A Macro Role for Imprinted Clusters of MicroRNAs in the Brain

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Abstract: Evidence suggests that ~95% of the human genome may produce noncoding RNAs (ncRNAs). Approximately 30% of parentally imprinted transcripts are ncRNAs that are found abundantly - and in some cases specifically - in the brain; however, the role ncRNA plays during development and day-to-day life is not apparent. Interestingly, ~1,300 transcripts show a consistent parental expression bias in the brain. This is in contrast to most tissues that show only rare imprinted transcripts. Furthermore, there are only two clusters of imprinted small nucleolar RNA (*snoRNA*) encoding genes in mammals, which are also expressed in the brain. These clusters, *Snrpn-Ube3a* (Human 15q11–q13/Murine 7qC) and *Dlk1-Dio3* (Human 14q32.2/Murine 12qF1) and rodent specific *Sfmbt2* (Murine 2qA1), form the focus of this review. These imprinted clusters are localized to imprinted regions that are associated with processes involved in neuronal plasticity and several neurodevelopmental disorders. Several miRNAs from the *Dlk1-Dio3* region are also involved in chromatin methylation and remodelling. The final loci of interest is the proximal region of murine chromosome 2 that contains *Sfmbt2* and an overlapping antisense transcript that is unique within mice and rats. This suggests that *Sfmbt2* may be in the process of becoming imprinted that is being driven by a cluster of intergenic miRNAs. Ultimately, imprinted clusters of ncRNA have the potential to offer novel insight into the understanding of the complex processes of cognition given their role in brain function.

Keywords: miRNA, snoRNA, Snrpn, Ube3a, Dlk1, Dio3, Sfmbt2, Imprinting, ncRNA.

Nervous systems are capable of generating and integrating information from multiple external and internal sources in real time using a complex network. This network is poorly understood, often shared across species, and hypothesized to have evolved over time. Further, its developmental course is directed by genetic endowment from the two parents, is highly sensitive to environmental influences, and the details involved remain poorly understood. Also, any accommodation of environmental effects during development may involve structural variations. A better understanding of structure-function relationship involving nervous system is needed to fully understand its normal and abnormal function. More recent results also argue that the nervous system developmental course may involve a number of special features. These include genomic imprinting and ncRNAs, which form the focus of this review. They may initiate and refine a process, establish short-term as well as long-term cellular memory, and an individual specific neural network. This review will attempt to assess the significance of such features using recent literature towards a better understanding of the development and functioning of the nervous system.

GENOMIC IMPRINTING

Genomic imprinting refers to the expression of genes dependent on the parental origin of the gene (allele). Unlike normal gene expression from both parentally contributed chromosomes, imprinting causes only the alleles from either the male or female parent to be expressed in the progeny. Although the parent of origin effect on some phenotypes was recognized about 40 years ago [1], successful genetic manipulations of the mouse helped clarify molecular mechanisms capable of altering the expression without changing DNA sequences [2]. These studies have illustrated a role for DNA methylation, histone modifications, and non-coding RNAs in the parent of origin dependent gene expression. Specifically, epigenetic mechanisms allow for the control of gene regulation at two levels. The first is an "on and off" control at the level of transcription that is mainly accomplished by cytosine methylation of DNA and specific modifications in histones. DNA methylation is the most extensively studied epigenetic modification and is primarily associated with transcriptional silencing, even though there is some evidence that it may also involve gene activation [3]. Furthermore, DNA methylation and histone modifications regulate gene expression at the level of chromatin in the regulation of transcription.

ncRNAS

The second level of epigenetic control, in contrast to DNA methylation and histone modifications, is achieved by small ncRNAs. They regulate gene expression through RNA silencing pathways that operate post-transcriptionally, allowing for the fine-tuning of gene expression [4]. Small ncRNAs include miRNAs and snoRNAs. Current estimates suggest that 95% of the human genome is transcribed and produces a vast variety of ncRNAs [5]. Recent estimates indicate that an individual miRNA may have up to a few hundred different target mRNAs, although not every interaction is physiologically relevant [6]. miRNAs are known to regulate gene expression post-transcriptionally by causing target degradation and/or translational interference of mRNA(s) [7]. Given their

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mechanism of function, miRNAs can act at both levels of epigenetic control, acting as either on–off switches or allowing for the fine-tune of gene expression profiles. In contrast to miRNAs, snoRNAs localize to the nucleolus and are generally involved in guiding rRNA modifications [8]. ncRNAs can be stably replicated *via* RNA-dependent RNA polymerase, suggesting that ncRNAs may be able to maintain patterns of gene silencing over several cell divisions [9]. Additionally, RNA silencing pathways may affect chromatin structure through RNA-directed modulation of DNA methylation and histone modifications [10]. They can also be transmitted through mitosis, suggesting that they may facilitate the transmission of cell-specific epigenetic signals.

EPIGENETIC PROGRAMMING: ALTERATION OF GENERATIONS

It is clear that during fertilization the oocyte and sperm bring together the maternal and paternal genomes; these two genomes come with "instructions" that are specific to the female and male gametes, respectively. These instructions often involve DNA methylation and histone modifications among other mechanisms. Furthermore, the dividing zygote and developing embryo undergo dynamic changes that include epigenetic reprogramming [11]. Chromatin organization contributes to the spatial context in which genes are developmentally regulated, such as the highly ordered chromatin of post-mitotic neuronal nuclei in imprinted regions [12, 13]. Imprinted genes are often found in clusters around differentially methylated regions (DMRs) characterized by hypermethylated DNA on one parental chromosome but hypomethylated DNA on the other parental chromosome [14]. The specific DNA methylation patterns are established again depending on the sex during germ cell development [15, 16]. This mechanism ensures mono-allelic expression in a parent-of-origin manner across generations. This feature appears to have evolved, potentially to assure genetic diversity by recombination during sexual reproduction and is similar in closely related species.

IMPRINTING IN NEURODEVELOPMENT

The significance of imprinting has been debated over the years. The current view is that imprinted genes may have an important role in general development, but particularly in neurodevelopment. For example, the overall effect of maternally expressed genes is to enhance brain size, while the overall effect of paternally expressed genes is to limit brain growth [17]. Together they are able to assure a proper balance. Additionally, some genes appear to be fully imprinted only in the adult brain [18]. This offers the speculation that imprinting is not only important for early life processes, but that it is also functionally important for so-called 'online' adult brain functions [19]. Intriguingly, thirty percent of parentally imprinted transcripts are ncRNA [20]. ncRNAs are found abundantly, and in some cases specifically, in the brain [21]. They have integral functions in neurodevelopment and long-term memory formation [22, 23]. Analysis of the parent of origin gene expression in the adult brain has revealed that the brain may be especially sensitive to epigenetic influences [24]. Approximately 1,300 transcripts in the embryonic and adult brain show a consistent parental expression bias [25, 26]. There is a predominantly maternally inherited expression bias in the embryonic brain, and the adult brain shows a paternal bias [24].

IMPRINTING AND ncRNA

There are currently only two known mammalian clusters of genes encoding snoRNAs, *Dlk1-Dio3* (Human 14q32.2/ Murine 12qF1) and *Snrpn-Ube3a* (Human 15q11q13/Murine 7qC). These clusters are neuronally expressed, involved in several neurodevelopmental disorders, and are localized to imprinted loci. Interestingly, the tandemly repeated C/D box snoRNA gene clusters in both loci are unique to eutherian mammals [27]. It was recently shown in both human and mouse brain tissue, that post-natal neurons undergo orchestrated chromatin decongestion at these two imprinted snoRNA clusters, but not at other highly transcribed, imprinted, or ncRNA containing loci [28]. Chromatin decondensation of imprinted snoRNAs was shown to be neuron-specific, developmentally regulated, and transcriptionally dependent on the ICRs. Prader-Willi syndrome human brain tissue and mouse neurons with a deletion in the ICR showed decreased nucleolar size, which demonstrates the important role snoRNA in neuron maturation. A final miRNA cluster of interest, *Sfmbt2*, is unique to rats and mice. These three clusters will be the primary focus in the following sections.

1. Dlk1-Dio3

Maternal *Dlk1-Dio3* (Human 14q32.21/Murine 12qF1) is not methylated at the DMR and expresses over forty

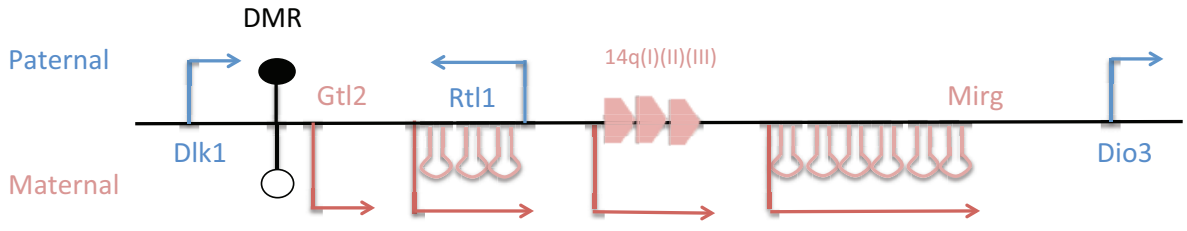

Fig. (1). A physical view of *Dlk1-Dio3* (Human 14q32.2/ Murine 12qF1). Maternal *Dlk1-Dio3* (Human 14q32.21/Murine 12qF1) expresses over forty miRNAs, which are contained in two clusters. The first maternal cluster is antisense to paternal *Rtl1*and the second maternal cluster contains *Mirg*, as well as a cluster of snoRNAs and *Gtl2*. Paternal *Dlk1-Dio3* expresses *Dlk1, Rtl1* and *Dio3* and is methylated at the DMR. Bars and arrows in blue indicate paternally expressed transcripts, whereas pink indicates maternally expressed transcripts. Not to scale.

miRNAs, which are contained in two clusters as well as a cluster of snoRNAs (Fig. **1**) [29]. These ncRNAs are expressed in embryo, placenta, and adult tissue, where their expression is confined mostly to the brain. Little is known about the function of the individual miRNAs from this cluster. So far, five miRNAs from the *Dlk1-Dio3* region were shown to have the unique role of being involved in chromatin methylation and remodeling, where they control imprinting of the *Rtl1* gene in that locus [30]. These miRNAs are also important for activity dependent dendritic re-modeling, with miR-134 specifically regulating the process [31]. These findings implicate that this large cluster of miRNAs may be involved in adaptive responses of neural circuits to the environment. Altering the dosage of the imprinted genes at the *Dlk1-Dio3* region causes a range of phenotypes from growth deficiencies and developmental defects in the embryo and placenta, to defects in adult metabolism and brain function [32]. This reveals a crucial role for *Dlk1-Dio3* not only in neurodevelopment, but also in 'online' brain function [19].

2. Snrpn-Ube3a

Paternal *Snrpn-Ube3a* (Human 15q11-q13/Murine 7qC) expresses a neuron-specific polycistronic transcript that includes two clusters of snoRNAs, HB/MBII-52 and 85 (Fig. **2**) [33-35]. Humans have 47 HBII-52 copies, and there are at least 130 copies of MBII-52 snoRNAs in the mouse. In contrast, maternal *Snrpn-Ube3a* is relatively inactive and is methylated at the DMR [36-38]. *Snrpn-Ube3a* transcriptional regulation is highly complex, involving multiple allele-specific epigenetic marks including DNA methylation, histone modification patterns, DNase hypersensitive sites, and ncRNAs [28,39-41].

Knockout mice lacking snoRNAs from the *Snrpn-Ube3a* locus have revealed that these snoRNAs do not abruptly shut down or turn on genes but rather mildly changes the expression of dozens of genes [42]. Interestingly, the *Snrpn-Ube3a* cluster has a role in alternative splicing [28]. The snoRNA clusters within *Snrpn-Ube3a* and *Dlk1-Dio3* have greater homology to one another than to any other mammalian snoRNAs and have minimal rRNA homology, with only three of the snoRNAs from *Snrpn-Ube3a* showing rRNA homology [43, 44]. This fact suggested that there is a unique role for these snoRNAs. Indeed, research has shown that the antisense box of H/MBII-52 has a sequence complementarity to exon Vb of the serotonin receptor *5htr2*c, which is a transmembrane receptor involved in cognition [45]. Failure to include exon Vb results in a non-functional receptor. Experiments have shown that MBII-52 increases exon Vb inclusion by blocking the action of the silencers in the pre-mRNA. The silencers located on the pre-mRNA can also be modified by 'traditional' RNA editing; however, the editing events change the amino acids of the receptor in exon Vb [45]. These silencer sites are located in a loop that is critical for protein function. Thus, the 'traditional' editing of the receptor pre-mRNA reduces its efficacy. The *5htr2c* mRNA containing the non-edited version of exon Vb encodes a receptor that produces the highest serotonin response. Therefore, MBII-52 promotes the generation of the most active receptor (Fig. **2**) [46]. Recent research has indicated that the MBII-52 expression unit generates several small ncRNAs that are most likely generated by further processing of the snoRNA termed psnoRNAs (for processed snoRNAs) [47]. The main product of the MBII-52 expression units appears to be psnoRNAs. As opposed to the snoRNA that is localized to the nucleolus, the psnoRNAs are present in the nucleoplasm where they could interact with pre-mRNA. A recent

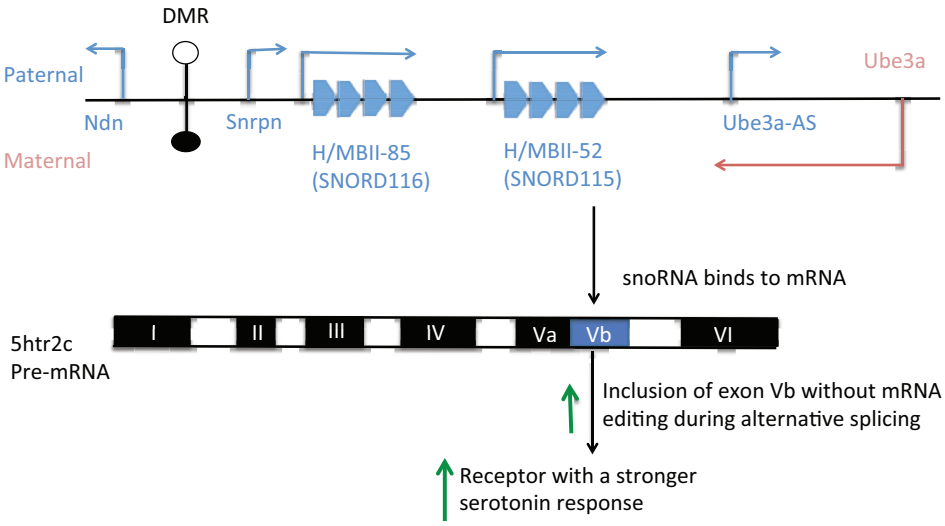

Fig. (2). A physical view of *Snrpn-Ube3a* (Human 15q11–q13/Murine 7qC). Paternal *Snrpn-Ube3a* (Human 15q11-q13/Murine 7qC) expresses a neuron-specific polycistronic transcript that includes the coding sequence for a splicesomal protein (SNRPN), two clusters of snoRNAs (HB/MBII-52 and 85, respectively the snoRNA families SNORD115 and 116), four single copy snoRNAs (HBII-436,13, 438a and 438b) and the antisense transcript to a maternally expressed ubiquitin ligase (UBE3A-AS)[33-35]. Humans have 47 HBII-52 copies and there are at least 130 copies of MBII-52 snoRNAs in the mouse. Maternal 15q11-q13 (Murine 7qC) is relatively inactive, as it is methylated at the DMR, and expresses UBE3A and ATP10A in the opposite orientation. H/MBII-52 is involved in the splicing and mRNA editing of the serotonin receptor 5htr2c. Bars and arrows in blue indicate paternally expressed transcripts, whereas pink indicates maternally expressed transcripts. Not to scale.

analysis of protein composition has shown that the psnoRNAs do not associate with the known structural C/D box snoRNA proteins, but rather with hnRNPs commonly implicated in splice site regulation [45, 46]. Furthermore, the MBII-52 psnoRNAs were implicated in the regulation of alternative splicing of five additional pre-mRNAs, DPM2, TAF1, RALGPS1, PBRM1, and CRHR1; some of which are involved in epigenetic and cell proliferative processes [47].

Snrpn-Ube3a is also involved in the classic sister imprinting disorders: Prader-Willi Syndrome and Angelman Syndrome. Prader-Willi syndrome is caused by a paternal *Snrpn-Ube3a* deficiency, whereas Angelman syndrome is caused by a maternal *Snrpn-Ube3a* deficiency [48, 49]. HBII-52 and HBII-85 deficiency appears to be the primary cause of Prader-Willi Syndromeas a paternal deletion results in the associated phenotypes [47, 50]. Mouse models of MBII-85 deficiency also replicate some of the Prader-Willi Syndrome phenoytpes including: post-natal growth deficits, a deficit in motor learning, and increased anxiety [42, 51]. On the opposite end of the spectrum, regardless of the mechanism, individuals with Angelman Syndrome do not express the maternal genes and overexpress the paternal genes (HBII-52 and HBII-85). Patients with Angelman syndrome have severe to profound mental deficits, including microcephaly, seizures, ataxia, and almost always a lack speech [52]. Additionally, 1-3% of autism cases are caused by a *Snrpn-Ube3a* duplication [53-55]. Overexpression of MBII-52 in a paternal duplication mouse model replicates abnormalities seen autism. The mice displayed poor social interaction, behavioral inflexibility, abnormal ultrasonic vocalizations, and increased anxiety [56]. An increase of MBII-52 within the duplicated region affects the *5htr2c* serotonin receptor, and correlates with altered intracellular Ca^{2+} responses elicited by a *5htr2c* agonist in the neurons of these mice. Additionally, a strong correlation amongst schizophrenia and psychotic illness in human carriers of maternally derived *Snrpn-Ube3a* duplications has been found, suggesting an excess of maternally expressed gene products in this region may be involved in the etiology of psychosis [57].

3. Sfmbt2

Genetic studies using reciprocal translocations have shown that the proximal region of murine chromosome 2 contains one or more imprinted genes [58]. Recently, the gene *Sfmbt2* was mapped to Murine 2qA1. *Sfmbt2* is expressed from the paternally inherited allele in early embryos and extra-embryonic tissues in mice [59]. When measuring allelic expression of 2qA1 it was found that *Sfmbt2* and an overlapping non-coding antisense transcript (Fig. **3**) are the only imprinted genes in a 3.9 Mb domain with approximately 20 genes, thus showing a very narrow imprinting region [60]. Currently, little is known about the function of *Sfmbt2* and its miRNAs. However, maternal disomy of the *Sfmbt2* region was shown to result in fetal and placental growth retardation, whereas paternal disomy was shown to result in normal fetal growth and placental overgrowth [59].

Like Murine *Sfmbt2*, C19MC is species specific and found only in primates [61-63]. In humans C19MC comprises of 46 pre-miRNA genes in a 400-700 bp long repeat flanked by an antisense-oriented Alu element. Most C19MC miRNAs are generated from the introns of a single large transcript, termed C19MC-HG, and are mainly expressed by the paternally inherited allele in the placenta [64, 65]. No experiments so far have examined whether C19MC is expressed and imprinted in the early human embryo. Labialle and Cavaille have observed that the *C19MC* and *Sfmbt2* clusters share the same ‘‘AAGUGC’’ seed sequence and speculate that, through convergent evolution, these evolutionarily distinct miRNA clusters may target the same mRNA target(s) in order to fine-tune gene expression during development [66].

An interesting feature of both mouse and rat imprinted *Sfmbt2* genes is the presence of a large cluster of miRNAs in intron 10. Other mammals, including the humans, lack this cluster of miRNAs and show bialleic *Sfmbt2* expression, suggesting that this region is not imprinted in other mammals. Further strengthening the argument for recent evolution of *Sfmbt2* imprinting is the demonstration that a more distant muroid rodent, *Peromyscus*, also lacks imprinting and the cluster of miRNAs [59]. These findings show that *Sfmbt2* imprinting appears to be unique to mice and rats, and is associated with the acquisition of a cluster of miRNAs in one of the introns. This suggests that *Sfmbt2* is a gene that is in the process of becoming imprinted, which is being driven by a cluster of intergenic miRNAs. As mentioned above, two other imprinted loci, *Dlk1-Dio3* and *Snrpn-Ube3a*, also contain large clusters of ncRNAs (miRNAs and snoRNAs) and possess classic germline DMRs. Wang *et al.* speculate that the lack of any noticeable germline methylation at *Sfmbt2* may reflect its youth as an imprinted domain, suggesting that imprinted regulation precedes establishment of differential methylation [60].

TOWARDS AN EPIGENETIC MECHANISM FOR COGNITION

In the field of genetics, a process is best understood by observing insults to it, as an examination of the effects may reveal the key components of the process. Given the observed phenotypes, imprinted ncRNAs have profound implications in our understanding of basic science, as their integral functions in neurodevelopment and long-term memory formation have already been observed [22, 23]. The dependence of the brain on epigenetic mechanisms for it’s complex traits presents a paradigm shift in our understanding of men-

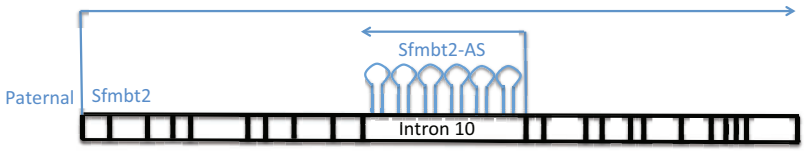

Fig. (3). A physical view of *Sfmbt2* (Murine 2qA1) and an overlapping non-coding antisense transcript contained in intron 10. There is currently no well-established DMR. [60]. Bars and arrows in blue indicate paternally expressed transcripts. Not to scale.

tal processes [67]. The most profound of which is the role of imprinted ncRNA in 'online' brain functions. Currently, the majority of the brain's workings remain a mystery and cognition is no exception to this puzzle. An understanding of the complex responsive network of spatial and temporal regulation created by the epigenetic landscape will shed light on some of the most unique aspects of our brain. It may come as no surprise that these imprinted ncRNAs will play a profound role in mechanisms of cognition. So far, we have discussed several lines of evidence supporting this hypothesis. First, is the brain specific expression and function. Given the unique presence and function observed so far, further research into these clusters will undoubtedly reveal many new roles. Second is the conserved evolutionary relationship between these clusters of imprinted ncRNA. Given that they have evolved only in eutherian mammals, they serve a function that is unique to this lineage. Indeed, the most unique and defining characteristic that comes to light given these two facts is complex brain function. Third is the fact that many, if not most, miRNAs might act as micromanagers of gene expression, subtly fine-tuning the expression of many target mRNAs simultaneously, often without detectable effects on target mRNA levels [6]. Given this observation and the limited work done on these clusters, it may come as no surprise that these clusters have many more targets in the brain that are involved a number of other complex traits. Given the evidence described so far, one may begin to speculate that these clusters are involved in cognition.

CONCLUSIONS

miRNAs are part of the regulatory process involved in gene expression and play a critical role in epigenetic effects and responding to environmental challenges. Furthermore, ncRNAs are an integral part of the epigenetic process and are often expressed mono-allelically depending on the parent of origin; they are known to play a critical role in a number of epigenetic disorders. More importantly they appear to be involved in diseases of fetal origin, including Fetal Alcohol Spectrum Disorders (Laufer *et al.*, unpublished results). ncRNAs appear to play a significant role in neurodevelopment; given the heterogeneity of cell types in brain tissue, epigenetic mechanisms in the brain must be viewed as a complex and spatiotemporally dynamic process. The dependence of the brain on epigenetic mechanisms for complex traits presents a paradigm shift in our understanding of mental processes; the most profound of which is the role of imprinted ncRNA in 'online' brain functions [67]. It may come as no surprise that these imprinted ncRNAs will play a profound role in mechanisms of cognition, but establishing their specific involvement will not be an easy process. Because these neural miRNAs are part of large miRNA clusters and since the knockout of individual miRNAs in the brain does not lead to easily discernable phenotypes, it has proven difficult for researchers to characterize them on an individuals basis. Specifically, all three of the ncRNA clusters cited are transcribed as a single polycistronic unit [33, 60, 68]. Thus, we argue the whole is greater than the sum of the parts and future research should focus on these clusters.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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