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REVIEW ARTICLE

RNA in Brain Disease: No Longer Just "The Messenger in the Middle"

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Abstract

RNA research has made great progress in recent years. A variety of unforeseen complexities have been identified, many with relevance to human brain disease. For example, neurologic illnesses may arise because of perturbations in distinct but interrelated tiers of RNA-based genetic regulation: pre-mRNA splicing; nonsplicing RNA modifications; and mRNA translational regulation. Furthermore, there is poor correlation between mRNA levels and protein levels in mammalian cells, due partly to complicated post-transcriptional regulation by hitherto unknown noncoding RNAs. Some noncoding RNAs have been shown to be involved in human brain diseases. Diseases potentially mediated by alterations in RNA processes include tauopathies, myotonic dystrophy, Alzheimer disease, brain cancer, and many others. Here we present an overview of new research highlighting functions for RNA that far surpass the "messenger in the middle" role and that identify RNA molecules as important agents in the human brain in health and in disease states.

Key Words: Alzheimer disease, Cancer, Microarray, mRNA, Proteomics, RNA, Tau.

INTRODUCTION

The Central Dogma

The "central dogma of molecular biology" was set forth by Francis Crick in 1958 (1). In this scheme, RNA is represented by messenger RNA (mRNA), the "messenger in the middle" between DNA and proteins. Two corollaries of the central dogma have been implicit in guiding subsequent research. First, transcriptional regulation (for which mRNA levels are an experimental surrogate) is seen as the important bottleneck of "gene expression." Second, genetic diseases are often characterized by a primary DNA "genotype" with a downstream protein-related "phenotype" (Fig. 1).

A reconsideration of the central dogma and corollaries may be required in light of recent research. Most biologists are aware of phenomena outside of the central dogma. For example, RNA can also serve as a template for DNA (i.e. reverse transcription), whereas a large majority of RNA molecules are never translated into protein. Additionally, in contrast to the central dogma, studies have now also demonstrated the apparent ability of prion conformations to propagate independently of RNA or DNA. It has been suggested that the central dogma is incomplete only in small details, critiqued merely as an "oversimplification" (2). However, new research suggests that the central dogma is deficient not only at the fringes of biology: fundamentally important misconceptions can derive from holding this progression as universal or "central."

RNA comprises an incredibly adaptable group of molecules with diverse cellular functions. This field is expanding rapidly, while moving into unexpected and exciting new directions. Recently discovered roles for RNA in brain diseases—including adult neurodegenerative diseases, childhood neurologic disorders, and brain cancers—underscore the potential importance of this field to the clinical neurosciences. Here a review of some newly discovered aspects of RNA biologic research is followed by a description of RNA-related perturbations associated with neurologic diseases. This review is meant to serve as an overview of this fascinating and fast-developing field, rather than an in-depth description of any single facet of RNA biology or disease.

RNA BIOLOGY—NEW CONCEPTS

Messenger RNAs Constitute a Small Percentage of Cellular RNA

As has been known for decades, mRNAs make up a very small percentage of total cellular RNA (~1%–2% as a percentage of dry weight). The majority of cellular RNA is composed of ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), although there are relatively few *types* of these RNAs. Hence, mRNAs, rRNAs, and tRNAs are the RNA subtypes with which most people are familiar.

Surprisingly, however, in mammalian cells there are a great many more types of different non-mRNA transcripts than was previously thought. These revelations derive in part from "tiling" microarray experiments by which researchers have determined the surprising proportion of DNA that is transcribed into RNA. It was thought until recently that only ~10% of DNA was transcribed. However, tiling array data indicate that >60% of mouse DNA is transcribed into RNA (3), with humans having an even higher percentage of DNA transcribed into RNA (4). Up to half of RNA transcripts are

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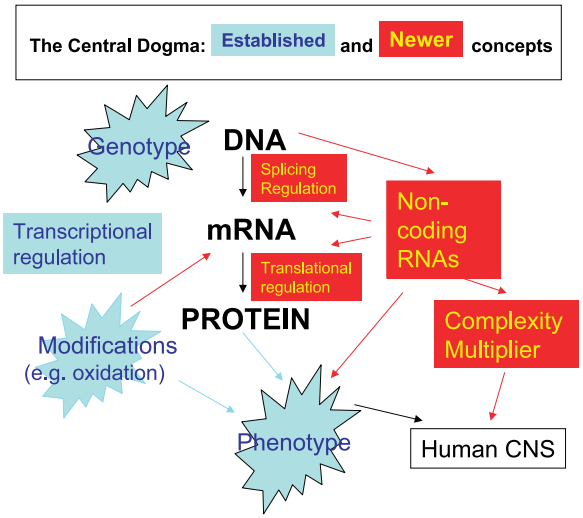

FIGURE 1. New research suggests that RNA can play important biologic roles other than the traditional ''messenger in the middle'' in both normal and disease tissues.

not polyadenylated and thus would escape most cDNA transcription/amplification protocols (5, 6). Because <2% of DNA spans areas known to include traditional exons (4), there are obviously many uncharacterized RNA transcripts that do not code for rRNA, tRNA, or proteins.

These amazing data, along with the tendency of "gene-like" areas of DNA to overlap, share borders, and contain novel regulatory elements, have prompted researchers to rethink the whole concept of a "gene" (3). The new definition of gene by the Sequence Ontology Consortium evades the traditional description of exons or proteins. Their new definition of gene is "A locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and/or other functional sequence regions" (3, 7). This definition reflects the fact that RNA transcripts other than those that code for proteins are thought increasingly to be very important.

Noncoding RNAs: A Diverse Category With Many New Entries

Noncoding RNAs (ncRNAs) comprise many distinct subtypes of RNAs unified only by their lack of a polypeptide-encoding open reading frame: rRNAs, tRNAs, small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs), some genetically mobile elements including a subset of retrotransposons, as well other groups and individual RNAs that defy nosologic classification. Table 1 presents a very brief overview of some ncRNAs.

Probably the most important thing to appreciate about ncRNAs is how little is currently known about them. Small regulatory RNAs, which are now known to play a fundamental role in biology in plants and humans, were the Breakthrough of the Year in the journal *Science* in 2002 (8). This distinction was bestowed because of the critical role these molecules play in regulating gene expression, yet specific details on this regulation remain largely unknown. The diversity of ncRNAs, in mammals as well as other metazoa, has only very recently been recognized. Only several hundred ncRNAs were known to exist in 1999, but by 2004 almost 5,000 had been predicted and annotated (4). In the meantime, whole categories of ncRNAs were discovered. Perhaps most notable are miRNAs, which are small regulatory RNAs that number probably >1,000 in humans (References 9 and 10 and see below). There is evidently an important role for these newly identified molecules in both physiologic and pathologic processes.

Messenger RNA Levels Correlate Very Poorly With Protein Levels in Mammals

Relatively few studies have examined the relationship between levels of mammalian cellular mRNAs and the proteins that they encode. This is an important correlation for at least 2 reasons. The first reason is a practical one: mRNA levels are considered in many experimental contexts to be a surrogate for gene expression (e.g. cDNA microarrays, Northern blots, serial analysis of gene expression, differential display, in situ hybridization, some polymerase chain reaction-based experiments, and massively parallel signature sequencing). Well over 100,000 scientific papers have been published using these techniques. One can hence assess whether or not it is valid to imply that changes in mRNA levels are expected to correlate with near-proportional changes in protein levels. In other words, is mRNA a good surrogate for "gene expression"?

Secondly, the correlation of mRNA and protein allows us to address an important theoretical question: is protein expression regulated primarily at the level of transcription, or is post-transcriptional regulation more important? One would hypothesize that if transcription were the primary

TABLE 1. Classes of Noncoding RNAs

MicroRNAs (miRNAs)
~22 nucleotides
High copy number per cell
Use evolutionarily ancient "machinery"
Probably >1,000 miRNAs exist
Small nucleolar RNAs (snoRNAs)
Reside in nucleolus
Target other RNAs for site-specific modifications (e.g. methylation, pseudouridylation)
~300 known
Modify many genes
Small nuclear RNAs (snRNAs)
Component of spliceosome
Involved in pre-mRNA splicing
Few in number, but very abundant
Transcribed pseudogenes
Transcribed retrotransposons (some are also translated)
Ribosomal RNAs (rRNAs)
Transfer RNAs (tRNAs)
Individual noncoding RNAs (ncRNAs) that evidently defy classification
BC-1
Many more!

point of gene expression regulation, there would exist a tight correlation between mRNA and protein levels. If post-transcriptional regulation is most important instead, then there should be a very weak correlation of mRNA levels and protein levels. New techniques, namely high-throughput mRNA expression profiling and high-throughput proteomics, have allowed researchers to assess in parallel how the levels of many different mRNAs correlate with the levels of the proteins that they encode within small tissue samples.

Studies from different laboratories have described a relatively poor to nonexistent correlation between mRNAs and their encoded proteins (11–21). Most correlations are very poor. Table 2 summarizes these experiments. The lack of mRNA-protein correlation also extends to yeast (22). Some of these findings may be explained partly by technical factors, and, to the best of our knowledge, this question has not been directly addressed using high-throughput means for both protein and mRNA in the context of the mammalian brain. The impact of important factors besides RNA translation (such as differential protein degradation) no doubt also play a role in these relationships; however, some of these experiments were performed with this variable in mind, using dynamic as well as steady-state conditions (15).

In summary, protein translation does not take place without any mRNA, however, beyond that a given mRNA level and its referent protein level tend to correlate weakly, if at all. These data address the 2 points made above: first, the use of mRNA as a surrogate for gene expression is a flawed experimental practice because mRNA levels correlate poorly with protein levels; and second, these data suggest that in the mammalian brain, as in other tissues, the most important regulation of gene expression takes place *after* transcription.

RNA Plays a Central Role in Post-Transcriptional Gene Expression Regulation

RNA is a central player in post-transcriptional gene expression regulation. Ribosomes, the nexus of translation, are ~60% RNA by mass in mammals. Recent data show that hitherto unknown RNA molecules coregulate translation. For example, miRNAs, in collaboration with Argonaute proteins, direct the suppression of specific mRNA translation (23). The method by which this occurs is the subject of some controversy, but it appears to involve the inhibition of translational initiation and/or the sequestration of miRNA-bound mRNAs into areas within the cells called P-bodies (24–27). miRNAs are further discussed below. A related family of small regulatory RNAs, the testes-enriched piRNAs, also appear to participate in the regulation of mRNA translation. Yet another novel ncRNA, the small cytoplasmic BC-1, has been implicated in translational regulation in hippocampal dendrites (28–30).

ncRNAs can also affect mRNAs after transcription but before translation. snoRNAs and snRNAs are classes of abundant RNAs that participate in pre-mRNA modification/editing and splicing, respectively. To take a single example, Kishore and Stamm (31) demonstrated that a snoRNA, HII-2, regulates the alternative splicing for the pre-mRNA for a neuronal serotonin receptor (5-HT2CR). In summary, it has long been recognized that ncRNAs (rRNA and tRNA) are central to the regulation of translation; however, newly discovered (and presumably as-yet undiscovered) ncRNAs also play an important role in post-transcriptional regulation.

The Human Brain: Noncoding RNAs a Complexity Multiplier?

RNA other than the protein-encoding transcripts may contribute to the phenotypic complexity of the human brain. Statistics about the human cerebral cortex afford some insights into why such a "complexity multiplier" would be useful from an evolutionary perspective. The complete human mRNA transcriptome is thought to comprise ~30,000 different protein-encoding gene units; the entire human DNA complement is ~3 billion base pairs in length. These figures can be compared against the staggering complexity of the human brain, which includes 100 billion neurons in the cerebral cortex alone, upon which are attached ~20,000 synapses each. Hence there are ~100 billion *times* more human cerebral cortical synapses than CNS protein-encoding genes, and overlaid on that are further immense complexities of neural networks (32)! Protein-encoding genes could conceivably yet provide all the necessary information to ensure that human brains receive their correct complement of neurons and synapses, manifested

TABLE 2. Studies in Mammalian Cells of mRNA/Protein Correlation

Species	Cells	Number of Genes Studied	mRNA-Protein Correlation	Ref
Human	Neutrophils (ex vivo)	>500	Very poor	11
Human	Lung cancer cell line	>1,000	Very poor	12
Human	Umbilical vein endothelial cells	>500	Very poor	13
Human	Monocytic Leukemia THP-1 cells	>500	Very poor	16
Human	Lung Adenocarcinoma	165	Extremely poor/nonexistent	19
Human	Liver	23	Poor to moderate (correlation coefficient 0.48)	21
Mouse	Leukemia cell lines	>400	mRNA drives "at most 40% of protein expression changes"	15
	Liver (ex vivo)	12		
Mouse	MCT3-E1 preosteoblasts	>2,500	Very poor	17
Mouse	MPRO leukemia cell line	123	Correlation coefficient = 0.58; termed "moderately well" correlated	20
Rat	Liver	>500	Poor; not systematically described	14

at the appropriate time points during development. Yet in light of the data showing that protein-encoding transcripts are a relatively small minority of the transcriptome, it is tempting to speculate that other transcribed genetic elements may play an important role in contributing to the regulation of the development and/or maintenance of the human CNS.

ncRNAs are well positioned to act as a complexity multiplier to increase the capacity of the DNA, mRNA, and proteins to develop such highly complex structures as the human CNS. It has been hypothesized that ncRNAs such as miRNAs play an important role in evolution (33, 34) and particularly also in primate evolution: primate-specific ncRNAs have been described (35). In any case it is important to recognize that evolution has no obligation to simplicity. No doubt there are complex systems of *cis*- and *trans*-regulation at almost every level of molecular neurobiology. Some examples of how the complex RNA biochemical regulation can play a role in neurologic diseases are presented below.

NEW INSIGHTS INTO THE ROLE OF RNA DYSFUNCTION IN HUMAN DISEASE

Important roles for RNA biology in human brain diseases have now been discovered in at least 3 different tiers of genetic regulation: pre-mRNA splicing; nonsplicing RNA modifications; and mRNA translational regulation. Examples are presented below. However, it should be noted that this is by no means a complete description of new disease-related ncRNAs (36). The intention of this overview is to give an idea of how RNA biology is expanding our expectations of human biology and human disease, particularly in light of the fact that most deadly diseases defy simple "genotype-phenotype" relationships. RNA processing is complex (Table 3) and only as these processes become better understood will we understand the full extent of their relation to the development of human illnesses.

TABLE 3. RNA Processes (Many Implicated in Neurologic Diseases)

Nucleus
Interactions with binding proteins
Chromatin interactions
Extensive processing
Editing
Base modification
5' cap
3' poly(A) tail
Splicing
Ribosomal maturation
Export
Cytoplasm
Transport to different cell compartments
Additional maturation/cleavage/processing
Interactions with RNA-binding proteins
Open reading frame regions
Untranslated regions
Cis (stem-loop) and *trans* (e.g. microRNAs) double-stranded RNA interactions
Ribozyme catalytic activity
Translation
Initiation
Elongation
Termination
Sequestration and/or inactivation
Degradation

Splicing

Pre-mRNA is processed in the nucleus in conjunction with RNAs and proteins collectively called the spliceosome (37). This process results in alternatively spliced mature mRNA transcripts, the contents of which change in response to developmental and/or environmental cues. Tauopathies can result from genetic mutations that cause abnormal tau pre-mRNA splicing (38–43). Tauopathies are often characterized clinically by relatively early-onset frontotemporal dementia, and, neuropathologically, tauopathy brains reveal intracellular deposits of tau protein-immunoreactive neurofibrillary pathology. The tau protein contains microtubule-binding motifs and is considered to help bind and bundle tubulin proteins into axonal microtubules (44). Several exons of the tau pre-mRNA are alternatively spliced: namely, exons 3 and 10 are excluded from cytoplasmic tau mRNA in fetal brain; these same exons are each present in roughly half of tau mRNA in the adult brain (39, 41, 44). A minority of tauopathies are caused by mutations near the exon-intron border at the 3' end of exon 9 in the tau gene, which in turn causes aberrant splicing of tau pre-mRNA (Reference 41 and see below). While rare, these mutations prove the potential importance of correct splicing of pre-mRNAs.

Myotonic dystrophy is a triplet repeat disorder caused usually by expansions of (CTG)*n* repeats in the 3′ untranslated region of the *DMPK* gene on human chromosome 19 (45–47). Myotonic dystrophy involves a complex phenotype that can encompass many organ systems, including the eye, brain, skeletal and cardiac muscle, and testes. The surprising fact is that the apparent defect in the different tissues is caused by problems that are associated with genes other than the *DMPK* gene itself—affected genes encode proteins such as a chloride channel, insulin receptor, cardiac troponin T, and tau protein, which themselves harbor no genetic mutation (48). The brains of patients with myotonic dystrophy can show neurodegeneration with tau-immunoreactive neurofibrillary pathology (49). The apparent pathogenetic mechanism underlying myotonic dystrophy is reviewed well elsewhere (48, 50). In brief, the triplet repeat expansion in the 3' untranslated region of the *DMPK* sequesters RNA binding proteins that are no longer able to interact with other pre-mRNA species. Those other pre-mRNAs are then misprocessed (mis-spliced) in the nucleus and are unable to correctly function in the cytoplasm. In the specific case of tau protein, the predominant isoforms of tau proteins that are present in the case of myotonic dystrophy patients with neurodegeneration resemble the fetal mRNA splice isoforms (51–53), apparently leading to the development of neurodegeneration in myotonic dystrophy. Thus, a *trans*-acting

RNA splicing post-transcriptional mechanism is responsible for neurodegeneration in this disease.

Frame Shift Alterations in RNA

Another potential means by which RNA may mediate alterations in cellular homeostasis is based on the manifestation of frame shift alterations during translation. Growing evidence suggests that ribosomal frame shifting may directly contribute to pathogenesis in a variety of age-related neurodegenerative disorders (54–57). In particular, frame shift alterations in ubiquitin B (UBB) and amyloid precursor protein (APP) are linked with the pathogenesis of Alzheimer disease (AD) (54–56). This frame shifting arises principally as the result of sequences within the mRNAs for UBB and APP, which increase the "slippery" nature of translation for these and other mRNAs (58). New reports suggest that glial fibrillary acidic protein (GFAP) may be another mRNA transcript prone to misreading in AD (59). Interestingly, translational misreading may preferentially occur in neurons vulnerable in AD (56, 59). This has led some investigators to suggest that molecular misreading contributes to the generation of misfolded proteins, which can then promote the inhibition of proteolytic pathways and induce protein aggregation (54, 57, 59). Together, these findings have stimulated interest not only in understanding the basis for dinucleotide deletions in disease-related genes but also in understanding the molecular basis for the potential toxicity induced by these and other "+1" proteins generated by deleterious frame shifts.

RNA Oxidation in Neurodegenerative Disorders

The role of oxidative stress in neurodegenerative disease has been a topic of intense interest (60). After exposure to reactive oxygen species, nucleic acids tend to become oxidized. To date >20 oxidized bases in nucleic acids have been characterized (61), with 8-hydroxy-2'-deoxyguanosine (8-OHG) being the most widely studied and characterized form of nucleic acid oxidation primarily because of the abundance of antibodies to 8-OHG and the stability of the 8-OHG adduct. It is important to point out that whereas 8-OHG and other forms of nucleic oxidation occur in both RNA and DNA, the bulk of what is known about the biochemistry of nucleic acid oxidation has been elucidated in studies of DNA (61).

Numerous studies have demonstrated that elevations in 8-OHG within DNA occurs in AD, with elevations in DNA oxidation preferentially occurring in the neuroanatomical regions most adversely affected in AD (62–65). In addition to 8-OHG alterations, studies have also demonstrated the presence of 8-hydroxyadenine, 4,6-diamino-5-formamidopyrimidine, and 5-hydroxycytosine in AD (66). Interestingly, DNA damage is associated with downregulation of rRNA and tRNA (8). These data suggest that there is a functional link between oxidative modification of DNA with RNA biology. In addition to the importance of understanding the links between DNA oxidation and RNA regulation, it is clear that a significant amount of investigation is needed to better understand which oxidized adducts are present in the RNA pool during the progression of AD and related neurodegenerative conditions. Additionally, future studies are needed to determine how the amount and different types of DNA oxidation correlate with the development and progression of the different forms of RNA oxidation.

In AD (whether or not in the context of Down syndrome), RNA oxidation is most severe in neurons compared with other cell types and appears to preferentially identify neurons possessing tau pathology (67). Interestingly, elevations in RNA oxidation are present in subjects with mild cognitive impairment (68), one of the earliest stages of AD, consistent with RNA oxidation being one of the earliest pathologic events in the development of AD. The increase in RNA oxidation can be observed in rRNAs within the ribosome complex, as well as non-rRNA pools such as mRNA and tRNAs (68, 69). So how can elevations in RNA oxidation potentially contribute to the development of neurophysiologic and neuropathologic alterations? To answer this question one must consider the potential effects of RNA oxidation in the different pools of RNA.

Each pool of RNA species (rRNA, tRNA, mRNA, and, presumably, the many various ncRNAs) must undergo complex processing before they are functional in a positive sense. Maintaining the proper processing of the different RNA molecules is absolutely necessary for maintaining sufficient levels of protein synthesis and limiting translational errors. Oxidative modifications of RNA molecules are likely to have deleterious effects on RNA processing and multiple levels. For example, the oxidation of RNA promotes specific RNA modifications including introduction of strand breaks, RNA cross-linking, and inducing specific base modifications, such as thymine glycol, 8-hydroxyguanine (8-OHG) and hydroxymethyluracil (70, 71). Such events may have adverse effects on RNA function, affecting the processing and metabolism of each type of RNA. The potential for other cellular sequelae is considerable. RNA oxidation may, for example, hamper the maturation of ribosomes, which is dependent on efficient rRNA processing. Impairments in rRNA oxidation could therefore contribute to the declines in ribosome function, which are observed in mild cognitive impairment and AD (68), by decreasing the amount of ribosomes and contributing the formation of dysfunctional ribosomes. With regards to mRNA oxidation, some studies suggest that RNA oxidation may preferentially suppress the translation of specific mRNAs (72). Quantitative analysis showed that the relative amounts of oxidized transcripts reach 50% to 70% for some RNA species (72). Such declines could in turn lead to decreases in the generation of essential proteins and thereby negatively impact cellular homeostasis. Lastly, the oxidation of tRNA that occurs in AD (68, 69) may contribute to translational error by negatively affecting translation during the elongation step or by promoting early termination of protein synthesis. Both of these events could lead to the generation of potentially toxic polypeptides or other by-products that might affect neuronal homeostasis. In future studies, it will be important to determine the basis for RNA oxidation in neurodegenerative conditions and to develop a

better understanding of the effects of such RNA oxidation on cellular homeostasis.

MicroRNA Dysfunction in Cancers, Including Brain Cancer

Along with other ncRNAs and proteins, miRNAs help to guide the regulation of mRNA translation. These ~22-nucleotide single-stranded ncRNAs exert their influence in mammals by recognizing particular mRNAs. miRNAs are thought to bind preferentially to areas in the 3′ untranslated region of mRNAs, and apparently miRNAs often recognize many different mRNAs (some recognize>1,000 different mRNAs!) (73). In effect, the miRNA guides a ribonucleoprotein particle, termed the miRNP, to the target mRNA, which causes that mRNA to not be effectively translated. Hence, in the presence of both the miRNA and the mRNA, polypeptide formation is decreased (for an excellent recent review of neuronal miRNAs, see Reference 10).

miRNAs are directly implicated in the pathogenesis of human cancer. Researchers from Carlo Croce's laboratory demonstrated a direct causal link for miRNAs in chronic lymphocytic leukemia (CLL), in which miR-15 and miR-16 may play a critical role in pathogenesis in the majority of human CLL cases (74). The deletion of these miRNAs has been shown to be important in disease progression and may constitute a tool for determining prognosis in CLL cases. The crucial breakthrough in understanding the relationship between miRNA deletions and CLL derived from the observation that miRNAs map to the CLL "minimal deletion" region of chromosome 13q14, and thus it became known which miRNAs may be altered in the course of the disease (75). Once this breakthrough was made, further insights were gained rapidly about the mechanisms involved (76). CLL research illustrates why it is important to know which miRNAs are altered in a disease state.

Three different laboratories have found miRNA expression patterns that are associated with brain malignancies. Chan et al (77) found that miR-21 is highly expressed in human glioma cells, and this is important because miR-21 exerts an antiapoptotic effect on cells from glioblastoma multiforme in human brains as well as other cell lines. A second group (78), also evaluating tissue from human glioblastoma multiforme tumors, found separate miRNAs that were upregulated (miR-221) or downregulated (miR-128 and miR-181 paralogs). Finally, it was demonstrated that miR-9 is expressed in neoplastic oligodendroglioma cells (both at the tissue level and via in situ hybridization at the cellular level), and the expression was increased in higher-grade tumors (79). More work is necessary in this area. However, miR-9 is a miRNA that is predicted to suppress the expression of >900 different mRNA transcripts (73, 80). It is tempting to speculate that the changes in the expression of these remarkably powerful genetic regulators play a role in the clinicobiologic behavior of these human brain tumors.

CONCLUSION

Recent RNA research has brought to light important and evolutionarily ancient biochemical processes and pathways. Among the new discoveries are details about the processing of ncRNA and the splicing and translation of mRNA. Future research in these areas will offer new insights into the complexity of the human CNS during both normal and disease states. Such can be the rewards of challenging scientific dogma—"central" or otherwise.

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