

Involvement of long noncoding RNAs in diseases affecting the central nervous system

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DNA sequences associated with protein-coding genes have been the primary focus of most genetic analyses of complex human diseases. Although we are rapidly gaining a comprehensive view of the etiology of certain central nervous system disorders, major gaps in our understanding persist. Recent studies have uncovered that many human genomic sequences are transcribed but not translated, generating an astounding diversity of noncoding RNAs (ncRNAs). This awareness should be taken into account when studying human diseases and may have profound implications on the development of novel biomarkers as well as therapies.

Introduction

The majority of transcription in humans results in the production of long non-protein-coding RNAs (lncRNAs), ranging from 200 bp up to several kilobases long. LncRNAs have only recently emerged as a major category of regulatory eukaryotic transcripts and can be classified into groups based on their location within genome. Natural antisense transcripts (NATs) arise from the opposite strand of protein-coding or non protein-coding genes¹⁻⁷ and can overlap with exons, promoter or other regulatory elements of their cognate genes. Another class originates from the introns, promoters or 3' termini of protein-coding genes.⁸⁻¹⁰ Finally, long intergenic RNAs (lincRNAs) are transcripts originating from genomic regions outside the boundaries of protein-coding genes.¹¹ Next generation sequencing of the human transcriptome has unearthed tens of thousands of lncRNAs from each class that are transcribed from specific cell and tissue types. As of February 2012, approximately 200 lncRNAs have been functionally annotated in the long noncoding RNA database (www.lncrnadb.com).¹² Although relatively few lncRNAs have been functionally characterized, increasing evidence suggests an important role for the thousands of uncharacterized remaining transcripts.

LncRNAs can have diverse functions including the ability to modulate transcription, regulate post-transcriptional RNA processing,¹³ translation,¹⁴ DNA methylation and chromatin

architecture through local (*cis*) and long distance (*trans*) mechanisms.¹⁵ Through interactions with transcription factors, co-activators and/or repressors, lncRNAs can affect different aspects of gene transcription to form a fine-tuned complex regulatory network. LncRNAs can modulate the activity of transcriptional machinery *in cis* in two main ways: by directly affecting transcription factors and their association with co-regulators¹⁶⁻¹⁸ or by orchestrating chromatin-remodeling events.¹⁹⁻²¹ Perhaps the most prominent mechanism involving lncRNAs is the recruitment of chromatin remodeling complexes, e.g., histone methyltransferases that methylate histones to either activate or repress gene transcription, in specific genomic loci. Interestingly, chromatin-remodeling proteins do not possess DNA-binding activity, suggesting that specific ncRNAs may be the critical link between these enzymes and the genome. In contrast to *cis*-acting lncRNAs, some transcripts exert their function by modulating gene expression on genomic loci other than the one from which they originate (*trans* activity).^{11,22,23}

Altered Expression of lncRNAs in CNS Diseases

Central nervous system (CNS) development, synaptic plasticity and the stress response are all tightly linked to epigenetic mechanisms that drive gene expression.²⁴ Temporal and spatial expression of lncRNAs appears to be important for proper neurological functioning through the precise regulation of epigenetic events. Many lncRNAs are expressed in the CNS where they have been shown to play fundamental roles.^{15,25}

MALAT-1 is a lncRNA that was originally described as being overexpressed in different metastatic tumors, however it has recently been found to play a role in synapse formation.²⁶ *MALAT-1* is expressed in a variety of tissues but is enriched in the brain where it controls the expression of a subset of genes significantly associated with synaptogenesis. One of the hallmarks of human brain evolution is the expanding complexity of synaptic networks and the controlled expression of specific lncRNAs in neuronal cells.^{27,28} Transcriptomics studies have revealed the dynamic expression profile of several lncRNAs during the maturation of neuronal cell subtypes.²⁹ Some studies revealed the presence of "brain-associated" lncRNAs that affect hippocampal development, oligodendrocyte myelination and aging in the brain.³⁰ Thus, it is not surprising that the alteration of lncRNAs

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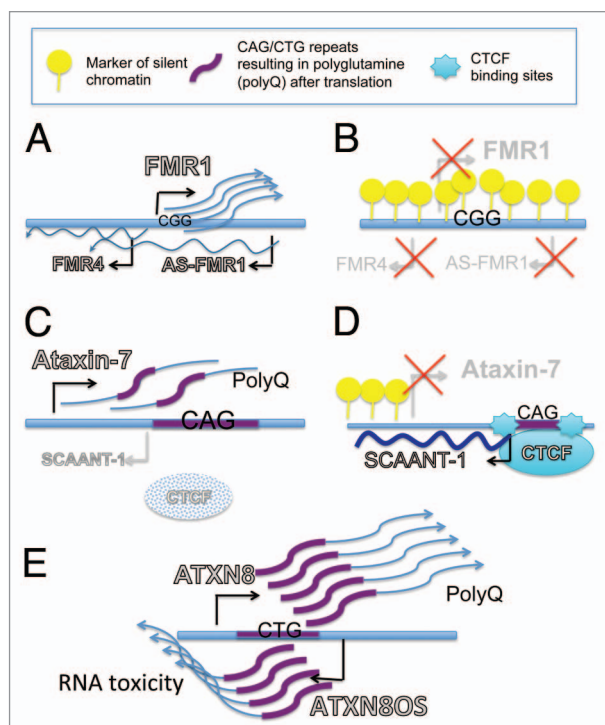


Figure 1. Microsatellite susceptible genes are featured by bidirectional transcription. (A and B) Fragile X Syndrome. (A) In general population the gene *FMR1* contains at the 5' UTR up to 54 CGG repeat that do not interfere with the expression of the gene. In this locus, in people not affected by Fragile X syndrome, antisense noncoding RNAs have been reported to be transcribed from intron 2 (*AS-FMR1*) and from the promoter (*FMR4*). (B) Amplification of CGG trinucleotides over 200 is associated with the onset of X-linked mental retardation and it's coupled with cytosine methylation that results in transcriptional silencing of the protein-coding gene *FMR1* and the non-protein coding *AS-FMR1* and *FMR4*. (C and D) Spinocerebellar Ataxia type 7. Spinocerebellar Ataxia 7 is caused by a CAG repeats expansion (37–400) located in proximity of the translation start site of Ataxin-7 protein leading to polyglutamine (polyQ) accumulation (C). Ataxin-7 gene is featured by CAG repeats flanked by CTCF-binding sites and by an alternative TSS of the antisense RNA *SCAANT1* (Spinocerebellar ataxia-7 antisense noncoding transcript 1) (D). In patients affected by Ataxia-7 the expansion of trinucleotides affects the binding of CTCF that causes reduced transcription of *SCAANT1* and results in higher expression of the aberrant Ataxin-7 protein (C). (E) SpinocerebellarAtaxia type 8. Spinocerebellar Ataxia type 8 arises when trinucleotide CTG in the *ATXN8* gene expand over 70 repeats (70–250). Bidirectional transcription has been detected in *ATXN8* gene producing protein coding gene *ATXN8* and the non-protein coding RNA *ATXN8OS*. CTG repeats in *ATXN8* mRNA results in accumulation of polyQ proteins while CTG repeats transcribed in antisense direction (CAG) are not translated and result in the production of toxic noncoding RNAs.

affects the basic functions of neuronal cells resulting in neurological and psychiatric disorders.^{31–33}

In addition, hundreds of small non-coding RNA molecules have been identified in the mammalian central nervous system. Among these are microRNAs (miRNAs) which have demonstrated importance in many aspects of neuronal function. Disruption of gene regulation mediated by miRNA has been implicated in a range of disorders such as neurodegenerative and neuropsychiatric conditions as well as in tumors of the CNS. The

focus of this review is to highlight the possible roles of the less explored long-noncoding RNAs in diseases of the CNS. For a comprehensive review of microRNA and other small RNA implicated in brain development and dysfunctions we refer the reader to other publications.^{34–37}

lncRNAs in microsatellite expansion diseases. Microsatellite expansion (or trinucleotide repeats) give rise to more than 30 neurological and neuromuscular diseases, including: Huntington disease (HD), Fragile X syndrome (FXS) and Spinocerebellar Ataxias.^{38,39} Transcriptomics efforts have revealed that antisense transcription is a common feature of mammalian genes that are actively transcribed from microsatellite disease loci.^{9,40,41} Transcription of lncRNAs containing repeats might alter their function and interfere with their ability to recruit protein complexes. The presence of nucleotide repeats might also alter the basal expression of lncRNAs, leading to an imbalance in sense-antisense transcript levels. Here, we highlight some of the recent findings showing that altered expression of specific lncRNAs might be involved in the etiology of several neurological disorders.

Fragile X syndrome. Fragile X syndrome (FXS) is the most common human heritable mental disorder. Patients display autistic symptoms and often suffer from epilepsy. A triplet nucleotide repeat expansion (> 200 repeats) in the 5' UTR of *FMR1*, the gene that encodes the crucial neuronal development protein, FMRP, is the causal factor in FXS (Fig. 1A and B). This repeat expansion results in the methylation of the promoter and subsequent silencing of the *FMR1* gene. Recent studies have suggested that noncoding RNAs may play a role in FXS as several lncRNAs are generated from the *FMR1* gene locus.^{9,10} The long antisense RNA originating from intron 2, *ASFMR1*, has multiple alternative splicing patterns and spans the long repeat sequence ending in the gene promoter.⁹ Another natural antisense transcript, *FMR4*, (–2 kb long, unspliced transcript) is initiated upstream of the *FMR1* start site.¹⁰ Previously, we have described that *FMR4* plays a role in cell survival as *FMR4* overexpression increases cell proliferation while *FMR4* downregulation induces apoptosis.¹⁰ We did not observe a cis acting effect on the expression of the *FMR1* gene and thus hypothesized that the ability of *FMR4* to interfere with proliferation pathways might occur in trans, however this has not yet been clarified. Moreover, *FMR4*, like *FMR1*, is silenced in Fragile X patients, thus its absence in the neurons of affected patients might contribute to the pathogenesis of this neurological disorder.

Spinocerebellar ataxias. Spinocerebellar ataxias are a diverse group of rare, slowly progressing, neurological disorders affecting the cerebellum. Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disorder caused by CAG repeat tract (37 ≥ 400 repeats) in the *ataxin-7* gene as compared with normal individuals (4–35 repeats).^{42,43} The expansion region is flanked by binding sites for the transcription factor CTCF⁴⁴ and by the TSS of the antisense transcript *SCAANT1* (spinocerebellar ataxia 7 antisense noncoding transcript 1)⁴⁴ (Fig. 1C and D). Evidence for CTCF binding in close proximity to the repeat sequences is emerging as a common occurrence in microsatellite expansion diseases. Importantly, CTCF binding is required for *SCAANT1* transcription and it has been shown

that the knockdown of *SCAANTI* or the absence of CTCF increases *ataxin-7* sense gene transcription. Based on this evidence, we can speculate that the overexpression of the antisense *SCAANTI* could be attempted to silence the aberrant expression of *Ataxin-7*. CTCF and lncRNA-mediated regulation of genomic architecture could play crucial roles in transcriptional deregulation at repeat loci. Interestingly, CTCF binding sites have also been detected in the promoter region of *FMRI*,^{9,45} suggesting that CTCF might regulate *FMRI* and *FMRI* locus-related noncoding RNA transcription or regional chromatin loop formation.⁴⁶

Spinocerebellar ataxia type 8 (SCA8) is characterized by a nucleotide repeat expansion in a region containing two transcripts expressed in opposite directions. Nucleotide repeat involves both the *ATXN8* protein coding gene and the *ATXN8OS* antisense noncoding gene.⁴⁰ Transcription of *ATXN8* protein-coding RNA containing CAG repeats is translated into a polyglutamine expansion protein while the untranslated antisense partner expresses CUG repeats (Fig. 1E). These findings suggest that SCA8 pathogenesis involves a toxic gain of function at both the RNA and protein levels. In fact, polyglutamine protein (PolyQ) inclusions have been found in SCA8 human tissues⁴⁰ and CUG expansions in the antisense transcript accumulate as ribonuclear inclusions in GABAergic neurons.⁴⁷ Despite the known role of the antisense transcripts in causing RNA toxicity, the authors speculate on the possibility that the noncoding transcripts might have additional roles in Spinocerebellar ataxia 8 that eventually regulate sense transcript expression.⁴⁸

In summary, it is becoming clear that repeat expansion diseases feature: (1) genetic instability and a strong tendency for repeat expansion through generations,⁴⁹ (2) binding sites for CTCF⁵⁰ and (3) bidirectional transcription encompassing the repeats.⁵¹

Huntington's disease. Investigation of potentially important accelerators of evolution have focused primarily on protein coding genes.^{52,53} However the availability of nearly complete genome sequences for several vertebrates provides useful information to find functional elements in the roughly 99% of the genome that does not have protein coding capacity.^{54,55} Many human accelerated regions (HARs) are associated with genes involved in transcriptional regulation and neurodevelopment. The HAR1 region contains long noncoding RNAs (*HARIF* and *HARIR*) and is expressed early during human embryonic neocortical development.²⁷ *HARIF* is specifically expressed in Cajal-Retzius neurons in the marginal zone during early development of the cortex and becomes expressed later on in other regions, e.g., in the hippocampal primordium, dentate gyrus, cerebellar cortex and olivary complex.²⁷ In the adult brain, *HARIF* is expressed in the frontal cortex, hippocampus, cerebellum, thalamus and hypothalamus.²⁷ Recently, it has been shown that in the brain of patients affected by Huntington's disease (HD), a trinucleotide expansion disease, the levels of lncRNAs *HARIF* and *HARIR* are decreased as compared with normal brains, suggesting a putative functional role for these lncRNAs in this disease. At the molecular level, the neurons of Huntington's disease patients are disrupted by aberrant nuclear localization of the master transcriptional repressor REST

(RE1-silencing transcription factor).⁵⁶ Furthermore, ChIP-seq data has revealed that the *HAR1* locus is under the control of REST⁵⁷ and that in the brain of HD HAR1-associated lncRNAs are part of widespread transcriptional dysfunction.

Long noncoding RNAs in imprinting-related diseases. Long noncoding RNAs have been implicated in the phenomenon of gene imprinting, where genes for only one allele (maternal or paternal) are actively transcribed. Imprinted genes are enriched in the brain and placenta and help mediate various cellular processes including cell growth and development.^{58,59} There is increasing evidence that imprinted genes are linked to neurodevelopmental processes through the regulation of cell survival, differentiation and maturation.^{59,60} Mental and neurological disorders are often influenced by deregulation of the imprinting processes. For example, genomic imprinting has been implicated in bipolar disorder, schizophrenia and autism.⁶¹⁻⁶³ Interestingly, imprinted genes are frequently associated with antisense transcripts,⁶⁴ which might play a role in gene silencing and could be causal factor in neurological disorders.^{3,4}

Angelman syndrome. Imprinted genes play a role in several human congenital syndromes such as Angelman syndrome (AS),⁶⁵ a disease characterized by severe neurologic abnormalities. The gene involved in this disorder, *Ube3a*, is biallelically expressed in most of the body's tissues, however in the brain, neurons express *Ube3a* only from the maternal allele.⁶⁶ Notably, in Angelman sufferers, the maternal *Ube3a* gene is deleted or mutated⁶⁶⁻⁶⁸ resulting in the absence of *Ube3a* expression in neurons. Currently, there are no effective drugs to treat this disorder, however activation of the dormant paternal allele has recently been achieved through treatment with topoisomerase inhibitors.⁶⁹ The activation of the silenced paternal allele is linked to a decrease in expression of the paternally expressed antisense transcript in neurons.⁶⁹ The mechanism behind this phenomenon remains unclear as the link between topoisomerase treatment, repression of the antisense transcript and re-expression of the gene is still obscure. These findings suggest that the re-expression of the paternal allele driven by a topoisomerase inhibitor, Topotecan, might be a potential treatment for Angelman syndrome.

Long noncoding RNAs and neuronal degeneration. **Alzheimer disease.** Long ncRNAs are involved in the pathogenesis of Alzheimer disease (AD),^{14,70} a neurodegenerative disorder partly characterized histologically by the deposition of amyloid plaques in the brain, due to increased levels of the β -secretase enzyme (BACE1). BACE1 cleaves the amyloid precursor protein (APP) and generates amyloid peptides which can aggregate and form plaques. A conserved antisense RNA, BACE1-AS, was originally identified from data produced by large-scale transcriptomics efforts from the FANTOM consortium as one of the sense-antisense pairs conserved between human and mouse.⁷¹ BACE1-AS is an approximately 2 kb RNA that is transcribed on chromosome 11, from the opposite strand of the BACE1 locus (11q 23.3). BACE1-AS shares 104 nucleotides of full complementarity to exon 6 of human BACE1 mRNA. It has been shown that BACE1-AS and BACE1 mRNA form a duplex that may act to alter the secondary or tertiary structure of BACE1 and to increase BACE1 mRNA stability.¹⁴ Alzheimer disease patients

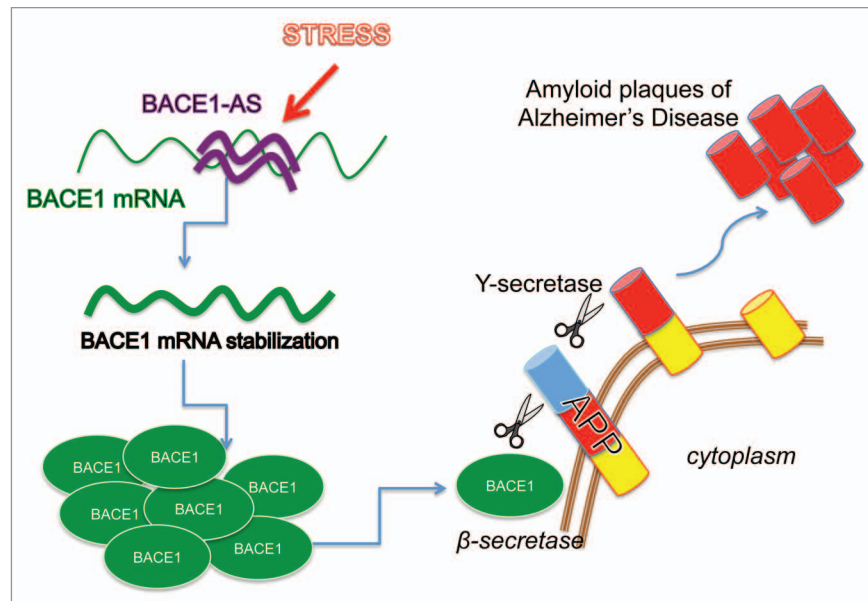


Figure 2. BACE1-AS contributes to β -secretase increased production in the brain of Alzheimer disease patients. Alzheimer disease associates with amyloid plaques in the brain of affected patients. The generation of these plaques derives by sequential cleavage of the amyloid precursor protein (APP) by β -secretase (BACE1) and γ -secretase. BACE1 is increased in affected patients due to the stabilization of the mRNA caused by the binding to its antisense noncoding RNA (BACE1-AS). Oxidative stress has been reported to cause the increase of BACE1-AS in brain resulting in the increased production of β -secretase enzyme.

display elevated levels of *BACE1-AS* in several brain regions as compared with controls.¹⁴ Furthermore, *BACE1-AS* increases significantly upon cellular stress causing an upregulation in *BACE1* mRNA and protein levels (Fig. 2). These findings are particularly interesting from a therapeutic standpoint as BACE1 remains a viable target for AD treatment.

Long noncoding RNAs in neurologic and neuropsychiatric diseases. Certain lncRNAs have been mapped to chromosomal regions associated with neurobehavioral diseases such as schizophrenia and autism.⁷² Many schizophrenic patients carry a translocation affecting two genes: *DISC1* and *DISC2* (disrupted in schizophrenia 1 and 2).⁷³ The *DISC1* protein is involved in intracellular transport, cell polarity and neuronal migration and disruption of its function may in part contribute to some neurological defects.²⁸ *DISC2* produces antisense non-protein coding transcripts that are transcribed from the 3' region of protein-coding *DISC1*. It has been proposed that *DISC2* ncRNA may be involved in the regulation of *DISC1* expression.⁷³ Interestingly, it has also been suggested that the *DISC1* and *DISC2* genes might also play a role in the development of unipolar and bipolar depressive disorders.⁷⁴ The same chromosomal translocation also disrupts another ncRNA gene, *PSZ11q14* (putative schizophrenia-associated gene from 11q14), which is downregulated in schizophrenia patients.⁷⁵ It is well recognized that neuropsychiatric conditions such as schizophrenia, depression and bipolar disorder are severe, multifactorial brain illnesses, thus it is not surprising that previously unrecognized noncoding RNAs can affect multiple pathways at different levels in these diseases.

The long ncRNA *Evf-2* is transcribed from the *Dlx5/6* locus and its transcription regulates that of *Dlx5*, *Dlx6* and *Gad1*^{16,76}

through the recruitment of transcription factors such as MeCP2. In vertebrates, *Dlx* genes play a critical role in neuronal development¹⁶ and the loss of *Evf2* noncoding RNA results in a decreased number of GABAergic interneurons in the early postnatal mouse hippocampus and dentate gyrus.⁷⁶ Schizophrenic patients show a consistent reduction of the enzyme responsible for GABA synthesis and the dysfunction of GABA-regulated circuits have been implicated in other neurological disorders such as autism and epilepsy.⁷⁷ Thus, the discovery that *Evf2* controls the development of GABAergic interneurons⁷⁶ raises the possibility of targeting this pathway to attenuate specific disease-associated symptoms.

Long noncoding RNAs in brain tumors. Gliomas. Gliomas are brain tumors that originate from glial cells (astrocytes, oligodendrocytes or ependymal cells). Among these, astrocytoma grade IV (glioblastoma multiforme, GBM), is the most common malignant brain tumor in adults and is one of the deadliest cancers. Despite the development of new surgical techniques and the use of antineoplastic drugs combined with irradiation, a cure for glioblastoma does not yet exist.

Glioblastomas are a heterogeneous group of cancers that show a number of clearly defined genetic lesions. For instance, in younger patients this tumor frequently features p53 mutations. p53 is a tumor suppressor protein encoded by a gene whose disruption is associated with more than 50% percent of human cancers. The p53 protein is known as the guardian of the genome because it acts as a checkpoint in the cell cycle that either prevents or initiates programmed cell death. Recent studies identified numerous long intergenic RNAs (lincRNAs) that are induced by the p53 tumor-suppressor pathway.^{11,78} When cells are subjected to stress, lincRNAs are among the transcripts specifically induced by p53. In particular, lincRNA-p21, is directly induced by p53

and plays a critical role in the p53 transcriptional response by targeting chromatin-modifying complexes to specific genomic loci.⁷⁸ While p53 is known to transcriptionally activate numerous genes, the mechanisms by which p53 leads to gene repression have remained elusive. Huarte et al. propose an elegant mechanism by which p53 globally downregulates a large subset of its targets. This study emphasizes the significance of lincRNA activation in the control of transcriptional programs that maintain cellular homeostasis. Since these lincRNAs are part of p53-mediated genome surveillance, it can be argued that tumors lacking functional p53, e.g., glioblastomas, might display altered expression of lincRNAs. Another recent study identified *PANDA*, a lincRNA that is 5 kb upstream of *CDKN1A* (p21). *PANDA* is induced by p53 and interacts with the transcription factor NF- κ B to limit the expression of pro-apoptotic genes.⁷⁹ Thus, the regulatory sequence upstream of *CDKN1A* drives the expression of multiple noncoding transcripts that coordinate the DNA damage response in healthy cells.

Inactivation, overexpression and copy number variations in the transcriptional repressor REST have been reported in several cancers. REST is a master negative regulator of neurogenesis that represses neuronal genes in non-neuronal tissues. Altered expression of REST may promote the loss of cellular identity and induce transformation through alterations in genomic stability, DNA methylation, chromatin remodeling and deregulation of onco- and tumor suppressor genes.⁸⁰ Abrajano et al. demonstrated that in glial cells both REST and CoREST modulate genes in key neuronal pathways.⁸⁰ This suggests that CoREST, like REST, might play a role in cellular transformation in the nervous system. Interestingly, the lincRNA *HOTAIR* serves as a scaffold for at least two distinct histone modification complexes: PRC1 (LSD1/CoREST/REST) and PRC2.⁸¹ *HOTAIR* interaction with these distinct complexes leads to the coordinated targeting of both to chromatin. Hence, we can speculate that the deregulation of REST in brain cancers might result in epigenetic alterations depending on the delocalization of this and potentially many other lincRNAs to their target loci. A better characterization of how REST and CoREST coordinate the epigenetic programming of neural cells can help us to better understand neural development and cancer.⁸²⁻⁸⁴

The polycomb repressive complex (PRC2) catalyzes the trimethylation of histone 3 on lysine 27 (H3K27me3), to silence chromatin, altering the degree of chromatin compaction and consequently the rate of gene transcription. The mechanism by which mammalian PRC2 is recruited to chromatin is unclear. In mammals, PRC2-targeted sequences consist mostly of CpG islands, but these sequences alone do not indicate a consensus response element.⁸⁵ Long ncRNAs are becoming recognized as important mediators of PRC2 genomic recruitment^{19,20,23,81,86,87} and such an interaction could be common to a large number of long ncRNAs.

Ezh2, the catalytic subunit of the PRC2 complex has been reported to interact with lincRNAs *Xist*,⁸⁶ *HOTAIR*,⁸⁸ *ANRIL*⁸⁷ and numerous lincRNAs.²² Mutations in Ezh2 have been observed in lymphoma⁸⁹ and its overexpression is an indicator of advanced and metastatic disease in many solid tumors⁹⁰ as well

as gliomas, according to a recent report.⁹¹ Thus, deregulation of both chromatin modifier enzymes and the lincRNAs that associate to them could easily lead to disruptions in chromatin regulation, potentially resulting in oncogenesis.

Imprinted lincRNAs and CNS tumors. It has long been suggested that chromosome 14q32 contains genes involved in the pathogenesis and progression of meningioma, a CNS tumor arising from arachnoid cells that line the brain and spinal cord. *Maternally expressed gene 3 (MEG3)*, is an imprinted gene located on chromosome 14q32 that produces a highly expressed noncoding RNA in normal human brains that is lost in high grade meningiomas.⁹² Interestingly, the expression of *MEG3* in human meningioma cell lines strongly suppresses tumor cell growth and activates p53-mediated apoptosis,⁹² suggesting a tumor-suppressive activity for this noncoding RNA. H19 is another example of an imprinted long noncoding RNA associated with CNS cancer. H19 is expressed during embryogenesis and the deregulation of H19 and other imprinted genes in this cluster are reported to promote cell transformation in a number of tumors, including gliomas. H19 is a target of the GLI1 transcription factor that mediates the Sonic Hedgehog signaling pathway and is amplified more than 50-fold in human gliomas.⁹³ Recently, a lincRNA antisense to H19 has been identified and its altered expression in human cancer cell lines.⁹⁴ The deregulation of this lincRNA in cancer further highlights the complexity of regulation within this gene cluster and may be relevant for CNS tumors.³⁰ Together, these examples demonstrate that lincRNAs are a rich source of candidate genes involved in normal health whose deregulation results in disparate diseases of the central nervous system.

Targeting of long noncoding RNAs. *RNA knockdown strategies.* Although the human genome is pervasively transcribed, the overwhelming majority produces non-protein coding transcripts that remain an unexplored field for drug discovery. RNA is a good candidate for drug design because it can adopt secondary structures that allow binding to specific ligands. The first RNA molecule was targeted in the late 1970s⁹⁵ when a short single-stranded sequence of DNA (antisense oligonucleotide, ASO), was developed to bind and block the translation of Rous sarcoma viral RNA. Since the application of the first unmodified antisense oligonucleotide molecules in 1978, numerous chemical modifications have been tested to improve the drug-like properties of ASOs. ASOs are used to knockdown genes through degradation of their respective mRNAs by RNase H (enzyme that cleaves RNA:RNA or RNA:DNA pairs) and inhibition of the translation process that does not require cleavage by RNase H. Stability, affinity and/or protein binding has been improved by the use of chemical modifications such as phosphorothioate backbones, morpholino oligomers, peptide nucleic acids (PNAs) or locked nucleic acids (LNAs). Multiple RNase H-dependent ASOs are in clinical trials, including a drug for high-grade glioma in Phase IIB trials.⁹⁶ The discovery of double-stranded small interfering RNAs (siRNAs) in the late 1990s added to the arsenal of RNA-targeting strategies and was successfully used in human cells a few years later.⁹⁷ The in vivo application of RNA interference to silence a specific transcript has shown promise but is limited by pharmacokinetic considerations and the poor ability of naked

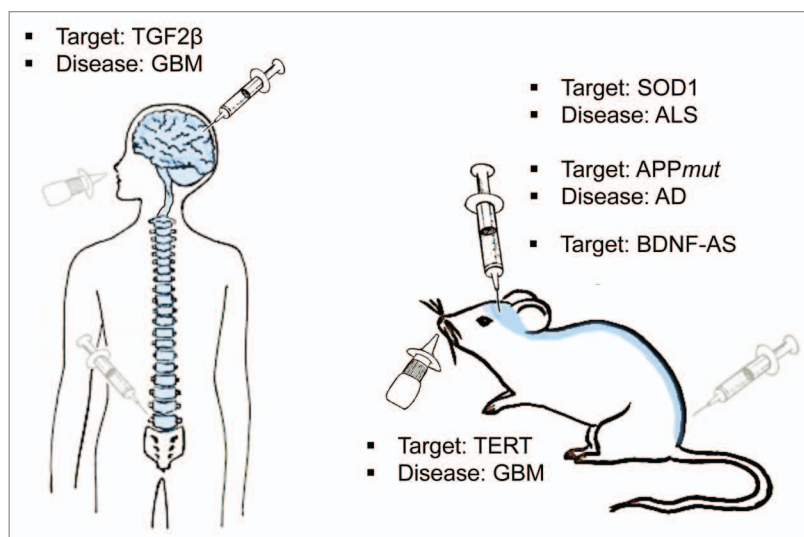


Figure 3. Administration of RNA-targeting drugs in the Central Nervous System. Several ways of administration have been developed to overcome the blood brain barrier and to deliver molecules that target the RNA in the brain. Intracranial delivery of oligonucleotides has been tested in rats and mice to target SOD1 and APP RNA to treat amyotrophic lateral sclerosis (ALS) and Alzheimer disease (AD) respectively. Recently, intracranial delivery of antagoNATs targeting BDNF-AS showed to increase BDNF levels in mice brain suggesting a challenging application to prevent neurodegeneration associated low levels of BDNF in AD. The same strategy was used to target TGF2 β in a study of phase II to delivery chemotherapy for glioblastoma (GBM). Intranasal administration of siRNA targeting the Telomerase (TERT) has been successfully used and will be potentially applied in the future to human being. Intrathecal administration, commonly used in pain relief cures, can be applied in the future for the delivery of drugs in the CSF (Blue line) to treat diseases affecting the CNS.

siRNAs to translocate through the cell membranes necessitating the development of novel delivery methods. Recently, we suggested a broadly applicable method to upregulate gene expression by use of AntagoNAT oligonucleotides targeted to derepress natural antisense (mostly lncRNAs) mediated inhibition (see below and ref. 98).

However, as with any therapeutic strategy, it is important to balance efficacy with possible adverse and off-target effects. In fact, despite encouraging results from preclinical and clinical studies and the significant progress which has been made in developing these agents as drugs, some hurdles may still exist. For example, several studies have determined that oligonucleotides can bind serum proteins affecting coagulation,⁹⁹ that they may cause activation of the immune system¹⁰⁰⁻¹⁰² and that their administration can lead to elevated transaminases, suggesting liver damage.¹⁰³ However, direct administration to CNS may bypass some or all of these latter potential concerns. More studies are nonetheless needed to fully assess risk profiles with centrally administered oligonucleotides.

Finally, while extremely challenging to identify, small molecules targeting RNA would arguably be the most attractive tools for regulating lncRNA activity. The interaction between small molecules and lncRNA can potentially disrupt the native conformation of the RNA, altering its activity and/or protein-binding ability. Gene therapy is a promising tool for the treatment of human diseases that cannot be cured by conventional treatments. The expression of genes epigenetically regulated by lncRNAs can be specifically modulated by targeting regulatory noncoding transcripts. This strategy has the particular advantage of inducing long lasting changes in gene expression through modulation

of epigenetic events. Furthermore, targeting lncRNAs can potentially restore the expression of a silenced gene which would be out of reach for conventional drugs.

Direct delivery of RNA-targeting drugs into the CNS. The blood brain barrier (BBB) protects the brain by forming a highly selective barrier that blocks the entry of large, hydrophilic molecules. The BBB also makes delivery of drugs affecting neurologic disorders challenging. Intravenous or intraperitoneal administration of drugs usually shows very low uptake into the brain. Our group and others have pursued a strategy to bypass this obstacle through intracerebroventricular^{104,105} or intrathecal administration of oligonucleotides into the cerebrospinal fluid (CSF).¹⁰⁶⁻¹¹¹

Administration of oligonucleotides directly into the brain (interstitial, intracranial or intracerebroventricular) has indeed been fairly successful in animal models and human (Fig. 3). For example, an oligonucleotide with a phosphorothioate backbone targeting superoxide dismutase 1 (*SOD1*) was continuously infused into the right lateral ventricle of rats via a surgically implanted catheter in the skull connected to a subcutaneously embedded osmotic pump.¹⁰⁹ Treatment showed a reduction in *SOD1* mRNA and protein levels in the brain and spinal cord. Mutated forms of *SOD1* are responsible for familial amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease caused by preferential loss of neurons in the upper and lower motor pathways, a disease for which current treatments are marginally beneficial. Another case where direct administration is promising is that of intracerebroventricular delivery of ASO's to treat Alzheimer disease. Point mutations in the human *APP* gene in the protein region targeted by BACE1, results in familial AD. Recent work by Chauhan et al. shows that mice injected directly

with antisense oligonucleotides directed at the mutated site of APP exhibit reduced β amyloid plaque deposition.¹⁰⁶

Recently, Modarresi et al. showed that the upregulation of BDNF (brain-derived neurotrophic factor) can be achieved in vivo by targeting BDNF-natural antisense transcript (*BDNF-AS*) with modified oligonucleotides called antagoNATs.⁹⁸ *BDNF-AS* represses the transcription of *BDNF* by altering the chromatin landscape at the gene locus, resulting in decreased BDNF protein levels. Oligonucleotides targeting *BDNF-AS* were administered to mice through intracerebroventricular delivery, using osmotic mini-pumps. After 28 days of continuous infusion of the AntagoNAT, *BDNF* mRNA levels were increased across fore-brain regions as compared with control oligonucleotide treated mice. Importantly, the neurotrophin BDNF is a growth factor that enhances the survival, development, differentiation and function of neurons.¹¹²⁻¹¹⁴ Several neurodegenerative, neurodevelopmental and psychiatric disorders¹¹⁵⁻¹¹⁷ show impaired levels of neurotrophins, suggesting that the upregulation of BDNF can have beneficial effects on several disorders. It has long been known that BDNF levels are decreased in AD brain¹¹⁸ and it also seems that the ratio of BDNF sense to BDNF antisense transcript concentration is markedly dysregulated in this disease.⁹⁸ In vitro and in vivo models of neurodegenerative disease show that *BDNF* prevents neuronal degeneration.^{115,119} Thus, its upregulation mediated by AntagoNATs might represent a potential neuroprotective agent useful to prevent neurodegeneration.

Intracranial delivery of oligonucleotides in an outpatient setting has been tested in a phase II clinical trial for human glioblastoma. Trabedersen[®], an antisense oligonucleotide targeting TGF- 2β , was infused intratumorally using convection-enhanced delivery (CED) via a subcutaneous port access system connected to an external pump to allow for outpatient treatment.⁹⁶ The treatment was safe and showed significant benefits compared with standard chemotherapy. The advantages of ventricular infusion with an implanted pump includes accurate continuous accurate delivery¹²⁰ and trapping of oligonucleotides inside the brain by the BBB.¹²¹

Intranasal delivery is a promising alternative to standard delivery systems as it provides a practical, painless method for delivering therapeutic agents to the brain (Fig. 3). Rats bearing brain tumors were treated intranasally with an oligonucleotide against Telomerase (TERT).¹²² The intranasally delivered treatment bypassed the blood-brain barrier, reached the tumor and inhibited tumor growth, leading to a prolonged lifespan in treated rats. This delivery approach appears to selectively kill tumor cells as toxic effects were not observed in normal brain tissues. These data support further research into the intranasal delivery of tumor-specific therapeutic agents for brain cancer patients.

Finally, while intrathecal administration (IT) of drugs is well established in anesthesia and pain management, recent data suggest that oligonucleotides introduced IT can be distributed to the brain through CSF suggesting an additional route of administration to treat CNS diseases.

As discussed previously in this review, the onset of many diseases affecting the CNS occurs during development and treatment

thus would ideally have to begin in the very early stages of the disease. Nevertheless, Michalon et al. for example, have published encouraging results showing that the early and chronic inhibition of the glutamate receptor mGlu5 in young FXS mouse model can “correct” a broad range of complex behavioral and neuro-anatomical defects associated to that disease. Importantly, the treatment was started in these mice at an age of 4–5 weeks, when the brain development is anatomically complete but highly plastic and when all behavioral and anatomical FXS phenotypes are evident.¹²³ These results may demonstrate the feasibility of treatments for developmental disorders, but there are a few important considerations in the design of therapies for these conditions. First, as in Michalon et al. study, treatment must be initiated early in the course of disease, which would generally imply very young pediatric patients. In addition, as most neurodevelopmental disorders have a strong genetic component, they can rarely be completely cured and patients would therefore require long-term treatment. Because of this, therapies and delivery strategies such as those discussed in this article should be considered for potential use over the course of many years.

Biological carriers for RNA-targeting therapies. Viruses are natural vehicles for the efficient transfer of genes into host cells. This characteristic makes viruses an attractive system for the delivery of therapeutic nucleic acids. Viral vectors that have recently been used in the laboratory and in clinical practice include adenoviruses, retroviruses and lentiviruses. Non-viral vectors have also been popular tools for gene delivery, because they are relatively safe and can be modified through the incorporation of ligands to target specific cell types.¹²⁴ This method of delivery includes three major vectors: polymer-, lipid- and peptide-based systems. However, the levels of gene expression mediated by these vectors are low as compared with viral vectors. A new twist on conventional viral-delivery systems is the virosome. Using virosomes, vesicles can be made from reconstituted virion-like phospholipid bilayers and unlike conventional liposomes, they are able to carry various drugs including nucleic acids.¹²⁵ Despite their low toxicity, in vivo application of this technology is sometimes limited by the risk of immunogenicity; to overcome this obstacle virosomes have been modified with polyethylene glycol (PEG).

Carrier-based strategies are currently being developed using organisms such as bacteria that can naturally evade the host immune system to enter the target cell.¹²⁶ Non-pathogenic bacteria such as *Lactococcus lactis* or *Streptococcus gordonii* could serve as vehicles for the production and delivery of biologically active proteins such as cytokines, enzymes or even siRNAs. To date, recombinant Salmonella strains have been engineered to deliver siRNAs to tumors to suppress tumor growth in vivo.^{127,128} In fact, Gram-negative bacteria such as Salmonella can colonize and grow within aerobic and anaerobic areas of tumors. The potential to selectively colonize hypoxic areas of tumors that cannot be treated by chemotherapy presents an exciting opportunity for cancer therapeutics.

Another strategy involves the use of the envelope from Gram-negative bacteria that is lysed with protein E,¹²⁹ to produce a “bacterial ghost”. Despite the absence of the cytoplasmic contents, the

surface enables encapsulation of a range of cargos and provides an intrinsic mechanism for targeting dendritic cells, macrophages and tumor cells.¹³⁰⁻¹³³

Essential components of the immune system such as macrophages can potentially be engineered to deliver therapeutic molecules due to their tendency to phagocytose nanoparticles and to target diseased tissues.^{134,135} Advances in the field of nanotechnology have led to preclinical studies in which ex-vivo-made nanoparticle-bearing macrophages are re-injected into the body as “Trojan horse” delivery carriers.¹³⁶ In this context, the formation of siRNA and/or oligonucleotide nanoparticles is an interesting strategy for systemic nucleic acid delivery.

Conclusions

It is becoming abundantly clear that focusing drug discovery efforts solely on conventional protein targets may not produce

the therapeutics needed urgently to combat CNS disorders. The last decade has yielded much insight into the important functional role of noncoding RNAs as well as the mechanisms of action of these transcripts. The direct involvement of long noncoding RNAs in the regulation of basic biological processes leads us to hypothesize that their altered expression and/or function can have profound effects on multiple cellular pathways and that they may constitute a large new class of viable biomarker and drug targets.

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