Exploring the dynamics of A-to-I editing in the mammalian brain

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LIST OF ACRONYMS AND ABREVIATIONS

^{3}H : tritium

5HT : serotonin

$5HT_{2C}$: serotonin $_{2C}$ receptor

ADAR : adenosine deaminase acting on RNA

Adx : adrenalectomized

Ala : Alanine

Alu : Alu-type genomic element

aMSH : alpha-melanocyte stimulating hormone

ANCOVA : analysis of covariance

ANOVA : analysis of variance

Arc : arcuate nucleus of the hypothalamus

A-to-I editing : adenosine-to-inosine RNA editing

BAT : brown adipose tissue

C57Bl/6 : c57 black 6 strain of mice

CADPS : Calcium activated protein for secretion

cDNA : complementary DNA

CP : choroid plexus

CSF : cerebral spinal fluid

Ctx : cortex

DA : dopamine

$\Delta\Delta$CT : delta-delta cycle threshold

DNA: deoxyribonucleic Acid

dsRNA : double-stranded ribonucleic acid

ECS : editing site complimentary sequence

ERK : Extracellular signal-related kinase

Exo I : exonuclease I

F5 : clotting factor 5

FLNA : Filamin A

FLNB : Filamin B

GABRA3 : Gamma-amino butyric acid receptor α-subunit 3

GIRK : G-protein coupled inwardly rectifying K^+ channel

Gli1 : Gioblastoma family zinc finger transcription factor

GluR-2 : AMPA-subtype glutamate receptor subunit 2

GluR-3 : AMPA-subtype glutamate receptor subunit 3

GluR-4 : AMPA-subtype glutamate receptor subunit 4

GluR-5 : Kainate-subtype glutamate receptor subunit 5

GluR-6 : Kainate-subtype glutamate receptor subunit 6

Gly : glycine

GPCR : G-protein coupled receptor

HBII-52 : Human brain specific snoRNA 52 (SNORD115)

HBII-85 : Human brain specific snoRNA 85 (SNORD116)

HCl : hydrochloric acid

Hip : hippocampus

HPA : hypothalamic-pituitary-adrenal axis

HPLC : high-performance liquid chromatography

HTMTA : high-throughput multiplex transcript analysis

IC : imprinting center

INI : isoleucine-asparagine-isoleucine isoform of the $5HT_{2C}$ receptor

Kv1.1 : voltage- gated potassium channel 1.1

MBII-52 : mouse brain specific snoRNA 52

MBII-85 : mouse brain specific snoRNA 85

mGluR4 : metabatropic glutamate receptor 4

mRNA : messenger RNA

NAc : nucleus accumbens

OB : olfactory bulb

P#: postnatal day

pCPA : parachlorophenylalanine

PCR : polymerase chain reaction

Pet1 : gene encoding the Fev transcription factor

PLA2 : Phospholipase A2

PMI : postmortem interval

POMC : pro-opiomelanocortin

PWS : Prader-Willi Syndrome

RNA : ribonucleic Acid

rRNA : ribosomal RNA

RT-PCR : reverse transcriptase polymerase chain reaction

SBB : standard binding buffer

SEM : standard error of the mean

SERT : serotonin transporter

SNORD : gene encoding a small nucleolar RNA

snoRNA : small nucleolar ribonucleic acid

snRNA : small nuclear RNA

SSRI : selective serotonin reuptake inhibitor

Str : striatum

TH : tryptophan hydroxylase

tRNA : transfer RNA

VGV : valine-glutamic acid-valine isoform of the $5HT_{2C}$ receptor

VSV : valine-serine-valine isoform of the $5HT_{2C}$ receptor

VTA : ventral tegmental area

WB : whole brain

Chapter I

INTRODUCTION

Beyond the central dogma

Life successfully evolved on earth because nucleic acids have formed a template for high-fidelity transfer of biological information through time in the form of genomes. The discrete components harboring information in the genome are genes and this information is coded in a sequential arrangement of covalently bound nucleic acids; the genome sequence. The genome sequence fundamentally dictates how an organism grows, reproduces, and interacts with the universe. Francis Crick first proposed the information flow used by peptide coding genes, a process he referred to as the "central dogma of molecular biology" [1]. In summary, it states that the deoxyribonucleic acid (DNA) polynucleotide sequence serves as a template for the synthesis of ribonucleic acid (RNA) polynucleotides. These RNAs then serve as templates for polypeptide synthesis, whereby the sequential incorporation of amino acids in the peptide is dictated by the RNA sequence. The "dogma" provides a useful framework for thinking about the transduction of genetic information, but should not diminish the importance of parallel processes that impart an even greater capacity to encode information in the genomic context. Interactions between proteins and nucleic acids have permitted the emergence of complex and dynamic systems that are conserved and selected by evolution generating a vast diversity of protein coding potential. The goal of the work described here is to better understand one such mechanism, known as adenosine-to-inosine RNA editing (A-to-I editing).

Organisms interact with the environment using a diverse milieu of physiological responses often involving changes in gene expression. The staggering variety of cellular

functions needed to support adaptive responses led early genomic researchers to hypothesize that the genomes of complex organisms like humans must harbor millions of protein coding genes. However, as progress was made in mapping, sequencing, and annotating the genomes of many organisms biologists were faced with the realization that the number of protein coding genes in multicellular organisms is typically on the order of thousands, not millions [2]. This insight yielded a new appreciation for mechanisms that generate molecular diversity from a finite genome. The need to conserve precious genomic information makes genome modification an unfavorable mechanism for generating functional diversity. Conversely, RNA is transiently expressed and represents a viable template for active molecular diversification; leaving the precious genome intact. Several mechanisms for generating protein diversity by RNA modification such as alternative splicing and RNA editing have been identified.

In most eukaryotic organisms, RNAs generated during transcription of genomic DNA do not typically encode a polypeptide in a single contiguous segment. Instead, these primary transcripts known as pre-messenger RNAs (pre-mRNAs) contain sequences encoding parts of a polypeptide interspersed within long regions of non-coding intervening sequences called introns. The individual units of regulatory and peptide-coding RNA between the introns are referred to as exons. During a process known as splicing, introns are removed and exons are joined together to form mature, peptide-coding messenger RNAs (mRNAs) [3]. The number of exons contained in a gene varies widely from a single exon to several hundred exons. Differential inclusion of these exons, known as alternative splicing, generates a diversity of mRNAs encoding peptides with unique primary structures all encoded by a single gene [4]. Generating protein diversity from a limited genome is invaluable for complex organisms; as such, a variety of mechanisms in addition to alternative splicing, like RNA editing, also have evolved for this purpose.

Generating of functional diversity by RNA editing

RNA editing refers to a number of post-transcriptional modification processes which alter the sequence of RNAs such that they differ from that of the genomic template from which they are derived. Several types of RNA editing have been described including insertion, excision, or covalent modification of nucleotides [5]. Editing can have a range of effects on RNA function including creation of new structural characteristics, modulation of RNA stability, or microRNA target selection [6, 7]. In the context of mRNAs, editing also can lead to the introduction of new stop codons or open reading frames, modulation of splice-site selection [8], or amino acid substitutions [9]. The work presented here is focused upon characterization of a particular form of RNA editing referred to as adenosine-to-inosine (A-to-I) editing. This RNA modification involves hydrolytic deamination of specific adenosine moieties at the C6 position; thereby converting adenosines to inosines [10]. A-to-I editing has been conserved throughout metazoan evolution where it is observed in species ranging from nematodes and insects, to fish and mammals [7].

There are a variety of potential effects of replacing an adenosine with inosine stemming from the fact that inosine forms a stable I-C base-pair with the same geometry as guanosine, albeit with one fewer hydrogen bond per pair. It is for this reason that inosine appears to function like guanosine in RNA [11]. Edited nucleotides can be detected indirectly by sequencing complementary DNA (cDNA); reverse transcriptase incorporates cytosine in the complementary strand for template inosine moieties during cDNA polymerization leading to disparity in the sequence of cDNA from that of genomic DNA [12]. The identification of these A-to-G discrepancies in cDNA libraries facilitated the discovery of hundreds of A-to-I editing events in many animal species. In addition to altering base incorporation during reverse transcription,

inosine contained in mRNA open reading frames base pair with cytosine in the anticodon loop of tRNAs which can alter the amino acid sequence of the peptide encoded by edited mRNAs [13]. The non-synonymous amino acid substitutions resulting from editing within codons can lead to profound functional changes for the encoded protein product. Several A-to-I editing events that predict changes in amino acid codons have been identified in a variety of animals, many of which are highly conserved in mammals.

The extensive conservation of specific sites selected for codon editing as well as anatomic editing profiles observed in different mammalian species is striking. The extensive conservation of such events suggests that the particular amino acids which are altered by editing have an important role in the encoded peptide's function. It also implies that the ability to edit these sites confers an advantage that has been maintained by evolution. Indeed, numerous studies have identified profound changes in protein function affecting overall physiology when only a single amino acid changes as a result of A-to-I editing [14]. For this reason, it is of paramount importance to identify the functional role of editing within different mRNA substrates as well as the dynamic processes that regulate A-to-I conversion in both healthy and in pathological states.

A-to-I editing is catalyzed by a group of enzymes known as adenosine deaminases that act on RNA or ADARs. These ADAR proteins facilitate hydrolytic deamination at carbon-6 of adenosines with the oxygen in a water molecule serving as the nucleophile. The ADAR enzymes seem to require that the edited ribonucleotides lie within an extended intermolecular duplex and interact with RNA via conserved double stranded RNA binding motifs which are characteristic of all ADAR enzymes [7]. However, more research is required to determine how precise targeting of deamination events to particular adenosines within such duplexes is directed. Through extensive homology searches, three highly-related proteins of the ADAR family have

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been identified in mammals. These proteins are referred to as ADAR1, ADAR2, and ADAR3. All three mammalian ADAR proteins have similar double-stranded RNA binding motifs presumed to facilitate targeting to double-stranded RNA regions, and in the case of ADAR1 and ADAR2, a conserved deaminase domain [7]. For ADAR3, there is no evidence of catalytic activity, suggesting that ADAR3 might play an alternative or supporting role in RNA processing [15]. While ADAR1 and ADAR2 display some overlapping substrate specificity, they also target unique adenosines within specific RNA substrates [16].

A-to-I editing is particularly interesting within the context of neuroscience for several reasons. The enrichment of inosine containing transcripts in the CNS, particularly in the primate, has been hypothesized to be a driving force in the evolution of the human brain [17]. ADAR proteins are enriched in neural tissues, particularly in neurons within the CNS, and many of the mRNAs that have been identified as substrates for A-to-I editing encode proteins which are important for nervous system function [7, 17]. These include voltage-gated [18] and ligand-gated ion channels [19], components of synaptic release machinery, regulatory proteins [20, 21], and at least two metabotropic neurotransmitter receptors [9, 22]. Table 1 contains a table summarizing many of the A-to-I editing events that have been identified in mammals.

By gaining a better understanding of the biological significance of codon-changing RNA editing events, new insights have been revealed about the key roles played by these proteins in fundamental neuronal processes. Unfortunately, determining the functional relevance of mRNA editing events that produce non-synonymous amino acid substitutions is limited by the functional assays available for interrogating protein function. The effect of such amino acid substitutions is typically studied by expressing cDNAs harboring either a guanosine or adenosine at the editing site under interrogation in an appropriate model system.

TABLE 1: **Summary of non-synonimous A to I editing sites in mammals.**

Editing site		Adar(s) preferred by substrate	Functional Consequences
GPCRs			
5HT2C	A site	Adar1[8,22]	Receptor activity[8], pharmacology[33]
	B site	Adar1[8,22]	
	C site	Adar2[8,22]	
	D site	Adar2[8,22]	
	E site	Adar1 and Adar2	
mGluR4	Q/R site	Adar2	Unknown
Ion channel subunits			
GABRA3	I/M	Adar1 and Adar2 [18]	Deactivation, trafficking[18]
Kv1.1	I/V	Adar2 [17]	Inactivation/deactivation kinetics[17]
GluR2	Q/R	Adar2[15]	Calcium ion permeability[11]
	R/G	Adar2[15]	Desensitization kinetics[11]
GluR3	R/G	Adar1[22]	Desensitization kinetics[99]
GluR4	R/G	Adar2[22]	Desensitization kinetics[99]
GluR5	Q/R	Adar1 and Adar2[22]	Calcium ion permeability[99]
GluR6	Q/R	Adar1 and Adar2[22]	Calcium ion permeability[100]
	I/V	Adar1 and Afar2[22]	Calcium ion permeability[100]
	Y/C	Adar2[22]	Unknown
Transcription factors			
Gli1	R/G	Adar1 and Adar2[20]	Kinase susceptibility[20]
Cytoskeletal associated			
FLNA	Q/R	Adar2[101]	Unknown
FLNB	Q/R	Adar2[101]	Unknown
Synaptic release machinery			
CADPS	E/G	Adar2	Unknown
Enzymes			
Adar2	-1	Adar2[22]	Splicing/Autoregulation[7]

Note: Adar1 and Adar2 both have some activity at each of these substrates in certain conditions but the enzyme(s) thought to be primarily responsible for editing *in vivo* is listed.

The best characterized RNA editing effects have generally involved ion channels because nuanced studies of their biophysical properties are permitted by various electrophysiological recording techniques. A seminal study with edited GluR-2 subunits of the AMPA-subtype of ionotropic glutamate receptor revealed that an A-to-I editing event leading to the substitution of glutamine with an arginine in the predicted ion pore conferred impermeability to calcium ions [12]. This change has profound ramifications for the effects of channel activation because calcium ions are highly regulated signaling molecules in neuronal cells. This observation fostered the remarkable realization that proteins harbor conserved switches that are actually exploited by RNA editing to confer dynamic functionality.

The first studies of editing *in vivo* were made possible by the development of gene-targeting techniques in mice. Initially, mice were generated harboring null mutations in each respective ADAR gene. In these studies it was observed that ADAR1-null homozygotes die at embryonic day 11.5 (E11.5), while ADAR2-null animals suffer from seizures and die between 3 and 5 weeks of age [16, 23]. These results indicated that RNA editing is indispensable for normal murine development and physiology, but initially failed to foster many useful insights about the particular role of different edited substrates. To determine the role played by editing at specific substrates, gene-targeting has been used to generate mice harboring a guanosine in the genome at the edited position to mimic the edited inosine containing transcript. To complement these studies it has also been useful to generate mice that express only the non-edited form of the transcript. This is generally achieved by disrupting the obligate duplex structure required for ADAR-mediated deamination without introducing nonsense or missense mutations into the gene. To date, mutant mice have been generated which exclusively express either the non-edited or edited isoforms of the GluR-2 subunit, the 5HT$_{2C}$ [24], and the voltage gated potassium channel

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subunit, Kv1.1, respectively. Interestingly, the neonatal lethality of the ADAR2-null phenotype could be rescued by co-expression of an edited GluR-2 (R) allele, indicating that the primary reason for lethality was an inability to edit GluR-2 transcripts [15,22]. As such, detailed phenotypic characterization of mutant mice bearing a non-edited GluR-2 allele was precluded by an early lethality similar to that observed in ADAR2-null animals. The lethality observed for both lines of mutant mice is presumed to result from the role this editing event plays in regulating calcium permeability[16]. Mutant mice solely expressing either the edited or non-edited isoforms of the $5HT_{2C}$ receptor [24, 25] or Kv1.1 transcripts reach adulthood and phenotypic characterization is ongoing. The primary goal of the work described here is to further address questions related to the functional significance and the dynamic regulation of RNA editing for $5HT_{2C}$ receptor transcripts.

Overview of the $_{2C}$-Subtype of 5HT Receptor

$5HT_{2C}$ receptor molecular biology

The $5HT_{2C}$ transcript represents an interesting substrate for studying the mechanisms regulating A-to-I editing, because it contains five editing sites in close relative proximity which are all located in a single duplex structure [9]. Despite the proximity of these five editing sites (referred to as sites A, B, E, C, and D respectively) they display differential susceptibility to deamination by ADAR1 and ADAR2 [16, 23]. Furthermore, different combinations of editing at these five sites can generate up to 32 RNA isoforms which can encode up to 24 unique protein isoforms differing by three amino acids predicted to lie in the second intracellular loop of the mature receptor protein. Comprehensive analysis of the functional differences between the 24 possible $5HT_{2C}$ isoforms has not been performed to date, but extensive analysis of some of the

most commonly expressed isoforms has yielded some useful information about the functional consequences of editing for $5HT_{2C}$ mRNAs.

$5HT_{2C}$ receptors are members of the A class of G-protein coupled receptors (GPCRs) encoded on the X-chromosome in mammals [26, 27]. The gene contains 6 exons and at least three isoforms are generated as a result of alternative splicing, only one of which is predicted to encode a functional GPCR [28]. As noted previously, even greater functional diversity is conferred to the $5HT_{2C}$ locus by five conserved A-to-I editing events that have been identified in exon 5 [29]. The genomically-encoded $5HT_{2C}$ receptor transcript predicts isoleucine, asparagine and isoleucine at amino acid positions 156, 158 and 160, respectively (coordinates relative to the initiator methionine in the human peptide sequence). In the case of $5HT_{2C}$, the protein isoforms resulting from differentially edited RNAs are generally referred to by the identity of amino acids encoded at these three positions, such that the non-edited RNA encodes the INI isoform. Likewise, a receptor encoded by an RNA harboring inosine (or guanosine) at each of these five sites encodes valine, glycine, and valine (VGV) at the analogous positions [30]. The best characterized functional differences between different edited isoforms of the $5HT_{2C}$ receptor are related to signaling activity [29]. The primary G-proteins activated by $5HT_{2C}$ receptors are thought to be those of the $G\alpha_{q/11}$ class which directly stimulate phospholipase C (PLC) [31]. While PLC activation is the most commonly used readout of $5HT_{2C}$ activity in heterologous systems, several studies provide evidence for potent efficacy on other effector
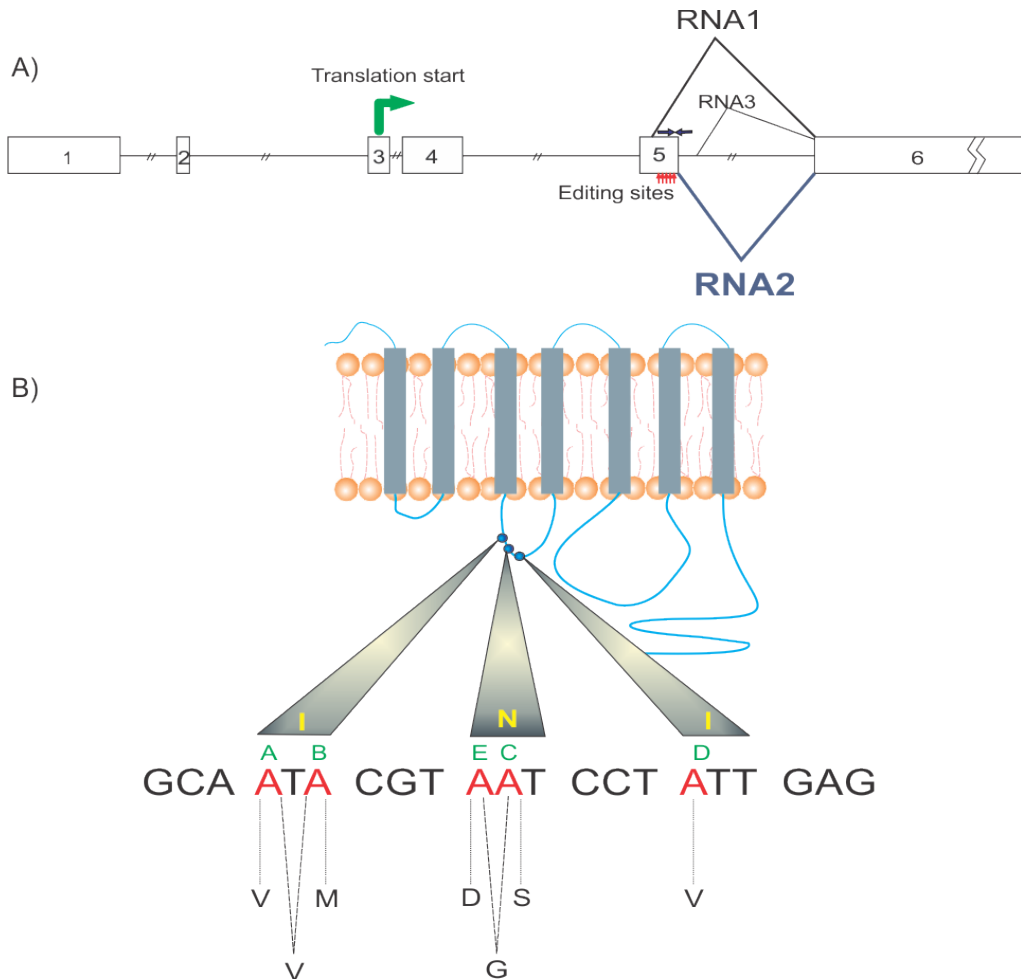
Figure 1. RNA processing of 5HT2C transcripts. A) The conserved genomic organization of the mammalian HTR2C gene located on Xq24. Relative size and locations of exons are indicated with boxes. The green arrow indicates the translation start site and red arrows indicate the locations of five A-to-I editing sites. The black arrows at the exon-intron junction of exon 5 indicate the location of nucleotides that forms the predicted hairpin duplex recognized by ADAR enzymes. Three different isoforms generated by alternative splicing have been reported. RNA2 encodes the functional $5HT_{2C}$ receptor. **B)** A schematic diagram showing the predicted topology of the $5HT_{2C}$ receptor. The predicted positions of the amino acid residues altered by editing are indicated by blue circles. The genomically encoded amino acids at these three positions are indicated in yellow. The genomic sequence of the edited region is shown with the substrate adenosines indicated in red and the names of the respective sites are indicated in green. Amino acid encoded after editing at each site or combination of sites is indicated.

pathways [32]. Importantly, $5HT_{2C}$ receptors activate GIRK channels *in vivo* [33] and phospholipase A2 (PLA2) via heterotrimeric G-protein-dependent mechanisms [34]. In addition, $5HT_{2C}$ receptors also appear to activate extracellular signal related kinase (ERK1 and ERK2) cascades through a G-protein independent, β-arrestin-dependent pathway [35].

Initial efforts to investigate the functional role of $5HT_{2C}$ editing were focused on elucidating the pharmacological properties of receptors encoded by different combinations of editing. The amino acids altered by editing are predicted to reside in the second intracellular loop of the receptor only a few residues away from the conserved DRY motif; a 'microswitch' thought to be important for G-protein activation [36]. The properties of the different edited isoforms of the $5HT_{2C}$ receptor were initially studied by comparing 5HT-stimulated phosphoinositide hydrolysis in cells expressing cDNAs encoding either the VSV or INI receptor isoforms. Efficient 5HT-stimulated PLC activation was observed in both cases, indicating that both isoforms couple similarly to parallel transduction pathways. However, the EC_{50} value for cells expressing the INI isoform indicated that the potency of 5HT to stimulated PLC activity is approximately 15-fold greater than cells expressing the VSV isoform, despite similar affinities for orthosteric ligands [9]. This finding attracted the attention of a number of GPCR pharmacologists who have contributed to characterization of the biophysical consequences of RNA editing for this receptor [27]. While these studies continue to provide additional insights about $5HT_{2C}$ signaling, the core finding with the greatest biological relevance is that agonist-independent (constitutive) activity is high for the INI isoform and essentially undetectable for the fully-edited (VGV) isoform of the receptor. The other edited isoforms have not been studied in detail, but they appear to display intermediate levels of constitutive activity that fall between that of INI and VGV. A similar rank order of potency is observed for the G-protein-dependent

activation of phospholipase A2 [37]. Interestingly, this generalization also seems to apply to the receptor's behavior in G-protein independent processes primarily thought to result from β-arrestin and calmodulin interactions [35, 38].

GPCRs are subjected to dynamic trafficking and inhibition as a response to agonist stimulation. This process is thought to be regulated primarily by direct interactions between GPCRs and cytoplasmic proteins known as β-arrestins. β-arrestins are recruited to active GPCRs where they block association with heterotrimeric G-proteins. In addition to physically blocking the G-protein binding site, GPCR-associated β-arrestins promote the formation of endocytotic vesicles which facilitate internalization of the GPCR [39]. In heterologous systems, INI receptors appear to have an increased tendency to be internalized into an endosomal compartment compared to VSV receptors, and VGV receptors largely appear to accumulate on the cell surface. These findings mirror the effects of editing observed for G-protein-dependent signaling pathways. In the case of the $5HT_{2C}$ receptor, β-arrestin binding not only facilitates internalization, but also stimulates ERK1/2 phosphorylation in what appears to a calmodulin-dependent process[35, 38]. This ERK1/2 phosphorylation elicits changes in gene expression that affect cell physiology depending on biological context. Taken together, it appears that a primary role of RNA editing at $5HT_{2C}$ receptor transcripts is to tune the constitutive activity of receptors to meet physiological needs.

Physiological roles of $5HT_{2C}$ receptors

$5HT_{2C}$ receptors are expressed throughout the central nervous system and mRNA can be detected in many parts of the brain with highest levels of expression observed in the ependymal tissue lining the ventricles known as choroid plexus [40] [41]. Studies in rodents also confirm an important roles for $5HT_{2C}$ receptors in the arcuate nucleus of the hypothalamus in the regulation

of feeding behavior and metabolism [42, 43]. Several lines of work have also indicated that $5HT_{2C}$ receptors expressed in the midbrain regulate dopamine (DA) release in the limbic forebrain modulating goal-directed behavior [44]. 5HT-based drug intervention is at the forefront of the struggle to treat a wide array of psychiatric and metabolic disorders including schizophrenia, depression, anxiety, addiction, and obesity and $5HT_{2C}$ receptors have established roles in the transduction of 5HT signals relevant to these disease processes [45].

$5HT_{2C}$ receptors regulate metabolism and feeding behavior

Regulating metabolism and feeding behavior is one of the most ubiquitous and fundamental functions facilitated by the mammalian nervous system. The brain maintains metabolic homeostasis through regulation of physical activity, autonomic tone, glucose homeostasis, and by providing the motivational drive to consume food. The notion that 5HT directly regulates feeding behavior dates back to initial studies indicating that it was a satiety hormone [46]. A concept exploited by the pharmaceutical industry beginning in the late 1960's when the drug fenfluramine came on the market for weight loss. The anorexigenic effects of fenfluramine have been attributed to its ability to increase extracellular 5HT levels [47]. Studies in which knockout mice lacking $5HT_{2C}$ receptors are treated with fenfluramine provided strong evidence that the $5HT_{2C}$ receptor plays a primary role in mediating the inhibition of feeding. The anorexigenic efficacy of fenfluramine was greatly attenuated in these $5HT_{2C}$-null animals, whereas untreated knockout-mice are hyperphagic and developed adult onset obesity [48] [49]. More recently, electrophysiological and molecular techniques have allowed for a more precise characterization of the receptor's role in feeding. In mice, pro-opiomelanocortin (POMC) neurons located within the arcuate nucleus (Arc) of the hypothalamus express the $5HT_{2C}$ receptor and that receptor activation promotes excitability and increased release of α-melanocyte

stimulating hormone (αMSH) [50]. Accumulating evidence implicating the $5HT_{2C}$ receptor as a key component in the regulation of feeding behavior has made it an attractive target for new anti-obesity drugs. The drug Lorcaserin, a selective $5HT_{2C}$ agonist, was approved for the treatment of obesity in 2012 and is currently marketed by Arena Pharmaceuticals.

Significant metabolic alterations in the animals which only express the VGV receptor isoform suggest that the role of this receptor in regulating metabolism is more complicated than previously appreciated. These animals display poor feeding in infancy, mirrored with hyperphagia, which is offset by elevated brown fat thermogenesis (BAT) in adulthood [25, 51]. Consistent with the hypothesis that efficient excitatory drive in POMC neurons, facilitated by $5HT_{2C}$ receptor signaling, is required for normal inhibition of feeding, the adult onset hyperphagia observed in VGV mice is similar to that observed in the $5HT_{2C}$ null animals [25]. Currently, it is not clear if this hyperphagia is directly related to deficient activation of POMC neurons in the Arc, or some indirect feedback mechanism related to the increased basal metabolic rate resulting, at least in part, from enhanced BAT. These results provide strong evidence that alterations in RNA editing at $5HT_{2C}$ transcripts can fundamentally disrupt metabolic homeostasis in mice.

$5HT_{2C}$ receptors regulate mesolimbic dopamine signaling

Animals possess the unique ability to physically interact with their environment to promote their own survival and reproduction. Goal-directed behavior and adaptive learning have evolved in vertebrates to facilitate the need to efficiently respond to the nearly infinite possible circumstances with which an animal may find itself. Mechanistically, goal-directed behavior and adaptive learning requires that an animal can identify a goal, perceive relevant sensory stimuli, generate possible motor responses, and anticipate the relative utility of these responses for

attaining the goal. The ability to encode reward upon the execution of effective motor sequences allows animals to modify and perfect behavioral sequences to efficiently achieve goals. The mesolimbic system, which includes; the nucleus accumbens (NAc), olfactory tubercle, ventral pallidum, mediodorsal thalamus, ventral tegmental area (VTA), lateral hypothalamus, limbic cortex, and amygdala provides the neural substrate for reward-associated learning and motivated behavior [52]. DA release in the NAc and olfactory tubercle is thought to be a key component in goal-directed selection of motor programs and in the neural coding of reward. Dopamine is supplied to the ventral striatum primarily by groups of neurons located in the midbrain known collectively as the VTA, and its release in the medial shell of the NAc and medial olfactory tubercle directly correlates with the rewarding effects of achieving goals as well as the reinforcing effects of drugs of abuse [53].

5HT is supplied to the structures of the mesolimbic system by neurons in the dorsal raphe and plays a significant role in the regulation of NAc DA release [54]. $5HT_{2C}$ receptor transcripts can be detected by *in situ* hybridization within the VTA and throughout the NAc [55] and autoradiography and immunohistochemistry have confirmed that $5HT_{2C}$ receptors are expressed in these brain areas [56, 57]. Studies in which selective $5HT_{2C}$ receptor agonists, antagonists, and inverse agonists are administered while monitoring DA levels in NAc via microdialysis suggest that active $5HT_{2C}$ receptors have a net inhibitory effect on both baseline and drug-induced DA release in the ventral striatum. Specifically, intra-accumbal infusion of an inverse agonist, but not antagonist, increases basal DA efflux in the NAc. Furthermore, acute decreases in basal accumbal DA levels are elicited by systemic infusion of a $5HT_{2C}$ agonist, and these effects can be attenuated by intra VTA infusion of the 2C-selective antagonist SB 242084 [58]. These results suggest that phasic activation of $5HT_{2C}$ receptors in the VTA attenuates DA levels

in the limbic forebrain and that constitutive activity of receptors in the NAc provide tonic suppression of DA release. Further supporting an inhibitory role for $5HT_{2C}$ receptors on DA release are the observations that systemic administration of either an inverse agonist or agonist results in a dose-dependent increase or decrease in the firing rate of dopaminergic neurons in the VTA, respectively [54].

$5HT_{2C}$ activation is predicted to have excitatory effects on cells expressing the receptor leading to the hypothesis that $5HT_{2C}$ receptors in the NAc and VTA negatively regulate DA release by activating inhibitory GABAergic interneurons which, in turn, directly inhibit dopaminergic neuron activity. In support of this model, $5HT_{2C}$ immunoreactivity has been observed primarily in GABAergic cell bodies within the VTA and NAc. Recently, several studies have attempted to evaluate the effects of various serotonin receptor ligands on the acquisition of stimulant addiction and on reinstatement of drug seeking behavior after extinction. The inhibitory role of $5HT_{2C}$ receptor agonists on DA release led to the hypothesis that such drugs could be effective in treating addiction. Indeed, several studies have indicated that systemic administration of $5HT_{2C}$-selective agonists decreases the acute hyperlocomotive response to stimulants and reduces self-administration and reinstatement of cocaine seeking behavior [59]. All of the subcortical $5HT_{2C}$ receptor populations analyzed seem to provide a net inhibitory drive on NAc DA release, but the differential responses to inverse agonists and antagonists, suggests that different populations of receptors play distinct roles in responding to 5HT. A potential mechanism for these functional differences is the possibility that select cell types or nuclei may express different edited isoforms of the $5HT_{2C}$ receptor, fulfilling the need for variable levels of constitutive activity either promoting tonic or phasic control of DA release. The important implications of this hypothesis obviate a need to better understand the regulation

of RNA editing at $5HT_{2C}$ transcripts as well as a detailed analysis of receptor isoform profiles expressed in discrete cell types within these brain regions.

Measuring RNA editing of $5HT_{2C}$ transcripts

The $5HT_{2C}$ transcript is unique among A-to-I editing substrates because it contains multiple adenosines in close proximity, some of which are selectively deaminated by only one of the two ADAR enzymes. ADAR1 and ADAR2 recognize an extended duplex structure in $5HT_{2C}$ pre-mRNA that forms between the edited region of exon 5 and a complementary region within intron 6, referred to as the editing complementary sequence (ECS) [9]. Despite the fact that all five sites lie within the same duplex, it appears that intrinsic selectivity exists for which sites are deaminated by ADAR1 and ADAR2, respectively [16, 23, 60]. This is remarkable considering that the enzymes have a short temporal window to access the editing site before splicing occurs and the ECS is removed from the pre-mRNA transcript. The current model is that ADAR1 efficiently edits the A and B sites and ADAR2 edits the C and D sites. This is based on *in vitro* editing assays where cells are transfected with a $5HT_{2C}$ minigene and either ADAR1 or ADAR2, resulting in selective editing at ADAR1 and ADAR2 sites. Further evidence is derived from the observation that in ADAR1 knockout mice, editing at the A and B sites is entirely ablated. Interestingly, editing at ADAR2 sites are increased in these animals implying that competition for access to the $5HT_{2C}$ editing site may play an important role in regulating the extent of editing [23]. One interpretation of this result is that ADARs bind the duplex in a mutually exclusive manner and editing can be regulated by reciprocal competition for access to the substrate duplex. This model is further supported by results from experiments that examined editing of $5HT_{2C}$ transcripts in mutant mice with disrupted autoregulation of ADAR2 expression. The ADAR2 pre-mRNA contains a conserved A-to-I editing site referred to as the -1 site. When this site is

edited it generates a non-canonical splice acceptor that leads to a frame shift resulting in a truncated protein lacking dsRNA binding and deaminase activity. This is presumed to serve as a negative autoregulatory feedback mechanism limiting the abundance of functional ADAR2-encoding transcripts. Mutant mice that cannot edit at the -1 site lose ADAR2 autoregulation resulting in a subsequent increase in ADAR2 protein. These mice edit more efficiently at the C and D sites and edit less efficiently at the A and B sites further supporting the model that competition exists between ADAR1 and ADAR2 for access to the editing site duplex[8]. Interestingly, transgenic mice which over express the most active form of ADAR2, driven by a strong, human cytomegalovirus promoter do not show alterations in editing at any of the five $5HT_{2C}$ sites [61]. This could result from insufficient increases in ADAR2 expression to affect the competition ratio at the editing site or from a deficiency in some unidentified RNA processing event required for appropriate expression and trafficking of the ADAR2 protein that was missing from the transgene. As an initial step to better understand the regulation of A-to-I editing in $5HT_{2C}$ transcripts, it was necessary to accurately characterize editing profiles at distinct developmental time points, in different tissues and in different mouse strains.

Most approaches for quantifying RNA editing rely on the fact that reverse transcriptase recognizes inosine as guanosine, incorporating cytosine in the newly synthesized complementary strand. In some cases, reverse transcription of RNA followed by primer extension designed to discriminate T from C in the cDNA is useful for accurately measuring the extent of editing in an RNA sample. However this approach faces several drawbacks including poor sensitivity for low abundance RNAs. Furthermore, it can only interrogate a single site at a time which is not ideal for substrates like the $5HT_{2C}$ transcript which contain multiple editing sites in close proximity to one another. These problems obviated the need to develop different approaches for quantifying

$5HT_{2C}$ editing profiles. The initial solution was to amplify the cDNA using PCR primers flanking the editing region and 'shotgun' subclone the resulting PCR amplicons into plasmids before transforming into E. *coli*. Bacterial clones harboring plasmids containing a single amplicon, corresponding to a single edited RNA species could be analyzed subsequently by conventional Sanger sequencing. By analyzing a large number of colonies an approximation could be made of the relative abundance of different edited isoforms [9]. This approach has the advantage of providing information about the combinations of editing that occur in $5HT_{2C}$ transcripts, as well as providing insights into how editing at each site might be related and which protein isoforms are encoded. The major problem with this method however, is that a very large number of clones must be analyzed to accurately assess the $5HT_{2C}$ editing profile. Despite this limitation, a number of groups proceeded to analyze editing in rodent and human tissues generating a variety of hypotheses relating editing at $5HT_{2C}$ transcripts to altered functions and disease states.

The most compelling early studies were related to tissue-specific patterns of editing in the brain. The editing profile in most brain regions appeared to be similar, such that transcripts encoding the VNV isoform made up close to 50% of the transcript population in mice while those encoding the VSV isoform predominate in human brain. Another universal finding was that while $5HT_{2C}$ receptors are highly expressed in the choroid plexus, editing at ADAR1 sites is essentially undetectable, owing to the undetectable levels of ADAR1 in this tissue. Interestingly, editing at the ADAR2 sites are not increased suggesting that the extent of ADAR2 editing at $5HT_{2C}$ transcripts is not exclusively regulated by competition with ADAR1in all cellular contexts [51].

5HT signaling pathways are implicated widely in the etiology of behavioral and psychiatric disorders and several 5HT receptors, including the $5HT_{2C}$ receptor, are thought to be important targets for pharmacological intervention. In humans, antipsychotic drugs used in the treatment of schizoaffective disorders interact with $5HT_{2C}$ receptors [62] and the $5HT_{2C}$ transcript is detected in several brain regions thought to be involved in psychiatric impairments associated with depression, anxiety disorders, and schizophrenia [40]. For these reasons, several groups have examined whether changes in $5HT_{2C}$ editing profiles are dynamic and could play a role psychiatric dysfunction. Initial hypotheses regarding $5HT_{2C}$ editing suggested that it might be altered in various pathological states. This stemmed from reports of altered editing efficiency at one or more sites for $5HT_{2C}$ transcripts isolated from depressed suicide victims [63]. Attempts to model these changes in mice also initially showed promise, yet as additional laboratories attempted to reproduce the findings of these studies, it became apparent that better tools for quantitative analysis of editing profiles would be required. The data provided in early studies was likely to be unreliable due to the fact that they were based on the analysis of an inadequate number of recombinant cDNA clones[30]. These studies reported editing profiles from inbred mouse strains which were inconsistent with those observed by our lab and others [51] as a result of statistical undersampling.

Several reports have provided evidence for alterations in editing within human disease cohorts and in rodents exposed to various pharmacologic and behavioral paradigms, however these reports have failed to produce consistent results and no reproducible alterations in editing have been associated with any particular disease cohort [30]. This is likely due to several factors including inconsistency in the precise brain regions analyzed, inherent diversity in human populations, and technical limitations that have made it difficult to accurately determine the

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profile of edited $5HT_{2C}$ transcript isoforms in a tissue sample. Recently, advances in sequencing technology have made it possible to accurately determine the editing profile for $5HT_{2C}$ transcripts in a tissue sample with unprecedented accuracy and reproducibility [51]. This new approach, based on the latest generation of massively parallel sequencing technology, promises to renew efforts towards understanding the role of A-to-I editing for numerous RNA species in the nervous system.

A recent study has taken advantage of more precise methodologies to quantify editing profiles in C57Bl/6 mice exposed to antidepressants and antipsychotic drugs [64]. This study reported a remarkably consistent reduction in editing at the A and B sites in the hippocampus in response to the serotonin-selective reuptake inhibitor (SSRI; fluoxetine) and a tricyclic antidepressant (amitriptyline) compared to control animals treated with saline. Interesting, these studies also included an experiment which indicated that the $5HT_{2C}$ editing profile of normal facility-reared C57Bl/6 mice that had never been exposed to any saline injection regimen had an editing profile that was nearly identical to the drug treatment groups [64]. This finding suggests that the drug treatment was preventing a change in editing that may have resulted from the stress associated with daily handling and saline injections.

Chapter II

INVESTIGATING THE DYNAMICS OF 5HT$_{2C}$ RNA EDITING PROFILES IN MICE

Studying RNA editing for 5HT$_{2C}$ transcripts presents a unique opportunity to examine feedback mechanisms that might lead to dynamic changes in A-to-I conversion. The murine 5HT system is accessible to manipulation by well characterized pharmacologic and genetic tools. We took advantage of some of these tools to test the hypothesis that perturbations in 5HT signaling can cause changes in 5HT$_{2C}$ editing profile. In addition to taking advantage of mouse models in which components of 5HT circuitry are manipulated, we also took advantage of stress paradigms which indirectly affect a variety of CNS circuits including those involving 5HT. However, the first step required to study 5HT$_{2C}$ editing was to establish a sensitive and precise method to measure RNA editing profiles. First, I will describe the approach we adopted to create an accurate, cost and labor efficient method for measuring editing profiles. This approach takes advantage of the passively parallel Genome Analyzer sequencing technology developed by Illumina (San Diego, CA). We validated this approach and used it to test the hypothesis that 5HT$_{2C}$ editing is malleable in response to pharmacologic, genetic, or physiological manipulation.

Materials and Methods

RNA analysis

RNA Extraction

Tissue was dissected and mechanically homogenized in TRI reagent (Sigma-Aldrich) using a Polytron homogenizer (Brinkman). RNA was isolated from the TRI reagent mixture according to manufacturer's instructions and stored at -80°C until further analysis.

High Throughput Multiplexed Transcript Analysis (HTMTA)

To characterize accurately the profile of edited 5HT$_{2C}$ isoforms present in a tissue sample it would be ideal to sequence every RNA species in the sample, thereby directly observing the distribution of edited 5HT$_{2C}$ RNA isoforms. As this is not a feasible approach, due to technological limitations, we indirectly quantified editing by sequencing cDNAs generated from 5HT$_{2C}$ transcripts. RNA editing profiles were determined by high-throughput sequence analysis (Figure 2) as previously described using random primers and avian myoblastosis virus reverse transcriptase (Promega) for initial cDNA synthesis followed by five cycles of PCR amplification with Phusion high-fidelity DNA polymerase (New England Biolabs) and 5HT$_{2C}$-specific primers containing either T3 or T7 RNA polymerase promoter extensions for the sense (5'-ATTAACCCTCACTAAAGGGAGCTGGACCGGTATGTAGCA-3') and antisense (5'-TAAT-ACGACTCACTATAGGGGGATACGAACTGATACACCTATAG-3') primers, respectively. Unincorporated single-stranded primers were digested with Exonuclease I (New England Bio-labs) and a second round of amplification (25 cycles) was performed with a common antisense primer (5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT<u>TAATACGACTCACTA-</u>

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TAGGG-3') matching the 20 nucleotide (nt) T7 RNA polymerase promoter (underlined) with a 34-nt adapter extension that can anneal to the Illumina flowcell surface (Adapter B) and one of twenty-four sense primers (5'-AATGATACGGCGACCACCGAGACTACACTC—TT—C-CTACACGACGCTCTTCCGATCTNNNNNNNATTAACCCTCACTAAAGGGA-3') matching the 20 nt T3 RNA polymerase promoter (underlined) containing both a 6-nt index sequence (NNNNNN) for sample identification and a 58-nt adapter extension (Adapter A) for high-throughput Illumina sequencing (Bentley, 2008). PCR amplicons (258 bp) were resolved on a 2% agarose gel, purified from excised gel slices using the Wizard SV Gel and PCR Purification Kit (Promega, Madison, WI) and mixed with up to 24 other products containing unique barcodes before single-end sequencing (100 bp) using the Illumina Hi-seq 2000 system (Illumina, San Diego, CA). Sequence reads were aligned to one of thirty-two $5HT_{2C}$ reference sequences (5'-NNNNNNATTAACCCTCACTAAAGGGAGCTGGACCGGTATGTAGCA**R**TRCGT**RR**TCC-T**R**TTGAGCATAGCCGG-3') where the five editing site(s) were represented by a purine (R) and only those sequences in which the index sequence was identifiable and the sequence reads precisely matched the reference sequences were included for further analysis. The relative expression of each edited $5HT_{2C}$ isoform was calculated as the percentage of reads matching a specific, edited isoform divided by the total number of $5HT_{2C}$ reads for all isoforms.

Quantitative Real-time PCR

Gene expression was analyzed using Taqman Probe-based real time PCR (Applied Biosystems). All experiments used a 6-carboxy-2',4,4',5',7,7'– hexachlorofluorescein (HEX)-labeled 18S rRNA probe and primer sets for an internal standard (4319413E). For analysis of $5HT_{2C}$ expression, a 6-carboxyfluorescein (FAM)-labeled probe and primer set (Mm00434127_m1)

which detected sequences common to all three $5HT_{2C}$ splice variants located near the 5'-end of the mature $5HT_{2C}$ mRNA transcript was employed. Factor V (F5) expression was used as a proxy measure for the extent of choroid plexus contamination in each RNA sample. F5 was detected using a FAM-labeled Taqman probe and primer set (Mm00484202_m1). For quantitative (q)RT-PCR, first strand cDNA was synthesized with random primers using the High-Capacity Reverse Transcription Kit and expression was analyzed according to manufacturer's instructions for Taqman real-time PCR (Applied Biosystems) on a CFX96 platform (Biorad, Hercules, CA) . Amplification efficiencies were determined by PCR miner algorithm [65] and data was analyzed by CFX manager (Biorad, Hercules, CA).

Drug Treatment

Mice received daily intraperitoneal injections of either 100 mg/kg para-chlorophenylalanine (pCPA) dissolved in sterile saline or vehicle alone for seven consecutive days at 8:30am. On the seventh day, the mice were decapitated and brain hemispheres were separated for chemical analysis or RNA extraction. A separate cohort of mice received daily intraperitoneal injections of either fluoxetine, amitriptyline, olanzapine, or vehicle for 28 consecutive days, as described [64].

Stress protocols

Restraint Stress

Physical stressors cause changes that directly affect physiology and behavior. To test the hypothesis that stress might affect RNA editing at $5HT_{2C}$ transcripts we used the well-validated stress model of tube restraint for 30 minutes [66]. All mice were either facility-reared or acquired after adrenalectomy from The Jackson Laboratory (Bar Harbor, ME). Beginning at 6-8 weeks of

age, the mice were subjected to 30 minutes of daily restraint in 50 ml conical tubes which had been modified to include holes for adequate ventilation during the restraint procedure. Tube restraint was performed daily at 8:30 am for durations ranging from 2 to 28 days, depending on the experiment.

Maternal Separation

To mimic early-life stress in humans, we utilized the maternal separation (MS) paradigm, an established animal model of vulnerability to development of enhanced stress responsiveness in adulthood [67-70]. Maternal separation began on postnatal day 1 (P1) and occurred daily for 8 hours until P14. C57BL/6J males and females were mated randomly at 8 weeks of age and males were removed from the cage following overnight pairings so that females were housed singly throughout pregnancy and parturition. Maternal separation consisted of first removing the dam, followed by the pups, from the home cage and placing them into separate clean cages with clean bedding. During MS, pups were kept in rodent incubators maintained at 30°C to preserve their nesting temperature. Control litters were left completely undisturbed from birth (P0)-P14. Animals were euthanized at P15 by cervical dislocation under anesthesia and specific brain regions were dissected (hippocampus, striatum, cortex and hypothalamus).

Brain Dissection

All dissections were performed as follows, unless otherwise noted. Brains were removed from the skull and placed on a cool dental wax surface. First the hypothalamus was identified by visual inspection and removed from the ventral surface of the brain for processing. Next the brain was sliced in the coronal plane at the anterior commissure using a sharp razor blade to separate the forebrain from the midbrain, while keeping the striatum and hippocampus intact.

The cortex was then removed from the caudal half exposing the hippocampus, which was removed for further processing. Next the striatum and frontal cortex were removed from the rostral portion of the brain for processing and the remainder of the brain tissue was discarded, unless otherwise noted.

Statistics

For standard statistical analysis, Student's T-test was used for all pairwise comparisons. Analysis of variance (ANOVA) was used for comparison of multiple groups and the Bonferroni post-hoc test was used for comparison of individual groups.
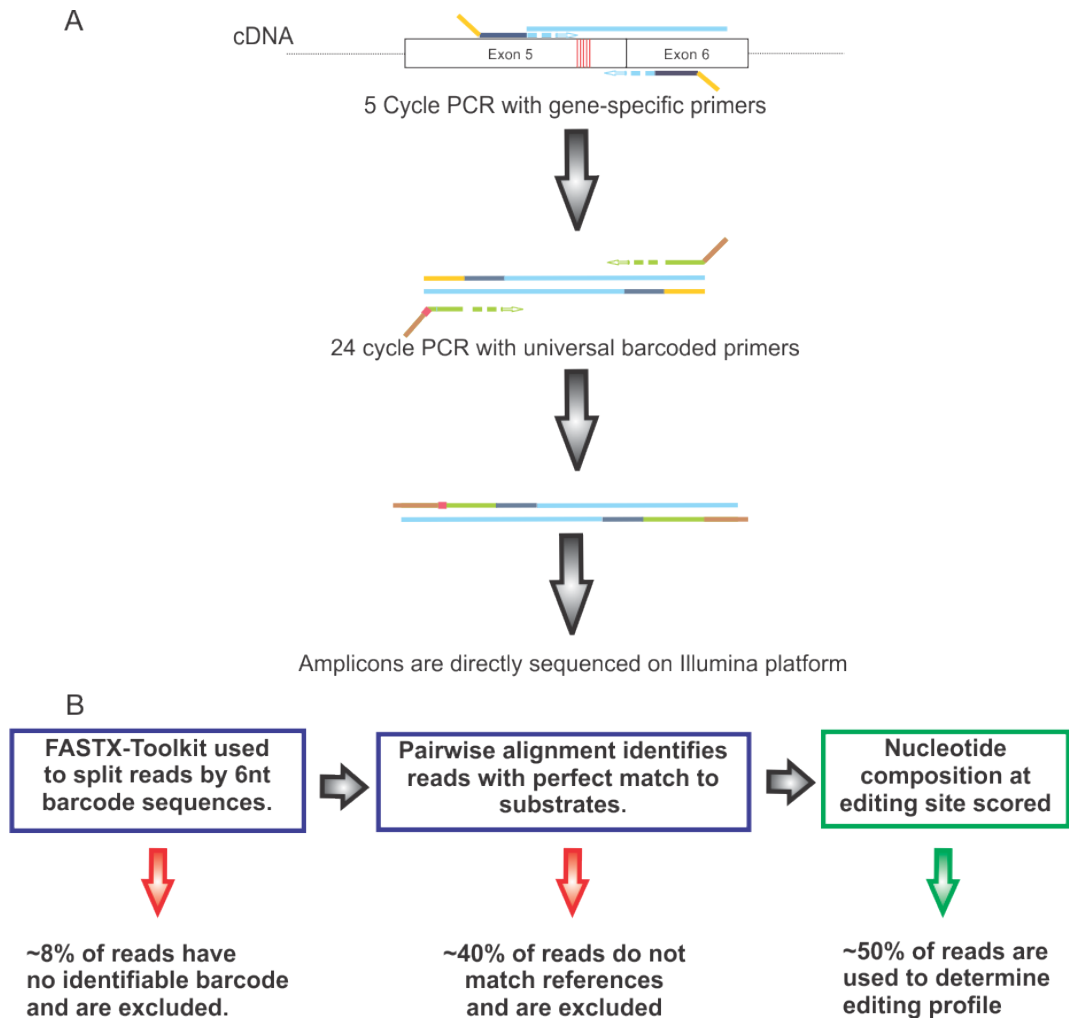
Figure 2. Methodology for quantifying RNA editing profiles using massively parallel sequencing. A) Summary of the high-throughput multiplex transcript analysis method used to determine the profile of edited transcript variants in an RNA sample. First, cDNA is synthesized and subjected to 5 cycles of PCR amplification with primers designed to amplify the transcript of interest. These primers contain 5' tails with T3 (sense) and T7 (antisense) promoter consensus sequences. Then, a second PCR reaction is performed using "universal barcoded primers" primers targeted to these 5' tail sequences. This set of primers contain 5' tail sequences that allow for sequencing on the Illumina Genome Analyzer platform as well as unique 5 nucleotide "barcode" identifier sequences that will be used for disambiguation of sequence data. **B)** Summary of the informatics protocol used in analysis of sequence data.

Results

Measuring RNA editing using high-throughput multiplexed transcript analysis (HTMTA)

Accurately measuring the profile of edited $5HT_{2C}$ transcript variants in a sample was historically difficult because the number of possible combinations of editing required extensive sampling of transcript sequences. PCR amplification is useful for generating a representative pool of DNA that can be directly bulk sequenced by the Sanger method. However, this sequencing method only provides a proximal representation of the extent of inosine conversion at each respective site and provides no information about the combinations of editing that might occur within individual transcripts. A method for simultaneous detection of editing status at all five sites is necessary for investigating linkage between editing at particular sites as well as to accurately predict the protein isoforms that are encoded. This was accomplished by 'shotgun subcloning' representative $5HT_{2C}$ cDNA pools into plasmid vectors that could then be grown in bacteria and directly sequenced. To estimate the distribution of different isoforms with the precision required to systematically study the RNA editing dynamics, a large number of clones had to be sequenced. Circumventing the need to subclone, pick, and sequence individual bacterial colonies we developed a method which takes advantage of parallel short read 'deep-sequencing' technology available on the Illumina Genome Analyzer platform. This technology allows for direct sequencing of any DNA fragment containing appropriate adapter sequences which facilitate application of DNA to the sequencing instrument. We originally described this targeted high-throughput multiplexed transcript analysis (HTMTA) process in 2010 [24]. Since then, additional multiplexing tools and protocol modifications have been made to make the technique more versatile and cost-efficient as described in the Materials and Methods. Once we established a protocol for amplifying and sequencing $5HT_{2C}$ transcripts on the Illumina platform

we set out to verify that this approach was both valid and reproducible. In order to do this we had to verify that our methods did not introduce any bias to the editing profile, establish the expected error rate, and demonstrate reproducibility. The following results provide strong evidence that HTMTA is indeed a useful and accurate way to make precise estimates about the editing profile of transcripts in an RNA sample.

Amplification Bias

Non-biased PCR requires that all potentially limiting reaction components are in excess to the template being amplified at every cycle of amplification. To verify that no component of the PCR that we use to generate the sequencing library was limiting and thus contributing to biased amplification we had to establish that the protocol is performed within the linear range of PCR amplification. No differences in editing profile were observed when a cDNA sample was subjected to either 19, 22, 25, 28, 31, or 34 cycles suggesting that the 28 cycles used in all subsequent experiments was well within the linear amplification range.

Error rate

The next step for validating this technique was to establish the error rate generated by polymerase error, sequencing errors, and RNA sampling. To determine the false discovery rate of our protocol we amplified the editing site from a single recombinant $5HT_{2C}$-INI clone using the HTMTA primers and sequenced the resulting amplicon. We observed a "non-A" base call at the editing sites with a frequency of 0.1%, indicating that our "error rate" for detecting editing events at each respective site is 0.1% and that our error rate for identifying predicted coding isoforms is 0.25%.
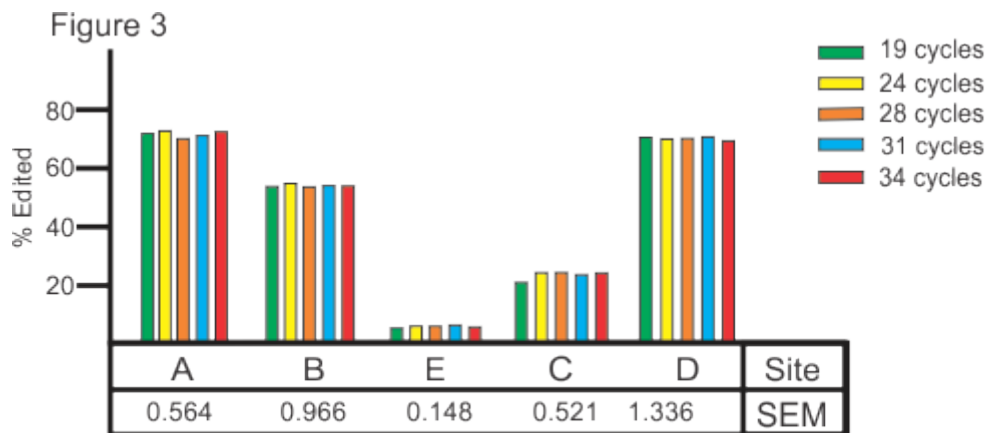
Figure 3: Determining linear range of PCR. Extent of editing detected at each site in an RNA sample extracted from a single 129S6 mouse brain as determined using a range of PCR cycle numbers from 19-24. Results indicate that all cycle protocols are within the linear range of PCR amplification.

Table 2: Error rate for HTMTA

Sample	Number of sequence reads	% Edited at each site				
		A	B	E	C	D
C57 #4	290,625	80.01	66.37	4.18	23.92	62.72
C57 #4	461,251	79.59	68.62	3.86	24.49	62.86
C57 #4	345,642	78.84	67.48	3.97	23.32	63.32
Mean		79.48	68.16	4.00	23.91	62.98
SEM		.341	.347	.092	.336	.152
INI Clone	1,610,268	.094	.041	.041	.028	.036
INI Clone	937,908	.097	.047	.045	.030	.041
INI Clone	1,438,506	.099	.047	.046	.027	.040
Mean		.096	.044	.044	.028	.039
SEM		.001	.002	.001	.001	.002

To assess the reproducibility and RNA sampling sufficiency, the HTMTA protocol was repeated three times using a single RNA sample generated from C57Bl/6 whole brain. The mean percentage of editing at each site (± SEM) observed in these studies was: A site= 79.48 ± 0.34; B site= 68.16 ± 0.35; E site= 4.01 ± 0.09; C site= 23.91 ± 0.34; and D site= 62.98 ± 0.15 (Figure 3 and Table 2). These results indicate that the extent of technical reproducibility is similar to the absolute error rate, providing strong evidence that the HTMTA paradigm is a reliable tool for measuring edited isoform profiles.

Establishing Base Line 5HT$_{2C}$ editing profiles

Previous efforts to establish the edited 5HT$_{2C}$ isoform profile in the brain yielded inconsistent results suggesting that a wide variability within brain regions and species may exist, even between littermates. One interpretation of these results is that sensitive and dynamic feedback systems regulate the expression of precise editing profiles in different physiological contexts. Conversely, it could also indicate that editing is a largely stochastic event and fitness is not greatly influenced by tight control of 5HT$_{2C}$ editing patterns. With a tool developed that allows efficient and accurate quantification of editing profiles, we began to interrogate 5HT$_{2C}$ editing patterns in a more systematic manner.

Reproducibility and inter-animal variability

As noted above, previous attempts to characterize 5HT$_{2C}$ editing profile in littermates yielded variable results, indicating high inter-animal variability. It was important to confirm this finding with the more accurate HTMTA approach to establish a baseline expectation for variability in whole brain editing profiles. We used five samples prepared from total RNA
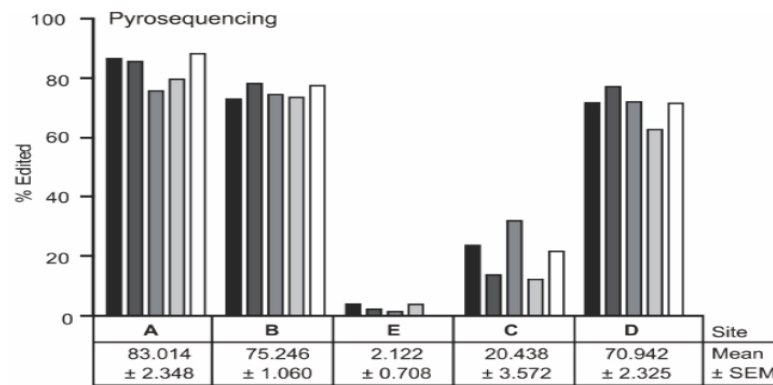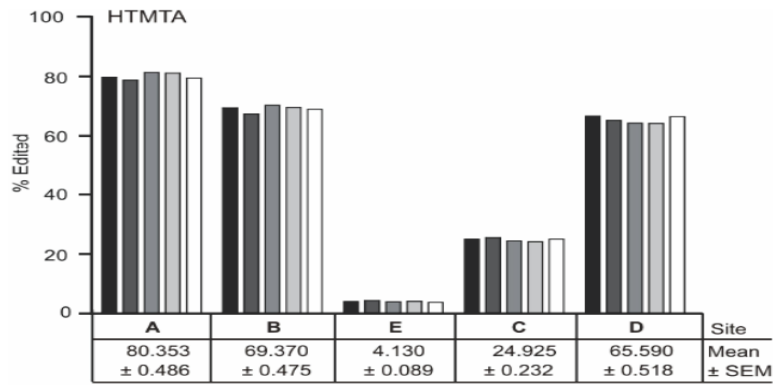
Figure 4. Comparison of RNA editing patterns using HTMTA and pyrosequencing methodologies. The extent of editing in five different whole brain RNA samples from male C57Bl/6 littermates was determined using HTMTA (*top*) or the pyrosequencing of 100 cDNA clones (*bottom*).

Isolated from the whole brains of 6 week old, male C57Bl/6 mice. Each RNA sample was analyzed by the traditional method of subcloning RT-PCR amplicons and pyrosequencing ~100 clones for each mouse. The editing profile of each sample was then quantified by the HTMTA method, which effectively sequences millions of clones. The results of the pyrosequencing approach, which only examined 100 clones per RNA sample, indicated significant inter-animal variability. The standard errors are 3% of the mean at the A site, 3% of the mean at the C site, 33% of the mean at the E site, 18% of the mean at the C site and 3% of the mean at the D site (Figure 4). However, when the same samples were analyzed by HTMTA, the results showed essentially no inter-animal variability with the highest standard error at the C site being 0.9% of the mean; the remaining sites had variability that was less than the 0.1% of the predicted error rate of the assay (Figure 4). These results indicated that whole brain editing profiles were actually remarkably similar in each C57Bl/6 mouse brain samples providing evidence for tight regulation of A-to-I editing for $5HT_{2C}$ transcripts.

Editing in inbred mice

Isogenic mouse lines have been generated by decades of commercial and academic facility breading. They are valuable tools for studying the influence of genes on physiology because they contain only one allele (albeit two copies) of every gene. This means each strain of mouse is essentially a genetically identical clone of its siblings and parents. In some cases, this has allowed for the identification of specific genes which influence or dictate relevant phenotypes. To examine potential strain-specific differences in $5HT_{2C}$ editing, we decided to look at whole brain editing profiles of several inbred mouse lines to see if different editing profiles correlated with any particular mouse strain. RNA extracts were prepared from the brains of normal facility-reared C57Bl/6J, Balb/cJ, DBA/2J, and 129S6 mice and $5HT_{2C}$ editing profiles were
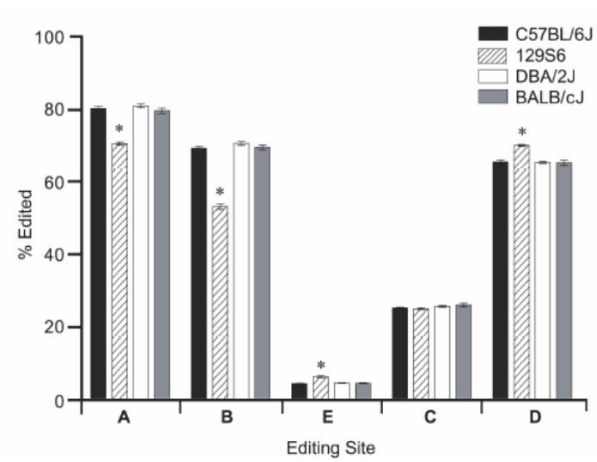
Figure 5. Analysis of 5HT$_{2C}$ mRNA profiles of different inbred mouse strains. HTMTA analysis of site-specific editing patterns from whole brain RNA isolated from C57Bl/6J, 129S6, DBA/2J, and BALB/cJ mouse strains (2-way ANOVA n=5, *p<.0001). Error bars indicate SEM.
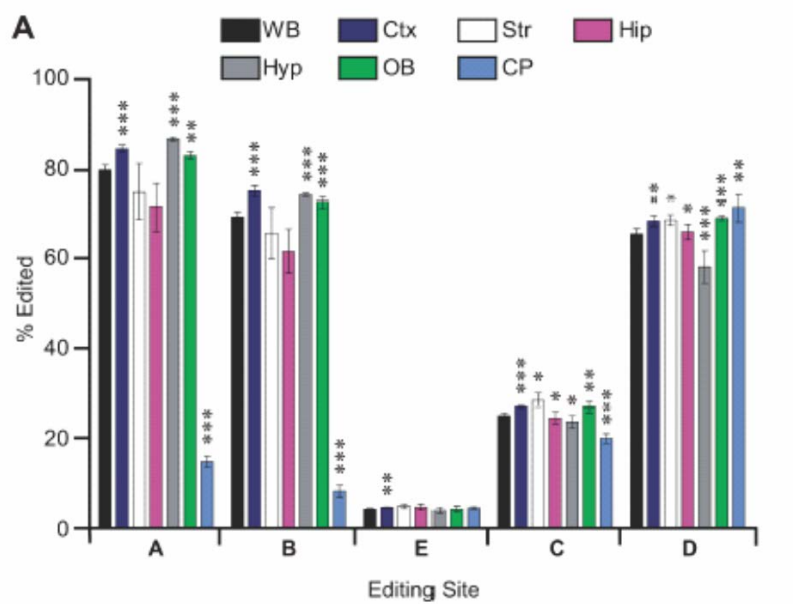
Figure 6. Analysis of brain region-specific 5HT$_{2C}$ RNA editing profiles in C57Bl/6J mice. 5HT$_{2C}$ editing profiles were determined by HTMTA from RNA isolated from whole brain (WB) and dissected brain regions including cortex (Ctx), striatum (Str), hippocampus (Hip), hypothalamus (Hyp), olfactory bulb (OB) and choroid plexus (CP) (means compared to whole brain editing profile by t-test; n$\geq$4, $*$p<0.05,$**$p<0.01, $***$p<0.001). Errors indicate SEM.

analyzed by HTMTA. No differences were observed between C57Bl/6J, Balb/cJ, and DBA/2J mice indicating that none of these mice harbored alleles that led to differences in whole brain $5HT_{2C}$ editing profiles. Significantly decreased editing at the A and B sites and increased D site editing was observed for 129S6 mice compared to the other three strains (Figure 5). It should be noted however, that this effect may reflect changes which are manifest in selective brain regions and characterization of editing profiles in discrete regions for each of these mouse strains may be needed for a better understanding of these inter-strain differences.

Editing profiles in various mouse brain regions

The previously described studies provided us with a new understanding concerning the relative inter-animal consistency for $5HT_{2C}$ editing profiles. To capitalize on this consistency, paired with the accuracy and reproducibility of this HTMTA paradigm, we focused upon quantification of editing profiles from more discrete brain regions in at least one of these isogenic mouse lines. For these experiments we dissected olfactory bulb, hypothalamus, hippocampus, striatum, frontal cortex, and choroid plexus from C57Bl/6J mice and compared the editing profiles to those observed in whole brain RNA extracts (Figure 6). While we observed slight differences in the relative levels of edited $5HT_{2C}$ isoforms in dissected brain regions from those observed in whole brain, the greatest differences were observed in RNA samples isolated from the choroid plexus. This ependymal tissue, which lines the ventricles, participates in cerebral spinal fluid (CSF) production and expresses the greatest levels of $5HT_{2C}$ mRNAs compared to surrounding neural tissues [41]. Importantly, ADAR1 expression is undetectable in choroid plexus by *in situ* hybridization [41, 71], which is reflected in a near complete deficiency of editing observed at the A and B sites. Likewise, slight elevations in the editing

efficiency are observed at the D site supporting the ADAR competition model for modulating site-selective editing efficiency. These findings provide a useful baseline for interrogating the potential dynamics of RNA editing for $5HT_{2C}$ receptor transcripts in response to physiologic, pharmacologic, and genetic manipulations.

Editing in animals with experimentally-manipulated 5HT systems

Measuring editing in the face of genetic manipulations of the 5HT system

Genetic manipulation of the 5HT system can be achieved by altering expression of enzymes, receptors, or transcription factors regulating different components of 5HT biosynthesis and signal transduction. Chronic manipulations of this kind can be expected to result in a variety of compensatory changes in circuit dynamics, gene expression and morphology. Genetic models with altered 5HT systems represent valuable experimental approaches for testing hypotheses relating to $5HT_{2C}$ RNA editing and associated feedback mechanisms. One possibility is that editing can be reduced in conditions of 5HT deficiency to provide compensatory signal tone in the form of receptor isoforms with increased constitutive activity. We tested this hypothesis in genetic models with ablated 5HT systems or altered 5HT uptake kinetics.

The most extreme case of central 5HT-deficiency is the Pet1-null mouse. This mouse was generated by targeted knockout of the gene encoding the Fev transcription factor (Pet1) [72]. This transcription factor is required for development of 5HT neurons; as such, neither serotonergic neurons nor 5HT is detected in the adult brain of these mice. We examined the $5HT_{2C}$ editing profiles in hippocampus and whole brain RNA samples from Pet1+/+, Pet1+/- , and Pet1-/- mice created on a 129sv background and observed no differences in editing for any of these groups (Figure 7). These results indicate that in the face of lifelong absence of input

Editing in 5HT mutants
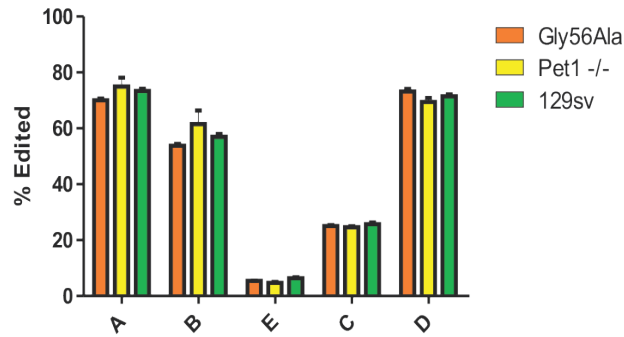
Figure 7. Whole brain editing profiles from animals with genetically-modified 5HT systems. HTMTA was used to determine $5HT_{2C}$ editing profiles in RNA isolated from whole brains of mice homozygous for a hyperactive serotonin transporter variant (Gly56Ala) and mice engineered to be homozygous null for the Fev1 transcription factor (Pet1-/-). A t-test revealed no significant differences in editing between the mutants and wild type mice at any of the five editing sites (n>3).Error bars indicate SEM.

from 5HT neurons, no alterations in editing are elicited, suggesting that $5HT_{2C}$ editing is not likely to be regulated by factors released by serotonergic neurons. However, interpretation of knockout studies can be complicated by the induction of compensatory mechanisms. To address this hypothesis further we sought a more nuanced model of 5HT signaling dysfunction in an attempt to reveal a role for 5HT signaling in the regulation of RNA editing.

We took advantage of a mouse model harboring a much less severe manipulation of the 5HT system to test the hypothesis that synaptic 5HT levels influence the expression of edited $5HT_{2C}$ isoforms. A serotonin transporter (SERT) variant identified in human autism patients, Gly56Ala, was knocked into the endogenous locus of 129S6 mice by hologous recombination in embryonic stem cells. These mice are viable and display normal growth patterns, but harbor a hypermorphic SERT allele which is predicted to clear 5HT more rapidly from the synapse [73]. These mutant animals provide an opportunity to analyze the effect of dysregulated synaptic 5HT rather than an absence of central 5HT. We compared whole brain editing profiles from mutant SERT Gly56Ala mice to those of wild-type animals, once again revealing no significant changes in editing. These experiments do not support the idea that $5HT_{2C}$ editing may represent a homeostatic mechanism whereby $5HT_{2C}$ receptor signaling is stabilized in the face of changing synaptic serotonergic input.

$5HT_{2C}$ editing after pharmacologic depletion of serotonin

In light of these findings, we thought it was important to examine whether acute depletion of 5HT could affect $5HT_{2C}$ editing profiles and circumvent potential compensatory mechanisms that may result from chronic 5HT dysregulation. For these studies, we injected C57Bl/6J mice with p-chlorophenylalanine, an irreversible inhibitor of tryptophan hydroxylase
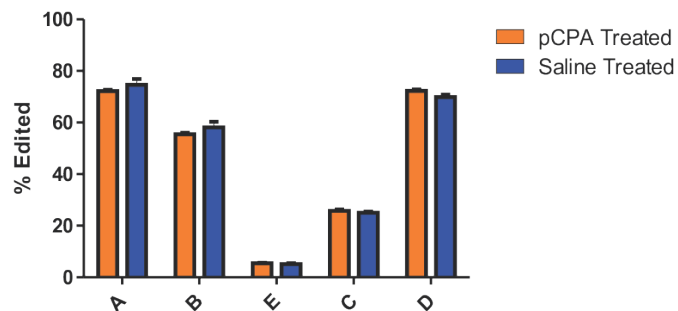
Figure 8. Quantitative analysis of 5HT$_{2C}$ RNA editing after 5HT depletion. Animals injected with pCPA or vehicle for 7 consecutive days were sacrificed and RNA was extracted from one cerebral hemisphere, while the other was subjected to HPLC analysis to determine tissue 5HT levels. Student's T-test revealed no significant differences between treatment groups (n=5). Error bars indicate SEM.

(TH), the rate limiting enzyme for 5HT synthesis [74]. After seven consecutive days of injections, whole brains were removed and the hemispheres separated. One hemisphere was used for HPLC analysis to determine the extent of 5HT depletion, while the other was processed for RNA editing analysis by HTMTA. Despite ~80% reduction in 5HT levels after drug treatment, no alterations in 5HT editing profiles were observed. These results further support the idea that if some active process is maintaining editing profiles in the narrow ranges observed for isogenic mice, it appears unlikely to require either serotonin or serotonergic neurons. Although these studies do not rule out the possibility that serotonergic tone might actively influence editing in discrete nuclei, further characterization of editing profiles in specific cell types and brain regions will be required to address such details of editing dynamics.

5HT$_{2C}$ editing in mice after exposure to common psychiatric medications

5HT$_{2C}$ receptors are thought to be important targets of antipsychotic, and antidepressant drugs which may act by altering 5HT signaling [62]. Recent studies have suggested that mice treated with these agents can affect 5HT$_{2C}$ editing profiles based upon analyses using a high-throughput sequencing strategy similar to that described in Figure 2[64]. The authors reported that when the edited isoform profiles were characterized in RNA samples isolated from the hippocampus of antidepressant-treated animals, a significant increase in A and B site editing was observed. This finding was based on the fact that the average extent of editing at the A and B sites was much higher in antidepressant-treated mice than control animals injected with vehicle (saline) alone. Upon careful examination of this observation, it became clear that the extent of A and B site editing in the hippocampus of saline-treated animals was actually much lower than untreated mice. In fact, the levels of editing reported for antidepressant treated mice in this study
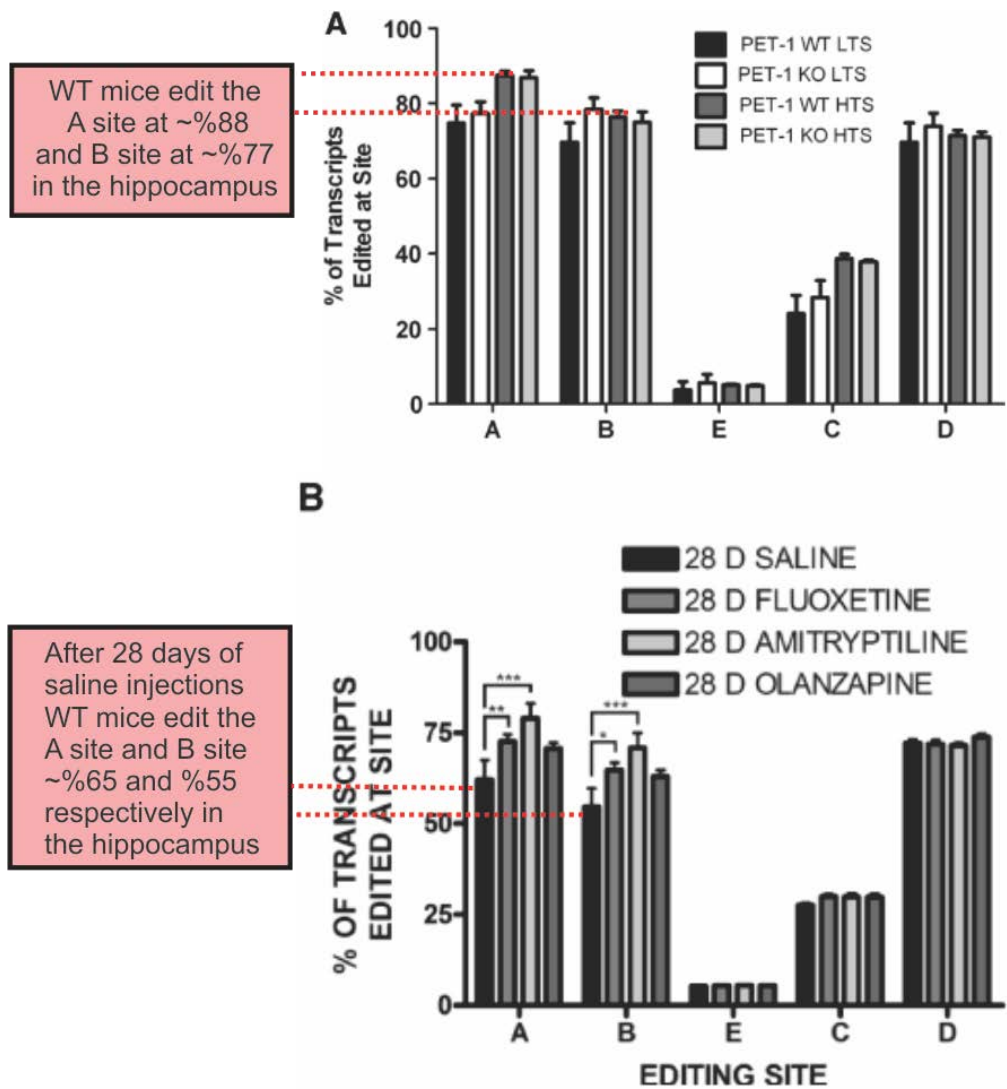
Figure 9. Effect of 28 day saline injection on hippocampus editing. A) Hippocampal editing profile of normal facility-reared wild-type mice as determined by high-throughput sequencing of RT-PCR amplicons. **B)** Hippocampal editing profile of mice treated with 28 consecutive days of saline show much lower editing at the A and B sites. The magnitude of this reduction is tempered when the saline injection also contains fluoxetine or amitriptyline, suggesting that the stress from daily handling and saline injection may modulate editing at the A and B sites and that this effect could be blocked by two classes of antidepressant drugs (adapted from Abbas, *et al* 2010 [63]).

were nearly identical to the levels displayed by untreated C57Bl/6 mice in our laboratory (*data not shown*). One explanation for this finding is that the antidepressants were having a protective effect on reduced editing of the A and B sites that was elicited by the stress from daily handling and saline injection. Indeed, handling and saline injections can potently activate the hypothalamic-pitutitary-adrenal (HPA) axis in mice [75] and these findings parallel extensive literature providing evidence that antidepressants promote resilience in the face of stress [76]. This paradigm provided an exciting avenue for testing the hypothesis that stress could modulate $5HT_{2C}$ editing profiles in the mouse brain and that antidepressant medications could work to restore $5HT_{2C}$ editing profiles to the unstressed state.

Effects of stress on $5HT_{2C}$ editing

The initial findings outlined above suggested that stress from daily handling and intraperitoneal injection could reduce $5HT_{2C}$ editing at the A and B sites, especially in the hippocampus. If such changes were reproduced using a better validated model of stress, it could provide new insights concerning the effects of stress on physiology and behavior and possibly a novel mechanism for antidepressant action in the central nervous system. In addition, it would provide a paradigm for dissecting the molecular mechanisms regulating $5HT_{2C}$ editing and the behavioral effects resulting from these changes.

Restraint stress time-course analysis

Tube restraint is considered a mild to moderate stressor for mice that leads to elevated glucocorticoids and stereotypical behaviors which vary depending on genetic background [77, 78]. In initial pilot studies, C57Bl/6 mice were subjected to 30 minutes of daily tube restraint at 8:30 am for 2 days, 7 days, 14 days, or 21 days. After final stress exposure, animals were

euthanized, RNA was isolated from dissected cortex, hippocampus, striatum, hypothalamus and amygdala and $5HT_{2C}$ editing profiles were determined by high-throughput sequence analysis. Results from these studies reproduced findings observed with daily injection of saline alone, supporting the hypothesis that stress can modulate $5HT_{2C}$ editing patterns (Figure 9). Dramatic reductions in A and B site editing were observed after 7 days of restraint, selectively in hippocampal tissue. These reductions reached a maximum after 14 days of restraint stress and began returning to baseline after 21 days of consecutive stress. One model implied by these results is that acclimation to a stressor parallels changes in editing where a molecular link between the psychocognitive effects of stress and changes in neurotransmitter receptor expression/signaling could provide important insights concerning stress adaptation.

To pursue these findings further, we performed more detailed time-course analyses to investigate the possibility that these effects were either transient or persistent. The hypothesis that editing changes as a result of stress, and then returns to normal after acclimation, was assessed by determining if editing returned to baseline after cessation of daily restraint (Figure 11). Since the most robust effects of restraint were observed after 14 consecutive days, we used 14-day restraint as a baseline and allowed animals to recover undisturbed for 24, 48, or 96 hours in normal housing. Results from this analysis indicated that animals stressed for 14 days still demonstrated the expected reductions in A and B site editing in the hippocampus after 24 hours of recovery. After 48 hours of recovery however, A and B site editing efficiency began returning to normal, and after 96 hours, the $5HT_{2C}$ editing profile in the hippocampus of stressed mice was indistinguishable from that observed in unstressed animals. These findings demonstrated that that the effects of uncontrollable stress on hippocampal $5HT_{2C}$ editing were transient manifestations
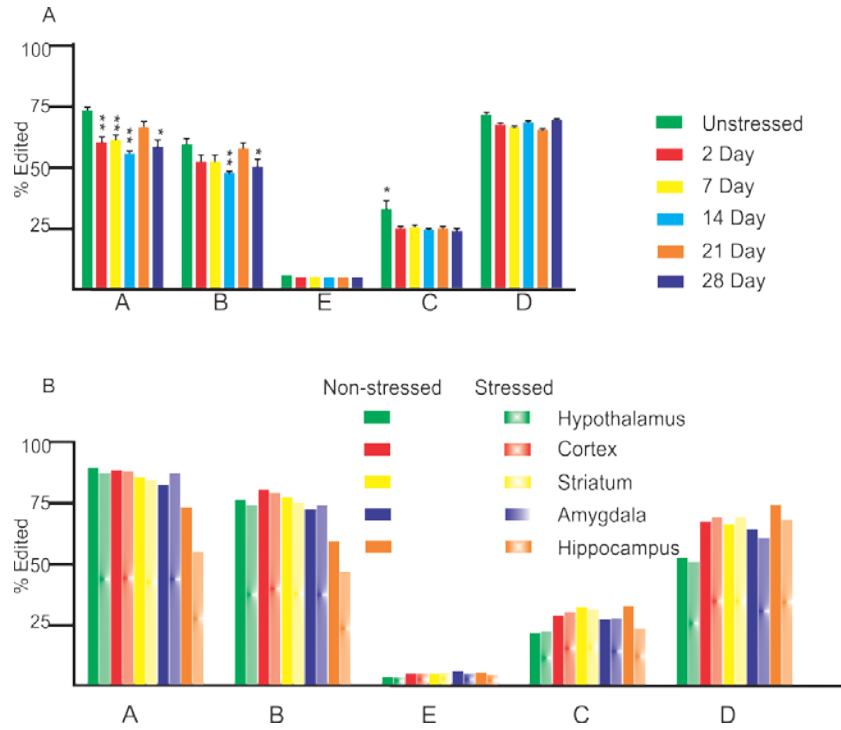
Figure 10. Effect of restraint stress on brain 5HT$_{2C}$ editing efficiency. A) Results from pilot studies investigating the effects of daily restraint stress on 5HT$_{2C}$ editing profiles. Editing efficiencies were determined by comparing the extent of editing after exposure to restraint stress to unstressed controls (2-way ANOVA n=4 * p<.01, **p<.001). **B)** Effect of 14 consecutive daily exposures to 30 minutes of tube restraint on site-selective 5HT$_{2C}$ editing in RNA isolated from dissected brain regions. Error bars indicate SEM
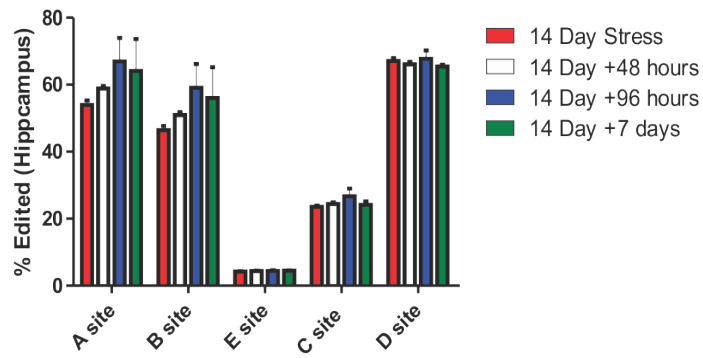
Figure 11. Dynamics of recovery from stress on 5HT$_{2C}$ editing profile in the hippocampus. 5HT$_{2C}$ editing profiles were determined in hippocampus of mice subjected to 14 days of restraint stress followed by up to 7 days of unstressed recovery. Error bars represent SEM. Statistical analysis is not shown because data is confounded by known artifacts as discussed in text.

that subside when the animals are no longer being exposed to the stressor or has learned to cope with the stressor in some other way.

Strain specificity of stress effects

Different stress coping mechanisms have been identified in inbred mouse strains of disparate genetic background. One example of such disparate stress coping behavior is observed when comparing stress-responsiveness in Balb/c and C57Bl/6 mice. C57Bl/6 animals generally respond to stress and novel environments with hyperactivity, whereas Balb/c mice respond with hypoactivity and a characteristic stretching and sniffing behavior [79]. To determine if the effects of stress on hippocampal $5HT_{2C}$ editing profiles were strain-specific and potentially related to different coping mechanisms, we exposed Balb/c animals to 14 days of consecutive tube restraint and characterized the $5HT_{2C}$ editing patterns in RNA samples isolated from distinct brain regions. Results from these analyses indicated significant reductions in $5HT_{2C}$ editing at the A and B sites, similar to that observed in stressed C57Bl/6 mice. Surprisingly, we also observed editing profiles in the hippocampus from some unstressed Balb/c mice that resembled the profile expected after stress exposure (Figure 12A). One potential explanation for this unexpected finding involves increased sensitivity to an unidentified environmental stressor experienced by some of the mice in their home cage which was leading to the changes in $5HT_{2C}$ editing patterns. Indeed some investigators have suggested that Balb/c mice represent a model of chronic anxiety [79]. A chronic anxious state might result in the observed alteration for editing profiles in Balb/c mice, even when they are not subjected to restraint.

Restraint stress effects on editing in adrenalectomized mice

After generating initial data concerning the temporal dynamics of stress-induced changes in A-to-I conversion, we wanted to identify the signaling mechanisms leading to the observed alterations in $5HT_{2C}$ editing. Stressors activate the hypothalamic-pituitary-adrenal (HPA) axis leading to elevated glucocorticoid release from the adrenal gland and many of the effects of stress on CNS function are thought to result from elevated glucocorticoid levels. Hippocampal neurons express high levels of glucocorticoid receptors and elevated glucocorticoids in mice have been correlated with changes in neuronal physiology, dendritic morphology, and reduced hippocampal volume [76]. Surgical removal of the adrenals has been shown to eliminate stress-induced glucocorticoid stimulation while preserving initial central responses to stress exposure. By subjecting adrenalectomized (Adx) mice to the tube restraint paradigm, we could address the possibility that changes in editing were dependent upon intact glucocorticoid signaling. Adrenalectomized animals were acquired from The Jackson Laboratory (Bar Harbor, Maine) and shipped to the Vanderbilt animal housing facility where they were allowed to acclimate for 7 days. After acclimation, a portion of the animals were sacrificed and dissected whole hippocampus was harvested for RNA isolation to determine whether adrenalectomy itself led to changes in baseline editing profiles. No differences between sham-Adx and Adx mice were observed (Figure 12B). The sham and Adx animals responded to tube restraint with similar behaviors including increased micturition, defecation, and periodic struggling. Furthermore, analysis of $5HT_{2C}$ editing patterns in the hippocampus revealed a similar reduction in A and B site editing after 14 days of daily restraint, suggesting a glucocorticoid-independent mechanism of stress that was driving the observed molecular plasticity.

A

Editing in restraint exposed Balb/c

B

Adrenalectomy + Stress

C

Pet1-KO and Stress
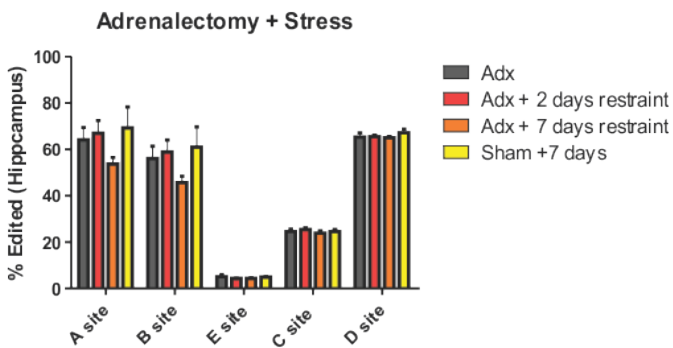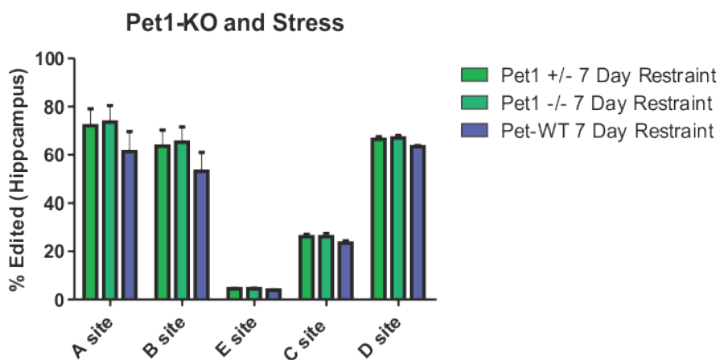
Figure 12. Results from initial quantitative analysis of 5HT$_{2C}$ editing patterns in response to genetic and surgical manipulation. Site-selective RNA editing patterns were quantified in Balb/c mice subject to 7 days of tube restraint (A), for control (sham) and adrenalectomized (Adx) C57Bl/6 animals after 2 or 7 days of tube restrain (B) and in mice null for the expression of Pet-1 (C). Error bars represent the SEM. Statistical analysis is not shown because data is confounded by known artifacts as discussed in text.

Effects of complete 5HT neuronal loss on stress induced editing dynamics

The apparent absence of a role for glucocorticoids in the observed stress-dependent alterations in $5HT_{2C}$ editing profiles motivated us to pursue an alternative hypothesis where changes in signaling from serotonergic neurons were driving reductions in site-selective $5HT_{2C}$ editing. For these studies, we took advantage of the Pet-1-null animals that have been shown to lack serotonergic neurons [72]. Homozygous Pet-1-null animals were subjected to 14 consecutive days of tube restraint (30 min/day) before analysis of hippocampal $5HT_{2C}$ editing patterns. Surprisingly, no changes in A or B site editing were observed in these mutant mice (Figure 12C), indicating that although stress-dependent changes on $5HT_{2C}$ editing were glucocorticoid-independent, they required the presence of 5HT neurons.

Effects of maternal separation on $5HT_{2C}$ editing profile

To determine whether other commonly employed experimental stressors also might affect $5HT_{2C}$ editing, we used perinatal maternal separation [80]. For these studies mice were removed from their mothers and individually housed for 6 hours each day from postnatal day 1 (P1) to P14. Mice were sacrificed on postnatal day 15 and the hippocampus was removed for quantitative analysis of 5HT2C editing. Results from this study indicated significant inter-animal variability, where some animals appeared to have reduced A and B site editing in the hippocampus, while others appeared to have normal editing profiles. The overall result indicated that the mean editing extent was not different between the two groups.

Monitoring choroid plexus contamination

At this point several inconsistencies in the data were beginning to draw our attention. For example, initial pilot studies indicated that the effects of stress on editing were maximal at 14
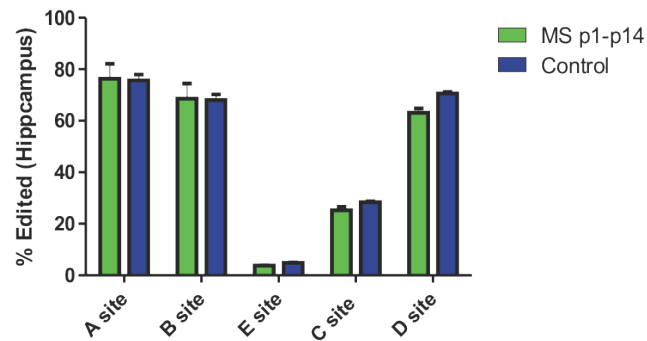
Figure 13. Quantitative analysis of early-life stress on 5HT$_{2C}$ editing patterns. The effects of daily maternal separation (MS) from P1-P14 on hippocampal 5HT$_{2C}$ RNA editing profiles were determined. Error bars represent SEM and a t-test comparing the mean extent of editing did not reveal a significant difference at any site. (p>.05) Error bars represent SEM.

days and then returned to normal, even in the face of continued daily restraint. However, the mice in the Abbas study still showed extensive reductions in A and B site editing, even after 28 days of saline injection [64]. Furthermore, no increases in ADAR2-selective D site editing were observed that could result from stress-induced decreases in ADAR1 activity/expression. Despite the reproducibility of our stress-induced changes in $5HT_{2C}$ editing in initial studies, as we attempted to employ larger mouse cohorts, inconsistent results were obtained. Many of the stressed animals appeared to have normal hippocampal editing profiles, while some unstressed mice displayed editing profiles similar to that observed after 14 days of tube restraint.

Several brain regions, including hippocampus, have close proximity to ventricles which contain choroid plexus tissue. This ependymal tissue, which plays a critical role in the production of cerebral spinal fluid (CSF) expresses high levels of $5HT_{2C}$ mRNA and receptor protein relative to surrounding neuronal tissue [41]. Furthermore, $5HT_{2C}$ editing patterns are unique in the choroid plexus (CP), where significantly reduced editing is observed at the A and B sites [81]. Several studies have observed profound alterations in hippocampal morphology and volume resulting from exposure to stress [82] [80]. For this reason, we wanted to test the possibility that our observed changes in $5HT_{2C}$ editing patterns did not result from stress-dependent alterations in hippocampal or choroid plexus morphology causing tissue contamination during routine brain dissection. To quantify the extent of choroid plexus contamination, we used real-time RT-PCR to quantify an RNA target that is highly-enriched in choroid plexus, but absent from surrounding brain tissue, coagulation factor V (F5) [41]. Results from this analysis revealed that the observed reduction in $5HT_{2C}$ editing at the A and B sites was correlated with the amount of F5 transcript present in the RNA sample (Figure 14), providing

evidence that the observed stress-dependent reductions in editing were actually an artifact of choroid plexus carryover during dissection and tissue isolation.
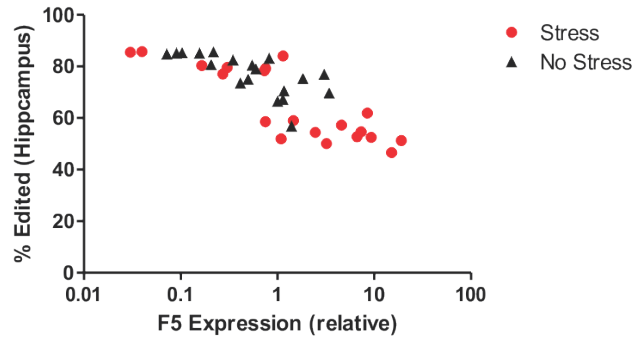
Figure 14. Expression of the choroid plexus marker, Coagulation Factor V (F5), in hippocampal tissue. Linear regression analysis reveals a significant correlation between % editing at the A site and F5 expression (p<.0001) revealing that choroid plexus contamination is a major factor contributing to the extent of editing observed in each sample.

Discussion

The results of these studies provide convincing evidence that $5HT_{2C}$ RNA editing is remarkably stable in adult animals and that the average editing profile in most brain regions is similar to that observed in the whole brain. The one major exception to this is the editing profile observed in choroid plexus tissue where an absence of ADAR1 expression leaves the A and B sites essentially non-edited [41]. The role of $5HT_{2C}$ receptors in choroid plexus has not been adequately explored and is not well understood. It is interesting to speculate a role for $5HT_{2C}$ constitutive activity in the choroid plexus that might oblige the high expression of constitutively active less-edited receptor isoforms in this tissue. More importantly however, is the observation that ventricular proximity and accidental contamination of samples with choroid plexus tissue can lead to inaccurate estimates of editing extent in brain tissue samples. This is an issue that needs to be considered when interpreting $5HT_{2C}$ editing data.

CHARACTERIZING 5HT$_{2C}$ EXPRESSION IN PRADER-WILLI SYNDROME

The 5HT$_{2C}$ Receptor and Prader-Willi Syndrome

A number of observations in rodents and humans have demonstrated that the 5HT$_{2C}$ receptor is a key regulator of feeding behavior and metabolism [50, 83]. The 5HT$_{2C}$ receptor is expressed by pro-opiomelanocortin (POMC) expressing neurons in the arcuate nucleus of the hypothalamus and receptor activation directly stimulates release of melanocortins from these neurons to potently suppress hunger [84]. Furthermore, the appetite suppressive action of the non-specific serotonin releaser, d-fenfluramine, appears to be mediated by 5HT$_{2C}$ receptors specifically in POMC neurons. This was confirmed in studies where hyperphagia and d-fenfluramine insensitivity in 5HT$_{2C}$-null animals was corrected by selectively re-expressing 5HT$_{2C}$ transcripts in POMC neurons using a POMC-Cre based system with a conditional STOP-FLOX-5HT$_{2C}$ allele [83]. These findings led to the development of Lorcaserin [85], a selective 5HT$_{2C}$ agonist currently marketed by Arena Pharmaceuticals for appetite suppression in obese patients. However, the efficacy of Lorcaserin as a therapeutic is tempered by an unfavorable side effect profile of this drug, requiring the use of low doses with marginal effects on appetite.

Mice engineered so that they solely express the VGV isoform of the 5HT$_{2C}$ receptor have been developed by several groups [24, 25] to study the importance of 5HT$_{2C}$ editing and the physiological consequences of solely expressing the receptor isoform with the least constitutive activity. These animals display several characteristics of a human genetic disorder known as Prader-Willi Syndrome (PWS), as well as a number of similarities to construct valid mouse models of PWS [51]. Some of the more prominent features observed are a failure to thrive and

severe hypotonia in infancy, adult onset hyperphagia, and neonatal lethality on a C57Bl/6 background. The precise mechanism conferring these phenotypes is unknown, but it may be related to abnormal regulation of $5HT_{2C}$ expression in the CNS, which is apparent in these animals. Specifically, mutant animals express $5HT_{2C}$ mRNA at similar levels to wild-type C57Bl/6 mice, whereas receptor protein levels were increased by 40-70 fold based upon western blotting, saturation binding, and autoradiographic methodologies [24, 25]. Likewise, VGV mice display heightened sensitivity to drugs targeting the $5HT_{2C}$ receptor. Administration of $5HT_{2C}$ agonists causes an immediate onset of seizures resulting in death at doses that only mildly suppress feeding in wild-type mice [25]. $5HT_{2C}$ antagonist administration in VGV animals results in pronounced hyperactivity reminiscent of the effects observed with high dose amphetamine treatment in wild-type animals [86]. When combined with previously identified alterations in $5HT_{2C}$-mediated behavior in mouse models of PWS (PWS-IC[del]) [87] observed increases in the editing of $5HT_{2C}$ transcripts in both a mouse model and human PWS patients [86][88] and the known involvement of the $5HT_{2C}$ receptor in controlling energy homeostasis and satiety [83, 89], the presence of PWS-like phenotypes in mice with altered $5HT_{2C}$ editing identifies the *htr2C* gene as the first locus outside the 15q11-13 imprinted region in which mutations can recapitulate multiple aspects of this human genetic disorder.

Prader-Willi Syndrome is a maternally-imprinted human disorder resulting from a loss of paternal gene expression on chromosome 15q11-13 which is characterized by a complex phenotype including cognitive deficits, infantile hypotonia and failure-to-thrive, short stature, hypogonadism and hyperphagia which can lead to morbid obesity [90, 91]. The diets of PWS patients must be carefully regulated or they will become morbidly obese by the time they reach adolescence [92]. The prevalence of PWS is estimated to be about 1/20,000 births worldwide and

the syndrome is attributed to the lack of expression of a subset of genes located within an imprinted region of chromosome 15 q11.2-q13 [90, 91]. Five protein coding genes, several antisense RNAs, and two unique putative small nucleolar RNA (snoRNA) clusters (SNORD115 and SNORD116) have been identified within this PWS locus [92] (Figure 15). However, the role each of these elements plays in the etiology of PWS is not clear, and no single mutation in any one of these genes has been associated with all of the features of the syndrome [93-96].

Recently, considerable attention has been focused on understanding the role that the non-protein coding snoRNA clusters might play in the disorder [97]. snoRNAs typically function by serving as a guide to direct the site-specific 2'-O-methylation of ribosomal RNAs (rRNAs) or small nuclear RNAs (snRNAs) by homology-dependent targeting mechanisms [98]. The snoRNAs SNORD115 (HBII-52) and SNORD116 (HBII-85) are present in 42 and 29 copies, respectively, within the human PWS region [92], and they are expressed widely in the brain [99]. No rRNA or snRNA targets have been identified for either of these snoRNAs, yet a homology search revealed that the methylation-targeting sequence for SNORD115 predicts that transcripts encoding the $5HT_{2C}$ receptor are potential targets for snoRNA-mediated 2'-O-methylation [100]. Interestingly, the sequence of SNORD115 predicts that it will facilitate 2'-O-methylation at a specific adenosine within the $5HT_{2C}$ transcript which is also one of the sites targeted for A-to-I editing (C site).

The studies described in this chapter are aimed at testing the hypothesis that alterations in $5HT_{2C}$ expression will be observed in the brains of PWS patients and that such changes may underlie the molecular etiology of this disorder. We used multiple approaches to measure $5HT_{2C}$ RNA levels, alternative splicing patterns, RNA editing profiles, 2'-O-methylation and receptor protein expression in dissected brain regions obtained from PWS patients and non-PWS controls.

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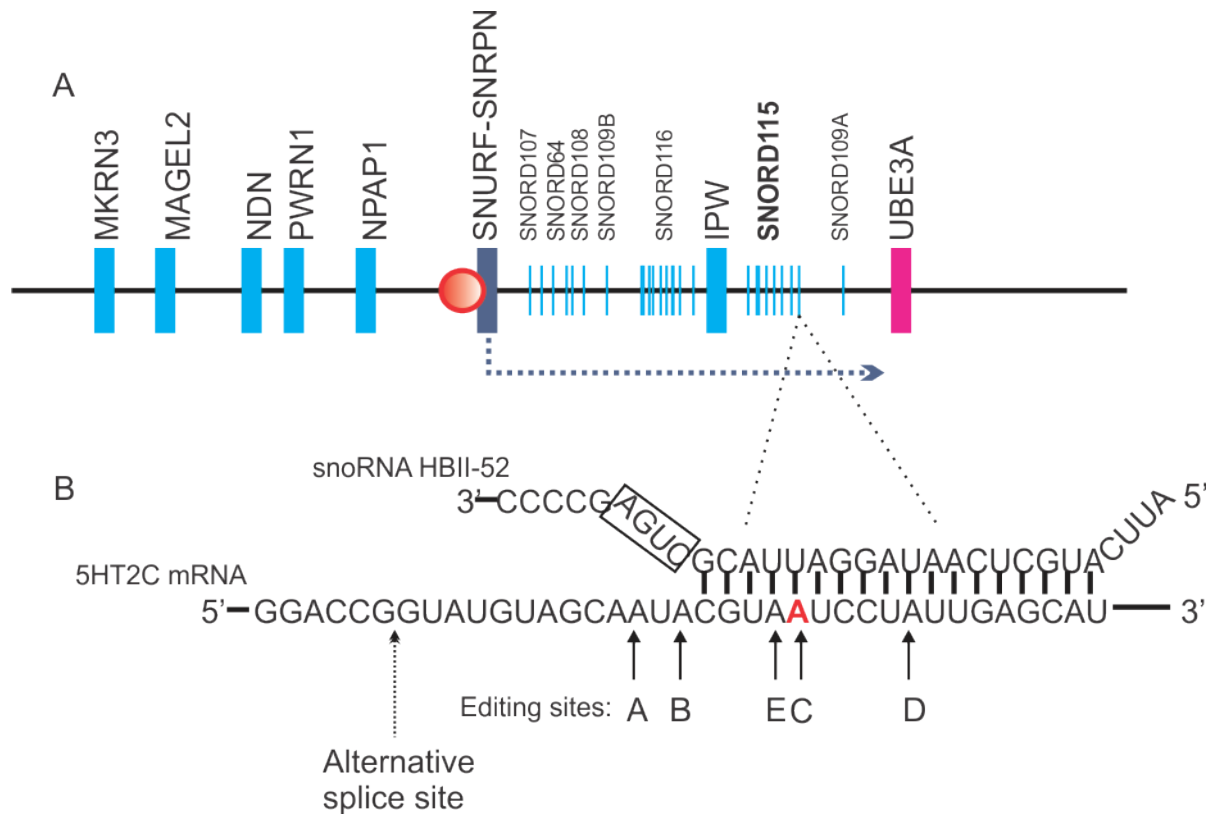
Figure 15. Schematic summary of the Prader-Willi Syndrome (PWS) locus and its putative interaction with 5HT$_{2C}$ transcripts. A) A schematic diagram of the chromosomal locus implicated in PWS 15q11-13 is presented. Large blue boxes represent maternally-imprinted genes while the red circle indicates the location of the imprinting center where CpG methylation occurs on the maternal chromosome to silence the maternally imprinted genes. Small blue lines indicate snoRNAs encoded in the 3'-untranslated region of the SNURF-SNRPN transcript. **B)** The human SNORD115 locus contains 48 copies of the snoRNA (HBII-52), each containing a region of sequence complementarity to the edited region of the 5HT$_{2C}$ transcript. This sequence complementarity predicts 2'-O-methylation of the C editing site (red) which is complementary to the base five nucleotides downstream from the conserved D-box element (rectangle) in the snoRNA. Loss of expression at this locus due to deletion, aberrant methylation, or uniparental (maternal) disomy results in PWS.

Methods

Human Tissue Sample Preparation

All human tissue samples were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). Tissue was processed by crushing into fine powder while frozen using a 'Cryo-cup' grinder and pestle (Biospec Products) and stored at -80° C until further processing for RNA, protein or membrane preparations was required.

Genotyping to confirm PWS diagnosis

PWS patients were diagnosed based upon physical examination and generally were not confirmed by subsequent molecular genetic analysis. To confirm diagnoses at a molecular level, genomic DNA samples from each individual were subjected to analysis by methylation-specific multiplex ligation-dependent probe amplification, as described previously [101].

RNA Characterization

RNA extraction

Frozen, powdered tissues were homogenized in Trizol® reagent (Life Technologies) and total RNA was isolated following the manufacturer's instructions.

Quantitative *RT-PCR*

$5HT_{2C}$ mRNA expression was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) amplification using the Assay-On-Demand primer/probe set for the human $5HT_{2C}$ transcript (Hs00168365_m1) and a Vic-labeled primer/probe set designed for the human large ribosomal protein transcript, RPLP0 (4326314E) which was used

as an internal loading control. To identify tissue samples contaminated with choroid plexus, qRT-PCR was used with a probe for the choroid plexus specific marker human coagulation factor V (F5) (Applied Biosystems, Hs00914120_m1). Gene amplification efficiencies were calculated using the PCR miner algorithm [65] and data was analyzed using CFX Manager (Biorad Laboratories).

RNA editing profiles determined by HTMTA

RNA editing profiles were determined by high-throughput sequence analysis as previously described [102] and presented in Chapter 1 (Figure 2).

Alternative splicing of 5HT$_{2C}$ pre-mRNA

5HT$_{2C}$ alternative splicing patterns were determined by semi-quantitative RT-PCR using primers which amplify all three splice variants (Sense: 5'-TATTTGTGCCCCGTCTGG-3'; antisense: 5'-CATGGTGCCCCTAGGACGTCT-3'). Resultant amplicons were resolved on a 2% agarose gel and ethidium bromide fluorescence was quantified by phosphorimager analysis using a Typhoon 9400 and ImageQuant TL software (GE Healthcare). The relative ratio of spliced isoforms was calculated by correcting fluorescence values by size ratios of the PCR amplicons to normalize for ethidium bromide binding.

Analysis of 2'-O-methylation in mouse brain RNA

A highly sensitive assay was developed to detect low levels of 2'-O-methylation for the adenosine at the "C" editing site in 5HT$_{2C}$ transcripts. This assay takes advantage of site directed RNAse H cleavage directed by a chimeric DNA-RNA oligonucleotide described by Yu *et al*. [103]. The chimeric oligonucleotide probe rArGr**GATTA**rCrGrUrArUrUrGrCrUrArCrArUrA-

rCrC was synthesized and PAGE purified by Integrated DNA Technologies (Coralville, Iowa) where bold letters represent deoxynucleosides and the ribonucleosides are shown with an "r" before each letter. Either water or 100 pmol of the chimeric probe was mixed with 5 ug of total mouse brain RNA extracted from mice genetically engineered to express only the non-edited INI isoform in 50 nM NaCl. The mixture was denatured by heating RNA to 90°C for 3 min and then annealed by allowing it to cool to room temperature followed by a 20 min incubation on ice. The annealed probe-RNA mixture and probe-free RNA mixture were treated with 5 units of RNAse H in 1x RNase H buffer (New England Biolabs) for 30min at 30°C, followed by RNase H heat inactivation by incubating at 65°C for 20 min. cDNA was then generated from each sample using a $5HT_{2C}$ gene specific primer (5'-CAATCACAGGGATAAGAACTGAAACTCCTATTG-3') and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. The cDNA was then amplified by PCR using OneTaq master mix (New England Biolabs) using the gene specific antisense primer used for reverse-transcription and one of two sense primers; either 5' of the putative methylation site (5'-TATTTGTGCCCC-GTCTGG-3') or 3' of the predicted cleavage site (5'-TTTCAATTCGCGGACTAAGGC-3'). Resulting PCR products were resolved on 2% agarose gels and 2'-O-methylation was quantified by ethidium bromide fluorescence of the 205 and 85 bp DNA fragments corresponding to 2'-O-methylated and non-methylated RNAs, respectively.

Receptor Binding

To quantify $5HT_{2C}$ receptor expression, saturation binding analyses was performed using frozen, powdered human tissues which were mechanically homogenized at 15000 rpm for 10 seconds (s) (Tissue Tearor; Biospec Products) in 2 ml of standard binding buffer (SBB; 50mM Tris–HCl, pH 7.4, 10mM $MgCl_2$, 0.1 mM EDTA) and then centrifuged at 25,000 x g for 10 min.

The supernatant was removed and the pellet was resuspended in 1 ml of SBB and transferred to 1.7 mL Eppendorf tubes. The tissue was then spun at 16,000 x g in a microcentrifuge for 5 min at 4°C. The supernatant was removed and the tissue pellets were frozen at −80°C until used for binding experiments. Total binding was determined using N^6-methyl-$[^3H]$-mesulergine (8-point curves were generated using $[^3H]$-mesulergine concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, and 8.0 nM) with non-specific binding measured using those same concentrations of $[^3H]$-mesulergine plus 8 μM ritanserin; both sets of conditions included 100 nM spiperone to block $5HT_{2A}$ receptor binding. Bradford protein assays were performed to quantify protein in each pellet (BioRad Laboratories). All incubations were performed in SBB for 1.5 h at room temperature. Incubations were terminated by rapid filtration using Whatman GF/C glass fiber filters, washed 3 times with cold 50mM Tris–HCl (pH 6.9 at room temperature, pH 7.4 at 4 °C). Samples were measured by liquid scintillation spectrometry in a Perkin-Elmer Tri-Carb 2800TR counter and data were analyzed using GraphPad Prism (Graphpad Software; San Diego, CA) using nonlinear regression to fit the experimental data to saturation binding equations (assuming depletion). Maximal binding (B_{max}) values were normalized to the protein concentration for each sample.

Results

Analysis of Genotype and Sample Integrity

Tissue samples from patients with Prader-Willi Syndrome were acquired from the NICHD Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) where the disorder was identified based upon clinical presentation and physical diagnosis (Table 3). To verify that PWS patients had molecular deficits consistent with the diagnosis of this syndrome, genomic DNA from each patient was isolated and subjected to analysis using methylation-specific multiplex ligation-dependent probe amplification to determine the integrity and methylation status of the PWS locus [101]. Results from this analysis revealed that two of the patients initially diagnosed with PWS contained neither deletions nor apparent methylation defects (patients 1556 and 4876; *data not shown*), suggesting alternative molecular mechanism(s) were contributing to the PWS-like phenotype in these individuals. A recent study using PWS samples from the NICHD Brain and Tissue Bank further confirmed that one of these patients (4876) expressed SNRPN, SNORD115, and SNORD116 transcripts which does not support a genetic diagnosis of Prader-Willi syndrome [104]. These non-PWS samples were excluded from further analysis, except where indicated.

The hippocampus is a C-shaped structure made of two rolled-up laminae, the cornu Ammonis (fields CA1, CA2/3) and the dentate gyrus. Its ventricular aspect is covered by the choroid plexus of the inferior horn, an ependymal tissue which plays an important role in the production of cerebrospinal fluid (CSF) and expresses very high levels of $5HT_{2C}$ transcript and receptor protein relative to surrounding neuronal tissue [105, 106].

Tissue ID	Diagnosis	Gender	Age	PMI	Ctx	Str	Hip
4725	Control	F	32.2	17	+	+	+
1136	Control	F	34.0	19	+	+	+
1648	Control	F	38.8	23	+	+	+
1830	Control	F	39.7	7	+	+	+
1363	Control	F	40.9	14	+	+	+
4636	Control	F	43.1	19	+	+	+
4228	Control	F	44.9	16	+	+	-
1795	Control	F	49.3	23	+	+	+
1789	Control	F	50.5	22	+	+	+
1568	Control	F	51.2	22	+	+	+
M3570M	PWS	F	32.4	24	+	+	-
M1199M	PWS	F	35.6	2	+	+	-
4769	PWS	F	37.2	20	+	-	-
M1889M	PWS	F	38.2	19	+	+	-
4805	PWS	F	39.1	16	+	+	+
1556	PWS	F	41.9	13	+	+	+
865	PWS	F	43.1	28	+	+	+
1290	PWS	F	44.3	29	+	+	+
4876	PWS	F	56.5	41	+	+	+
5016	PWS	F	59.5	9	+	+	+
1545	Control	M	45.3	20	+	+	+
4639	Control	M	45.3	21	+	+	+
1428	Control	M	45.5	14	+	+	+
5024	Control	M	56.0	10	+	+	+
1113	Control	M	56.8	17	+	+	+
1940	Control	M	57.2	20	+	+	+
1447	PWS	M	45.0	3	+	+	+
1510	PWS	M	56.3	19	+	+	+

Table 3. Summary of control and PWS brain samples for analysis of 5HT$_{2C}$ RNA and protein expression. All human tissue samples were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). Diagnosis of PWS was based upon clinical analysis of phenotype and the age (years) at time of death and post-mortem interval (PMI; hours) are indicated. The availability of indicated brain regions from each patient is designated by a plus (+) or minus (-) sign. Ctx, cortex; Str, striatum, Hip, hippocampus.

[107] [108]. Importantly, the $5HT_{2C}$ editing profile in choroid plexus is significantly different from surrounding brain regions, where editing at the A and B sites is dramatically reduced [81], suggesting that small levels of choroid plexus contamination in the hippocampus samples could significantly affect $5HT_{2C}$ editing profiles, complicating any interpretation of results. For this reason, we chose to exclude any tissue samples that contained high levels of choroid plexus contamination from further analysis. To determine the extent of choroid plexus contamination, we employed real-time RT-PCR to quantify human coagulation factor V (F5) mRNA, an RNA target that is highly enriched in choroid plexus, but absent from surrounding brain tissue [109]. Results from this analysis indicated that F5 mRNA expression was high in a majority of the hippocampus samples isolated from both control and PWS patients, varying by up to four orders of magnitude between samples. Comparisons between the extent of editing at the A and B sites for $5HT_{2C}$ transcripts and F5 RNA levels showed a significant correlation ($R^2 = 0.4884$; $p \leq 0.0002$), indicating that choroid plexus contamination was a major driver of $5HT_{2C}$ editing profiles in the acquired hippocampal RNAs (Figure 16A). However, only background levels of F5 mRNA were detected by qRT-PCR in any of the striatal or cortical tissue samples (*data not shown*). As an additional strategy, we also quantified the level of $5HT_{2C}$ mRNA and receptor protein expression in hippocampus samples as markers of choroid plexus contamination, since previous studies have demonstrated that $5HT_{2C}$ expression in the choroid plexus is higher than surrounding brain regions [105-108, 110]. Analyses of $5HT_{2C}$ mRNA and receptor protein levels using qRT-PCR and saturation binding with [3H]-mesulergine, respectively, revealed a significant correlation between $5HT_{2C}$ expression and F5 mRNA levels (Figure 16B, C), further supporting the hypothesis that hippocampal RNA editing profiles are strongly tied to choroid

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plexus contamination. For this reason, we excluded the analysis of hippocampal tissue for comparisons between patient groups.
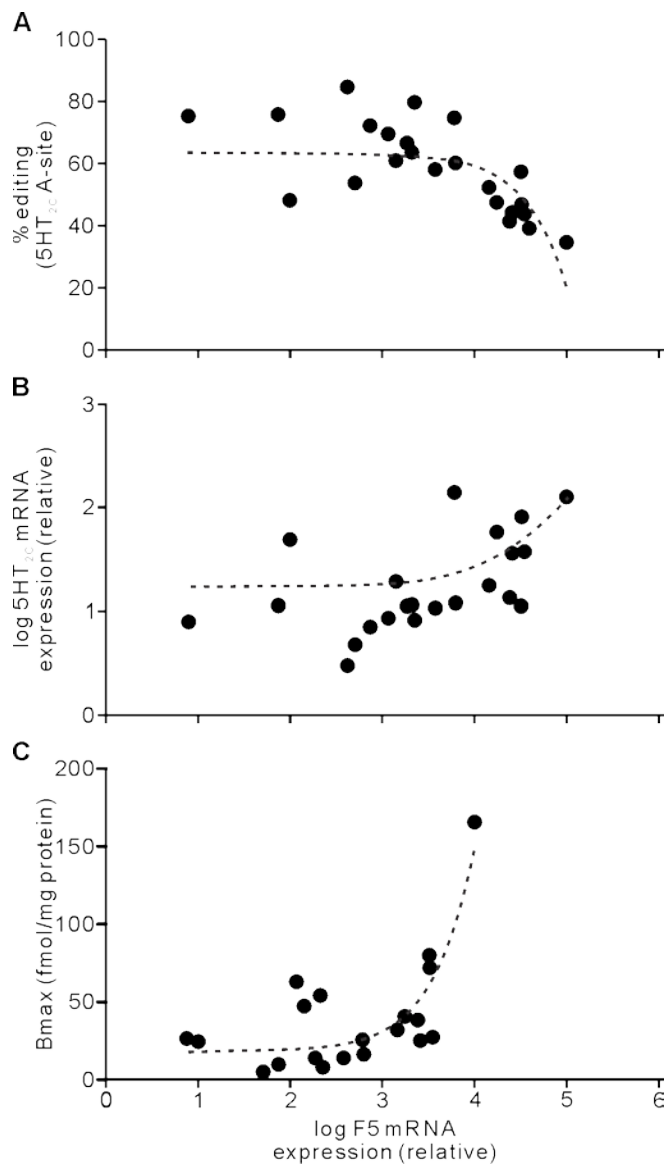
Figure 16. Analysis of choroid plexus contamination in hippocampus samples. A) The extent of editing at the A-site for $5HT_{2C}$ transcripts in each hippocampus sample (PWS and control) was compared to the relative mRNA expression for a choroid plexus marker, coagulation factor V (F5). The best-fit curve, determined by linear regression analysis, is indicated with a dashed line (R^2= 0.4884; $p \leq 0.0002$). **B)** Correlation between relative $5HT_{2C}$ and F5 mRNA expression (R^2= 0.3561; $p \leq 0.0034$). **C)** Correlation between $5HT_{2C}$-specific total binding (B_{max}) values and F5 mRNA expression (R^2= 0.7042; $p \leq 0.0001$).

RNA expression and alternative splicing

Alternative splicing of $5HT_{2C}$ pre-mRNAs can generate three distinct mRNA species from the use of different 5'-splice sites located in exon 5 and intron 5 (Figure 17). Use of the proximal and distal 5'-splice sites results in the generation of mRNAs encoding truncated non-functional receptor, $5HT_{2C}$-tr (RNA1) [9], and $5HT_{2C}$-COOHΔ (RNA3) [81], respectively, whereas use of the intermediate splice junction generates RNA2 which encodes the full-length, functional $5HT_{2C}$ receptor. Previous analyses of $5HT_{2C}$ transcripts in mice sole expressing the VGV isoform of receptor revealed alterations in the alternative splicing pattern for $5HT_{2C}$ pre-mRNAs with an increase in the steady-state level of RNA2 and a corresponding decrease in RNA1 [24], consistent with observations that editing at the E, C and D sites promotes the production of RNA2 at the expense of RNA1 [111]. Recent characterization of the receptor isoform encoded by RNA 1 has revealed that although this truncated gene product does not function as a G-protein coupled receptor, it can dimerize with the full length receptor protein (RNA 2 gene product) to retain receptor heterodimers in the endoplasmic reticulum [112]. RNA3 is rarely detected and the functional relevance of this transcript variant has not been examined. Previous reports have provided evidence that one of the snoRNAs encoded in the PWS locus SNORD115 and its mouse homologue MBII-52 play a role in the regulation of pre-mRNA splicing of the $5HT_{2C}$ transcript [88]. These studies predict that SNORD115 promotes the formation of RNA2 at the expense of RNA1 by partially blocking a 'splice silencer' located within exon 5. Since PWS patients lack expression of SNORD115, it is anticipated that the relative expression of $5HT_{2C}$ RNAs 1 and 2 would be increased and decreased, respectively, in PWS patients compared to normal controls.

Relative $5HT_{2C}$ RNA expression was determined by qRT-PCR using a Taqman probe which spanned the exon 3-4 splice junction to detect all three alternatively splice isoforms. These studies did not indicate a significant difference in steady-state $5HT_{2C}$ mRNA levels between control and PWS patients (Figure 18). RNA expression was variable within each group, possibly reflecting a combination of factors including region-specific dynamic regulation in human populations. The high variability in mRNA expression was especially prominent in the striatum which could result from variations in spatial expression between striatal sub-regions. The steady-state level of $5HT_{2C}$ transcript expression in the cortex was very low compared that observed in the striatum, consistent with previous *in situ* hybridization analyses [105-108].

Consistent with the model proposed by Kishore *et al* [88], analyses of alternative splicing revealed a significant reduction in RNA2 with a concomitant increase in RNA1 in tissue samples from PWS patients (Figure 19). Interestingly, we see an even greater reduction in the relative expression of RNA2 to RNA1 in the PWS-like patients who received a clinical diagnosis of PWS, but were not found to harbor a canonical genetic defect within the PWS locus. It is not clear how decreased expression of RNA2 is related to the phenotype, but the two PWS-like patients displayed pronounced cognitive and metabolic dysfunction resembling that typical of PWS.
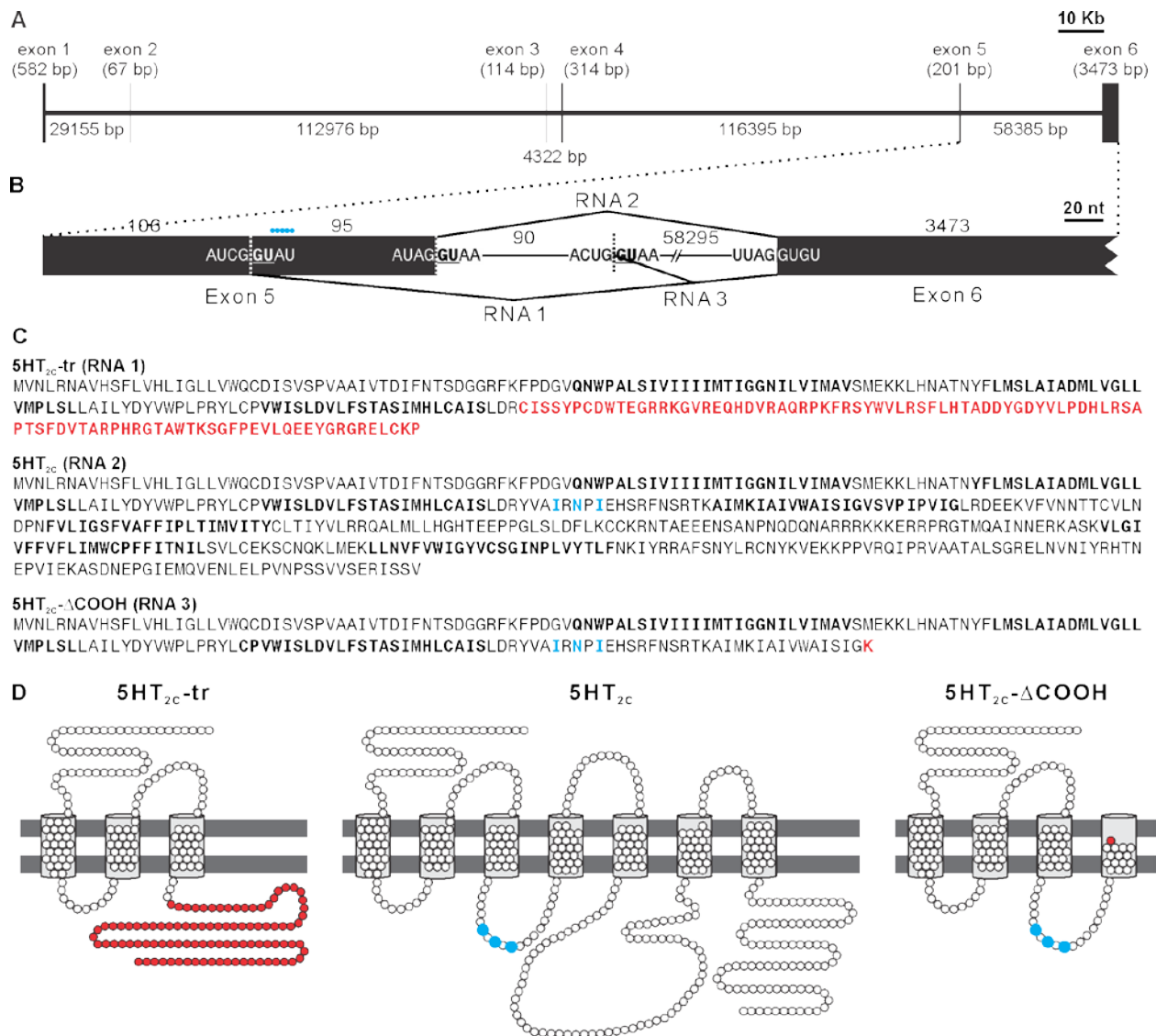
Figure 17. Alternative splicing of 5HT₂C transcripts. A) A scaled diagram of the human serotonin 2C receptor gene (Htr2c), located at position 114584078-114910061 of the X chromosome, is presented; the sizes of exons and introns are indicated in base pairs (bp). **B)** Schematic diagram of three alternative splicing events in $5HT_{2C}$ pre-mRNA to generate distinct, mature mRNAs (RNA1, RNA2 and RNA3). Sequences surrounding the alternative splice junctions are presented with a horizontal dashed line, the conserved GU at alternative 5'-splice sites is underlined, the position of the editing sites are indicated with small blue circles and the lengths of relevant exon and intron regions are presented in nucleotides (nt). **C)** Amino acid sequences encoded by alternatively spliced 5HT2C mRNAs. The predicted transmembrane-spanning domains are indicated in bold and the genomically-encoded amino acids at the sites affected by RNA editing are shown in blue. Novel amino acids resulting from splicing to an alternate reading frame are indicated with red lettering. **D)** Predicted topology of $5HT_{2C}$ receptors encoded by alternatively spliced mRNA variants. The amino acid residues affected by RNA editing are shown in blue and novel amino acids resulting from splicing to an alternate reading frame are indicated with red circles.

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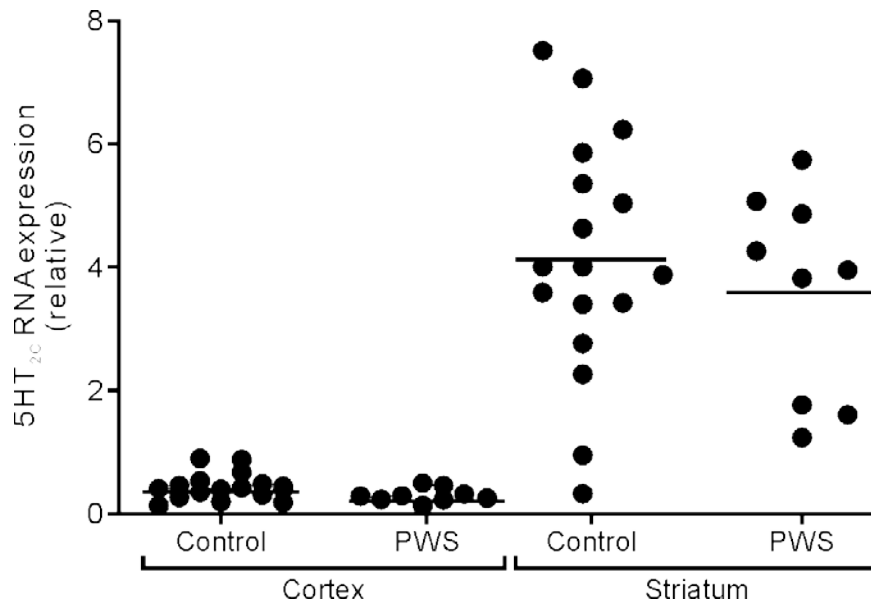
Figure 18. Quantitative analysis of relative 5HT$_{2C}$ mRNA expression. Relative 5HT$_{2C}$ mRNA expression levels from PWS and control brain regions were determined by quantitative RT-PCR; the mean value for each group is indicated with a horizontal bar.
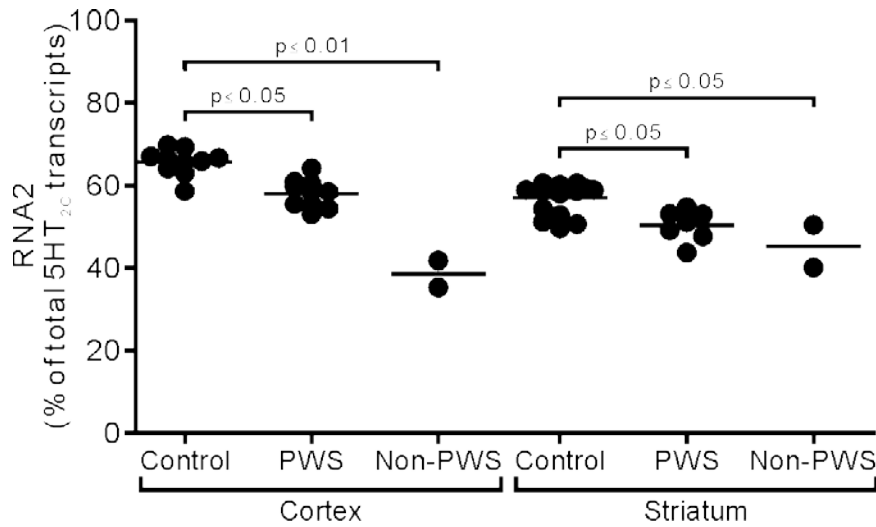
Figure 19. Quantitative analysis of the relative expression levels for 5HT$_{2C}$ alternative splicing variants. Expression of the major spliced isoforms of 5HT$_{2C}$ receptor mRNA were quantified by end-point PCR from multiple brain regions isolated from control and PWS patients. Two individuals that were initially diagnosed with PWS based upon clinical evaluation, but lacked a detectable defect in the PWS locus (non-PWS), are also shown. Data are presented as the percentage of RNA2 transcripts relative to total 5HT$_{2C}$ mRNA expression; the mean value for each group is indicated with a horizontal bar and compared using Student's t-test.

Analysis of RNA Editing Profiles

To assess whether $5HT_{2C}$ pre-mRNA editing is altered in PWS, we quantified RNA editing profiles using a high-throughput sequencing strategy as described in Chapter 1. The data from these studies initially was compared by examining site-selective modification at each of the five editing sites (A, B, E, C and D) in RNA samples isolated from PWS and control patients (Figure 20). This analysis did not reveal any significant differences in the frequency of editing at any site for all brain regions analyzed. Since combinatorial patterns of editing can give rise to 32 mRNA isoforms encoding 24 distinct receptor species, we also analyzed the data to examine potential changes in isoform expression at both the RNA (Figure 21) and protein (Figure 22) levels. A significant reduction in the percentage of transcripts encoding the VNV receptor isoform was observed in both the striatum and cortex (striatum $p \leq 0.01$, cortex $p \leq 0.05$) (Figures 21, 22). This transcript profile predicts that PWS patients express a decreased percentage of protein isoforms containing valine, asparagine and valine (VNV) at amino acid positions 156,158, and 160, respectively (Figure 1 and 22) . Interestingly, similar changes in editing profile have been reported for PWS-IC$^{del+/-}$ mice, a construct valid mouse model of PWS [113]. While it is unlikely that such a small change could lead to the profound phenotype observed in PWS, it is important to note that observed difference in the $5HT_{2C}$ editing profile for PWS patients could reflect a much larger change in discrete subpopulations of neurons.
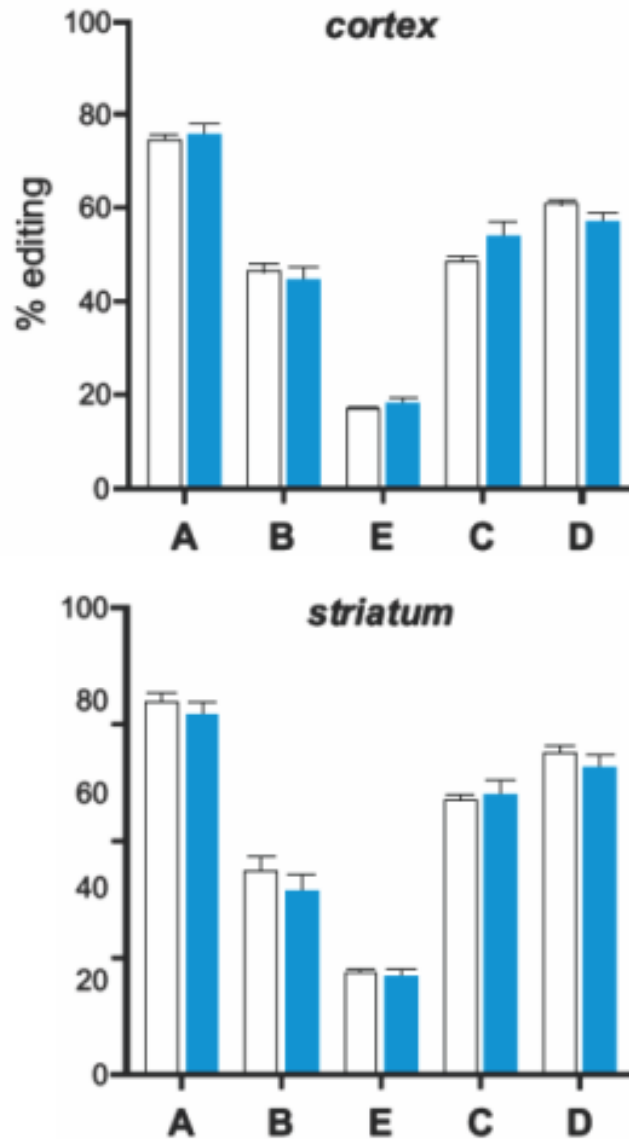
Figure 20. Quantitative analysis of site-selective 5HT$_{2C}$ editing observed in RNA samples isolated from the cortex and striatum of control (white) and PWS patients (blue). Error bars represent the mean $\pm$ SEM; n>12. No significant differences at any of the editing sites were observed using Student's t-test.
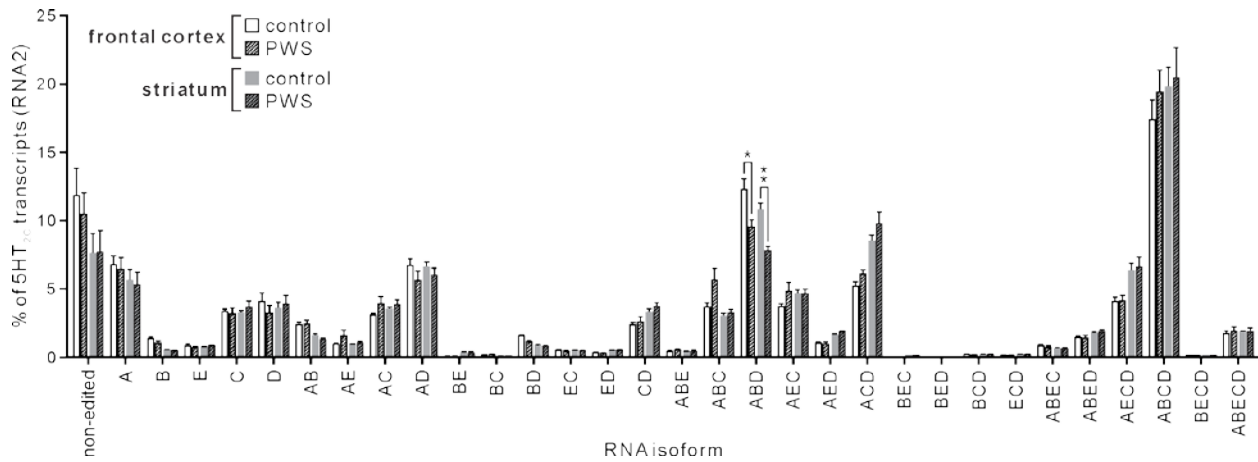
Figure 21. Sequence analysis of brain region-specific 5HT$_{2C}$ RNA editing profiles. Editing profiles in dissected brain regions were determined by high-throughput sequence analysis and the thirty-two possible 5HT$_{2C}$ RNA isoforms resulting from RNA editing are represented as the percentage of total 5HT$_{2C}$ sequence reads for control and PWS patients in each brain region. Means ± SEM were compared by two-way ANOVA; *p<0.05, **p<0.01.
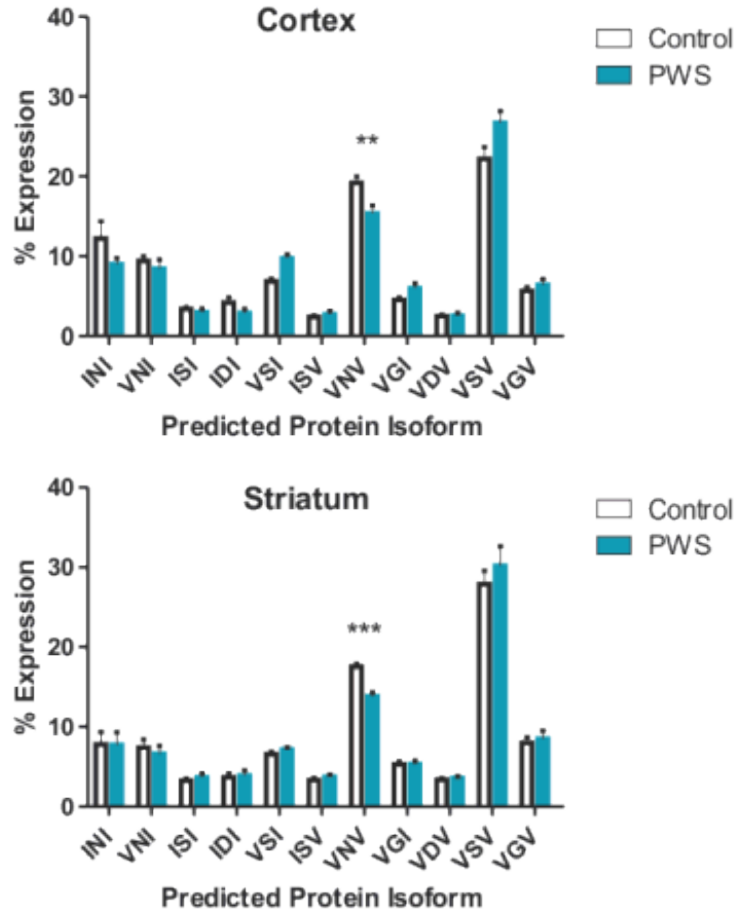
Figure 22. Comparisons of the most common 5HT$_{2C}$ protein isoforms predicted by the RNA editing profile in controls and PWS patients. Predicted amino acids at the editing sites are shown with the single letter amino acid code for the major receptor isoforms and are represented as the predicted percentage of total 5HT$_{2C}$ receptors for control and PWS patients in each brain region. Means ± SEM were compared by Student's t-test; *p<0.01, ***p<0.001.

Analysis of 5HT$_{2C}$ Receptor Protein Density

Previous studies of mutant mice solely expressing the fully-edited (VGV) isoform of the 5HT$_{2C}$ receptor revealed a number of phenotypic alterations similar to those observed in patients diagnosed with PWS. In addition, a number of molecular alterations also were identified in mutant animals including a dramatic increase in 5HT$_{2C}$ receptor density, despite any observed differences in steady-state 5HT$_{2C}$ mRNA levels [24]. The mechanistic explanation for this phenotype is currently under investigation, but may result from differences in translation efficiency or constitutive activity-dependent alterations in subcellular localization that could affect receptor isoform stability [114]. A saturation binding strategy was employed to quantify the receptor density for protein samples isolated from the striatum and cortex of PWS and control patients. No specific binding was detected in cortex samples, consistent with the low-level of steady-state 5HT$_{2C}$ mRNA expression previously observed in this brain region (Figure 17). In the striatum however, 5HT$_{2C}$ receptor density in PWS samples was two-fold greater than that observed in control patients ($p \leq 0.01$) (Figure 23). Considerable inter-individual variation also was observed for receptor density in the striatum, similar to the variability identified in analyses of 5HT$_{2C}$ mRNA levels (Figure 16).

Analysis of 2'-O-methylation

SNORD115, and its mouse orthologue (MBII-52), are members of the box C/D family of snoRNAs that are responsible for directing the 2'-O-methylation of specific ribose moieties in pre-ribosomal RNA transcripts and U small nuclear RNAs [115]. SNORD115 is complementary to an 18 nucleotide segment of 5HT$_{2C}$ mRNA containing three of five editing sites (E, C and D), predicting 2'-O-methylation of the ribose for the adenosine at the C-site [100],

Figure 23. Quantitative analysis of 5HT$_{2C}$-specific binding. Saturation binding isotherms were used to determine 5HT$_{2C}$ receptor density in total membranes prepared from brain regions isolated from control and PWS patients; the mean value for each group is indicated with a horizontal bar and compared using Student's t-test.

an editing position that determines the identity of amino acid 159 for the $5HT_{2C}$ receptor and significantly affects the function of encoded protein isoforms [29, 34]. Previous studies using synthetic RNAs have indicated that ribose 2'-O-methylation leads to a >200-fold decrease in the rate of editing for the modified adenosine nucleoside suggesting that this snoRNA, which is absent in PWS, may serve to inhibit A-to-I conversion at the edited C-site. Several attempts to detect 2'-O-methylation at this site using a reverse transcriptase-dependent primer-extension strategy have been unsuccessful, presumably due to insufficient sensitivity for this experimental approach [30]. To increase the sensitivity of 2'-O-methylation detection, we used an RT-PCR-based approach, as previously described by Yu et al [103]. In this paradigm, the extent of site-specific 2'-O-methylation is quantified using an RNA-DNA chimeric probe to direct ribonuclease (RNase) H cleavage at the modified nucleoside. RNAse H cleavage is inhibited in the presence of 2'-O-methylation, allowing RT-PCR amplification of cleaved and uncleaved RNA templates (Figure 24A). Because site-selective 2'-O-methylation requires precise complementarity between the hybrid probe and mRNA target, it was not impossible to directly assess methylation of human $5HT_{2C}$ mRNAs due to the presence of multiple nucleoside alterations resulting from RNA editing events within the region of the probe. As an alternative approach, we assessed methylation of $5HT_{2C}$ transcripts in RNA isolated from whole brains of mutant mice engineered to express only the non-edited (INI) isoform of the $5HT_{2C}$ receptor. This allowed the design of a chimeric probe for a single edited isoform that selectively targeted RNAse H to detect 2'-O-methylation of the ribose for the adenosine at the C-site. Use of this approach with 5'-end labeled, synthetic RNAs (31 nt), with and without a 2'-O-methyl modification, generated RT-PCR amplicons of the expected size that displayed a low level of incomplete digestion for the unmodified target (Figure 24B). Parallel analyses with mutant mouse RNA, revealed efficient

amplification of the cleaved RT-PCR product (85 bp), but only background levels of the non-cleaved product (205 bp) were observed in the presence of the chimeric probe, indicating no detectable 2'-O-methylation (Figure 24C) at steady-state. However, this assay cannot rule out the possibility that transient methylation events may occur in the pre-mRNA which could impact splicing or inhibit the efficiency of editing at the C site.
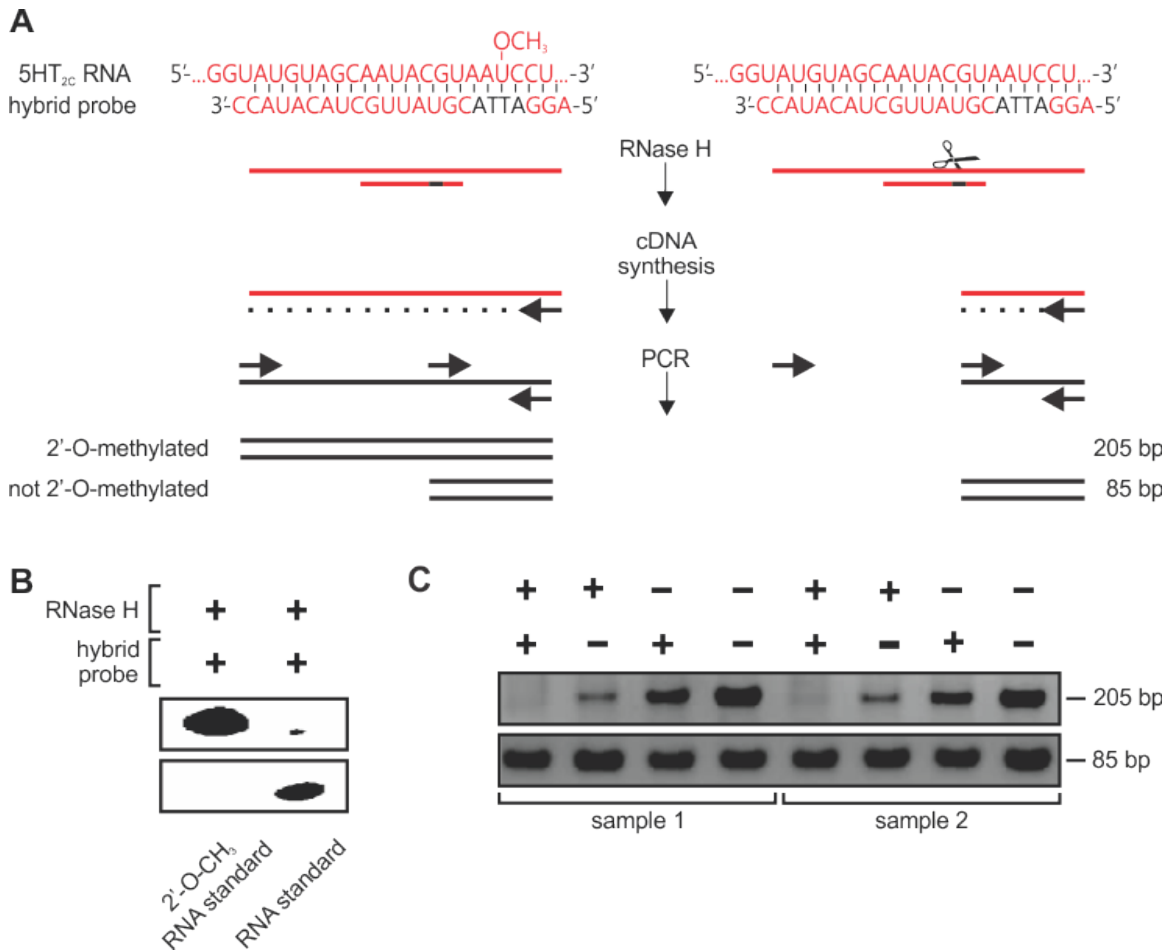
Figure 24. **Analysis of site-selective 2'-O-methylation in 5HT$_{2C}$ transcripts. A**) Schematic diagram depicting an experimental strategy to detect 2'-O-methylation in 5HT$_{2C}$ transcripts using a hybrid DNA/RNA oligonucleotide probe. A portion of the 5HT$_{2C}$ mRNA is shown with the predicted position of SNORD115-mediated 2'-O-methylation (-OCH$_3$), as well as the hybrid DNA/RNA oligonucleotide probe designed to direct RNAse H-mediated cleavage in non-modified 5HT$_{2C}$ transcripts; RNA residues are indicated in red and DNA residues are indicated in black. After incubation with RNase H, RT-PCR amplification of RNAs corresponding to cleaved (non-modified) and non-cleaved (2'-O-methylated) forms of 5HT$_{2C}$ transcripts were amplified with sense primers located up- and downstream from the predicted cleavage site and a single antisense primer (bold arrows), giving rise to PCR amplicons of 205 and 85 bp. **B**) 2'-O-methylation analysis of synthetic 5HT$_{2C}$ RNA standards (31 nt) corresponding to non-modified and 2'-O-methylated transcripts were digested with RNase H in the presence of the hybrid DNA/RNA oligonucleotide probe and the reaction products were resolved by denaturing polyacrylamide gel electrophoresis to generated the expected 31 and 11 nucleotide products. **C**) 2'-O-methylation analysis of whole brain RNA isolated from mutant mice solely expressing the non-edited (INI) isoform of the 5HT$_{2C}$ receptor. Representative data from two animals is shown where the expected migration positions for the RT-PCR products derived from 2'-O-methylated (85 bp) and non-modified (205 bp) 5HT$_{2C}$ transcripts are presented.

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Discussion

Previous studies have shown that the maternally imprinted snoRNA, SNORD115, can alter both the splicing and editing of $5HT_{2C}$ transcripts in a heterologous expression system [88], providing a provoking and straightforward mechanism by which to link the $5HT_{2C}$ gene expression with the Prader-Willi locus on chromosome 15q11-13. These observations are consistent with analyses indicating that $5HT_{2C}$ RNA editing is increased in autopsy samples from PWS patients [88] and a mouse model (PWS-IC^{del}) that also demonstrates alterations in $5HT_{2C}$-related behaviors including reactivity to palatable foodstuffs and impulsivity [116]. In the present study, $5HT_{2C}$ RNA editing was minimally affected by PWS diagnosis, although the possibility that this RNA modification is differentially regulated in other brain regions or specific cell types cannot be ruled out. However, a significant reduction in transcripts encoding the VNV isoform of the receptor and an increase in transcripts encoding the VSV isoform (Figure 22) was observed. These findings are consistent with previous analyses of PWS IC$^{del+/-}$ mice which also showed an increase in VSV-encoding transcripts [113]. Interestingly, these differences primarily result from changes in editing at the C site, the adenosine predicted to be targeted for 2'-O-methlyation by SNORD115 [100].

Previous studies have shown that 2'-O-methylation dramatically reduces the efficiency of ADAR-mediated deamination [117, 118], yet stable methylation of the ribose at the C site in $5HT_{2C}$ mRNAs from mutant mice could not be detected. Several clinical cases have been identified that either suggest deletion of SNORD115 is not sufficient to cause a PWS-like phenotype [119] or that a specific paternal deletion of the SNORD116 (HBII-85) snoRNA cluster can result in PWS [120-122]. While most recent clinical and mouse studies have focused upon the SNORD116 snoRNA cluster as playing a causal role in PWS [95, 96, 119-121], the

targets of these imprinted snoRNAs remain largely unknown, raising the possibility that alternative mechanisms exist by which genes located within the 15q11-13 locus can alter $5HT_{2C}$ receptor function independent of SNORD115 expression.

The role of SNORD115 is not well understood, and several reports suggest disparate models related to its function and biology [123-125]. Studies in heterologous systems have provided evidence that it not only affects the splicing of $5HT_{2C}$ transcripts, but also the splicing of other pre-mRNA transcripts [123]. The present studies suggest that SNORD115 promotes the production of RNA2 at the expense of RNA1. In support of these hypotheses, reduced RNA2 and increased RNA1 levels were observed in RNA samples from PWS patients lacking SNORD115. The functional consequences of increased expression for the truncated RNA1 gene product have been examined recently to show that this alternatively spliced isoform may serve to decrease the accumulation of functional $5HT_{2C}$ receptors at the cell surface [112] by nuclear retention of heterodimers in the endoplasmic reticulum. Reductions in surface expression are predicted to reduce $5HT_{2C}$ signaling in POMC neurons, thereby increasing food consumption by decreasing the anorexigenic effects of serotonin, consistent with the hyperphagia observed in PWS.

Although limited to a single brain region, analysis of receptor density in the striatum of PWS patients demonstrated a nearly two-fold increase, with no concomitant increase in steady-state mRNA levels (Figures 18, 23). By contrast, mutant solely expressing the fully-edited (VGV) isoform of the $5HT_{2C}$ receptor displayed a 40-70-fold increase in receptor protein [24, 112]. It is not clear how increased $5HT_{2C}$ receptor density may contribute to the etiology of PWS, yet it appears to be a common feature for PWS patients and mutant VGV mice which phenocopy many aspects of this disorder [24]. While the magnitude of these differences cannot

be compared directly, it should be noted that such differences could result from alterations in basal $5HT_{2C}$ signaling. The major $5HT_{2C}$ RNA isoforms expressed in mouse and human brains encode the VNV and VSV isoforms of the receptor, respectively (Figures 21 and 22), displaying significantly different signaling properties relating to G-protein coupling and constitutive activity.

The present studies have shown changes in $5HT_{2C}$ alternative splicing (Figure 18), RNA editing (Figure 21) and receptor density (Figure 23) for RNA samples isolated from dissected brain regions of PWS patients compared to controls, indicating that genes within the PWS locus either directly or indirectly modulate $5HT_{2C}$ expression. Observations that mutant mouse models of this disorder share aspects of altered $5HT_{2C}$ expression [113] and changes in $5HT_{2C}$-mediated behavior [87], as well as studies indicating that introduction of editing alterations in mutant mice can phenocopy several aspects of PWS [24] further supports a relationship between $5HT_{2C}$ receptor expression and the Prader-Willi critical region. Given the complex relationship between editing, splicing and receptor density observed in VGV mutant mice [24], it is not surprising that changes in all three aspects of $5HT_{2C}$ expression also were observed in PWS patients. Although multiple mouse models lacking expression of one or more PWS candidate genes have implicated several loci with aspects of the PWS phenotype, these models vary widely in their capacity to recapitulate the human disorder [93-96, 126], suggesting that individual genes or gene combinations may be responsible for only a subset of syndrome phenotypes. The $5HT_{2C}$ receptor has been implicated directly in the anorectic modulation of food intake using both genetic models and pharmacological approaches [43, 48, 50], thereby making it a promising target for therapeutic intervention in Prader-Willi Syndrome.

Chapter IV

COMPARATIVE ANALYSIS OF A-TO-I EDITING IN HUMAN AND NON-HUMAN

PRIMATE BRAINS

5HT$_{2C}$ editing profiles in humans and non-human primates

One of the most interesting observations made while analyzing 5HT$_{2C}$ editing profiles in human tissue samples was the remarkable variability observed between individuals. This contrasts with observations in rodents reared in a laboratory setting that display near identical editing profiles regardless of experimental exposure (Chapter 1) [51, 127]. The observed variability in the human population provides an opportunity to investigate the interrelatedness of site-selective editing for different transcripts across development, as well in discrete anatomical regions. It is fascinating to consider the prospect that these inter-individual differences result from active processes regulating A-to-I conversion and that editing could be dynamically-tuned throughout life to provide physiological advantage. Alternatively, differences in editing between transcripts and brain regions could be relatively stochastic, dictated by a molecular balance encoded by the genomic differences represented by this cohort of individuals. In an effort to differentiate between these possibilities, we examined editing profiles for several editing targets in human brain samples and compared them to equivalent RNA samples isolated from the Rhesus macaque (*Macaca mulatta*) raised in a controlled laboratory environment in hopes of better discriminating the effects of environment and genetics on A-to-I editing.

In addition to 5HT$_{2C}$ RNAs, numerous additional transcripts in the nervous system are modified by A-to-I editing to introduce non-synonymous amino acid alterations that affect protein function. For these studies, we selected several of these editing sites for further analysis

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including: 1) the Q607R editing site on transcripts encoding the GluR-2 subunit of the AMPA-subtype of ionotropic glutamate receptor [128]; 2) the R/G site of transcripts encoding the GluR-4 subunit of the AMPA receptor [129-130]; 3) the Q124R site on transcripts encoding type 4-metabotropic glutamate receptor (mGluR4) (Chapter 2); 4) the I342V site on transcript encoding the α_3-subunit of the ionotropic γ-amino butyric acid receptor (GABRA3) [131]; and 5) the E1250G site in transcripts encoding Ca^{2+}-dependent activator protein for secretion (CAPS1) [132]. Each of these substrates is widely expressed throughout the brain and encodes a protein critical for nervous system function including components of the synaptic signaling machinery ranging from pre-synaptic modulation (mGluR4), postsynaptic inhibition (GABRA3) and postsynaptic excitation (GluR2/4), to vesicle fusion and release (CAPS1). The functional consequences resulting from editing-dependent amino acid alterations for some of these RNAs have been well characterized [16, 19, 29, 34, 131] while others are the subjects of ongoing research. To probe the interrelationship of RNA editing at multiple sites from dissected brain regions in two primate species, simultaneous quantification of RNA editing profiles may be performed in each RNA sample, enabling us to gain mechanistic insights from patterns of editing within individuals and across brain regions. We carried out these studies by measuring the extent of editing for each of these seven substrate transcripts in matched hippocampus, striatum, and frontal cortex across the human cohort used in the PWS studies in addition to a cohort of 12 rhesus macaques.

<center>**Methods**</center>

Quantitative analysis of editing profiles

The human RNA samples analyzed for these studies are the same PWS and control samples analyzed in Chapter III. Primers designed to amplify each respective editing site of interest were used in the initial five cycles of RT-PCR amplification as described for $5HT_{2C}$ transcripts in Chapter II. For this study, primers were designed to selectively amplify the edited region of the transcripts encoded by the following genes: GABRA3, GRM4, GRIA2, GRIA4, and CADPS. Due to unique sequences resulting from alternative exon inclusion, we also could discriminate between the alternatively spliced GRIA4-Flip and GRIA4-Flop isoforms providing additional information about potential relationships between splicing and editing.

RNA editing profiles were quantified using next-generation sequencing as schematized in Figure 2 [102]. Amplified products for each RNA target were pooled, separated in a 2% agarose gel and purified from excised gel slices using the Wizard SV Gel and PCR Purification Kit (Promega). The concentration of gel-purified fragments was determined by spectrophotometry (A_{260}) and ~20 ng of each sample was pooled with up to 24 other products containing unique barcodes and subjected to single-end sequencing using the MiSeq platform (Illumina).

Informatics

The FASTX-Toolkit was used to resolve the sequencing reads according to each of 24 different barcodes incorporated during the second round of PCR amplification [102]. For each read, pairwise alignments were made against all reference sequences allowing for only one A-to-G mismatch at the editing site and assigned to match its corresponding candidate gene. The

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extent of editing at each site was calculated as the percentage of edited (G) reads for each substrate divided by the total number of reads (G + A) for each target.

Statistical Analysis

Analysis of covariance was performed by quantifying the extent of site-selective editing by the patient diagnosis, age, sex, and PMI. Correlations were analyzed for statistical significance using Student's t-test for disease diagnosis and gender or linear regression analysis for continuous variables including ADAR expression, age and PMI. No significant effect was identified for any of these variables. Linear regression was also used to compare the extent of editing in different brain regions and the extent of editing for different substrates.

Quantification of ADAR mRNA expression

Substrate-specific Taqman probes and primer sets were selected to detect steady-state mRNA expression levels for ADAR1 (Hs00241666_m1 and Rh02801420_m1) and ADAR2 (Hs00953724_m1 Rh00955199_m1) (Applied Biosystems). Real-time RT-PCR was performed as described in Chapter 2 and $\Delta\Delta C_t$ values were determined by comparing cycle threshold for each ADAR transcript to an 18S ribosomal RNA internal control (4319413E).

Results

Covariant analysis of CADPS, GluR2, GluR4, GABRA3, and mGluR4

We quantified the extent of editing for several substrates by high-throughput sequence analysis in matched hippocampus, striatum, and frontal cortex samples from both humans and Rhesus macaques. After data collection, we performed analyses of covariance indicating that gender, age, postmortem interval or PWS diagnosis did not differentially affect editing profiles. None of these factors appear to correlate with the extent of editing for any of these substrates in the brain regions analyzed (ANCOVA, $p \geq 0.05$).

Differential regulation of A-to-I editing in different substrates

Previous studies have demonstrated characteristic editing profiles in facility-housed inbred rodents for specific ADAR targets within dissected brain regions [51, 64, 133], yet the extent of editing can vary widely between different RNA targets within the same brain region, For example the transcript encoding the GluR-2 subunit of the AMPA-subtype of glutamate receptor (GRIA2), is edited nearly 100% of the time in rodents (Q/R site), while other ADAR2 substrates such as the D site in $5HT_{2C}$ transcripts is edited only ~60% of the time [51, 64, 133]. Similarly, another ADAR2 substrate, the mRNA encoding the type-4 metabotropic glutamate receptor (GRM4), is edited around 10-15% in most tissues analyzed (Figure 25). Such dissimilarities in editing could be explained by cell-specific differences in the expression/activity of ADAR proteins, but not for ADAR targets expressed within the same neuron. An alternative interpretation of these observations is that RNA substrates have unique intrinsic propensities to undergo hydrolytic deamination and are therefore autonomously regulated by differential affinity and/or availability. By simultaneously monitoring the editing efficiency of multiple substrates

across diverse populations of individuals, we can make inferences about the co-regulation of substrates across brain regions and learn more about the nature of how A-to-I editing is regulated on a substrate-to-substrate basis.
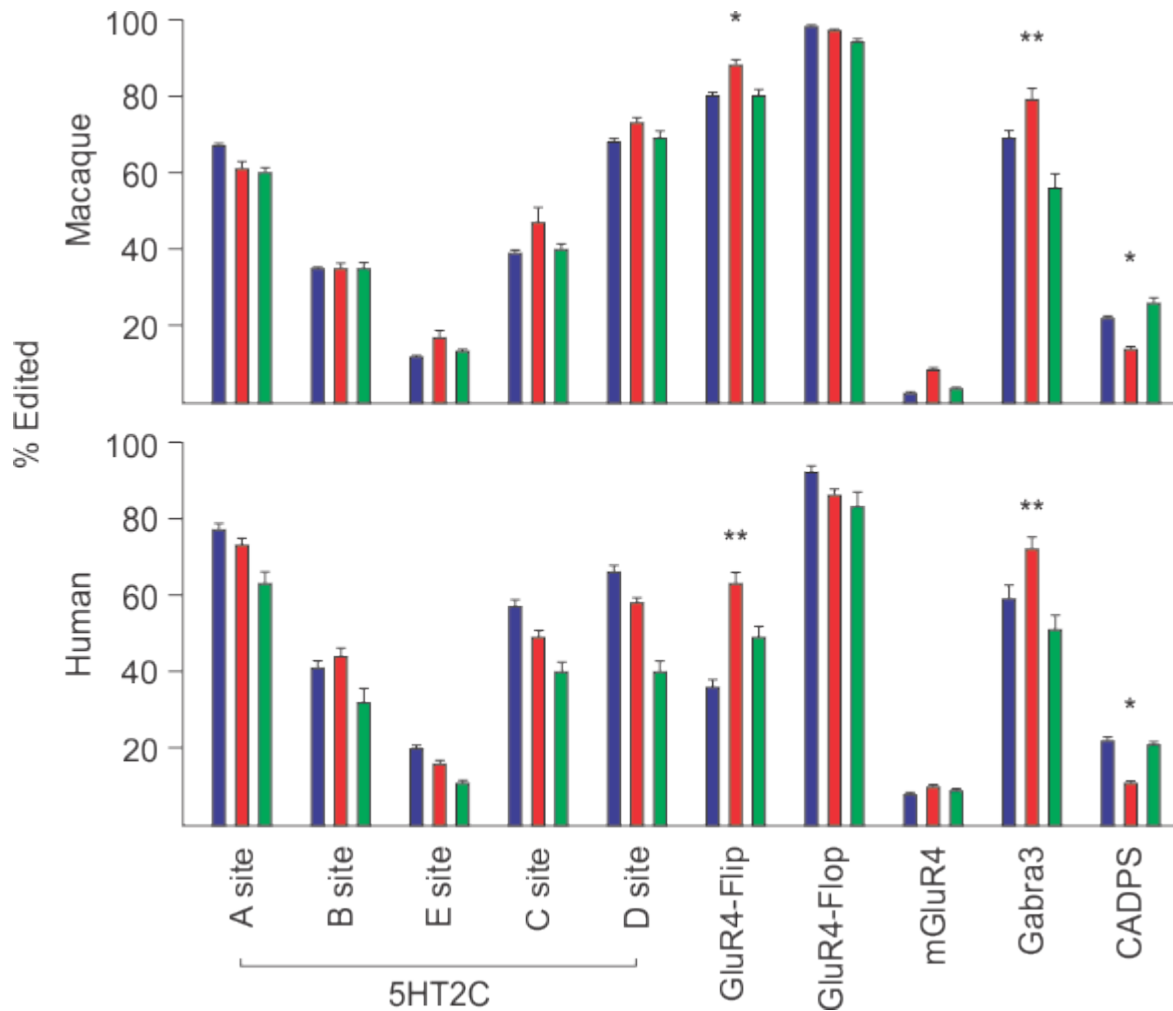
Figure 25. Quantitative analysis of editing efficiency for ADAR targets in samples isolated from the striatum, cortex, and hippocampus of humans and macaques. A) The extent of site-selective editing is shown for RNA samples isolated from the macaque putamen, motor cortex, and hippocampus. (n=12). Error bars represent the mean ± SEM; *p ≤ 0.01, **p ≤ 0.001. **B)** The extent of site-selective editing is shown for RNA samples isolated from human striatum (blue), cortex (red), and hippocampus (green). (n=28).

Overall patterns of editing in the brain

Editing was quantified for multiple RNA substrates in dissected brain regions isolated from human and macaque cohorts. The values for $5HT_{2C}$ editing profiles in three and eight hippocampal samples isolated from macaque and human brain, respectively, was excluded from the dataset due to choroid plexus contamination as described in Chapter III (Figure 16). The mean extent of editing observed at each respective substrate in these two species was generally similar, however considerably more variability was observed in the human population compared to the macaque cohort for each substrate (Figures 25 and 26). Several editing patterns emerged that appeared to be highly conserved in both rhesus macaques and humans. In both species, the extent of GluR4-Flip, mGluR4, and GABRA3 editing was significantly higher in the cortex then either the striatum or hippocampus (2-way ANOVA, $p \leq 0.01$). Editing patterns for GluR4-Flip, Gabra3, and CADPS were conserved across species, while editing in the cortex was significantly higher for GluR4-Flip and GABRA3, while CADPS editing was significantly lower in cortical tissue. Importantly, the extensive variability in editing observed in the RNA samples isolated from the human cohort was largely driven by a few individuals who displayed profound deficiencies in editing at a few, or in some cases all, of the substrates analyzed. In both species, the greatest variability in editing was observed for transcripts encoding the α_3-subunit of the GABA receptor (GABRA3). While site-selective editing patterns were found to be similar in humans and macaques, editing for GluR4-Flip and mGluR4 RNAs was much higher and lower, respectively, in all three brain regions of the macaque compared to corresponding human samples (Figure 26). These results suggest an evolutionary divergence in site-selective editing patterns, yet the functional significance of these findings is difficult to interpret due to a limited understanding concerning the precise role(s) played by these edited isoforms *in vivo*.
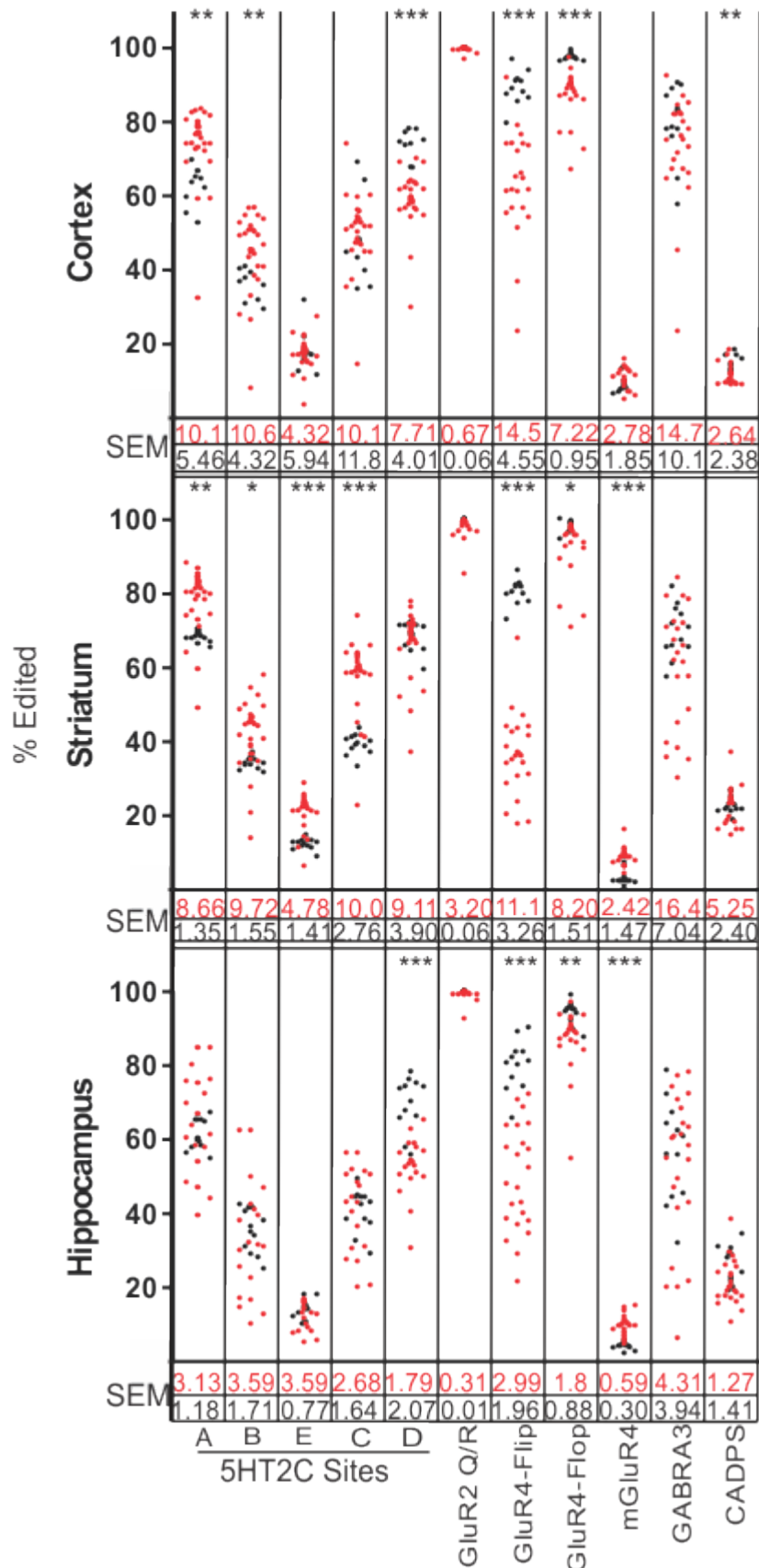
Figure 26. Quantitative analysis of region-specific, site-selective RNA editing patterns in human (■) and macaque (■) brain. The percentage of editing for site-selective modification of ADAR targets in dissected brain regions is shown; SEM values for the human (red) and macaque (black) cohort for each transcript are provided (human n=28, macaque, n=12).

Conversely, another pattern observed in both species was that CADPS editing was significantly lower in the cortex than in hippocampus or striatum ($p \leq 0.01$) (Figure 25). These results show that although editing is regulated in a region-specific manner in the brain, this pattern of RNA modification is conserved between humans and macaques. The fact that evolution has retained these patterns across primate species suggests that the extent of editing for these substrates is important for conferring evolutionary fitness.

Co-regulation of RNA editing

When profiling editing patterns in the human cohort, a higher level of variability was observed than previously identified for other species (Chapter 1). This variability was largely driven by a few individuals with large deficiencies in editing at specific substrates, or in some cases, a deficiency in editing for all mRNAs examined. We took advantage of this variation to examine potential co-regulation of editing across substrates and brain regions.

As observed previously, editing at each of the five sites for $5HT_{2C}$ transcripts was highly co-regulated [134], where the extent of editing at any one site generally predicted editing efficiencies at the other sites. These observations suggested an interdependence of editing within $5HT_{2C}$ mRNAs that may result from cooperation or a common rate-limiting step that controls editing at each of the five sites. This finding is not surprising considering that each of the $5HT_{2C}$ editing sites is located in close proximity within the same RNA substrate. Similar correlations between the editing of $5HT_{2C}$ transcripts and GABRA3 RNAs were also identified, specifically in the cortex (Pearson correlation $p \leq 0.0001$, $r^2 = 0.8$) (Figures 27 and 28). This relationship was not

Figure 27: Correlational analysis of 5HT$_{2C}$ editing patterns across human brain regions. Pair-wise comparisons of the percentage of site-selective editing for 5HT$_{2C}$ transcripts across human brain regions is presented; $p \leq 0.02$, $r^2 \geq 0.39$ for each site by linear regression analysis.

Figure 28. Correlational analysis of RNA editing patterns across human brain regions. Pair-wise comparisons of the percentage of site-selective editing for several ADAR substrates across human brain regions is presented; Linear regression analysis identified significant correlations for GABRA3 editing in the striatum and cortex, and mGluR4 in striatum and hippocampus ($p \leq 0.01$, $r^2 \geq 0.4$; *highlighted in red*).

Figure 29: Correlational analysis of 5HT$_{2C}$ editing patterns across macaque brain regions. Pair-wise comparisons of the percentage of site-selective editing for 5HT$_{2C}$ transcripts across macaque brain regions is presented.

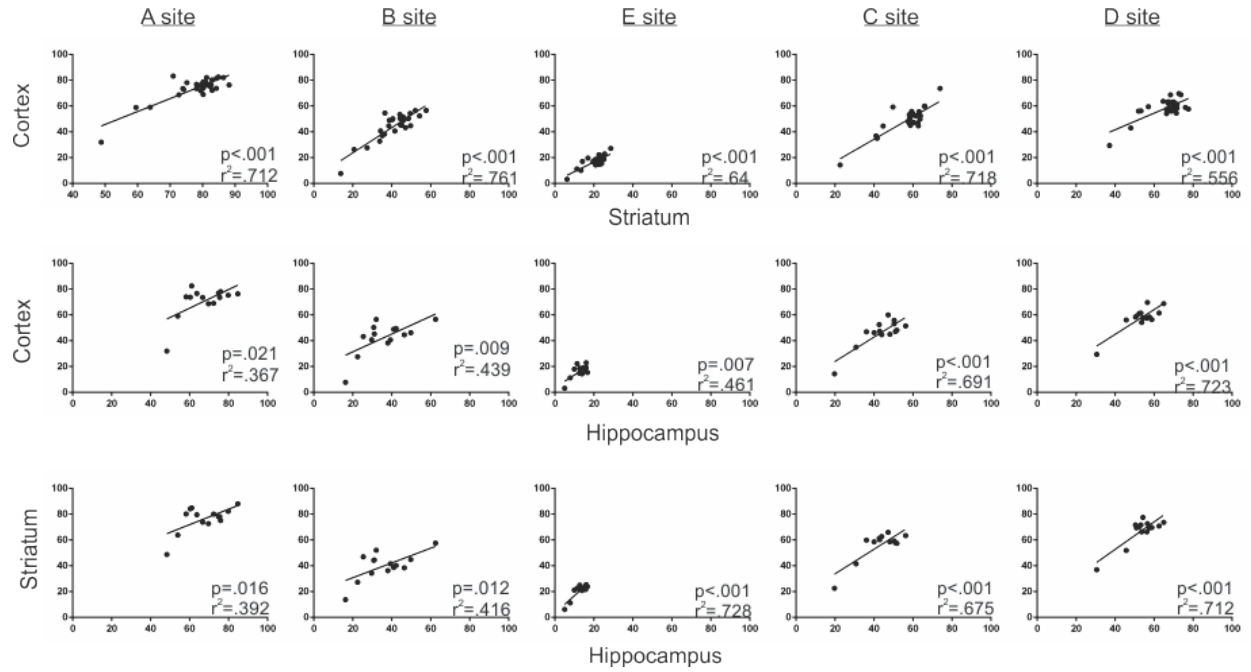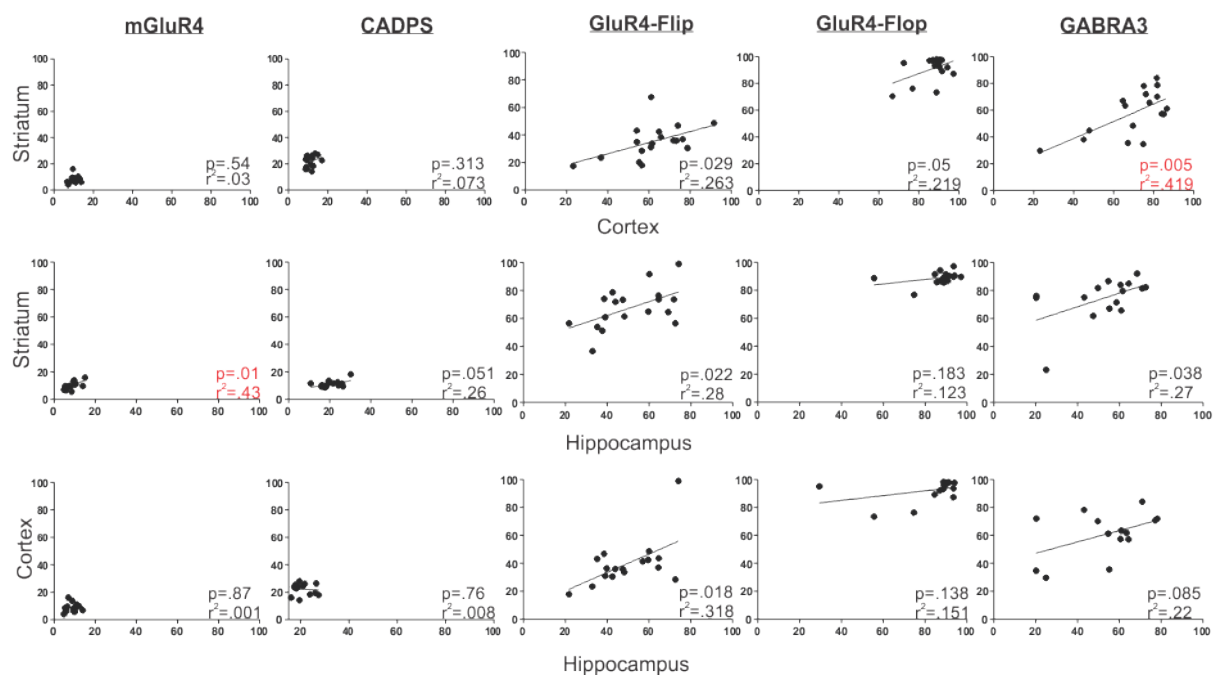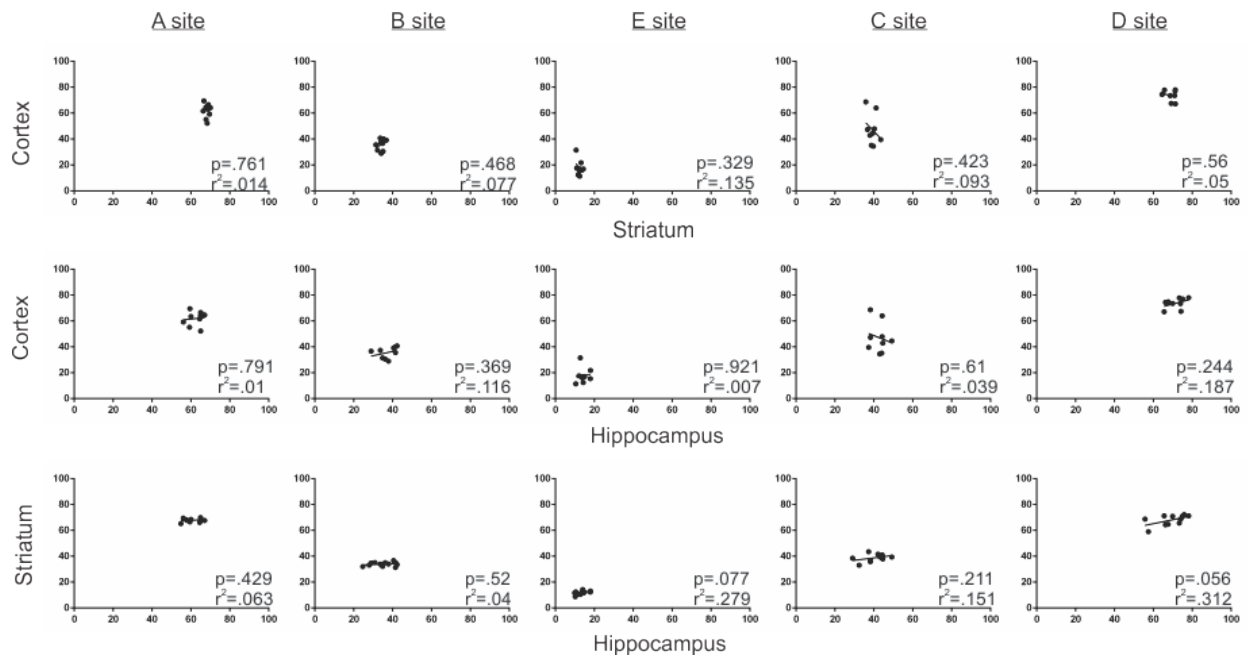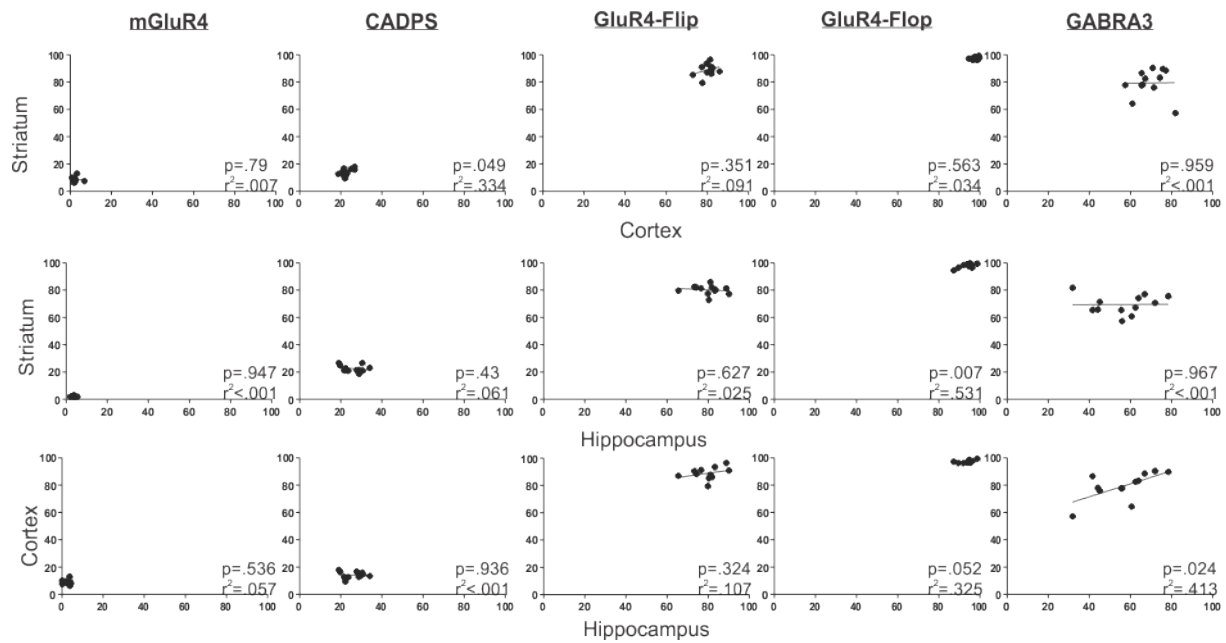**Figure 30. Correlational analysis of RNA editing patterns across macaque brain regions.** Pairwise comparisons of the percentage of site-selective editing for several ADAR substrates across macaque brain regions is presented; Linear regression analysis identified significant correlations for CADPS editing in striatum and cortex, and GABRA3 editing in cortex and hippocampus ($p \leq 0.05$, $r^2 \geq 0.3$).

observed in striatum or hippocampus, despite similar levels of variability suggesting that the mechanisms or cell types regulating editing for these substrates are similar in cortex, but distinct in the hippocampus and striatum. This result implies that the stochastic events conferring variability in RNA editing for these two substrates may be similar in cerebral cortex.

Another interesting relationship was the direct correlation observed for editing of GluR4-Flip and GluR4-Flop transcripts. The extent of editing at GluR4-Flip turned out to be a strong predictor of the extent of editing at GluR4-Flop transcripts, specifically in the cortex (Pearson correlation $p \leq 0.001$, $r^2 = 0.62$). This finding could be related to the fact that these transcripts are generated by alternative splicing of the same pre-mRNA, and thus are subject to similar regulatory mechanisms. This effect was not observed in hippocampus or striatum further supporting the hypothesis that region- or cell-specific processes regulate A-to-I RNA editing for certain substrate RNAs.

The finding that editing patterns for some substrates show significant correlations within specific brain regions, but not in others, could be explained by differences in the co-expression of these transcripts within the same cells in those respective brain areas. Detailed characterization of the co-expression profiles for these transcripts would be required to determine whether these relationships are driven primarily by cellular context or by substrate-specific mechanisms. While not mutually exclusive, these two possibilities carry unique implications regarding the mechanisms regulating editing. It would be interesting to test the hypothesis that editing for one ADAR2 substrate could be increased while editing of another ADAR2 substrate would be decreased in the same cell. Unfortunately, the difficulty of single-cell transcript analysis in postmortem human tissue precludes direct assessment of this model.

Global regulation of editing across brain regions

The variability in site-selective editing observed in the human cohort also has allowed us to address the hypothesis that editing for some substrates is regulated globally across different brain regions. We examined whether editing in one brain region could accurately predict the editing patterns in another brain region within the same individual. An analysis of co-dependency was performed for site-selective editing for each substrate across brain regions by linear regression analysis and we considered any regression with a non-zero slope ($p \leq 0.01$ and an $r^2 \geq 0.4$) sufficient to support the hypothesis that similar mechanisms may be actively regulating editing of the substrate in discrete brain regions. Editing of $5HT_{2C}$ receptor transcripts appears to be uniquely co-regulated in the cortex and striatum as determined by linear regression analysis (Pearson correlation $p \leq 0.0001$, $r^2 \geq 0.5$ for each site) (Figure 27). We also observed evidence for co-regulation of editing at GABRA3 transcripts in cortex and striatum ($p \leq 0.005$, $r^2 = 0.42$), albeit to a lesser extent than observed for $5HT_{2C}$ editing. These results indicate that the editing of $5HT_{2C}$ and GABRA3 transcripts may be globally regulated by similar mechanisms in these two brain regions.

We also observed a significant correlation for GRIA2 editing patterns across brain regions (Figure 28), but these results are difficult to interpret due to the limited range; as ~98-99% of the transcripts were edited in all tissues from all individuals with the exception of a single patient (#1199). This individual had the lowest levels of GRIA2 editing in both striatum and cortex, implying some primary deficiency in editing for this substrate. Unfortunately, hippocampal tissue was not available from this patient, so we were unable to confirm this finding in a third brain region. Attempts to reproduce these findings in the rhesus macaque cohort were precluded by the relatively low levels of inter-animal variability (Figure 26). Despite significant

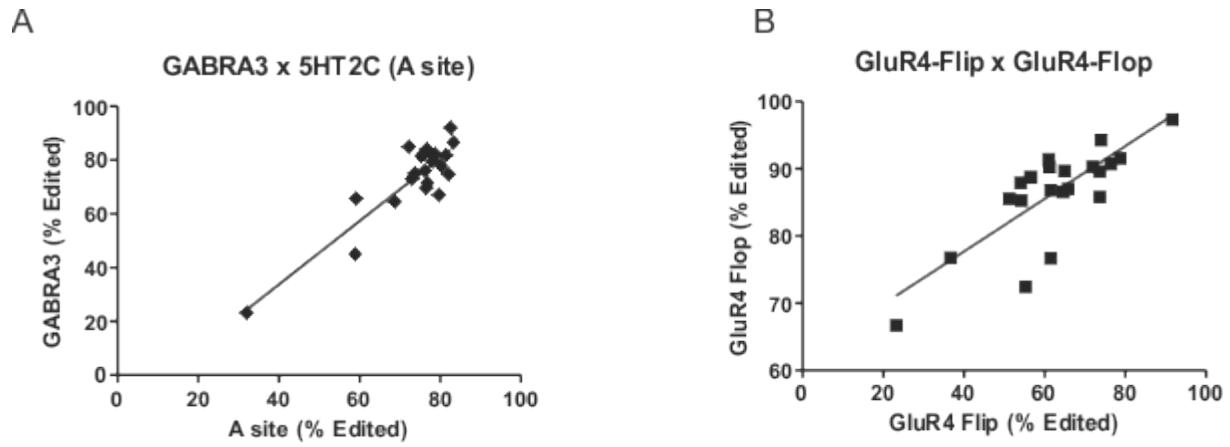**Figure 31. Pair-wise correlational analysis of editing patterns for multiple ADAR substrates in human cortex. A)** GABRA3 editing directly correlates with $5HT_{2C}$ editing in the cortex (A site shown, $p \leq 0.0001$ $r^2 = 0.8$) **B)** The editing of GluR4-Flip and GluR4-Flop RNAs correlate in the cortex ($p \leq 0.0001$ $r^2 = 0.62$), but not in the hippocampus or striatum.

genetic diversity in this region. Attempts to reproduce these findings in the rhesus macaque cohort were precluded by the relatively low levels of inter-animal variability. Despite significant genetic diversity in this population (Dr. Judy Cameron, *personal communication*), editing patterns varied little between individuals. These animals were raised in a highly-controlled environment, compared to the diverse environments experienced by individuals in the human cohort. These observations support the notion that environmental factors may influence the extent of editing in humans, although we cannot rule out contributions of genetic diversity.

Analysis of RNA editing patterns in the Rhesus macaque

The Rhesus macaque (*Macaca mullata*) is a valuable model organism for biomedical research and one of the best studied non-human primates. Relatively few studies have investigated RNA editing in primates, so little is known about the relative conservation of editing patterns between macaques and humans. Detailed analysis of RNA editing in rhesus monkeys can provide insights related to the genetic contribution to editing profile that are precluded in inbred rodent lines with limited genetic diversity. We obtained matched RNA samples extracted from motor cortex, putamen, and hippocampus from 12 rhesus macaques and performed high-throughput sequence analysis for the same set of substrates analyzed in human tissue samples: $5HT_{2C}$, GluR-2, GluR-4, mGluR4, GABRA3, and CADPS. The animals used in this study were ovariectomized adult females classified as either control (n=6) or hypoactive (80% physical activity, n=6) by continuous monitoring using an accelerometer [135]. Covariant analysis did not reveal any effect of physical activity on editing profiles (*data not shown*). As mentioned previously, extensive conservation of editing patterns were found between individual animals, as

well as some similarity to the patterns observed in humans. The major difference that was readily observed involved the extent of intra-individual variability (as measured by standard deviation from the mean) which was generally much less than that observed in the human cohort (Figure 26). Making comparisons between macaques raised in a highly controlled environment and humans living under diverse circumstances allows us to infer the effect of environment on relevant variables. The similarity in editing patterns observed in macaques compared to variable patterns in the human cohort supports the idea that site-selective editing may be dynamically influenced by the environment.

ADAR expression and editing profiles

To test the hypothesis that site-selective editing patterns are simply regulated by ADAR expression, we measured steady-state mRNA expression levels for ADAR1 and ADAR2 in each tissue sample by quantitative RT-PCR. ADAR expression levels were highly variable between individuals and brain regions and no significant correlations were observed between site-selective editing patterns and either the level of ADAR mRNA expression or the ADAR1:ADAR2 ratio for any of the substrates included in these studies. These results suggest that ADAR mRNA expression does not play a primary role in regulating editing patterns *in vivo,* although the level of ADAR protein or ADAR activity in each tissue sample represents a superior variable for comparison. The results of these studies did reveal a surprising relationship between mRNA expression levels for ADAR1 and ADAR2 (Figure 32) where a significant correlation was observed between the ratio of ADAR1 to ADAR2 that varied significantly between brain regions. Interestingly, these fixed ratios for ADAR1:ADAR2 mRNA expression appear to be largely conserved in humans and Rhesus macaques.
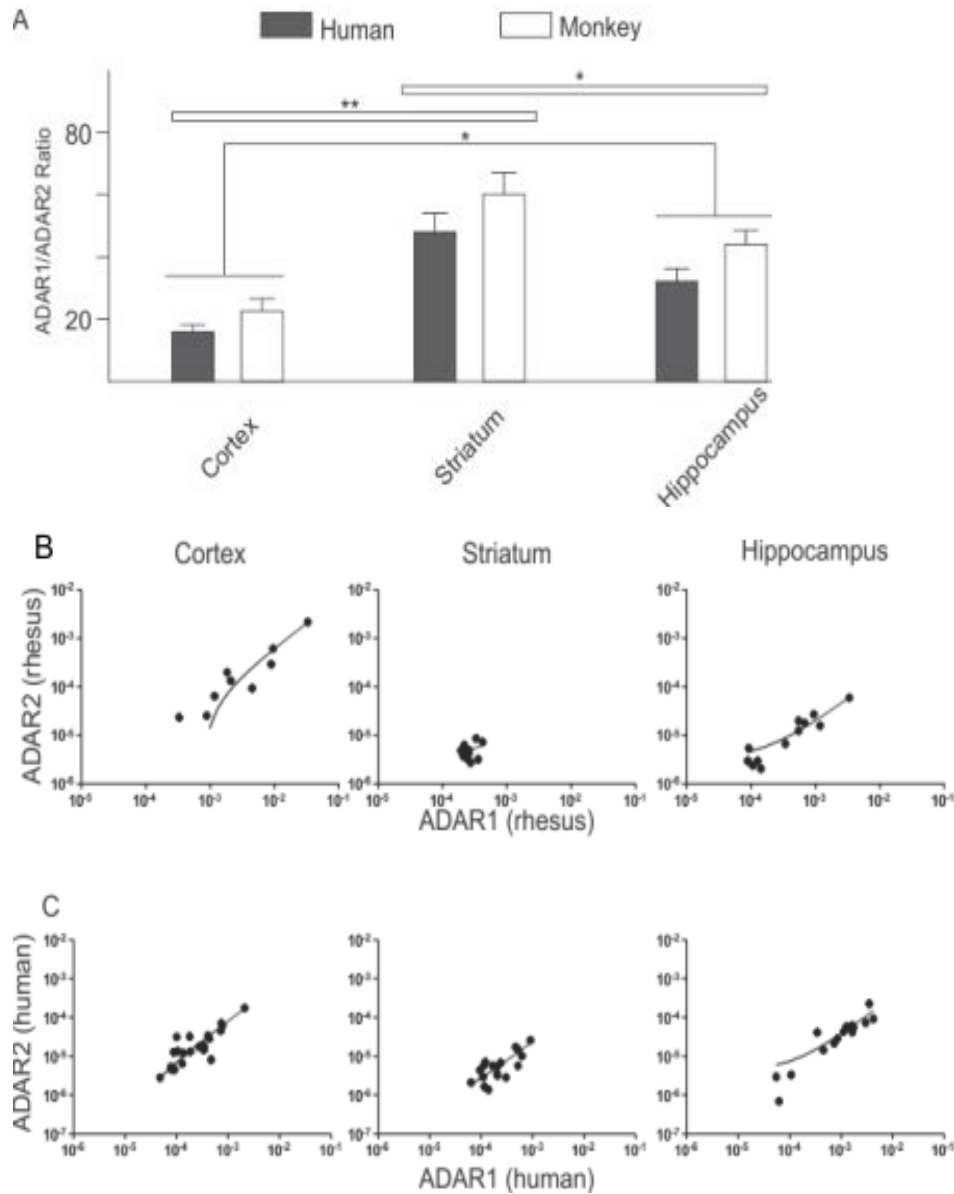
Figure 32. Region-specific ADAR1 and ADAR2 expression in human and rhesus macaque brain. A) The mean ADAR1:ADAR2 ratio in each brain region is shown; and no significant differences were observed between species. Asterisks represent significant differences between mean combined ratios of both species in each brain region as determined by 1-way ANOVA with Bonferroni post-test (*$p \leq 0.01$, **$p \leq 0.001$). **B)** Linear regression analysis of ADAR1 and ADAR2 expression in rhesus macaque reveals a significant correlation between ADAR expression in cortex ($p \leq 0.0001$, $r^2 = 0.93$) and hippocampus ($p \leq 0.0001$, $r^2 = 0.92$). **C)** Linear regression analysis of ADAR1 and ADAR2 expression in human reveals a significant correlation between ADAR expression in cortex ($p < .0001$, $r^2 = 0.91$), striatum ($p \leq .0001$, $r^2 = .72$), and hippocampus ($p \leq .0004$, $r^2 = 0.62$).

Discussion

One of the most interesting observations made while analyzing A-to-I editing in human brain tissue samples was the remarkable inter-individual variability. This contrasts with the observations made in rodents reared in a laboratory setting which display similar editing profiles regardless of experimental exposure. The variability in this human cohort provides an opportunity to examine the hypotheses that either environment or genetics are capable of influencing the profile of edited RNA isoforms expressed. An exciting prospect to consider is one where the processes regulating editing can be dynamically tuned throughout life in response to altering physiological cues. Alternatively, the observed differences could be stochastic and dictated by a molecular balance coded in the diverse genomes undoubtedly represented by this cohort of individuals. In an effort to differentiate between these models, we quantified editing profiles for several ADAR substrates in matched brain samples across brain regions in human populations. Additionally, we assessed editing in non-human primates raised in a controlled laboratory environment to better discern the effects of environment and genetics.

The results of these studies indicate that there are global mechanisms responsible for regulating editing for each of these substrates. This is clearly demonstrated in cases for certain individuals with global deficiencies in A-to-I editing that are seen across both ADAR substrates and brain regions. Conversely, several examples of context-specific regulation of editing also were observed, as some individuals demonstrated deficiencies in site-selective editing for only a few substrates in specific brain regions. Taken together, these results imply that several distinct levels of regulation exist which can affect the efficiency of editing either globally or can be limited to specific contexts.

These studies clearly show that evolution has conserved mechanisms for maintaining precise site-specific editing profiles for ADAR targets across brain regions in adult mammals. Divergent patterns observed in some human individuals suggest that while editing often falls within characteristic ranges, it can vary widely. The fact that evolution has conserved editing patterns within specific ranges across species, suggests that the differences in editing observed for some human individuals are likely to have subsequent consequences for CNS function [136]. It will be important to identify the neurological consequences of altered editing profiles by studying larger human cohorts with detailed psychiatric and neurological histories. These further studies, in large human populations, will also facilitate discovery of gene-environment interactions and factors driving certain "abnormal" RNA editing profiles. Identification of factors capable of influencing site-specific editing profiles will be essential for building a more complete models of CNS function and potentially even exploited for therapeutic purposes.

Chapter V

CONCLUSIONS

Through the course of these studies a number of insights have been revealed about the nature of A-to-I editing in the CNS. It is apparently a process under tight control in specific brain regions. Unfortunately, failure to induce alterations in editing profiles in response to genetic or pharmacological manipulation *in vivo* precludes a more detailed interrogation of the precise mechanisms conferring dynamic regulation. However, some of the results of this work lend themselves to the development of new models related to how editing might be regulated. A primary observation is that some substrates are edited nearly 100% of the time while others vary widely. Presumably, many of these transcripts are expressed in overlapping cell populations. These observations indicate that substrate-specific factors play a role in the regulation of A-to-I conversion. These differences could result from differences in ADAR activity, or due to the intrinsic affinity of specific substrates for the editing machinery, rendering ADAR availability rate-limiting in some cases and not in others. A third model involves substrate competition model where substrates compete for and potentially even chelate ADARs in relevant contexts: A number of transposable elements, including Alu elements in primates, are excellent non-protein coding substrates for ADARs [137]. Alu-rich RNAs and could potentially be employed to lure ADARs away from functional substrates effectively regulating the pool of active enzymes available. This is an attractive model but its contribution *in vivo* is currently only a matter of speculation. Additional layers of complexity emerge when considering the effects of editing on the function of particular gene products. Rather than a bimodal process where a substrate's function is tuned in one direction or the other by RNA editing, proteins function as multimeric complexes that can consist of subunits encoded by both edited and non-edited transcripts.

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This complexity is especially evident in the case of $5HT_{2C}$, the gene products of which appear to function as dimers [138]. An editing-heterodimerization model provides an opportunity to express over 500 unique receptor complexes. Although, based on the RNA profiles observed in most tissues, combinations between the most common 4 or 5 isoforms would be expected to predominate. Recent studies even suggest that the incorporation of certain edited isoforms into dimeric complexes could be regulated in-trans by the peptide encoded by the alternatively spliced $5HT_{2C}$ gene product RNA1 [28]. The potential diversity in signaling properties of the many heterodimer combinations that can be created forces us to consider a staggeringly complex network of functional regulation. Evolution has apparently seized upon the great functional diversity afforded by a $5HT_{2C}$ editing-splicing-dimerization network and may employ the receptor in a variety tasks [27]. The fact that this system appears to be relatively well conserved from rodents to primates highlights the importance of this particular editing network for proper CNS function.

The initial discovery of RNA editing at GluR2 AMPA receptor subunits painted a primarily developmental picture regarding the functional significance of editing. This is because ADAR transcript levels increase during the course of development and GluR2 transcripts appeared to be edited rarely early in development and essentially 100% of the time in adults. These findings led to the speculation that editing was a bimodal process where the proteins encoded by edited transcripts would be favored later in development as more ADAR enzyme is expressed. The discovery of characteristic intermediate editing at multiple sites on $5HT_{2C}$ transcripts added complexity to this model because it implied that evolution had selected functions for both edited and non-edited proteins in adult animals. The conserved nature of editing at $5HT_{2C}$ transcripts and others tells us that that a balanced expression of each

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functionally distinct isoform is required for normal physiology. This opened up the possibility that editing at some substrates could independently vary from cell to cell, individual to individual and within an individual over time. Several studies were initially launched to test the hypothesis that $5HT_{2C}$ editing is altered in the brains of people with psychiatric disorders. Unfortunately, these early studies were under powered and suffered important technical limitations that prevented meaningful systematic characterization of $5HT_{2C}$ editing.

The development of massively parallel sequencing technology opened the door for HTMTA, which provided the first opportunity to accurately assess editing profiles for several substrates in a large number of samples. The studies described here have allowed for the identification of signature editing patterns in different brain regions that are common across species. The editing profiles in humans described above indicate that, for many of the substrates analyzed, editing efficiency is maintained within specific ranges depending on anatomical context rather than simply developmental stage. This implies that the mechanisms regulating editing are specific to each substrate and dependent on factors other than overall ADAR expression level. Interestingly, significant deviations from these signature editing profiles were be observed in some individuals in the human cohort. The fact that these deviations were expressed in multiple brain regions of certain individuals implies that global mechanisms for regulating editing efficiency are at play. Attempts to identify similar deviations in primates were unsuccessful, potentially owing to the greater similarity in genetic backgrounds or the common environmental conditions (chow, medication, etc.) compared to the human population.

Only a handful of human individuals had editing profiles that fell outside of a single standard deviation from the mean editing at most sites. This confirms results from other recent studies showing that editing does not vary much between individuals [139], while adding new

evidence that editing profiles can deviate significantly in certain individuals as a result of yet unidentified factors. Importantly, these variations seem to play out on a global scale (in multiple brain regions) and in some cases in multiple substrates rather than only a single substrate in a discrete brain region. In these cases editing is presumably influenced by some up-stream effectors, genetic or environmental, which push editing profiles outside of the 'normal' characteristic range. This is the first work providing a comprehensive analysis of editing at multiple substrates in multiple brain regions in humans so included below is a detailed descriptive summary of editing at each respective transcript analyzed in these studies.

Editing patterns in the human brain

$5HT_{2C}$ editing in the human brain

$5HT_{2C}$ transcripts are thought to be edited by both ADAR enzymes in most tissues. The apparent lack of ADAR1 expression in choroid plexus [109] presumably accounts for the lack of editing at the A and B sites in this tissue. In these studies, we analyzed editing profiles in striatum, cortex, and hippocampus. Our results indicate that the mean extent of editing in the striatum and cortex is slightly higher than in the hippocampus (2-way ANOVA with Bonferroni post-test $p \leq 0.01$ at each site with the exception of the E and D sites when comparing cortex to hippocampus). The high variability in editing observed at this transcript was largely driven by three individuals with patient IDs 1113, 1199, and 1556. These individuals had low levels of $5HT_{2C}$ editing in all three brain regions indicating global deficiency in editing at $5HT_{2C}$ transcripts. A more detailed discussion about these three patients is included at the end of this chapter.

GABRA3 editing in the human brain

The highest variability observed for any editing site in this study was the GABRA3 editing site which results in a substitution of isoleucine for valine at residue 342 (I342V). GABRA3 editing is highest in the cortex where editing occurs approximately 75% of the time in most human samples and lower levels (~60%) editing is observed in the striatum and hippocampus. This substrate had the largest variability and a large number of individuals with values greater than one standard deviation from the mean in every brain region, indicating that editing at this site is highly dynamic across individuals as well as across tissues (Figure 26).

GluR4 editing in the human brain

GluR4 transcripts are subject to alternative splicing to generate the functionally distinct "Flip" and "Flop" isoforms [140]. These splice isoforms are expressed in partially exclusive cell populations in each of the brain regions analyzed [141]. As a result of these studies, a characteristic pattern of GluR4 editing emerged whereby the Flop isoform is more likely to be edited than the Flip isoform. This pattern is expressed in all three tissue types and is most pronounced in the striatum where less than half of the Flip transcripts are edited and nearly all of the Flop isoforms are edited. This could reflect a direct effect of editing on splicing or simply a result of cell-type bias in editing efficiency and expression.

mGluR4 editing in the human brain

The functional significance of editing at mGluR4 encoding transcripts has not been elucidated. This editing event predicts a glycine substitution for the genomically encoded arginine at amino acid 124 (R124G). This amino acid substitution occurs in the N-terminal extracellular domain where it has been proposed to be involved in receptor dimerization [142].

mGluR4 transcripts are infrequently edited in all tissues analyzed where the observed frequency of editing was typically ~10%. There was no clear indication that the editing pattern observed in one brain region predicted the extent of editing in others. Meaningful interpretation of the overall results is precluded by the limited current understanding of how this editing event affects mGluR4 function, as such, elucidating the functional impact of mGluR4 editing is a subject of ongoing work.

CADPS editing in human brain

The CADPS transcript encodes a protein thought to be important for docking and Ca^{2+}-activated secretion of dense core vesicles. The lowest levels of CADPS editing were observed in cortex tissue samples where the extent of editing was ~10-20%, while editing in the striatum and hippocampus was more extensive (Figure 25). Analysis of editing of tissue from Rhesus macaque confirms that this pattern is conserved in other primate species (Figure 25). There was no evidence that the site-specific editing of other ADAR substrates or editing in other brain region significantly correlated with CADPS editing. The functional role of CADPS editing is not understood and additional work to characterize the effects of this amino acid substitution will be required for meaningful interpretations about the physiological significance of these patterns.

GluR2 editing in human brain

GluR-2 (GRIA2) editing, resulting in the substitution of glutamine 607 for arginine (Q607R), was the first A-to-I editing event to be characterized [12]. Editing of this transcript is low in the developing murine nervous system, but high by adulthood [133]. Studies in a variety of mammals have revealed a highly conserved nearly 100% efficiency of editing for this

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transcript in adults. In agreement these observations, we quantified GluR-2 editing to be in excess of 99% in most of the human tissue samples analyzed for these studies (Figure 25). Interestingly, one individual (patient ID #1199) expressed a marked deficiency in editing at all substrates in each brain region analyzed. This included GluR2, where only 84%-92% of GluR2 transcripts were edited depending on the brain region analyzed. This patient was a 35 year old female diagnosed with PWS. Unfortunately, additional patient history for this individual was not available.

Global editing deficiency in three human patients

As previously mentioned, much of the variability in editing was driven by the observation that three patients had global deficiencies in editing at the transcripts analyzed in these studies; patients 1199, 1113, and 1556. Patients 1199 and 1556 were females (ages 35 and 41, respectively) who had been diagnosed with PWS although patient 1556's diagnosis was subsequently refuted by molecular diagnostic techniques described in chapter III. Patient 1113 was a 56 year old male "control" whom was reportedly a heavy smoker and died of complications related to a respiratory infection. Two of these individuals; 1199 and 1113, had editing levels more than one standard deviation below the mean for GluR4, GABRA3, and $5HT_{2C}$ transcripts in all three brain regions examined. Interestingly, patient 1556 had a similar pattern of editing deficiency in hippocampal and striatal tissue, but had normal editing (less than one standard deviation from the mean) for all other substrates (GluR2, GluR4, mGluR4, GABRA3 and CADPS) in the striatum. These results indicate that common genetic or physiological factors influence the editing of $5HT_{2C}$, GluR4, and GABRA3 transcripts across different brain regions. It also suggests that GluR2, mGluR4, and CADPS might be regulated

differently, since normal editing patterns for these substrates were observed in each of these patients.

Conclusions

This dissertation provides a more complete framework for understanding the nature of A-to-I editing. It is now clear that evolution has conserved mechanisms for maintaining precise context-specific expression profiles for certain edited transcripts in adult primates. Expression of different $5HT_{2C}$ editing profiles appears to be relatively unaffected by acute treatment with antidepressant and antipsychotic drugs, or repeated stress exposure in mice, and variations in physical activity in primates. However, divergent patterns observed in some human individuals suggest that while editing often falls within characteristic ranges, it can vary widely under certain circumstances. The fact that evolution has conserved editing within specific ranges across species suggests that the differences in editing observed in some human individuals included in these studies are likely to have consequences for CNS function. It will be important to identify the neurological consequences of altered editing profiles by studying larger human cohorts with detailed psychiatric and neurological histories. These further studies in large human populations will also facilitate discovery of gene X environment interactions and factors driving certain "abnormal" RNA editing profiles. Identification of factors capable of influencing editing profiles will provide important insights about previously unappreciated gene expression feedback processes. Once identified and better understood, these mechanisms will be used for building more complete models of CNS function and potentially even exploited for therapeutic purposes.

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