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An emerging role for microRNAs in sexually dimorphic neurobiological systems

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Abstract

Over the past 20 years our understanding of the basic mechanisms of gene regulation has vastly expanded due to the unexpected roles of small regulatory RNAs, in particular, microRNAs (miRNAs). miRNAs add another layer of complexity to the regulation of effector molecules for nearly every physiological process, making them excellent candidate molecules as therapeutic targets, biomarkers, and disease predictors. Hormonal contributions to mature miRNA expression, biosynthetic processing, and downstream functions have only just begun to be investigated. Elucidating the physiological consequences of miRNA sexual dimorphism, and their associated regulatory processes, may be key towards understanding both normal and pathological processes in the brain. This short review provides a basic overview of miRNA biosynthesis, their role in normal brain development, and potential links to neurological diseases. We conclude with a brief discussion of the current knowledge of sex-specific miRNA processes in both the brain and the heart to conceptually integrate the relevance of miRNAs with the overarching theme ("sex differences in health and disease: brain and heart connections") of this special topics issue.

Introduction

MicroRNAs (miRNAs) have emerged over the last decade as powerful regulatory agents for virtually all fundamental biological processes, and therefore functional dysregulation of miRNAs has been implicated as a causative factor in a variety of pathogenic conditions. Belonging to a larger class of non-coding regulatory RNA molecules, these tiny ~22-nucleotide (nt) RNA species participate in the cell as highly effective gene silencers through the post-transcriptional regulation of their target gene mRNA transcripts [1–3]. Pioneering work in the laboratories of Victor Ambros [4–6], Gary Ruvkun [7], and David Baulcombe [8, 9] first identified these small regulatory RNAs in *C. elegans* and various plant species, but evolutionary conserved miRNAs have since been described in all animal species studied to date, including humans [10, 11] (see Table 1 for current nomenclature conventions). While our understanding of the intracellular processing of miRNAs and the basics of miRNA:target gene interactions has increased significantly in the past few years, many questions remain about how miRNAs are regulated, especially with respect to age and hormones.

miRNA Biosynthesis

The majority of miRNAs in humans and higher vertebrates have promoter sequences that are autonomously situated between other genes and are often found in clusters, with multiple miRNA promoter sequences lying in close proximity to one another [11]. The canonical

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miRNA biosynthetic pathway in mammals begins with the generation of a primary transcript (pri-miRNA) encoded from these promoters and initiated by RNA Polymerase II, similar to the biosynthetic pathways for protein coding messenger RNA (mRNA) [12, 13]. First strand pri-mRNA transcription results in a relatively long (i.e. 100 – 1000 bp) double-stranded molecule with a classical hairpin loop that results from its high degree of self-complementarity. The subsequent processing of the pri-miRNA is accomplished through successive cleavage events orchestrated by two RNase III enzymes: the nuclear enzyme drosha and the cytoplasmic enzyme dicer. Drosha forms a heterodimer with DiGeorge syndrome Critical Region 8 (DGCR8), a RNA binding protein that positions the catalytic domain of drosha near the base of the pri-miRNA hairpin loop [14, 15]. Drosha cleaves the pri-miRNA hairpin yielding a ~70 nt stem-loop precursor miRNA (pre-miRNA) that is exported out of the nucleus in a RAN-GTP dependent manner by the cotransporter Exportin 5 [16, 17]. Dicer completes the biosynthetic process by cleaving pre-miRNA precursors into the canonical ~22 nt duplex miRNA.

Interestingly, a small fraction of miRNA coding sequences, termed “mirtrons”, have their precursor sequences encoded within the intronic region of another gene, allowing them to bypass drosha processing. In those cases, transcription of the pre-miRNA is dependent on the upstream regulation of its “host” gene. Mirtrons are more commonly found in lower organisms that have an abundance of genes containing short introns (i.e. similar in size to that of a pre-miRNA), such as insects and nematodes [18, 19]. One explanation for this evolutionary switch in miRNA processing might be that higher organisms evolved the more complex two-step processing pathway (drosha-dicer) to better regulate miRNA activity, and their respective target genes, within more highly specialized cell types, such as neurons. Alternatively, the small percentage of evolutionarily retained mirtrons in higher organisms may provide an alternate pathway for mature miRNA synthesis and another level of regulation during complex early developmental processes. This scenario would facilitate the immediate miRNA-mediated post-transcriptional modulation of embryonic stem cell target genes that determine cell fate specification and differentiation pathways, such as Hox genes.

While the biochemical events required for processing of the pri-miRNA and pre-miRNA sequences are becoming more transparent, the precise molecular mechanisms regulating transformation of the miRNA duplex to a single-stranded effector remain unclear. Most studies suggest that one strand of the duplex, termed the “guide” strand, is preferentially selected for incorporation into the RNA-induced silencing complex (RISC), while the opposite “passenger” strand (formerly designated as miR*) is typically degraded [20–22]. The RISC complex is a ribonucleoprotein aggregate that contains two primary functional units: the mature guide strand miRNA and a member of the Argonaute (Ago) family of proteins. Because the guide and passenger strands are inverse complements it is reasonable to predict that their target genes would be vastly different, making the strand selection process a critical regulatory step for directing the functional consequences of miRNA-mediated gene repression. However, the mechanisms dictating the process of strand selection remain unresolved. One model suggests that the miRNA is incorporated into the Ago protein as a duplex; the duplex is then unwound based on the thermodynamic stability of the first 1–4 nucleotides at the 5′ end; the least thermodynamically stable strand is retained in the Ago protein as the “guide”; and finally, the passenger strand is discarded [23, 24]. One important assumption for this model is that it requires active ATP-dependent loading of the miRNA duplex into the Ago protein. An alternative model posits that the miRNA duplex is unwound by specialized RNA helicases prior to guide strand incorporation into the Ago protein and then the single-stranded effector can be spontaneously incorporated in an ATP-independent manner [25]. Evidence for this model is based largely on theoretical predictions about the inherent properties of RNA helicases and the thermodynamic properties governing the miRNA asymmetrical strand selection.

Although several putative RNA helicases have been proposed to support this model, none have yet been shown to be sufficient and required at a specific step in the RISC assembly process.

miRNA-mediated gene silencing

A highly conserved protein from the Argonaute superfamily is at the core of all RISC complexes. Single-stranded miRNAs associated with a particular Ago protein direct the ribonucleoprotein RISC complex to a specified target gene, accomplished through miRNA recognition of a partial complementary binding sequence on the target mRNA 3′UTR. There are four identified Ago protein subfamilies in humans (AGO1-4), but AGO2 is the only one that has demonstrated endonuclease ("slicer") activity [26] thereby, defining it as a critical component in vertebrate RNA silencing complexes (see [27] for an excellent review of Ago proteins). Alternatively AGO1, 3, and 4 induce translational repression of their target genes in the absence of mRNA cleavage, which raises the fundamental question of what mechanisms confer miRNA specificity for a given Ago protein. Indeed, the highly conserved structure of the Ago protein domains suggest that a great deal of overlap exists among the members of the Ago superfamily, allowing for multiple small RNA molecules, including siRNAs, to bind more than one Ago protein subtype. In plants, some evidence suggests that the length of the mature miRNA guide strand (21 nt – 22 nt) and the nucleotide identity at the 5′ end are factors determining specificity for Ago proteins. Nonetheless translational repression, as opposed to mRNA cleavage, is now widely recognized as the primary mechanism by which miRNAs achieve gene silencing in animals.

Sex differences in miRNA silencing mechanisms remain a relatively unexplored area of research. Although several studies have described the effects of sex steroid hormones, primarily 17β-estradiol (E_2), but also testosterone (T), on steady-state mature miRNA expression and some of the processing enzymes, such as drosha and dicer, none have examined whether protein constituents of the RISC complex or mechanisms of miRNA silencing action are regulated by sex steroid hormones.

miRNAs in neurodevelopment

Whole-genome sequencing data from a variety of species have underscored the importance of post-transcriptional and post-translational modifications needed to achieve extensive phenotypic diversity. miRNAs have the ability to fine-tune the downstream physiological effects of gene transcription by regulating the effectors (i.e. proteins) of those genes. Central nervous system (CNS) development, in particular, requires a precise temporal orchestration of events that is uniquely suited for the fine-tuning attributes of miRNAs. The importance of miRNAs in embryonic development was originally demonstrated using transgenic animal models that manipulated miRNA biosynthetic processing enzymes, such as dicer and DGCR8. There is a single gene that encodes dicer in *C. elegans*, mice, and humans and depletion of dicer results in severe developmental consequences. Studies in dicer-mutant zebrafish showed they had disrupted embryonic morphogenesis and neural differentiation [28]. More specifically, the brains lacked ventricles, neuronal positioning was disrupted suggesting migration defects, and many neurons had defasciculated axons [28]. Strikingly, injections of miRNAs from the miR-430 family (miR-430a/b/c) reversed many of the brain morphogenic defects that resulted from dicer deletion in the zebrafish, revealing a direct connection between mature miRNAs and dicer during development. The partial rescue of neuronal defects in this study also provided some of the first evidence for tissue-specific effects of miRNAs [28, 29]. Global dicer deletion in mice is embryonic lethal [30] prompting the generation of tissue-specific conditional dicer-null mouse models. In the developing neocortex, the absence of dicer resulted in a smaller cortex, improper cortical

layering, increased apoptosis, as well as an overall reduction in neural progenitor cells and oligodendrocytes [31–33]. Dicer is also a critical enzyme for mediating the effective maturation and maintenance of olfactory, purkinje, dopaminergic and forebrain neurons [34–37]. One important consideration is that dicer, an RNase III enzyme, mediates the biosynthetic processing of all small regulatory RNAs, including siRNA, and could possibly be an important regulator of other, as yet unidentified, physiological processes. Therefore, caution must be exercised when drawing conclusions about the direct cause of the phenotypic manifestations observed in dicer-null animals. Deletion of DGCR8 in murine embryonic stem cells also results in a phenotype that reflects a loss of miRNA function and disrupted miRNA biosynthesis including excess accumulation of primary miRNA transcripts, lack of mature miRNA expression, abnormal expression of differentiation markers, and early arrested development. Although lethal in early embryonic development, the magnitude of neuronal defects observed in DCGR8-null mice is not as striking as those in dicer-null mice [38], suggesting that other compensatory mechanisms can override the phenotypic consequences of DGCR8 deletion.

The discrete biological functions of individual miRNAs during neuronal development are less well understood. Lsy-6 was the first specific miRNA recognized to have a role in nervous system development *in vivo*, where it was shown to regulate left/right asymmetrical patterning of the taste receptor neurons in *C. elegans* [39]. Two other miRNAs, miR-9 and miR-10, are highly expressed in the brain and have been shown to play important roles in the brain development of many species including humans, rodents, zebrafish and drosophila, demonstrating a high degree of evolutionary conservation among these miRNAs. Specifically, miR-9 promotes migration and proliferation in human neural progenitor cells by targeting stathmin, a gene required for microtuble assembly [40] and peripheral nervous system sensory organ development in drosophila [41]. Further, miR-9 is significantly reduced in the *presenilin-1* null mouse model, which exhibits severe CNS developmental defects, during specific stages of development compared with wild-type mice [42]. Another important miRNA during development is miR-10, which targets members of the HOX gene family; a highly conserved group of transcription factors that coordinate anterior-posterior body axis alignment in zebrafish and other species during development [43].

In addition to posttranscriptional regulation of mRNA, alternative pre-mRNA splicing is another well-described mechanism for increasing phenotypic diversity with a limited pool of protein-coding genes. Recent studies have shown that miRNAs actively participate in the regulation of alternative splicing events in neurons by targeting genes that are involved in pre-mRNA splicing processes. For instance, the neuronal-specific miRNA, miR-124, was shown to directly target the RNA-binding protein PTBP1, which reduces neuron-specific alternative splicing [44]. Taken together, these studies revealed that specific miRNAs and their processing enzymes are critical for proper gene expression and brain function throughout development, however once the brain is fully formed, constant monitoring of ongoing cellular process is yet another critical function for miRNAs in the brain.

miRNAs in neuronal cell maintenance

miRNAs vital role in neuronal maintenance processes position them as attractive targets for therapeutic strategies to combat a variety of neurodegenerative conditions. Ongoing cellular maintenance processes are critical facilitators of neuronal plasticity, which results from dynamic changes in dendritic spine density. Specific miRNAs, such as miR-29a/b and miR-134, have been identified as participants in this process by regulating components of actin polymerization that are required for dendritic spine formation and remodeling. In hippocampal neurons, miR-29a/b targeted expression of the protein ARPc3, which is a subunit of the ARP2/3 actin nucleation complex [45]. Additionally, miR-134 decreased

dendritic spine size by targeting a synaptic kinase, LIMPK1, that enhances actin polymerization in hippocampus neurons [46]. Further evidence for miRNA participation in ongoing neuronal maintenance has been elucidated in studies investigating miR-124, which is highly enriched in neuronal tissues. In both chick and xenopus, miR-124 was not required for neuronal determination [47, 48]. Rather, Sanuki and colleagues showed that miR-124 promoted neuronal maturation and axonal growth in embryonic and adult mice demonstrating its ongoing role in CNS maintenance [49].

miRNAs beyond early development

The role of miRNAs beyond early developmental periods is just beginning to be investigated. Mature miRNA expression is age-dependent and accordingly, miRNAs regulate both early developmental gene expression changes as well as those that occur throughout the lifespan in various species [50–53]. A deep sequencing study recently highlighted 75 miRNAs that were differentially expressed in the brain with age, 71 of which were downregulated, including miR-124 and miR-34a [53]. As mentioned above, miR-124 is highly expressed in differentiating and mature neurons and its possible primary function is to suppress non-neuronal genes and promote the expression of neuronal-specific phenotypes in adult neurons, as demonstrated by the induction of a neuronal-like phenotype in HeLa cells following overexpression of miR-124 [54, 55]. miRNAs that are important for continuing brain development are not restricted to rodents and zebrafish. A predictive bioinformatics analysis has recently suggested that miRNAs are responsible for differences in gene expression from birth to adult in the primate hippocampus. Importantly, the miRNAs of interest in this study were also expressed in the human hippocampus, yet some miRNAs were specific to the non-human primate [56]. A recent comparison of human, chimpanzee and macaque using Affymetrix microarrays found that gene expression patterns diverged at a faster evolutionary pace in humans compared to macaques and chimpanzees, suggesting that miRNAs might have significantly contributed to the evolution of advanced forebrain architecture in humans [57] and that miRNA-mediated gene regulation could have been the driving force behind the evolutionary development in the human brain [57].

miRNAs in the aging brain

Gene expression profiles of aging human and primate brains have shown that steady state mRNA levels are altered with age particularly for those genes involved with regulating metabolism, synaptic activity, dendritic growth, and cell survival [58–61]. Overall, there is a global decrease in gene expression in the aging brain [58, 59, 61], which can result in a cellular environment that promotes disease. The exact cause of the observed age-related global decrease in gene expression has not been determined, though reports suggest that altered transcription factor expression and/or oxidative damage to gene promoter regions may be contributing factors [62]. Additionally, overall changes in gene expression could potentially be attributed to global changes in RNA processing, for which, regulatory RNAs have come to the forefront as important regulators of age-related gene expression and neurological disease [53, 63–65]. miRNAs were first discovered as important regulators of embryonic development in *C. elegans*, and as aging often recapitulates development, it follows that one of the first pieces of evidence that miRNAs are crucial for normal aging was also described in *C. elegans* [51, 66]. However, more recent mammalian studies have provided greater insight into the role of miRNAs in brain aging. In mice, global miRNA expression is increased with age, corresponding precisely with overall decreases in mRNA expression [53, 63]. Interestingly, some of the miRNAs downregulated with age have been shown to target genes involved with oxidative phosphorylation [63]. Further, microarray data obtained from studies in our laboratory revealed that 21 miRNAs were differentially regulated by age alone in the hippocampus of female rats (Table 2, unpublished data).

Target pathway prediction programs, such as DIANA-mirpath [67], indicated that these 21 miRNAs could regulate genes involved in multiple cellular signaling pathways, but especially those important for regulating neuronal plasticity (Fig. 1). Human and primate studies have revealed additional levels of complexity for miRNA interaction in the aging brain. Somel and colleagues used macaque and human prefrontal cortex brain samples to track changes in miRNA, mRNA, and protein expression over the lifetime [65]. Their study showed that miRNA expression is highly dynamic during developmental and aging processes, reflecting inverse expression patterns for both mRNA and protein however, the underlying mechanisms behind these age-related changes in miRNA/mRNA expression remain to be elucidated. One hypothesis is that a combination of synergistic factors including adaptive responses to environmental stressors, oxidative DNA damage, altered hormone/growth factor levels, and perhaps circadian rhythms, can all contribute to changing gene expression profiles with age [65]. A significant limitation from the available miRNA/mRNA expression datasets is that they almost all used prefrontal cortex as basis for the study, which may not accurately represent how aging occurs in the other brain regions. Indeed, each brain region has a unique expression profile and associated physiological function. Another important consideration is that cognitive aging, and associated functional decline, are not equivalent for men and women. For example, specific neurological diseases, such as Alzheimer Disease (AD), depression, and anxiety, all present with a bias towards females, indicating that sex hormones and/or chromosomal contributions also play a role in the molecular processes governing these conditions [68–70].

miRNAs in neurodegenerative disorders

miRNA expression profiles have been reported for a variety of neurodegenerative disorders, however the best studied to date is AD. The precise role of miRNAs in the pathogenic progression of AD is not clear, although mature miRNAs are differentially expressed in diseased brains [71–75]. Specific miRNAs that are commonly dysregulated in many AD patients include miR-7, miR-9, miR-26, miR-29, miR-34, miR-125a/b, miR-181, and miR-495. Interestingly, all of these miRNAs have also been shown to be regulated in the brain by age alone ([65], Table 1), making their contribution to age-related pathological conditions difficult to discern. Several of these miRNAs are also predicted, or have been shown to directly target, β-site APP-cleaving enzyme 1 (BACE1) and amyloid precursor protein (APP). These proteins are critical in the accumulation of β-amyloid plaques, one hallmark of AD pathology, thought to be a critical component of neuronal death and cognitive impairments. Recent studies of sporadic AD patients have shown that there are increased BACE1 protein levels and enzymatic activity in the brain [71, 76], yet no corresponding increase in BACE1 mRNA, suggesting the potential for miRNA-mediated post-transcriptional regulation of BACE1 [71]. Two predicted regulators of BACE1 are miR-29a/b and miR-9, both of which have reduced expression levels in AD brains [71]. miR-9 expression is also reduced in Huntington's disease, which has similar pathologies to AD [77], suggesting that certain miRNAs might have important roles in specific cellular pathways that have a high propensity for generating diseased states when dysregulated. Due to the correlative nature of the available datasets from AD patients, it is not possible to determine if miRNAs are causing AD, or if their altered expression profiles are merely another consequence of AD pathogenesis. A thorough understanding of how miRNAs contribute to the normal aging process is required to determine if they play a causative role in AD or any other neurodegenerative disorders.

Empirical determination of miRNA targets is crucial towards understanding their impact and specific role in the progression of neurological diseases, however this has proved to be one of the key challenges in the miRNA field. One of the best experimental strategies currently available to identify miRNA gene targets is High-throughput Sequencing of RNA isolated

by Crosslinking Immunoprecipitation (HITS-CLIP). HITS-CLIP is a novel technique that isolates mRNA bound to the RISC complex by UV crosslinking and immunoprecipitation [78]. Once the mRNA is isolated, RNA sequencing can be used to determine what transcripts are bound to the RISC complex. Elucidation of the exact miRNA target genes can then be determined by examining the 3′ UTR of the isolated mRNA transcripts for seed sequence matches. Using a HITS-CLIP approach on AD brain samples would reveal all of the mRNAs that are being repressed by the RISC complex, thereby providing valuable insight into the role of differentially expressed miRNAs in AD affected brains.

Another major obstacle for treating AD patients is the lack of available early diagnostic markers. Diagnosis at the onset of AD would enable more targeted and rigorous treatment possibilities with the ultimate goal of slowing disease progression. Notably, miRNAs have recently been proposed for use as potential biomarkers for AD, as well as other diseases, such as various cancers, since many of the miRNAs dysregulated in AD and aging are also expressed in the plasma of cancer patients [79, 80]. Importantly, these miRNAs are enclosed in exosomes that protect them from degradation, making them a potentially reliable biomarker for many diseases.

miRNAs in mental illness

Mental health disorders such as depression, anxiety, and schizophrenia are often highly correlated with advanced age [81–83]. Collectively, mental health disorders are characterized by disrupted neuronal communication, and typically involve the dysfunction of many different brain regions, making these disorders extraordinarily complex to diagnose and treat. Communication between neurons requires tight temporal regulation of multiple genes, which can be finely controlled by miRNAs. In support of this idea, dendrites and synapses express mature miRNAs as well as the components required for miRNA biogenesis and repression, indicating that miRNAs are likely involved in the regulation of neuronal communication [46, 84–86]. miRNA expression is decreased overall in patients diagnosed with depression, whereas miRNA expression is generally increased in those with schizophrenia, suggesting that small RNAs may have a role in these diseases. Much like in AD, it is not yet known if altered expression patterns of miRNAs in schizophrenic patients are responsible for inducing the pathology or are a resulting consequence. The individual miRNAs that are differentially expressed in these patients are predicted to target genes involved in mediating synaptic plasticity [87, 88]. In particular, abnormal expression of miR-132 and miR-137 were positively associated with schizophrenia [88, 89] and previous studies have demonstrated a vital role for miR-132 in promoting dendritic growth, dendritic spine formation, and synaptic integration [88, 90–92]. Studies have also shown that members of the miR-34 family are dysregulated in depression and anxiety disorder [93, 94]. For instance, miR-34c repressed corticotropin-releasing factor receptor type 1 (CRFR1) after acute and chronic stress, resulting in neurons that were less responsive to corticotropin-releasing factor (CRF) [93]. Desensitization and/or hyper-reactivity of the hypothalamo-pituitary-adrenal (HPA) axis, of which CRF is a key neuropeptide, has been proposed as an important contributor to the development of affective disorders, such as depression and anxiety [95]. miR-34 is also an age-regulated miRNA, highlighting a potential molecular mechanism for how the aging process contributes to the manifestation of these disorders [65], however no study to date has directly examined the role of age-regulated miRNAs in the context of mental health disorders. Taken together, these studies suggest that specific miRNAs may be a common underlying factor in the etiology of mental illness and the study of their target genes can reveal the molecular pathways involved.

Hormone regulation of miRNAs

miRNAs are estimated to control the translation of more than a third of all protein-coding genes [96], many of which are implicated in sex-biased diseases. Manifestations of such sex-specific morphological and pathophysiological phenotypes are often rooted in differences in circulating gonadal steroid hormones, such as androgens and estrogens, although chromosomal differences can also be contributing factors [97]. Hormonal regulation of steady-state mature miRNA expression was first reported in *Drosophila melanogaster* [98, 99]. In this species, the hormones ecdysone (Ecd) and juvenile hormone (JH) exert opposing actions on the progression from larval-to-pupal, and pupal-to-adult, stages of development. Sempere and colleagues [98] found that two miRNAs, *lin-4* and *let-7*, mediated the expression of genes (*lin-14*, *lin-28*, and *lin-41*, respectively) required for facilitating the successful transitions between these developmental stages. Moreover, the steady-state expression of *lin-4* and *let-7* were dependent on increased levels of Ecd, which peak just prior to the larval-pupal transition [98]. A follow up study identified 3 additional miRs (mir-100, mir-125, and mir-34) in *D. melanogaster* that were responsive to Ecd and/or JH [99], raising the possibility that hormones might also influence miRNAs in vertebrate and mammalian species.

Ecdysone is a steroid hormone and, in addition to its role in regulating molting, it is the primary sex hormone produced in *D. melanogaster* [100]. The Ecd receptor is a member of the highly conserved superfamily of steroid receptors, which collectively function as ligand-activated transcription factors in both vertebrate and invertebrate animals. In mammals, T and its primary androgenic metabolite, 5α-dihydrotestosterone (DHT), exert their actions through high affinity androgen receptors (AR), while E_2, the estrogenic metabolite of T, exerts its action through estrogen receptors (ER). Classically these receptors directly alter gene transcription by binding to cognate DNA response elements in the nucleus [101] therefore, these classical mechanisms of steroid hormone action are termed “genomic”. Alternatively, non-genomic effectors of hormone:receptor complexes include multiple kinases and phospholipases that can activate intracellular second messenger cascades.

Coincident with reports of Ecd regulating miRNAs in *D. melanogaster*, Calin and colleagues [102] observed decreased expression of miR-15 and miR-16 in cases of chronic lymphocytic leukemia. Their study suggested that miRNAs might play an important role in cancer pathogenesis and prompted several subsequent miRNA expression profiling studies in other types of cancer. Convergence of the evidence for miRNAs putative role in cancer, along with the evidence suggesting hormones could regulate them, made breast cancer an attractive model system to explore whether there was a definitive mechanistic link between hormones and miRNAs. Indeed, our current understanding of hormonal regulation of miRNAs in mammals has been most thoroughly derived from studies using breast cancer cell lines as model systems and microarray technology as endpoint detection strategies. These broad-based screening approaches have yielded an enormous amount of data, yet there have been few in-depth studies investigating the roles of individual miRNAs and their respective biological functions. Unfortunately, even among studies that used the same well-characterized MCF-7 breast cancer cell line as a model, many of these efforts have also yielded contradictory and ambiguous results, possibly due to variations in E_2 treatment paradigms and differing microarray platform technologies [103].

Collectively, early studies demonstrated that miRNA expression profiles could potentially be used as markers to accurately predict the phenotype of a breast cancer tumor [104, 105]. For instance, 29 miRNAs were significantly different (either up or downregulated) between normal and tumorigenic tissue samples [104] and 43 were specifically upregulated in ER-positive compared to ER-negative tumors [105]. Interestingly, miR30s (miR-30a-5p, 30b,

30c, 30d) were significantly downregulated in both ERα- and progesterone receptor- (PR) negative breast cancer tumors [104]. Breast tumor phenotypes that do not express hormone receptors (mainly ERα) typically do not respond as well to conventional therapies and predict a poorer prognosis compared with ER-positive tumors. The significant decrease of mature miRNAs from the miR-30 family that is observed in hormone receptor-negative tumors could indicate that hormones normally regulate these miRNAs, and/or that they target a specific subset of oncogenes that are preferentially expressed in hormone receptor-negative cell types. Importantly, ERβ, which acts in opposition to many ERα-mediated effects in breast cancer tumors, inhibits pri-miRNA synthesis of miR-30a [106], highlighting another mechanism for ERβ-mediated antagonism of ERα signaling pathways. Notably, this was one of the first reports showing a direct effect of ERβ on miRNA regulation in any system.

In contrast to the number of studies describing hormonal regulation of miRNAs in classically steroid-responsive tissues, such as breast [103], ovary [107, 108], uterus [109–112], prostate [113], and testis [108], there are a paucity of studies investigating the effects of hormones on miRNAs in the brain of any species. In the teleost fish, *Hippoglossus hippoglossus* (i.e. Atlantic halibut) miRNA expression in the brain varied according to both age and sex, but few of the observed sexually dimorphic miRNAs were validated using qRT-PCR [114]. Specifically noted was a significant increase in miR-451, and a decrease in miR-9, in 3yr-old females compared with males; however, because the potential mRNA targets are still unknown the biological significance of this sex difference is not readily apparent. Further, Morgan and Bale [115] recently demonstrated that 7 miRNAs were significantly different between male and female rat brains at postnatal day (PND) 0. Remarkably, a single injection of an aromatase inhibitor, which prevents T-to-E_2 conversion, was sufficient to induce a female-like miRNA profile in the PND 0 male brains, suggesting that some miRNAs in the rat brain preferentially target genes that regulate sexual differentiation [115]. One limitation of both of these studies was that the entire brain was used in the assay samples, which perhaps diluted the magnitude of results that might have been observed from any brain-region specific effects. A more targeted approach analyzing 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 [116]. Consistent with those findings, studies from our laboratory demonstrated that specific miRNAs in the hippocampus, paraventricular nucleus, and central amygdala were E_2-responsive, with the largest effects observed in the hippocampus (unpublished data). Importantly, not only were these miRNAs E_2- responsive, but a few were also differentially regulated by E_2 dependent on age. Further analysis using algorithmic target prediction programs (i.e. Targetscan [117, 118], microRNA.org [119], and MicroCosm [120]) revealed that these miRNAs potentially target genes important for mediating learning and memory, the stress response, and maintaining synaptic connections, all of which are physiological processes with well-documented sexually dimorphic phenotypes. Although these miRNAs have not yet been analyzed in males, we can infer that they would have sexually dimorphic expression patterns due to the inherent differences in circulating E_2 levels between males and females.

Sex differences in miRNA expression in the brain would be especially relevant to examine in the context of sexually dimorphic neurological diseases, such as stroke, AD, and schizophrenia. To date, several studies have examined the miRNA expression profiles in post-mortem brains from patients afflicted with AD and/or schizophrenia, but only one differentiated the data by sex [121]. They found that miR-30b, which is estrogen responsive, was expressed at significantly lower levels in female schizophrenic patients compared to males, however a putative target for miR-30b in those patients was not determined [121]. Another study examined sequence variations in mature or precursor miRNAs in 193 male

schizophrenic patients compared with age-matched controls [122]. They hypothesized that schizophrenia might be associated with point mutations in miRNAs leading to altered or ineffective downstream gene targeting. Importantly, they limited their analysis to miRNAs encoded on the X-chromosome, due mainly to the propensity for males to develop schizophrenia and their associated decreased fertility rates. Their results showed that eight variants in miRNA genes, all of which they described as “ultra-rare”, were present in schizophrenic patients, but only one was found in the controls [122]. Overall, this study provided evidence for chromosome-based sex differences in miRNAs that are potentially independent of hormonal influence. A similar study examined the influence of miRNAs on the posttranscriptional regulation of X-linked inhibitor of apoptosis (XIAP), a caspase inhibitor that is thought to contribute to the sex differences observed following cerebral ischemia [123]. Importantly, the baseline and stroke-induced sex differences in XIAP were independent of ovarian steroid hormones, suggesting the observed sex effects were due solely to differences in chromosomal contribution. Siegel and colleagues verified that miR-23a bound directly to the 3′UTR of XIAP and miR-23a knock-down increased XIAP mRNA expression levels in HeLa cells [123]. Moreover, miR-23a sex differences in the brain were absent at baseline, yet following cerebral ischemia there was a significant increase in miR-23a expression in females [123]. Taken together, these data indicated that the sex-specific miR-23a regulation of XIAP was crucial for the hormone-independent neuroprotective effects seen in the female ischemic brain.

Data from breast cancer model systems laid a valuable foundation for understanding how the hormonal control of miRNA activity acts as an underlying mechanism to regulate mammalian gene expression patterns in normal physiological processes. Similar to the lack of studies investigating the hormonal regulation of miRNAs in the brain, there has, to our knowledge, only been one report describing a miRNA sex differences in the heart [124]. Connexins are critical proteins that contribute to the formation of gap junctions in the heart, facilitating cell-cell communication pathways. Abnormal expression of connexin 43, a contributing factor to sudden cardiac death, may be important in women, for which cardiac arrhythmia is more common compared to men. In one study, female adult rat ventricular myocytes had greater Cx43 mRNA and protein expression at baseline compared with male rats, suggesting the potential for increased gap junction formation and/or function [124]. Further, female ventricular myocytes had a greater increase in total, as well as phosphorylated, Cx43 protein expression compared with males following stimulation with phenylephrine (PE, an α-adrenergic receptor agonist). These results provided a basis for the sex differences observed in cardiac arrhythmias and raised the possibility that miRNAs could target Cx43 in a sexually dimorphic manner. Indeed, the authors revealed that miR-1, a muscle-specific miRNA previously reported as having a role in intensifying arrhythmic phenotypes [125], was differentially expressed in males and females after PE treatment [124]. Apart from this single study, no other work to date has been done to identify sex-specific miRNA regulation of cardiac function.

Conclusions

The field of miRNAs is still very young, just over a decade since their discovery, and the amount of knowledge we have gained in that short period of time has expanded exponentially. A recent PubMed search using the term “microRNA” revealed nearly 20,000 published reports, with the majority of those occurring in just the last few years (Fig. 2). Conversely, adding the terms “hormones” and “brain” yielded a mere 45 publications, most of which do not describe any empirical testing of how hormones affect miRNAs in the brain. Generally it is assumed that the fundamental cellular processes underlying all posttranscriptional and posttranslational events are equivalent for both sexes. With regard to general miRNA processing, there have not been any studies designed to determine whether

the RISC complex is assembled and functions the same way in both sexes, whether there are sex differences in polyribosome occupancy of miRNAs, whether there are temporal differences in miRNA turnover between sexes, or potential sexual dimorphism in miRNA biosynthetic processing. The lack of such studies represents a significant gap in our knowledge base and underscores an important direction for future efforts. Imperative to this is the need for direct hormonal manipulations in animal models in order to dissect the relative contributions of individual hormones to miRNA-mediated physiological processes. In this review we cite studies detailing the contribution of miRNAs to neuronal development, cellular maintenance, and the etiology neurological diseases. Importantly, sex steroid hormones play an essential role in maintaining a fine balance between homeostatic and pathological outcomes for all of these processes. From sexual differentiation in the developing brain to the presentation of sex disparities in mental health and AD, it is clear that both hormones and miRNAs are central regulators of these processes, and the overlap between the regulatory processes governing each is vastly understudied.

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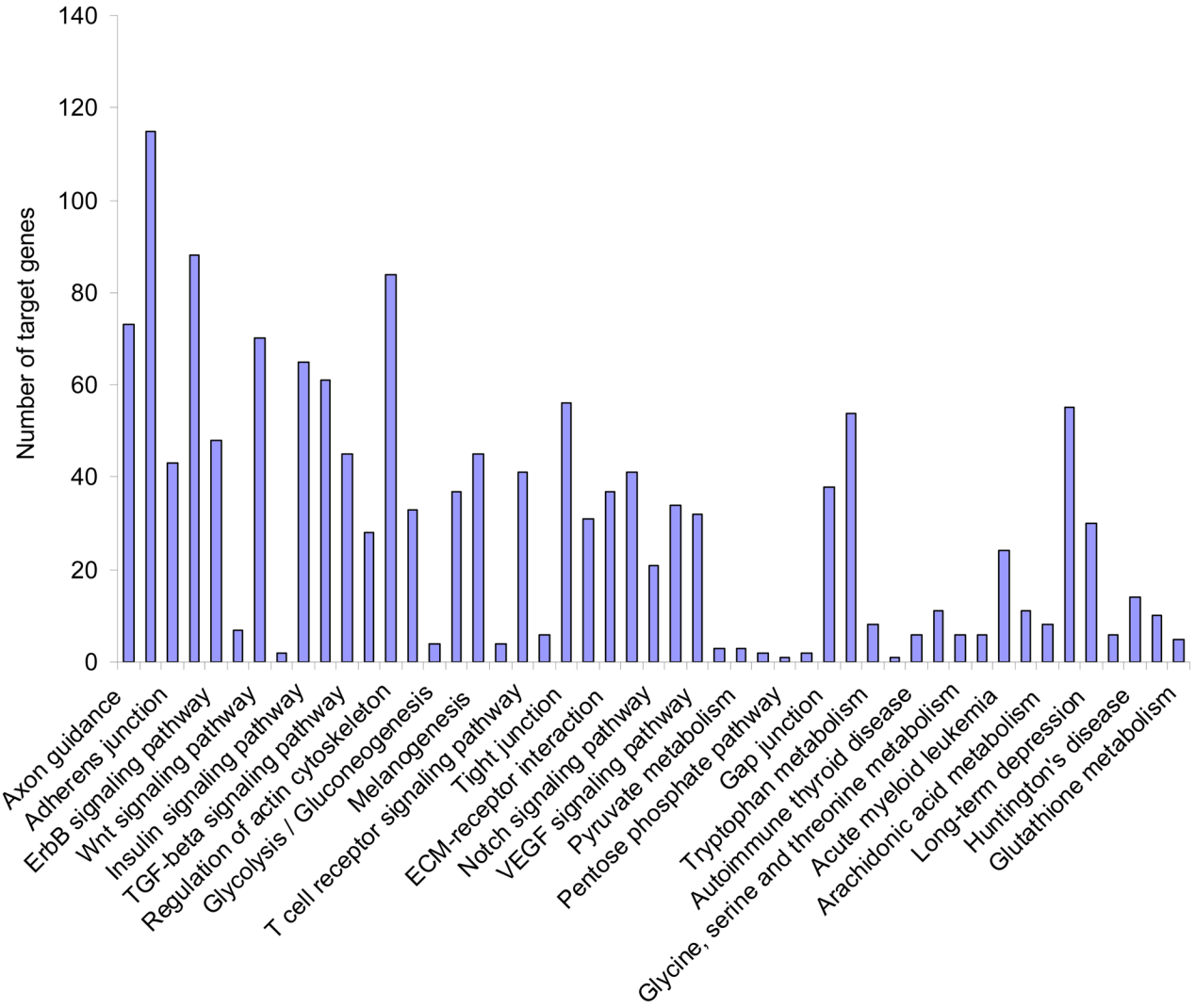

Fig. 1. Cellular pathway analysis of predicted miRNA target genes

Putative miRNA target gene cellular pathways altered by age were generated using the software program DIANA-miRpath [67]. Mature miRNA expression levels were analyzed from the hippocampus of female Fisher 344 rats at 3 and 18 mo. of age. All animals were ovariectomized 7 days prior to tissue collection and total RNA isolation. miRNA expression levels were analyzed using a rat microRNA microarray platform (LC Biosciences, Sanger 18.0 miRbase probes). Loyola University Institutional Animal Care and Use Committee approved all animal experiments.

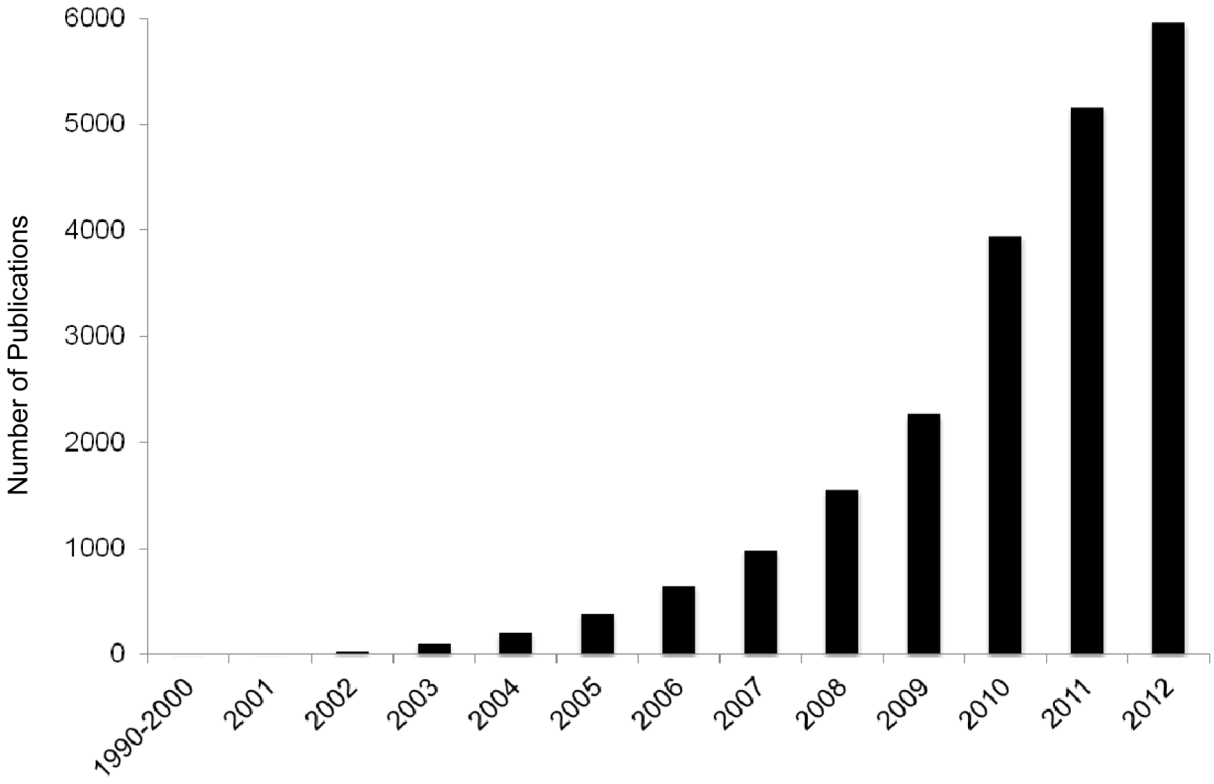

Fig. 2. Number of miRNA publications listed in PUBMED

The number of total publication listed in PUBMED database according to year of publication. Search term used was “microRNA”. Total publications include all types of articles.

Table 1

Nomenclature guidelines for miRNAs. Reference [44] and www.mirbase.org

ANNOTATION	CRITERIA
First 3 lower case letters	Denotes species (i.e. hsa = human, rno = rat, dme = drosophila)
mir/miR	Lower case “r” indicates pri- or pre- forms. Upper case “R” indicates mature sequence
Number	Number is assigned based on date of sequence validation (i.e. lower numbers were discovered first)
Lower case letter after number	Denotes closely related sequences (i.e. miR-20a, miR-20b)
-3p/-5p	Indicates the arm of the precursor hairpin from which the mature sequence was derived.

Note – the ‘minor’ miRNA product (usually transcribed from the -3p end and expressed at lower concentrations in the cell) was previously designated with an asterisk (*). This annotation will no longer be recognized beginning with miRBase17 and will be replaced with the -3p/-5p annotation.

Table 2

miRNAs signifcantly altered by age alone in the rat female hippocampus

Female Fisher 344 rats at 3 and 18 mo. old were ovariectomized. Brains were removed 7 days post-ovariectomy, hippocampus microdissected, total RNA isolated, and processed for mature miRNA expression using a rat microRNA microarray platform (Sanger 18.0 miRBase probes, LC Sciences).

miRNA	p-value
miR-218a*	1.95E-03
miR-495*	3.23E-03
miR-26a*	3.26E-03
miR-423-3p	2.08E-02
miR-382	2.12E-02
miR-376b-3p	2.88E-02
miR-379	2.95E-02
let-7d-3p	3.03E-02
miR-125b-5p*	3.46E-02
miR-137	3.97E-02
miR-539	4.15E-02
miR-7a*	5.92E-02
miR-21	5.82E-03
miR-219-2-3p	7.52E-03
miR-150	1.87E-02
miR-329	2.56E-02
miR-181b*	4.04E-02
miR-29a*	4.28E-02
miR-323	5.08E-02
miR-34c	6.25E-02
miR-132	6.92E-02

An * indicates miRNAs that were also significantly altered by E_2 treatment.