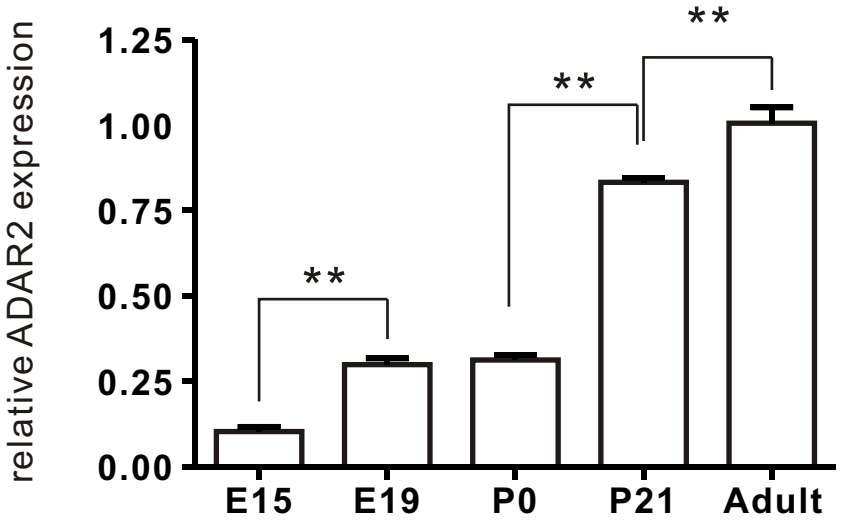
our laboratory took the advantage of a Taqman-based real-time RT-PCR analysis. As shown in Figure 20, ADAR2 mRNA expression was detected as early as E15 and increased till P21, after which no further changes in ADAR2 mRNA were observed (Figure 20A, personal communication from Michelle Jacobs). To examine ADAR2 autoediting during brain development, the RT-PCR analyses (described in figure 15) were performed on brain RNA harvested from different developmental stages. Results from this analysis demonstrated little 47 nucleotide inclusion at E15, yet use of the proximal 3'- splice site increased with developmental increases in total ADAR2 mature mRNA expression (Figure 20B), suggesting that the modulation of ADAR2 expression by autoediting is developmentally regulated. To further assess ADAR2 autoediting during development, brain ADAR2 protein expression in wild-type and ΔECS mice at the age of P0 was compared, and no significant difference between the two genotypes was observed (Figure 20C), suggesting that ADAR2 autoediting may be not be important for regulating ADAR2 protein expression during the embryonic and neonatal development.

Discussion

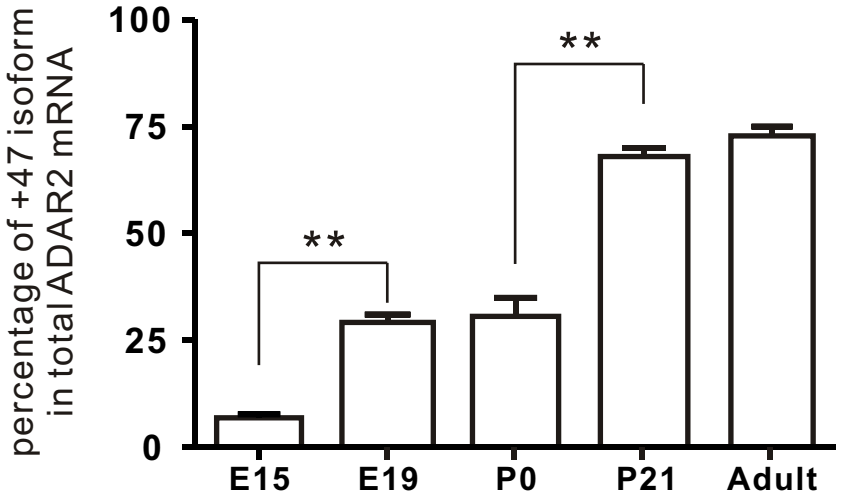
Characterized by the presence of both dsRBDs and a catalytic adenosine deaminase domain, members of the ADAR family convert adenosine into inosine within double-stranded region of RNA, thereby modulating the genetic information passed through mRNA transcripts. A-to-I editing events have been shown to alter the coding sequences of mRNAs encoding AMPA and kainate

Figure 20 Developmental regulation of ADAR2 expression and autoediting in the brain of wild-type and ΔECS mice.
A. ADAR2 mRNA expressions in the brain at different developmental stages were quantified by real-time RT-PCR and normalized (as described in the Material and method) with the expression in the adult brain set up as 1. (courtesy from Michelle Jacobs, Vanderbilt University)
B. Quantitative analysis of ADAR2 alternative splicing during brain development. The percentage of total ADAR2 transcripts containing the 47-nt cassette but not exon 4 (3/5 +47) is quantified in total RNA isolated from the brains at different developmental stages, using the fluorescent RT-PCR analysis as described in the Material and method (mean±SEM; n=3).
C. Quantitative Western blotting analysis of ADAR2 protein levels in wild-type and ΔECS mice at the age of P0. The fold increase of ADAR2 protein expression in ΔECS versus wild-type tissues was quantified after normalization to the internal β-actin control (mean±SEM; n=5; **, $p<0.01$; *, $p<0.05$).

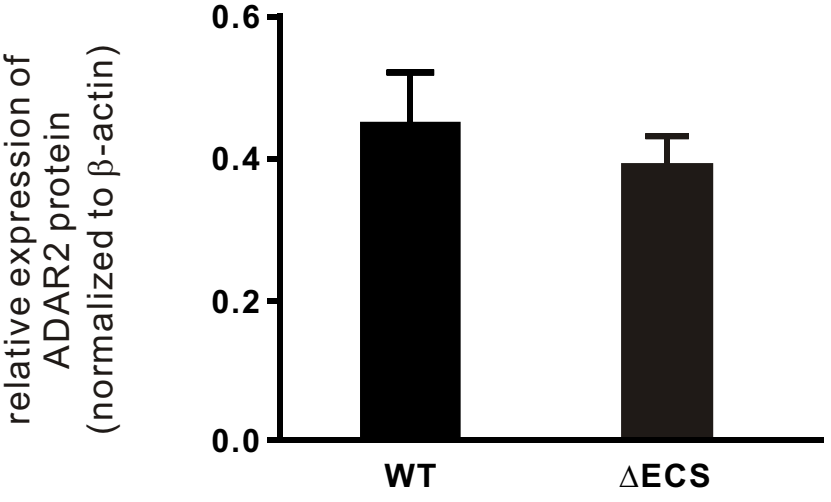
A


relative ADAR2 expression
1.25
1.00
0.75
0.50
0.25
0.00
**
**
**
E15
E19
P0
P21
Adult

B


percentage of +47 isoform in total ADAR2 mRNA
100
75
50
25
0
**
**
E15
E19
P0
P21
Adult

C


relative expression of ADAR2 protein (normalized to β-actin)
0.6
0.4
0.2
0.0
WT
ΔECS

ionotropic glutamate receptor subunits, the 5-HT_{2C} receptor and the K_v1.1 potassium channel subunit, resulting in the production of multiple protein isoforms with profoundly altered functional properties (Burns et al., 1997; Seeburg and Hartner, 2003). ADARs have the potential to affect more than the coding potentials of mRNA transcripts. In *C. elegans*, ADARs have been linked to the RNAi pathway (Tonkin and Bass, 2003). RNAs hyperedited by ADARs are selectively retained in the nuclei of mammalian cells, indicating that ADAR activity may serve to control the stability and nuclear export of transcripts (Kumar and Carmichael, 1997; Prasanth et al., 2005). Moreover, the discovery of numerous A-to-I editing events in the UTRs of human, mouse and C. elegans mRNAs further suggests a role for ADARs in regulating mRNA stability or translation efficiency (Morse et al., 2002; Morse and Bass, 1999).

The exact mechanism by which site-specific adenosine modification is achieved in a naturally occurring substrate is still unknown. It has been shown that ADARs edit adenosines within any RNA duplex of sufficient length regardless of sequence; consequently, ADAR site-selectivity is predominantly determined by bulges and loops that separate base-paired sequence within imperfect RNA duplexes (Lehmann and Bass, 1999). However, when expressed at high levels, ADARs can edit adenosines in duplexes as short as 15 bp in length, so unregulated ADAR activity could lead to aberrant and deleterious mRNA sequence alterations (Herbert and Rich, 2001). *In vitro* experiments have suggested that ADAR overexpression may actually result in the inhibition of other dsRNA-binding proteins by competitively binding to their substrates (Chen et al.,

2000). Two lines of transgenic mice generated in our laboratory, in which the CMV promoter drives the constitutive expression of either the wild-type or a catalytically inactive rADAR2 protein are severely obese due to hyperphagia, demonstrating a detrimental physiological consequence of unregulated ADAR expression that may result from aberrant dsRNA binding and competition with other dsRNA-binding proteins (Unpublished data from Dr. Singh).

Just as excess ADAR activity can be harmful, lack of ADAR activity also has deleterious consequences. Genetically modified mice lacking ADARs die during either embryonic development (ADAR1) or shortly after birth (ADAR2), demonstrating the critical physiological importance of A-to-I RNA editing (Hartner et al., 2004; Higuchi et al., 2000; Seeburg and Hartner, 2003; Wang et al., 2000). While the GluR-2 Q/R site in wild-type mice is edited to nearly 100%, mice harboring a single non-editable GluR-2 allele developed seizures and die by three weeks of age (Brusa et al., 1995). In addition, clinical studies have associated alterations in ADAR editing with a number of CNS disorders including depression, schizophrenia, seizure susceptibility and amyotrophic lateral sclerosis (Gurevich et al., 2002; Iwamoto and Kato, 2003; Kawahara et al., 2004; Kwak and Kawahara, 2005; Niswender et al., 2001; Sodhi et al., 2001; Vissel et al., 2001). All of these studies demonstrate that an optimal level of A-to-I editing is required for normal CNS function. Using computer-based algorithms, newly identified ADAR targets in the coding and non-coding regions of human and mouse transcriptsomes have increased the number of known A-to-I modification events by at least an order of magnitude (Athanasiadis et al., 2004; Blow et al.,

2004; Clutterbuck et al., 2005; Kim et al., 2004; Levanon et al., 2004; Levanon et al., 2005; Morse et al., 2002). Identification of these new ADAR targets strongly suggests that ADARs may play important roles in a broader range of biological process than previously speculated and underscores the need for an organism to tightly regulate ADAR activity.

Several lines of evidence have provided evidence for diverse cellular mechanism to modulate ADAR2 activity. ADAR2 is preferentially concentrated in the nucleolus, but can shuttle readily from the nucleolus to the nucleoplasm in response to increased expression of ADAR2 targets (Desterro et al., 2003). Translocation from the nucleolus to the nucleoplasm results in increased editing for ADAR2 substrates, suggesting that ADAR2 activity can be functionally regulated by subnuclear sequestration (Desterro et al., 2003; Sansam et al., 2003). In addition, two alternative splicing events also have been demonstrated to affect ADAR2 activity in the cell. One such splicing event, in the region encoding the deaminase domain, increases the enzymatic activity of ADAR2 by two-fold (Gerber et al., 1997); whereas the other splicing event is dependent upon the ability of ADAR2 to edit its own pre-mRNA, thereby promoting the insertion of a 47-nucleotide cassette near the 5'-end of the open reading frame to abort translation prematurely (Rueter et al., 1999). To determine whether ADAR2 autoediting and subsequent alternative splicing represent important regulatory mechanisms for modulating ADAR2 protein expression and activity, our laboratory has generated mutant mice in which the ability of ADAR2 to edit its own transcript has been selectively ablated.

To eliminate the ability of ADAR2 to generate a proximal 3'-splice site preceding exon 5, we disrupted the predicted RNA duplex required for A-to-I conversion using Cre-mediated recombination to remove the evolutionarily conserved ECS region in intron 4 (Dawson et al., 2004; Rueter et al., 1999; Slavov and Gardiner, 2002). In the current study, deletion of the ECS region in mice efficiently eliminated editing at the -1 site (Figure 14) and inclusion of the 47-nucleotide cassette in mature ADAR2 transcripts (Figure 15), indicating that the ECS element is essential for the -1 site editing and subsequent alternative splicing *in vivo*. Ablation of -1 site editing promoted the use of the distal 3'-splice site immediately preceding exon 5 (Figure 14), to allow exclusion of the 47-nucleotide cassette and increase the relative expression of the ADAR2 mRNA isoform (3/5 -47) that encodes the full-length, functional protein (Figure 17). Accordingly, increased ADAR2 protein expression in multiple tissues (Figure 16) and a concomitant increase in the editing of ADAR2 substrates were observed in ΔECS mice (Table 4), strongly supporting the hypothesis that ADAR2 autoediting serves as a negative feedback mechanism for modulating ADAR2 protein expression.

The possibility that the increased ADAR2 expression in ΔECS mice resulted from alterations in the steady-state level of ADAR2 transcripts, via changes in RNA stability or transcription, were ruled out by real-time PCR analysis; no change in the level of ADAR2-derived RNAs was observed in most tissues, with the exception of the brain, where the level of mature ADAR2 mRNA was decreased by 30% when compared to control animals (Figure 17C). Decreased

levels of GluR-2 mRNA have been reported previously in mice with a deletion of the ECS element required for GluR-2 editing (Q/R site), due to nuclear accumulation of incompletely processed primary GluR-2 transcripts (Brusa et al., 1995). A similar decrease in GluR-2 mRNA expression was also observed in ADAR2-null mice, where a low level of GluR-2 editing (Q/R site) was observed (Higuchi et al., 2000), indicating that RNA editing may be a prerequisite for efficient splicing and processing of GluR-2 pre-mRNAs (Higuchi et al., 2000). Ribonuclease protection analysis of ADAR2 RNA isoforms from wild-type mouse brain indicated that the transcripts encoding the full-length functional protein (3/5 -47) represented 26% of the total ADAR2 transcript population, which include both mature and unprocessed ADAR2 mRNA (Figure 17B). Since quantitative RT-PCR analysis further indicated that the same ADAR2 mRNA isoform (3/5 -47) comprised 26% of the total mature mRNA, these data would suggest that unprocessed precursors in wild-type animals represents only a small percentage of the total ADAR2-derived RNAs. By contrast, analysis of ΔECS mice revealed no detectable RNA isoforms with the 47-nucleotide cassette and only 2.5% of the transcripts containing exon 4 (Figure 15), indicating that the 332 nt protected species observed from RNase protection analysis of ΔECS mice (Figure 17; 22% of total ADAR2 RNAs) almost exclusively represented ADAR2 pre-mRNA. These data indicated that the ablation of ADAR2 autoediting results in an accumulation of unprocessed RNA precursors, as with mutant mice in which GluR-2 (Q/R site) editing was eliminated. The hypothesized inefficient RNA processing due to lack of A-to-I editing could explain the decreased level of mature ADAR2 transcripts in

RNA isolated from ΔECS mouse brain (Figure 17). Further experiments directly quantify the relative level of ADAR2 pre-mRNA will provide more information about how RNA editing at the -1 site affects on ADAR2 transcript maturation.

Consistent with the increased expression of ADAR2 protein, editing efficiencies at ADAR2 specific sites are elevated in the brain of ΔECS mice. In addition, a decrease in ADAR1-mediated editing was also observed at the A-site of the 5-HT_{2C} receptor transcripts, which is the only known substrate containing both ADAR1- and ADAR2-specific sites within close proximity (13 nt). Previous studies had shown that editing at the D- site, a specific ADAR2 site, was elevated in mice lacking ADAR1 expression, and *in vitro* experiments also demonstrated that increased expression of ADAR1 or ADAR2 can decrease the editing efficiency of the D- or A-sites on 5-HT_{2C} receptor RNA duplex, respectively, suggesting a competition between ADAR1 and ADAR2 on 5-HT_{2C} R transcripts (Chen et al., 2000; Hartner et al., 2004). Our *in vivo* data is consistent with this hypothesis as the ΔECS mice showed decreased editing of the ADAR1-selective A-site on 5-HT_{2C} R transcripts. Interestingly, no alteration was detected for the editing at the +60 site of the GluR-2 transcripts, even though a specific ADAR2 site (Q/R site) is located within the same predicted RNA duplex. The +60 and the Q/R sites in GluR-2 pre-mRNA are 60 nt apart compared with the A- and D-sites in the 5-HT_{2C}R transcript which are within 13 nt of one another (Figure 4). Since previous studies have demonstrated that the minimal length of an RNA duplex for ADAR binding is 15-20 bp (Nishikura et al., 1991), we reasoned that while ADAR1 and ADAR2 can bind independently on the GluR-2 duplex without

interfering with each other, whereas their binding sites overlap on in the 5-HT_{2C} receptor pre-mRNA.

Despite significant alterations in the editing of ADAR substrates in ΔECS mice (Table 4), no overt phenotypic alterations were observed in mutant mice from birth through adulthood. Since all altered editing events are limited to the CNS (Table 4), we assessed alterations in animal behavior using a gross examination based upon the neurological screen developed by Irwin (Irwin, 1968). In addition, body weight analysis was performed on the wild-type and ΔECS mice from the age of 4 to 35 weeks and revealed no differences between the two genotypes. However, it is too early to draw any conclusion about how ablation of ADAR2 autoregulation affects mouse physiology. For mouse growth rate studies, animals were maintained on breeder's chow, a relatively high fat diet which could promote growth in mice and mask any differences between wild-type and mutant animals. Repeating these studies with an altered diet could provide give us a more conclusive answer on whether ΔECS mice have deficits in growth compared to wild-type animals.

Comparisons of ADAR2 genes between multiple vertebrate species (rat, mouse, chicken, zebrafish, pufferfish and humans) have revealed >90% intronic sequence conservation in the predicted region of the inverted repeats, with a majority of the nucleotide differences clustered in predicted bulge regions within the duplex (Dawson et al., 2004; Rueter et al., 1999; Slavov and Gardiner, 2002) (Figure 11). Furthermore, alternative 3'-splice site selection has also been shown to be conserved among these species (Slavov and Gardiner, 2002), suggesting

that ADAR2 autoediting and alternative splicing represent an important biological process to modulate ADAR2 protein expression in vertebrates. Recent studies of the *Drosophila* ADAR gene (dADAR) have demonstrated that the dADAR enzyme is capable of editing its own mRNA to generate a protein isoform with a serine-to-glycine substitution close to the dADAR active site (Keegan et al., 2005). This single amino acid alteration appears to restrict dADAR function, since the glycine-containing isoform encoded by the edited dADAR mRNA is less active than the genomically-encoded protein product. Ubiquitous or muscle-specific expression of a dADAR transcript that cannot be modified by A-to-I conversion results in increased editing of a voltage-gated calcium channel transcript (Ca alpha 1D) and subsequent lethality (Keegan et al., 2005), demonstrating that the ability of dADAR to edit its own mRNA is critical for *Drosophila* viability. In an example of what appears to be convergent evolution, dADAR and ADAR2 have developed completely different autoediting strategies to modulate ADAR activity. The difference in phenotypic consequences between these species could result from the fact that the transgenic dADAR2 expression in drosophila could be much higher than mouse ADAR2 expression in ΔECS mice. In addition, despite multiple unbiased approaches undertaken to identify more ADAR substrates in vertebrates, only two novel nonsynomous A-to-I conversions were discovered in transcripts encoding proteins that are putatively important in the CNS (Levanon et al., 2005). However, many more transcripts encoding proteins involved in rapid neurotransmission have been found to undergo A-to-I editing in drosophila (Hoopengardner et al., 2003), suggesting that the role of

ADAR in drosophila CNS may be more critical than that in vertebrates. Alternatively, compensatory alterations in vertebrates could serve to circumvent the adverse phenotypic consequences that may result from increases in ADAR2 expression and alterations in editing patterns for ΔECS mice.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

A-to-I RNA editing is the most widespread RNA editing modification identified in metazoa. Mice lacking ADAR1 expression die during embryonic development and ADAR2-null mice died from postnatal seizure, demonstrating the critical physiological significance of ADAR function (Hartner et al., 2004; Higuchi et al., 2000; Seeburg and Hartner, 2003). In mammals, ADAR-mediated RNA editing has been shown to alter the coding potentials of transcripts encoding proteins that are important to normal CNS functions, including 5-HT_{2C} receptor, iGluRs and K_V1.1. In Drosophila, most known ADAR substrates are transcripts encoding channels, receptors and synaptic proteins that are involved in fast electrochemical transmission (Hoopengardner et al., 2003). Moreover, A-to-I RNA editing has also been shown to alter the splicing pattern of pre-mRNAs encoding a non-receptor protein tyrosine phosphatase and ADAR2 (Beghini et al., 2000; Rueter et al., 1999). In addition to affecting RNA coding potential and splicing pattern, ADARs also have been linked to the RNAi pathway in C. elegans (Tonkin and Bass, 2003). Systematic screening for novel A-to-I conversion using either computational algorithms or a massive sequencing approach revealed numerous novel A-to-I editing sites in human, mice and

chicken transcriptsome (Blow et al., 2004; Levanon et al., 2004; Levanon et al., 2005). The majority of these newly-identified A-to-I conversions are predicted to occur within the non-coding region of RNA transcripts. Interestingly, previous studies have demonstrated that edited RNA may be selectively retained in the nuclei in mammalian cells and subsequently degraded by inosine-specific ribonuclease (Kumar and Carmichael, 1997; Scadden and O'Connell, 2005; Scadden and Smith, 2001). More recently, the 3'UTR of cationic amino acid transporter 2 (CAT2) transcribed nuclear RNA (CTN-RNA) was found to be subjected to ADAR editing and play an important role in nuclear retentions of the transcript (Prasanth et al., 2005), suggesting the A-to-I conversions in the UTRs of transcripts may be involved in controlling the RNA stability and nuclear transport.

The ability of ADARs to produce alterations in the coding potentials of RNA transcripts, introduce alternative splicing patterns or modulate RNA stability could be deleterious to the organism if it were non-specific or unregulated. Therefore a number of mechanisms have been evolved to tightly regulate ADAR activity. Numerous studies have demonstrated that ADAR1 expression and activity are regulated at the transcriptional, post-transcriptional as well as post-translational levels. The transcription of ADAR1 is controlled by one of the two alternative promoters, Pi and Pc, which drive the production of transcripts encoding an IFN-inducible 150kD protein that can shuttle between the nucleus and cytoplasm, or a constitutive 110kD nuclear protein respectively (George and Samuel, 1999; Patterson and Samuel, 1995; Patterson et al., 1995; Thomis et al., 1992). The

IFN-inducible transcripts undergo alternative splicing modifications, which generates multiple mRNA isoforms encoding proteins that possess comparable catalytic activity but differ in the spacer regions between dsRBDs and deaminase domains (Liu et al., 1997). Two post-translational mechanisms have been identified to regulate ADAR1 activity. Firstly, it has been demonstrated that ADAR1 is highly concentrated in the nucleolus of mammalian cells and the expression of an editing-competent ADAR substrate can relocate the endogenous ADAR1 from the nucleolus to nucleoplasm where the substrate transcripts accumulate, suggesting that the nucleolus can serve as a storage spot for ADAR1, while the editing reaction takes place in the nucleoplasm (Desterro et al., 2003). Interestingly, recent studies have also demonstrated that the ADAR1 is modified by SUMO-1 in the nucleolus, and sumoylation decreases ADAR1 editing activity (Pinto Desterro et al., 2005). Therefore, it is possible that these two mechanisms may act synergistically to restrain ADAR1 activity in the nucleolus and regulate the availability of ADAR1 protein at sit of action *in vivo*.

In comparison to studies on ADAR1, limited information exists regarding the regulation of ADAR2 expression and activity. Similar to ADAR1, ADAR2 activity appears to be modulated by nucleolar sequestration (Desterro et al., 2003; Sansam et al., 2003); however, since ADAR2 protein doesn't have the motif required for SUMO-1 mediated modification, it is unlikely that ADAR2 activity is affected by sumoylation (Pinto Desterro et al., 2005). In addition to subnuclear localization, ADAR2 expression and activity are also regulated by alternative splicing. One such splicing event results in the insertion of 30 (human) or 10

(mouse, rat) amino acids in the deaminase domain of ADAR2 and leads to the production of protein with increased activity. However, it is not clear if this alternative splicing is regulated by any cellular or molecular cues. The other alternative splicing event in the 5'-end of the coding region has been identified as a subsequent event of ADAR2 editing on its own pre-mRNA. This alternative splicing results in the insertion of a 47nt cassette in the open reading frame, causing a frame shift and eventually resulting in premature translation termination (Rueter et al., 1999). In the current studies, we have demonstrated that the ADAR2 autoediting and subsequent alternative splicing represent a negative-feedback regulatory mechanism by which ADAR2 expression and activity are modulated *in vivo*. In the ΔECS mice, where ADAR2 autoediting has been selectively ablated, ADAR2 protein expression increases in multiple tissues, and ADAR2-selective RNA editing is also elevated in the brain. The observed increases in ADAR2 protein expression correlate with the extent of ADAR2 autoediting observed in wild-type tissues, indicating that ADAR2 autoediting is a key regulator of ADAR2 protein expression and activity *in vivo*.

It is commonly believed that the process of negative feedback provides a noise-reduction mechanism for biological systems and can serve to minimize the effects of fluctuations caused by cellular disturbance (Becskei and Serrano, 2000; Kaern et al., 2005; Paulsson, 2004; Simpson et al., 2003). The ability of ADAR2 to edit its own pre-mRNA not only provides a mechanism to limit ADAR2 protein expression, but also serves as a strategy by which A-to-I RNA editing activity may be modulated in response to changes in the expression of ADAR substrates

or other RNA binding proteins. It has been demonstrated that different substrates can compete with one another for ADAR binding and activity (Dawson et al., 2004). In a situation where ADAR substrates expression is increased, editing at the -1 site in ADAR2 pre-mRNA would be inhibited, resulting in the increased production of full-length, functional ADAR2 protein. In this aspect, the ADAR2 autoediting could serve as a sensor to monitor the expression of numerous ADAR substrates in the nucleus, thereby modulating ADAR2 activity to maintain steady-state levels of A-to-I conversion (Figure 21).

Future directions

Genetically modified mice lacking ADAR2 autoregulation (ΔECS mice) provide an excellent model for studying the self-regulatory mechanisms modulating ADAR2 expression and activity *in vivo*. Further molecular analysis and behavioral characterization of these mutant animals will provide further insight regarding the regulation of A-to-I RNA editing in different tissues for distinct ADAR substrates and the physiological significance of ADAR2 autoregulation.

Editing efficiency of recently identified substrates

Four codon-altering A-to-I RNA editing events were recently identified in genes encoding FLNA, BLCAP, CYFIP2 and IGFBP7 (Clutterbuck et al., 2005; Levanon et al., 2005). Unlike previously identified ADAR substrates in mammals, none of these RNAs encodes a receptor protein. Among the four substrates, FLNA and

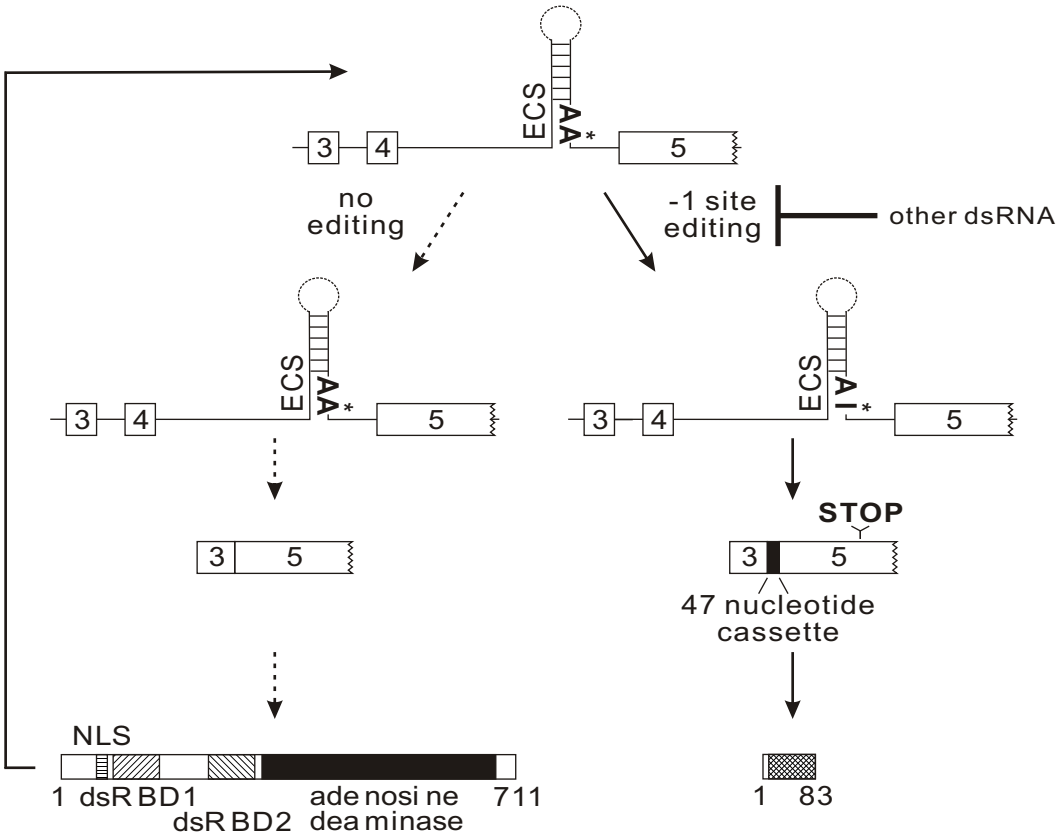


Figure 21 Model of ADAR2 autoregulation.
A schematic diagram of the biosynthetic processes involved in the production of ADAR2 is presented, showing a region of the ADAR2 pre-mRNA between exon 3 and 5, the predicted RNA duplex required for A-to-I editing, and the position of the -1 site (*). The RNA processing pathway lacking -1 site editing, leading to the production of full-length (711aa) ADAR2 is indicated with dashed arrows; the functional domains in ADAR2 (NLS, nuclear localization signal; dsRBD, double-stranded RNA binding domains and adenosine deaminase domain) are indicated. The biosynthetic pathway involving A-to-I conversion at the -1 site (*), leading to the production of a predicted 9 kD (83aa) protein, is indicated with solid arrows; the hatched box represents the amino acid sequences encoded by an altered reading frame resulting from proximal 3'-splice acceptor use and inclusion of an additional 47nt in the mature ADAR2 transcript.

CYFIP2 are strongly expressed in the CNS and transcripts isolated from mouse brain contain both edited and non-edited RNA isoforms (Levanon et al., 2005). FLNA has been shown to cross-links actin into orthogonal networks in the cortical cytoplasm (Hartwig and Stossel, 1975) and participates in the anchoring of membrane proteins (van der Flier and Sonnenberg, 2001). A-to-I modification in FLNA transcripts result in a Q/R substitution within the 22nd immunoglobulin-like domain, which has been previously shown to be important for binding to the small GTPase Rac1 (Ohta et al., 1999), and interact with the *Drosophila* homologue of CYFIP2 (Schenck et al., 2003). BLCAP encodes a 10kD globular protein containing two highly hydrophobic regions, a proline-rich area that resembles the src homology 3 domain binding site and a SPXX motif which is usually found in transcription factors (Gromova et al., 2002). Differential expression of BLCAP mRNA has been found in bladder cancer and renal cancer cell lines (Gromova et al., 2002; Rae et al., 2000). Multiple editing sites have been predicted in the 5'-UTR and the N-terminal region of the protein and verified only in RNA isolated from the brain. The three editing sites in the coding region all represent non-synonymous amino acid alterations (Q/R, Y/C and K/R) and are well conserved from *C. elegans* to human, suggesting a biological relevance for RNA editing in regulating the function of this gene (Clutterbuck et al., 2005). As we have shown that extent of editing is altered in the brains of ΔECS mice for multiple ADAR substrates, it will be worthwhile to not only compare the editing of FLNA, CYFIP2 and BLCAP RNAs isolated from the brain of wild-type and ΔECS mice, but also

subsequent studies to determine the effects of such A-to-I modifications on the function of the encoded protein products.

The RNA editing event in IGFBP-7 transcripts is of particular interest since this RNA modification takes place in the lung (Levanon et al., 2005), since ΔECS mice show the greatest increase of ADAR2 protein expression in the lung, quantification of IGFBP-7 RNA editing will give some insight on whether ADAR2 is responsible for this RNA modification and serve as a preface to determine how ADAR2 autoediting can affect IGFBP-7 activity and lung function.

Regulation of A-to-I RNA editing in peripheral tissues

The absence of the I/V site editing of K_v1.1 RNAs in peripheral tissues, despite the presence of ADAR2 protein, suggests that there may be other regulatory mechanisms to modulate A-to-I RNA editing in tissues other than the brain. The coexpression of ADAR3 and K_v1.1 in some areas of the brain suggests that ADAR3 may serve as a modulator in ADAR2-mediated K_v1.1RNA editing (I/V site) in the brain. However, we can not rule out the possibility that a peripheral tissue-specific inhibitory factor could suppress I/V editing or that the expression of ADAR2 protein in peripheral tissues does not reach a threshold for efficient K_v1.1 editing. Recent studies have demonstrated that RNA editing in BLCAP transcripts is also brain specific, despite the ubiquitous expression of this gene (Clutterbuck et al., 2005), indicating that further investigation is needed to address the possible mechanisms regulating ADAR2 editing in peripheral tissues.

Functional consequence of altered K_v1.1 RNA editing in ΔECS mice

The substitution of isoleucine residue with a valine moiety fasten the K_v1 potassium channel re-sensitization process by 20 fold in a heterologous expression system, suggesting that I/V site editing in transcripts encoding the α subunit of this channel is important in regulating the homomeric K_v1 channels kinetics (Bhalla et al., 2004). In ΔECS mice, editing at the I/V site of K_v1.1 transcripts was elevated by 10%, presumably leading to a faster recovery from inactivation in comparison to the K_v1 channels in the wild-type animals. The function of K_v1 voltage-gated potassium channel has been implicated in learning process and amphetamine-elicited neurobehavioral effects (Kourrich et al., 2005; Pirisino et al., 2005). Mice lacking the $K_v\beta$1.1 subunit have a reduced K^+ current inactivation in hippocampal CA1 pyramidal neurons and demonstrate impaired learning ability in both water maze test and social transmission of food preference task (Giese et al., 1998). Since faster recovery from inactivation, resulting from I/V site editing, could lead to a similar reduced K+ current inactivation to that observed in the $K_v\beta$1.1-deficient mice, ΔECS mice may have similar learning defects due to increased editing in the α subunit of K_v1 channel. Therefore, electrophysiological characterizations on CA1 pyramidal cells in the hippocampal slices and behavior analyses on the learning behavior for wild-type and ΔECS mice should be performed to provide further information regarding the physiological and behavioral consequences of altered K_v1.1 RNA editing due to ADAR2 overexpression.

Potential compensatory mechanisms to ADAR2 autoregulation deficiency

Studies described in this thesis have demonstrated that although ΔECS mice reveal obvious changes in ADAR2 expression and activity, no behavioral alteration has been identified. ADAR2 autoediting is conserved from pufferfish to human (Slavov and Gardiner, 2002), suggesting an evolutionary significance of this RNA modification in a wide array of species. It is possible that the absence of behavioral alterations in ΔECS mice results from activation of compensatory mechanisms to counteract the adverse consequence due to overexpression of ADAR2. As demonstrated in Figure 20, ADAR2 autoediting is developmentally regulated and the relative abundance of mRNA isoforms containing the 47nt cassette gradually increases from 15% (E15) to 75% (adult). Since ADAR2 autoediting is a key regulator of ADAR2 protein expression, and the increase in ADAR2 protein in ΔECS mice can be predicted by extends of 47nt inclusion in the wild-type counterparts (Figure 16), it is likely that ADAR2 protein expression will increase gradually elevated during postnatal development in ΔECS mice. Therefore, compensatory mechanisms may be activated and adapt to the ADAR2 protein overexpression during postnatal development in ΔECS animals.

To prevent the development of possible adaptive compensation during postnatal development, we can take advantage of the cre-loxP system for the inducible ablation of ADAR2 autoediting using Cre recombinase under the control of an inducible promoter containing Tetracycline- or estrogen-response elements. Mice carrying the lox-ECS-lox allele (Figure 13) can be mated with inducible cre

transgenic mice to generate offspring (lox-ECS-lox/iCre) in which the ECS region can be removed in a temporally-inducible fashion. Cre recombinase expression can be activated in adult lox-ECS-lox/iCre mice, and molecular/behavioral analysis can be performed on mice with/without Cre recombinase expression to reveal the molecular and physiological consequence resulting from sudden ablation of ADAR2 autoediting.

APPENDIX

Irwin screen examination

I. Physical factors and gross appearance

a. Coat color
   - A=albino
   - Ag=Agouti
   - Bl=Black

b. Presence of whiskers
   - 0=none
   - 1=a few
   - 2=most, but not a full set
   - 3=a full set

c. Appearance of fur
   - 0=un-groomed and disheveled
   - 1=somewhat disheveled
   - 2=well-groomed

d. Piloerection
   - 0=none
   - 1=most hairs standing on end

e. Patches of missing fur on face
   - 0=none
   - 1=some
   - 2=extensive

f. Patches of missing fur on body
   - 0=none
   - 1=some
   - 2=extensive

g. wounds
   - 0=none
   - 1=signs of previous wounding
   - 2=slight wounds present
   - 3=moderate wounds present
   - 4=extensive wounds present

II. Observation of behavior in a novel environment

a. Transfer behavior
   - 0=coma
   - 1=prolonged freeze (>10sec.), then slight movement
   - 2=extended freeze, then moderate movement
   - 3=brief freeze (a few seconds), then active movement

4=momentary freeze, then swift movement
5=no freeze, immediate movement
6=extremely excited (“manic”)

b. Body position
   0=completely flat
   1=lying on side
   2=lying on back
   3=sitting or standing
   4=rearing on hind legs
   5=repeated vertical leaping
c. Spontaneous activity
   0=none, resting
   1=casual scratch, groom, slow movement
   2=vigorous scratch, groom, meoderate movement
   3=vigorous rapid/dart movement
   4=extremely vigorous, rapid/dart movement
d. Respiration rate
   0=gasping, irregular
   1=slow, shallow
   2=normal
   3=hyperventilation
e. Tremor
   0=none
   1=mild
   2=marked
f. Palpebral closure
   0=Eyes wide open
   1=Eyes ½ closed
   2=Eyes closed
g. Piloerection
   0=none
   1=coat stood on end
h. Gait
   0=normal
   1=fluid but abnormal
   2=limited movement only
   3=incapacity
i. Pelvic elevation
   0=markedly flattened
   1=barely touches
   2=normal (3mm elevation)
   3=elevated (more than 3mm elevation)
j. Tail elevation
   0=dragging
   1=horizontally extended
   2=elevated

k. Urination
    0=none
    1=little
    2=moderate
    3=extensive

III. Reflexes and reactions to simple stimuli

a. Touch escape
    0=no response
    1=mild (escape response to firm stroke)
    2=moderate (rapid response to light stroke)
    3=vigorous (escape response to approach)
b. Positional passivity
    0=struggles when restrained by tail
    1=struggles when restrained by neck (finger grip, no scruffed)
    2=struggles when held supine (on back)
    3=struggles when restrained by hind legs
    4=Does not struggle
c. Trunk curl
    0=absent
    1=present
d. Reaching reflex
    0=None
    1=Upon nose contact
    2=Upon vibrasse contact
    3=before vibrasse contact (18mm)
    4=Early vigorous extension (25mm)
e. Body tone
    0=Flaccid, no return of cavity to normal
    1=Slight resistance
    2=Extreme resistance, board like
f. Pinna reflex
    0=None
    1=Active retraction, moderately brisk flick
    2=hyperactive, repetitive flick
g. Preyer reflex
    0=None
    1=Active retraction, moderately brisk flick
    2=Hyperactive, repetitive flick

IV. Measures recorded during supine restraint

a. Skin color
    0=Blancked
    1=Pink
    2=Bright, deep red flush
b. Limb tone

0=Slow, bradycardia
1=Normal
2=Fast, tachycardia

c. Abdominal tone
   0=No resistance
   1=Slight resistance
   2=Moderate resistance
   3=Marked resistance
   4=extreme resistance
d. Righting reflex
   0=No impairment
   1-10=Number of seconds required to right
e. Air righting reflex
   0=No impairment
   1-10=Number of seconds required to right

V. Grip strength, motor coordination, and locomotor activity

a. Grip strength
   0=None
   1=Slight grip, semi-effective
   2=Moderate grip, effective
   3=Active grip, effective
   4=Unusually effective
b. Wire maneuver
   0=Active grip with hindlegs
   1=Difficulty to grasp with hindlegs
   2=Unable to grasp with hindlegs
   3=Unable to lift hindlegs, falls within seconds
   4=Falls immediately
c. Wire hang
   0=Active grip with hindlegs
   1=Difficulty to grasp with hindlegs
   2=Unable to grasp with hindlegs
   3=Unable to lift hindlegs, falls within seconds
   4=Falls immediately
d. Inverted screen
   0-60=Numbers of seconds before falling

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