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Regulatory mechanisms of long noncoding RNAs in vertebrate central nervous system development and function

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Abstract

Long noncoding RNAs (lncRNAs) have emerged as an important class of molecules that regulate gene expression at epigenetic, transcriptional, and post-transcriptional levels through a wide array of mechanisms. This regulation is of particular importance in the central nervous system (CNS), where precise modulation of gene expression is required for proper neuronal and glial production, connection and function. There are relatively few functional studies that characterize lncRNA mechanisms, but possible functions can often be inferred based on existing examples and the lncRNA's relative genomic position. In this review, we will discuss mechanisms of lncRNAs as predicted by genomic contexts and the possible impact on CNS development, function, and disease pathogenesis. There is no doubt that investigation of the mechanistic role of lncRNAs will open a new and exciting direction in studying CNS development and function.

Recent advances in sequencing technology and genome-wide sequencing projects have overturned the protein-centric view of the cell by revealing that there is extensive non-protein coding transcription throughout eukaryotic genomes (Carninci et al., 2005; Katayama et al., 2005; Birney et al., 2007; Bernstein et al., 2012; Djebali et al., 2012). In fact, 80% of the human genome is likely linked to a biochemical function, with many functional regions assigned outside of traditional protein-coding loci (Bernstein et al., 2012). Only about 1.5% of DNA is protein-coding, indicating that the remaining transcriptional activity contributes to a large population of noncoding RNAs (ncRNAs) (Bertone et al., 2004; Kapranov et al., 2007). These transcripts can take several forms – housekeeping RNAs, such as ribosomal RNAs and transfer RNAs; small ncRNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs; and long ncRNAs (lncRNAs). While the housekeeping and small ncRNAs have been characterized, the mechanisms by which lncRNAs regulate gene expression and cellular functions remain poorly understood. In this review, we will highlight new discoveries of lncRNA functions in gene expression regulation with an emphasis on how these functions can influence the development of the central nervous system (CNS) and contribute to neurological disorders.

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Features of lncRNAs

lncRNAs are classified as transcripts >200 nucleotides (nt) in length that show little or no evidence of an open reading frame (ORF) (Kapranov et al., 2007). They can be transcribed from either strand and can be classified as sense, antisense, bidirectional, intronic, or intergenic with respect to nearby protein-coding genes (Huang et al., 2012). Many, but not all, lncRNAs are processed as typical mRNAs including 5' capping, polyadenylation, and histone modifications (Okazaki et al., 2002; Carninci et al., 2005; Cheng et al., 2005; Kapranov et al., 2007; Ponjavic et al., 2007; Derrien et al., 2012). Splicing of lncRNAs is less efficient than that of protein coding genes and may not occur at all in some cases (Tilgner et al., 2012). There is a heavy bias toward transcripts with two exons (42% of lncRNAs compared to 6% of protein-coding genes) (Derrien et al., 2012). Some lncRNAs are exported from the nucleus and perform important functions in the cytoplasm, but the majority are found in the nucleus, particularly associated with chromatin (Derrien et al., 2012). Though there is evidence for numerous lncRNA loci, only a fraction have been functionally characterized (Carninci et al., 2005; Katayama et al., 2005; Birney et al., 2007; Harrow et al., 2012). Those that have been characterized participate in a striking diversity of cellular processes (Ponting et al., 2009; Clark and Mattick, 2011; Wang and Chang, 2011; Qureshi and Mehler, 2012; Rinn and Chang, 2012).

There was initial debate that lncRNAs simply represented transcriptional noise, as evidenced by their lack of sequence conservation and the low fidelity of RNA polymerase II transcription initiation (Ponjavic et al., 2007; Struhl, 2007). In addition, even though there are many lncRNA transcripts identified through sequencing and array projects, they are transcribed at much lower levels than individual mRNAs (Mattick and Makunin, 2006). However, the low sequence conservation of lncRNAs has been shown to maintain lncRNA secondary structure rather than primary sequence (Ulitsky et al., 2011), which would also help illuminate how diverse lncRNAs can interact with the same protein complexes (Khalil et al., 2009; Guttman et al., 2011; Moran et al., 2012b). Furthermore, part of the ENCODE project probed the possibility that lncRNAs contain very short ORFs that encode small peptides, and showed that the vast majority of transcripts classified as lncRNAs are not translated, supporting a role for these transcripts as ncRNA rather than protein precursors (Banfai et al., 2012; Derrien et al., 2012). Finally, though direct functional studies are lacking, lncRNAs have been shown to be developmentally regulated (Blackshaw et al., 2004; Rinn et al., 2007), expressed in specific cell types (Mercer et al., 2008), localized to specific subcellular compartments (Sone et al., 2007; Bond and Fox, 2009; Ip and Nakagawa, 2011), associated with chromatin signatures indicating transcriptional regulation (Guttman et al., 2009), under evolutionary constraint (Pollard et al., 2006; Ponjavic et al., 2007; Guttman et al., 2009), and associated with diseases (Qureshi and Mehler, 2011; Wapinski and Chang, 2011), all of which support a meaningful role of lncRNAs.

lncRNAs in the central nervous system

The vertebrate CNS contains an enormous diversity of neuronal and glial cell types that differentiate and form networks through an intricate developmental program that must coordinate intrinsic and external stimuli to achieve proper form and function. Cells in the CNS are highly transcriptionally active and exhibit strong expression of ncRNAs, with 5,458 of a total 9,747 lncRNA transcripts detected in the human brain using a microarray, including specific expression of ~40% of the most highly and differentially expressed lncRNAs (Derrien et al., 2012). In mouse embryonic stem cells, shRNA-mediated knockdown of 93% of long intergenic ncRNAs (lincRNAs) resulted in significant gene expression changes, implying that the majority of lincRNAs are functional (Guttman et al., 2011). In addition, lncRNAs can work through various mechanisms to exert precise control

over both individual genes and large gene networks, allowing the spatiotemporal precision necessary to execute complex neurobiological processes.

Though ncRNAs are present in species from bacteria to humans, expansion of ncRNAs has been linked to evolutionary complexity. The study by Taft et al. provides a beautiful illustration of the link between the proportion of non-protein coding DNA sequence in the genome and evolutionary complexity, with vertebrates containing the highest proportion of non-protein coding sequence (Taft et al., 2007). miRNAs have already been linked to brain complexity and have an important role in brain cellular diversity (Berezikov et al., 2006); as lncRNAs continue to be characterized they will doubtless follow suit.

Large-scale studies surveying lncRNAs in the nervous system have provided further evidence of their importance. *In situ* hybridization has shown that many lncRNAs are expressed in specific anatomical regions, cell types, or subcellular compartments in the mouse brain (Mercer et al., 2008). In a study of intergenic lncRNAs, those in the brain were found to be highly conserved between mouse and humans, enriched in predicted RNA secondary structure, and preferentially located near protein-coding genes that are also implicated in nervous system development or function (Ponjavic et al., 2009). lncRNAs have also been shown in both human and mouse cells to influence pluripotency or differentiation specifically in neural cells (Mercer et al., 2010; Lin et al., 2011; Ng et al., 2012). In addition, lncRNAs have been implicated in neurodevelopmental, neurodegenerative, neuroimmunological, and neuro-oncological disorders, underlining their importance in CNS development and function (Table 1) (Mehler and Mattick, 2007; Qureshi et al., 2010; Lin et al., 2011; Qureshi and Mehler, 2011; Ziats and Rennert, 2012). In this review, we will highlight the roles of lncRNAs in nervous system development by connecting their genomic context with their possible cellular functions.

lncRNAs associated with genomic contexts

It has become increasingly clear that eukaryotic genomic organization is highly complex, involving convergent, divergent, overlapping, and antisense transcription of protein-coding and ncRNAs (Carninci et al., 2005; Katayama et al., 2005; Kapranov et al., 2007; Derrien et al., 2012; Djebali et al., 2012). Many lncRNAs exhibit multiple splicing isoforms and transcription start sites, use of which may contribute to cell type- or developmental stage-specific events (Liu et al., 2006; Amaral et al., 2009; Spigoni et al., 2010). While several methods of classifying lncRNAs based on function have been proposed (Wilusz et al., 2009; Wang and Chang, 2011), this review will continue to classify lncRNAs based on genomic context as this information is easily accessible, while many cellular functions need to be further revealed.

lincRNAs

lncRNAs that are not found near any protein-coding locus (within <10kb) are considered long intergenic noncoding RNAs (lincRNAs) (Guttman et al., 2009; Ponting et al., 2009), the most numerous of the lncRNA types (Derrien et al., 2012) (Fig. 1). lincRNAs have been shown to have effects both in *cis* (Orom and Shiekhattar, 2011) and in *trans* gene expression regulation (Guttman et al., 2009; Guttman et al., 2011).

lincRNAs in cis regulation—In *cis*, lincRNAs can have enhancer-like functions (Orom et al., 2010). Knockdown of these enhancer lincRNAs (also termed activating ncRNAs or ncRNA-a) with siRNAs resulted in decreased expression of neighboring genes, indicating that the enhancer-like effect was ncRNA-a-dependent (Orom and Shiekhattar, 2011). While the exact mechanism by which enhancer lincRNAs perform their function is unknown, the working model is that the ncRNA transcribed from the enhancer locus acts as a scaffold to

mediate chromatin looping and assembly of transcriptional machinery (Orom and Shiekhattar, 2011). A second class of lincRNAs functioning in *cis*, called eRNA, is transcribed from the enhancer region of a gene and promotes transcription of nearby genes (Kim et al., 2010). Although the exact mechanism of eRNA function is unknown, the expression of eRNAs may promote transcription of nearby genes by opening chromatin structure, recruiting RNA polymerase II to the promoter, or by facilitating associations between enhancer and promoter regions (Kim et al., 2010).

MALAT1 (also known as *NEAT2*) has been shown to interact with splicing factors and is implicated in nervous system development as it is expressed during later stages of neuronal and oligodendrocyte development (Mercer et al., 2010). Knockdown of *MALAT1* in cultured hippocampal neurons results in decreased synaptic density, while overexpression has opposite effects (Bernard et al., 2010). Contrary to *in vitro* studies, a *Malat1* knockout mouse was generated and showed no general effects on transcription or splicing (Zhang et al., 2012). However, some genes located near the *Malat1* locus were upregulated in mutant cortices, supporting a role for *Malat1* in *cis* regulation (Zhang et al., 2012). The authors attribute the *cis* effect of *Malat1* to transcriptional interference or histone modification, but do not eliminate the possibility that the *Malat1* locus contains DNA elements that contribute to *cis* regulation (Zhang et al., 2012). Due to the identity of *Malat1* as a lincRNA (>10kb from other loci), it is unlikely that *Malat1* works through transcriptional interference, as other examples of transcriptional interference occur over much shorter distances (Bumgarner et al., 2012; Hongay et al., 2006; Petruk et al., 2006).

In the CNS, a study of transcription in the mouse neocortical layers revealed a set of 66 lincRNA loci that were patterned across the layers (Belgard et al., 2011). These transcripts were more likely to be correlated with the expression of their protein coding neighbors than unpatterned lincRNAs, supporting a role for some of these lincRNAs in *cis* regulation (Belgard et al., 2011). Further, these lincRNAs were enriched for transcription across enhancers, providing support for eRNA functions in *cis*. Specific lincRNAs functioning in *cis* in the CNS remain to be characterized.

lincRNAs in trans regulation—lincRNAs have also been shown through short hairpin RNA (shRNA) knockdown methods to influence global gene expression in *trans* (Guttman et al., 2011). Several studies have shown that many lincRNAs physically associate with chromatin modifiers, including readers, writers, and erasers, to influence gene expression at various loci (Khalil et al., 2009; Guttman et al., 2011). Similar to lincRNA action in *cis*, the proposed mechanism for lincRNAs acting in *trans* describes the ncRNA as a scaffold that aids in the assembly of large ribonucleoprotein complexes and their targeting to specific loci to execute cell-type-specific gene expression programs (Guttman et al., 2011).

Several lincRNAs that work in *trans* have been identified with important roles in the nervous system. *Gomafu* (also known as *RNCR2* or *MIAT*) is expressed in differentiating neurons and oligodendrocytes (Mercer et al., 2010) as well as mitotic and postmitotic retinal precursors (Rapicavoli et al., 2010) and forms a punctate pattern of subnuclear domains that are not associated with any known nuclear bodies (Sone et al., 2007). The mechanism of *Gomafu* action is unknown, but it has been shown to interact with splicing factors and may influence alternative splicing patterns to drive differentiation (Tsuiji et al., 2011).

The lincRNA *Neat1* is an architectural component of paraspeckles, a nuclear body involved in edited RNA retention (Fox and Lamond, 2010). *Neat1* has been shown to be downregulated in neural and oligodendrocyte progenitors and upregulated in their progeny in mice (Mercer et al., 2010). Knockdown of *Neat1* disrupts paraspeckles, causing export of the retained RNA to the cytoplasm (Chen and Carmichael, 2009). Because its nuclear

localization and function in RNA retention are independent of genomic location, *Neat1* works in *trans*.

In the developing mouse retina, lincRNA *TUG1* is important for photoreceptor development (Young et al., 2005). Overexpression of *TUG1* in the rat retina showed no obvious phenotype, but siRNA-mediated knockdown resulted in mislocalization and improper formation of rod photoreceptor cells (Young et al., 2005). Cells electroporated with the *TUG1* siRNA constructs exhibited a decrease in rod-specific rhodopsin and an increase in cone-specific peanut agglutinin, as well as complementary changes in gene expression (Young et al., 2005). Microarray analysis of wild type and *TUG1* knock down retinas revealed global down regulation of the rod-specific gene expression program in *trans*, indicating that the normal role of *TUG1* is to promote photoreceptor differentiation into rod cells (Young et al., 2005).

Natural antisense transcripts

Natural antisense transcripts (NATs) are lncRNAs that are transcribed overlapping with and in the antisense orientation to protein coding genes or ncRNAs (Fig. 1). While the importance of NATs has been known for many years (Knee and Murphy, 1997), it was not until recently that studies by the FANTOM consortium discovered that antisense transcription is widespread in the mammalian genome and presents a powerful means of regulation (Carninci et al., 2005; Katayama et al., 2005). Further studies in human cells have also found that antisense transcription occurs in genomic contexts distinct from sense transcription, nonrandomly across the genome, and differs among cell types, indicating its importance in gene regulation (He et al., 2008).

NATs in cis regulation—Most NATs that have been described work in *cis* through various mechanisms. Splicing of the sense mRNA can be regulated by binding of the complementary NAT to block recognition of a splice site (Enerly et al., 2005; Werner and Sayer, 2009). NATs can create endo-siRNAs by binding the sense mRNA to generate regions of double stranded RNA (Tam et al., 2008; Watanabe et al., 2008). These endo-siRNAs can take either the sense or the antisense orientation to silence the coding mRNA or the NAT (Werner et al., 2009). More recent work has suggested a further mechanism for NATs in which they bind to the sense mRNA and mask miRNA binding sites, thus blocking miRNA-induced gene repression (Faghihi et al., 2010). NATs can also use their sequence complementarity to form a DNA:RNA triplex, creating a roadblock for the transcriptional machinery, which results in downregulation of the sense gene expression (Shearwin et al., 2005). Furthermore, NATs can recruit epigenetic modifiers to the locus from which they are transcribed to cause silencing or activation of neighboring genes (Morris, 2011; Magistri et al., 2012). Finally, NATs can help their complementary protein-coding mRNA associate with active polysomes to promote translation (Carrieri et al., 2012).

In the nervous system, many important genes have antisense transcription (Mercer et al., 2008). Among these genes is *BDNF*, which is involved in the developing and adult nervous system in processes such as cell survival and differentiation, axon growth and pathfinding, and synaptic transmission as well as several neuropsychiatric and neurodegenerative diseases (Bibel and Barde, 2000; Binder and Scharfman, 2004). Upon examination of the *BDNF* locus in humans, it was found that there are 12 isoforms of a lncRNA transcribed antisense to the *BDNF* mRNA (Pruunsild et al., 2007). At least 4 of these *BDNF-AS* transcripts are coexpressed with *BDNF* in the brain, where they form RNA:RNA duplexes (Pruunsild et al., 2007). A recent study found that inhibition of *BDNF-AS* results in upregulated *BDNF* mRNA, decreased chromatin silencing marks at the *BDNF* locus, increased BDNF protein, and increased neurite outgrowth and neuronal differentiation

(Modarresi et al., 2012). The formation of RNA:RNA duplexes suggests that *BDNF-AS* could act through generation of endo-siRNAs or by blocking splicing. Moreover, the observation of changed chromatin marks suggests that *BDNF-AS* could also regulate its sense partner through recruitment of chromatin modifiers.

The transcription factor Emx2 has been implicated in arealization, precursor proliferation, and lamination in the developing cerebral cortex (Mallamaci et al., 1998; Bishop et al., 2000; Heins et al., 2001). Antisense transcription from the *Emx2* locus produces several antisense isoforms, one of which (*Emx2OS*) is expressed in the developing CNS (Spigoni et al., 2010). In differentiating neural precursors, high levels of *Emx2OS* cause destabilization of *Emx2* mRNA by Dicer1, while low levels are necessary for proper *Emx2* transcription (Spigoni et al., 2010). It was also found that *Emx2* sense transcription is required for proper *Emx2OS* expression (Spigoni et al., 2010).

Analysis of the locus containing *Nrgn* and *Camk2n1* revealed multiple overlapping and antisense transcripts that were found to be developmentally regulated during corticogenesis (Ling et al., 2011). These NATs, *Nrgn-AS* and *Camk2n1-AS*, were localized in the cytoplasm and formed double stranded RNA complexes with their sense transcripts. The detailed mechanism is unknown, but the formation of dsRNA may prevent translation of their sense counterparts or serve as sources of endo-siRNAs and regulate the spatiotemporal expression of *Nrgn* and *Camk2n1* during corticogenesis (Ling et al., 2011).

The BACE1 protein is critical in Alzheimer's disease and the *BACE1* mRNA has a highly conserved antisense transcript (*BACE1-AS*) that is upregulated in the disease state (Faghihi et al., 2008a). *BACE1-AS* stabilizes its sense partner by blocking the binding site for a miRNA, resulting in upregulated *BACE1* expression (Faghihi et al., 2010). Knockdown of *BACE1-AS* causes reduced levels of beta-amyloid, suggesting that the antisense transcript could be used as both an early biomarker and a therapeutic target (Modarresi et al., 2011).

The gene *Uchl1* encodes a deubiquitinating enzyme that plays roles in brain function as well as Parkinson's disease and Alzheimer's disease pathogenesis (Setsuie and Wada, 2007). In a search for sense-antisense gene pairs in the brain, antisense *Uchl1* was identified, which overlaps with the first two exons of *Uchl1* (Carrieri et al., 2012). Through knockdown and overexpression experiments, this antisense lncRNA was found together with *Uchl1* in the cytoplasm and promote its association with active polysomes to increase translation (Carrieri et al., 2012).

Other genes with NATs include *Nkx2.2*, a transcription factor known to direct differentiation of neural stem cells into the oligodendrocyte lineage, and *Sox4*, a transcription factor important for specification and differentiation of neurons and glia in the developing CNS (Cheung et al., 2000; Tochitani and Hayashizaki, 2008; Ling et al., 2009). Another interesting study found that some sense-antisense transcript pairs are coexpressed in synaptoneurosomal preparations, indicating that they are coexpressed in the dendrites of pyramidal neurons and may be involved in regulation of synaptic plasticity (Smalheiser et al., 2008).

NATs in trans regulation—NATs can also work in *trans*, meaning they are transcribed from a genomic locus different from the one they target, but maintain sequence complementarity (Wang et al., 2006). A study of ESTs across many species estimates that there are 2,896 transcriptional units in humans that are involved in *trans* sense/antisense pairs, one fourth of which (>700) involve noncoding RNAs (Li et al., 2008). Of the *trans* sense/antisense pairs involving ncRNAs, a majority were correlated to inhibition or stabilization of complementary sense transcripts, while the remainder could be involved in

regulation of translation, splicing, RNA editing, trafficking, or subcellular localization, similar to their *cis*-acting counterparts (Li et al., 2008). For example, the mouse *Msh4* meiotic recombination gene forms a dsRNA complex with *Hspa5* mRNA and ultimately causes cell death (Hirano and Noda, 2004).

In the human nervous system, a duplicated and inverted locus produces an RNA transcript with antisense homology to the *NOS2A* mRNA (Korneev et al., 2008). This *trans*-NAT undergoes expression changes reciprocal to the *NOS2A* mRNA during differentiation of human embryonic stem cells into neurospheres, suggesting it may negatively regulate its sense partner and play an important role in neuronal differentiation (Korneev et al., 2008). This antisense transcript was also shown to be expressed in human brain tumors, including glioblastomas and meningiomas, indicating a possible role in tumor formation (Korneev et al., 2008).

In the human genome, several regions have been identified to show significant evolutionary acceleration compared to the chimpanzee genome, many of which are associated with genes involved in neurodevelopment (Chimpanzee Sequencing and Analysis Consortium, 2005; Nielsen et al., 2005). One of these regions, *HAR1*, encodes a lncRNA, *HAR1F*, which is antisense to another lncRNA, *HAR1R*, and is expressed in the developing human cortex (Pollard et al., 2006). *HAR1F* is coexpressed with reelin in Cajal-Retzius neurons and forms a stable secondary structure, suggesting a role in establishing the lamination of the cortex (Pollard et al., 2006). However, whether *HAR1F* influences reelin or reelin receptor expression is unknown. *HAR1R* is expressed at lower levels and later stages in brain development and may have a role in *HAR1F* downregulation by antisense inhibition (Pollard et al., 2006).

The rodent-specific *BC1* and the primate-specific *BC200* are highly expressed NATs in the brain, where they repress translation initiation in dendrites in an activity-dependent manner (Muslimov et al., 1998; Wang et al., 2002). Because the 200 nt cut off of lncRNAs is arbitrary and meant to distinguish lncRNAs from small ncRNAs, *BC1* is treated here as a lncRNA, even though it produces a transcript of 152 nt, due to its function in *trans* regulation of translation initiation. In addition, *BC200* was found to be upregulated in Alzheimer's Disease brains, and may contribute to the complex etiology of the disease (Mus et al., 2007).

In summary, it appears that the NAT lncRNAs work through multiple mechanisms, both in *cis* and in *trans*, to ensure proper expression levels of its sense partner in both CNS development and function.

Overlapping transcripts

One additional type of lncRNA is an overlapping transcript, which is transcribed in the same direction as and contains a protein-coding gene (Fig. 1). Possible mechanisms of overlapping transcript regulation include *cis*-acting promoter competition (Conte et al., 2002) or synergistic expression through opening of chromatin structure and deposition of histone marks that activate downstream transcription (Wagner and Carpenter, 2012).

lncRNA *Sox2ot*, which overlaps with *Sox2* within an intron, has several isoforms that are dynamically regulated and expressed both in conjunction with *Sox2* and independently, indicating a possible role in regulation of *Sox2* (Amaral et al., 2009). *In situ* hybridization for *Sox2ot* in the adult mouse brain revealed a striking overlap of expression with the *Sox2* protein-coding gene in areas of adult neurogenesis (Mercer et al., 2008).

The overlapping lncRNA *Evf2* is transcribed in the opposite direction of *Dlx6* and contains *Dlx6* within an intron (Feng et al., 2006). From this genomic location, *Evf2* can recruit positive and negative transcription factors to the nearby enhancer to regulate the expression of *Dlx5* and *Dlx6* (Bond et al., 2009). *Evf2* has been shown to form a stable complex with the transcription factor Dlx2 *in vivo*, indicating that it has direct influence on transcriptional activity (Feng et al., 2006). Mice mutant in *Evf2* had decreased numbers of postnatal GABAergic interneurons and reduced synaptic inhibition in the adult hippocampus, which may be implicated in disorders such as schizophrenia, autism, Tourette's syndrome, and epilepsy (Bond et al., 2009). Knockdown of *Evf2* by insertion of an early polyadenylation signal resulted in increased transcription of both *Dlx5* and *Dlx6*, but ectopic *Evf2* could only rescue the *Dlx5* level, indicating that the lincRNA works through different mechanisms to regulate transcription of each of the genes (Bond et al., 2009).

As 52 overlapping sense lncRNAs have been identified in the human genome (Derrien et al., 2012), the importance of this class of lncRNAs is emerging.

Bidirectional lncRNAs

Bidirectional or divergent lncRNAs have transcription start sites in close proximity to a protein-coding gene but are transcribed in the opposite direction (Fig. 1). Transcription of bidirectional lncRNAs is often concordant with expression of the nearby protein-coding gene, suggesting a *cis*-regulatory role of the lncRNAs, possibly in maintaining an open chromatin structure through pervasive expression (Wei et al., 2011) or through shared promoters (Ho et al., 2012). However, some lncRNAs have a discordant expression relationship with the nearby protein-coding gene (Mercer et al., 2008), implying a repressive role of the lncRNA in *cis*, possibly through promoter competition (Trinklein et al., 2004), or an independent role in *trans* (Engstrom et al., 2006). For the majority of bidirectional pairs, the orientation is conserved between human and mouse, implying functional importance in evolution (Trinklein et al., 2004).

In humans, a primate-specific bidirectional lncRNA, *FMR4*, is silenced in fragile-X syndrome patients and appears to have an anti-apoptotic function (Khalil et al., 2008). The *Sox8* locus is important in oligodendrocyte lineage specification and also hosts a bidirectional lncRNA, *Sox8OT* (Mercer et al., 2010). A biochemical function for *Sox8OT* has not been identified, but it shows concordant expression with its sense partner and may share a promoter (Mercer et al., 2010). The bidirectional lncRNA *Six3OS* has also been found to work in *trans* to regulate retinal differentiation (Alfano et al., 2005; Geng et al., 2007; Rapicavoli et al., 2011). Knockdown or overexpression of *Six3OS* does not affect *Six3* expression in the mouse retina, indicating there is not an interaction in *cis* on the host gene (Rapicavoli et al., 2011). However, as discovered through protein microarray and confirmed by RNA immunoprecipitation, *Six3OS* does interact with histone modification enzymes, suggesting that *Six3OS* can regulate Six3 function by facilitating chromatin modifications of Six3 target genes (Rapicavoli et al., 2011).

Other genes important in retinal development that have bidirectionally transcribed lncRNAs include *Otx2* and *Vax2* (Alfano et al., 2005; Meola et al., 2012). Functionality of *Otx2OS* has not been confirmed, but its expression pattern suggests possible repression of *Otx2* (Alfano et al., 2005). *Vax2OS1* represses cell cycle progression and differentiation in photoreceptor progenitors (Meola et al., 2012). The most likely mechanism for *Vax2OS1* is in *trans* since it is expressed in different cell types than *Vax2* and does not affect *Vax2* transcription (Meola et al., 2012).

Until now, there are 3,417 bidirectional lncRNAs annotated in the human genome (Derrien et al., 2012) and 51 identified in the mouse brain (Mercer et al., 2008). Fully functionally characterization of bidirectional lncRNAs remains an active research direction.

Sense intronic lncRNAs

A final class of lncRNAs is long intronic RNAs that are transcribed in the same direction as their protein-coding host gene (Fig. 1). Unlike their NAT counterparts, these sense intronic lncRNAs do not have antisense complementarity to their host gene and therefore must work through different mechanisms. The most well-known example of a sense intronic lncRNA is COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), which is transcribed from an intron of *FLOWERING LOCUS C (FLC)* (Heo and Sung, 2011). COLDAIR epigenetically regulates the *FLC* locus by recruiting the polycomb repressive complex 2 (PRC2) to silence FLC expression (Heo and Sung, 2011). Though this example comes from *Arabidopsis*, the mechanism may be conserved as lncRNAs in the nervous system are known to interact with PRC2 and other epigenetic regulators (Johnson et al., 2009; Ng et al., 2012). Another mechanism of sense intronic lncRNAs is coordination of gene expression programs through *trans* activation of diverse genes (Hill et al., 2006). In the mouse brain, sense intronic lncRNAs were found both co-expressed with their host genes and expressed independently, indicating that they could have an activating or repressive effect on the host locus or could work in *trans* (Mercer et al., 2008). One possible sense intronic lncRNA, *Svet1*, has been identified, but its function is unknown. *Svet1*, which maps to an intron of the *Unc5d* putative Netrin receptor gene, is expressed early in development in the subventricular zone and later in the upper layer neurons of the mouse cortex (Tarabykin et al., 2001). Sasaki et al. attributed *Svet1* to a splicing product of the *Unc5d* mRNA as it is only found in the nucleus, but did not eliminate the possibility that *Svet1* is an independent transcript (Sasaki et al., 2008).

There are 593 sense intronic lncRNAs annotated in the human genome (Derrien et al., 2012) and 182 shown to be expressed in the adult mouse brain (Mercer et al., 2008); while less prevalent than other types of lncRNAs, sense intronic lncRNAs may still be shown to have interesting and important functions in the nervous system.

lncRNAs independent of genomic context

Pseudogenes

Previously thought of as junk DNA or evolutionary relics, pseudogenes have been shown to have functionality in recent studies (Tam et al., 2008; Watanabe et al., 2008; Hawkins and Morris, 2010; Muro and Andrade-Navarro, 2010; Poliseno et al., 2010; Han et al., 2011). Pseudogenes can be formed through spontaneous mutation that prevents transcription or translation, gene duplication due to uneven crossing over, or reintegration of an mRNA transcript (reviewed in Pink et al., 2011). The recent GENCODE project has annotated 11,224 pseudogenes in the human genome, of which 863 were transcribed and associated with active chromatin marks (Pei et al., 2012). There are several proposed mechanisms of pseudogene action. First, they can regulate the expression of their parent gene by competing for mRNA stability factors (Chiefari et al., 2010). Pseudogenes can also be a source of *trans*NATs (Hawkins and Morris, 2010; Muro and Andrade-Navarro, 2010; Muro et al., 2011). Several studies have shown that endo-siRNAs originating from pseudogenes binding their parent mRNA can regulate mRNA expression through RNA interference (Tam et al., 2008; Watanabe et al., 2008; Poliseno et al., 2010). Finally, an additional function of pseudogenes is providing decoy miRNA binding sites, resulting in increased expression of the parent mRNA (Poliseno et al., 2010).

While a large proportion of transcribed pseudogenes were found to be expressed in the brain (Pei et al., 2012), few functional studies have been performed. An RNA-seq study conducted during the differentiation of induced pluripotent stem cells into neurons found that expression of 1,371 pseudogenes changed significantly between the two cell types (Lin et al., 2011). Mentioned above, the NAT to *NOS2A* is derived from a pseudogene of *NOS2A* and may suppress the parent gene expression (Korneev et al., 2008). Aside from this example, the functions and mechanisms of pseudogenes in the nervous system are yet to be characterized.

Imprinted lncRNAs

The best known lncRNAs involved in imprinting are *inactive X-specific transcript (Xist)* and *X (inactive)-specific transcript, antisense (TsiX)*. These lncRNAs are required for X chromosome inactivation in females, an epigenetic event that maintains equal X chromosome expression between the mammalian sexes (Leeb et al., 2009). More generally, lncRNAs are frequently found within imprinted clusters and are often implicated in the *cis* regulation of imprinted genes (Santoro and Barlow, 2011). The most widely studied mechanism of lncRNA-driven silencing is through recruitment of chromatin-modifying enzymes to the locus from which the lncRNA is transcribed (Mohammad et al., 2009). Another possible mechanism is transcriptional interference, in which constant transcription from a lncRNA promoter can block transcription from a coding gene promoter on the opposite strand (Callen et al., 2004; Pauler et al., 2007).

The Prader-Willi syndrome/Angelman syndrome locus in humans is differentially imprinted on the maternal and paternal chromosome in neurons, perturbation of which results in neurodevelopmental disorders (Buiting, 2010). The *UBE3A* gene encodes a ubiquitin E3 ligase that is expressed maternally, while the paternal allele expresses a lncRNA antisense transcript, *UBE3A-ATS* (*LNCAT* in mouse) (Le Meur et al., 2005). The causal link between *UBE3A-ATS* expression and *UBE3A* paternal silencing in neurons has recently been demonstrated, though it is still unknown whether the silencing is mediated through the RNA itself or through transcriptional interference (Meng et al., 2012).

The lncRNA *MEG3* is part of the imprinted *DLK1-MEG3* locus that is expressed from the maternal allele but suppressed at the paternal allele in humans and mice (Hagan et al., 2009). Knockout of *Meg3* in mice followed by microarray analysis revealed that *Meg3* is involved in many processes in brain development, including calcium, Notch, and Wnt signaling, long-term potentiation, and angiogenesis (Gordon et al., 2010). In the mouse brain, knockout of *Meg3* resulted in increased microvessel formation (Gordon et al., 2010). *Meg3* activates the tumor suppressor p53 *in vitro* and is lost in several tumor types, indicating that it works as a tumor suppressor as well as playing roles in brain development, though the biochemical mechanism is unknown (Gordon et al., 2010; Wang et al., 2012).

lncRNAs in neurological diseases

Many lncRNAs are implicated in a wide variety of neurological diseases. In neurodevelopmental disorders, *Ube3a-ATS* is transcribed from the Angelman Syndrome and Prader-Willi Syndrome locus and *FMR4* is silenced in Fragile-X Syndrome (Khalil et al., 2008; Meng et al., 2012). In addition, the *DISC* locus, including *DISC1* and its NAT *DISC2*, is disrupted in schizophrenia and affective disorders (Millar et al., 2004). *DISC1* regulates neuron proliferation, migration, and neurite outgrowth during cortical development (Kamiya et al., 2012). Like many NATs, *DISC2* is thought to regulate the expression of its sense partner, thereby contributing to disease etiology (Brandon et al., 2009).

BACE1-AS and *BC1/BC200* have been implicated in Alzheimer's disease, while *ATXN8OS* is associated with neurodegenerative spinocerebellar ataxia type 8 (Mus et al., 2007; Faghihi et al., 2008b; Daughters et al., 2009). In addition, spinocerebellar ataxia type 7 is a neurological disorder characterized by cerebellar and retinal degeneration caused by a repeat expansion in the *ataxin-7* gene (Martin et al., 1994). A convergently transcribed and slightly overlapping NAT, *SCAANT1* has been found to repress *ataxin-7* transcription in *cis* (Sopher et al., 2011). Repeat expansion of *ataxin-7* reduces *SCAANT1* promoter activity, thereby derepressing *ataxin-7* and contributing to the disease state (Sopher et al., 2011). Moreover, *anti-NOS2A* is upregulated in human brain tumors and can influence the efficacy of chemotherapeutic drugs, while *MEG3* is lost in several tumor types (Broholm et al., 2003; Gordon et al., 2010; Zhou et al., 2012).

As more lncRNAs associated with neurological disorders are identified and functions of more lncRNAs are characterized, lncRNAs may become a new therapeutic target for the treatment of neurological diseases.

Perspectives

With the release of the latest lncRNA information from GENCODE (Derrien et al., 2012; Djebali et al., 2012; Pei et al., 2012), one of the greatest obstacles in lncRNA biology – annotation – has been largely overcome. The field must now move toward detailed functional and mechanistic studies. As evidenced by the current dearth of functionally defined lncRNAs, this is not an easy task.

Generation of knockout models of lncRNAs may be instructive to learn about lncRNA function, but the results must be analyzed with care as knockout of lncRNAs that work in *cis* could produce effects beyond the action of the lncRNA itself. Some lncRNAs may also function as precursors for small RNAs, such as miRNAs or small nucleolar RNAs (snoRNAs), which could complicate functional studies (Derrien et al., 2012). It will be important to demonstrate recovery of phenotype with the lncRNA in *trans* in these cases to determine if the transcript functions as a lncRNA or if the lncRNA is merely a precursor. A risk of generating lncRNA knockout animal models is the lack of identifiable phenotypes due to its fine-tuned regulation of gene expression and unknown functions. In addition, fusion of lncRNAs to an IRES-GFP sequence is a complementary approach to knockdown lncRNAs, as these constructs mislocalize to the cytoplasm and produce a dominant negative effect (Rapicavoli et al., 2010). Again, these results must be interpreted with care as cytoplasmic localization of some lncRNAs may have off-target effects.

As many lncRNAs have been implicated in epigenetic regulation (Khalil et al., 2009; Tsai et al., 2010; Magistri et al., 2012; Ng et al., 2012), it will be helpful to know where in the genome the lncRNA is binding. Chromatin isolation by RNA purification (ChIRP) allows genome-wide mapping of lncRNA occupancy based on the lncRNA sequence (Chu et al., 2011; Chu et al., 2012). This technique is especially powerful because it depends only on the lncRNA sequence, not the structural or functional domains.

In addition to learning where in the genome lncRNAs are binding, it will be important to know what proteins they are interacting with. RNA coimmunoprecipitation followed by deep sequencing (RIP-seq) is a method used to determine all of the lncRNAs that are interacting with a single protein, which can be used to infer lncRNA function (Moran et al., 2012a). A complementary method for pulldown of a specific lncRNA involves insertion of a sequence that creates a stem-loop structure at the 3' end of the lncRNA and is recognized by the bacteriophage MS2 coat-binding protein (Gong et al., 2012). Finally, lncRNAs can be hybridized to protein microarrays to identify lncRNA:protein interactions (Hu et al., 2009;

Rapicavoli et al., 2011). Subsequent analysis of the proteins and RNAs that co-purify with the lncRNA will give clues to the lncRNA function.

Another obstacle to determining lncRNA function is the lack of methods to determine secondary structure. Parallel analysis of RNA structure (PARS) is a technique that has been used in yeast to profile the genome-wide secondary structure of RNAs (Kertesz et al., 2010). The technique involves treatment of RNA with structure-specific enzymes followed by deep sequencing to identify the resulting fragments. PARS has the potential to be modified for use with lncRNAs and could give insights to conservation of lncRNA secondary structure across different species or allow categorization of secondary structures capable of interacting with different proteins.

Finally, many strategies currently used to target mRNAs or small ncRNAs could be applied to lncRNAs, such as antisense and catalytic oligonucleotides. For example, shRNA and siRNA knockdown of lncRNAs have become successful research tools and could present a potential lncRNA-targeting therapeutic strategy (Bernard et al., 2010; Orom et al., 2010; Poliseno et al., 2010; Rapicavoli et al., 2010; Kotake et al., 2011; Flockhart et al., 2012; Modarresi et al., 2012). Catalytic oligonucleotides, such as antagomirzymes (also called LNAzymes) have been used *in vitro* and *in vivo* to inhibit miRNA function through sequence complementarity and a catalytic core that cleaves the target RNA (Jadhav et al., 2009; Suryawanshi et al., 2012). For both of these tools, a targeted delivery method would be required to observe specific effects of lncRNA knockdown in the CNS.

Conclusions

It has become abundantly clear that noncoding transcription plays an important role in the functional genome. lncRNAs are a pervasive class of ncRNAs transcribed from various genomic orientations, providing a wide array of regulatory mechanisms that can work through fine-scale modulation of gene expression or large-scale regulation of developmental programs. The precise temporal and spatial control of lncRNAs is particularly important in the CNS, where lncRNAs have been implicated in every stage of neurodevelopment as well as a growing number of CNS pathologies (Table 1). However, despite their apparent importance, a strikingly small number of lncRNAs have been mechanistically characterized due to their diverse roles and often multiple target loci. With the latest ENCODE annotation of lncRNAs and new tools for precipitation, purification, and knockdown of lncRNAs, it is time to move toward functional characterization of individual lncRNAs. As we learn more about how lncRNAs are working, we can further our understanding of the complex molecular interactions of the extensive non-protein-coding components of our genome in regulating the development and function of the CNS.

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Highlights

- About 98% of the human genome contains transcripts of noncoding RNAs.
- Many long noncoding RNAs (lncRNAs) are expressed in the central nervous system.
- lncRNAs can be classified as sense, antisense, bidirectional, intronic, intergenic
- lncRNAs regulate coding gene expression using many mechanisms in *cis* and *trans*.
- Dysregulation of lncRNAs is associated with human neurological disorders.

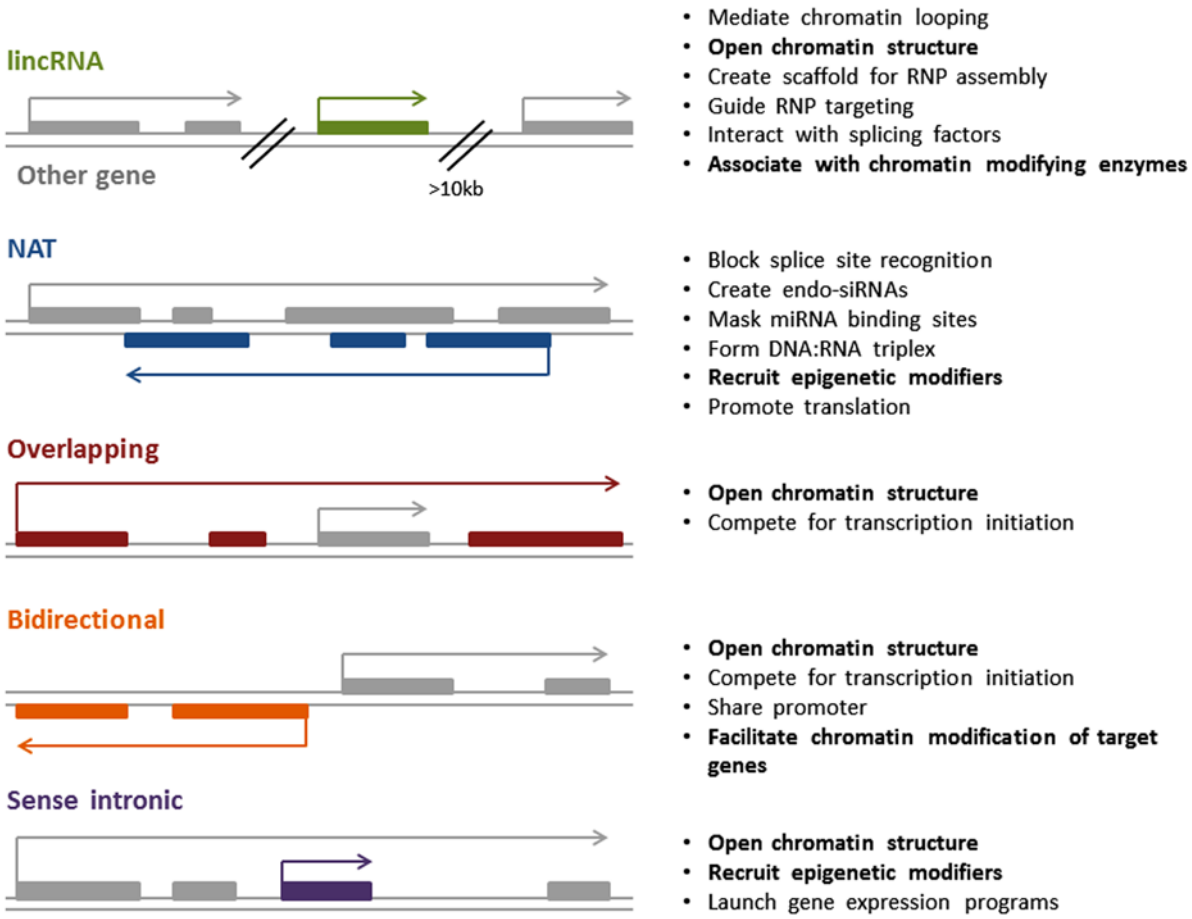

Figure 1. Genomic contexts of lncRNAs

Schematic diagram demonstrating the various genomic positions of lncRNA types (in color) with respect to other genes (in grey). Possible mechanisms of each type are shown on the right, with thematic functions in bold.

Table 1

Summary of long noncoding RNAs with characterized functions in the central nervous system.

Name	Type	Role	Species	References
Otx2OS	bidirectional	Expressed in the developing retina in a partially overlapping pattern with *Otx2*; expression pattern suggests repression of *Otx2*	*Mus musculus*	Alfano et al., 2005
Six3OS	bidirectional	Acts as a scaffold to direct histone modifiers to *Six3* target genes; binds coregulators of *Six3* to modulate target gene expression	*Homo sapiens, Mus musculus, Gallus gallus*	Alfano et al., 2005; Geng et al., 2007; Rapicavoli et al., 2011
Vax2os1	bidirectional	Retina-specific, overexpression delays cell cycle of photoreceptor progenitors during development	*Mus musculus*	Alfano et al., 2005; Meola et al., 2012
FMR4	bidirectional	Primate-specific, silenced in fragile-X syndrome patients; knockdown in HEK-293T and HeLa cells results in delayed cell cycle and increased apoptosis; overexpression promotes proliferation	*Homo sapiens, Macaca mulatta*	Khalil et al., 2008
Sox8OT	bidirectional	Concordant expression with *Sox8*, may promote oligodendrocyte lineage commitment	*Homo sapiens, Rattus norvegicus, Mus musculus*	Mercer et al., 2010
Lncat (Ube3a-ATS)	imprinted	Silences the paternal allele of Ube3a, involved in Prader-Willi and Angelman Syndromes	*Homo sapiens, Mus musculus*	Le Meur et al., 2005; Meng et al., 2012
MEG3	imprinted	Part of the imprinted *DLK1-MEG3* locus; knockout leads to increased microvessel formation in the brain; loss may contribute to tumor formation	*Homo sapiens, Mus musculus, Bos taurus*	Hagan et al., 2009; Gordon et al., 2010; Wang et al., 2012
Gomafu	lincRNA	Competes for splicing factors to inhibit splicing reactions; represses amacrine and Müller glial differentiation in the retina	*Homo sapiens, mus musculus, Gallus gallus, Xenopus tropicalis*	Sone et al., 2007; Rapicavoli et al., 2010; Ip and Nakagawa, 2011
MALAT1	lincRNA	Expressed late in neuron and oligodendrocyte differentiation; promotes synapse formation and dendrite growth	*Homo sapiens, Mus musculus*	Bernard et al., 2010; Mercer et al., 2010
Neat1	lincRNA	Architectural component of paraspeckles; promotes differentiation and maturation of neurons and oligodendrocytes	*Homo sapiens, Mus musculus*	Mercer et al., 2010; Ip and Nakagawa, 2011
TUG1	lincRNA	Required for photoreceptor formation in the developing retina, promotes rod differentiation and inhibits cone differentiation	*Homo sapiens, Mus musculus, Rattus norvegicus, Bos taurus, Canis lupus familiaris*	Young et al., 2005
HAR1F	NAT	Primate-specific lncRNA expressed in Cajal-Retzius neurons in the developing neocortex; predicted to promote cortical neuron specification and migration	*Homo sapiens*	Pollard et al., 2006
BC1/BC200	NAT	Brain-specific transcript that represses translation in dendrites to allow spatial translation regulation in postsynaptic microdomains; dysregulated and implicated in Alzheimer's disease	*Homo sapiens, Mus musculus*	Ohashi et al., 2000; Wang et al., 2002; Mus et al., 2007
Sox4AS	NAT	Expressed with and independently of *Sox4* throughout brain development; no function known	*Mus musculus*	Ling et al., 2009
NrgnAS	NAT	Forms cytoplasmic dsRNA aggregates with *Nrgn*; may be involved in LTP	*Mus musculus*	Ling et al., 2011
Camk2n1AS	NAT	Forms cytoplasmic dsRNA aggregates with *Camk2n1*; may be involved in LTP	*Mus musculus*	Ling et al., 2011

Name	Type	Role	Species	References
BACE1-AS	NAT	Upregulated in Alzheimer's brains; promotes *BACE1* stability by blocking a miRNA binding site	*Homo sapiens*	Faghihi et al., 2008; Modarresi et al., 2011
BDNF-AS	NAT	Coexpressed with *BDNF* in the brain; forms dsRNA duplexes to repress *BDNF* expression	*Homo sapiens Homo*	Pruunsild et al., 2007; Modarresi et al., 2012
Emx2OS	NAT	In differentiating neural precursors, high levels destabilize *Emx2*, while low levels are required for proper *Emx2* transcription	*sapiens, Mus musculus*	Spigoni et al., 2010
Nkx2.2AS	NAT	Cytoplasmic localization, can increase *Nkx2.2* mRNA levels; overexpression in NSCs promotes differentiation along the oligodendrocyte lineage	*Homo sapiens, Mus musculus*	Tochitani and Hayashizaki, 2008
Disc2	NAT	Translocation breakpoint segregates with susceptibility for schizophrenia; may regulate *Disc1* sense gene expression	*Homo sapiens*	Millar et al., 2004; Brandon et al., 2009
ATXN8OS	NAT	Repeat expansion associated with spinocerebellar ataxia type 8 causes accumulation of RNA foci and sequestration of Mbnl1, causing dysregulation of Mbnl1 target pathways	*Homo sapiens, Mus musculus*	Ikeda et al., 2008; Daughters et al., 2009
anti-NOS2A	NAT	Undergoes reciprocal changes to *NOS2A* during hESC differentiation into neurons, indicating repression of *NOS2A*	*Homo sapiens, Lymnaea stagnalis*	Korneev et al., 2008
SCAANT1	NAT	Represses *ataxin-7* expression in *cis*; implicated in spinocerebellar ataxia type 7	*Homo sapiens*	Sopher et al., 2011
Evf2	overlapping	Influences expression of Dlx5, Dlx6, and Gad1 in the developing ventral forebrain; promotes generation of GABAergic interneurons in the hippocampus and dentate gyrus, loss results in reduced synaptic inhibition	*Homo sapiens, Mus musculus*	Feng et al., 2006; Bond et al., 2009
Sox2OT	overlapping	May regulate *Sox2* in neurogenesis to promote neural differentiation, also has independent roles	*Homo sapiens, Mus musculus, Gallus gallus, Danio rerio*	Amaral et al., 2009
Svet1	sense intronic	Expressed early in development in the proliferating cells of the SVZ and later in upper layer neurons, function unknown	*Mus musculus*	Tarabykin et al., 2001; Sasaki et al., 2008