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Long Noncoding RNAs: Central to Nervous System Development

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Summary

The development of the central nervous system (CNS) is a complex orchestration of stem cells, transcription factors, growth/differentiation factors, and epigenetic control. Noncoding RNAs have been identified, classified, and studied for their functional roles in many systems including the CNS. In particular, the class of long noncoding RNAs (lncRNAs) has generated both enthusiasm and skepticism due to the unexpected discovery, the diversity of mechanisms, and the lower level of expression than found in protein-coding RNAs. Here we describe evidence supporting the role of lncRNAs in driving CNS-specific differentiation. It is clear that lncRNAs exhibit a functional diversity that makes their study and compartmentalization more challenging than other classes of noncoding RNAs. We predict, however, that lncRNAs will be essential for the characterization of discrete neuronal cell types in the age of single-cell transcriptomics and that these regulatory RNAs contribute to the multitude of functional mechanisms during CNS differentiation that will rival the diversities of protein-based mechanisms.

An Introduction to Noncoding RNAs in CNS Development

Some of the more important distinguishing features of mammalian genomes are the complex mechanisms by which genes are regulated in coordinate fashion during development. This orchestration is most evident in the complexity, organization, and function of the mammalian brain. Here, the synchronized regulation by precise attenuation or activation of functional genetic components establishes a staggering variety of cell types, and, remarkably, enables the integration of these diverse cell types into the functional circuitry of the brain. A shared characteristic of many traumatic brain injuries or neurodegenerative disorders is a specific effect on discrete neuronal subtypes, demonstrating that individual neuronal subtypes are likely to be metabolically distinct from each other. Certain neuronal subtypes may be acutely sensitive to disease progression by mechanisms such as enhanced sensitivity to oxidative stress (e.g. dopaminergic neurons in Parkinson's disease; Saxena and Caroni, 2011). Distinct cell-specific mechanisms highlight the functional diversity of individual neurons, as exemplified by how presynaptic alternative splicing regulates receptor function postsynaptically (Aoto et al., 2013). Understanding how each specific neuronal cell type is established can enable directed differentiation and facilitate the design of therapeutic transplants. While a broad range of mechanisms involving traditional protein-coding genes have been explored, a new and exciting component is

the class of noncoding RNAs, including microRNAs and lncRNAs, defined below. The fundamental role of molecular RNA in life processes and evolution has been considered for over 45 years, as was discussed in a prescient article by Darnell and Doolittle (1986). Cech and Steitz (2014) more recently summarized the broad range of molecular activities attributable to RNA in the context of understanding noncoding RNA-based cellular mechanisms. It has long been assumed that a large proportion of all protein-coding genes is expressed in brain--in one case the expression of 76% of all known genes was detected in mid-fetal human brain (M. B. Johnson et al., 2009), supporting the hypothesis that some large fraction of the genome is required for human CNS development. This hypothesis is now considered to include noncoding RNAs (Bae et al., 2015). The goal of this review is to outline a fundamental biological question: how do noncoding RNAs contribute to the diversity of cell types observed during neuronal development? By expanding our understanding of the specific classes of regulatory events affected by lncRNAs that endogenously define neuronal cell lineages, this knowledge can be exploited to study cellular differentiation or to generate precisely tailored neurons for targeted replacement therapies.

Classes of Noncoding RNA

Aside from the more structural non-coding RNAs (ribosomal, small nuclear, and telomerase are all examples), the first unexpected group of noncoding RNAs identified in the post-genomic era was microRNAs, short (17-23 nt) RNAs produced by cleavage of hairpin precursor RNAs with Dicer with mature forms found in ribonucleoprotein complexes including Ago2 (Bartel, 2004). It was clear from the beginning that these could not be protein-coding RNAs because they are too short. The major mechanism for microRNAs is the regulation of mRNA stability and/or translation by a sequence-dependent interaction with cognate mRNAs. Among the best-studied examples of microRNAs regulating key aspects of CNS development are miR-124 and miR-9 (Krichevsky et al., 2006; Visvanathan et al., 2007). It has even been proposed that ectopic expression of these microRNAs promotes direct neurogenesis from fibroblasts by reprogramming (Yoo et al., 2011). However, the topic of microRNAs in CNS development has been considered in depth by a number of reviews (Díaz et al., 2014; Fiore et al., 2008), and so we will not consider them further here but we will instead focus on a broader class of noncoding RNAs.

Another recently identified class of noncoding RNAs are found as covalently-closed circles (Circular RNAs; circRNAs). These have been found to be abundant in the mammalian brain (Rybak-Wolf et al., 2015) and dynamically expressed in the periphery during development (Memczak et al., 2015). Interestingly, sequences of circRNA are consistent with their derivation from otherwise well characterized cellular genes encoding synaptic-related proteins and, since large numbers of circRNAs change in abundance during synaptogenesis, it has been suggested that circRNAs function to regulate synaptic function (You et al., 2015). To date, tens of thousands of distinct circRNA candidates have been identified, with a strong enrichment for these molecules in nervous system tissues (Rybak-Wolf et al., 2015). Consistent with a functional role in the brain, circRNAs are also dynamically regulated during both neural development as well as spatially across brain regions, often independently of the linear mRNA forms encoded by the same gene (Rybak-Wolf et al.,

2015). Early studies into circRNA molecular mechanisms suggested that these molecules may act as non-productive binding partners for microRNAs to attenuate their function (Hansen et al., 2013). However, this does not appear to be a common unifying mechanism for circular RNAs (Guo et al., 2014; Thomson & Dinger, 2016). These are tantalizing findings but at this point relatively little has been reported for the function of circRNAs during CNS development.

Long noncoding RNAs (lncRNAs) are uniquely suited to influence cell fate decisions and contribute to the general heterogeneity of neuronal cell types in the mammalian brain. Similar to circRNAs, lncRNAs are a tissue- and cell-type-specific class of regulatory RNA genes with exquisite spatio-temporal restriction in the brain that are likely to contribute to specific cellular identities. lncRNAs represent a recently described, and expansive class of RNA genes that are likely to contribute to the establishment of specific cellular fates. Despite their number, and perhaps as a consequence of this, we are only beginning to discern the myriad roles for these RNA genes.

Each of these types of non-coding RNA has the potential to participate in differentiation processes in the CNS. However, lncRNAs have been found to exhibit properties consistent with the identification of specific cells as well as possessing mechanisms consistent with driving cellular pathways. Below we will summarize several examples of lncRNAs that have been studied during brain development.

Characteristics of lncRNAs

The defining characteristic of lncRNAs is the lack of discernable protein coding potential. Generally, this extends beyond a lack of open reading frame, and most successful lncRNA identification pipelines incorporate a measure of the evolutionary conservation of codon structure. Using tools originally developed to identify putative coding regions of the genome [(Lin et al., 2011) as e.g.], many lncRNA cataloging efforts rely on the inference that a low probability of conserved coding potential, combined with a lack of discernable open reading frame, suggests that a given locus would not produce a functional protein product. Additional evidence that a given gene is non-coding may come from alignment to known protein domain databases such as PFAM (Finn et al., 2009) or UniProt (UniProt Consortium, 2015). To reduce the noise, filters can be applied to assembled transcripts including a minimum requirement of 200 bp in length, and at least two exons as evidence for active splicing of the putative lncRNA gene. It is important to note that lncRNAs appear to be a homogeneous class of RNAs but effectively they are quite diverse. The definition of lncRNAs is technical rather than mechanistic, like that for microRNAs.

Prior to their codification as a distinct class, a number of lncRNAs were identified in expansive cDNA and EST libraries (Nordström et al., 2009; Okazaki et al., 2002). With the advent of high-throughput RNA sequencing (RNAseq) however, the number of identified lncRNAs in mammalian species began to grow rapidly (Cabili et al., 2011; Guttman et al., 2009). Current estimates suggest that the number of lncRNA genes in the human genome is rapidly approaching the number of protein coding genes [15,941 vs. 19,815 respectively; GENCODE v24 (Harrow et al., 2012)]. Put in this perspective, it is not surprising that most lncRNA genes have not been evaluated for a functional role within the cell.

It is understood that lncRNAs are less conserved than protein coding genes. However, this statement mainly reflects the observation that lncRNAs exhibit less primary sequence conservation than protein coding genes. In many cases, primary sequence conservation may not be a true reflection of evolutionary pressure, and other RNA characteristics may be preserved over evolution with no detectable pressure on primary sequence (e.g. RNA secondary structure or the act of transcription). lncRNAs exhibit surprising preservation of other properties that suggest they may have conserved functions, and in general, their activities may be independent of their primary sequence. Within primates 80% of human lncRNAs have a readily identifiable ortholog in chimpanzee, identified as expressed within a syntenic region (Washietl et al., 2014). This number decreases to 35 and 38% of human lncRNAs with an identified ortholog in rat or mouse respectively. Interestingly however, the tissue-specific expression patterns for lncRNAs are also preserved across evolution, suggesting that lncRNAs are subject to regulatory pressures that are under positive selection (Washietl et al., 2014). While these conserved properties do not directly imply a functional role for any lncRNA gene, they suggest that the presence of these genes and their conserved regulation may be important even as proxy signals for biological activity within a cell.

Another important characteristic of lncRNAs is their relatively low expression level as compared to protein coding genes when measured in bulk tissue or cell expression studies. Relative to protein-coding genes of comparable expression levels, lncRNAs are much more tissue-restricted in their expression profiles (Cabili et al., 2011; Washietl et al., 2014), and recent work has demonstrated that many lncRNAs are expressed in a significantly more cell-type-specific manner than protein coding genes. Indeed, a large cohort of novel lncRNA genes was assembled using RNAseq from distinct, sorted populations of cortical projection neurons (Molyneaux et al., 2015) suggesting that lncRNAs may contribute to the establishment or specific activities of neuronal cell subtypes. Consistent with this, and with the staggering complexity and heterogeneity of the mammalian CNS, the brain is host to the largest repertoire of lncRNA genes relative to other somatic tissues (Cabili et al., 2011; Qureshi and Mehler, 2012). It is this tissue and cell-type specificity that makes lncRNAs ideal candidates for focused studies involving cellular specification and development in the mammalian CNS.

Another open question in lncRNA biology is whether the functional activity of lncRNAs is restricted to *in cis* genomic regulation of neighboring genes, or whether lncRNAs can function *in trans* away from their sites of transcription. The original gene regulation hypothesis by Jacob and Monod (1961) envisioned diffusible proteins acting *in trans* and DNA sequence elements acting *in cis*, but both of these concepts translate nicely into mechanisms involving RNA components as well. A number of mechanisms have been proposed for RNA operating *in cis* or *trans* (Rinn and Chang, 2012; Ulitsky and Bartel, 2013). In support of *cis*-regulatory mechanisms, it has been observed that lncRNA genes tend to be found in close genomic proximity to known developmental regulator genes (Cabili et al., 2011; Ulitsky and Bartel, 2013; Ulitsky et al., 2011). Furthermore, lncRNA expression and activities of adjacent genes are closely related to nearby enhancer activity, suggesting a potential overlap between these classes of regulatory elements. Conversely, several lncRNAs have been observed to have functional activity in the cytoplasm (Kim et al., 2007; Kino et al., 2010; Yoon et al., 2012), and

many lncRNAs have exclusive expression outside of the nucleus (Cabili et al., 2015), suggesting that lncRNA activities need not be restricted to their genomic neighborhood. In reality, activities both *in cis* and *in trans* have been described for lncRNA genes, and the answer to this question may be that both *cis*- and *trans*-regulation by lncRNAs are possible. Importantly, both activities may be possible for a single lncRNA gene (Bond et al., 2009), suggesting that any attempt to describe a 'uniform' mechanism to this class of genes may prove futile. A myriad of functional activities have already been described for lncRNAs in a variety of neural contexts, highlighting the diverse potential roles for these genes in CNS development.

LncRNA mechanisms in CNS differentiation

Recent studies have identified a broad array of molecular mechanisms by which lncRNAs regulate or influence CNS differentiation. The fact that there is no single, unifying mechanism, as there is for microRNAs, for example, is likely one reason why it has been so difficult to understand lncRNAs on a genome-wide scale. As outlined in the following examples, each lncRNA must be painstakingly studied on an individual basis before its mechanism is revealed. This fact may explain why so much skepticism has been expressed about the role of lncRNAs in the absence of protein-coding capacity or simple sequence conservation. Within the developing brain, many lncRNAs exhibit exquisite cell specific localization (Goff et al., 2015; Qureshi et al., 2010; Ramos et al., 2013), are regulated during neural lineage specification (Mercer et al., 2010; Molyneaux et al., 2015) and exhibit strikingly restricted expression patterns within the cerebral cortex (Ayoub et al., 2011; Belgard et al., 2011; Goff et al., 2015; Molyneaux et al., 2015; Sauvageau et al., 2013), suggesting the potential to influence cell fate decisions. Several recent studies have demonstrated the requirement for lncRNA genomic loci in the establishment of specific neuronal cell populations, or the proper regulation of neuronal precursor differentiation. lncRNAs have been shown to affect neuronal development (Sauvageau et al., 2013) and are thought to be important players in various neurological disorders (Qureshi et al., 2010; Schonrock and Götz, 2012). These few examples highlight the extent of the impact lncRNA genes have on normal brain development and suggest that much work remains to fully describe the roles for these regulatory RNA genes within the CNS. Figure 1 highlights a sample of the thematic mechanisms for lncRNAs described in these examples.

Regulation during development

A number of lncRNAs have been identified as being regulated during CNS development. One study found 17 noncoding RNAs with expression levels changing during fate specification of neural progenitor/stem cells (NPSCs), 64 during NPSC differentiation into GABAergic neurons, and 100 during oligodendrocyte specification (Mercer et al., 2010). While only correlative, these observations lay valuable groundwork for subsequent mechanistic studies. They also demonstrate that cell type specification is directly associated with the appearance of specific pools of noncoding RNAs, suggesting that there is likely to be a cause-and-effect relationship between cellular differentiation and regulation of lncRNAs. More recent mechanistic studies provided evidence for lncRNAs directly affecting cellular differentiation.

Regulation of transcription and/or splicing

One lncRNA, Malat1 (Bernard et al., 2010), was found to associate with nuclear speckles, which are complexes associated with storage or assembly of transcription and/or splicing factors. This punctate appearance of Malat1 is strong in neurons but is nearly absent in non-neuronal cells of the CNS. Malat1 is quite stable after a block in transcription but the location appears to become more diffuse in nuclei in the absence of transcription. The speckles positive for Malat1 are also enriched in specific functional complexes, including the SF2/ASF splicing complex and SR-related transcription factors. Knockdown of Malat1 interrupts recruitment of SF2/ASF to active transcription sites. A screen for genes affected by Malat1 knockdown revealed a number of functional gene sets including synaptogenesis. This was confirmed in primary hippocampal neurons where knockdown of Malat1 decreased, and Malat1 overexpression increased the density of synapses. Developmentally, Malat1 expression corresponds with periods of synaptogenesis. Therefore, a specific lncRNA is capable of regulating synaptogenesis by organizing with splicing and/or transcription factors in nuclear loci known to harbor complex assembly. These results predict a *trans*-acting role in assembling transcription and/or splicing complexes required for synaptogenesis.

Cis-acting mechanisms

A balance of excitatory and inhibitory neurons is key to brain function. Orchestrated developmental pathways carefully regulate the differentiation, cellular migration, and functional integration of neuronal precursors. In the case of inhibitory neurons, the primary source of cells is the medial ganglionic eminence (MGE). The cells resulting from this pathway are predominantly GABAergic. Major developmental regulatory factors include the Dlx class of homeobox proteins. Dlx1 and Dlx2 regulate expression of Dlx5 and Dlx6 from opposing strands of a region of mouse chromosome 6 that includes two lncRNAs—Evf1 and Evf2. Evf2 is a target of SHH signaling and is part of an ultraconserved intergenic regulatory region that is believed to control Dlx5 and Dlx6. Bond and colleagues (2009) constructed a mouse with a triple-poly(A) site insertion shortly following initiation of Evf2 transcription, intending to interfere with Evf2 transcription. No Evf2 lncRNA could be detected by in situ hybridization, but the insertion led to increased levels of Dlx6, and, to a lesser extent, Dlx5 without affecting the nearby Evf1. These increases could be enhanced by in utero electroporation of Evf2-expressing plasmids, consistent with at least a partial effect in *trans*. Since Dlx1/2, Mecp2 and HDAC1 have been found to be associated with regulatory sites in this gene cluster, CHIP-qPCR was used to show decreased binding of Dlx1/2 and Mecp2 to this region with little effect on HDAC1. This suggests that Evf2 lncRNA participates in the recruitment of these factors to regulatory sequences. The reduced inhibitory Mecp2 is consistent with increases in Dlx6 and Dlx5. The differential result in separate regulatory regions predicts that additional binding events or sequence recognition likely provides finer control. Most importantly, reduced Evf2 lncRNA correlated with reduced numbers of hippocampal interneurons, Gad1 expression, and synaptic inhibition. The reduction in Gad1 was corrected later in development but the loss of synaptic inhibition persisted later in life. Evf2, therefore, is essential for timely development of inhibitory interneurons and the persistent creation of functional inhibitory synapses. The mechanism appears to at least partially occur in *cis*, because ectopic expression of Evf2 did not rescue Dlx6 dysregulation caused by a transcriptional block of

endogenous Evf2, but rather it exacerbated the effect. However, the apparent recruitment of inhibitory factors such as Mecp2 and Hdac1 are more consistent with an effect *in trans*, assuming that these factors interact with Evf2 RNA. While the precise mechanism is complex, it is clear that expression of Evf2 is required for producing sufficient inhibitory synapses in brain development.

Antisense mechanisms

A different lncRNA encoded within the Dlx cluster appears to function as an antisense attenuator. Dlx1as was first identified as being expressed in the subventricular zone (Liu et al., 1997). Dlx1as is transcribed from the opposite strand overlapping a portion of Dlx1. Krause and colleagues (2013) inserted four poly(A) cleavage sites into a Dlx1as intron so that it should not affect Dlx1 mRNA expression and found that a lack of Dlx1as was associated with increased Dlx1 mRNA. Interestingly, such Dlx1as knockouts were fertile, appeared normal, but had no detectable change in the numbers of GABAergic neurons. Therefore, while the biological impact of Dlx1as may be subtle and it may primarily act as an antisense attenuator for Dlx1 mRNA, the mechanism of action seems to be limited to serving as an antisense RNA.

Interaction with regulatory proteins

The regulation of NPSC between self-renewal and differentiation is not well understood, but one group proposed that one or more lncRNAs participate in this process. Daniel Lim's group at UCSF searched for novel RNAs enriched in ventricular-subventricular zone (V-SVZ) cells and reported Pnky (formerly Inc-Pou3f2) as one such example (Ramos et al., 2013). Analysis shows that Pnky is transcribed from bivalent genomic regions, is specifically expressed in V-SVZ and not other NPSC populations, and is enriched in nucleus (Ramos et al., 2015). In cultures derived from primary V-SVZ, Pnky is expressed but diminishes during differentiation of neuronal cells. When Pnky is reduced by transfection with specific shRNAs, neuron production increases and lineage tracing shows a concomitant reduction in multipotent precursor cells, consistent with the hypothesis that Pnky expression maintains NPSC in a proliferative state. Pnky lncRNA was found to bind PTBP1, a protein known to participate in splicing during neuronal differentiation. Knockdown of either Pnky or PTBP1 produced overlapping sites of differentially spliced transcripts. Therefore, in the case of Pnky, lncRNAs may have a role in regulating differentiation decisions in neuronal precursor cells by interacting with mRNA splicing regulatory schemes.

Requirement during cortical differentiation

Pantr2, originally named *Brn1b*, is a lncRNA gene that, similar to *Pnky*, affects the differentiation potential of cortical NPSC during corticogenesis, and as a consequence, affects proper lamination and organization of the developing neocortex. *Pantr2* knockout mice exhibit a significant and specific loss of basal progenitors (intermediate precursor cells) within the subventricular zone of the cortex during development (Sauvageau et al., 2013). As a result, these mice demonstrate a significant reduction in callosal projection neurons of the upper cortical layers, which are principally derived from IPCs. The requirement for lncRNA genes such as *Pnky* and *Pantr2* for the proper development of specific neuronal populations suggests that lncRNAs can have a direct effect on neuronal cell fate decisions.

Indirect mechanisms

LncRNA genes can modulate the activity of their targets using a variety of direct or indirect mechanisms, including the recruitment of regulatory complexes to specific genomic loci. One such lncRNA, which uses an indirect mechanism, is Six3OS. This RNA gene is important for proper development and cell fate selection in the developing retina (Geng et al., 2007; Rapicavoli et al., 2011). Six3OS is co-expressed with the neighboring Six3 protein coding gene and both are highly specific for the retina and hypothalamus (Geng et al., 2007). Modulation of Six3OS affected the relative proportion of bipolar cells and Muller glia (Rapicavoli et al., 2011). Interestingly, it was demonstrated that Six3OS affects the activity of the Six3 gene indirectly, without modulating its expression levels. The authors observed that this occurs *in trans*, through a scaffolding interaction between the PRC2 subunit Ezh2 and various members of the *eyes absent* family of proteins. They conclude that Six3OS coordinates the regulation of Six3 genomic targets in a competitive manner to indirectly modulate Six3 activity.

Direct activity in transcriptional regulation

In a more direct regulatory scheme, lncRNA genes may act cooperatively with specific factors to modulate their activity directly. One such neuronal lncRNA influences target gene regulation by binding with a traditional protein transcription factor. RMST (rhabdomyosarcoma 2-associated transcript) is expressed selectively in brain under the control of the CNS-specific repressive factor REST (Ng et al., 2013). By physically interacting with Sox2, RMST alters the genomic binding site sequence requirements to affect the set of regulated genes and in particular the combined molecules target genes involved in neurogenesis. The mechanism of RMST demonstrates that lncRNA may function as a coregulator, binding with and affecting the regulatory activity of a “traditional” transcription factor.

Interpreting knockout experiments

In contrast to the examples listed above, some groups conclude that select lncRNAs contribute less to differentiation mechanisms than mRNAs. For example, a knockout of Visc-2 (also known as C130071C03Rik) had no detectable effect on several measures of cellular differentiation or behavior (Oliver et al., 2015). The Visc-2 lncRNA is sequence conserved among mammals and its transcription unit overlaps another lncRNA, Visc-1, as well as a microRNA, miR-9-2. The knockout strategy replaced the first exon of Visc-1 as well as the single exon of Visc-2, leaving neighboring genes unaffected but reducing expression of miR-9-2 by 30%. Previous work identified a promoter near miR-9-2 that should have been unaffected by the Visc-2 deletion (Laneve et al., 2010), but the possibility remains that the overlapping Visc-1 exon sequences may contribute to miR-9-2 levels. Surprisingly, there was little change in levels of Visc-1 lncRNA, presumably due to the use of cryptic transcription start sites and alternative 5' exon sequences. However, even with the clear loss of Visc-2 lncRNA, there was no detectable change in survival, gross anatomy, cortical layers, or markers of precursors. Similarly there was no effect in a series of behavioral tests. The relative abundance of Visc-2 lncRNA in cortex was interpreted as a predictor of function, however, the lack of a detectable effect in the knockout was interpreted by the authors as proof that Visc-2 had little or no function in cortical development. The possibility

remains that it is not the transcript acting *in trans* but rather the transcription event acting *in cis* that produces function. In this knockout strategy, levels of Visc-2 lncRNA were undetectable, but transcription from a start site may have been present--as evidenced by the detection of Visc-1 lncRNA. Perhaps the presence of active transcription is itself required for function of this genetic locus as has been described for other lncRNA loci (Yin et al., 2015). This study highlights the difficulty of knocking out lncRNA genes or of interpreting results. With such a variety of mechanisms utilized by this class of genes, it is difficult to ensure that the activity has been interrupted by replacing sequences or preventing the production of mature lncRNA.

Encoding of micropeptides

While the primary definition of lncRNAs is that the RNA is incapable of encoding a polypeptide, recent work suggests that even this property may need to be flexibly interpreted when examining lncRNA function. For example, some lncRNAs may encode cryptic micropeptides (Slavoff et al., 2013; Sun et al., 2013; Bazzini et al., 2014; Anderson et al., 2015). At least one small peptide product (46 amino acids) of a lncRNA has been found to function in regulating Ca^{+2} -mediated contraction (Anderson et al., 2015). This implies that a subset of lncRNAs ought to be associated with polysomes and some of these could be translated into such micropeptides.

To investigate this possibility within CNS development, we re-analyzed polysome fractionation/RNAseq data from a recent study of translational control in mouse cortex (Liu et al., submitted; GEO accession GSE77647), using transcript definitions from the GenCode consortium containing 5,263 transcript-level records identified as "lncRNA." Filtering for lncRNAs that were unchanged in total abundance between E13 and E16, we identified only 32 lincRNA transcripts associated with polysomes that differed by time point. Of these, 8 were enriched in E13 polysomes and 24 were enriched in E16 polysomes. Because the unfractionated RNA levels were unchanged, the differential association with polysomes was unlikely to be due to changes in overall expression levels. The presence of polysome-associated lncRNAs and the observation that a fraction of them is differentially associated with polysomes over CNS development suggests that the functions, if any, for these RNAs may involve association with the translation machinery, either to engage in active translation of an encoded polypeptide, or a regulatory interaction with the translation machinery. One possibility, as outlined above, is that they encode micropeptides and some of these products may be functional. Indeed, only 2-3 of the 32 differentially-associated lncRNAs had any reasonable ORF consistent with a micropeptide (30-90 codons). A total of 6 had small but positive phyloCSF scores indicating the possible conservation of a small ORF. However, if the longest ORF that is generally found in lncRNAs is on the order of 30-50 codons (90-150 nt), we would expect that translating lncRNAs would be associated with monosomes, which accommodate translation up to ~590 nt (Heyer and Moore, 2016). The more likely possibility is that these lncRNAs are not bound with the polysome as a translation substrate but instead may be a component of ribonucleoprotein complexes that are in turn associated with ribosomes or ribosome-binding complexes. Indeed, such a mechanism has recently been described for lncRNA genes that enhance translation (Zucchelli et al., 2015a; 2015b). It is clear that translation is regulated during development (Kraushar et al., 2014; 2015) and RNA-containing complexes would provide an efficient interface to select mRNA targets for regulation in a sequence-

dependent manner. In either case, the possibility of unexpected translational mechanisms at work during CNS development extends the scope of lncRNA mechanisms. More analysis is required to identify specific mechanisms underlying these associations with polysomes.

LncRNA in CNS disorders or degeneration

Support for lncRNAs functioning during CNS development is complemented by evidence linking genetic variants with disease or degeneration. Given the vast number of lncRNA genes, and the diversity of mechanisms through which they may influence cellular physiology, it is not surprising that many lncRNA genes have been associated with disorders of the central nervous system. Mutations in lncRNA gene loci have been identified in a handful of neurological disorders such as Alzheimer's disease (Faghihi et al., 2008; Lee et al., 2015; T. Liu et al., 2014; Magistri et al., 2015), Huntington's disease (Johnson, 2011; Johnson et al., 2010), Angelman syndrome (Brant et al., 2014) and various other imprinting disorders (Meng et al., 2015; Santoro et al., 2013), autism, stroke (Dharap et al., 2013), and spinocerebellar ataxia (Daughters et al., 2009; Mutsuddi et al., 2004). lncRNA genes are also associated with neuropsychiatric disorders as well (Barry et al., 2014; Rao et al., 2015; Ren et al., 2015). An important consideration when predicting the scope of lncRNA impact on CNS disorders is that the majority (~88%) of mutations with strong genetic associations to disease occur in regions of the genome that do not code for proteins (Hindorff et al., 2009). These brief examples demonstrate that lncRNAs are required for either development of the CNS or its continued health during life.

Conclusions and Predictions

With this summary of lncRNA characteristics and the diverse examples of lncRNA mechanisms active in CNS development, it should be clear that lncRNAs must be integral to models of developmental pathways. lncRNAs use a dizzying array of strategies to regulate epigenetic status, transcription (directly or *in trans*), post-transcriptional steps including splicing, and translation, at least. The flexibility of RNA as a folding substrate, its ability to interact with proteins, and the ability to hybridize to nucleic acids using both Watson-Crick or Hoogsteen pairing, suggests that the possible mechanisms for lncRNA-mediated regulation may be staggeringly diverse.

Following upon the hypothesis that RNA was the first information-carrying and catalytic molecule of life (Darnell and Doolittle, 1986), perhaps it is not surprising that noncoding RNAs are capable of such diverse, seemingly unconserved functions. Perhaps noncoding RNAs are remnants of adaptive mechanisms whereby early RNAs exerted sole control over newer polypeptides and DNA. In this case, it would be reasonable to presume that noncoding RNAs became integral to the process of multicellularity and cellular differentiation. Just as RNA satisfies all aspects of Jacob and Monod's hypothesis of gene regulation, there is no *a priori* reason to doubt that RNA participates in the establishment of cellular identity. We are left, then, with a conundrum. Is the vast diversity of lncRNA expression in different cells of the CNS the *cause* of cellular specificity or the *result* of differentiation mechanisms? We argue that lncRNAs are more likely to be a driving force in differentiation, based on the wide variety of examples cited above.

No matter the reason, the fact that lncRNAs are highly specific for different neurons and non-neuronal cells of the CNS provides a valuable tool. With the advent of techniques for single-cell RNAseq, we are acquiring the necessary resolution to evaluate precisely how lncRNAs relate to cell state specification. We propose that lncRNAs will be more robust discriminators or classifiers of cell identity than mRNAs. To take full advantage of this, single-cell sequencing depths and RNA capture rates must be sufficient to reliably detect lncRNAs which are less abundant than mRNAs. However, there is evidence that the predominant lncRNAs unique to each cell type are present at higher abundances than in heterogeneous populations of cells (Liu et al., 2016). Either way, we predict that lncRNAs will be particularly valuable for classifying CNS cells as single-cell RNAseq becomes more widely used.

Finally, if one or a few key transcription factors are capable of inducing direct differentiation of somatic or pluripotent cells (D'Alessio et al., 2015), might a handful of cell-type specific lncRNAs act similarly? Since it is possible to use transfected mRNAs to reprogram somatic cells to pluripotency (Warren et al., 2010), one might predict that ectopic expression of lncRNAs may be similarly effective for directing or facilitating differentiation. Other noncoding RNAs (microRNAs) can be used to promote induced neurogenesis (Yoo et al., 2011). Based on the examples described above, we predict that lncRNAs, at least in combinations with transcription factors, will likely refine cellular specificity, or enhance efficiency of cellular reprogramming.

The sudden appearance of tightly-controlled, mechanistic studies of lncRNAs in CNS development demonstrates that we are only beginning to appreciate this class of regulators. The fact that lncRNA mechanisms fit well into predictions made early in the study of nucleic acids shows, however, that it is us who have come late to the party, not lncRNAs.

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Figure legend

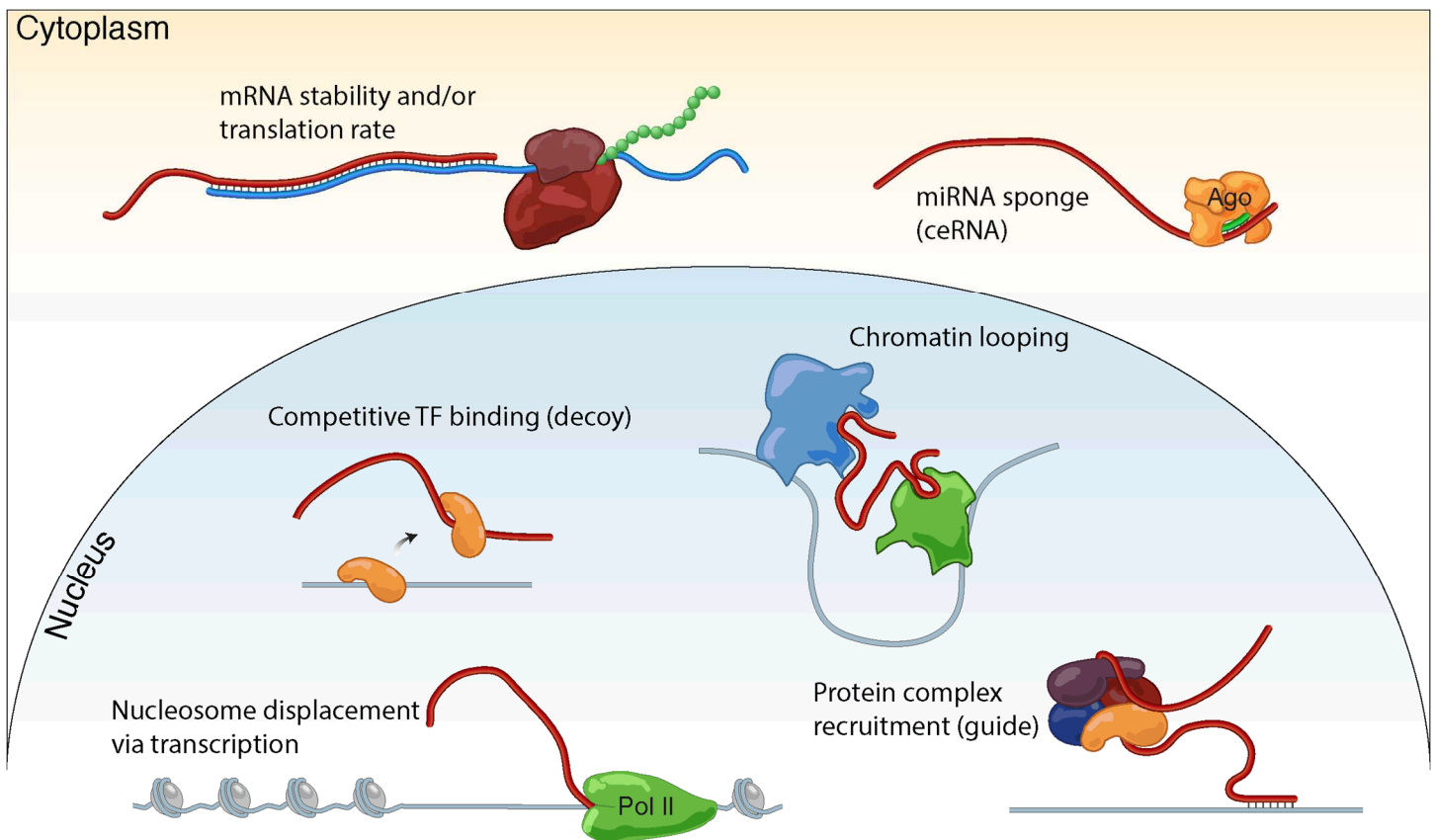


Figure 1. Illustrated examples of lncRNA mechanisms. Among the many mechanisms used by lncRNAs during CNS development are: Interactions with transcription factors (TF), such as through decoy binding or competitive inhibition; Chromatin modulation by nucleosome displacement or chromatin looping; Recruitment of protein complexes to affect transcription or epigenetic regulation; Interaction with the translation machinery; mRNA stabilization; or Binding with microRNAs to interfere with their activity.