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microRNAs and the adolescent brain: filling the knowledge gap

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Abstract

Over two decades ago the discovery of microRNAs (miRNA) broadened our understanding of the diverse molecular pathways mediating post-transcriptional control over gene expression. These small non-coding RNAs dynamically fluctuate, temporally and spatially, throughout the lifespan of all organisms. The fundamental role that miRNAs have in shaping embryonic neurodevelopment provides strong evidence that adolescent brain remodeling could be rooted in the changing miRNA landscape of the cell. Few studies have directly measured miRNA gene expression changes in the brain across pubertal development, and even less is known about the functional impact of those miRNAs on the maturational processes that occur in the developing adolescent brain. This review summarizes miRNA biogenesis and function in the brain in the context of normal (i.e. not diseased) physiology. These landmark studies can guide predictions about the role of miRNAs in facilitating maturation of the adolescent brain. However, there are clear indicators that adolescence/puberty is a unique life stage, suggesting miRNA function during adolescence is distinct from those in any other previously described system.

Introduction

Adolescence refers to the transitional period between childhood and adulthood. This postnatal developmental period encompasses both psychological and physiological changes - those driven by sociological and environmental factors and those that are biologically driven. These factors are intimately intertwined, as biological changes can have a profound influence over behavior, and conversely, environmental factors can have a profound influence on the progression of normal biological development. On the other hand, puberty refers to the biological processes governing maturation of the gonads and gametes leading to the attainment of reproductive competency. This process varies according to species and can even vary within a species dependent on specific strain, as observed in laboratory rodents. In addition, the process of puberty can be disrupted by numerous environmental factors such as circadian timing, psychological and/or physiological stress, and perturbations of prenatal hormones. Adolescent maturation of the brain is thought to be a protracted period of time

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spanning pre-pubertal and post-pubertal development, and adolescent brain maturation in humans is estimated to continue until approximately 25 years of age.

Many parallels can be drawn between embryonic brain development and adolescent brain development including a renewed surge of neurogenesis, increased synaptic formation, increased dendritic pruning, and the strengthening or creation of functional neural circuits (Catts et al., 2013; Ernst et al., 2009; Holder and Blaustein, 2014; Luna et al., 2015; Spear, 2013). Widespread changes in gene expression profiles during pubertal development are one of the major molecular mechanisms governing all of these processes (Harris et al., 2009). Gene expression is controlled by complex regulatory systems, part of which includes the actions of microRNAs (miRNA). These small non-coding RNAs regulate gene expression post transcription, thereby acting downstream from the regulatory actions exerted by transcription factors at gene promoters and splicing factors at nascent RNA transcripts.

Although miRNAs were discovered more than two decades ago, our understanding of the mechanisms regulating their biogenesis, expression, and function is still in its infancy (Feinbaum and Ambros, 1999; Lee et al., 1993; Olsen and Ambros, 1999; Wightman et al., 1993). In particular, very little research has focused on changes in miRNA expression during the adolescent period. This review will delineate the current state of knowledge regarding miRNA biogenesis and processing and then discuss specific miRNAs that are known to have an important role in neurodevelopmental processes. We will also review studies describing how hormonal factors contribute to the regulation of miRNA biogenesis and expression, as dynamic changes in circulating gonadal steroid hormones are a hallmark of pubertal development. Taken together, these studies can provide a framework in which we can begin to predict the role these miRNAs play during parallel changes that occur in the maturation of the adolescent brain.

microRNA Biogenesis

There are 3 key steps in the biogenesis of miRNAs. At each step, distinct nucleotide motifs are recognized by a variety of accessory proteins to selectively modulate the biogenesis of specific subsets of miRNAs. The first step in miRNA biogenesis is similar to that of protein-coding mRNA; a primary transcript is generated from the DNA template by RNA Polymerase II, or in some cases RNA Polymerase III (Fig. 1). An estimated 30% of miRNAs are transcribed by RNA Polymerase III, but the significance of this observation is still unclear (Borchert et al., 2006; Corcoran et al., 2009; Lee et al., 2004a). The DNA coding regions for these miRNAs can be located between (intergenic) or within (intragenic) a protein-coding gene (Borchert et al., 2006; Cai et al., 2004; Corcoran et al., 2009; Kim and Kim, 2007; Lee et al., 2004a; Morlando et al., 2008; Ruby et al., 2007). Intergenic miRNAs typically have a unique promoter region that drives the generation of the primary transcript, however intragenic miRNAs are often under the same regulatory constraints as their “host” gene (Berezikov et al., 2007; Corcoran et al., 2009; Fujita and Iba, 2008; Monteys et al., 2010; Morlando et al., 2008; Zhou et al., 2007). Intragenic or intronic miRNAs (also known as mirtrons) make up a small percentage of miRNA sequences in mammals, but are highly abundant in lower organisms (Berezikov et al., 2007; Kim and Kim, 2007; Monteys et al., 2010; Ruby et al., 2007). Interestingly, not all mirtrons follow the same expression patterns

as their host gene and recent evidence suggests that some have their own promoters from which they can be independently transcribed (Monteys et al., 2010; Ruby et al., 2007). The transcription of a miRNA gene leads to the production of a long RNA transcript termed a primary miRNA (pri-miRNA) (Lee et al., 2002; Morlando et al., 2008; Pawlicki and Steitz, 2008). The pri-miRNA transcript can be hundreds to thousands of base pairs long and forms one or more hairpin secondary structures, containing the nucleotide sequence of the mature miRNA in the stem section of the structure.

The second step of intergenic miRNA biogenesis results in cleavage of the hairpin structure by a large protein complex called the microprocessor. At the core of this processor is the RNase III enzyme DROSHA and its partner DGCR8 (di-George syndrome critical region 8, also called partner of DROSHA (PASHA)). Together, these two vital proteins drive the transition from the pri-miRNA transcript to precursor miRNA (pre-miRNA) (Fig. 1). DGCR8 binds to the hairpin structure on the pri-miRNA and acts as a molecular ruler for DROSHA, so that the RNase III enzyme cleaves at the correct 5’ and 3’ ends of the hairpin (approximately 11 nt from the stem-loop junction) (Han et al., 2006; Nguyen et al., 2015). The resulting pre-miRNA is a stem-loop structure ~ 70 nt whose 3’ end is 2 nt longer than the 5’ end, a structural feature critical for subsequent export to the cytoplasm and processing of the mature miRNA. DROSHA has at least 20 identified accessory proteins that could potentially serve as selection factors to process specific miRNA subsets (Denli et al., 2004). DROSHA can also cleave the pri-miRNA in the absence of any other proteins, as shown using purified DROSHA *in vitro*, however the efficiency of the reaction is very low underscoring the critical requirement for a multiprotein microprocessor complex (Han et al., 2006). How these microprocessor proteins change across the lifespan, particularly during pubertal development, represents a large gap in our current understanding of miRNAs. This second step in miRNA biogenesis takes place inside the cell nucleus, and is thought to occur shortly after the generation of the primary transcript. In fact, some evidence suggests that cleavage of the pri-miRNA to pre-miRNA is a co-transcriptional event (Morlando et al., 2008; Pawlicki and Steitz, 2008). Notably, mirtrons do not require DROSHA/DGCR8 for pre-miRNA formation. Instead, the pre-miRNA is cleaved as a result of intron splicing components, similar to what occurs with the processing for other types of RNAs (Ruby et al., 2007). This raises the possibility that pubertal changes in spliceosome components could have a broad impact on the regulation of gene expression through the increased generation of intron-derived miRNAs. Ultimately, a pre-miRNA hairpin structure is formed for further downstream processing regardless of whether the originating coding sequence is intra- or intergenic.

The final step of miRNA biogenesis occurs outside the nucleus in the cytoplasm of the cell (Fig. 1). The pre-miRNA stem-loop structure is exported out of the nucleus bound to the double-stranded RNA binding protein, Exportin-5 (Lund et al., 2004; Yi et al., 2003). Export of the pre-miRNA is rapid and more than 90% can be cleared from the nucleus within 30 minutes under experimental conditions (Bohnsack et al., 2004; Lund et al., 2004). Exportin-5 transport of pre-miRNA is RanGTP-dependent, similar to tRNA and other small RNAs (Bohnsack et al., 2004; Yi et al., 2003). Once in the cytoplasm, the pre-miRNA is then cleaved by the RNase III enzyme, DICER. DICER cleaves the hairpin pre-miRNA to form a RNA duplex (Lee et al., 2004b) and, unlike DROSHA, can efficiently cleave RNAs

without its binding partners. DICER forms herterodimeric complexes with other RNA binding proteins, such as Tar RNA binding protein (TRBP) and LIN28, and these interactions can regulate DICER activity to inhibit or promote processing of pre-miRNAs (Chendrimada et al., 2005; Lee and Doudna, 2012; Lee et al., 2013).

microRNA Function

Once the miRNA duplex has been formed, the leading strand (also called the Guide) is selected for incorporation into the RNA-induced silencing complex (RISC), while the lagging strand (also called the Passenger) is typically degraded (Okamura et al., 2008). However, in some circumstances the lagging strand is also incorporated into the RISC complex and represses mRNA translation. For example, both the guide (miR-9-5p) and passenger (miR-9-3p) strands for the brain-enriched miR-9 are highly expressed in the brain, suggesting that a miR-9 duplex could have dual roles with each strand repressing distinct mRNA pools (Fig. 1) (Guo and Lu, 2010; Okamura et al., 2008; Yang et al., 2011). The RISC complex consists of an argonaute protein in association with a single-stranded mature miRNA. (Wang et al., 2010; Yoda et al., 2010). In humans, there are four identified argonaute proteins (AGO1, AGO2, AGO3, and AGO4) and AGO2 is distinct from the others in its ability to cleave RNA (Meister et al., 2004). AGO2 can also associate with DICER to facilitate processing of the pre-miRNA to the miRNA duplex (Chendrimada et al., 2005).

The ultimate functional outcome of miRNA activity is a reduced abundance of translated protein from a target mRNA. The guide miRNA directs the RISC complex to a complementary sequence on the target mRNA, typically located on the 3’UTR, which results in translational inhibition and mRNA destabilization. Unlike in plants, animal miRNAs do not share perfect complementary binding with their target mRNA sequences. The first 2-8 nucleotides on the 5’ end of the miRNA, termed the seed sequence, determine the specificity of binding to a particular mRNA sequence (Baek et al., 2008). This relatively loose base pairing allows for considerable promiscuity, such that miRNAs will bind to multiple target mRNAs. Experimental evidence has shown that overexpression of a single miRNA can result in the repression of hundreds of genes (Baek et al., 2008; Wang et al., 2010). Similarly, a single mRNA target can harbor seed sequences for multiple miRNAs.

A major question in the field has centered on defining the dominant mechanism for miRNA-mediated reduction in protein abundance. Data from early studies clearly showed that miRNAs repress translation and this was initially considered to be the likely dominant mechanism in mammals (Olsen and Ambros, 1999; Wightman et al., 1993). However, a series of subsequent papers demonstrated that miRNAs could mediate destabilization of the mRNA (Bagga et al., 2005; Krutzfeldt et al., 2005; Lim et al., 2005), and this was achieved largely through deadenylation of the target mRNA (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006). Recent work showed that the RISC complex induced recruitment of the CCR4-NOT (Carbon Catabolite Repressor protein 4-Negative On TATA) deadenylation complex to remove the poly-A tail of the mRNA, which signals exoribonuclease-mediated degradation (Nishihara et al., 2013). The question then centered on the ordering of each process, and two seminal papers demonstrated that translational repression preceded mRNA destabilization and decay (Bazzini et al., 2012; Djuranovic et

al., 2012), again suggesting that translational repression was a dominant mechanism. Notably, translational repression still occurred even when mRNA targets were prevented from deadenylation processes (Bazzini et al., 2012). However, these studies used Zebrafish and *D. melanogaster* as model organisms and focused on just a few select miRNAs. Most recently, Eichhorn and colleagues used a comprehensive approach to study multiple cell types in various growth conditions (arrested growth or translationally inhibited) and found that mRNA destabilization was broadly observed in all cases (Eichhorn et al., 2014). Although miRNA translational repression was still observed, the biological consequences were negligible prior to the miRNA-induced mRNA destabilization. Therefore, the primary effect of miRNA action in mammalian cells appears to be a reduction in protein as a direct result of mRNA destabilization.

The overall impact of miRNAs on protein expression levels is relatively modest, despite numerous studies demonstrating the importance of miRNAs in posttranscriptional gene regulation. This phenomenon is likely due to the many other regulatory factors that can affect a particular protein (Kozomara et al., 2014; Li et al., 2009; Mukherji et al., 2011). Therefore, it is hypothesized that the main role for miRNAs is to “fine-tune” protein expression; a subtle change that might be necessary to maintain homeostasis in the face of changing cellular conditions (Li et al., 2009). However, there are instances where miRNAs can act as switches of protein expression. Mukherji and colleagues demonstrated that miRNAs could act as both a switch (i.e. on/off) and fine tuner, with the mRNA expression being the dependent variable (Mukherji et al., 2011). For example, they showed that a particular miRNA was highly repressive when the target mRNA expression was below a certain threshold, however as the mRNA expression reached threshold, the miRNA would only modestly repress the expression, thereby acting to fine-tune protein levels (Mukherji et al., 2011).

microRNAs and Embryonic Development

The flux of miRNA expression over a lifetime indicates they are involved in dynamic biological processes that require spatio-temporal regulation. The earliest described phenotypic consequences of miRNA actions were related to their control of specific developmental stages in embryonic and early postnatal time periods in *C. elegans* (Lee et al., 1993; Wightman et al., 1993). Heterochronic genes are key regulators of developmental timing and miRNAs participate in complex feedback loops to achieve the precisely-timed expression of these genes. For example, two heterochronic genes that are vitally important for developmental progression in *C. elegans* are *Lin14*, which encodes a transcription factor important for cell fate determination, and *Lin28*, which encodes a miRNA binding protein. Mutations in either of these genes disrupt normal development and they both have multiple binding sites for the miRNAs lin-4 and let-7 in their 3’UTR (Grosshans et al., 2005; Moss et al., 1997; Reinhart et al., 2000). Importantly, there is a pattern of increased lin-4 expression coupled with decreased Lin14 protein at key developmental transitions (Feinbaum and Ambros, 1999), demonstrating the precisely timed interplay between this miRNA and its target gene. A feedback loop has also been identified between Lin28 and let-7, whereby Lin28 protein binds to the terminal loop of pre-let-7 and inhibits processing of the mature let-7 miRNA (Hagan et al., 2009; Viswanathan et al., 2009). This sets up a regulatory

negative feedback loop necessary for proper developmental maturation in the animal, because let-7 post-transcriptionally inhibits *Lin28*. Lin28 is also involved in a positive feedback loop with let-7 and c-myc. In this case, c-myc is a gene target of let-7 and, as Lin28 inhibits let-7, more c-myc protein is translated, which then stimulates the transcription of *Lin28* (Dangi-Garimella et al., 2009; Viswanathan et al., 2009). These feedback loops have been described for other miRNAs and their gene targets demonstrating a sophisticated mechanism for miRNAs to amplify their effects in the context of the cellular environment. The Lin28/let-7 system is conserved in mammalian species and has also been investigated in the context of adolescent development, which will be discussed in later sections.

microRNAs in the Brain

Genetic animal models with deletions of key miRNA biogenesis enzymes were among the first studies to show the importance of miRNAs in brain function and development (Giraldez et al., 2005). For instance, dicer-null zebrafish had failed brain development, yet somatic axis formation was not disturbed in the embryo (Giraldez et al., 2005). This represented an important distinction for the role of miRNAs during embryonic development between vertebrate and non-vertebrate animals. Importantly, the authors were able to rescue normal phenotypic brain development by introducing a single miRNA (miR-430), providing strong evidence that miRNAs are required for normal brain maturation (Giraldez et al., 2005). Mice lacking either *Dicer1* or *Dgcr8* were also generated and both knockout models resulted in an embryonic lethal phenotype (Babiarz et al., 2011). However, prior to lethality these mice displayed several abnormal brain morphologies: a deformed hippocampus, reduced size in the cortex, and increased neuronal cell death (Babiarz et al., 2011). Taken together these studies demonstrated the importance of miRNAs for normal embryonic brain development, yet the specific miRNAs and their gene targets were undetermined.

Since then, genome-wide sequencing coupled with high throughput cross-linking techniques have identified a collection of miRNAs and their target genes that are critical for neuronal development (Abernathy and Yoo, 2015; Follert et al., 2014; Iyengar et al., 2014; Krichevsky et al., 2006; Lagos-Quintana et al., 2002). Specifically, miR-124 and miR-9/9-3p have been identified as two of the most prominent neuronal-enriched miRNAs (Kapsimali et al., 2007), as miR-124 is a strong marker of mature neurons and miR-9 is a marker for differentiating neurons. Interestingly, miR-124 decreases the expression of non-neuronal genes, indicating that it plays an important role in cell fate determination among brain progenitor cells (Conaco et al., 2006; Yoo et al., 2011). In a similar manner, miR-9/9-3p has been shown to regulate the expression of neuronal genes through the repression of REST and coREST (Packer et al., 2008). Remarkably, Yoo and colleagues directly reprogrammed human fibroblasts to form neurons, bypassing cellular dedifferentiation, simply by overexpressing miR-124 and miR-9 (Yoo et al., 2011). Together, these studies emphasized that just a few miRNAs are sufficient and necessary for generating neuronal cells.

Cellular Localization of microRNAs in Neurons

Genome-wide profiling studies have all demonstrated a shift in miRNA expression as the brain develops in multiple species including zebrafish, mice, non-human primates, and

humans (Kapsimali et al., 2007; Manakov et al., 2009; Miska et al., 2004; Smith et al., 2010; Somel et al., 2011). These fluctuations in miRNA levels correlate fairly well with their predicted target mRNA and protein expression profiles, suggesting an overall major impact of miRNA action during brain development. One of the first described brain enriched miRNAs was miR-124, which was initially identified as a brain-specific miRNA since it was highly expressed in the brain and not in other tissues (Bak et al., 2008; Hu et al., 2011; Hua et al., 2009; Sempere et al., 2004). Subsequent profiling studies showed that miRNAs are brain region and cell type specific (Table 1) (Amar et al., 2012; Herzer et al., 2012; Juhila et al., 2011; Olsen et al., 2009; Parsons et al., 2008). Analysis of miRNA expression in primary neuronal cell cultures and use of fluorescent in-situ hybridization (FISH) revealed a group of miRNAs that are specific to neurons: miR-124, miR-9, miR-128, miR-29, let-7, and miR-26 (He et al., 2012; Kye et al., 2007).

The studies of miRNA expression in neurons also showed that miRNAs were not only localized to the soma (body) of the neuron, but also to the dendrites and synapses, suggesting that miRNAs could regulate local protein synthesis at these distant sites (Kye et al., 2007; Lugli et al., 2008). While miRNAs were overall found to be equally distributed between the soma and dendrites, there were some miRNAs that were preferentially localized to the dendrites (Kye et al., 2007). In support of these studies, argonaute proteins were also localized to the dendrites demonstrating that the key components of RISC were anatomically situated to facilitate miRNA regulation of mRNA targets in dendritic branches. Surprisingly, one of the key miRNA biogenesis proteins, DICER, was also localized to the dendrites and pre-miRNAs were discovered in the synaptic fractions (Barbato et al., 2007; Lugli et al., 2008). Together, these results provided evidence that the pre-miRNAs are actively trafficked to the dendrites where they are further processed to their mature forms through local DICER cleavage.

microRNA Facilitation of Synaptic Plasticity

Critical to brain function is the formation of synaptic connections between neurons for cell-cell communication. Synaptic plasticity is the process of forming and destroying synaptic connections between neurons, which increases at key developmental stages and is one of the major molecular mechanisms underlying cognitive and behavioral changes coincident with puberty (Selemon, 2013; Stolyarova and Izquierdo, 2015). An important genetic tool has been the ability to delete genes temporally and in a cell-specific manner (i.e. conditional knock out). The generation of conditional knock out *Dicer1* mouse models was used was used to demonstrate that miRNAs continue to be critical mediators of postnatal brain function and development (Konopka et al., 2010). Konopka and colleagues demonstrated increased expression of *Bdnf* and *Mmp-9* (matrix metallopeptidase 9), two key regulators of synaptic plasticity, following loss of forebrain neuronal *Dicer1* expression in adult mice (Konopka et al., 2010). These gene expression changes were correlated with functional improvements on a variety of learning and memory tasks and morphological changes in dendritic spines. The authors concluded that a subset of mature miRNAs had remarkably stable half-lives, potentially explaining these unexpected results. However, these results also raise the intriguing possibility that certain miRNAs are responsible for inhibiting genes that facilitate cognition and this could partly explain age-related memory decline. Moreover,

these results highlight the importance of examining miRNAs in the context of specific life stages and the danger of generalizing findings from embryos or adults to those of adolescents.

Validated miRNAs important for synaptic plasticity include miR-132 and miR-134. Specifically, miR-132 is regulated by BDNF, via CREB activation, and its overexpression increased the likelihood of synaptic transmission in adult mouse hippocampal neurons (Lambert et al., 2010). Moreover, deletion of the locus encoding miR-132 in adult mouse hippocampal neurons resulted in decreased dendritic arborization and synaptic spine density (Magill et al., 2010). Together these studies demonstrated that miR-132 is a key regulator of synaptic plasticity in the adult brain, however the suite of affected target genes important for mediating those morphological changes was not identified. By contrast, miR-134 was shown to target the serine/threonine kinase, LIMK1 (LIM domain kinase1), which negatively regulates dendritic spine volume in rat hippocampal neurons (Schratt et al., 2006). During embryonic development, miR-134 increases in the rat hippocampus and peaks by postnatal day (PND) 13, but its expression at successive time points between embryogenesis and adulthood has not been assessed. miR-134 tends to localize to synaptic sites on the dendrites (Schratt et al., 2006), which provides local control over synaptic plasticity. Consistent with the negative role of miR-134 on dendritic spine volume, high expression levels impairs synaptogenesis and memory in adults. This was demonstrated in a mouse model where *Sirt1* (Sirtuin1) was deleted specifically in the brain. *Sirt1* deletion increased expression of miR-134 in the hippocampus, which led to decreased protein levels of BDNF and CREB, reduced long-term potentiation, and poor performance on behavioral memory tests. (Gao et al., 2010). Together, these data suggest that miR-134 plays an integral role in orchestrating the correct timing of synaptic plasticity and implicates it as an important factor during pubertal brain development.

Hormone Regulation of microRNAs

Hormonal changes at puberty are a driving force for many phenotypic and behavioral changes that occur during this developmental period. The first observations for hormonal regulation of miRNAs were in *D. melanogaster*. Those studies demonstrated that ecdysone and juvenile hormone modulated the expression of select miRNAs at critical points during fly development (Sempere et al., 2002; Sempere et al., 2003), providing evidence that hormones could regulate miRNAs in other species at specific developmental stages. The morphological processes coincident with brain development during puberty are analogous in many ways to tumor growth, with increased cellular proliferation (gray and white matter volume), changes in cellular morphology (akin to metastatic tumors) and increased blood perfusion (observed in pubertal girls) (Holder and Blaustein, 2014; Satterthwaite et al., 2014). Therefore, much of the knowledge we have gleaned from miRNAs in cancer models could potentially be used to predict the role of miRNAs during pubertal development. In particular, data from breast cancer model systems have laid a valuable foundation for understanding how the hormonal control of miRNA activity acts as an underlying mechanism to regulate mammalian gene expression patterns. Those findings will not be discussed in detail here and we instead refer the reader to recent reviews that have summarized the findings from breast cancer cell models (Fletcher et al., 2014; Klinge,

2012). However, it is important to note that different patterns of hormonal miRNA regulation have been observed under normal physiological conditions (compared with diseased) and the hormonal regulation of miRNAs in the brain do not necessarily match those seen in other tissues, once again highlighting the unique tissue-specific regulation of miRNAs.

Sexual Dimorphism and Hormonal Effects on microRNAs in the Brain

The number of studies describing hormonal effects on miRNAs in the brain are greatly outweighed by those in other steroid-responsive tissues, such as breast, ovary, uterus, prostate, and testis (Burney et al., 2009; Di et al., 2014; Fitzgerald et al., 2016; Jackson et al., 2014; Klinge, 2012; Maalouf et al., 2014; Mo et al., 2013; Narayanan et al., 2010; Nothnick, 2016; Nothnick and Healy, 2010; Pan et al., 2008; Pan et al., 2007; Sangiao-Alvarellos et al., 2015; Tong et al., 2014; Waltering et al., 2011). Nevertheless, sexually dimorphic brain miRNA expression patterns have been identified in both diseased and normal physiological contexts for a variety of vertebrate and non-vertebrate species (Khan et al., 2015; Morrison et al., 2014; Pak et al., 2013). These observations raised the possibilities that there were discrete sex chromosome effects on miRNA expression (Feng et al., 2009; Siegel et al., 2011), organizational effects of prenatal hormones (Bizuayehu et al., 2012; Morgan and Bale, 2011), and/or acute effects exerted by circulating gonadal steroid hormones (Rao et al., 2013; Rao et al., 2015). Evidence supporting a critical role for prenatal hormones was first demonstrated by Morgan and Bale (Morgan and Bale, 2011). Their study identified 7 miRNAs that were significantly different between male and female rat brains at PND0. Remarkably, a single injection of an aromatase inhibitor, which prevents conversion of testosterone-to-estrogens, was sufficient to induce a female-like miRNA profile in the PND0 male brains, suggesting that some miRNAs in the rat brain preferentially target genes that regulate sexual differentiation (Morgan and Bale, 2011). Targeted analysis of adult mouse hippocampus, cerebellum, and cortex showed that there were significant sex differences in all brain regions, with the greatest number of sexually dimorphic miRNAs being expressed in the hippocampus (Koturbash et al., 2011). However, that study did not directly manipulate hormone levels to determine if circulating gonadal steroid hormones were an underlying cause for the observed sex differences.

One of the first reports to directly examine the effects of gonadal steroid hormones on miRNA expression in the brain used a miRNA microarray approach (Sanger miRBase release 18) (Rao et al., 2013). In that study, 3- and 18-month old rats were ovariectomized (OVX) and administered vehicle or 17β-estradiol (E2) at 7 days post-OVX. A total of 34 miRNAs were regulated by E2 treatment in the dorsal hippocampus, and 9 were differentially regulated by E2 dependent on age. However, E2 did not exert the same regulatory effects on these miRNAs in every brain region, as the ventral hippocampus, paraventricular nucleus, and amygdala all showed unique miRNA expression profiles (Rao et al., 2013). Collectively, these data indicated that at least some of the tissue specific effects of E2 are due to miRNA-mediated posttranscriptional modulation of mRNA gene targets. To our knowledge, there have not been any published studies to date describing a similar regulatory role of progesterone, the other major ovarian steroid hormone, on miRNA expression in the postnatal brain.

microRNAs and Adolescence

There is a large gap in our knowledge when it comes to puberty-specific changes in miRNA expression. Moreover, it is currently unclear whether miRNA expression changes would have a major impact on the maturation of the adolescent brain, as there are many well established regulatory mechanisms that appear to be independent of miRNAs. Further complicating this lack of knowledge is the fact that most research into this dynamic period of development can be difficult to interpret due to the lack of standardized approaches, confusion about nomenclature, and the challenges of making broad generalizations across different model organisms.

To date, there have been 3 published manuscripts that specifically measured changes in miRNA expression in the brain throughout the pubertal transition. Two of these manuscripts focused primarily on the Lin28/let-7 system in the hypothalamus of mice (Grieco et al., 2013), rats, and monkeys (Sangiao-Alvarellos et al., 2013). *Lin28* has 2 mammalian homologs: *Lin28a* and *Lin28b*, which were identified in genome-wide screens as potential candidates important for regulating the age of menarche in girls (Elks et al., 2010; He et al., 2009; Ong et al., 2009; Perry et al., 2014; Zhu et al., 2010). Both studies observed significant changes in the expression profiles of *Lin28a* and *Lin28b* from neonatal/juvenile to adult ages (Grieco et al., 2013; Sangiao-Alvarellos et al., 2013). Interestingly, *Lin28b* significantly declined in male rats between early puberty to puberty/adult, but this observation was not consistent in female rats, female monkeys, or in female mice. Similarly, *Lin28a* was significantly higher in juvenile female mice (PND15) compared to peripubertal mice, but again, this effect was not consistent with the female or male rats, or the female monkeys. There were also no differences observed in mice across pubertal development in the let-7 family of miRNAs predicted to target *Lin28a* and *Lin28b* (let-7a and let-7g). Similarly, let-7a did not change across pubertal development in the rat, but the levels were significantly lower during all stages of puberty compared to adults (Grieco et al., 2013; Sangiao-Alvarellos et al., 2013). The miRNA let-7b had the largest magnitude increase between two time points measured (puberty and adult) in male and female rats, increasing by nearly 400%. However, let-7b changed only modestly during peripubertal development (male-specific), suggesting that the large increase observed between puberty and adult ages happened closer to the completion, rather than the onset, of puberty. Strikingly, miRNA expression levels during puberty were also altered in both male and female rats when pubertal onset was delayed by experimental manipulations of prenatal hormones, nutritional status, or photoperiod (Sangiao-Alvarellos et al., 2013). Significant changes in miR-9, miR-132, and miR-145 expression levels were also observed and, while none of them showed altered expression levels across pubertal development, there were significant differences between neonatal to adult ages and differences associated with experimentally delayed pubertal onset. Taken together, these studies implicate the Lin28/let-7 system as important not only for embryonic development, but also for postnatal neurodevelopment during puberty.

The only other published study that directly measured miRNA expression across pubertal development used a rat model of “binge-pattern” alcohol exposure (Prins et al., 2014). Risky behaviors are increased during adolescence and the dramatic surge in binge-pattern alcohol

use over the past two decades is a major national health concern (Patrick and Schulenberg, 2013; Silveri, 2012; White and Hingson, 2013). Prins and colleagues measured 4 alcohol-sensitive miRNAs (miR-10a-5p, miR-26a, miR-103, and miR-495) in the rat hippocampus that were predicted to target *Bdnf* and *Sirt1* - two genes implicated in mediating hippocampal synaptic plasticity. Contrary to the expression levels observed for let-7, each of the miRNAs tested had brain region-specific patterns of expression between early-, mid-, and late-puberty (Figs. 2, 3 show normal developmental changes in untreated animals). Moreover, the normal developmental profile of these miRNAs was dramatically altered following binge-pattern alcohol exposure (Prins et al., 2014). The changes persisted into adulthood despite a long-period of alcohol abstinence, suggesting that alcohol permanently skewed the trajectory of normal pubertal brain maturation. Correlative changes in the predicted target genes *Bdnf* and *Sirt1* were also observed, but no functional or morphological parameters were measured in that study.

Conclusions

Collectively, these studies raise some important considerations. First, species differences are readily apparent and broad conclusions based on a single species must be interpreted with caution. Species-specific gene regulatory mechanisms might dominate over the actions of a particular miRNA, despite the evolutionarily well conserved sequences of many miRNAs and their predicted target genes. Second, changes in miRNA expression levels do not necessarily correlate well with mRNA levels of the target gene. An inherent confound exists when measuring the miRNA/mRNA simultaneously, as the events of translational repression, mRNA destabilization, and miRNA stability/turnover are temporally distinct, making “snapshot” data difficult to interpret. Third, multiple other factors regulate gene expression and miRNAs are primarily recognized as responsible for fine-tuning cellular processes, suggesting that small, and perhaps statistically insignificant, changes could still have biologically relevant consequences. Finally, these studies reinforce the concept that sex differences and the effects of circulating hormones on miRNA function in the brain should not be overlooked.

The conspicuous lack of studies investigating miRNAs during pubertal development points to many critical knowledge gaps that will require intensive research efforts. Genome-wide RNA sequencing screens across multiple time points during the pubertal transition are necessary to correlate changes in non-coding RNAs with those of putative protein-coding gene targets. Next, the validation of those predicted gene targets, through highly rigorous assays like RIPiT-SEQ or HITS-CLIP is essential (Licatalosi et al., 2008; Ule et al., 2003). This will allow for a systematic interrogation of the functional consequences of miRNA action on the transitional phenotype of the brain from childhood to adulthood, which is more important than mere identification of miRNAs and gene targets. Finally, once the role of miRNAs in adolescent brain development have been established under normal physiological conditions we can begin to uncover the basis of mental health disorders that tend to emerge during this time period, and works toward developing precisely targeted therapeutic strategies.

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Highlights

This review gives a brief summary of miRNA biogenesis and function in the brain.

The impact of hormones on miRNA function are discussed.

miRNA function during puberty could be distinct from other developmental stages.

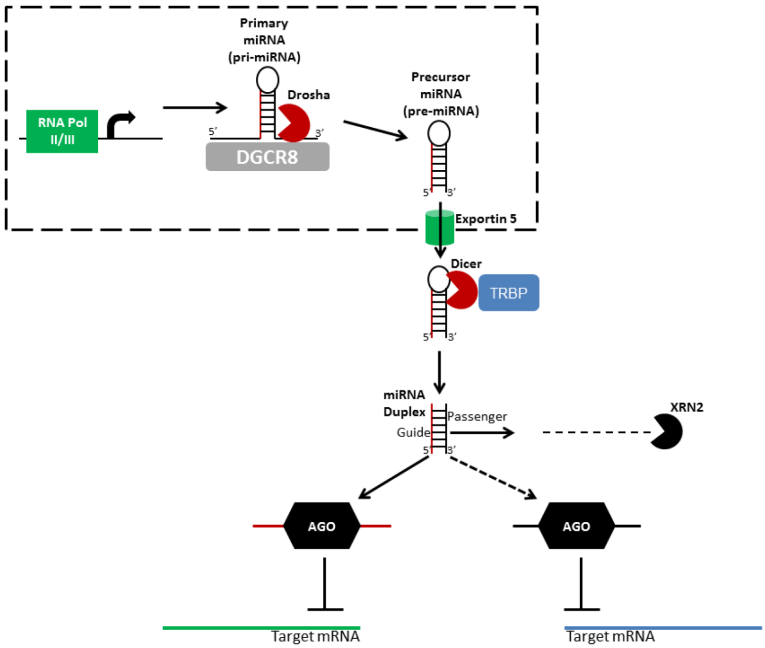

Figure 1. Diagram depicting the canonical miRNA biogenesis pathway

Dashed-line box denotes the cell nucleus. DGCR8 = diGeorge syndrome critical region 8; TRBP = Tar RNA binding protein; XRN = 5'-3' Exoribonuclease 2; AGO = argonaute

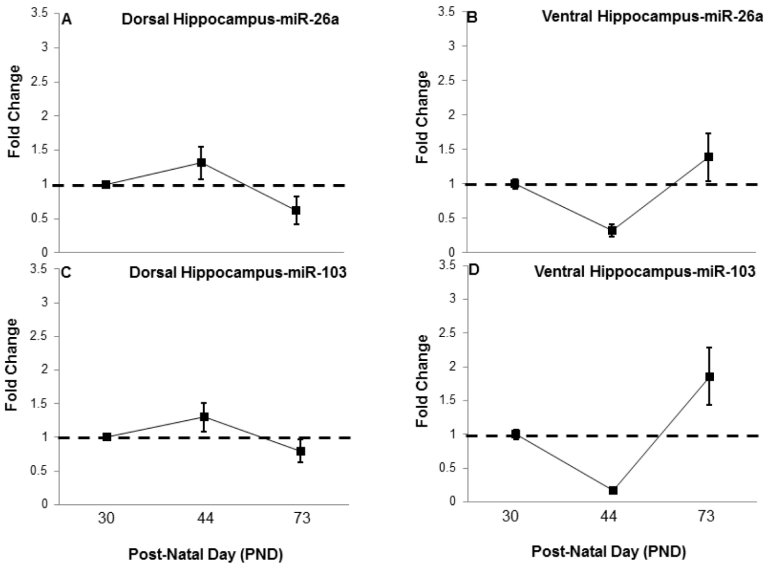

Figure 2. Mature miRNA expression levels are brain-region and age-dependent during pubertal development

Mature miRNAs were measured by RT-qPCR in the dorsal and ventral hippocampus of untreated male Wistar rats at postnatal day (PND) 30, 44, and 73. Expression of the miRNAs are normalized to PND 30 animals (dashed line). miR-26a in the dorsal (A) and ventral (B) hippocampus. miR-103 in the dorsal (C) and ventral (D) hippocampus. Adapted from Prins et al., 2014, *PLOS ONE*.

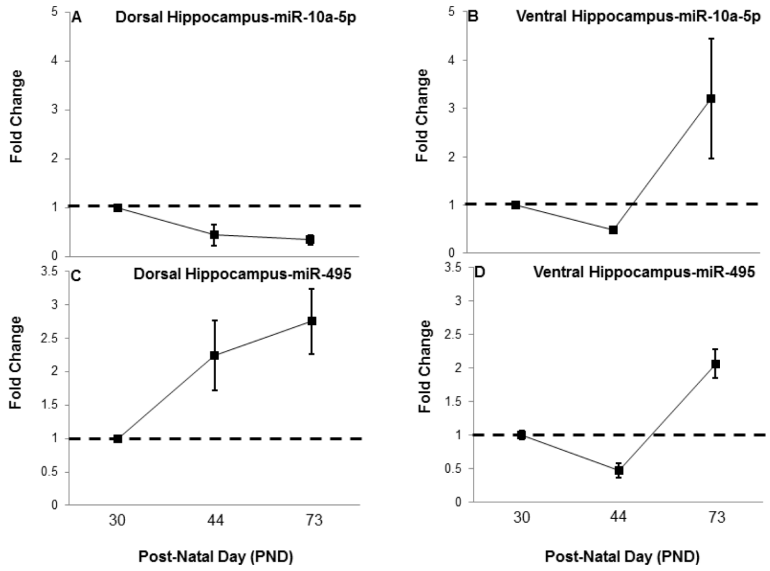

Figure 3. Mature miRNA expression levels are brain-region and age-dependent during pubertal development

Mature miRNAs were measured by RT-qPCR in the dorsal and ventral hippocampus of untreated male Wistar rats at postnatal day (PND) 30, 44, and 73. Expression of the miRNAs are normalized to PND 30 animals (dashed line). miR-10a -5p in the dorsal (A) and ventral (B) hippocampus. miR-495 in the dorsal (C) and ventral (D) hippocampus. Adapted from Prins et al., 2014, *PLOS ONE*.

Table 1

microRNAs highly expressed in the brain

Brain Cell Type	microRNA
Neuron	miR-9/9-3p, miR-124, miR-132, miR-134, miR-125a, miR-7a/b, miR-128
Astrocyte	miR-21, miR-34b/c, miR-210, miR-222
Oligodentrocyte	miR-20a/b, miR-219-2-5/3-p
Microglia	miR-126, miR-150, miR-200c