Regulation and Functional Consequences of $mGlu_4$ RNA Editing

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For my parents, Bruce and Cathryn Hofmann.

I could only do this because of you.

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List of Abbreviations

Abbreviation	Definition
5-FU	5-Fluorouracil
5-HT_{2C}	5-hydroxytryptamine receptor 2C
7TM	7 transmembrane
ACPD	(1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid
ACPT-I	(1S,3R,4S)-1-Aminocyclopentane-1,3,4-tricarboxylic acid
ADAR	Adenosine Deaminases Acting on RNA
ADHD	Attention Defecit/Hyperactivity Disorder
AED	Anti-Epileptic Drugs
AGS	Aicardi-Goutieres syndrome
AIMP2	Aminoacyl TRNA Synthetase Complex Interacting Multifunctional Protein 2
ALS	amyotrophic lateral sclerosis
Alu	mobile, repetitive genetic element of the SINE class and specific to primates
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism Spectrum Disorder
ATD	Amino Terminal Domain
A-to-I	Adenosine-to-Inosine
BodV	Borna disease virus-1
BRET	Bioluminescence Resonance Energy Transfer
CA	Cinnabarinic Acid
CaBP1	Calcium binding protein 1
Cadps1	Calcium-Dependent Activator Protein for Secretion 1
CALM1	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CaSR	Ca^{2+} Sensing Receptor
$Ca_V1.3$	Calcium channel voltage-dependent L type Cav1.3 alpha 1D subunit
CBX4	Chromobox protein homolog 4
cDNA	Complementary DNA
CDS	Coding Sequence
CFA	complete Freund's adjuvant
CLIP	Genetically modified Human O^6-methylguanosine-DNA alkyl transferase (hAGT)
CPP	Conditioned Place Preference
CPu	Caudate Putamen
CNS	Central Nervous System
CODA-RET	Complemented Donor Acceptor resonance energy transfer
CRD	Cysteine Rich Domain
CREB	cAMP-response element binding protein
C-to-U	Cytosine-to-Uridine
Cyfip2	Cytoplasmic FMR1 Interacting Protein 2
DBI	Diffuse Brain Injury
DCV	Dense Core Vesicle
DDX15	Pre-mRNA-splicing factor ATP-dependent RNA helicase ddx-15
DNA	Deoxyribonucleic Acid
DOI	2,5-Dimethoxy-4-iodoamphetamine
DSH	dyschromatosis symmetrica hereditaria
dsRBD	double stranded RNA binding Domain
dsRMB	double stranded RNA binding motif
DUSP1	Dual Specificity Phosphatase 1
DZF	domain associated with zinc fingers

EAE	Experimental Autoimmune Encephalitis
ECS	Editing Complementary Sequence
EGR1	Early Growth Response 1
ELFN1	Extracellular Leucine Rich Repeat and Fibronectin Type III Domain Containing 1
ELFN2	Extracellular Leucine Rich Repeat and Fibronectin Type III Domain Containing 2
ER	Endoplasmic Reticulum
ESS	Evolutionary Selected Sites
FLNA	Filamin-A
FRET	Förster Resonance Energy Transfer
GABA	gamma-Aminobutyric acid
GDP	Guanosine-5'-Biphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GIRK	G Protein-Coupled Inwardly-Rectifying K^+ Channel
GPCR	G Protein-Coupled Receptor
Gria2	Glutamate Ionotropic Receptor AMPA Type Subunit 2
GRIP	glutamate receptor interacting protein
GRK	G protein Receptor Kinase
GRM4	Metabotropic glutamate receptor 4, human gene
Grm4	Metabotropic glutamate receptor 4, mouse gene
GTEx	The Genotype-Tissue Expression (GTEx) project
GTP	Guanosine-5'-Triphosphate
GWAS	Genome Wide Association Study
HD	Heptahelical Domain
HEK293T	Human Embryonic Kidney 239 w/ T Antigen
HELA	Henrietta Lacks (Human Cervical Cancer cell line)
HEPG2	Human Caucasian hepatocyte carcinoma
HTMTA	High Throughput Multiplexed Transcript Analysis
IFIH1/ MDA5	Interferon Induced With Helicase C Domain 1
IL10	Interleukin 10
TBI	Traumatic Brain Injury
IL12	Interleukin 12
iL2	Intracellular Loop 2
IL23	Interleukin 23
iL3	Intracellular Loop 3
ILF2	Interleukin enhancer-binding factor 2
ILF3	Interleukin enhancer-binding factor 2
INS	Rat Insulinoma Cell Line
IP6	Inositol Hexaphosphate
JNK	c-Jun N-terminal kinase
K562	Human Caucasian chronic myelogenous leukaemia
KA	Kainic Acid
KO	Knockout
$K_V1.1$	Potassium voltage-gated channel subfamily A member 1
L-AP4	L-2-amino-4-phosphonobutyric acid
LB1	Top lobe of mGlu LBD
LB2	Bottom Lobe of mGlu LBD
LBD	Ligand Binding Domain
L-DOPA	l-3,4-dihydroxyphenylalanine
LPS	Lipopolysaccharide
LRET	Lanthanide Resonance Energy Transfer

L-SOP	L-serine-O-phosphate
LTD	long-term depression
LTP	long-term potentiation
MAP1B	Microtuble-associated protein 1B
MAP4	(S)-2-Amino-2-methyl-4-phosphonobutanoic acid
MAPK	mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MG-63	Human osteosarcoma cell line
mGlu	Metabotropic Glutamate Receptor
MHCII	major histocompatibility complex class II
miRNA	microRNA
mRNA	messenger RNA
MS	Multiple Sclerosis
MSG	monosodium L-glutamate
MUNC-18	Syntaxin-binding protein 1 (STXBP1)
NAc	Nucleus Accumbens
NAM	Negative Allosteric Modulator
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NMDA	N-methyl-D-aspartate
NOVA1	RNA-binding protein Nova-1
NOVA2	RNA-binding protein Nova-2
NRT	Nucleus reticularis thalami
OL	Oligodendroglial
OPRM1	Opioid Receptor Mu 1
P54(nrb)	54 kD nuclear RNA-binding protein
PAM	Positive Allosteric Modulator
PBP	periplasmic amino acid binding proteins
PD	Parkinson’s Disease
PHCCC	N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide
PI3K	Phosphoinositide 3-kinases
PICK1	Protein Interacting With PRKCA 1
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PKR	Interferon-induced, double-stranded RNA-activated protein kinase
PLC	Phospholipase C
PPF	paired Pulse Facilitation
Pre-mRNA	Pre-messenger RNA transcript
RADAR	rigorously annotated database of A-to-I RNA editing
RANTES	RANTES chemokine
RBP	RNA-binding proteins
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
RPS14	Ribosomal Protein S14
scRNAseq	Single Cell RNA sequencing
SDCDP	Syntentin 1
SE	Status Epilepticus
SF9	Spodoptera frugiperda pupal ovarian tissue cell line
SH-SY5Y	Human neuroblastoma cell line
SINE	short interspaced nuclear elements

SNAP	Genetically modified Human O^6-methylguanosine-DNA alkyl transferase (hAGT)
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF Attachment Protein Receptor
SNP	Single Nucleotide Polymorphism
SNr/c	Substantia Nigra pars reticulate/compacta
SRE	splicing regulatory elements
SRSF9	Serine/arginine-rich splicing factor 9
STN	Subthalamic Nucleus
STRBP	Spermatid perinuclear RNA-binding protein
STX1BP	Syntaxin 1-B
STY1	Mitogen-activated protein kinase sty1
SUMO-1	Small ubiquitin-related modifier 1
SYN1	Synapsin 1
TDP-43	TAR DNA-binding protein 43
TGF-β	Transforming growth factor beta
TMD	Transmembrane Domain
tRNA	Transfer RNA
TTX	Tetrodotoxin
U20S	Human Osteosarcoma
UTR	Untranslated Region
VBT	Ventrobasal Thalamus
VFT	Venus Fly Trap
WWP2	NEDD4-like E3 ubiquitin-protein ligase WWP2
ZFR	Zinc finger RNA-binding protein

Chapter I: Introduction

Historic Context

Following the landmark discovery of the structure of DNA by Watson and Crick in 1953 [1], a flurry of manuscripts were published elucidating not only the structural and sequence components of DNA we refer to as a gene, but, 22 years later, it was determined that was a subset of RNA, called messenger RNA (mRNA), carried the genetic information encoded by DNA to ribosomes, acting as the instructions for protein synthesis [2-10]. These mRNAs contained unique structural features, such as a 5' 7-methyl-guanosine cap [11,12] and a poly-adenylated 3' tail [13-15] , that modulate the stability of transcripts and were thought to be encoded directly by the sequence of DNA nucleotides from which they were transcribed.

In 1977, a shocking publication revealed that mRNA transcripts within the cytoplasm are actually stitched together from distinct areas of a gene, exons, after the removal of non-coding sequences, introns, creating a sequence which coded for the resultant protein–a process which could encode more than one protein from any one particular gene by the alternative splicing of select exons [16,17]. Indeed, this was the first discovery of an event which altered the sequence of nucleotides found within the DNA and, importantly, altered the sequence of amino acids within the resulting protein. Less than a decade later, another landmark discovery showed that mammalian transcripts were subject to base-specific modifications, demonstrating another method to alter the nucleotide sequence directly encoded by DNA [18]. Unlike RNA splicing, which affects the coding of larger stretches of amino acid sequence in a resultant protein, base-specific modification by RNA editing allows for alteration of specific codons encoding individual amino acids.

Adenosine-to-Inosine (A-to-I) editing is the most common RNA editing event within the mammalian central nervous system[19] and highly studied due to its ability to result in nonsynonymous amino acid substitutions, most commonly in synaptic proteins. To date, there

have been 29 such recoding events reported in human mRNA transcripts which are evolutionarily conserved in rodents, according to the RNA editing database *RADAR* [20]. Only 14 of these conserved 29 sites have a known function, and research into the effects of RNA editing is ongoing[21]. One of these conserved sites, discovered in 2013, predicts the substitution of Glutamine (Q) for Arginine (R) at amino acid 124 in metabotropic glutamate receptor 4 ($mGlu_4$) [22]. As chance would have it, the family of metabotropic glutamate receptors was discovered concurrently with mammalian RNA editing [23-25], and this thesis now stands at the convergence of these previously separate fields.

This work provides the first in-depth analysis of the extent of A-to-I editing of $mGlu_4$ transcripts, the *cis* and *trans* factors required for its regulation, and proposes a function for this conserved amino acid alteration. The following thesis is divided into four chapters: the first providing an extensive literature review of both A-to-I editing and mGlu receptor fields, the second encompassing the extent of editing of $mGlu_4$ transcripts and *cis*- and *trans*-acting regulatory factors, the third investigating the functional consequences of the Q124R amino acid substitution within the resulting protein, and the fourth providing implications for future research.

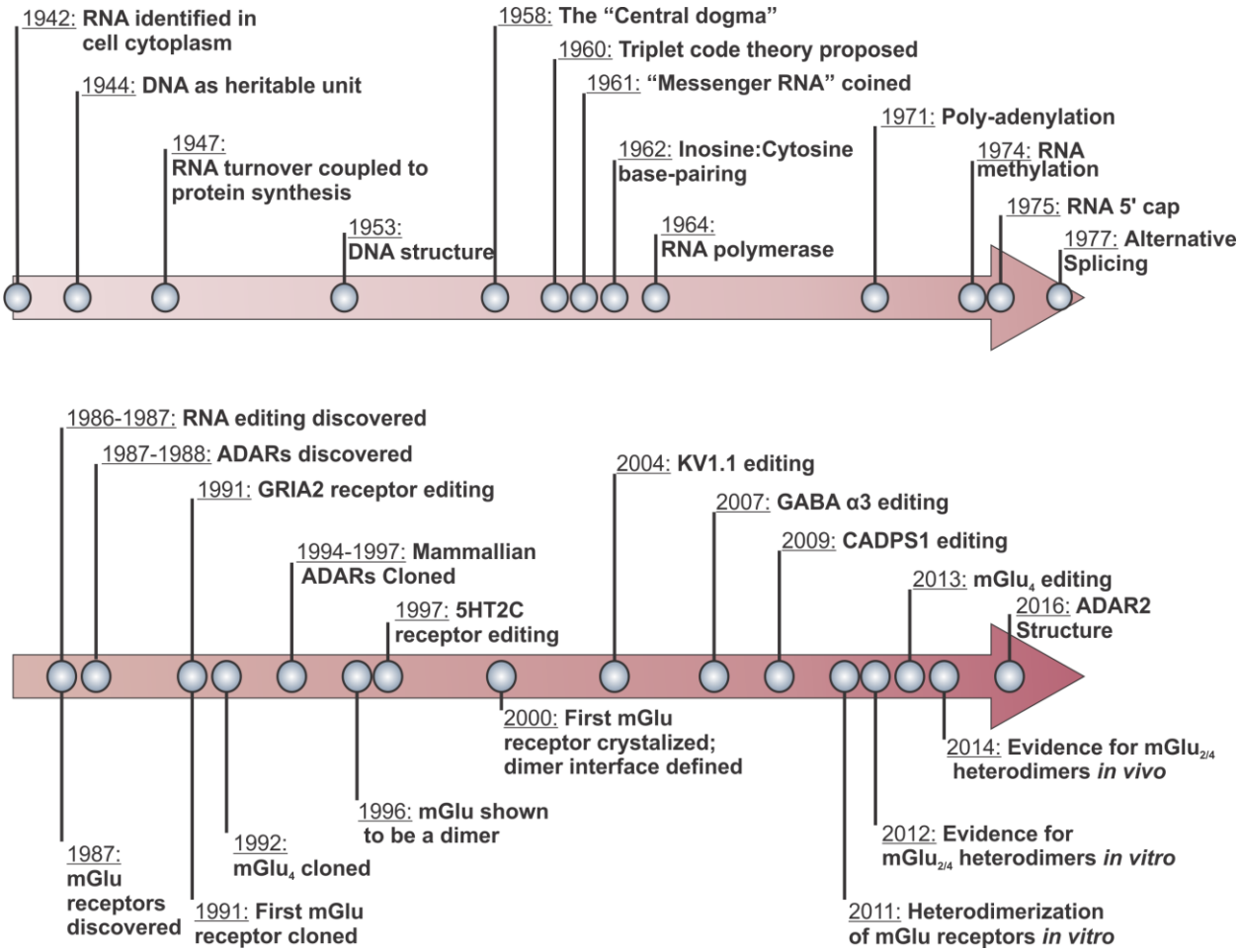

Figure 1: Major discoveries in RNA processing and metabotropic glutamate receptor biology

Timeline denoting the major discoveries in RNA processing and mGlu receptor biology. RNA processing discoveries are shown on the top half of the timeline and mGlu receptor discoveries are shown on the bottom half. Glutamate was recognized as a neurotransmitter in the 1970s and was suspected to act at 3 types of ionotropic receptors [26]. mGlu receptors were first discovered in 1987, concurrently with the discovery of RNA A-to-I Editing.

Adenosine-to-Inosine RNA Editing: Literature Review

Prior to maturation, precursor RNA molecules are subject to a host of co-transcriptional and post-transcriptional processing events. RNA editing consists of changes to the nucleotide sequence of an RNA molecule which alters its sequence from that which is encoded by the DNA; importantly, this does not include common RNA processing events such as capping, poly-adenylation, or splicing [27]. Two major types of RNA editing have been described: insertion/deletion editing [28], and base-specific deamination[29]. The insertion and deletion of RNA nucleotides has been described in trypanosomes, a member of kinetoplastid protozoa [28], but not within vertebrate species. Common forms of vertebrate RNA editing include the conversion of cytidine to uracil (C-to-U) and adenosine to inosine (A-to-I) by deamination [29]. Of these, Adenosine to Inosine (A-to-I) RNA editing is the most common form of RNA nucleotide-specific modifications in vertebrates, encompassing as much as ~94-99% of transcriptome modifications [19], and will be the focus of this literature review.

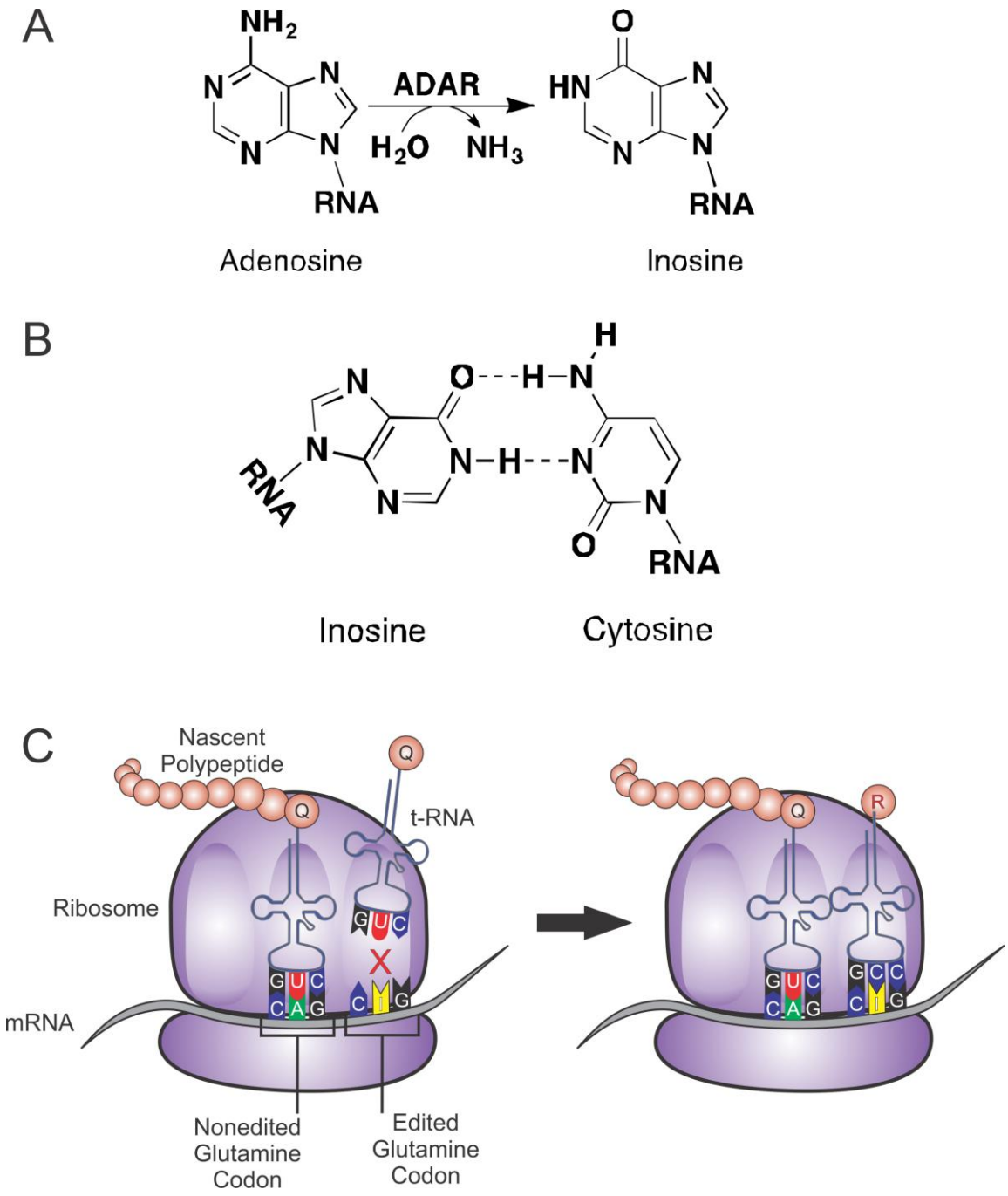

Figure 2: Site Specific Recoding by A-to-I Editing

A) Hydrolytic deamination of carbon 6 of adenosine residues by ADAR enzymes yields inosine. **B)** Inosine base pairs preferentially with cytosine. Two hydrogen bonds are formed in an I:C base pair. **C)** Inosine is recognized by the cellular machinery as a guanosine, and A-to-I editing is functionally an adenosine to guanosine mutation. When this occurs within the coding sequence of an mRNA transcript (shown in gray), recognition by t-RNA anticodons in the ribosome cause the resulting amino acid sequence to be “recoded”. Nucleotides are shown in black (Guanosine, “G”), Red (Uracil, “U”), Green (Adenosine, “A”), Blue (Cytosine, “C”), and yellow (Inosine, “I”).

A-to-I Editing

The site-specific deamination of adenosine (A) nucleotides to inosine is catalyzed by a family of three Adenosine Deaminase that Act on RNA (ADAR) enzymes[29]. Contrary to adenosine, inosine preferentially base pairs with cytosine (C) nucleotides rather than uridine (U); [30] for this reason, A-to-I editing has historically been considered functionally an adenosine to guanosine (G) transition. Indeed, A-to-I editing sites were historically discovered by identifying "A" to "G" discrepancies between DNA and RNA templates by Sanger sequencing [31]. Adenosine to inosine conversion can affect the structure, export, splicing, expression, and coding potential of RNA transcripts [29,32].

The formation of an extended RNA duplex structure is required for ADAR binding and modulates the specificity and efficiency of A-to-I catalysis [18,33-38]. These RNA structures are formed by the intermolecular base pairing interactions of neighboring imperfect, inverted repeats often found within exons and neighboring introns of an RNA transcript [33,34,39-42]. Various computational methods, including inverted repeat analysis, special sequence conservation, and RNA folding algorithms, have been used to propose these putative structures; however, few have been validated by mutational analysis [33,34,40-43]. In 2004, the first entirely exonic RNA duplex was described and validated in transcripts encoding the potassium channel $K_V1.1$ [43]. Only one subsequent exonic duplex has been validated since, though several others have been proposed [44-48]. The sequence opposite a targeted adenosine within a dsRNA substrate is often called the Editing Complementary Sequence (ECS); though often found in introns, these sequences are highly conserved [49].

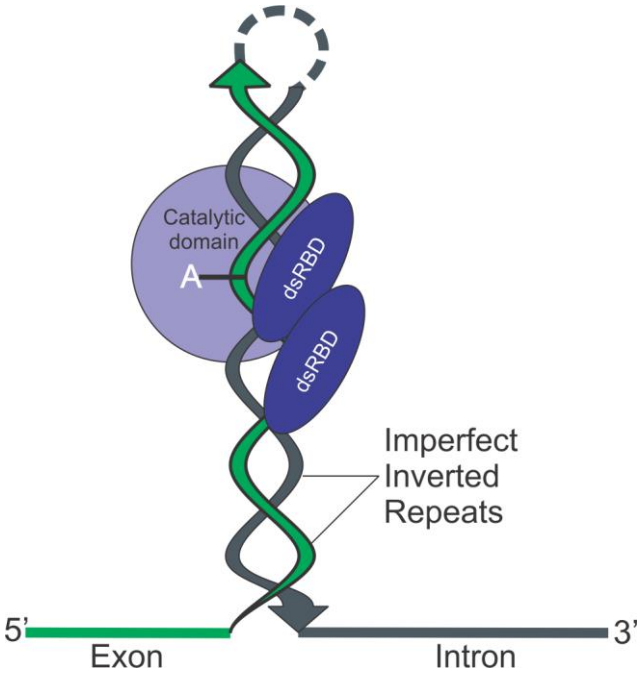

Figure 3: Double Stranded RNA (dsRNA) Duplex

Double stranded RNA duplexes are paramount to editing by ADAR enzymes. Depicted in the cartoon above is an example duplex formed between two inverted repeats (denoted by filled arrows) between an exon (green) and neighboring intron (dark gray). The hashed loop between these repeats denotes the sequence which is not involved in duplex formation and can vary greatly in sequence length and structure. As described in the following sections, ADARs recognize dsRNA structures through dsRNA binding domains (dsRBD) (dark blue) and catalyze deamination by "flipping out" an adenosine residue from a dsRNA duplex into the catalytic site of the enzyme (light purple).

ADAR Protein Family

The mammalian ADAR enzymes share a common domain architecture of multiple (2-3) conserved N-terminal double stranded RNA Binding Motifs (dsRBM), a nuclear localization signal (NLS), and a C-terminal catalytic deaminase domain (Figure 4) [50].The dsRBMs are composed of a highly conserved αββββα structural motif and may act independently of each other to bind dsRNA substrates [51]. The catalytic domain is highly conserved amongst the ADAR enzyme family and amongst vertebrate species [29]. This domain catalyzes the hydrolytic deamination of adenosines to form inosine. ADAR1 and 2 have been demonstrated to have overlapping, yet distinct, editing

footprints–sites may be edited by only one ADAR, or by both to a varying extent [52-54]. Despite the high degree of conservation between the deaminase domains, only ADAR1 and ADAR2 are thought to be catalytically active. ADAR3 can bind dsRNA substrates[55]; however, no ADAR3-specific substrates have been identified *in vivo* and this protein was unable to edit synthetic constructs *in vitro* [53,54,56,57]. The presence of a NLS leads to the accumulation of these enzymes in the nucleus where they can bind to and act on pre-mRNA substrates [58 59,60]. Apart from their structural similarities, ADAR enzymes contain several unique structural elements highlighting their functional differences.

ADAR1 uniquely exists in two catalytically active isoforms as the result of alternative splicing, a shorter p110 isoform and a longer p150 isoform formed by the inclusion of an additional upstream exon. Contrary to the constitutive expression of the p110 isoform, p150 expression is induced by interferon [61]. ADAR1 p110 serves as the primary splice isoform of editing within the Central Nervous System (CNS), whereas interferon-inducible ADAR1 p150 is a crucial regulator within the innate immune response [62]. In addition to an NLS, the p150 isoform contains a Nuclear Export Signal (NES), allowing for accumulation of this ADAR1 splice variant within the cytosol [61,63]. Both splice isoforms are crucial for tissue development and homeostasis; expression of the p150 isoform is critical to intestinal tissue homeostasis and B cell development while the expression of ADAR1 p110 is essential in proper kidney patterning [62].

ADAR1 also contains α and/or β Z-DNA binding domains, although only the Zα domain has been demonstrated to bind DNA [64]. Binding of Z-DNA through this domain may localize ADAR1 to the DNA at sites of active transcription for editing [65]. The introduction of Z forming sequences in RNA targets did not affect the extent of editing of the minimal substrate RG14X8a; however, mutation of the Z-DNA binding domain reduced editing of the substrate by 28% [65]. Binding of Z-DNA by ADAR1 may play a crucial role in certain neurologic behavioral functions. ADAR P150, which contains both Z-DNA binding domains, was shown to be essential for fear

extinction in the mouse prefrontal cortex by reducing levels of Z-DNA at over 100 genomic loci, an event which was also linked to increases in A-to-I editing levels of those transcripts [66].

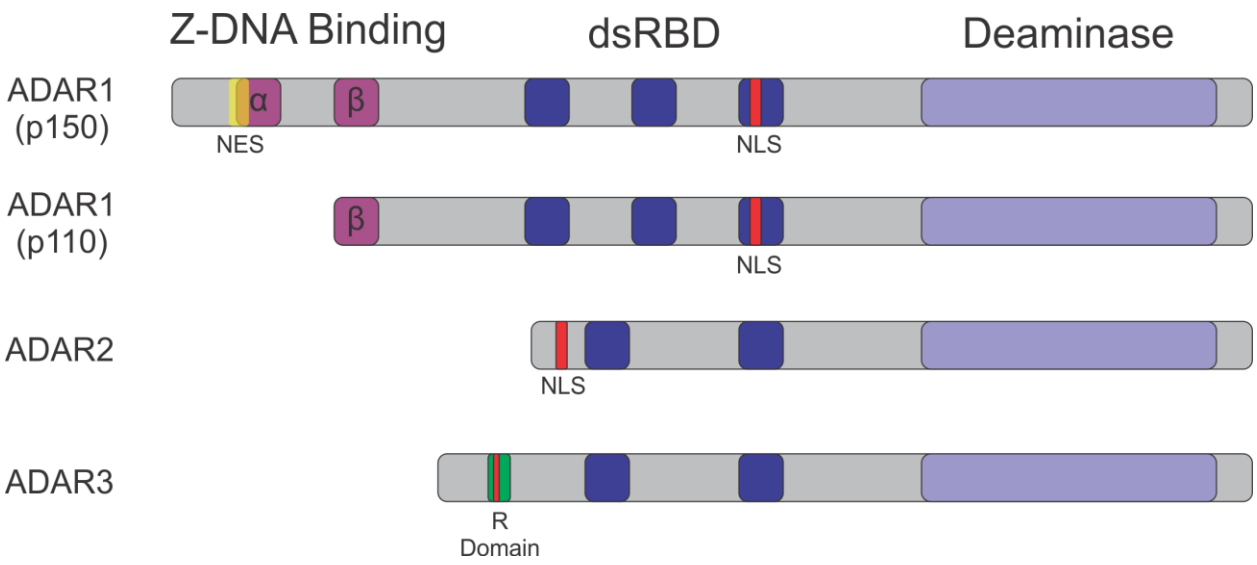

Figure 4: ADAR Protein Structure

Domain architecture of Adenosine Deaminases that Act on RNA (ADARs). Protein peptide chains are denoted in gray. Each ADAR protein contains a conserved deaminase domain (shown in light purple), multiple double stranded RNA binding domains (dsRBDs) (shown in blue), and a nuclear localization signal (NLS) (shown in red). ADAR1 contains two z-DNA binding domains (shown in dark purple) and nuclear export signal (shown in yellow). ADAR3 contains an R domain (shown in green) with which it can bind ssRNA.

ADAR2 is unique in that it contains a buried inositol hexaphosphate (IP6) molecule within the core of the enzyme[67]. This molecule is absent in other ADAR enzymes despite serving an essential structural and catalytic role in ADAR2 function [67]. ADAR2 pre-mRNA transcripts are also subject to alternative splicing; however, only one of these resulting protein isoforms is catalytically active. Alternative splicing of ADAR2 transcripts is regulated in a feedback loop by the enzyme's own activity, a process termed auto-editing. In this case, ADAR2 protein selectively deaminates an adenosine within constitutively expressed ADAR2 mRNAs, generating a novel "AI" splice site. Alternative splicing of ADAR2 transcripts using this novel splice site introduces an additional 47

nucleotide exon containing a premature stop codon, resulting in reduced ADAR2 expression and enzyme specific editing [68,69].

Apart from its unique catalytic inactivity, ADAR3 is also the only ADAR that can bind both single stranded and double stranded RNA templates. Binding of ssRNA occurs through an encoded arginine rich single stranded RNA binding domain (R domain) [56]. The function of this R domain remains unknown; however, it has been demonstrated to contain a NLS [58].

ADAR1 and 2 are ubiquitously expressed within mammals [70-74]; however, editing occurs to a higher extent within the mammalian central nervous system (CNS) [75]. Elevated and essential levels of RNA editing have also been observed in peripheral tissues, specifically within the cardiovascular system [42]. ADAR3 localizes specifically to the CNS [76], suggesting a possible role of ADAR3 in the regulation of RNA editing despite its lack of observed catalytic activity. It has been suggested that ADAR3 could regulate RNA editing by antagonizing the binding of ADAR1 or 2 to double-stranded RNA substrates [77]; however, this fails to explain the relatively high levels of editing observed within the CNS compared to peripheral tissues where ADAR3 is not expressed [56,77,78] . Nonetheless, ADAR3 is likely important to neuronal function as mice which do not express ADAR3 (to be discussed further in “Animal Models” section) have deficits in memory tasks [79]. ADAR3 has also been shown to influence the expression of neuronal immediate early genes DUSP1 and EGR1 by directly binding of their mRNA transcripts, presumably inhibiting miRNA or RBP binding[55].

Mechanism of Catalysis

The catalytic center of ADAR enzymes sits within a highly positive electrostatic pocket where amino acid side chains form direct interactions primarily with the backbone of dsRNA substrates [67,80]. Catalysis proceeds through a base flipping mechanism in which a loop within the

catalytic domain inserts itself into the minor grove of the RNA duplex and flips out an adenosine nucleotide from the RNA duplex into the enzyme active site [80,81]. A highly conserved glutamate residue (ADAR1 E1008; ADAR2 E488) occupies the space vacated by the displaced adenosine and stabilizes the RNA duplex; protonation of this residue enhances the catalytic rate of deamination [82,83]. Within the active site, dehydrogenation of a Zinc-coordinated water molecule by a neighboring glutamate residue (E396 of ADAR2) generates a nucleophilic, reactive Zinc-hydroxide. E396 then acts as a proton shuttle to allow the newly formed nucleophilic hydroxide to displace ammonia from adenosine, generating inosine [67,84]. Of note, Tryptophan375 of human ADAR2 was computationally predicted to sterically hinder cytosine within the active site, possibly explaining why, despite structural similarity to cytidine deaminases, ADARs specifically deaminate only adenosines [67].

Human ADAR Mutations and Disease

Mutations in the genes encoding both catalytically active ADARs, ADAR1 and 2, have been linked to human disease states. 131 ADAR1 mutations have been identified in patients with Dyschromatosis symmetrica hereditaria (DSH) [85], a rare, autosomal dominant disorder characterized by areas of hyper- and hypo-pigmented macules on the hands, feet, and face [86]. An additional 9 mutations in ADAR1 have been associated with Aicardi-Goutières syndrome (AGS) [85], a rare, autosomal recessive disorder characterized by encephalopathy and skin inflammation. Mutations causing DSH and AGS are not localized to any single functional domain of ADAR1, underscoring the importance of ADAR1 in normal homeostatic functioning [85]. ADAR1 may be associated with other inflammatory pathologies. Increases in ADAR1 expression and activity have been linked to systemic lupus erythematosus [85]. Clinical outcomes have not been associated with mutations in ADAR2; however, decreased expression of ADAR2 has been associated with neurologic disease states such as amyotrophic lateral sclerosis (ALS) [87,88], ischemia [89], astrocytoma [90], Huntington's Disease, schizophrenia [91,92], spinal cord injury [93], and

Alzheimer's disease [91]. ADAR2 function is also be associated with immunologic disease states and infection as viruses may hijack RNA editing to bypass the immune system. The Borna disease virus (BoDV) utilizes RNA editing by ADAR2 in order to evade innate immune detection during infection in human oligodendroglial (OL) cells [94]. Global hypo-editing has been observed in patients with Autism Spectrum Disorder (ASD) [95]. Alterations in the extent of A-to-I editing holds immense potential to influence the pathogenesis of numerous other neurologic disorders due to the enrichment of editing sites within genes implicated in human brain-related diseases [96].

RNA editing may play an important role in tissues outside of the CNS as well. Within the cardiovascular system, editing of Filamin A (FLNA1) pre-mRNAs occurs to a high extent (>90% of transcripts) and regulates proper cardiac function [42]. A reduction in the extent of FLNA1 editing, which predicts a single glutamine (Q) to arginine (R) transition, is associated with cardiovascular disease[42]. A-to-I editing has additionally gained attention for both its association with various cancers, through recoding of individual proteins as well as global patterns under-editing or over-editing, and its enormous capacity to diversify the proteome [90,97-102]. In addition to the high mutation rate of cancer cells, extraneous editing has the capacity to further re-code the proteome within tumor cells, potentially enhancing metastasis or immune cell evasion[99].

Genetic mutations of human ADAR3 have not been associated with a clinical phenotype; however, ADAR3 expression correlated with positive outcomes in human glioma patients, suggesting a tumor suppressive role of the enzyme[103]. Furthermore, expression of ADAR3 positively correlates with the extent of GRIA2^{Q607R} editing, demonstrating a potential role in the regulation of ADAR2-catalyzed-deamination *in vivo* [103].

Animal Models

The implications of RNA editing have been explored in multiple genetically modified mouse models. Commonly, studies of the effects of editing at specific substrates use mouse

models which generate an “always edited” or “never edited” isoform of the protein in question. These substrate-specific models fall outside the scope of this review, but more information may be found in the following review by Jinnah et al[21]. To study the physiologic effects of A-to-I editing and to assess the contribution of individual ADAR enzymes, genetic knockout (KO) or knock in mouse models have been generated for all three ADARs individually as well as mice which lack both ADAR1 and 2 and are entirely editing deficient.

ADAR1 functions as a critical regulator of hematopoiesis, renal and intestinal tissue homeostasis and of the innate immune response [104,105]. In mouse models, genetic knockout of ADAR1 (*Adar1*$^{-/-}$) or mutational disruption of its catalytic activity (*Adar1*$^{E861A/E861A}$) results in embryonic lethality [106-108]. Genetically modified mice expressing solely the p110 splice isoform of ADAR1 display a similar embryonic lethality, demonstrating the critical role of ADAR1 p150 in proper homeostasis [62,109]. Prior to embryonic lethality, *Adar1*$^{-/-}$ mice display a disintegration of proper liver tissue structure, defects in hematopoiesis, and overproduction of type I IFN [105,106,110].[62,105]. Embryonic lethality likely results from the critical role of ADAR1 in suppressing the innate immune response. ADAR1 critically destabilizes host dsRNA structures to help cells distinguish invading viral double-stranded RNAs from endogenous dsRNA structures [104,105]. Concurrent knockout of either interferon induced with helicase C domain 1 (IFIH1/MDA5) or mitochondrial antiviral-signaling protein (MAVS) proteins of the innate immune response delays lethality in *Adar1*$^{-/-}$ mice to shortly after birth [62,108,111,112]. The critical role of ADAR1 persists into adulthood; ADAR1 deletion in adult mice likewise leads to hyper-inflammation and disruption of tissue homeostasis [62].

Interestingly, while a genetic knock-in mutation which inactivates ADAR1 (*Adar1*$^{E861A/E861A}$) results in embryonic lethality, mice with a concurrent knock out of IFIH1 (*Adar1*$^{E861A/E861A}$ *Ifih1*$^{-/-}$) survive longer than *Adar1*$^{-/-}$ *Ifih1*$^{-/-}$ animals[108,113], suggesting a possible editing-independent function of ADAR1. While editing-independent functions of ADAR enzymes have been suggested

[114-118], a comparison of *Adar1*$^{-/-}$ *Ifih1*$^{-/-}$ and *Adar1*$^{E861A/E861A}$ *Ifih1*$^{-/-}$ mice demonstrated only subtle phenotypic differences, suggesting the main function of ADAR1 is suppression of the innate immune response [113].

Genetic knockout of ADAR2 in mouse models (*Adar2*$^{-/-}$) leads to an extreme susceptibility to seizures in young animals and early postnatal lethality due the ADAR2-mediated recoding of a single amino acid within transcripts encoding the Gria2 subunit of the AMPA receptor [119,120]. This nonsynonymous glutamine (Q) to arginine (R) transition (Q607R) occurs in nearly 100% of GRIA2 mRNA transcripts and alters the permeability of the resulting heterotetrameric AMPA channel, preventing flux of the divalent cation calcium (Ca^{2+}). This recoding event is equally essential in the developed brain of adult animals [89]. A concurrent genomic mutation recoding Q607 of GRIA2 to R (*Adar2*$^{-/-}$ *Gria2*$^{R/R}$) rescues the early postnatal lethality in *Adar2*$^{-/-}$ mice suggesting a singular critical role of this enzyme in mammalian homeostasis [120].

One explanation for the seemingly singular critical role of ADAR1 and 2 is compensation for the loss of enzyme function by either other ADAR enzymes or other cellular mechanisms [21]. In agreement with this hypothesis, editing-deficient mice lacking ADAR1 and ADAR2 (*Adar2*$^{-/-}$ *Gria2*$^{R/R}$ *Adar1*$^{-/-}$ *Mavs*$^{-/-}$) have a high mortality by postnatal day 15, despite harboring concurrent genetic alterations that rescue either *Adar2*$^{-/-}$ or *Adar1*$^{-/-}$ mice individually [45]. However, this early lethality may be due to editing-independent functions of ADAR1. Editing-deficient mice in which a mutation renders ADAR1 catalytically dead (*Adar1*$^{E861A/E861A}$ *Ifih*$^{-/-}$ *Adar2*$^{-/-}$ *Gria*$^{R/R}$), rather than *Adar1*$^{-/-}$ mice, are viable to adulthood with no overt phenotypic abnormalities [53]. The above described mouse models do not rule out the hypothesis that additional, yet unknown, cellular mechanisms may be able to compensate for deficiencies in A-to-I editing.

Mice expressing a dsRNA-binding-deficient ADAR3 that lacks the dsRBDs encoded by exon3, *Adar3*^{EXON3}, are viable but display deficiencies in memory tasks and anxiety-related

phenotypes, suggesting a critical role of the ADAR3 enzyme in proper neuronal function [79]. The mechanism by which ADAR3 modulates neuronal function is still unclear. Inhibition of ADAR2-mediated GRIA2 transcript editing was observed in a U87 cell line dependent on the expression of ADAR3[77]. This inhibition was dependent on the presence of dsRBDs, suggesting that ADAR3 acts as a negative regulator of ADAR2 activity through competition for dsRNA binding sites, inhibition of ADAR2 activity, or facilitation of GRIA2 splicing thereby removing ADAR2 binding sites[77]. This *in vitro* evidence implies that dysregulation of ADAR3 function may affect downstream editing levels *in vivo* [77,79]. *Adar3*^{EXON3} mice, however, did not display significantly altered levels of editing of the well-known editing targets *Blcap*, *Gabra3*, and *5-Ht2cr* [79].

Extensive Transcriptome Editing, Poor Conservation

Historically, A-to-I editing events were discovered serendipitously through the identification of adenosine to guanosine discrepancies in DNA and RNA sequences, respectively, by Sanger sequencing [31]. Today, millions of A-to-I editing sites are catalogued in mass across entire transcriptomes using high throughput sequencing methods [22,121-124]. As many as 100 million unique A-to-I edit sites are estimated to exist across the human transcriptome [124]. Editing sites will likely continue to be discovered as the identification of editing sites, especially those edited to either extreme (<20%, >80%), is highly dependent on the read depth of sequencing [19,122,125]. Gene-specific, targeted approaches with greater read depth improve the quantification of the extent editing within transcripts and enhance the sensitivity to detect sites edited only in small proportions of transcripts.

A majority of A-to-I edit sites occur within introns or repetitive transposable short interspaced nuclear elements (SINE) of the 3' UTR of mRNA transcripts [122-124,126-131]. *Alu* repeats are a type of SINE which are specific to humans and primates and extremely common in these species; *Alu* elements comprise ~11% of the human genome [132]. Global editing levels observed in primates and humans are significantly higher than other mammals, likely due in part to the high

degree of repetitive *Alu* transposable elements [133,134]; however, this does not entirely explain the increased editing levels. Human transcripts are edited to a 2-fold higher extent on average than those of primates despite the high degree of Alu element conservation between these species [134]. A majority (~83%) of the 1932 Alu elements unique to humans compared to primates were determined to be editable (within 2500bp of an *Alu* of reverse orientation) and significantly enriched in genes essential for nervous system function [134]. Editing within these noncoding elements could influence transcript stability through the alteration of RNA binding protein (RBP) binding sites [115] or miRNA binding [135]. Together, this suggests a potential mechanism by which humans have expanded the regulation of key neuronal genes beyond that of their close genetic ancestors to increase neuronal function and cognitive capabilities [134].

Despite the potential functional role of these sites as alluded to in the described studies, debate still exists within the field as to whether these events are meaningful or necessary physiologically [54,123], as these editing events are poorly conserved across species [78,123,131,136]. Furthermore, high levels of editing appear more in newly evolutionarily derived *Alu* elements as opposed to older *Alu* elements and editing seen within exons, suggesting that high levels of editing may even be harmful [123].

Recoding by A-to-I Editing

A-to-I editing events are more common in introns but may occur in exons as well. Editing events within the coding sequence of mRNA transcripts are much smaller in number [54,78,123,131,136] ; however, these events have incredible potential to recode specific amino acids within select proteins, often with considerable consequence [33,34,39,40,42-44,120,137]. Degenerate mRNA codons encoding 12 of the 20 canonical amino acids are convertible by A-to-I editing; their conversion often results in substantial nonsynonymous amino acid substitutions causing alterations in size, charge, and key chemical groups (Ex. –OH in serine/threonine) in residues critical for protein function and enzyme catalysis [57]. All three stop codons are able to be edited. In some instances,

this could lead to ablation of the stop codon and the incorporation of tryptophan, reminiscent of a readthrough mutation.

Encoded Amino Acid	Nonsynonymous A-to-I Editing	Synonymous A-to-I Editing
Lysine (AAA/G)	Glutamic Acid (IAA/G, IAI)	NA
	Glycine (IIA/I/G)	
	Arginine (AIA/G)	
Asparagine (AAU/C)	Aspartic Acid (IAU/C)	
	Glycine (IIA/I/G)	
	Serine (AIU/C)	
Isoleucine (AUA/U/C)	Valine (IUA/I/U/C)	
	Start/Methionine (AUI)	
Tyrosine (UAU/C)	Cysteine (UIU/C)	
Histidine (CAU/C)	Arginine (CIA/I/G)	
Aspartic Acid (GAU/C)	Glycine (GIU/C)	
Start/Methionine (AUG)	Valine (IUG)	
StopCodon (UAA, UGA)	Tryptophan (UGI,UII)	Stop Codon (UIA, UAI)
Threonine (ACA/U/C/G)	Alanine (ICA/I/U/G/C)	Threonine (ACI)
Arginine (AGA/G, CGA)	Glycine (IGA/I/G)	Arginine (AGI, CGI)
Serine (AGU/C, UCA)	Glycine (IGU/UCI)	Serine (UCI)
Glutamine (CAA/G)	Arginine (CIA/I/G)	Glutamine (CAI)
Glutamic Acid (GAA/G)	Glycine (GIA/I/G)	Glutamic Acid (GAI)
Glycine (CGA)	NA	Glycine (GGI)
Alanine (GCA)		Alanine (CGI)
Proline (CCA)		Proline (CCI)
Valine (GUA)		Valine (GUI)
Leucine (C/UUA)		Leucine (UUI, CUI)
Phenylalanine	No Editable Adenosines	
Tryptophan		
Cysteine		

Table 1: Re-codeable Codons by A-to-I Editing

Inosine base pairs preferentially with cytosine. For this reason, A-to-I editing is functionally an adenosine to guanosine conversion when read by the ribosome, as shown in Figure 2. Codons for 17 of 20 amino acids contain adenosines and may be edited, but only 12 amino acids may be recoded. Codons for 7 of these amino acids always result in recoding whereas codons for 5 amino acids and the stop codon are dependent on the position of the modified adenosine.

Editing of adenosines within coding sequences is unique in its extent, specificity, and conservation. Contrary to those in repetitive elements, editing events in coding sequences are often highly conserved across multiple species, some even from invertebrate species to humans [78,131,136]. While a large proportion of primate and human editing likely arose from the increased presence of repetitive genetic elements (Alu repeats), a majority of editing sites within coding sequences are not within *Alu* elements and therefore did not arise from *Alu* insertion [131]. dsRNA substrates formed by repetitive elements display generally low levels of nonspecific editing; however, the editing of dsRNA substrates of CDS editing sites occurs to a greater extent with high specificity [48]. Furthermore, the intronic elements (ECS sequences) within intron/exon dsRNA which are substrates to CDS site deamination display remarkable conservation, despite the poor conservation of intronic sequence across species, underscoring the importance these dsRNA structures and editing of these substrates [49]. While the editing footprints of ADAR1 and 2 overlap to some extent, ADAR1 functions as the primary editing enzyme of repetitive regions whereas the more highly conserved sites within non-repetitive regions are primarily edited by ADAR2 [52-54,78].

By comparing RNAseq data from human, mouse, rat, cow, opossum, and platypus, Pinto et al. demonstrated the high conservation of 59 editing sites, comprising only 0.004% of human editing sites, which they named “Evolutionary Selected Sites” (ESS) [136]. A vast majority (38/59) of these conserved sites occur within the coding sequences of mRNA transcripts (17 intron, 2 miRNA, 2 3’ UTR). The extreme level of evolutionary conservation suggests some important and advantageous function of recoding by RNA editing. Interestingly, a disproportionate number of those proteins recoded by A-to-I editing are neurotransmitter receptors, membrane ion channels, or other synapse-related proteins expressed within the CNS [48,53,78,96,131,136,138-141] including ionotropic glutamate [33,34] and GABA receptor subunits [44], the 2C subtype of serotonin receptor ($5HT_{2C}$) [39], the $K_V1.1$ subtype of voltage gated potassium channel [43], the $Ca_V1.3$ ion channel [142], and the vesicular release accessory protein CADPS1 [96] as notable examples. Additionally,

transcript recoding by RNA editing is generally restricted to the CNS [75]. Recoding of neuronal-specific proteins is not reserved only to membrane ion channels and receptors; editing within introns, miRNAs, and neuron-specific RNA binding proteins such as NOVA1 expand the potential influence of RNA editing as a regulator of neuronal function[46,136].

Despite the plethora of known protein-recoding events by RNA editing, the functional consequences for only a few substrates have been discovered. Within these substrates, recoding alters amino acids within critical domains for neuronal protein function, resulting in significant functional outcomes.

The creation of entirely editing-deficient animals has demonstrated that RNA editing is not essential for mammalian homeostasis[53]; however, this does not imply these sites do not serve important functions. The creation of "always edited" or "never edited" mouse models have demonstrated the significant influence of the editing of specific substrates [21]. Genetic models of transcript-specific editing dysregulation have demonstrated critical roles for the serotonin 5-HT_{2C} receptor in metabolic and depressive phenotypes, Cadps1 editing in the dense core vesicle (DCV) exocytosis from chromaffin cells and neurons, and FLNA1 editing in cardiovascular fitness [21]. While these models are not representative of the complex and dynamic landscape of RNA editing for any one transcript, they aid in identifying potential phenotypic outcomes by providing a model of opposing ends of the editing spectrum-animals capable of editing presumably exist between these two extremes.

Fate of Highly Edited Transcripts

Edited RNA transcripts are subject to additional regulation following deamination. Licht et al. demonstrated that by slowing RNA splicing, increases in editing were observed in pre-mRNA transcripts within the nucleus, but remained at static levels within the cytosol, suggesting a mechanism to limit the nuclear export of inosine containing transcripts. Indeed, other studies have

shown the retention of highly edited transcripts within the nucleus [143-145]. Using extracts from HELA cells, Zhang et al. identified the nuclear RNA binding protein p54 (nrb) which binds specifically to inosine-containing RNAs, retaining them in the nucleus [143]. Inosine-containing transcript binding was dependent on concentration, suggesting that transcripts with fewer inosines are less efficiently retained in the nucleus [120,143]. Certain edited transcripts must make it to the cytosol. In *C. elegans*, edited, inosine-containing human and worm mRNA transcripts were identified on polysomes, suggesting translocation to the cytosol [146]. Additionally, editing of GRIA2 is essential for proper channel function and survival[120], suggesting the translocation and translation of these transcripts.

Following export to the cytosol, some inosine-containing transcripts may not be translated by the ribosome. Using mass spectrometry to identify peptide products from short, edited minigenes, Licht *et al* found that some inosine-containing mRNA codons lead to translational stalling and truncation of the encoded peptides [147]. This effect was dependent on the number and position of inosines within the codon with termination rates ranging from 0%-84%. Most codons containing only 1 inosine, as is the case for many conserved RNA editing substrates, showed low levels (~3%) of transcript truncation. Translational stalling has also been described using more native, full length constructs. 5-HT_{2C} receptor transcripts contain 5 editing sites in close proximity. In HEK293T cells transfected with 5-HT_{2C} transcripts encoding full length constructs, the presence of inosines at all 5 positions ablated expression of the protein [148]. Two inosines in close proximity were sufficient to limit translation; either two inosines in one codon or one inosine in two subsequent codons significantly reduced translation of the 5-HT_{2C} receptor [148]. In human brain samples, a precipitous drop was observed in the ribo-seq signal of edited transcripts following the position of the editing site, presumably signaling translation stalling and/or altered translation kinetics of edited transcripts *in vivo* [147].

While historically A-to-I editing has always been considered functionally an “A” to “G” conversion to the cell machinery, it may not always be interpreted this way by the ribosome. Inosine:cytosine base pairs are generally less stable than guanosine:cytosine base pairs; I:C base pairs contain only two hydrogen bonds, in comparison to the three H-bonds of a G:C base pair [149] and their relative strength is dependent on their placement within a codon [148]. More recent studies have shown inosine may base pair with other nucleotides with an order of stability of I:C>I:A>I:T=I>G [147,150]. In the model system described above by Licht et al, it was found that inosine may be read as “A” or “U” in small percentages; all of these transitions, however, occurred in low percentages and only when inosine was present in the first position of the codon [147]. Thus, it is still generally considered that inosine acts as a guanosine when read by the ribosome, though more extensive studies are needed to determine to confirm this long-held assumption.

Species, Tissue, and Developmental Differences in Editing Levels

The landscape of RNA editing exhibits immense spatiotemporal complexity with regulation observed that is specific to species, tissue, region, and cell-type [78,125,131,151,152]. Editing levels vary to the greatest extent between species, likely due to differences in the stability of dsRNA substrates [78]. This observation agrees with an evolutionary model proposed by Rob Reenan in which editing begins with the generation of an original RNA duplex structure. The specificity and extent of editing gradually change with variations in the nucleotide sequence, and thus structure, of the duplex during evolution [151]. Within a particular organism, editing levels display both tissue-specific and region-specific regulation. Editing sites within CDSs which lead to recoding exhibit higher tissue-specific variation in the extent of editing than other non-recoding sites within non-repetitive elements [78], suggesting some level of tissue-specific regulation for these sites. Interestingly, the expression of ADAR enzymes cannot fully account for these tissue-specific editing levels. In an analysis of mammalian RNA editing using both mouse and human GTEx RNAseq data, ADAR expression was unable to account for the tissue specificity of editing. Of the

variation in coding sites, only 6% and 25% was explained by ADAR1 and ADAR2 expression, respectively [78].

Within the brain, select transcripts also display regional differences in the extent of editing [39,152-156]. For transcripts with multiple editing sites, such as 5 editing sites within 5-HT_{2C} receptor transcripts, region-specific levels of editing may be unique to each editing site [154]. Despite differences in editing levels between species, conservation in the spatial patterns of editing suggest conserved patterns of spatial regulation [153]. Region-specific differences in editing levels cannot be explained simply by the difference in expression levels of ADAR1 or 2; strikingly, this variance could be explained by expression of the catalytically inactive ADAR3 [152]. Furthermore, within individual brain regions, cell-type specific variation has been identified between neuronal, microglial, endothelial, astrocytic, and oligodendrocytic cell types within the brain; neurons and astrocytes display the highest levels of editing [125,152]. It remains to be shown conclusivley, however, whether editing levels significantly vary between individual cells of a particular subtype. scRNAseq analysis of human cortical surgical biopsies revealed a bimodal distribution of editing levels, suggestive of “all or none” A-to-I editing within individual cells[125]. Upon merging the scRNAseq reads from human cortical surgical biopsies to mimic an ensemble tissue, the “all or none” distribution of editing levels became unimodal, suggesting that studies determining changes in editing at the anatomic level may be missing critical cell-specific alterations[125]. The extremely limited number of cells used (446 total cells), however, limits the conclusions to be drawn from this study[125].

The extent of A-to-I editing increases temporally over the course of development, though debate still surrounds the mechanism by which these differences arise [78,131,152,157]. Increases in the extent of RNA editing are associated temporally with neuronal maturation [131,152,158]. The expression of ADAR enzymes increases over development and generally correlates with increased editing levels in human and mouse brain tissues; however, the small increases in

expression fail to completely account for increases in editing over development [78,131,152,157]. A recent study in mice showed that while the expression of ADAR2 transcripts increased over development so did ADAR2 autoediting[152]. Nonedited ADAR2 transcripts encoding the active enzyme remained constant over development, suggesting that expression of active ADAR2 remains constant but activity increases [152]. The expression of *trans* regulators of ADAR activity are likewise temporally associated with neuronal maturations and may explain, at least in part, developmental increases in editing activity. During development, there is an increase in the expression of pro-editing regulators of ADAR2 nucleolar localization, PIN1 and Importin-alpha4, [158,159] and a concurrent downregulation of negative regulators, including the RNA binding proteins RPS14, DDX15 and SRSF9 [160]. Debate exists, though, as to whether developmental changes in regional cell composition are the major contributor to these changes in editing [125,131,152]. Findings of "all or nothing" editing[125] within cells certainly suggests cellular composition could have a strong influence on editing levels observed at the anatomic level, but all cell types in the brain show some developmental increases in editing, suggestive of shared developmental mechanisms regulating editing in these cell types [152]. Additional single-cell-resolution studies are needed to determine the spatiotemporal regualtion of RNA editing.

Why edit at all?

The poor conservation of the vast majority of RNA editing sites and the seemingly singular purpose of editing suggested by ADAR KO animals have led many to ask, why edit at all? The answer to this question is unknown but several theories have been put forward.

The potential for RNA editing to diversify the transcriptome is immense. As noted by Barak et al, the differential editing of an *Alu*-containing transcript, which could contain ~30 editing sites, leads to astronomical transcript diversity, generating over 1 trillion (2^{30}) transcript isoforms. While a function for this diversity has yet to be discovered, the authors note that the complexity

generated is potentially greater than that of alternative splicing, although, in many cases, editing of these regions will not alter the sequence of the encoded protein [161]. It has even been argued that higher levels of human editing lead to higher transcriptome diversification and regulation and could explain the markedly increased cognitive abilities of humans over chimpanzees, whose genomes are 98.8% similar to our own [134]. Alterations in RNA editing have been linked to human neurologic disease states (see above section “Human ADAR Mutations and Disease”); however, an effect of editing in cognition is not observed within editing-deficient mouse models.

Some argue that RNA editing events tend to occur at sites where an A to G conversion occurred early in evolution, and A-to-I conversion by RNA editing helps preserve an ancient form of the genome [19,136,162].This model, however, fails to explain the differential spatiotemporal regulation of editing of unique substrates and the enrichment of editing activity and substrates within the central nervous system. The final argument posits RNA editing as an important regulator for increasing proteome diversity beyond that which is encoded by the DNA, allowing for the alteration of specific amino acids within key synaptic plasticity proteins to a degree in tune with the cells needs [31,49,151]. The conservation of these recoding events, including intronic ECSs, spatial patters of editing, and significant functional effects, denotes an important need for recoding by RNA editing [49,78,136,153].

Regulation

dsRNA substrates are required for recognition and catalysis by ADAR enzymes; however, only certain adenosines, or a unique adenosine, within a dsRNA substrate are specifically deaminated, often to differing extents. Furthermore, ADAR1 and 2 have been demonstrated to have overlapping yet distinct specificity for select adenosine residues [52-54]. This specificity has been shown to involve several *cis* and *trans* factors acting in concert to regulate levels of RNA editing.

Cis-regulatory factors and Site Specific Deamination

Double stranded RNA (dsRNA) Template

Sequence and structural differences within the required dsRNA substrate influence the specificity and efficiency of editing. A minimum length of 15-20 base pairs of dsRNA is required for recognition by ADARs; editing efficiency increases with increased duplex length, plateauing at ~100bp [35]. Increases in dsRNA length, however, also increase the promiscuity of deamination observed along the duplex [35,163]. Long, nearly-perfect dsRNA duplexes are edited to varying extents at 50% of available adenosines [163]. The presence of RNA secondary structural elements, i.e. internal loops and bulges, further modulate the specificity [164] and efficiency [165,166] of RNA editing. Internal loops of 6 nucleotides or greater are equivalent to helix termini, demonstrating a possible mechanism by which to increase editing specificity along an RNA duplex by effectively segregating longer RNA duplexes into smaller dsRNA helices [164]. Even small alterations within the dsRNA sequence and/or structure can affect the efficiency of editing. Within a minigene encoding the rat GRIA2 minimal RNA duplex, correcting the base pairing of only two single-nucleotide bulges decreased the extent editing of the R/G site by rADAR2 from 75% to 40% and altered the localization of ADAR binding to the mutant dsRNA construct [167].

Enzyme Influence on Specificity

How ADAR enzymes select specific substrates and adenosine nucleotides is not completely understood, but both structural and sequence elements influence this specificity. ADARs display unique structural preferences within a dsRNA substrate; ADAR2 was able to deaminate adenosines located within 1−2 nt of the ends of dsRNA substrates, [52] whereas ADAR1 was unable to edit nucleotides within up to 8 nucleotides from 3’ end or 3 nt of the 5‘ end of a dsRNA substrate [163]. At the sequence level, ADAR enzymes display unique preferences for adenosines with distinct 5’ and 3’ neighboring nucleotides as well as for nucleotides directly

opposing the edited adenosine. ADAR1 preferentially acts on adenosines with a 5' neighbor preference with an order of A=U>C>G, while 3' nucleotides does not affect efficiency [163,168]. ADAR2 has a similar 5' neighbor preference of U ≈ A > C = G and additionally demonstrates a 3' neighbor preference of U = G > C = A; these 3' and 5' neighboring nucleotides equally influence ADAR2 editing efficiency[52,168]. Both ADAR1 and ADAR2 more efficiently edited adenosines opposing cytosine or uridine in comparison to adenosine or guanosine nucleotides [165,169]. Alteration of solely the opposing nucleotide from a cytosine to a guanosine has been shown to significantly lower the editing efficiency in some substrates [44,165,169].

The specificity of ADAR enzymes appears to be determined largely by the catalytic domain. In a study using chimeric proteins, in which the catalytic domains of human ADAR1 and 2 were swapped, editing profiles were largely unchanged [169]. 5' and 3' neighbor preferences are also determined principally by the catalytic domain [168]. Within the structure of the hADAR2 catalytic domain, residues within the catalytic loop which penetrates the dsRNA substrate cause steric hindrance with neighboring 5' nucleotides, perhaps explaining the neighboring sequence preference of ADAR enzymes [80]. Additionally, this structure reveals the presence of an ADAR2-specific RNA binding loop within catalytic domain that could explain the enzyme's unique selectivity compared to ADAR1. Mutation of the residues within this loop significantly lowered ADAR2 editing efficiency [80].

While the catalytic domain is critical for selectivity, the presence of dsRBDs influence the specificity and efficiency of editing, perhaps even being required for catalysis in some cases. Editing was completely ablated in a Drosophila ADAR (dADAR) construct lacking only its N terminus, but retaining its dsRBDs and catalytic domain [170], suggesting a critical function of this domain. However, this does not seem to be a requirement in all species, or, perhaps, all substrates. dADAR is a homolog of mammalian ADAR2; constructs consisting of only the mammalian ADAR2 deaminase domain retained selectivity for some adenosines, albeit with

reduced efficiency [67]. Furthermore, inhibition of hADAR2 dsRBD binding through chemical modification of the RNA recognition site decreases site-specific editing [171]. dsRBDs influence the specificity of ADAR enzymes rather than acting as pan-dsRNA binding domains. While a chimeric ADAR1 protein with the dsRBD of ADAR2 retained editing selectivity [169], substitution of the dsRBD of interferon-induced, double-stranded RNA-activated protein kinase (PKR) ablates editing of endogenous constructs [172]. Furthermore, using an assay in which the proteins protected dsRNA constructs from directed hydroxyl radical cleavage, the RNA binding sites of human ADAR2 (hADAR2) dsRBDs and the dsRBD of PKR were shown to be distinct, demonstrating that it is not the mere presence of a dsRBD that determines the specificity of binding, but the amino acid content of the domain [171]. Additional factors are likely involved in determining site selectivity and efficiency. In an event that is not yet understood, ADARs can bind RNA substrates without catalyzing editing at all [173].

ADAR Dimerization

Editing efficiency by a constant concentration of ADAR enzyme decreases as the concentration of dsRNA substrate increases; this phenomenon, called substrate inhibition, suggests that ADAR dimerization is required for editing [174]. In this model, increasing substrate concentration results in an increased number of ADAR binding sites and decreased dimer formation. ADAR dimerization has been evaluated using yeast two hybrid models, size exclusion chromotography and Bioluminescence Resonance Energy Transfer (BRET)/ Förster Resonance Energy Transfer (FRET) experiments. Yeast two hybrid assays function by fusing proteins of interest to separate, individual domains of the yeast *Gal4* transcription factor. Functional dimerization of proteins of interest in yeast cells brings Gal4 transcription factors in proximity to activate the reporter *Gal4* gene leading ultimately to a color changing reaction. BRET and FRET experiments utilizes the tagging or fusion of bioluminescent/fluorescent proteins or molecules to

proteins of interest. Excitation of a “”donor” molecule can transfer energy to an “acceptor” only when in extremely close proximity, releasing a unique wavelength which demonstrates dimerization. These experimental models have indeed demonstrated that ADAR 1 and 2 are able to form both homodimers and heterodimers [90,170,174-179]. Furthermore, the removal of dsRBDs in rat and Drosophila ADAR constructs significantly reduced dimerization which occurred with a concomitant reduction in editing efficiency[170,176]. ADAR3 has only been demonstrated to form homodimers[175]. Interestingly, in an assay identifying dimeric complexes by size exclusion chromatography, recombinant ADAR3 remained monomeric while endogenous ADAR3 isolated from mouse brain tissue formed homodimers, suggesting a mechanism for ADAR3 dimerization unique to brain tissue[175]. While it is known that ADAR enzymes can dimerize, conflicting reports exist describing this dimerization as either dependent [170,174,179] or independent [175,176,178] of dsRNA binding.

Recently, the x-ray crystallographic structure of the hADAR2 dimer, containing the deaminase domain and dsRBD1 complexed with dsRNA, was solved[179]. Analysis of this structure revealed an asymmetric dimer, with catalytic subunits of each monomer centered on the editing site and their respective dsRBDs extending in opposite directions along the dsRNA substrate [179]. Only one monomer within the dimer possessed an adenosine residue within the active site; a previously unknown dimerization loop (amino acids 501-509) within ADAR2 occupied the active site of the opposite subunit, suggesting that ADARs may use one subunit to bind RNA while employing the second for catalysis [179]. This dimerization loop contains 3 highly conserved residues (T501, W502, D503) which significantly decreased dimer formation and editing efficiency when mutated selectively to alanine, further demonstrating the ability of ADAR dimer formation to regulate editing efficiency [179].

This model of dimerization with a single active subunit could explain how the titration of inactive ADAR enzymes competitively inhibits site-selective modification by active ADARs

[90,170,175,177,179]. It is possible that that inactive ADARs compete with active enzymes for dsRNA binding sites; however, it is interesting to speculate that hetero vs homodimer formation may influence editing levels, dependent on which ADAR monomer is the active subunit in a given dimer. In transiently transfected SF9 cells, dimers composed of a wild-type and catalytically dead mutant monomer edited synthetic constructs with roughly ~50% efficiency of wild type dimers while editing of 5-HT_{2C} RNA decreased to ~30% of the wild-type homodimer activity [175], suggesting dimerization, or competitive inhibition, may affect different substrates or sites differently. In both of these models, the proper balance of the three ADAR enzymes is potentially critical to site-specific deamination.

*Trans-r*egulation of A-to-I RNA Editing

As the interplay between dsRNA structure and ADAR activity modulates editing levels in *cis*, so too does this occur in *trans* by a host of factor regulating ADAR activity and dsRNA formation. The extent of RNA editing within the transcriptome is expansive and not surprisingly numerous proteins have been shown to affect editing levels. ADAR enzymes can be regulated in *trans* either through alterations in expression, direct protein-protein interactions, competitive inhibition of RNA binding, or direct post translational modification [78,160,180-185]. These *trans* regulatory proteins impact the editing levels of unique subsets of editing sites, providing a dynamic regulatory environment [78,180] which can be influenced by neuronal activation [160].

The binding of ADAR enzymes to dsRNA regulates the specificity and efficiency of deamination; thus, it is not surprising that modifications or protein binding, which inhibit interactions with dsRNA, can effect editing levels. A recent study identified a series of DZF-domain-containing RNA binding Proteins (RBPs) (ILF3, ILF2, STRBP, and ZFR) which antagonize ADAR1- and ADAR2-mediated editing by a mechanism dependent on RNA binding, suggesting these proteins directly compete for RNA binding sites [185]. It is also important to note

that A-to-I modification is but one form of RNA modification. Other RNA modifications may further antagonize ADAR binding and catalysis. Levels of RNA editing negatively correlate with levels of another common RNA modification, m^6 adenosine methylation[184]. M^6A writer proteins are not thought to interact with ADAR, however, suggesting that methylation of adenosines may inhibit catalysis or binding by ADARs[184].

ADAR can also be antagonized directly through protein-protein interactions. The splicing factor SRSF9 has been shown to inhibit ADAR2-mediated RNA editing independently of recognition of its canonical splice recognition site, suggesting it inhibits ADAR function through direct inhibition rather than direct competition for RNA binding sites[182]. SFRS9 expression is negatively correlated with editing levels, with the lowest expression within the CNS and highest expression in the muscle, where editing levels are lowest[182]. This protein alone seems to regulate the tissue-selective editing of $Ca_V1.3$ substrates[183]. Other regulatory proteins may affect editing levels by influencing ADAR expression. The protein AIMP2, which functions as a non-enzymatic component of the aminoacyl-tRNA synthetase complex, inhibits ADAR1 and 2 by promoting their degradation [78]. Protein-protein interactions may also enhance editing efficiency. The nuclear proteins DSS1 and TDP-43 are the only known enhancers of RNA editing activity. TDP-43 increases editing levels by stimulating transcription of ADAR1 [181]. While the mechanism of editing enhancement by DSS1 is unknown, it does not appear to function through a direct interaction with ADAR enzymes [180].

RNA editing occurs co-transcriptionally, with dsRNA structures often requiring ECSs within introns for editing. Nuclear shuttling of ADARs promotes co-localization of editing enzymes with pre-mRNA substrates and critically regulates enzymatic activity [158,186,187]. Nuclear localization is established by active transport and further influenced by post-translational modification. Nuclear transporter proteins Importin-α4 and Importin-α5 modulate the translocation of ADAR2 into the nucleus, an event coupled to increases in enzymatic activity [58,158]. In a SH-SY5Y neuroblastoma

cell model, editing of a co-transfected ADAR2 minigene construct was increased 2.2 fold by overexpression of Importin-α4 [158]. Nuclear localization is further influenced by direct modification of ADAR2. Phosphorylation of two serines, 26 and 31, of the ADAR2 N-terminus regulates the binding of PIN1, which sequesters ADAR2 in the nucleolus and prevents its degradation[159]. Remaining cytosolic ADAR2 is ubiquitinated by WWP2, leading to its rapid degradation [159]. *In vitro*, siRNA knockdown of PIN1 decreased the editing of co-transfected GRIA2 minigenes by ~50%, demonstrating its critical role in editing efficiency. PIN1 and Importin-α4 interactions with ADAR2 increase over neuronal development contemporaneously with increases in editing levels in cultured primary cortical neurons, possibly influencing the increase in editing activity observed during development [158].

While the mechanism remains unknown, expression of PIN1 was required for normal localization of ADAR1 in MEF cells, underscoring this protein's key role in regulating both catalytically active ADAR enzymes [159]. Additionally, hADAR1 can be sumoylated at lysine 418 by SUMO-1; this post-translation modification reduces ADAR1 editing activity without affecting localization[188]. While ADAR1 and ADAR2 activity is restricted to the nucleus, ADAR2 activity is primarily localized within the nucleolus [186,189]. SUMO-1 also localizes predominantly to the nucleolus and may function to turn off ADAR1 activity in this subcellular compartment [188,189].

Interplay with RNA Splicing

A-to-I editing and RNA splicing both occur co-transcriptionally and may influence the progression of one another[68,190-192]. Many edited pre-mRNA substrates contain their editing complementary sequences (ECS) within downstream introns, though some dsRNA substrates are entirely exonic[43,44]. In an *in vitro* system, splicing efficiency was inhibited in minigene constructs by the introduction of additional intronic elements of increasing size; this decreased splicing efficiency increased the extent of editing in pre-mRNAs substrates with intronic ECSs,

suggesting that splicing affects dsRNA substrate formation and influences editing levels [193]. No dependence on splicing was observed for substrates with exonic ECSs [193]. Analysis of RNAseq data in mouse brain tissue revealed a negative correlation between splicing efficiency and the extent of editing for mRNA transcripts with intronic ECSs but not exonic ECSs, suggesting interplay between these two co-transcriptional processes also occurs *in vivo* [45]. Furthermore, genetic knockout of splicing factors NOVA1 and NOVA2 was shown to alter global editing levels in mouse models [45]. Together, these data suggest that there exists two separate pools of editable transcripts: those that can only be edited co-transcriptionally within the nucleus, and those that can be edited either co-transcriptionally or post-transcriptionally; even, perhaps, extranuclearly.

RNA editing may also influence RNA splicing. In HepG2 and K562 cell lines, siRNA knockdown of ADAR1 P110 altered global splicing patterns with enrichment of genes involved in RNA processing [194]. RNA editing may affect splicing, either by directly generating or disrupting a slice donor/acceptor site. As previously discussed, ADAR2 edits its own mRNA transcript, generating a novel splice site [68]; however, the generation or ablation of donor/acceptor splice sites is rare. More commonly, editing disrupts splicing regulatory elements (SREs) [194]. It can be appreciated from the influence these two processes exert on one another that the interplay between these two co-transcriptional events requires careful coordination. The C-terminal tail of RNA polymerase II may play a role in coordinating the two events [195,196] although no direct interactions with ADARs have been shown.

Dynamic Regulation

RNA editing is a dynamic process, allowing cells to modulate protein activity through recoding in response to the cell's needs. Editing levels are regulated in response to various external stimuli, including forebrain ischemia in rats[89], thymine deficiency in culture mouse cortical neurons[197], metabolic state in mouse pancreatic islets [198 199 200], hypoxia in lymphoblastoid cells [201], anti-depressant treatment HELA cells[202], and neuronal activation [203 204]. In rat cortical neurons,

the extent of editing for select substrates was dependent on neuronal activity. In the short term (6 hour treatment), neural activation by K^+-induced depolarization decreased levels of editing, whereas neuronal inhibition by the sodium channel blocker TTX increased editing levels [203]. Conversely, long term, chronic activation (48 hour treatment) had the opposite effect: chronic activation increased editing, whereas chronic blockade decreased editing [203], suggesting that changes in RNA editing are responsive to not just to stimuli, but to the delivery of that stimulus. Dynamic regulation of editing has also been demonstrated *in vivo*. In mice, serotonin deprivation decreased editing of the C and C' editing sites within 5-HT_{2C} mRNA transcripts, leading to the generation of receptors more sensitive to serotonin; however, stimulation by the 5-HT_{2C} agonist DOI increased editing at the C' site, reducing G protein-coupling within the forebrain neocortex [204].

Stimulus-driven regulation of RNA editing is not confined to the CNS. In mouse pancreatic islets, high fat diet increased the expression of ADAR2 and increased editing levels of GRIA2 transcripts [198,199].Conversely, the opposite effect was observed in response to fasting–GRIA2 editing was then rescued upon feeding. This editing may perform an important function within islets; in rat INS cells, an *in vitro* immortalized cell line derived from pancreatic β cells, knockdown of ADAR2 impaired glucose-stimulated insulin release due to reduced expression of the exocytosis-regulating proteins MUNC18 and synaptotagmin-7 [200]. This effect was dependent on RNA editing as overexpression of only catalytically active ADAR2 and not an editing-deficient ADAR2 recued the impaired exocytosis in ADAR2 knockdown cells[200].

Perhaps most interestingly, the effects of high fat diet were specific to islets and not observed in other tissues including the brain, underscoring the tissue-specific regulation of editing in response to specific stimuli [198]. Indeed, regulation of editing efficiency may occur by multiple stimuli exerting effects through different mechanisms. For example, the regulation of ADAR2 expression was dependent on Ca^{2+}[203] and cAMP-response element binding protein (CREB) [89]

within neurons but enzyme activity within pancreatic islets was dependent on c-Jun N-terminal kinase (JNK) signaling [199] .

Summary and Conclusions

The scope of known RNA editing has changed immensely over the last decade. The advent of high-throughput sequencing has increased the number of known sites from a few to hundreds of millions. Of these sites, 0.004% account for those predicted to recode proteins; however, these sites are extremely well conserved and often result in significant functional alterations in the resulting proteins. Affected proteins are critical for synaptic function and their editing rates are spatiotemporally regulated and responsive to neuronal stimulation. Conserved recoding by RNA editing functions is an important, highly regulated mechanism by which cells can modulate the function of proteins. The functional implications of many of these conserved sites, however, is lacking. Elucidation of the functional consequences of each protein recoded by RNA editing is necessary to comprehensively understand the role of A-to-I editing within homeostatic function as well as in disease states presenting with altered levels of editing. Transcripts encoding the metabotropic glutamate receptor 4 ($mGlu_4$) are subject to RNA editing at two unique sites, predicting nonsynonymous amino acid changes within the the resulting receptor. To date, no functional consequence of this recoding has been discovered, nor has any publication described its extent of editing, conservation, or regulation.

Metabotropic Glutamate Receptors: Literature Review

The main excitatory neurotransmitter within the mammalian CNS, glutamate, mediates its effects through two separate classes of receptors: ionotropic and metabotropic receptors [26]. Ionotropic receptors function as ligand-gated cation channels which promote rapid depolarization and action potential firing of neurons in response to glutamate binding. These channels are separated into four subtypes (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), Kainate, Delta) based upon both their sequence homology and response to pharmacologic compounds[205]. While ionotropic receptors are necessary for fast glutamatergic transmission, metabotropic glutamate (mGlu) receptors function as G protein-coupled receptors (GPCRs) and mediate their effects through second messengers in a slower, often more prolonged response to glutamate binding. This dual action of glutamate as an excitatory and modulatory neurotransmitter underlies its role in the modification of the strength or efficacy of synaptic transmission in response to activity, called synaptic plasticity, a mechanism important for learning and memory[206-209]. This review will focus on the metabotropic class of glutamate receptors. For more information regarding ionotropic receptors, see the following review (Traynelis et al **2010** *Pharmacol Rev*) cited here [205]. Additional information regarding glutamate as a neurotransmitter may be found in Meldrum et al **2000** *J Nutr* [26].

mGlu receptors belong to the superfamily of G protein-coupled receptors–the largest protein superfamily in the mammalian genome with over 800 known GPCRs [210]. These membrane-bound receptors share a 7 α-helical membrane-spanning domain architecture and for this reason are often referred to as “7-transmembrane” or “7TM” receptors interchangeably. A plethora of diverse ligands, including proteins, light, small molecules and peptides, activate these receptors, stimulating coupled heterotrimeric G proteins. G proteins are composed of three subunits: Gα, β, and γ. GPCRs act as Guanine Exchange Factors (GEFs) to facilitate the exchange of Guanosine-5’-Diphosphate (GDP) for Guanosine-5’-Triphosphate (GTP) within Gα

subunits, causing the heterotrimeric G protein to dissociate from the receptor and into a monomeric Gα subunit and dimeric Gβγ subunit complex; the free, GTP-bound Gα protein and βγ subunit complex can then initiate unique signaling cascades through direct interaction with downstream effector proteins. GPCRs are classified by sequence homology into 5 families in mammals: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin[210]. Many classical neurotransmitters, including serotonin, acetylcholine, noradrenaline, cannabinoids, dopamine, opioid, and histamine, mediate their effects through the most common receptor class–rhodopsin like, or class A, receptors.

mGlu receptors belong to the much smaller C class of GPCRs together with the structurally related calcium sensing receptor (CaSR), γ-amino-butyric-acid (GABA) type B receptor, taste and retinoic acid receptors, as well as several orphan receptors [211]. In contrast to Class A GPCRs, Class C receptors bind endogenous agonists within a large, extracellular amino terminal domain (ATD). Class C ATDs are thought to have evolved from bacterial periplasmic binding proteins (PBP), which share a high degree of structural and amino acid sequence homology to these domains; early models of Class C receptors were generated based on existing, well characterized PBP structures [212].

Metabotropic glutamate receptors were first discovered in 1987 by Sugiyama et al [23]. To date, a family of 8 mGlu receptors have been discovered [25,213-215]; these receptors are further subdivided into three groups based on sequence homology and G protein coupling (Figure 5). Group I receptors ($mGlu_1$, $mGlu_5$) are predominantly postsynaptic and activate phosphoinositol hydrolysis and Ca^{2+} mobilization through activation of $G\alpha_q$. Activation of Group II ($mGlu_2$, $mGlu_3$) and III ($mGlu_4$, $mGlu_6$, $mGlu_7$, $mGlu_8$) receptors primarily stimulate a reduction in intracellular cyclic AMP (cAMP) through activation of $G\alpha_{i/o}$. While group II receptors may be expressed both pre- and post-synaptically, group III receptors are predominantly presynaptic. All mGlu receptors function constitutively as dimers (Figure 5).

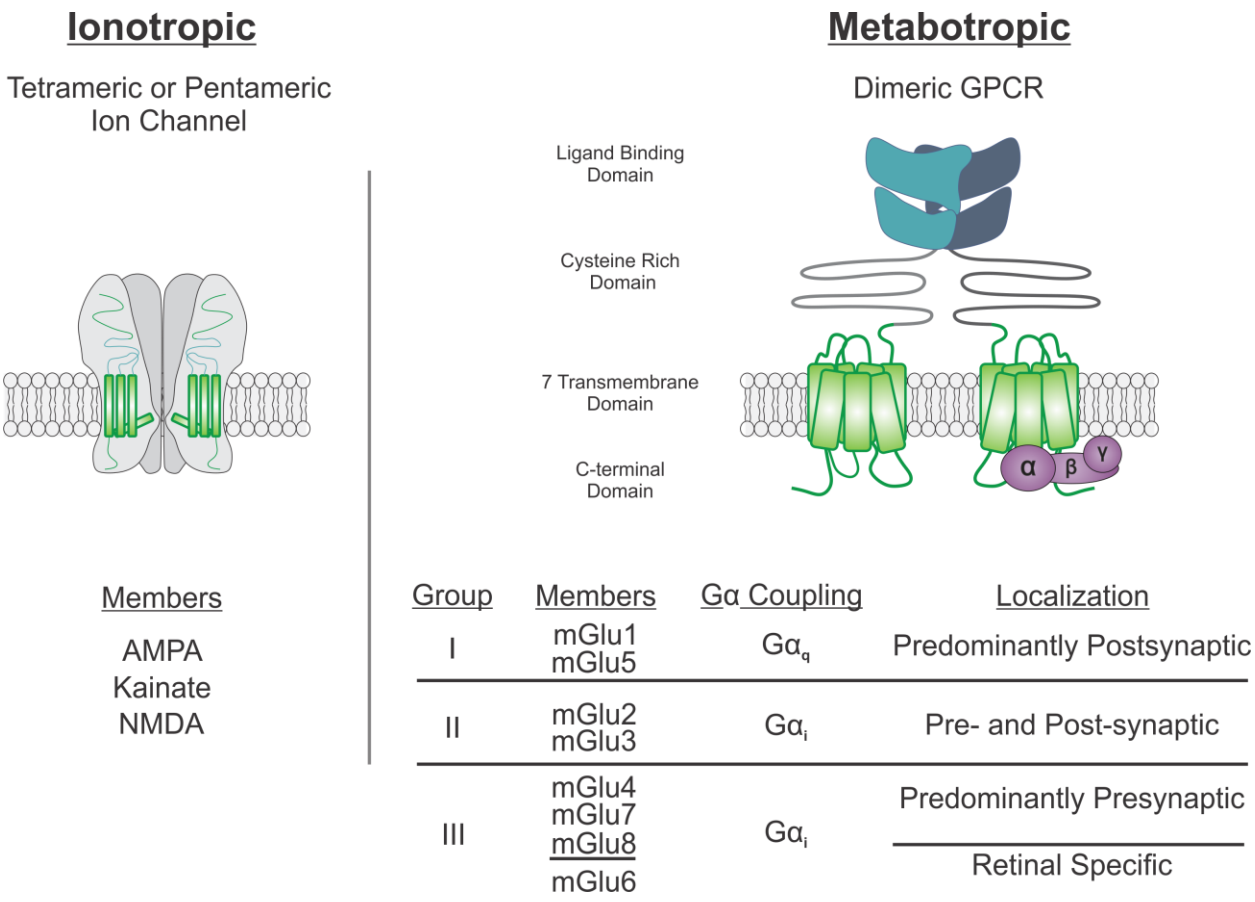

Figure 5: Glutamate Receptor Classes and Structure

L-Glutamate exerts its effects as an excitatory neurotransmitter through ionotropic and metabotropic receptors. The cartoon above depicts the architecture of these different receptor types and the different members of each class of receptor.

mGlu Receptor Structure

Ligand Binding Domain

Two domains comprise the large, extracellular ATD: the ligand binding domain (LBD) and the cysteine rich domain (CRD). Amino acid residues lining the inner surfaces of the top and bottom lobe of the LBD coordinate the binding of orthosteric agonists, including the endogenous agonists L-glutamate and L-serine O-phosphate (L-SOP) [216-218]. Both LBDs within an mGlu dimer exist in an equilibrium between open and closed states, leading to 3 receptor conformations: open-open, open-closed, or closed-closed[219-221]. Orthosteric agonist binding stabilizes the closed

confirmation of the LBD in which the top (LB1) and bottom (LB2) lobes enclose around the bound orthosteric ligand in a mechanism reminiscent of a Venus fly trap; indeed, for this reason the LBD is often referred to as the Venus fly trap domain (VFT). Agonist binding to one LBD (open-closed conformation) is sufficient for submaximal activation of the receptor; however, closure of both LBDs (closed-closed conformation) is required for full activation of the receptor [222,223]. Conversely, orthosteric antagonists stabilize the inactive (open-open) confirmation of the LBD. Though several functional studies suggest positive cooperativity of agonist binding within the LBDs [222-225], analysis of radioligand binding in $mGlu_1$ receptors suggests that agonist binding to one LBD decreases the agonist affinity of the other [226].

The LBD is a hot spot for binding of and modulation by cations and anions. Cl^- ions can bind to two unique sites; one site within the top lobe (LB1), and another formed between LB1 and LB2 subunits. Cl^- may act as an agonist and potentiator of $mGlu_4$ signaling, possibly due to its interactions with residues Ser229 and Tyr230 which are critical for glutamate binding [227,228]. Di- and trivalent cations (Ca^{2+}, Mg^{2+}, Gd^{3+}) also bind within the LBD [216,220,229]. Ca^{2+} and Gd^{3+} potently activate mGlu receptors even in the absence of glutamate [229-232] and Ca^{2+} can also potentiate signaling in response to glutamate [229-232]. Ca^{2+} is thought to bind within LB1 and modulate receptor sensitivity through interactions with a conserved serine residue within a critical helix for receptor activation, the C helix [216,229]. Mg^+ may occupy the same Ca^{2+} binding site, as co-administration of Mg^{2+} inhibits the stimulatory effects of Ca^{2+} [232]. While Gd^{3+} would be unlikely to be found in physiologically relevant levels within the body, it has been used in the crystallization of mGlu receptors and modulates their activity[220]. Gd^{3+} may occupy a unique binding site formed between the acidic surfaces of the LB2 domains of each protomer. These acidic patches form an interface upon receptor activation which Gd^{3+} binding may stabilize, possibly explaining activation of mGlu receptors by this trivalent cation [220]. mGlu receptors are likely exposed to a relatively constant concentration of Ca^{2+} and the kinetics of Ca^{2+}-induced activation are much slower than those

induced by agonists, suggesting ions, specifically Ca^{2+}, may provide a tonic level of activation/potentiation [230]. This tonic stimulation in the absence of agonist may explain why certain antagonists are observed to have inverse agonist activity [227,228].

Additionally, the LBD contains 4 conserved cysteine residues. One cysteine occurs within a disordered loop region between helices B and C of the ligand binding domain and forms an interprotomer disulfide bond with a homologous conserved cysteine within the opposing protomer, covalently linking the two mGlu promoters [233-235]. Two cysteines form an intraprotomer disulfide bond which is likely required for proper folding and ligand binding [216,233]. The final, conserved cysteine resides within a patch of residues on the bottom surface of LB2, the most conserved region of the LBD, and forms an intraprotomer bond the CRD; this disulfide bond in essential for linking the agonist-induced activation of the LBD to G protein activation [236,237].

Cysteine Rich Domain

The LBD is connected to the 7-transmembrane domain via the CRD. In addition to the intraprotomer disulfide bond formed between the LBD and the CRD, this domain contains 8 other essential cysteine residues which form 4 intraprotomer disulfide bonds within the CRD. These conserved disulfide bonds add a critical rigidity to this structure necessary to transmit ligand-induced activation of the LBD into a conformational change within the 7- transmembrane domain; deletion of the CRD or mutation of any conserved cysteine residue ablates signaling in mGlu receptors [236,238,239]. The CRDs of monomeric subunits are distant from one another within the relaxed receptor, but are brought into close proximity upon activation [217]. Furthermore, the creation of a disulfide bond between the CRDs of two mGlu protomers results in a constitutively active receptor that is decoupled from the LBD; basal activity may be decreased with the use of negative allosteric modulators, but not by orthosteric antagonists [238].

7 Transmembrane Domain

The mGlu transmembrane domain (TMD), also called the Heptahelical domain (HD), is composed of 7 membrane spanning α-helices which anchor the ATD to the cell membrane. Despite the similarity in transmembrane-spanning architecture to class A GPCRs, the amino acid sequence is not highly conserved between Class A and C receptors [210,211,240]. While the LBD binds orthosteric agonists and antagonists, most positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), with the exception of the cations mentioned previously, bind within the TMD [240-242]. In $mGlu_4$, PAMs may occupy one of, or a combination of, two binding sites: one homologous to the agonist binding pocket of class A receptors and the other homologous to a more deeply situated Na^+ binding pocket [242]. These PAMs display unique properties consistent with which pocket they bind; binding to the Na^+ binding pocket potentiates agonist induced response whereas binding to the homologous class A agonist pocket drives allosteric agonism [242].

In contrast to Class A GPCRs, intracellular loop 2 (il2), not il3, regulates the specificity of G protein coupling [225,243,244]. il3 still serves a critical function, however, in G protein activation; mutation of a single phenylalanine within this intracellular loop, F781S in $mGlu_4$, is sufficient to ablate G protein coupling and is more conserved than il2 across mGlu receptors [244]. For certain mGlu receptors, il2 is also an important site for regulation by G protein Receptor Kinases (GRK) [245]. Activation of all G protein subclasses may not proceed by the same mechanism; activation of $G\alpha_i$ requires that both TMDs functionally couple G protein whereas $G\alpha_q$ may couple to only one TMD [225,246].

C-Terminus

The C-terminus is important for G protein coupling, regulation, and managing protein-protein interactions in some mGlu receptors [247]; however, studies demonstrating a critical importance of this domain in $mGlu_4$ function are lacking. It has been suggested that this domain

may serve as a binding site for signaling and anchoring proteins [248-250] and is subject to alternative splicing [251]. These items will be discussed further in the “Localization, signaling cascade, and regulation” section of this review.

Dimerization and the LB1 interface

Dimer formation is paramount to proper ligand-induced mGlu receptor function [252]; individual mGlu protomers are even covalently linked by a conserved cysteine residue within the disordered loop atop the LBDs [233-235,253]. In contrast to the closely related $GABA_B$ receptor which may form a “dimer of dimers”, mGlu receptors do not seem to form higher order oligomers [222,254-256]. Furthermore, the LBD has been shown to be critical for dimerization to occur [216,224,235,257-259]. Crystallographic analyses of the mGlu1, 3, 5, and 7 ATD structures have extensively mapped this dimeric interface. Within the relaxed state of the receptor, the LBDs of each protomer are positioned in opposing orientation to one another with contacts between the two domains being overwhelmingly hydrophobic in nature and primarily occurring within the B and C helices situated in the hinge region of the top lobe (LB1) of the LBD [216,217,259-261]. This interface is often referred to as the LB1 interface. The conserved interprotomer disulfide bond is not required for receptor dimerization or activation as the hydrophobic interface alone suffices for dimerization.[233-235,253].

Dimerization occurs early on in the trafficking pathway in the endoplasmic reticulum (ER) and is important for proper receptor trafficking [262,263]. Dimerization is also essential for response to orthosteric ligands. When reconstituted in lipid nanodisks, only dimeric and not monomeric $mGlu_2$ receptors were able to couple G protein in response to agonist [252]. Conversely, compounds with purely allosteric activity, i.e. PAMs, in dimeric receptors functioned as agonists in either isolated monomeric receptors, or mutant receptors lacking the ATD, suggesting that dimerization is essential for response to orthosteric agonists and that, under unstimulated conditions, the LBD locks the TMDs in an inactive conformation [217,224,252]. Furthermore, the LB1 dimer interface is critical in the maintenance of this dimer state and receptor activation. Mutations within the B helix

of the dimer interface in $mGlu_2$ were shown in single molecule FRET studies not only to weaken the dimer interface but also to increase the proportion of receptors in the active state, even in the absence of agonist [224]. $mGlu_1$ receptors with mutations in helix B displayed signaling defects despite binding [^{3}H]-quisqualate [259].

Receptor Activation

mGlu receptor activation begins with stabilization of the closure of either one or both LBD domains by orthosteric agonist binding; closure of both LBDs is necessary for full activation [221-223]. This dual ligand-binding requirement contrasts the closely related Class C receptor, $GABA_B$, which exclusively binds agonist within one protomer, $GABA_{B1}$, of the heterodimeric receptor [264]. Closure induces a reorientation of the LBD domains in which the open face of the LBDs rotate downward towards the plasma membrane. The hydrophobic LB1 interface is maintained; however, it is reoriented by roughly 70° [216] and additional interprotomer contacts form between the LB2 domains [216,220,260]. This reorientation induces inter- and intra-subunit rearrangements within the receptor. The CRDs and TMDs of opposing protomers are brought into closer proximity to one another [216,217,237,238,260,265], forming additional inter-CRD and inter-TMD contacts in the active state of the receptor [217,260,266]. Intra-subunit rearrangements within the TMD occur on a longer timescale than intersubunit rearrangements, suggesting the conformational change within the TMD occurs after intersubunit rearrangement [265].

While two TMDs are required for agonist-induced activation [237,258], only one TMD transitions to an activate state in a given signaling event [265,267,268]. This is similar the $GABA_B$ receptor in which the $GABA_{B1}$ subunit, which binds orthosteric ligands, aids in the activation of the $GABA_{B2}$ subunit which exclusively couples G protein[264]. Activation can occur either in *cis*, in which LBD closure activates the TMD of the same protomer, or in *trans*, where LBD closure activates the TMD of the opposing protomer. In a set of elegant experiments by Brock et al., it

was shown that activation of an $mGlu_5$ homodimer occurs equally well by either *cis* or *trans* activation, with each activation pathway occurring ~50% of the time[237].

Adding additional complexity to mGlu signaling dynamics, there is considerable crosstalk between mGlu receptor subunits. The binding of negative or positive allosteric modulators to the TMD can influence the potency of agonist-induced LBD closure, suggesting communication between the two domains [221]. Furthermore, reorientation of the LBD is dependent on G protein binding [221].

mGlu Heterodimers

While once believed to function only as homodimers, recent studies have begun to elucidate the reality, capacity, and functional consequences of mGlu heterodimerization. An early study using $mGlu_1$ and $mGlu_5$ chimeric receptors which enrich for heterodimeric receptor combinations demonstrated that these receptors could form functional heterodimers, but it was unknown whether dimerization of these chimeric constructs was driven by the chimeric receptor expression system[267]. The utilization of FRET technology prominently influenced the study of receptor dimerization [255] and in 2011, a seminal paper was published by Doumazane et al. using fluorescently labeled mGlu receptors detailing the immense capacity of mGlu heterodimerization [254]. This study was the first of many to demonstrate that, in addition to homodimerization, heterodimerization occurs *in vitro* in two main groups: within group I receptors and inter-/intra-group heterodimerization within group II and III receptors [224,254,257,269-273]. Heterodimerization increases the number of possible mGlu receptor combinations within the CNS from 7 combinations to 18 (excluding $mGlu_6$,). The propensity for $mGlu_6$ heterodimerization is not well described; studies often exclude the $mGlu_6$ receptor due to its retinal specific localization [274]. Multiple mGlus are co-expressed in both GABAergic and glutamatergic neuronal subtypes [257,275,276], suggesting that many of the possible heterodimeric combinations are probable *in vivo*. *In vitro* characterization of $mGlu_{2/4}$, $mGlu_{2/7}$, $mGlu_{1/5}$, and $mGlu_{2/3}$ heterodimers has been

described in the literature; however, only $mGlu_{2/4}$, $mGlu_{1/5}$, and $mGlu_{2/7}$ have been shown to interact *in vivo* by co-immunoprecipitation from brain tissue [224,253,269,270]. Furthermore, the existence of only $mGlu_{2/4}$ heterodimers has been observed electrophysiologically by the use of subtype selective pharmacologic compounds[277]. It has been shown that the ATD alone is sufficient to account for differences in dimerization propensity between the different mGlu receptor subtypes [257].

Importantly, the inherent asymmetry of heterodimer formation creates receptors with pharmacology unique from homodimeric receptors[224,269-273]. Studies in immortalized cell lines have demonstrated that the potency of glutamate and the group II selective agonist LY379268 is much greater at $mGlu_{2/4}$ heterodimers than at $mGlu_4$ homodimers, resembling that of $mGlu_2$ homodimers [270,271,273]. However, L-AP4, a specific, potent agonist of group III mGlu receptors, has only partial efficacy at $mGlu_{2/4}$ heterodimers [270]. Furthermore, the function of subtype-specific allosteric modulators can diverge within heterodimers; while the $mGlu_4$ specific PAMs PHCCC, VU0418506, VU0155041 and Lu AF21934 can all potentiate signaling at $mGlu_4$ homodimers, only Lu AF21934 and VU0155041 can potentiate agonist-induced response at $mGlu_{2/4}$ heterodimers [270,278]. It is not known whether this dimer-specific pharmacology is driven by the unique PAM binding site occupied by these two classes of compounds, or by potential stoichiometric binding requirements for efficacy.

Asymmetry within heterodimers extends to G protein coupling as well; $mGlu_{2/4}$ heterodimers were shown to activate G protein almost exclusively through $mGlu_4$ subunits. Activation through the $mGlu_2$ subunit required either an $mGlu_4$ NAM or $mGlu_2$ PAM to direct activation through this protomer [272]. Furthermore, this asymmetric activation was shown to uphold in combinations of $mGlu_2$ with all other group III mGlu receptors ($mGlu_6$, $mGlu_7$, $mGlu_8$). However, asymmetric activation may not occur in all heterodimers; $mGlu_{1/5}$ heterodimers, like those of $mGlu_5$ homodimers, were able to activate G protein through the TMD of either protomer [253,267].

Contrary to an $mGlu_{2/4}$ heterodimer which is formed from a group II and III mGlu receptor, $mGlu_{1/5}$ heterodimers are composed of mGlu receptors from group I, possibly explaining its symmetric G protein coupling, though further experiments are needed to delineate the specific structural motifs and pharmacologic influences directing specific subunit activation. Moreover, heterodimeric receptors may be primed to asymmetric activation by nature of heterodimerization alone. Single molecule imaging experiments in $mGlu_{2/7}$ heterodimers suggest that heterodimerization with a non-ligand bound $mGlu_2$ increases the apparent affinity for LSP4-2022 1000-fold in the $mGlu_7$ subunit by inducing $mGlu_7$ to occupy a unique transition state [269]. Excitingly, this would offer a possible explanation as to how the low affinity $mGlu_7$ receptor could be activated at physiologic concentrations of glutamate; however, additional studies have not observed any such interactions with $mGlu_2$ and $mGlu_7$ [270-272]. While the ATD dictates the propensity of mGlu heterodimerization[257], the TMD contributes to heterodimer-specific differences in receptor activation; specifically, transmembrane helix 4 (TM4) and TM6[279].

Of additional note, studies have suggested the formation of mGlu receptor complexes with Class A GPCRs [280-282], though more work is needed to conclusively demonstrate the existence and relevance of these proposed heterodimers. These heterodimers fall outside the scope of this review and will not be discussed further. For more information, the reader is directed to the recent review on oligomeric receptor complexes in the CNS by Dasiel Borroto-Escuela and Kjell Fuxe cited here [283].

$mGlu_4$

Localization, signaling cascade, and regulation

$mGlu_4$ is expressed throughout the mammalian CNS as well as in several other tissues. Expression has been identified in the pancreas, stomach, gastrointestinal tract/colon, breast, bladder, skin, adrenal gland, kidney, upper respiratory tract epithelia, and in dendritic cells [284-287]. Higher levels of expression are observed within the central nervous system and the receptor

shows the most pronounced expression within the cerebellum where it localizes within the granule cells and parallel fibers[288]. $mGlu_4$ expression has also been observed in the hippocampus, hypothalamus, caudate nucleus, cortex, putamen, and spinal cord [213,276,288-293]. Expression is not limited to neurons, but has also been observed in astrocytes, oligodendrocytes, and microglia [294-297].

Three splice variants of the $mGlu_4$ receptor have been described–the $mGlu_{4a}$ receptor is the canonical $mGlu_4$ isoform. cDNA of the splice isoform $mGlu_{4b}$ was identified in a rat cerebellar lysate and differs from the canonical sequence in the coding and length of C-terminal domain of the receptor; the last 64 amino acids of $mGlu_{4a}$ are replaced by 135 unique residues in $mGlu_{4b}$. Both $mGlu_{4a}$ and $mGlu_{4b}$ splice isoforms displayed similar agonist preference, and coupled to $G\alpha_i$ in SF9 insect cells [251]. These splice isoforms may be differentially regulated; *In situ* hybridization data demonstrated that $mGlu_{4b}$ is specifically upregulated in rat cerebellar cortex following ischemia whereas $mGlu_{4a}$ is downregulated [298]. It should be noted, however, that no other study has been able to confirm the presence of $mGlu_{4b}$ transcripts, and it is unclear whether the donor/acceptor splice sites for such a transcript exists [299,300]. An additional splice isoform, often referred to as taste-$mGlu_4$ and which lacks approximately 50% of the N-terminal domain, is expressed only in the epithelium of the tongue and contributes to the sensation of umami taste [301]. Activation of taste-$mGlu_4$ stimulates $G\alpha_i$ and induces reductions in intracellular cAMP; however, much higher concentrations of the agonists L-AP4 or mono-sodium glutamate (MSG) are required for activation [301].

Within neurons, $mGlu_4$ localizes predominantly presynaptically on glutamatergic and GABAergic terminals where it functions as an autoreceptor or heteroreceptor, respectively, modulating the release of neurotransmitter [302-304]. As do all group III receptors, $mGlu_4$ couples to $G\alpha_{i/o}$ and stimulates decreases in the intracellular concentration of cAMP by inhibition of adenylyl cyclase [213,289]. The βγ subunits of the $G\alpha_{i/o}$ heterotrimeric complex, however, can serve multiple

additional functions. βγ subunits are able to directly inhibit voltage-gated Ca^{2+} ion channels [305,306] and activate G protein coupled inwardly rectifying K^+ channels (GIRKs) [307]. Decreased extracellular Ca^{2+} uptake and increased intracellular K^+ extrusion results in hyperpolarization of the synapse, inhibiting further vesicle release. In addition, βγ subunits can directly inhibit the SNARE complex, blocking the release of fused synaptic vesicles [308,309]. The mGlu stimulated suppression of neurotransmitter release can be excitatory, when occurring at GABAergic neuron terminals, or inhibitory when occurring at glutamatergic terminals. $mGlu_4$ distinctively localizes postsynaptically within the hippocampus, suggesting its function may be unique within this anatomic region [290].

$mGlu_4$ displays some promiscuity in its G protein coupling. Within the synapses of the cerebellar parallel fibers, $mGlu_4$ activation has been linked to the activation of $G\alpha_q$ and PLC/PKC pathways [310,311]. Studies have demonstrated that $mGlu_4$ can elicit its effects through additional GPCR signaling pathways. Apart from $G\alpha_{i/o}$ signaling, $mGlu_4$ activation has also been linked to the activation of the phosphoinositide 3-kinase (PI3-K) [312,313], c-Jun N-terminal kinases (JNK) [314], and mitogen-activated protein kinase (MAPK) [310,313] signaling pathways. $mGlu_4$ can also regulate neurotransmitter release by a mechanism that is independent of ligand binding and downstream signaling cascades. A key protein in the priming and release of release of synaptic vesicles, MUNC-18, interacts directly with $mGlu_4$ [304,315,316]. At resting state, $mGlu_4$ serves as a reservoir of bound MUNC-18; upon depolarization, increased intracellular Ca^{2+} activates calmodulin (CaM) which displaces the $mGlu_4$-bound MUNC-18, suggesting a possible mechanism allowing for the disinhibition of MUNC-18 and regulation of short term facilitation [315].

Within the synaptic membrane, mGlu receptors do not exist in isolation but rather extend into both intracellular and extracellular space allowing for the coordination of a network of protein-protein interactions to modulate signaling, trafficking, and localization [247]. The postsynaptic proteins ELFN1 and ELFN2 extend across the synapse, form transsynaptic interactions with

presynaptic group III mGlu receptors and allosterically modulate their activity [317,318]. Transsynaptic expression of these proteins increases $mGlu_4$ surface expression while simultaneously decreasing glutamate potency and maximal response in HEK293 cells [317,318]. ELFN2 KO mice display multiple neuropsychiatric manifestations as well as downregulation of multiple mGlu receptors; however, treatment with the $mGlu_4$ specific PAM VU0155041 rescued locomotor, epileptic, and sociability phenotypes, suggesting a crucial role of $mGlu_4$ specifically in these phenotypes [318]. A plethora of intracellular proteins have also been suggested to interact directly with $mGlu_4$ receptors, though functional evidence for most is lacking. Those proteins include: PKA [310], PKC [310,311,319,320], synaptosomal-associated protein 25 (SNAP-25), synapsin 1 [316], syntaxin [316], calmodulin [321,322], Calcium binding protein 1 (CaBP1) [319], syntentin, glutamate receptor interacting protein (GRIP) [249], microtubule-associated protein 1B (MAP1B) [323], Protein Interacting With PRKCA 1 (PICK1) [248-250], and chromobox protein homolog 4 (CBX4) [324]. Further experiments are needed to determine the potential functional consequences, if any, of interactions with these specific proteins.

Currently, little is known regarding the regulation of $mGlu_4$ expression, trafficking, and recycling. Conflicting reports have been published regarding $mGlu_4$ internalization. In HEK cells expressing a C-terminally tagged $mGlu_4$- Green Fluorescent Protein (GFP) construct, the receptor was rapidly internalized in response to L-AP4 and but returned to localization consistent with untreated cells after 10 minutes; this was dependent upon the action of G protein-coupled receptor kinase-2 (GRK2) [325]. In a separate study, L-AP4 and glutamate failed to induce internalization of a cMyc-$mGlu_4$ receptor in HEK cells, even increasing expression slightly in response to L-AP4; however, heterologous desensitization and internalization was induced by activation of PKC [320]. Reports regarding $mGlu_4$ mRNA expression are equally scant and conflicting. Subcutaneous injection (2mg/kg) twice a day for two days with the $mGlu_4$ PAM TCN238 negatively regulated *GRM4* expression in the hippocampus but not the frontal cortex of

Wistar Rats [326]. However, within the nucleus accumbens (NAc) and caudate putamen (CPu), $mGlu_4$ expression increased in response to pharmacologic activation [327]. In primary rat cerebellar granule neurons, the half-life of $mGlu_4$ transcripts was only 4 hours, suggesting that $mGlu_4$ mRNA transcripts may be regulated by active degradation under normal conditions. Furthermore, expression of these transcripts was dependent on active protein synthesis [328].

Associated Disease/Disorders

Grm4$^{-/-}$ Animals

Genetic knock-out mice which do not express $mGlu_4$ protein (*Grm4*$^{-/-}$ mice) are generally phenotypically normal throughout development and into adulthood, performing normally in numerous behavioral assays [329-333]; however, *Grm4*$^{-/-}$ mice display motor learning abnormalities suggestive of problems with the processing of spatial information [331,333-335], likely associated with its high expression within the cerebellum. Additionally, *Grm4*$^{-/-}$ mice have a significantly reduced acoustic startle response and paired pulse inhibition, suggesting problems in sensorimotor gating, a process by which the brain filters out relevant from irrelevant information, possibly due to the loss of $mGlu_4$ expression within the hippocampus and basal ganglia [329]. *Grm4*$^{-/-}$ mice also demonstrate increases in the acquisition of cued fear, a measure of hippocampal and amygdala-dependent associative learning and memory [332]. This means *Grm4*$^{-/-}$ mice acquire a form of associative learning and memory better than wild type mice.

Presynaptic group III mGlus modulate neurotransmitter release from glutamatergic and GABAergic neurons, and *Grm4*$^{-/-}$ mice can aid in determining anatomical regions and synapses where $mGlu_4$ specifically plays a critical functional role. Impairments in paired pulse facilitation (PPF) of cerebellar parallel fiber-Purkinje cell synapses have been observed in *Grm4*$^{-/-}$ mice, possibly explaining the motor abnormalities observed in these animals [334]. Interestingly, despite issues with spatial learning and memory, PPF increased in the dentate gyrus and CA1 of the hippocampus in *Grm4*$^{-/-}$ mice [335]. $mGlu_4$ may further play an important functional role in modulating

neurotransmitter release within the thalamocortical circuitry. Analysis of extracellular neurotransmitter levels in *Grm4*$^{-/-}$ mice by fiber voltammetry determined that *Grm4*$^{-/-}$ mice had a significant increase in both basal and evoked glutamate in the ventrobasal thalamus (VBT), nucleus reticularis thalami (NRT), and cortical layers IV-VI [336]. Basal and evoked GABA levels were likewise significantly higher within the VBT compared to wild type mice; however, in contrast to glutamate, basal and evoked GABA levels were lower in cortical layers IV-VI of *Grm4*$^{-/-}$ mice and no change was observed in the NRT [336]. No differences in extracellular concentrations of either glutamate or GABA were observed in cortical layers I-III [336].

Critical functions of $mGlu_4$ are not restricted to neurons; in a model of Experimental Autoimmune Encephalomyelitis, a rodent model commonly used to simulate Multiple-Sclerosis (MS) [337], *Grm4*$^{-/-}$ mice develop more severe disease phenotypes and faster disease progression than wild type mice, suggesting a critical extra-neuronal role for $mGlu_4$ within dendritic cells of the immune system [287,338].

Central Nervous System Disorders

Neurologic/Psychiatric disorders

Genome wide association studies (GWAS) in human populations have implicated $mGlu_4$ in several neurologic diseases and disorders. *GRM4* SNPs have been associated with Bipolar Disorder, schizophrenia [339], Major Depressive disorder [340], and ADHD [341]. Interestingly, all of the identified $mGlu_4$ SNPs associated with these disorders occur within introns and are not predicted to alter the coding sequence of the receptor; however, SNPs within intronic elements may still affect receptor function by altering expression [340,341], splicing, or base-specific RNA modifications potentially contributing to these disease states.

Furthermore, pharmacologic activation of $mGlu_4$ alleviates symptoms in rodent models of autism, schizophrenia, anxiety, and depression. In *Opm1*$^{-/-}$ mice, a model of autism spectrum

disorder, $mGlu_4$ expression is significantly decreased within the CPu and NAc of the reward/social circuitry [327,342]. Treatment with either behavioral intervention or the $mGlu_4$-specific PAM VU0155041 improved behavioral outcomes in *Opm1*$^{-/-}$ mice. Moreover, behavioral intervention partially normalized $mGlu_4$ expression in affected brain regions [327,342]. Within the Ashkenazi Jewish population, $mGlu_4$ was one of six genes strongly associated with both Bipolar 1 disorder and schizophrenia [339]. In rodent models of schizophrenia, treatment with $mGlu_4$-selective agonists (LSP1-2111, LSP4-2022) and PAMs (Lu AF21934, Lu AF32615, ADX88178) alleviated phenotypes in mouse models consistent with positive and negative symptoms of the disorder [343-346]. Pharmacologic activation may further be used to treat mood disorders such as anxiety and depression. The use of group III mGlu agonists have validated these receptors as potential targets for mood disorders such as anxiety and depression [347-350]. Specific targeting of the $mGlu_4$ receptor, with orthosteric agonists or PAMs, may be sufficient in treating anxiety [345,351,352] and depression [353].

Pain

Pain affects as many as 75 million Americans [354]. Many of the medications used to treat acute and chronic pain have limited efficacy and may have unwanted, harmful side effects such as the addictive properties of opioids [355]. $mGlu_4$ is an attractive target in the management of pain due to its expression within the amygdala and dorsal horn of the spinal cord [293,356-358]. Studies in *Grm4*$^{-/-}$ mice suggest $mGlu_4$ may modulate only certain types of pain. For example, *Grm4*$^{-/-}$ mice have an increased sensitivity to mechanical compression and more quickly develop nociceptive behaviors in the inflammatory phase of the formalin test, a common model of acute pain stimulated by the subcutaneous injection of formalin into a rodent's paw, in comparison to wild type mice, but are unchanged in their response to punctate mechanical and thermal stimuli [358]. Intrathecal injection of the group III agonists L-AP4 or ACPT-I has been demonstrated in rodent models to depress signaling within the spinal cord, reducing sensitivity to noxious stimuli [357,359].

Due to the advent of subtype-selective compounds, the role of $mGlu_4$ has also been addressed more directly. In mice, both systemic infusion and direct intra-amygdala infusion of the $mGlu_4$ agonist LSP4-2022 reduced hypersensitivity and pain related behaviors in a complete Freund's adjuvant (CFA)-induced pain model, while an effect was not observed in control animals [358,360]; notably, this effect was significantly reduced in *Grm4*$^{-/-}$ mice [358]. Allosteric modulation of $mGlu_4$ may also prove efficacious in pain management to various stimuli. Intrathecal injection of PHCCC inhibited hypersensitivity to mechanical stimuli in inflammatory and neuropathic rat pain models[359]. Furthermore, in L5 spinal nerve ligated rats, a model of neuropathic pain, intrathecal administration of VU0155041 dose dependently increased pain thresholds to both mechanical and thermal stimuli [361].

Epilepsy

Epilepsies are common neurologic disorders characterized by recurrent, unprovoked seizures that affects ~1% of the population [362]. Roughly 1/3 of those individuals have refractory epilepsy, which is not well controlled by current drug regimens [362]. Genome wide association studies (GWAS), immunohistochemical, and pharmacologic studies strongly suggest a role for $mGlu_4$ in epilepsy, and the *GRM4* gene (human chromosome 6, band p21.3) resides within a known susceptibility locus known for juvenile myoclonic epilepsy [300,363]. GWAS analysis has discovered a moderate but significant association of the *GRM4* SNP, rs2029461, with both generalized [364] and juvenile myoclonic epilepsy [365-367]. An additional SNP, rs2451334, is significantly associated with response to anti-epileptic drugs (AEDs), underscoring the importance of $mGlu_4$ in epilepsy treatment [364].

Alterations in $mGlu_4$ expression have likewise been associated with status epilepticus (SE). Within a patient suffering chronic focal encephalitis, several mGlu receptors were found to exhibit significantly decreased expression; however, most striking was the cortical expression of $mGlu_4$, which was 20-fold lower than those of temporal lobe epilepsy patients and 200-fold lower

than control brain tissues [368]. Upregulation of $mGlu_4$ in SE has also been described; in surgically resected hippocampal samples from patients with temporal lobe epilepsy, $mGlu_4$ mRNA and protein expression was significantly increased within the dentate gyrus and CA4 regions of the hippocampus [369]. It remains to be determined whether these alterations are causal or a result of SE in these patients; however, in kainate-induced SE in rats, $mGlu_4$ expression was upregulated within the CA3 region of the hippocampus at P10, suggesting $mGlu_4$ expression may be regulated in response to SE, perhaps to protective against further excessive neuroexcitation [370]. The contribution of $mGlu_4$ to SE may be dependent on seizure type. *Grm4*$^{-/-}$ mice were resistant to absence seizures induced by the administration of a $GABA_A$ antagonist [371] but suffered significantly more severe SE symptoms and more resulting hippocampal cell loss following SE induced using the $GABA_A$ antagonist pilocarpine[372].

Pharmacologic targeting of $mGlu_4$ offers potential novel treatment avenues for epilepsy, although more research is needed to determine if, when, and how receptor activation vs. inactivation is required. Administration of group III receptor agonists may have pro- and anticonvulsant effects; administration of L-AP4 or L-SOP are protective during SE; however, administration of L-SOP briefly acts as a proconvulsant before becoming protective [373]. The functional contribution of $mGlu_4$ within the pro- or anticonvulsant phases of treatment are not yet known. It additionally remains to be determined whether allosteric modulation of $mGlu_4$ would be efficacious in the treatment of SE. The administration of $mGlu_4$-specific agonists is beneficial in the treatment of SE [374,375]; however, treatment with the $mGlu_4$ PAM PHCCC may increase seizure severity [376].

Neuroprotection / NeuroInflammation

$mGlu_4$ activation is broadly neuroprotective through both direct and indirect mechanisms which limit neuroinflammation. $mGlu_4$ activation is protective against cell death in neurons [295,328,377], oligodendrocytes [294], and microglia [295]. Direct pharmacologic stimulation of $mGlu_4$ offers

neuroprotection. *In vitro*, $mGlu_4$ activation increased the viability of primary rat cerebellar granule neurons in response to low K^+ [328], as well as hippocampal neurons in response to staurosporine and glutamate-induced toxicity [377]. Moreover, cultured cerebellar neurons were protected from NMDA-induced toxicity by the agonist cinnabarinic Acid (CA) and the $mGlu_4$-specific PAM PHCCC; however, this could be due to effects mediated through astrocytes in co-culture conditions [378]. Indeed, neurons may also garner protection through $mGlu_4$ activation indirectly. The group III agonist L-AP4 decreases microglia-induced neuronal toxicity *in vitro*[295]. Furthermore, administration of the group III agonists L-AP4 and DCG-IV to MPP^+-treated astrocyte cultures significantly reduced the toxicity of the resulting conditioned media when added to neurons in culture in comparison to that of MPP^+-treated astrocytes without group III agonist administration[296]. This indirect neuroprotection may be due to a restoration of glutamate uptake in MPP^+-treated astrocytes[296]. Protection of oligodendrocytes may additionally be attributed to activation of astrocytic $mGlu_4$ as it has been shown that protection of oligodendrocytes against kainic acid (KA) induced toxicity was dependent on the presence of co-cultured astrocytes [294]. Interestingly, the anti-inflammatory effect was dependent of $G\alpha_{i/o}$ signaling in astrocytes [297], but independent of $G\alpha_{i/o}$ mediated signaling in microglia [379] and dendritic cells [338], suggesting possibly divergent mechanisms for $mGlu_4$ stimulated anti-inflammatory actions in these cell types.

The indirect protection afforded by $mGlu_4$ activation may manifest through the inhibition of inflammatory signaling. Inflammation induced by Lipopolysaccharide (LPS) increased $mGlu_4$ expression in *in vitro* astrocyte cultures [294] and stimulation of $mGlu_4$ decreased expression of the pro-inflammatory chemokine RANTES [297]. In isolated primary microglia, activation of $mGlu_4$ limits inflammatory signaling and presentation of major histocompatibility complex class II (MHCII) proteins [379,380]. *In vivo* models have likewise demonstrated the critical influence of $mGlu_4$ signaling in neuroinflammation; *Grm4*$^{-/-}$ mice are considerably more susceptible to Experimental Autoimmune Encephalitis (EAE) [287,381]. This experimental model resembles the

neuroinflammation observed in multiple sclerosis (MS) and is induced by immunization of encephalitic peptides, such as myelin basic protein [337,382]. During EAE, peripheral dendritic cells infiltrate CNS tissue and activate T-cells, exacerbating inflammation [382]. $mGlu_4$ expressed within those dendritic cells critically determines the T-cell response by enhancing the expansion of T-regulatory and T-helper cells while limiting expansion of pro-inflammatory of Th-17 cells [287,381]. In cultured dendritic cells, $mGlu_4$ activation using the PAM ADX88178 increased levels of the anti-inflammatory molecules IL10 and TGF-β [338]. Furthermore, *in vivo* prophylactic treatment with Group III agonist L-AP4, cinnabarinic acid (CA), or the $mGlu_4$ PAM PHCCC significantly reduced the severity of EAE symptoms [287,297,381].

Neuroinflammation is a hallmark of neurologic conditions such as neurodegeneration [383], traumatic brain injury (TBI) [384], and Parkinson's disease [385]. Pharmacologic activation of $mGlu_4$ is efficacious in preventing dopaminergic cells loss in Parkinson's disease, which is discussed more in the following section. Other neuroinflammatory disorders could benefit, however, from $mGlu_4$ activation. Following a diffuse brain injury (DBI), $mGlu_4$ expression is significantly upregulated in rat cerebellar cortex [386,387]. While more studies with $mGlu_4$-specific compounds are needed, treatment with L-AP4 significantly improved the performance of injured animals on days 3 and 7 in the balance beam and inclined plane tasks following DBI compared to saline treated animals [387], demonstrating the potential of Group III mGlu receptor activation in neuroinflammation.

Parkinson's disease (PD)

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder, affecting 1.5-2% of the population over 60, and is characterized by death of the dopaminergic neurons of the substantia nigra[388]. Dopaminergic cell death in the substantia nigra reduces the release of dopamine in the basal ganglia, affecting two pathways in controlling movement. Decreased dopamine release decreases activation of the direct pathway and disinhibits the indirect pathway to cause a suppression of movement. The current first-in-line

therapy, L-DOPA/Carbidopa, is efficacious but also limited both by waning efficacy over disease progression and by its burdensome dyskinetic side effects[389]. Pharmacologic activation of the $mGlu_4$ receptor offers an exciting treatment avenue to alleviate symptoms, enhance L-DOPA efficacy, and reduce dopaminergic neuron death, thereby acting as a disease modifying therapy. Interest in targeting the $mGlu_4$ receptor stems from its presence in excitatory glutamatergic projections from the cortex to the striatum [270,292,390-393](corticostriatal projections), from the subthalamic nucleus to the substantia nigra [291,394] (STN-SNr/c projections), and in inhibitory GABAergic projections from the striatum to the globus palidus (striatopalidal projections) [291,299,395-397]. Activation of $mGlu_4$ in these synapses can restore the direct/indirect pathway balance by limiting the output of the indirect pathway [389]. Group III mGlu receptor agonists and $mGlu_4$-specific PAMs have shown efficacy in various rodent models of PD [278,393,395-406] as well as primate PD models [292,407]. Of particular interest, $mGlu_4$ activation relieves the increased glutamatergic tone onto dopaminergic neurons of the substantia nigra, reducing dopaminergic cell death, and increasing the efficacy of L-DOPA treatment [378,399,407-411]. Recent findings suggest that while striatopalidal synapses express mainly $mGlu_4$ homodimers, corticostriatal synapses express mainly function $mGlu_{2/4}$ heterodimers [270]. VU0418506, an $mGlu_4$ specific PAM which does not potentiate $mGlu_{2/4}$ heterodimers, was efficacious in treating rodent models of PD [278], suggesting that potentiation of $mGlu_4$ homodimers alone, or perhaps potentiation of the striatopalidal synapse alone, is sufficient for pharmacologic rescue of PD symptoms.

Addiction

Drug addiction is a worldwide epidemic with estimated costs of up to $740 billion in the US alone[412]. In the past 20 years, our knowledge of the molecular mechanisms underlying addiction has evolved to elucidate multiple drug-specific molecular mechanisms converging on a similar pathway: dopamine release in the NAc [412,413]. Multiple mGlu receptors exert effects on addiction [414], but only more recently has the role of $mGlu_4$ been addressed. Though the exact

neural networks of $mGlu_4$ function in addiction are not well mapped, recent studies demonstrate $mGlu_4$ activation could reduce the efficacy of certain drugs of abuse and curb relapse.

$mGlu_4$ may function as a regulator of the motor stimulator effects of ethanol. $Grm4^{-/-}$ mice were seemingly immune to the motor stimulatory effects of ethanol despite showing similar drinking preferences to their wild type litter mates [415]. In a mouse model of chronic alcohol consumption, C57BL/6 mice that consumed alcohol had a 2.2-fold reduction in $mGlu_4$ mRNA expression within the NAc [416]. In human subjects, however, $mGlu_4$ expression was elevated within 16 brain regions in human chronic ethanol users [417]. It is unknown whether these expression changes are region- or species-specific, as $mGlu_4$ expression within the human NAc has not yet been described. Furthermore, pharmacologic activation of $mGlu_4$ may serve as a potential avenue to limit alcohol consumption. Treatment with the agonist LSP2-9166, which activates $mGlu_4$ and $mGlu_7$, reduced ethanol intake and significantly reduced reinstatement of drug seeking after 2 week cessation period in a rat model of alcohol consumption [418]. Further work is required to determine the contributions of $mGlu_4$ and $mGlu_7$ to the effects of LSP2-9166, and the molecular mechanisms underlying its effects.

$mGlu_4$ activation may also regulate aspects of cocaine and morphine addiction. Cocaine use in rats increased the expression of both $mGlu_4$ and $mGlu_7$ within the hippocampus and NAc while decreasing expression in the amygdala [419]. Within mice, however, $mGlu_4$ expression was unchanged in both the NAc and CPu following cessation from cocaine administration [420], again raising questions regarding whether these alteration are species-specific, or model-specific, effects. In rat models of self-administration, systemic administration of the agonist LSP2-9166 or the $mGlu_4$-specific PAM Lu AF21934 decreased sensitization to cocaine [419,421]. Sensitization to nicotine was unaffected by $mGlu_4$ activation, underscoring that $mGlu_4$ activation as a therapeutic target in addiction may be compound or mechanism-specific [421]. In mice, cessation from morphine downregulated $mGlu_4$ expression within the CPu and NAc [420]. Treatment with the agonist LSP2-

9166 decreased the rewarding properties of morphine and decreased reinstatement of drug seeking behavior, but this effect is suggested to be due to activation of $mGlu_7$ due to ablation of these effects with co-administration of the $mGlu_7$ NAM, XAP044 [422]. However, intra-accumbal injection of VU0155041 was sufficient to dose dependently decrease the instatement of conditioned place preference (CPP) in a Wistar Rats [423]. Furthermore, pharmacologic activation of $mGlu_4$ during the cessation period may help improve social functioning aspects disrupted by drug abuse and additionally may restore $mGlu_4$ expression within the NAc [420].

Peripheral pharmacologic avenues

Glucagon Release

Conflicting reports have been published regarding $mGlu_4$ expression and function in pancreatic islets. Both $mGlu_4$ and $mGlu_8$ receptors have been demonstrated to be expressed within the α-cells of the pancreas and to limit the release of glucagon upon activation [286,424,425]; however, the lack of highly selective agonists in these studies limits the ability to conclude whether either of these receptors alone, or perhaps in concert, serve to regulate pancreatic glucagon release. Further confounding the interpretation of this data, Cabrera et al. observed that treatment of isolated, perfused mouse and human islets with the group III agonists, *trans*-ACPD and ACPT-1, or antagonist CPPG did not alter glucagon release in comparison to untreated islets[426]. Moreover, the glucagon response of islets from *Grm4*$^{-/-}$ mice were not distinguishable from those of their wild type littermates[426]. Further studies are needed to validate the potential of $mGlu_4$ as a pharmacologic target for modulating glucagon release.

$mGlu_4$ in Cancer

In recent years, an association of $mGlu_4$ in the progression of numerous cancers has emerged. Most well described is the association of $mGlu_4$ with osteosarcoma, originally identified by Savage et al in 2013 [427]. This finding was later confirmed in two GWAS studies [428,429] and further demonstrated in osteosarcoma cell models [324,430]. GWAS studies identified a single

nucleotide polymorphism (SNP), rs1906953 (C>T), which correlated with osteoarcoma prognosis and metastasis; however, these studies in separate Chinese populations reached opposing conclusions. Jiang et al found that, within the Chinese Han population, those homozygous for the thymidine at rs1906953, the TT genotype, had decreased survival rates compared to those with a CC genotype, with heterozygous CT individuals demonstrating median survival rates between the two [428]. Conversely, Wang et al. found in the Chinese Guangxi population that CC individuals had the lowest rates of survival, with TT individuals faring best [429]. rs1906953 (C>T) is not predicted to alter the coding sequence of $mGlu_4$ protein, but rather *In vitro* data suggests that a C to T transition at this locus may decrease transcription of GRM4 by reducing nucleoprotein binding [341]. In the Chinese Guangxi population, $mGlu_4$ expression was significantly enriched in osteosarcomas compared to surrounding tissues and correlated with metastasis and Enneking Stage, a measure of progression in bone cancer [431]. Despite this, overexpression of $mGlu_4$ was demonstrated to decrease proliferation, migration and invasion in MG-63 and U2OS osteosarcoma cell lines [324]. A recent study by Kansara et al. suggests that the effects of $mGlu_4$ in osteosarcoma may not be due to $mGlu_4$ expression within the tumor, but in infiltrating $CD45^+$ $CD11c^+$ MHC^+ myeloid cells[432]. In a ^{45}Ca-induced model of osteosarcoma, $Grm4^{-/-}$ mice developed tumors faster than wild type mice and their dendritic cells displayed increased expression of pro-inflammatory markers IL12 and IL23 [432].

$mGlu_4$ has also been associated with cancers of the brain [312], colon [433], bladder [434], kidney [435], and breast [285]. $mGlu_4$ expression has been linked to the development of cerebellar tumors, or medulloblastomas. This is thought to arise from the expression of $mGlu_4$ within neural stem cells and its influence on neural cell proliferation and differentiation [312,314,436-438]. The role of $mGlu_4$ within healthy bladder, kidney, and breast tissues has yet to be defined, but it is likely to be unique within these different tissues. While increased $mGlu_4$ expression was positively correlated with poorer

overall outcomes in bone [431], bladder [434], and renal cancer [435], the opposite was true of outcomes in patients with breast cancer [285] and medulloblastoma [312].

The expression of $mGlu_4$ within cancerous tissues is an exciting prospect as a new target against this potentially devastating disease. *In vitro* data suggests that pharmacologic activation of $mGlu_4$ could inhibit tumor growth in certain cancers. The $mGlu_4$-specific PAMs PHCCC and VU0155041 decreased the *in vitro* proliferation of medulloblastoma [312] and bladder cancer [434] cell lines, respectively. Furthermore, pharmacologic activation of $mGlu_4$ may increase the efficacy of current chemotherapeutic agents; within a 5-FU resistant colorectal cancer cell line, *in vitro* co-administration of the $mGlu_4$ antagonist MAP4 increased cytotoxicity of 5-FU in resistant cells [433]. Perhaps most excitingly, the $mGlu_4$ PAM, PHCCC, was able to reduce tumor size in xenograft mouse models of osteosarcoma and medulloblastoma [312,432]. Within the mouse osteosarcoma xenograft model, a 10mg/kg dose of PHCCC was as efficacious in reducing tumor growth as a 5mg/kg dose of the common osteosarcoma treatment doxorubicin, but was not associated with weight loss in treated mice [432], underscoring its potential for *in vivo* efficacy. Additionally, the correlation of $mGlu_4$ expression with disease severity highlights the potential for receptor expression as a clinical biomarker in several cancers.

Summary and Conclusions

Metabotropic Glutamate Receptor 4 ($mGlu_4$) is one of eight mGlu receptors within the Class C G protein-coupled receptor superfamily. It is localized primarily to the presynaptic membrane of neurons where it functions as an autoreceptor and heteroreceptor controlling synaptic release of neurotransmitter. Several studies have demonstrated roles of $mGlu_4$ signaling in addiction, T-cell maturation, glucagon release, breast cancer metastasis, neuroinflammation, pain, autism, schizophrenia, epilepsy, anxiety, depression and Parkinson's disease. $mGlu_4$ is implicated in numerous disorders and has been proposed to be a promising, druggable target;

however, more remains to be understood about its regulation and pharmacology, specifically by RNA editing and dimerization with other mGlu family receptors.

Chapter II: Regulation of A-to-I editing of $mGlu_4$ mRNA Transcripts

Preface

This chapter includes text and figures that were originally published in *RNA*[439]. Colleen M. Niswender, Sheridan Carrington, Andrew N. Keller, and Karen J. Gregory contributed as authors.

Introduction

Millions of editing sites have been discovered across mammalian transcriptomes; these occur primarily within 5' and 3' regulatory elements of mRNA transcripts and are poorly conserved across species [22,96,127]. However, there is a much smaller, number of adenosines within coding sequences that are highly conserved and undergo substantial editing [136]. In the brain, these editing events occur in transcripts encoding proteins critical for neuronal signaling and excitability, including ionotropic glutamate and GABA receptor subunits, the $K_V1.1$ potassium channel, and the 5-HT_{2C} serotonin receptor [39,40,43,44,440-442]. As inosine is read as guanosine by the cellular translational machinery, these A-to-I editing events often involve non-synonymous codon changes in mRNA, resulting in the production of proteins with altered amino acid sequences and potentially unique functional properties. A-to-I editing is generally restricted to the central nervous system [75], where it can vary between species, anatomic regions and even individual cell types [78,125,131,151,152], suggesting that editing levels may be regulated to the specific cellular or anatomical needs.

The conversion of A-to-I is a widespread co-transcriptional modification resulting from the hydrolytic deamination of selective adenosine residues catalyzed by a family of Adenosine Deaminases that Act on RNA (ADARs) [29]. Localized to the nucleus, ADARs target select adenosines by binding to double-stranded (ds) RNA substrates, often formed via inverted repeats between exons and neighboring introns of pre-mRNA transcripts. While all three ADAR enzymes can bind ds-RNA, ADAR3 is thought to be catalytically inactive [56]. Additionally, ADAR1 is

expressed as two main splice variants: a constitutive form, ADAR p110, and an inducible form, ADAR p150 [61]. ADAR1 p110 catalyzes site specific deamination within the central nervous system (CNS), whereas the interferon-inducible ADAR1 p150 is a crucial regulator within the innate immune response [62]. ADAR1 and ADAR2 have been demonstrated to have an overlapping ability to edit certain adenosines, while acting specifically at others [29,50].

Using existing RNA sequencing datasets to identify novel editing sites across the human transcriptome, Ramaswami et al. first discovered adenosine-to-inosine (A-to-I) editing of $mGlu_4$ pre-mRNA transcripts in 2013 [22]. A-to-I editing of $mGlu_4$ transcripts predicts a glutamine (Q) for arginine (R) at position Q124; this amino acid resides within the critical "helix B" of the N-terminal, glutamate binding domain of the receptor [216]. mGlu receptors are obligate dimers, and helices B and C are critical in forming the mGlu dimer interface in both the resting and activated states of the receptor [216,217,220,252,260]. Several studies have demonstrated the importance of helix B in modulating receptor dimerization, trafficking, and activation [224,259].

The present analysis was designed to identify and accurately quantify editing sites within the $mGlu_4$ ligand-binding domain. While the existence of the Q124R editing site in $mGlu_4$ transcripts has been known for several years, no studies have extensively characterized A-to-I editing of $mGlu_4$ transcripts [22,45,153]. Additionally, critical *cis* and *trans* elements involved in the regulation of $mGlu_4$ editing within the mammalian brain were identified. Finally, the conservation of this editing event amongst mGlu receptors and across species was addressed. Similar experimental paradigms have been used in the characterization of past recoding editing events [39,43,44].

Results

A-to-I editing alters two amino acids in the $mGlu_4$ dimer interface

RNAseq is a powerful tool to identify and quantify A-to-I editing sites across the transcriptome. However, low read depth for certain transcripts can lead to a failure to detect less frequently edited adenosines as well as improperly quantify those detected. We scanned the rat $mGlu_4$ coding sequence using Sanger sequencing for evidence of RNA editing; however, artifacts, or "noise", observed by Sanger sequencing may be mistaken for evidence of RNA editing. Therefore, we employed a targeted approach, High Throughput Multiplexed Transcript Analysis (HTMTA) [154], to validate potential editing sites observed by Sanger sequencing methods occurring within regions encoding N-terminal domain of the receptor. Using HTMA, we probed rat (NM_022666.1) $mGlu_4$ transcripts from nucleotides 1022-1108, 1221-1330, 1339-1459 (encoding T50-D92, V123-V165, A166-V198) for novel editing sites. A rat model was used due to its common use in the study of $mGlu_4$ pharmacology. Reads were restricted due to read length limitations of the Illumina sequencing platform. After validating two editing sites which lay within the sequence encoding a putative dsRNA editing substrate in rat $mGlu_4$ transcripts, the homologous region of human transcripts (NM_000841.4) from nucleotides 729-854 (encoding P93-R135)was probed. Using this technique, we quantified the editing levels of the Q124R editing site in multiple brain regions of both rat (~27.6%) and human (~10.3%) tissues (Figure 6A, B). In addition to this previously discovered Q124R site, we identified a novel editing site located 15 nucleotides downstream of this position (editing percentages in brain of 1.6% rat, 2.1% human), predicting a lysine to arginine substitution at position 129 of the resulting protein (Figure 6A). No additional sites were observed within human *GRM4* transcripts across the predicted RNA duplex region.

Together, transcripts edited at these two sites comprise roughly ~11.7% and 22.3%-35.1% of the transcript pool in human and rat brain samples, respectively. Using HTMTA, we identified

and quantified the 4 unique transcript isoforms resulting from editing at all combinations of the two sites (Figure 6B). Edited transcript levels appeared region-specific in the rat brain, with editing levels in cerebellum and hypothalamus varying significantly from cortex, hippocampus, and striatum. Though the Q124R transcript isoform was the most prevalent edited transcript in all rat brain regions, levels were 10-11% higher in the cerebellum and hypothalamus than in the cortex ($p<0.02$) and striatum ($p<0.01$). K129R and Q124R/K129R transcripts were likewise increased 0.17-0.25 % in hypothalamus ($p<0.05$) compared to cortex and hippocampus and increased 0.97-1.2% compared to all other regions ($p<0.0001$), respectively. Conversely, these transcripts were significantly decreased in cerebellum, with 0.27%-0.52% lower levels of K129R transcripts compared to cortex or hippocampus ($p<0.02$) and 0.53-0.76% Q124 K129R in cerebellum compared to all other tissues ($p<0.01$). For both human and rat samples, no significant differences were found between the levels of edited transcripts in cortex, hippocampus, and striatum within species (Figure 6B). Interestingly, while Q124R edited transcripts were on average 2.7-fold higher (16.5%, $p<0.0001$) in rat brain regions compared to human, the opposite was true for those edited at K129R alone, which was 2.9-fold higher (0.9%, $p=0.04$) in human transcripts. In contrast, transcripts edited at both sites were not significantly different between species in the hippocampus and cortex but showed a significant 2.2-fold increase (0.64%, $p=0.02$) in rat striatum (Figure 6C).

RNA editing of $mGlu_4$ transcripts predicts the substitution of amino acids within the B helix of the resulting receptor protein (Figure 6D). An alignment of the mGlu receptors' B and C helix peptide sequences from rat (Figure 6D) and human sequences (Figure 6E) reveals that the B helix is well conserved across the mGlu receptors as well as across species. Q124 of $mGlu_4$ is completely conserved in group III mGlu receptors; however, an arginine is present in the group I and II mGlu receptors. K129 of $mGlu_4$ is conserved in group III receptors, except for $mGlu_6$, but is less conserved than the Q124 position across the mGlu receptor family.

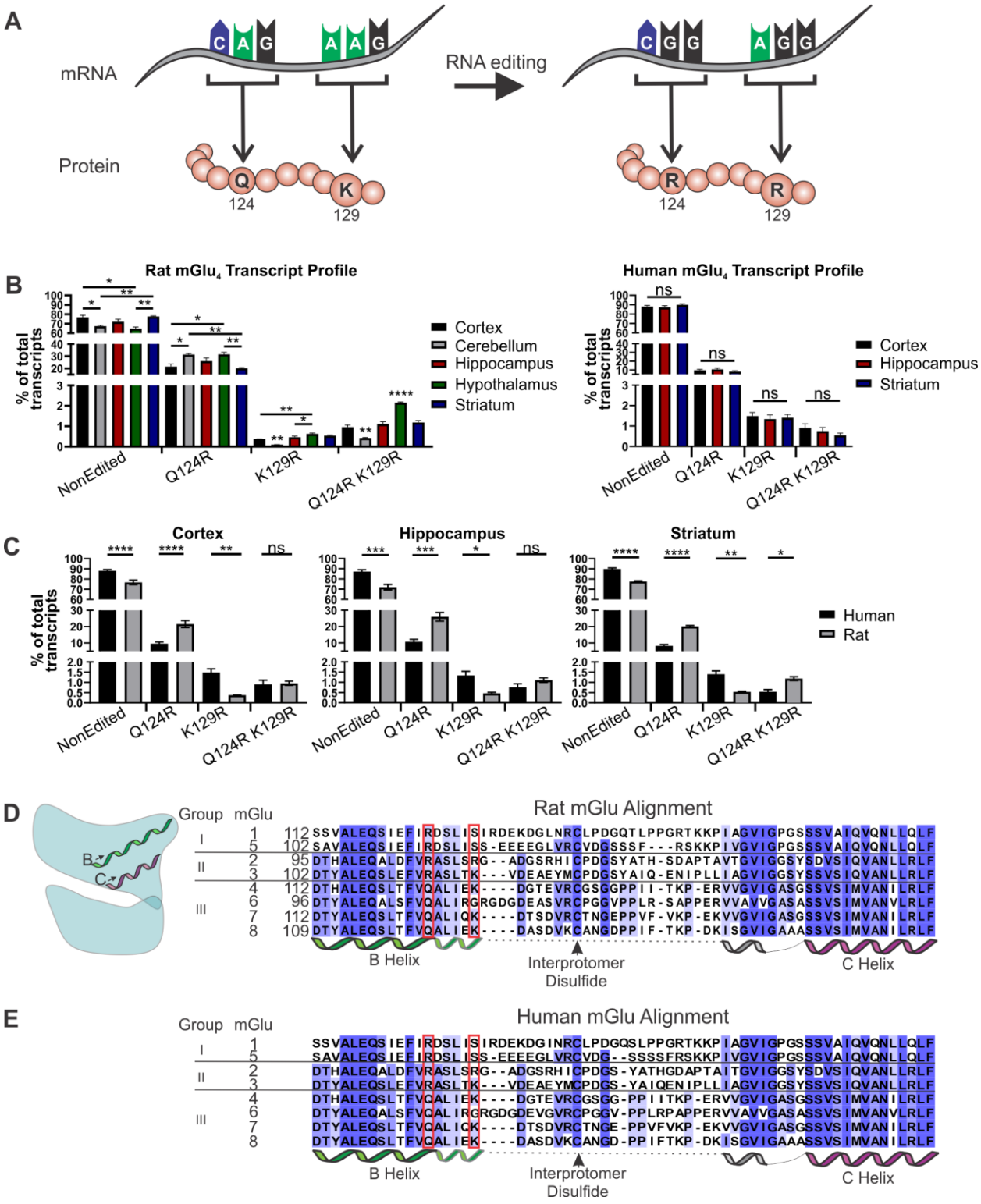

Figure 6: A-to-I editing of mGlu$_4$ transcripts reveals conservation of editing between rodents and humans.

A) Cartoon depiction of mRNA codons altered by RNA editing and their predicted translation by the ribosome. Editing is depicted as changing an adenosine to guanosine because A-to-I editing is functionally an “A” to ”G” conversion for the ribosome. **B)** Transcript isoforms expressed as a percentage of the total $mGlu_4$ transcript pool. Mean ± S.E.M. Significance tested by One-way ANOVA w/ Tukey’s post hoc test (ns, $p>0.05$; *, $p\leq0.05$; **, $p\leq0.01$; ***, $p\leq0.001$; ****, $p\leq0.0001$, rat n=3, human n=6-8). **C)** Re-plot of the data in 1B to highlight the differences in human and rat brain regions. Mean ± S.E.M. Significance tested by One-way ANOVA w/ Sidak’s post hoc test. **D)** Editing alters 2 amino acids (Q124R, K129R) highlighted in red within the B helix of the ligand binding domain. The helices of both monomers come together to comprise the mGlu dimer interface. Helix B and C are denoted below the alignment. The portion of helix B which is maintained in both active and inactive receptor states is outlined in black below the alignment. The helix without outline represents the amino acids incorporated into helix B in the relaxed state of the receptor.

Co-regulation of multiple editing sites in $mGlu_4$ transcripts

The serotonin 5-HT_{2C} receptor contains 5 editing sites (A, B, E, C, D) closely interspaced within a single RNA duplex [39,443]. Previous studies have suggested that a correlation between editing at these sites demonstrates a co-regulation either of multiple sites within the same transcript or across different brain regions [153,444]. Given the proximity of the $mGlu_4$ editing sites, we hypothesized that the Q124R and K129R sites could be regulated by a similar mechanism. In both human striatum (r=0.80, p=0.03) and hippocampus (r=0.75, p=0.03), the levels of Q124R editing were predictive of the extent of editing at K129R within the same subject (Figure 7A, Table 2), consistent with these sites being co-regulated in these tissues, possibly controlled by a similar mechanism that determines the extent of editing at both sites. Interestingly, this correlation was not significant in the cortex. In addition, the total extent of $mGlu_4$ editing in the striatum correlated with the total extent of editing in the hippocampus, suggesting a potentially similar regulation of editing of the $mGlu_4$ substrate within these two brain regions (p=0.036) (Figure 7B, Table 2); in contrast, a significant correlation was not observed between the extent of editing in the cortex and either striatal or hippocampal regions. This correlation was not significant when examining the extent of editing of either the Q124R or K129R sites alone; however, this is likely due to a limited samples size, and comparisons of editing in human striatal and hippocampal samples at Q124R (p=0.055) and K129R (p=0.051) sites approached statistical significance. These results suggest not only that the extent of editing of the Q124R and K129R sites may be co-regulated within a particular brain region, but that regulation may be distinct in other tissues.

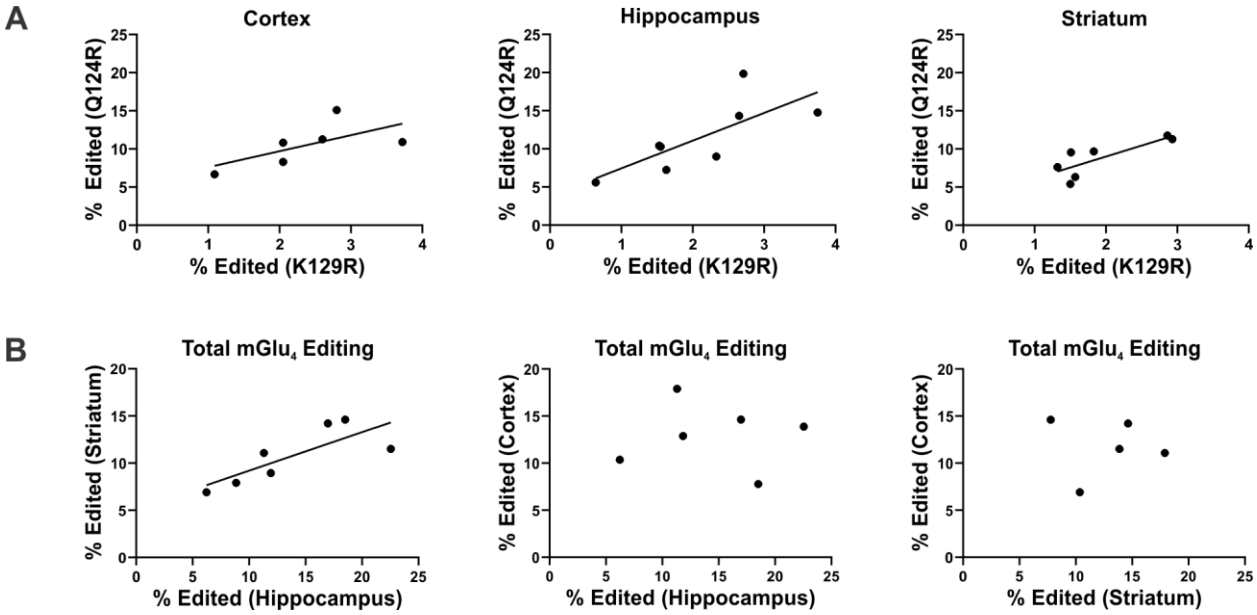

Figure 7: Co-Regulation of $mGlu_4$ editing.

A) Pearson correlation of total editing of the Q124R site vs the K129R site within individuals. **B)** Correlation of total editing levels between distinct brain regions of individual subjects.

		Linear regression			Pearson		
Edit Site	Tissue	R^2	slope	p	r	R^2	p
Q124R Vs. K129R	Cortex	0.416	2.100	0.167	0.645	0.416	0.167
	Hippocampus	0.565	3.632	0.032	0.751	0.565	0.032
	striatum	0.644	2.881	0.030	0.803	0.644	0.030
Total $mGlu_4$ Editing	Ctx Vs. Hipp	0.001	0.299	0.954	-0.031	0.001	0.954
	Str Vs. Hipp	0.619	0.408	0.036	0.787	0.619	0.036
	Ctx Vs. Str	0.001	-0.027	0.956	-0.034	0.001	0.956

Table 2: Pearson correlation and linear regression analysis summary table.
This table contains the values of analysis shown in Figure 7.

The $mGlu_4$ RNA duplex is entirely exonic

An extended RNA duplex is essential for ADAR binding and catalytic A-to-I conversion. Using the protein folding algorithm *mfold*, a putative fold was generated using 9000 base pairs of the $mGlu_4$ human pre-mRNA sequence (NC_000006.12) surrounding the editing sites. A similar fold was generated using rat mRNA sequence (NM_0226666_1) by constraining the input sequence to that of the putative duplex in human mRNA (Figure 8A). The Rattus norvegicus $mGlu_4$ mRNA sequence attained from NCBI (NM_022666.1) contained a (CGG) codon while the genomic reference (AC_000088.1) for the same species denotes a (CAG) codon for amino acid 124 of the receptor. We believe that an edited mRNA sequence was submitted in this case. Folds were developed using this sequence after modification of the (CGG) codon to (CAG).

Surprisingly, these studies predicted putative 127 bp folds containing both editing sites which were composed entirely of exonic sequence. The majority of validated RNA editing substrates are comprised of inverted repeats between exons and neighboring introns. These putative folds agreed with the observation that human and rat $mGlu_4$ cDNA constructs, which lack intronic elements, were edited at both Q124R and K129R positions when co-transfected with ADAR enzyme constructs in HEK293T cells (Figure 8B). To validate the putative rat RNA duplex, the minimal sequence encoding the putative duplex was expressed as a minigene either alone or when co-transfected with ADAR cDNA constructs in HEK293T cells (Figure 8C). This minimal sequence was sufficient for editing of both sites and the specificity of editing by ADAR1 and ADAR2 was consistent with the results for full length cDNA transcripts. There are two splice isoforms of ADAR1, p110 and p150. Only the constitutively expressed ADAR1 p110 was used for analysis as it serves as the primary splice isoform of editing within the central nervous system (CNS), whereas interferon-inducible ADAR1 p150 is a crucial regulator within the innate immune response [62]. These splice variants share identical deaminase and double-stranded RNA binding domains both of which confer the specificity and efficiency of the enzyme [169]. ADAR3 was

excluded from analysis as it has not been shown to be catalytically active. As expected in cells that do not endogenously express editing enzymes [34,44,72,76], there was no editing observed when minigene constructs were co-transfected with an empty vector control (Figure 8B, 8C), demonstrating that any editing observed was due to the co-transfected ADAR construct.

"Compensatory" or "destabilizing" mutations were designed either adjacent to or across from the editing sites within the proposed duplex; either set of mutations alone was predicted by *mfold* to destabilize the RNA secondary structure, whereas minigenes with both destabilizing and compensatory mutations were predicted to fold similarly to the wild-type construct. Consistent with a destabilized structure preventing ADAR binding, no editing was observed in constructs with "destabilizing" or "compensatory" mutations alone by either ADAR1 or ADAR2. Editing was rescued in the re-stabilized structure bearing both destabilizing and compensatory mutations, with similar ADAR specificity when compared to the wild-type minigene. No significant differences were detected between the extent of editing at either site by ADAR1 in the wild type and re-stabilized minigene. ADAR2-meditated editing of the Q124R site was rescued to ~70% of the levels seen in the wild-type duplex; this incomplete rescue is likely due to alterations in the nucleotide sequence which, along with the critical requirement of RNA secondary structure, determine site-selective editing [163,168].

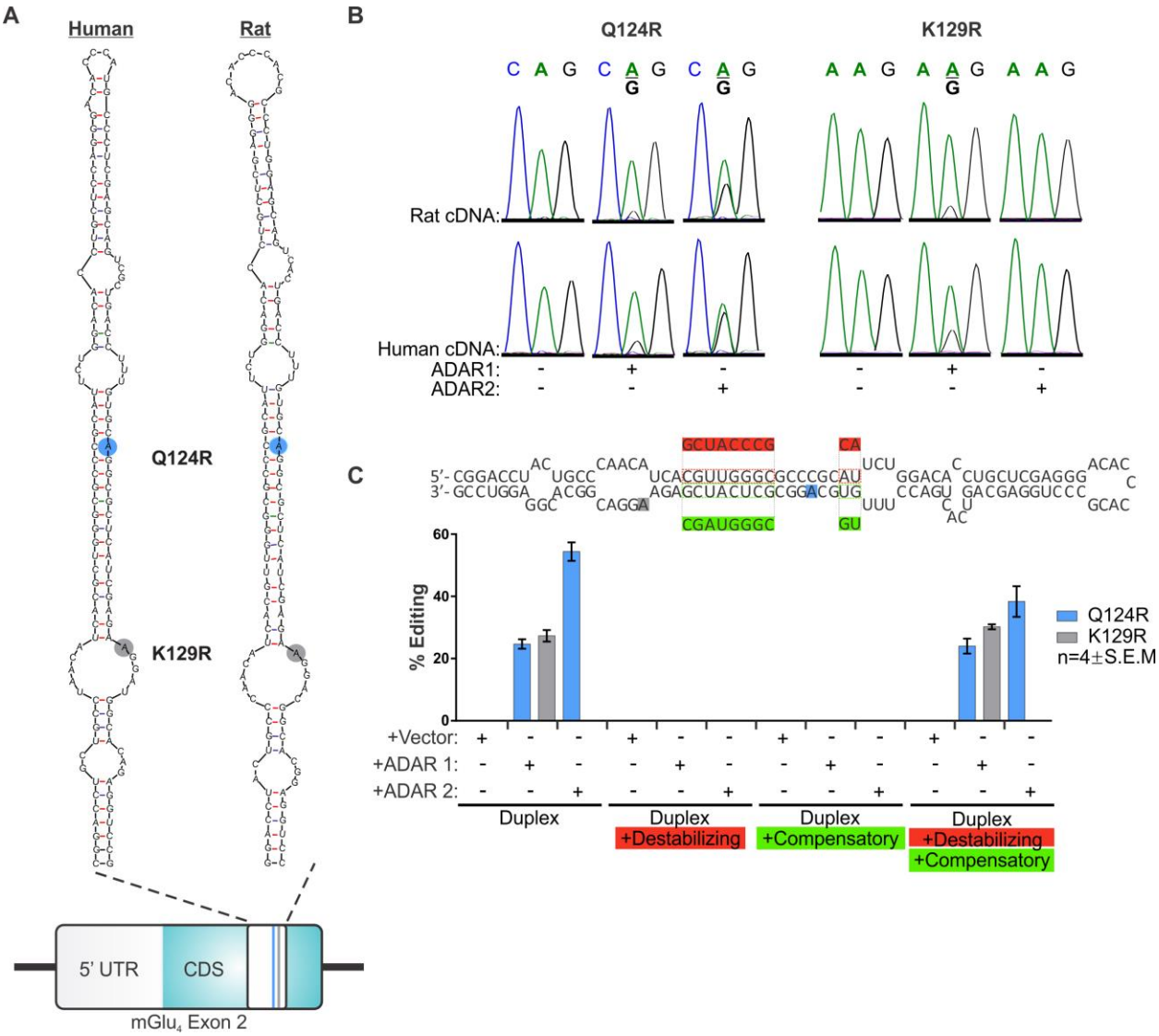

Figure 8: An mGlu$_4$ RNA duplex composed of exonic sequence is sufficient for editing.

A) Predicted mGlu$_4$ RNA duplex generated using *mfold*, composed of nucleotides 729-855 (Human, NM_000841.4), and 1132-1258 (Rat, NM_022666.1). **B)** Editing of rat and human cDNA constructs co-transfected into HEK293T cells with either empty vector, ADAR1, or ADAR2. Editing can be seen by the dual "A" and "G" chromatogram peaks. **C)** Predicted rat mGlu$_4$ duplex expressed as a minigene co-transfected into HEK293T cells ± ADAR1, ADAR2, or vector control. Editing percentages are calculated by measuring the peak heights of Sanger sequencing chromatogram peaks. Mean ± S.E.M. n=4. Q124R and K129R editing site positions within the duplex are denoted by blue and gray colors, respectively. The nucleotide sequence on the opposing side of the proposed duplex to the editing site (outlined in red hashes) was mutated (mutant sequence highlighted solid red) to destabilize the structure. The nucleotide sequence surrounding the editing site (outlined in green) was mutated (mutant sequence in solid green) with compensatory changes to restabilize the RNA duplex.

The $mGlu_4$ RNA structure is conserved in multiple mammalian species

Transcripts encoding additional members of the group III mGlu receptors, rat $mGlu_6$, rat $mGlu_7$ and $mGlu_8$, share 74.8%, 75.0% and 74.2% nucleotide identity, respectively, across the predicted rat $mGlu_4$ duplex region. These transcripts all share the Q124 (CAG) codon while $mGlu_7$ and $mGlu_8$ also share the K129 (AAG/AAA) codon (Figure 9A). Analysis of the rat $mGlu_6$, $mGlu_7$, and $mGlu_8$ RNA sequences in *mfold* failed to generate extended RNA duplex structures similar to that of $mGlu_4$. No editing was observed at either codon (Figure 9B) in $mGlu_7$ or $mGlu_8$ transcripts amplified from rat hippocampus. In contrast to the lack of conservation of editing among the group III mGlu receptors, the $mGlu_4$ duplex region is highly conserved among species, with sequences sharing 78.0%-100% nucleotide identity with human transcripts within this region of the mRNA. This high level of sequence conservation amongst species is not surprising, as exonic sequences are often highly conserved; however, extended duplex structures resembling the human and rat duplex were observed for some, but not all, species using *mfold* (Figure 9D). Folds were highly conserved for mammals but not reptiles, suggesting that editing of $mGlu_4$ transcripts evolved in a common ancestor of mammalian species. This agrees with the suggested model that RNA editing begins with the formation of a basic secondary structure, followed by small variations that lead to the generation of species-specific editing levels and, in some cases, additional species-specific sites [151]. Editing has been shown to be conserved in multiple mammalian $mGlu_4$ transcripts including human (Figure 6A), macaque [153], rat (Figure 6A, 8B), and mouse [45] brain samples (Figure 9C, D).

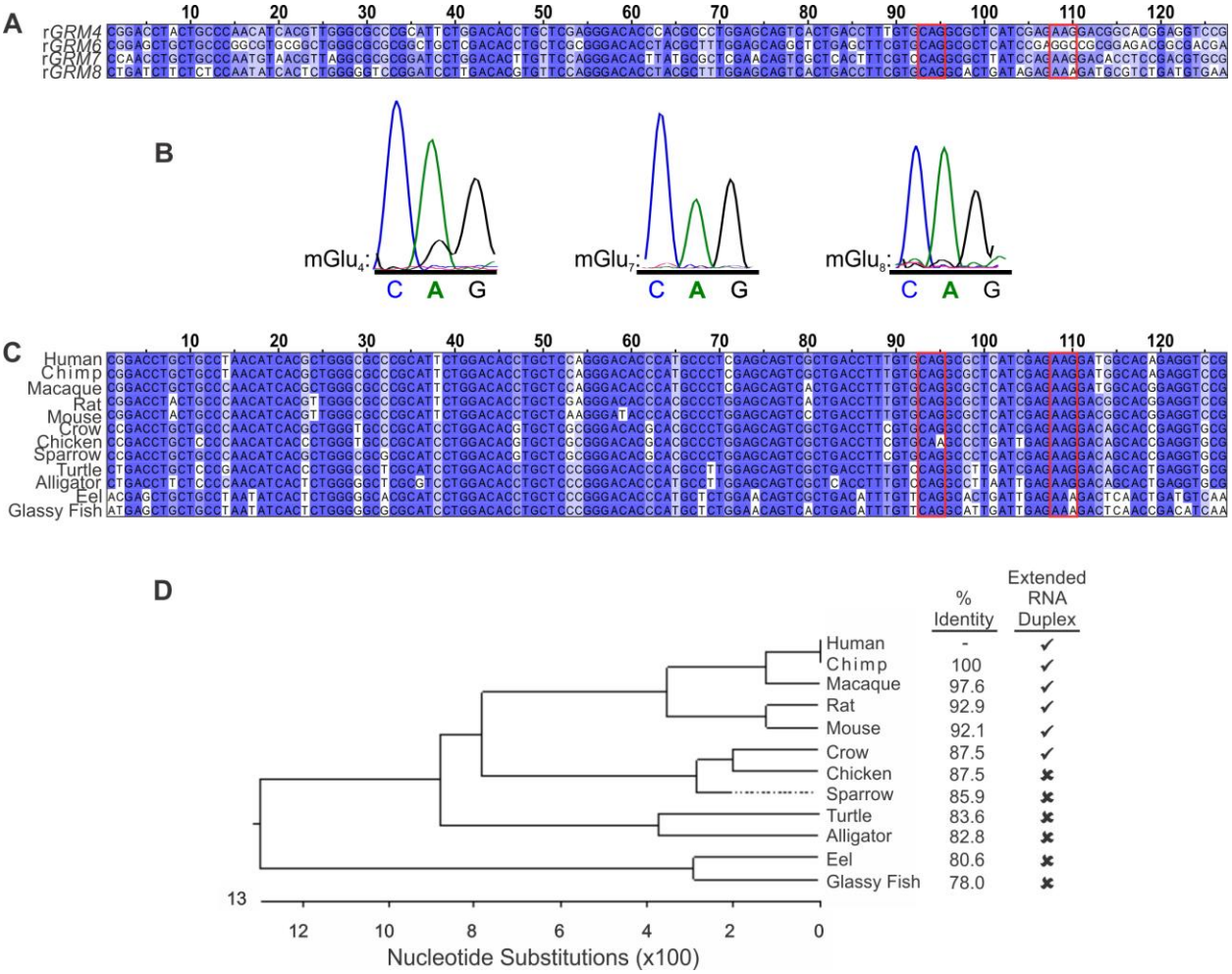

Figure 9: Mammalian conservation of the mGlu$_4$ duplex.

A) Alignment of rat mGlu$_6$, mGlu$_7$ and mGlu$_8$ mRNA sequences to the mGlu$_4$ duplex. **B)** Sanger sequencing data demonstrating a dual adenosine and guanosine chromatogram peak characteristic of RNA edited transcripts in mGlu$_4$ samples amplified from rat cerebellum but not mGlu$_7$ or mGlu$_8$ transcripts amplified from rat hippocampus. **C)** Alignment of the mGlu$_4$ duplex across multiple species. Q124R and K129R codons altered by editing are outlined in red boxes. Nucleotides are colored by percent conservation. Darker blue represents higher % conservation of the highlighted nucleotide. Conservation demonstrated in 4 groups from darkest blue to white (>80%, >60%, >40, <40%.) **D)** Cladogram of mGlu$_4$ duplex sequence generated using DNAstar software (DNASTAR Inc., Madison WI, USA). Percent identity is shown in comparison to the human sequence. Sequences producing a similar extended RNA duplex to human are noted by a check mark.

Discussion

The study of RNA editing has evolved rapidly over the last decade. What was once thought to be a rare phenomenon discovered often serendipitously by comparing individual RNA and DNA sequences has transformed into high-throughput analyses determining editing patterns across entire transcriptomes. Millions of edit sites are now known to occur throughout mammalian transcripts, most of which occur in non-coding regions and are not conserved between species. While this has led to some debate in the field as to whether all RNA editing events are biologically relevant, there is a consensus that editing sites that are conserved across species and have the potential to cause non-synonymous amino acid substitutions are functionally important and warrant further study [54,127,136,445].

While the editing of $mGlu_4$ transcripts has been reported previously, this has mostly been in the context of measuring overall editing patterns of multiple substrates in large RNAseq data sets [22,45,153]. Our analysis is the most robust, targeted HTS approach to analyze the editing of $mGlu_4$ transcripts across multiple brain regions in rat and human samples. Our data demonstrate the existence of the novel editing site, K129R, as well as region-specific editing patterns in rat brain samples. Mean levels of editing varied significantly in hypothalamic and cerebellar regions of rat brain samples but appeared to be static in both humans and rats within the cortex, hippocampus, and striatum. While the mean levels of editing across transcripts appeared similar, correlation analysis comparing editing levels across human brain suggests more variation in region-specific editing levels within individuals, specifically in the striatum and hippocampus vs. cortex, than the mean values imply. Interestingly, previous work reported that editing of the Q124R site in macaque samples was decreased in the striatum compared to the cortex, suggesting that, while editing of this site is conserved across multiple species, the extent of editing and spatial editing patterns vary between species.

While the extent of mGlu4 editing is low (~10% in human brain samples), this does not necessarily default to low importance. The Q124R site of $mGlu_4$ is one of 59 Evolutionary Selected Sites (ESS), which, due to their high conservation of editing across species, are thought to have been conserved due to the beneficial effect of protein recoding by editing [136]. 10 of these 59 ESSs display editing levels <20% in human subjects [136]; this includes the $K_V1.1$ ion channel [139], the E site of the $5\text{-}HT_{2C}$ receptor [153], and the SNARE accessory protein CADPS1 [137] which have demonstrated phenotypic outcomes due to RNA editing in rodent models [21]. The effects of RNA editing are determined not only by the extent of editing but the type of amino acid substitution. Additionally, the variance of editing levels for individual transcripts between and within unique cell sub-types remains unknown. Increased editing of transcripts within select cell types or circuits may provide a critical phenotypic function within these circuits. Ultimately, the gross phenotypic consequences and importance of $mGlu_4$ editing cannot be determined until “always edited” or “always nonedited” animal models are generated, as is common in the study of RNA editing events [21]. Of additional note, while generally most pronounced in the CNS, RNA editing may serve a critical functional role within peripheral tissues. This has been shown to be the case with a Q-to-R transition in filamin A (FLNA) which affects cardiovascular function as shown in a mouse model [42]. It is possible that $mGlu_4$ editing displays a unique profile or serves a critical functional role in a particular peripheral tissue in which $mGlu_4$ is expressed, including the pancreas, stomach, gastrointestinal tract/colon, breast, bladder, skin, adrenal gland, kidney, upper respiratory tract epithelia, and dendritic cells [284-287]. $mGlu_4$ is most prominently expressed within the CNS where the extent of RNA editing is generally much higher than peripheral tissues. Additionally, evidence of functional roles of the $mGlu_4$ receptor in peripheral tissues is lacking. For these reasons, we focused out efforts on determining $mGlu_4$ editing levels in brain regions specifically.

In addition to describing the editing profile of these transcripts, our work is the first to demonstrate the minimal RNA nucleotide sequence requirements for RNA editing of $mGlu_4$ transcripts and site-specificity of A-to-I catalysis by ADAR enzymes. The proposed intron-less structure is specific to $mGlu_4$ among the group III mGlu receptors and highly conserved across multiple mammalian species. This is only the third such editable substrate to be discovered and validated for which the RNA structure is composed entirely of exonic sequence, implying that it could be subject to editing outside of the nucleus [43,44]. ADAR enzymes are normally localized to the nucleus, but certain splice variants of ADAR1 can be expressed in the cytoplasm in response to viral infection, inflammation, and interferon induction [59,61,186,187]. The extent of editing of RNA substrates composed of exonic and intronic sequence is highly correlated with splicing efficiency, whereas no correlation has been observed for those substrates composed entirely of exonic sequence [193]. In mice, no significant correlation was observed between editing levels and splicing efficiency of $mGlu_4$ transcripts, further validating the strictly exonic sequence composition of the $mGlu_4$ RNA duplex [45]. Our results show that, *in vitro*, both ADAR1 and ADAR2 are able to edit the Q124R site, whereas only ADAR1 can edit the K129R site. Moreover, ADAR1 can edit both Q124R and K129R sites to approximately equal extents. This is in direct contrast to the significantly higher levels of editing at the Q124R site compared to K129R observed in human and rat tissues, leading us to speculate that ADAR2 is the predominant enzyme acting on $mGlu_4$ substrates *in vivo*. In agreement with this hypothesis, a recent publication by Licht et al (2019) did not observe editing at the Q124R position in *Adar2*$^{-/-}$ animals [45]. Additionally, Licht et al. did not identify editing at the K129R site; however, this study was performed in whole brain samples of p14 mice after enriching specifically for nascent transcripts [45]. Editing sites display unique developmental increases in editing percentages and levels of K129R editing may not be observable until later developmental stages [157]. Mutational disruption of base pairing immediately 5’ and 3’ of the Q124R editing site ablated editing which was restored upon the introduction of complementary mutations which restabilized the proposed base pairing. The duplex generated

using *mFold* is a putative structure; however, these results suggest that base pairing within the central stalk of the putative duplex surrounding the editing site is critical for A-to-I catalysis of $mGlu_4$ transcripts and that a 16 nucleotide region ending 56 nucleotides upstream of this region is most likely the editing complementary sequence (ECS), or RNA sequence directly opposing the editing site. Additionally, ADAR1-mediated editing of Q124R was fully rescued in the restabilized helix; however, ADAR2-mediated editing was restored to only ~70% of the levels seen in the wild-type duplex, suggesting the importance of both structural and sequence elements in editing efficiency of $mGlu_4$ transcripts by ADAR2.

Methods

Tissue collection

Rat tissue was collected from 3 untreated, 3-5-week-old Sprague Dawley rats. Following euthanasia, brain regions were dissected, flash frozen in $N_{2\ (l)}$, and stored at -80C until further processing. Samples were homogenized in 1ml of Trizol® reagent (Invitrogen) by sonication (Sonic Dismembrator 100, Fisher Scientific) and processed according to the manufacturer's instructions. Human control RNA from tissues obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland in Baltimore, MD) were processed for use in a previous study [153].

High-throughput sequencing

RNA samples were analyzed for quantity and quality by Nanodrop (Company). cDNA was generated by random hexanucleotide primer, single strand synthesis using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Editing profiles were determined by high-throughput multiplexed transcript analysis (HTMTA) as described previously [153,154,446].

Bioinformatics

The RNA sequencing reads were composed of multiplexed samples, identifiable via a series of 6-nucleotide barcodes. We leveraged SeqKit [447], a powerful and flexible FastQ file toolkit, that supports regular expression-based fuzzy matching for identifying variants within otherwise fixed sequence. Within our workflow, we first demultiplexed the FastQ reads into their respective sample bins, and then performed an exact alignment of each barcoded read for the gene reference sequence out to 20-nucleotide 3' of the known RNA editing variant position, allowing for variant nucleotides at the known position. Reads that did not match the adapter sequence exactly were discarded. The nucleotide frequency at each adenosine residue within the reference sequence was measured and used to determine an overall error rate for the polymerase of

0.243%. An adenosine at the corresponding position in the reference sequence was considered "not edited" while a guanosine above the error rate cutoff was considered "edited".

mfold

Sequences encoding either the human $mGlu_4$ or rat $mGlu_{4,\ 7,}$ and $_8$ pre-mRNA were input into the *mfold* RNA folding form (http://unafold.rna.albany.edu/?q=*mfold*/RNA-Folding-Form) [448] and set to default constraints. An initial input of 9,000bp flanking the human Q124R edit site was used to determine an initial putative fold. Rat $mGlu_{4,\ 7,}$ and $_8$ sequences were then constrained to sequence homologous to this initial putative duplex and folded under default folding constraints. Mutations introduced into $mGlu_4$ minigenes were evaluated in *mfold* to predict destabilization or re-stabilization of the duplex.

ClustalW/Tcoffee

$mGlu_4$ transcripts from multiple species [Alligator (XM_006025093.3), Sea Turtle (XM_0278259655.1), Amoenefish (XM_023275611.1), Crow (XM_088644711.2), Chicken (XM_015298989.2), Human (NM_000841.4), Glassy Fish (XM_028409706.1), Macaque (XM_0015136121.1), Mouse (XM_001291045.1), Chimpanzee (XM_0094510151.3), Rat (NM_022666.1), Eel (XM_026333481.1), Sparrow (XM_005492784.3), Human mlu7 (NM_00844.4), Human mGlu8 (NM_00845.2), Rat mGlu7 (NM_031040.1), Rat mGlu8 (NM_022202.1), Rat mGlu6 (NM_022920.1)] were collected from the National Center for Biotechnology Information (NCBI). Alignments were conducted using T-COFFEE (Version 11.00.d625267) set to default settings. Output ClustalW alignment files were visualized and % identity to the human sequence was determined using JalView [449].

In vitro editing assay

HEK293T cells plated in 6-well culture dishes were transfected with either an $mGlu_4$ minigene construct alone, or co-transfected with ADAR1 P110 or FLAG-ADAR2b. 48 hours post transfection, cells were rinsed with HBSS and lysed in 1ml of Trizol®. RNA was extracted

according to the manufacturer's instructions and DNAsed using Turbo DNAfree (Life Technologies). To further limit genomic contamination, cDNA was made using the High Capacity cDNA Kit according to the manufacturer's protocol except an $mGlu_4$ specific primer with a unique sequence overhang was substituted in place of the random hexanucleotide primers. Primers complementary to $mGlu_4$ and the unique nucleotide sequence were used to amplify $mGlu_4$ minigenes for Sanger sequencing. Percentage editing was determined by analysis of chromatogram peak heights in ImageJ. Samples without discernable "A" or "G" peak were given a value of "0".

Chapter III: Functional Consequences of $mGlu_4$ Recoding by RNA Editing

Preface

This chapter includes text and figures that were originally published in *RNA*[439]. Colleen M Niswender, Sheridan Carrington, Andrew N. Keller, and Karen J. Gregory contributed as authors.

Introduction

The major excitatory neurotransmitter in the mammalian central nervous system, L-glutamate, mediates its effects through two classes of receptors: ionotropic and metabotropic [26]. While ionotropic receptors function as ligand-gated ion channels mediating excitatory synaptic signaling, metabotropic glutamate (mGlu) receptors are class C G protein-coupled receptors that modulate neuronal plasticity, long-term potentiation (LTP), and long-term depression (LTD) through second messengers and effector proteins. This dual action of glutamate as an excitatory and modulatory neurotransmitter underlies the mechanisms for learning, memory, and synaptic plasticity [206,207]. The eight members of the mGlu receptor family are separated into three groups determined by their sequence homology as well as their downstream heterotrimeric G protein coupling profile. Group I mGlu receptors ($mGlu_1$ and $mGlu_5$) are predominantly expressed postsynaptically in neurons and function to increase neuronal excitability by signaling through $G\alpha_q$. Group II ($mGlu_2$, $mGlu_3$) receptors are expressed in both pre and postsynaptic locations and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$, $mGlu_8$) receptors are predominantly expressed presynaptically [247]. Both group II and III receptors signal primarily through $G\alpha_{i/o}$ to inhibit neurotransmitter release, an effect which is inhibitory at glutamatergic presynaptic terminals and excitatory at GABAergic presynaptic terminals [302-304].

$mGlu_4$, a group III mGlu receptor, is expressed throughout the human brain in multiple regions including the hippocampus, hypothalamus, caudate nucleus, cortex, putamen, and cerebellum [288,289]. Several studies have implicated this receptor in multiple motor system

phenotypes, neurological disorders, and disease states including learning and memory of motor tasks, spatial memory, inflammation, glucagon release, cancer progression, addiction, pain, the motor stimulatory effects of alcohol, and Parkinson's disease (PD) [278,284,287,312,358,380,415,418,424,450]. Expression of $mGlu_4$ is associated with various cancers; *in vitro* evidence has demonstrated that pharmacologic activation of $mGlu_4$ in cancers of the breast, bone, and brain, or inhibition of the receptor in colon cancer, can inhibit tumor growth [284,285,312,433,451]. Furthermore, inhibition of meduloblastoma and bladder cancer tumors *in vivo* in mouse xenograft models underscores the potential of $mGlu_4$ activation in cancer treatment [312,434]. Activation of the $mGlu_4$ receptor not only improves symptoms of PD in animal models but also to protects dopaminergic neurons in the substantia nigra pars compacta (SNc) from excessive excitatory drive from neurons of the subthalamic nucleus (STN) [278,395,452,453].

Despite the array of evidence demonstrating the physiologic relevance, "druggability", and pharmacologic importance of $mGlu_4$, more remains to be discovered regarding the regulation of this receptor. For example, it has recently been shown that RNA encoding $mGlu_4$ can undergo a post-transcriptional process known as RNA editing.

Results

$mGlu_4$ structural modeling

RNA editing of $mGlu_4$ substrates converts an encoded, conserved glutamine (Q) residue in group III mGlu receptors to the equivalently conserved arginine (R) in groups I and II (Figure 6D). We next sought to understand how RNA editing might influence 3D protein structure. We created 3D homology models of the extracellular domains of $mGlu_4$ in active and inactive states based on x-ray crystallography and cryo-electron microscopy structures of the near full-length $mGlu_5$ (Koehl et al., 2019). RNA editing altered residues at the top and outer surface of the extracellular domain. Q124 contributed to the dimer interface formed by the B helix between the two mGlu subunits (Figure 10). This interface was composed of several highly conserved, hydrophobic residues as well as several less conserved, polar residues, with the B helix of each protomer immediately adjacent to an unstructured loop region, which contains the disulfide link between the dimers. K129 was found within the unstructured loop region in the inactive state and the end of the B helix in the active conformation, with the side chain accessible to solvent in both instances. Given that this region shows low similarity between mGlu subtypes, varying in both length and composition, we focused modeling efforts on Q124. We created homology models of the $mGlu_4$ dimer in both active and inactive states to predict the effects of amino acid substitution of Q124 by RNA editing where neither, one, or both protomers were edited. Within the active state, the positioning of Q124 at the B helix dimer interface was relatively static, with the side chain predicted to form an H bond with E128 within the same protomer (Figure 10B). However, when one protomer was edited, the single R124 showed greater conformational diversity with the side chain having the potential to form an H bond across the dimer interface with E128 of the Q124-containing protomer (Figure 10C). When both protomers were edited to Arg, greater conformational diversity was seen with multiple H bonding partners predicted across the dimer interface (Figure 10D). Additionally, the Q124R substitution was predicted to alter dimer stability

within the resting state. The nonedited Q124 residues were predicted to form a direct polar interaction (H-bonds) with each other between the protomers (Figure 10B). Substitution for arginine in one protomer allowed the guanidinium group of Arg to occupy the interface between helix B/B', with the potential to coordinate multiple polar interactions (H-bonds and salt bridges, Figure 10C). A change of Q124R in both protomers was predicted to cause a repulsion between the two positively charged Arg residues, where neither residue was observed to occupy the interface between helix B/B' in our molecular predictions. Therefore, we postulated that the single residue edit creates a more stable interface in the resting state, when compared to the nonedited and double Q124R edit, and that editing in both protomers would create a more stable dimer interface when the structure was in the active state. Collectively, the modeling predicted that $mGlu_4$ dimerization would likely be influenced, which may also impact heteromerization with other mGlu receptors with a Q rather than R in the homologous 124 position.

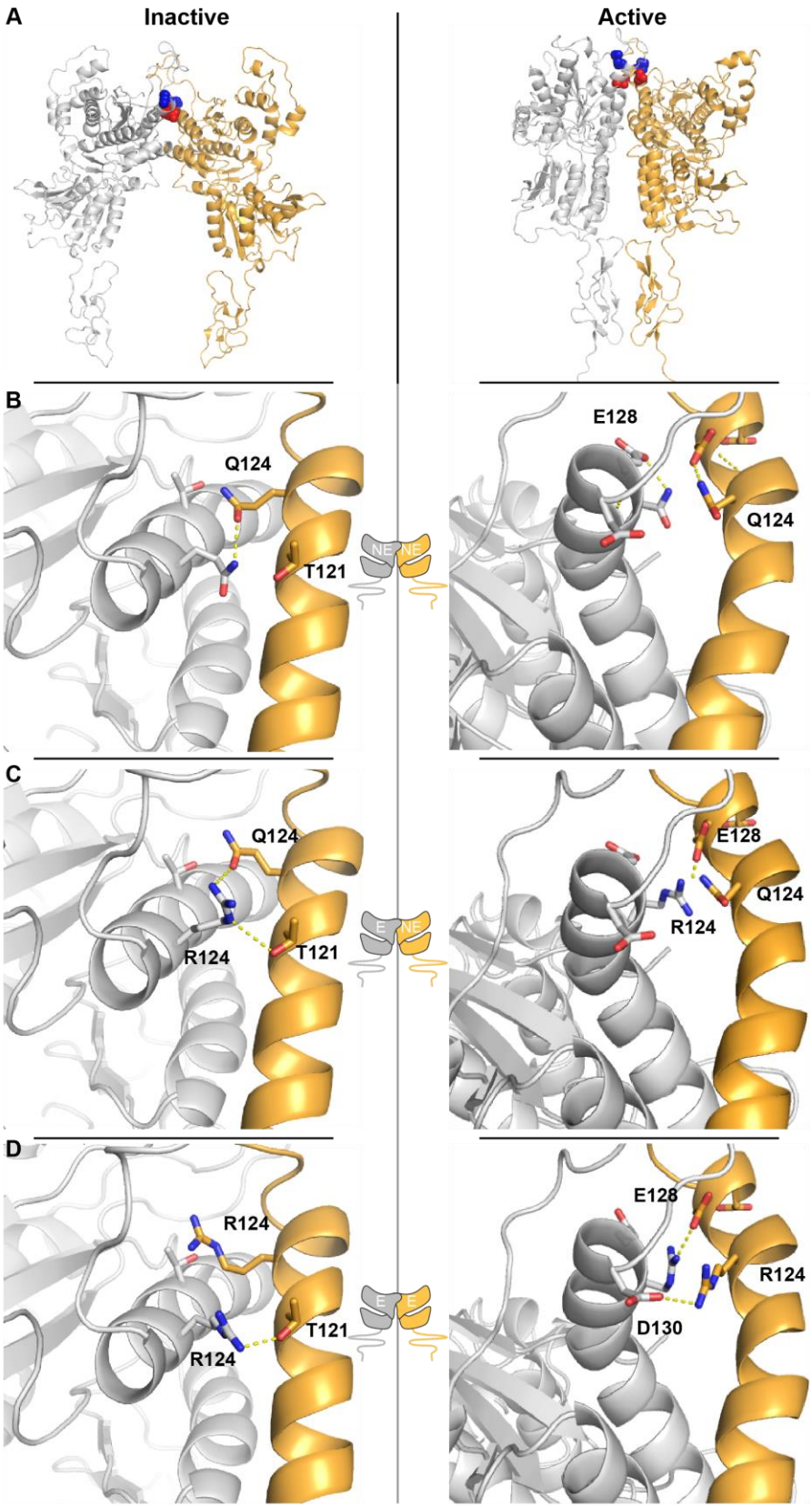

Figure 10: Structural modelling of the Q124R edit site in an $mGlu_4$ dimer

A) $mGlu_4$ homology model of the extracellular domains based on inactive and active $mGlu_5$ structures (PDB: 6N50 and 6N4X). The positions of Q124 (red) and K129 (blue) are shown in spheres. Ribbon representation of Helix B and B' (gold helix, all other secondary structure removed for clarity) of the opposite protomer in dimers with two nonedited protomers **(B)**, a nonedited and an edited protomer **(C)** or two Q124R edited $mGlu_4$ protomers **(D)**. Dashed yellow lines show potential H-bonding interactions between side chains.

Editing of both dimer subunits does not alter $G\alpha_{i/o}$ activation

The Q124 residue within the B helix is situated on the exposed outer face of the N-terminal domain, removed from the core ligand binding pocket. Therefore, Q124R substitution was not expected to alter orthosteric agonist binding; however, it was predicted to potentially stabilize the dimer interface within the active state of the receptor when present in both protomers. Therefore, we empirically determined if editing induced non-synonymous amino acid substitutions that would alter the response to orthosteric agonists. We expressed nonedited, Q124R, K129R, and Q124R/K129R isoforms alone in HEK293 cells, which do not endogenously express $mGlu_4$, and measured receptor activity via co-expressed G Protein Gated Inwardly Rectifying Potassium Channels (GIRK). This strategy should generate surface-expressed mGlu dimers that are composed of two identical $mGlu_4$ protomers. A thallium (Tl^+) flux assay was used to assess the activation of the G protein, $G_{i/o}$. In this assay, mGlu receptor activation is indirectly accessed by the activation of GIRK channels through dissociated βγ subunits of the activated $G_{i/o}$ heterotrimeric G protein, increasing the rate of entry of extracellularly-applied Tl^+ and accumulation of intracellular fluorescence of a Tl^+ sensitive dye. In response to agonist, significant differences were not detected between any of the receptor variants expressed alone (Figure 11A, B). In addition, all $mGlu_4$ edited isoforms appeared to respond identically to the $mGlu_4$-specific positive allosteric modulators (PAMs) ADX88178 and VU0155041.

The Q124R site was edited to a much greater extent than the K129R site in humans and rats (Figure 6B) and was edited by both ADAR1 and ADAR2 (Figure 7B,C); transcriptomic studies have revealed that ADAR2 is the primary editing enzyme of highly conserved re-coding sites within mammalian transcripts whereas ADAR1 specific sites are not as conserved [53,54,78].For this reason, we focused our studies on the functional effects of the Q124R substitution. In HEK293-GIRK cells stably expressing either rat nonedited or Q124R $mGlu_4$ isoforms, the edited receptor

responded identically to its non-edited counterpart in response to a battery of seven unique $mGlu_4$ agonists, including the endogenous ligands glutamate and L-SOP as well as several synthetic ligands. Potency (pEC_{50}) values obtained for each agonist displayed a nearly perfect correlation with an R^2= 0.9936 ($p<0.0001$) between the edited and nonedited receptors. These receptors likewise responded identically to several $mGlu_4$-specific PAMs and partial agonists, again demonstrating a near perfect correlation in the leftward fold shift of the agonist response for both L-AP4 (R^2=0.9726, $p<0.0001$) and glutamate (R^2=0.9969, $p<0.0001$) when pretreated with one of seven unique compounds (Figure 11C, Table 3).

Allosteric Modulation by the Transsynaptic ELFN1

Receptors in neurons do not exist in isolation. Synapses are highly structured environments with accessory proteins modulating the trafficking, localization, and activation of synaptic receptors. The postsynaptically expressed protein ELFN1 (Extracellular Leucine Rich Repeat and Fibronectin Domain III Containing 1) interacts specifically with, and allosterically inhibits agonist-induced efficacy of, group III mGlu receptors [317]. ELFN proteins act transsynaptically to allosterically modulate presynaptic mGlu receptors through their extracellularly exposed N-terminal domain, which is composed of two subdomains: the cysteine rich domain (CRD) and the N-terminal domain that binds orthosteric agonist. Due to the location of the editing sites within the extracellular $mGlu_4$ N-terminal domain, we hypothesized that these alterations could change interaction or modulation of various edited isoforms by transsynaptic proteins. We used a co-culture assay using HEK293-GIRK cells stably expressing either rat nonedited or Q124R $mGlu_4$ isoforms (Figure 11E). The maximal response of the nonedited receptor was significantly decreased in response to glutamate specific to the ELFN1 condition over a vector alone control (Figure 11F, G), suggesting the co-culture conditions were sufficient for allosteric modulation of $mGlu_4$ receptors by ELFN1. The reduction in maximal response of the

Q124R receptor did not reach statistical significance (p=0.096). We also did not observe differences in response in the presence of $mGlu_4$-specific PAMs (Figure 11G).

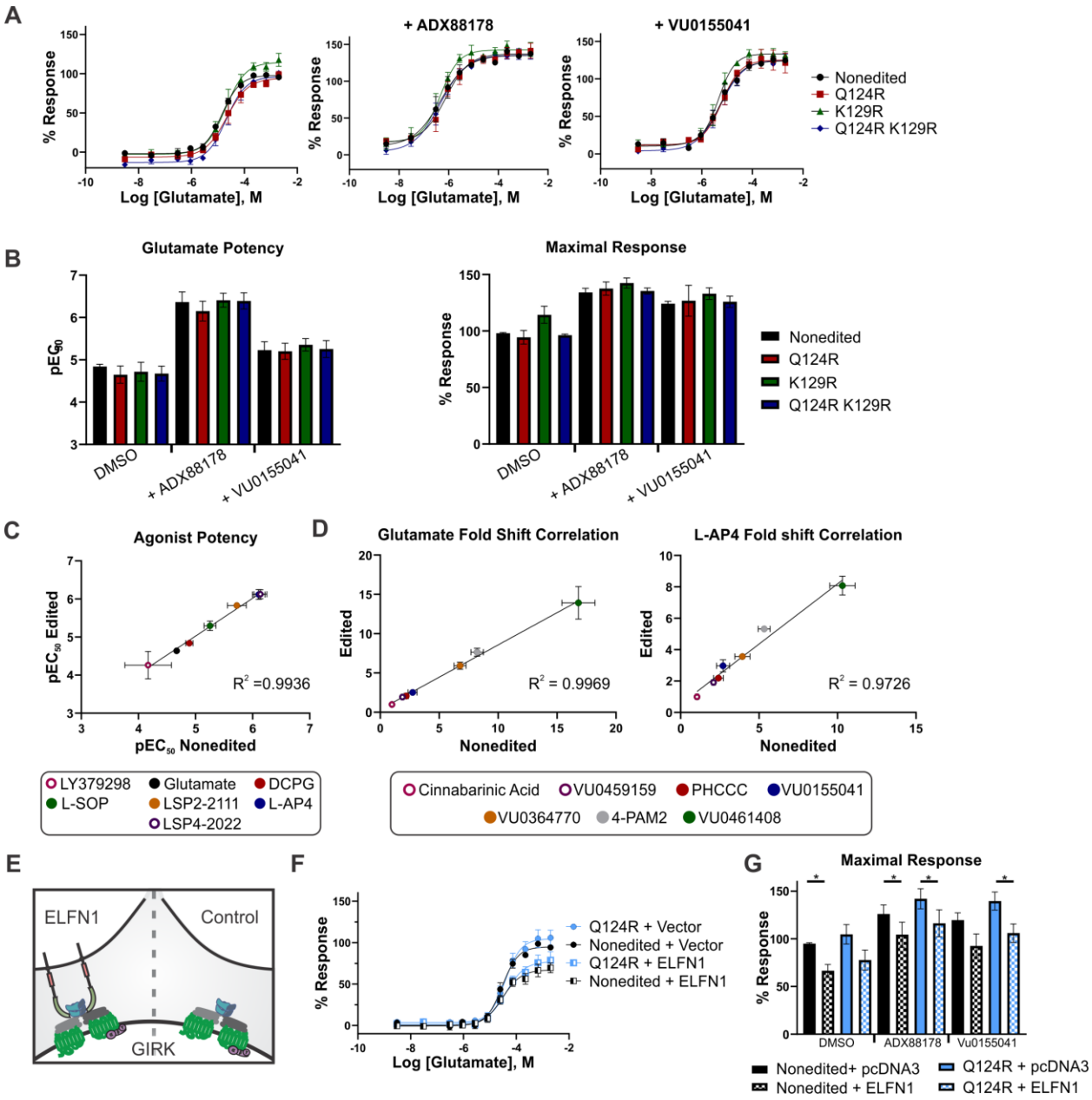

Figure 11: $G\alpha_{i/o}$ signaling in dimers with two edited monomers is indistinguishable from nonedited receptors.

A) 12-point concentration-response curves to glutamate ± the PAM VU0155041 (30µM) or ADX88178 (30µM), measuring Tl^+ flux induced by $mGlu_4$ nonedited and edited isoform activation after transient transfection into HEK-GIRK cells. Mean ± SEM. n=3. Blank (WT vehicle signal) was subtracted from all values and normalized to Nonedited DMSO max response. Analyzed by One-way ANOVA. **B)** pEC_{50} and maximal response values from the non-linear regression curves shown in 5A. Linear regression analysis of the potency and fold shift (pEC_{50}) of various $mGlu_4$ **(C)** agonists and **(D)** PAMs in polyclonal cells expressing either non-edited or Q124R edited $mGlu_4$ receptor. **E)** Schematic representation of the co-culture assay used to measure allosteric modulation of receptor isoforms by ELFN1. **F)** Concentration-response curves for cells expressing $mGlu_4$ edited isoforms co-cultured with ELFN1 or control cells. n=4. Mean ± S.E.M. **G)** Maximal

receptor response of data represented in 5F. Analyzed by paired t-test between vector control versus ELFN1 for nonedited or Q124R $mGlu_4$.

A

	Agonist						
	Glutamate	L-AP4	DCPG	L-SOP	LSP2-2111	LSP4-2022	LY379298
Nonedited	4.67±0.02	6.11±0.11	4.89±0.06	5.25±0.10	5.72±0.17	6.14±0.11	4.17±0.41
Edited (Q124R)	4.64±0.05	6.12±0.13	4.84±0.08	5.30±0.13	5.83±0.06	6.12±0.12	4.26±0.36

B

			+ 10uM Positive Allosteric Modulator					
Agonist	Isoform	DMSO	PHCCC	VU0461408	VU0155041	VU0364770	VU0459159	4-PAM2
Glutamate	Nonedited	4.74±0.03	5.08±0.03	5.96±0.00	5.17±0.03	5.57±0.01	5.01±0.01	5.65±0.01
	Edited	4.72±0.03	5.04±0.03	5.86±0.05	5.12±0.03	5.49±0.01	5.01±0.02	5.60±0.01
L-AP4	Nonedited	6.41±0.06	6.78±0.07	7.42±0.07	6.83±0.01	7.00±0.02	6.73±0.04	7.13±0.09
	Edited	6.39±0.04	6.73±0.02	7.29±0.06	6.85±0.05	6.94±0.03	6.67±0.01	7.11±0.05

Table 3: $mGlu_4$ agonist and PAM potency (pEC_{50}) and fold shift values.

This table contains the values of analysis shown in figure 11C.

Q124R substitution in one subunit does not alter $G_{i/o}$ activation

Q124R edited $mGlu_4$ mRNA isoforms account for ~12% and 30% of the transcript pool in humans and rats, respectively. Assuming each cell expresses mixed populations of transcripts, it is most likely that an edited $mGlu_4$ protein monomer would dimerize with a non-edited counterpart. We hypothesized that editing of only one monomer in an $mGlu_4$ receptor could alter the signaling characteristics of the resulting heterodimer. Observing specific heterodimer populations is challenging due to the presence of multiple surface-expressed receptor populations in co-transfection models. To address this, we took advantage of the quality control system of $GABA_B$ receptors, which requires two monomers with complementary C-terminal coiled-coil domains to dimerize and mask the encoded ER retention motif in order to traffic to the surface. Chimeric $mGlu_4$ constructs fused with these unique tails, labeled Gb1 and Gb2, allowed for the surface expression of dimers comprised of edited and non-edited monomers while preventing surface expression of homodimer populations (shown schematically in Figure 12A). The addition of GABA C-terminal tails significantly decreased (28 ± 19%, p=0.029) the maximal response to glutamate in comparison to the wild type construct (Figure 12B, C); however, the glutamate pEC_{50} was not significantly different between the constructs (Figure 12B), suggesting that the presence of GABA tails does not significantly alter function, but may limit surface expression. This could be due to restrictions on dimer assembly and the requirement of two subunits with unique C-terminal tails–a limitation which is absent in the wild type receptor. A dose-dependent increase in signal was not observed when Gb1 and Gb2 tailed receptor constructs were expressed alone (Figure 12B). Similar to the use of non-chimeric receptors, significant differences were not observed between dimer pairs with Q124R substitutions in both protomers compared to nonedited receptor homodimers. Differences in maximal responses or glutamate pEC_{50} were not observed when restricting surface receptors to an edited/nonedited dimer pair (Figure 12D, E, F).

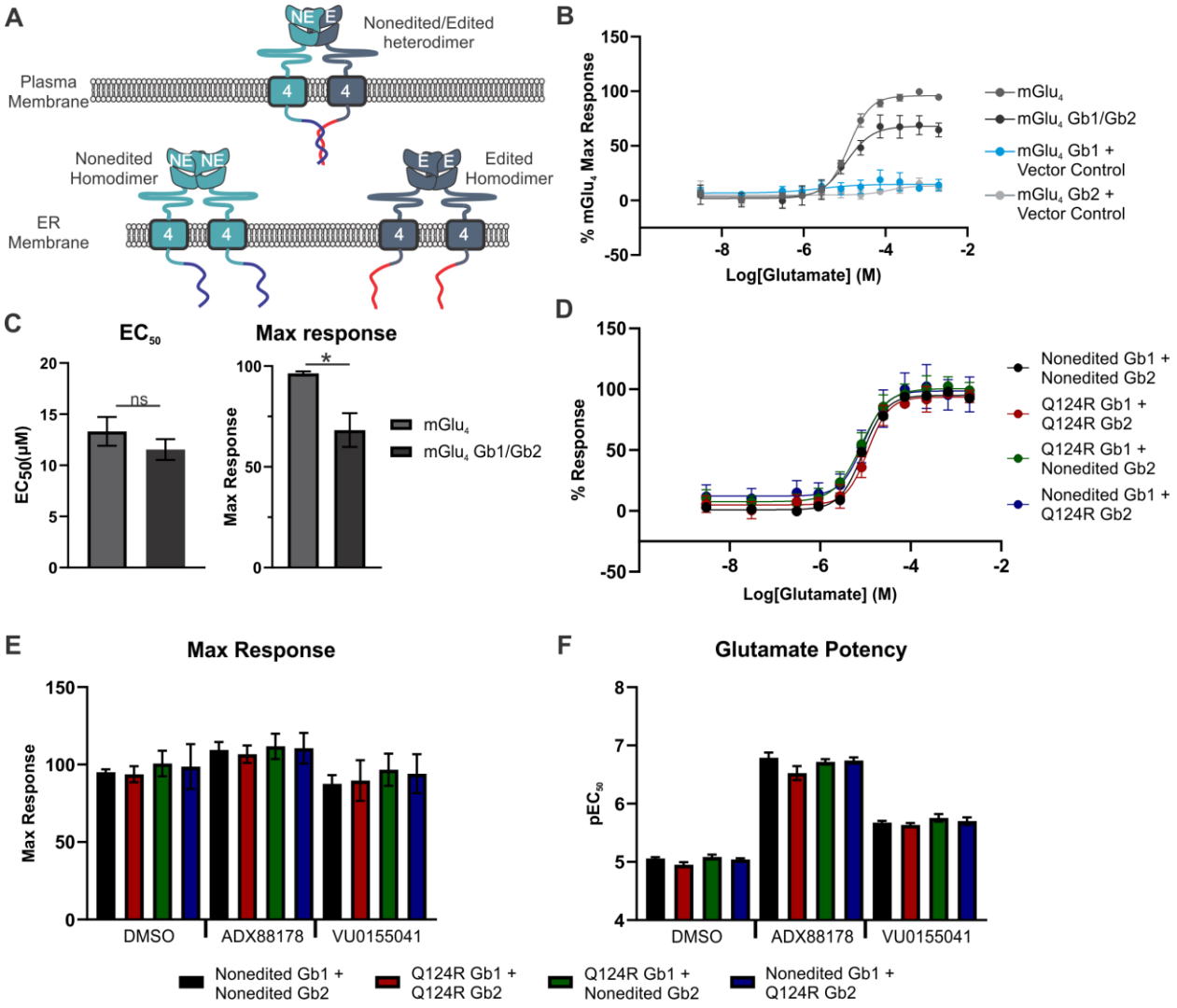

Figure 12: Response of Edited/Nonedited $mGlu_4$ receptor dimers.

A) Schematic representation of the use of $GABA_B$ receptor C-terminal tails to restrict surface expression exclusively to heterodimers. **B)** Concentration-response curves comparing cells transfected with equal microgram amounts of an $mGlu_4$ control construct, $mGlu_4$ $GABA_B$ tailed receptor constructs together, or tailed constructs alone plus an empty vector control. All constructs in this subfigure are nonedited at the Q124 residue. **C)** Max response for the Gb1/Gb2 receptor combination was lower in comparison to wild type $mGlu_4$ in the DMSO-matched condition. I don't think you need this sentence (*, p≤0.05; **, p≤0.01). **D)** Concentration-response curves for HEK293A-GIRK cells co-transfected with chimeric $mGlu_4$ constructs restricting surface dimer populations to those of two nonedited receptors or Q124R edited / nonedited receptor heterodimers. Mean ± S.E.M. n=3. **E)** Maximal response values and **(F)** glutamate potency (pEC_{50}) from the non-linear regression curves shown in 12D. Each receptor pair is compared to the nonedited receptor condition using a one-way paired ANOVA with Sidak's Post Hoc analysis.

Q124R substitution decreases heterodimerization with $mGlu_2$ and $mGlu_7$

While it was once thought that mGlu receptors only formed homodimers, recent studies have suggested that not only do these mGlu receptors form heterodimers both *in vivo* and *in vitro*, but that this can dramatically alter receptor responsiveness to both orthosteric ligands as well as allosteric modulators [254,257,269,270,272,273]. Due to the position of the editing sites along the dimer interface for mGlu receptors and based on our modeling, we sought to determine how the Q124R substitution altered the propensity of $mGlu_4$ to homodimerize versus heterodimerize with other mGlu partners. Truncating $mGlu_4$ peptides before the transmembrane domain results in a disulfide-bound $mGlu_4$ dimer that is secreted from the cell and retains similar binding affinities to its full length counterpart [454]. We took advantage of a Myc-tagged, secretable, extracellular fragment ("prey") in a co-transfection assay with a full length, HA-tagged ("bait") receptor in order to isolate and specifically measure heterodimer populations at the cell surface. In this assay, homodimer populations would be secreted and removed by washing. Heterodimer populations at the surface could then be measured specifically using unique epitope tags on the N-termini of "bait" and "prey" receptors (Figure 13A). Both nonedited and edited, truncated $mGlu_4$ constructs were expressed, processed, and secreted at similar levels (Figure 13B). Cell lysates were reduced by the addition of DTT and represent monomeric $mGlu_4$ ATD constructs. "Media" blot samples have not been DTT-treated and demonstrate dimerization of the ATD constructs, shown by the bottom band of the "media" blot. The top band of the media blot may represent binding of the ATD dimer to a serum protein and is not expected to affect dimerization. mGlu receptors dimerize within the ER and are covalently bound before reaching the cell surface where serum proteins are present[262]. Interestingly, editing of the Q124R site did not alter levels of dimerization with the full length nonedited $mGlu_4$ construct; however, a significant decrease was observed in dimerization propensity with $mGlu_2$ (35.8 ± 4.3%, $p<0.05$) and $mGlu_7$ (28.1± 3.8%, $p<0.05$) receptors. For all other mGlu receptors, the propensity to dimerize with $mGlu_4$ was unaffected by editing (Figure 13C). It can further be seen by pooling data from heterodimer populations where

editing did not have an effect that there is a clear order of dimerization preference for $mGlu_4$, with decreased preference for the group I receptors ($mGlu_1$, $mGlu_5$), and an increased propensity to heterodimerize with group II receptors ($mGlu_2$, $mGlu_3$) in agreement with previous studies (Figure 13D) [254,257]. In addition, our assay included the $mGlu_8$ receptor, and $mGlu_4$ demonstrated a substantial preference for heterodimerization with $mGlu_8$ compared to homodimerization, with a significant, 57% ($p<0.0001$) increase in the propensity to heterodimerize.

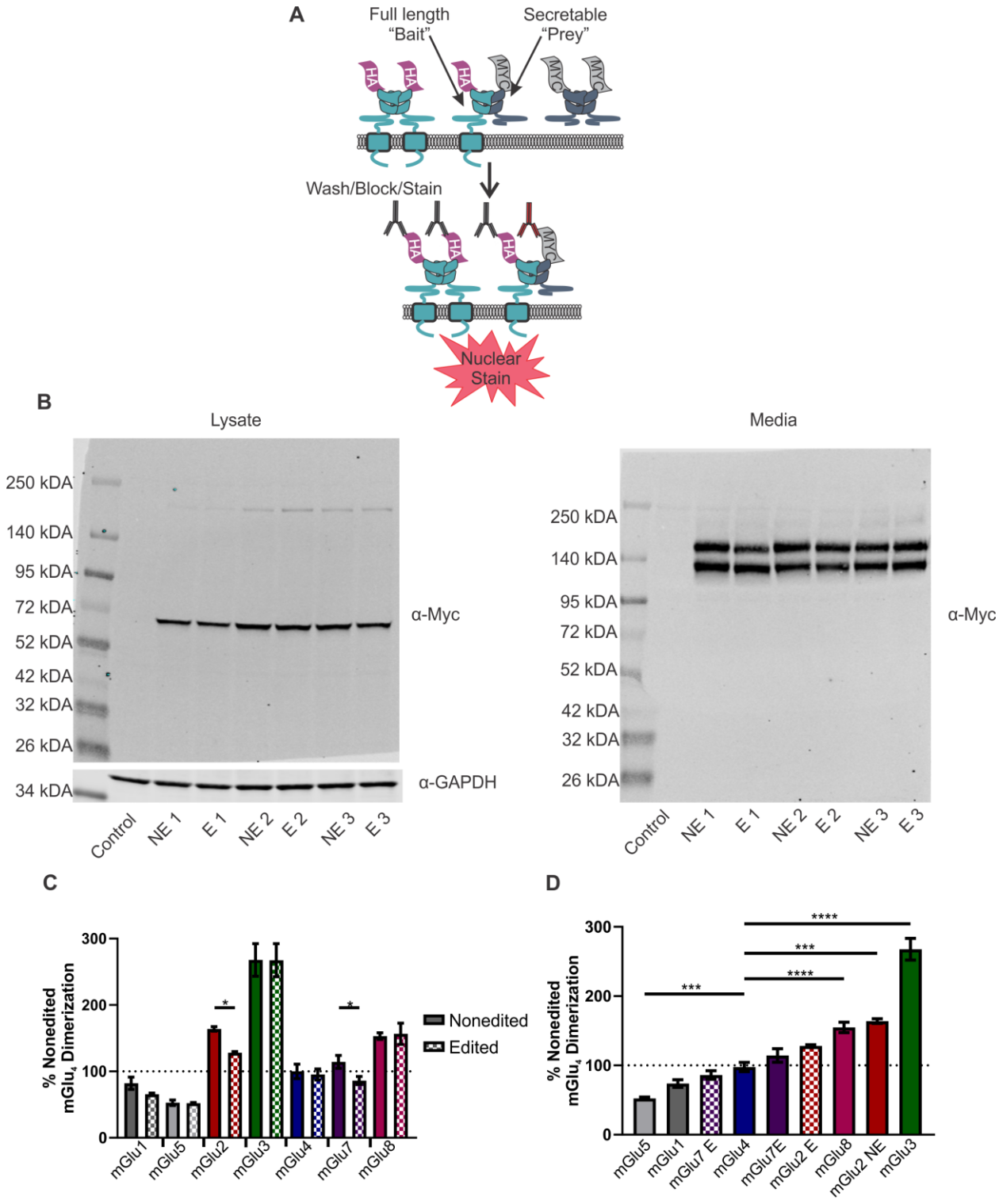

Figure 13: Q124R substitution decreases heterodimerization with $mGlu_2$ and $mGlu_7$.

A) Schematic representing the “bait” and “prey” method to measure mGlu dimerization propensity. HA or MYC signal are measured in separate wells and normalized to DRAQ5 cell stain to account for cell number. **B)** Western blot analysis of truncated, nonedited and edited $mGlu_4$ receptor constructs from concentrated media or whole cell lysates. **C)** $mGlu_4$ dimerization propensity assessed by the ratio of the secretable $mGlu_4$ (MYC) signal to full length (HA) signal. Data are normalized to the dimerization levels of nonedited $mGlu_4$. Mean ± S.E.M. Significance assessed by paired t-test. (*, $p \leq 0.05$). **D)** Re-plot of the data in 8C after pooling data for receptors where editing did not affect dimerization showing the order of $mGlu_4$ dimerization propensity. Mean ± S.E.M. Significance assessed by ANOVA w/ Dunnet’s post hoc. (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Discussion

The predicted amino acid substitutions introduced by RNA editing occur within the B helix of the $mGlu_4$ receptor which, along with helix C, form the hydrophobic dimer interface of mGlu receptors–an area also critical in receptor activation [216,217,224,259,260]. These helices are thought to comprise the only interface within the resting state which shifts upon activation to include additional contacts between the LBD and 7TM domains. While there are many conserved hydrophobic contacts in the mGlu dimer interface, there are several polar interactions which are less conserved across mGlu receptors, including the Q124 site of $mGlu_4$. The conservation of this residue suggests a critical functional importance of this position in the protein, but perhaps one that differs between group III and group I/II mGlu receptors. Several studies have observed that non-synonymous substitutions induced by RNA editing generally occur in regions less conserved than average [455]. Previous studies analyzing the crystallographic structure of $mGlu_1$ posit that R124, homologous to Q124 of $mGlu_4$, resides at the interface of both inactive and active receptor states. This residue sits at the C-terminus of the B helix within the active state [216]. In the resting state of the receptor, there is an extension of the helix to include additional residues such as K129 (Figure 6B, 10A). These interdomain movements are thought to be conserved among mGlu receptors [217]. In cryoEM structures of a full length $mGlu_5$ extracellular domain in the active and inactive state, Koehl et al (2019) suggested that Arg114, homologous to Q124 of $mGlu_4$, releases from an interaction with E111 in the inactive state to interact with E121 of the active state [260]. This interaction was proposed to stabilize the active state of the receptor. A function has not been suggested for the K129R site, which is edited an average of only 2.1% of human transcripts and 1.6% in rats; therefore, we decided to focus our efforts on elucidating the functional consequences of the Q124R site which is edited at 10.3% in humans and 27.6% in rats.

Facing the multitude of evidence suggesting a critical function of helix B and, specifically, of those residues homologous to Q124, it was surprising not to observe any significant differences

in signaling of these edited receptor isoforms when present in either only one or both protomers of a dimer. The substitution of a glutamine for arginine is a subtle change. Both residues are capable of polar interactions; however, the arginine may form a salt bridge whereas glutamine cannot. While this is generally a conservative substitution, the consequences can be severe; the Q84R substitution in Tribbles homologue 3 (TBR3) was associated with insulin resistance in human populations [456] and R1131Q in the kinase domain of the human insulin receptor significantly reduced phosphorylation [457]. A similarly conservative substitution of surface exposed lysines to arginines in GFP significantly increased protein stability [458]. Furthermore, mutations in this helix in $mGlu_2$ were shown in single molecule FRET studies not only to weaken the dimer interface but also increase the proportion of receptors in the active state, even in the absence of agonist [224]. $mGlu_1$ receptors with mutations in helix B displayed signaling defects despite binding [^{3}H]-quisqualate [259]. Using the $mGlu_5$ structure as a guide, we modeled the $mGlu_4$ dimer to predict the effects of amino acid substitution by RNA editing. Residue 124 was predicted to make distinct binding interactions depending on the editing status of each protomer and whether the receptor was in an active or resting conformation, with the major prediction being to alter the stability of the dimer interface. This prediction was consistent with the lack of effect of RNA editing on receptor activation in response to agonist. It should be noted that the Tl^+ flux assay measures activation of the βγ subunits in a $G_{i/o}$ heterotrimeric G protein. $mGlu_4$ has also been shown to couple to $G\alpha_q$ in cerebellar parallel fiber-molecular layer interneuron synapses [310]. Apart from G protein coupling, $mGlu_4$ activation has also been linked to the activation of PI3-kinase [312,313], c-Jun NH_2-terminal kinase (JNK) [314], and mitogen-activated protein kinase (MAPK) [310,313] signaling pathways. Additional studies are needed to determine whether RNA editing influences these other signaling pathways.

mGlu receptors are often co-expressed within the same neurons and can not only form homodimer receptors, but heterodimers as well, immensely increasing the potential variation and

complexity of mGlu receptor signaling. Our structural model of the $mGlu_4$ receptor found that Q124R substitution within a single monomer resulted in the most stable dimer interface in the resting state. This suggests that Q124R substitution would increase the propensity for edited $mGlu_4$ protomers to dimerize with other mGlu receptors with a Q rather than R in the homologous 124 position. Contrary to our predictions, $mGlu_4$ constructs with a Q124R substitution did not show an increased propensity to dimerize with nonedited $mGlu_4$ protomers, which have a Q at position 124, in our dimerization assay (Figure 13C).

Our results suggest that Q124R substitution in $mGlu_4$ decreases the receptors' propensity to heterodimerize specifically with $mGlu_2$ and $mGlu_7$, which have an encoded R and Q, respectively, at the homologous position (Figure 7C). Additionally, the structural model suggests residue 124 can interact with Glu128 or Asp130 of the opposite $mGlu_4$ protomer. Alignment of the helices comprising the dimer interface from all mGlu receptors shows the subtype-specific amino acids that are implicated in the dimer interface (Figure 6D). Residues unique to $mGlu_2$ and $mGlu_7$, which cause altered dimerization propensity with edited $mGlu_4$ constructs, are not readily apparent, although altered interactions across the dimer interface with $mGlu_4$-Q124R involves non-conserved residues. It is possible, however, that dimer formation occurs in the ER while each protomer is in a pre/semi-folded state, limiting the utility of using such structural comparisons to understand these data [262].

Both $mGlu_2$ and $mGlu_7$ are expressed presynaptically and have been shown to co-localize with $mGlu_4$ [257,270,275,276]. $mGlu_{2/4}$ heterodimers have been documented *in vitro* and *in vivo,* with heterodimerization altering the receptors' responses to endogenous and synthetic orthosteric agonists as well as allosteric modulators [254,270,273]. Additionally, it has long been postulated that $mGlu_4$ and $mGlu_7$, based on their overlapping expression within striatopallidal projections [275,276], could form heterodimers within this region and have been shown to interact *in vitro* [254,257]. Additional studies are needed to determine whether $mGlu_{4/7}$ heterodimers exist *in vivo* as well as

the effect of this heterodimerization on downstream signaling in response to endogenous and synthetic ligands.

In extensive modeling by the Levitz lab, it was suggested that, for heterodimers to occur, two mGlu receptors must have either equal or increased propensity for heterodimerization as for homodimerization [257]. Further, the proportion of homo- vs- heterodimer populations present in a cell expressing two or more mGlu receptors was relatively stable across multiple molar concentration of those receptors but highly dependent on the K_d of their interaction. Heterodimerization of $mGlu_4$ with $mGlu_2$ and $mGlu_7$ was decreased by 28-30%, which could alter the proportion of select heterodimer populations at the surface while increasing the proportion of homodimeric population. This could be especially important in cells expressing more than two mGlu receptors; in single-cell RNA sequencing (scRNAseq) analysis of mouse cortex, over 50% of glutamatergic neurons expressed at least 4-5 mGlu receptors and at least 2-3 mGlu receptors were expressed in GABAergic neurons [257]. We found that nonedited $mGlu_4$ exhibited similar levels of dimerization with $mGlu_2$ and $mGlu_8$; however, editing decreased dimerization with $mGlu_2$ but not $mGlu_8$, potentially switching the preference for $mGlu_4$ dimerization ($mGlu_{4\ (NonEdited)/2}$>$mGlu_{4/8}$>$mGlu_{4\ (edited)/2}$). We also found that $mGlu_4$ and $mGlu_7$ had similar levels of dimerization, again with editing of $mGlu_4$ decreasding heterodimerization with $mGlu_7$ but not homodimerization with $mGlu_4$ subunits. Of note, this assay was carried out in a condition of either entirely nonedited or entirely edited $mGlu_4$ constructs. The effect on dimerization within cells expressing both constructs at varying percentages would likely be more subtle, but also more attunable to the cell's specific needs. Additionally, studies have reported background levels of dimerization of $mGlu_4$ with $mGlu_1$ and $mGlu_5$ [254,257]. Levels of dimerization observed within our assay may, therefore, represent an increased background above other assay formats, although the order of dimerization propensity is similar to those previously published.

Editing is dynamically regulated by neuronal stimulation, hypoxia, stress, and energy/nutrient status, suggesting neurons can potentially modulate their editing status in response to their specific signaling needs [459]. This is especially interesting in the context of disease states in which RNA editing levels are known to be altered, such as cancer [99,285,326,451], amyotrophic lateral sclerosis (ALS, [460]), spinal cord injury [93], Alzheimer's disease [461], arthritis [462], hypoxia [201], and rheumatoid arthritis [462].

In summary, A-to-I editing of $mGlu_4$ transcripts results in the non-synonymous substitution of two amino acids within the dimer interface of the resulting receptor, increasing proteome diversity. The RNA secondary structure necessary for editing presumably evolved in a common mammalian ancestor and is well conserved. The amino acid substitutions induced by RNA editing did not cause alterations in $G\alpha_{i/o}$ activation as assessed by Tl^+ flux assay in response to various endogenous and synthetic agonists, or allosteric modulators. Furthermore, Q124R substitution by RNA editing was demonstrated to alter the propensity of $mGlu_4$ to heterodimerize with the group II and III mGlu receptors, $mGlu_2$ and $mGlu_7$, respectively.

Methods

Cell Culture

All cells were maintained in a 37C incubator with 5% CO_2. HEK293-GIRK parental cells were passaged in media (50% DMEM, 50% F12, supplemented with 10% FBS, 20mM HEPES, 1mM Na Pyruvate, 2mM Glutamax, 0.1mM non-essential amino acids, 1X antibiotic/antimycotic) under G418 (700ug/ml) selection to maintain GIRK expression. HEK293-GIRK cells stably expressing rat $mGlu_4$ constructs were additionally maintained under puromycin (600ng/ml) selection. HEK293A polyclonal cells stably expressing either ELFN1 or empty vector were passaged in media (DMEM, supplemented with 10% FBS, 20mM HEPES, 1mM Na Pyruvate, 2mM Glutamax, 0.1mM non-essential amino acids, 1X antibiotic/antimycotic) under G418 (700ug/ml) selection to maintain expression. Cells were transfected using Fugene6 transfection reagent (Promega) according to the manufacturer's protocol.

Tl^+ Flux assays

Cells were plated in black-walled, clear-bottomed, amine-coated 384-well plates (Ref#356719, Corning) at 15,000cells/20ul/well in assay media (DMEM, supplemented with 10% dialyzed FBS, 20mM HEPES, 1mM Na Pyruvate, 1X antibiotic/antimycotic) devoid of exogenous glutamate. For experiments involving ELFN1, stable cell lines were mixed to a ratio of 2:1 ELFN1- or vector-expressing cells: $mGlu_4$-expressing cells before plating. Assay dye loading, compound addition, and experimental measurement procedures have been described previously [270]. This assay was developed in our laboratory and first described in [463]. In short, glutamate stimulates the activation of $G_{i/o}$ heterotrimeric G proteins via transfected mGlu receptors. Dissociated β/γ subunits then directly stimulate the opening of GIRK channels, increasing the rate of influx of extracellularly applied Tl^+ and leading to an increase in fluorescence of the intracellular Tl^+-sensitive dye, Fluozin-2. HEK293 cells stably expressing GIRK channels demonstrate a background level of Tl^+ flux; however, that flux is not modulated in response to glutamate. Basal flux, defined by the rate

of Tl^+ entry in cells into the absence of glutamate, is subtracted from all values to obtain the agonist-induced signal. Signal obtained from different experimental days is normalized to the percent response of a control protein, in this case, the nonedited receptor condition. Percent response is calculated by expressing fluorescent values as a percentage of the maximal fluorescent response obtained to glutamate at saturation for a particular control condition.

Western Blot

On day 1 following transfection, media of transfected cells was replaced with Opti-MEM (Gibco, 11058-021) containing 2% (v/v) added FBS serum. On Day 2, medium was collected and centrifuged at 500xg to remove any floating cells and debris. The supernatant medium was collected and concentrated to ~250ul using an Amicon Ultracel-50K (Millipore, UFC805024) according to the manufacturer instructions. Cells were rinsed and lifted by scraping in ice-cold PBS and collected by centrifugation at 500xg. Cells pellets were lysed in RIPA buffer (Sigma, R0278) with 1X Complete Protease Inhibitor (Roche, 04693124001) on ice for 30 minutes. The supernatant solution was separated from insoluble cell debris by centrifugation at 20,000xg for 20 minutes. Protein concentration was assessed by BCA (Thermo Scientific, 23225). Proteins were separated by SDS-PAGE (BioRad, 465-1095) and transferred to a nitrocellulose membrane using the iBlot2 transfer system (ThermoFisher) at 20 volts for 8 minutes. Membranes were blocked using Intercept TBS blocking buffer (LiCor, 927-60001) and incubated with rabbit anti-Myc (Cell Signaling, 71D10) diluted 1:1000 in blocking buffer overnight at 4°C. Membranes were washed in TBST (Sigma, T5912) and incubated for 1 hour at room temp with goat anti-rabbit 800CW (LiCor, 926-3211) diluted 1:5,000 in blocking buffer. Membranes were again washed with TBST and imaged using a Licor Odyssey scanner. Membranes were then re-blocked for 30 minutes and incubated with mouse anti-GAPDH (ThermoFisher, MA-5-15738) for 1 hour at room temp. Following washing with TBST, membranes were incubated with 1:10,000 diluted goat anti-mouse 680LT (LiCor, 926-68020), washed with TBST, and imaged using a LiCor Odyssey scanner.

Analysis was conducted using Image Studio Lite (LiCor). Fluorescent values for Myc signal were normalized to those of GAPDH.

Dimerization Assay

Black-walled, clear-bottom 96-well plates (Corning, #3764) were coated with Poly-D-Lysine hydrobromide solution (Sigma, P64075mg) for 24 hours prior to cell plating according to the manufacturer's protocol. HEK293-GIRK parental cells transiently transfected with a 2:1 ug ratio of plasmid encoding the truncated, secretable MYC-tagged $mGlu_4$ to HA-tagged full length mGlu construct were plated at 100,000 cells/well in assay media. The following day, cells were washed in PBS, fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 20 minutes at room temperature, and washed 4X5 minutes in PBS. After blocking for 1.5 hours in Intercept (PBS) Blocking Buffer (LiCor), cells were stained with either 1:1000 Rabbit anti-HA (Abcam, Ab9110) or 1:1000 Mouse Anti-MYC (Cell Signaling, 9B11 mAB) and 1:1000 DRAQ5 (Thermo Fisher) overnight at 4C with rocking. Cells were washed 5X5 minutes in PBS-T (0.01%Tween-20), stained with 1:15,000 IR Dye 800CW Donkey anti Mouse (LiCor) or 1:15,000 IR Dye 800CW Donkey anti Rabbit (LiCor). Fluorescent labeling of Myc and HA tags was determined in separate wells due to overlap in spectra of the secondary antibodies used for detection. Within each well, fluorescent values for MYC or HA signal were normalized to that of the DRAQ5 nuclear stain. 3-6 technical replicates' of normalized values were averaged for each condition and the average normalized HA or Myc signal of un-transfected control cells was subtracted as a blank from all values. Propensity to dimerize was determined by dividing the Myc signal of the secretable $mGlu_4$ ATD by that of the HA signal for the full length, co-transfected mGlu receptor. Data were normalized across days by normalizing all conditions to that of the response of non-edited $mGlu_4$.

Chapter IV: Future Directions

Reframing Recoding by A-to-I Editing

The advent of high-throughput sequencing has led to the discovery that RNA editing is much broader in its scope than originally believed. Many of the substrates of A-to-I editing were discovered within mRNA coding sequences and predict the recoding of the encoded protein; however, we now know that events of protein recoding by RNA editing represent only 0.004% of editing events. This incredible disparity could lead to the interpretation that these events are not specific, but a side effect of vestigial, transcriptome-wide editing. Additionally, while genetic knockout of ADAR1 or 2 in mouse models results in lethality, the concurrent genetic manipulation of a single gene can rescue this lethality, suggesting a unimodal critical functionality of these two enzymes. This information alone may lead many to question the functional importance of RNA editing. I have been asked this question over the duration of my degree many times and even asked whether a single amino acid changes can significantly alter protein function. I believe that these views of the importance of RNA editing stem from an incorrect interpretation of these data and unreal expectations of the functional consequences of endogenously-controlled amino acid alterations.

The deamination induced by RNA editing may provide more than one function. Comparing the relatively few conserved recoding sites to the vast number of total RNA events frames these recoding events as a less significant function of ADAR enzymes; however, recoding by RNA editing and the less-conserved editing of repetitive elements may occur for distinct functions. Non-conserved editing occurs within repetitive elements, is mainly induced by deamination mediated by ADAR1, and varies widely in editing specificity and extent within individual substrates. Recoding events occur mainly in non-repetitive sequences, are mainly catalyzed by ADAR2, and demonstrate a far-increased specificity and extent of editing. Comprising a small percentage of

total editing events does not reduce the functional importance of recoding by RNA editing, nor do these largely unique processes need to be compared.

Furthermore, the high level of evolutionary conservation of RNA editing highlights its importance. The dsRNA substrates required for editing of some of these substrates require complementary sequence within introns, which are abnormally well conserved across species in comparison other intronic sequences. While KO animal models suggest that recoding of a majority of RNA substrates is not essential for survival, they have nonetheless resisted the selective pressures of evolution, suggesting their functional importance. So what can we expect then from single non-synonymous amino acid changes? There are many examples of single nonsynonymous mutations linked to human disease states (Rett syndrome[464], Tay Sachs[465], Sickle Cell Anemia[466], cancer[467]). Additionally, many recoding events alter amino acids within critical domains for neuronal protein function, including the pore domain of AMPA and $K_V1.1$ ion channels [43], the G protein-binding loop of the serotonin 5-HT_{2C} receptor [39], and the calmodulin binding site of $Ca_V1.3$ ion channel [142]; however, RNA editing is an endogenous process, resulting in amino acid substitutions within generally less conserved areas of the resulting proteins. By its very nature, these effects are likely to be small allosteric changes. Indeed, most RNA editing events that result in amino acid substitutions, apart from the Gria2 subunit of the AMPA receptor, modulate protein activity rather than cause large on/off effects. From all of the evidence, it is most likely that RNA editing serves (as many have stated) as a method by which cells may expand their proteome beyond the capabilities encoded within the genome. This process would allow for cells to specifically modulate the function of key synaptic proteins to their individual needs. Is this process essential for life? Apart from two discrete functions, the answer is largely no. That does not mean it's not functionally important.

RNA Editing: Unanswered Questions

Critical questions regarding A-to-I editing remain unanswered or insufficiently answered, limiting our understanding of the functional role of RNA editing in normal mammalian homeostasis. The extent of recoding by RNA editing has been described largely by the analysis of RNAs isolated from dissected tissues; however, it is unknown whether editing levels attained at the anatomical level are proportional to those within a single cell [125,131,152]. In one study, analysis of single cells isolated from human cortical surgical biopsies showed "all or none" A-to-I editing within individual cells[125]. Within the context of $mGlu_4$ editing, levels of K129R editing (~2%) determined at the anatomic level appear low, but this could represent a mixed population of cells with great disparity in editing levels. K129R editing within certain cell subsets could serve a critical function in that unique cell or within a larger neural circuit. scRNAseq studies with higher resolution and from a greater population of cells will be needed in the future to determine to what extent transcripts are differentially edited between individual cell types of a particular anatomical region and importantly within individual cells of a particular subtype.

Within individual cells, it is still unknown whether the proportions of edited receptor transcripts within the cellular RNA pool reflect the proportions of resultant recoded proteins. Radioligand binding data suggests a 4000-7000% increase in serotonin receptor expression in mutant mice expressing solely the "VGV" isoform of the $5\text{-}HT_{2C}$ receptor compared to wild type littermate controls which express all edited variants of the receptor [446]. As Jinnah et al have stated, this enormous increase in protein expression, despite invariable mRNA transcript abundance, suggests unique post-transcriptional mechanisms to regulate protein expression [21]. Furthermore, this suggests that the proportion of the total protein pool by a recoded isoform may not be reflective of mRNA abundance.

Cell-specific editing levels and protein isoform expression/stability are essential to understanding the role of RNA editing. The functional consequences of editing are typically

assessed in *in vitro* or *in vivo* models in which the protein of interest is either “always edited” or “always nonedited” [21]. These models can be useful and presumably demonstrate phenotypes at the extremes of a spectrum of editing. However, if edited transcripts comprise only a proportion of transcripts within a cell, the effects will likely be much smaller, modulatory effects than observed in an “always edited” system. Additionally, all tissues and cell types express only edited or nonedited receptors in these models, masking the regional and spatiotemporal specificity of homeostatic editing levels. Finally, these genetic models alter genomically encoded adenosines to guanosine, resulting in mRNA transcripts with guanosine rather than inosine. The incorporation of inosine may result in nuclear retention of mRNA transcripts or translational stalling [143-145,147]. Additionally, inosine occasionally base pairs with A or U rather than C within tRNA anticodons, leading to a more complex proteome resulting from an inosine-containing transcripts[147,148,150]. For this reason, it is critically important to determine the abundance of differential protein isoforms generated from inosine-containing transcripts in a native tissue or setting.

Functional Effects of $mGlu_4$ RNA Editing

Higher Precision *In Vitro* Methods and Single Molecule Imaging

In the signaling assays described in this thesis, either edited or nonedited receptors were heterologously overexpressed *in vitro*; with such a receptor excess, it can difficult to determine whether there is indeed no functional effect of the Q124R substitution on receptor signaling, or whether the saturation of receptor within our assay platform masked differences within these receptor dimer pairs. Within the last decade, more sensitive fluorescence-based technologies based on traditional Fluorescence and Bioluminescence Resonance Energy Transfer (FRET and BRET) techniques have been developed which offer remarkable specificity in determining the molecular dynamics of mGlu receptor dimerization and activation.

Dimerization

The study of mGlu receptor dimerization is complicated by the formation both homo- and hetero-dimers, resulting in multiple surface-expressed populations of dimeric receptor pairs. To aptly study dimerization, researchers must design ways to specifically measure individual dimer pairs within multiple populations of receptors. In my work, I employed a system in which truncated, secretable $mGlu_4$ constructs were co-expressed with full length mGlu receptors (Figure 13A). Two recent advances in fluorescent labeling techniques may offer increased sensitivity in the assessment of mGlu receptor dimerization and could be used to validate the effect of Q124R substitution on $mGlu_4$ heterodimerization as well as more sensitively quantify changes in dimerization propensity.

One such method is single-molecule subunit counting. In brief, this technique involves the solubilization of co-expressed, dimerized mGlu receptors which bear a C-terminal fused GFP. After immobilization of the solubilized receptors on a cover slip, individual dimers can be directly counted[224]. This method, pioneered by the Levitz and Isacoff labs, was used to show that mutations in helix B of $mGlu_2$ weakened the dimer interface of these receptors [224].

Another powerful technique involves the use of N-terminally fused SNAP and CLIP tags. These enzymatic tags have significantly advanced our capabilities to monitor the molecular dynamics of mGlu receptors, even down to the level of single molecules. SNAP and CLIP tags are relatively small (~20 KDa) proteins generated through mutation of a human DNA repair enzyme, 0^6-alkylguanine-DNA-alkyltransferase, to allow for the covalent labeling of either subunit specifically with unique benzylguanine- or benzylcytosine-tethered-substrates, respectively. The fusion of these proteins atop the mGlu receptor LBD provide significant advantages to the study of mGlu dimers; labeling of SNAP or CLIP subunits with specific FRET or Lanthanide Resonance Energy Transfer (LRET) donor and acceptor fluorophores allows for the identification of heterodimeric complexes which can produce a FRET signal while other dimer receptor

combinations cannot. This incredibly selective technique has allowed for the identification of heterodimeric/oligomeric populations and importantly the study of their molecular dynamics.

Activation of the LBD

Upon activation by agonist, the mGlu ligand binding domains pivot in relation to one another. In SNAP and CLIP-tagged receptors, this subunit rearrangement causes a transition from a high FRET to low FRET state which can be measured on a sub-millisecond time scale. Using this technique, several labs have begun probing the rapid transitions of mGlu receptors between resting and active states [219,224,266,269]. These transitions are proportional to agonist response and provide a concentration-response similar to other activity assays; however, this information is related to the primary activation of mGlu receptors, i.e., movement of the LBD. This provides a unique measure of receptor activation that is decoupled from G protein activation and could provide additional insight into mGlu receptor dynamics. Monitoring the activation of $mGlu_4$ receptors by the dynamics of the LBD may provide unique insights into the effects of RNA editing. RNA editing alters amino acids within the B helix of the LBD and effects at this domain of the protein may be masked by the CRD and TMD.

Isolating Heterodimeric Signal

More sensitive assay platforms may be required to more accurately and completely probe the functional consequences of A-to-I editing of $mGlu_4$ RNA mRNA transcripts. In my work, I have used chimeric mGlu receptors which bear the C-terminal tails of the $GABA_B$ receptor to restrict surface expression to only heterodimeric receptors (Figure 12A); however, it is possible that this system may be “leaky”, allowing for some contribution from homodimeric receptors at the surface. This system is also artificial in that it does not allow for homeostatic processes in which mGlu receptors dimerize and traffic to the surface.

The transitional FRET states of mGlu LBDs provides another mechanism by which to study signaling specifically in heterodimeric populations. Receptor activation can be assessed using the transition to a lower FRET state as a measure of agonist-induced activation. A benefit to this method is that SNAP and CLIP subunit can be uniquely labeled, which restricts FRET signal to only those dimers composed of one SNAP and one CLIP subunit, i.e. heterodimers, even when multiple populations are expressed. This method allows for the normal assembly and trafficking of mGlu receptors.

The CODA-RET system, developed by the Javitch group, also isolates signal from heterodimers amongst multiple receptor populations, but assesses G protein activation. In this experimental paradigm, a chimeric G protein fused with mVenus is co-transfected with two GPCRs, each fused on their intracellular surface one half of a luciferase molecule; only when these two GPCRs interact in close proximity, i.e. form heterodimers, can the two luciferase halves come together to form a bioluminescent molecule [468]. Furthermore, only when the G protein is recruited by receptor activation can energy transfer from luciferase to mVenus. By measuring this energy transfer (BRET signal), the CODA-RET system measures signaling specifically from heterodimer populations. The increased sensitivity of this system could allow for the detection of more subtle alterations within heterodimers with one edited $mGlu_4$ receptor. While also using chimeric receptors, this format has the added advantage of not disrupting receptor trafficking as in the $GABA_B$ tailed experiments.

GRM4 “Always Edited” or “Never Edited” Animal Models

While an “always edited” or “never edited” model of $mGlu_4$ editing is artificial in its representation of transcript abundance, it could help determine the effects of $mGlu_4$ editing within a gross physiologic context, as has been realized for other mouse models of editable substrates [21]. Within this body of work, the $mGlu_4$ mRNA duplex has been defined and validated which can guide genetic strategies to alter coding of these transcripts. With this knowledge, genetically

modified mouse models could be generated which solely express either of the nonedited or Q124R $mGlu_4$ transcripts isoforms.

Generation of a “never edited” animal must involve destabilization of this duplex due to the lack of glutamine (CAA/G) and lysine (AAA/G) codons that lack editable adenosines. Due to the exonic nature of the RNA duplex, destabilization must be completed with caution through the use of missense mutations to avoid the introduction of novel editing sites within the dsRNA substrate and to prevent alteration in the amino acid sequence of the resulting $mGlu_4$ receptor. The generation of an “always edited” mouse model specific to the Q124R isoform is possible by genetic alteration of the glutamine (CAG) codon to that of arginine (CGG), but may also require destabilization of the dsRNA duplex to eliminate extraneous editing of the K129R site. It has not been determined whether deamination at a particular adenosine within the $mGlu_4$ substrate can affect deamination of other sites. The *in vitro* editing assay and minimal essential $mGlu_4$ minigene described in this work can function as an effective platform to test mutational combinations for duplex disruption which limit the introduction of nonspecific duplex editing prior to generating expensive genetically modified animals.

Initial phenotypic profiling studies in GRM4 “always edited” or “never edited” animals should be directed to processes where $mGlu_4$ function has been implicated. The role of $mGlu_4$ has been most extensively defined within the central nervous system where, coincidentally, levels of RNA editing are highest. $mGlu_4$ activity has been implicated in a number of neurologic phenotypes by profiling *Grm4*$^{-/-}$ mice, namely locomotor activity, learning and memory, and neuroinflammatory models.

$mGlu_4$, $mGlu_{2/4}$, and $mGlu_{4/7}$ Expression at Specific Synapses

Q124R substitution in the $mGlu_4$ LBD reduced heterodimerization with $mGlu_2$ and $mGlu_7$ (Figure 13); however, glutamate-induced response was not effected in either dimer pair (Figure 14). Further investigation is needed to determine whether alterations in the propensity of $mGlu_4$

heterodimerization alter synaptic function. Electrophysiology could serve as a powerful and specific technique for assessing these effects *in vivo*. The genetic mouse models of "always edited" or "never edited" $mGlu_4$ animals discussed above would be optimal for these studies. A viral transduction model in $Grm4^{-/-}$ mice could also be used and provide a simpler, single mouse model for the characterization of additional edited isoforms (K129R, Q129R K129R). Essential to the delineation of editing specific effects within these dimer pairs is the identification of synapses where $mGlu_4$ and other mGlu receptors are co-expressed. The predominant mGlu receptor modulating activity of the parallel fiber-Purkinje cell synapses of the cerebellum is $mGlu_4$ [334], making this synapse a strong candidate for functional studies of the effects of Q124R substitution within $mGlu_4$ homodimers *in vivo.* More recent studies have shown that $mGlu_2$ and $mGlu_4$ form functional $mGlu_{2/4}$ heterodimers at corticostriatal synapses[270] and thalamo-mPFC synapses [277]. It remains unknown whether $mGlu_4$ and $mGlu_7$ form functional heterodimers *in vivo*; however, these two receptors do demonstrate overlapping expression within striatopallidal projections [275,276].

Final Summary

A-to-I RNA editing wields an immense power to modify the mammalian transcriptome. Though deamination occurs at up to 100 million adenosines, only a small subset of these sites are highly conserved and predict the alteration of the amino acid sequence of key synaptic excitability proteins. Despite this immense power to reshape the transcriptome, there is still much unknown about the importance of RNA editing, specifically regarding the functional consequences of recoding in individual synaptic proteins. One of these recoded proteins, $mGlu_4$ is an important modulator of neurologic signaling and may be a promising drug target. This thesis work elucidates the extent and regulation of editing of $mGlu_4$ transcripts at tissue-level resolution within the CNS which predicts the recoding of two conserved amino acids within the resulting receptor's dimer interface. This work has important implications for the field of RNA editing and mGlu receptors alike. By describing the regulation and consequences of $mGlu_4$ editing, further light is shed on the

implications of transcriptome regulation by deamination. Additionally, we have described a process by which heterodimerization of $mGlu_4$ could be controlled by the endogenous and modulatable process of RNA editing. Most importantly, this work lays an important fundamental ground work on which to design future studies to address the physiologic contributions and consequences of A-to-I RNA editing of $mGlu_4$ transcripts.

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