Regulation of Micrornas by 17β-Estradiol in the Aging Female Brain

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LOYOLA UNIVERSITY CHICAGO

REGULATION OF MICRORNAS BY 17β-ESTRADIOL
IN THE AGING FEMALE BRAIN

A DISSERTATION SUMBITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY
YATHINDAR S. RAO
CHICAGO, IL
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CHAPTER I

STATEMENT OF THE PROBLEM

Advancements in medicine and better lifestyles have increased the human life expectancy in the past century[1]. Women in particular are living longer than men, about 5 years more (81 vs 76 years respectively in the United States). The shift in life expectancy has resulted in women living a third of their lives with very low levels of circulating estrogens due to the onset of menopause remaining constant over the same time period. Longer lifespans have also resulted in an increase in age related disorders, especially cognitive disorders such as Alzheimer’s, dementia, and mood disorders. Disorders of the brain present an enormous personal and financial cost to individuals and their families.

Estradiol (17β-estradiol, E2) is the major circulated sex steroid hormone in women, and it has been shown to have many beneficial physiological effects especially in the brain. It is suggested that E2 is the reason that women have significantly decreased risks for acquiring cognitive diseases. However the beneficial effect of E2 is lost after menopause, a period of time in a women’s life when their circulating E2 levels decline.
The declining levels of E2 are correlated with an increased risk for cardiovascular disease, osteoporosis, and cognitive decline.

Hormone Therapy (HT) is used to increase the E2 levels in women who are going through the menopausal transition to alleviate the negative symptoms associated with menopause and decrease the risk of chronic diseases. Clinical and basic science research had previously shown that HT provided protection from cognitive decline and reduced the risk of dementia [2,3,4]. However the evidence was far from concrete and therefore the Women’s Health Initiative (WHI) clinical trial was designed to study the safety and effectiveness of HT. The results of the WHI demonstrated that HT had no beneficial effects for cognitive decline and, in some cases, increased the risk of dementia in post-menopausal women. The findings of the WHI convinced many physicians to stop prescribing HT to their patients and caused a lot concern among patients. However, further analysis of WHI data showed that when HT was given to women who were just starting menopause, they experienced beneficial effects including a decreased risk for cognitive decline and a reduced risk of dementia. The results suggested that there was a “critical window” for the treatment of menopausal women, past which HT would not be effective.

Hormone therapy is still a controversial treatment and is currently not widely used. However, the developments of alternative therapies or recommendations for the use of HT are notably lacking. The insufficient progress is the result of a paucity of knowledge of E2 molecular actions in the brain and how they are altered with aging.
Estradiol is known to regulate the transcription of genes through its nuclear receptors the estrogen receptors. However the new millennium brought forth a new mechanism for the regulation of gene expression: a group of small regulatory non-coding RNAs called microRNAs (miRNAs). miRNAs regulate a myriad of cellular processes by repressing the translation of protein-coding genes. Recent studies have demonstrated that miRNAs are critical regulators of brain development and function. Just like any gene, the expressions of miRNAs are modulated by many different extracellular signals, including E2. However the vast majority of studies of E2 regulation of miRNAs have only been performed using cancer tumors or cell lines as a model system, there has been no research to date addressing how aging might alter that regulation.

Hypothesis

In the present study, I hypothesized that estrogen, specifically 17β-estradiol, differentially regulates mature miRNA expression in the brain with advanced aging resulting in decreased expression levels target genes. To test this hypothesis, I developed three specific aims:

Specific Aims

1. Define the factors responsible for the critical window of time for altered E2 regulation of miRNA expression in the brain. The "Timing Hypothesis" suggests that E2 replacement therapy has positive effects on neurological function, but only within a restricted period of time. We hypothesize that this is partially due to the age dependent differential effects of E2-mediated miRNA expression patterns we observed in female
rats. This aim will determine whether aging or increased lengths of E2 deprivation are responsible for the alterations in miRNA expression. Chapters III and IV will explore this aim.

2. Elucidate the precise ER subtype mediating E2-mediated miRNA expression. The two ER subtypes have different regional distributions and functions in the brain. For example, ERβ has been shown to be a positive regulator of mood and antagonize the actions of ERα. This aim will determine the precise ER mediating the effects of E2 on miRNAs and determine the mechanism of E2 on miRNA expression. Chapters IV and VI will explore this aim.

3. Identify the gene targets of E2 regulated miRNAs in the brain. Ultimately, the functional significance of these changes in miRNA expression is dependent on the specific mRNA targets of the miRNA. This aim will use bioinformatic analysis to identify the target genes for the miRNAs regulated by E2. Chapter III will explore this aim.

Impact

Knowledge of the molecular actions of estrogen in the brain is required to develop novel therapies for post-menopausal women and provide a foundation for understanding the clinical results of hormone therapy. The results of this dissertation provide evidence of the direct regulation of miRNAs by E2 in the brain and identify a set of miRNAs that are regulated in an age-dependent manner. These data suggest that miRNAs could be a
mechanistic explanation for the observed altered efficacy of HT in post-menopausal women
CHAPTER II

REVIEW OF THE LITERATURE

Menopause

Menopause is the termination of the menstrual cycle and thereby marks the end of the reproductive years for a woman. The average age of menopause is about 51 years of age and is preceded by the loss of ovarian follicles which results in decreased production of 17β-estradiol (estradiol, E2), the major circulating estrogen in women. The increase in life expectancy has resulted in women living more than half their lives without circulating E2, thereby increasing their risk of developing these chronic cognitive disorders. The loss of circulating E2 is considered to be a major contributing factor for increased risk of chronic diseases such as osteoporosis, cardiovascular diseases, and cognitive disorders in post-menopausal women.

Estradiol is a known regulator of many neurobiological processes including memory and mood. Post-menopausal women are at increased risk for developing mild cognitive impairment, dementia, and Alzheimer’s disease (AD) [4], all of which affect learning and memory processes. Additionally, post-menopausal women are at increased risk for
developing mood disorders such major depressive disorder (MDD) [5,6,7], and women with MDD have also been shown to have low circulating levels of E2[8]. Conversely, women with high circulating levels of E2 have been shown to have a blunted activation of brain regions associated with the stress response when given a stress challenge, which would result in a decreased risk for acquiring a mood disorder [9].

Hormone Therapy

The treatment of post-menopausal women to prevent these chronic cognitive disorders is through use of hormone therapy (HT), the goal of which is to increase the circulating E2 levels. The HT used in the United States is a conjugated equine estrogen (CEE) sold under the brand name Premarin, which is a 50:50 combination of estrone (another type of estrogen) and E2. CEE is also given in conjunction with synthetic progesterone, medroxyprogesterone acetate (MPA), to women who still have an intact uterus (i.e. have not had prior hysterectomy) to prevent endometrial cancer.

Small clinical trials initially supported the idea of increased E2 levels providing beneficial outcomes in post-menopausal women. For instance, the risk of dementia was decreased with HT and women reported less depressive symptoms while receiving HT [10,11]. Basic science studies using animal models also supported the use of HT. Estradiol treatment has been shown to be neuro-protective in animals following an experimentally-induced stroke [12]. Further, removal of endogenous circulating E2 by
ovariectomy in animal models resulted in decreased performance on learning and memory tasks, and the deficits in performance were rescued by E2 treatment [13]. Finally, depressive behaviors were decreased in rats given E2 treatment [14]. While there was mounting evidence for the benefits of HT, there were also studies that showed no beneficial effect of HT in postmenopausal women [15]. In addition, there was not much known about the possible health risks associated with women taking HT. Therefore a larger study was needed to settle the question of the effectiveness and safety of HT.

The Women’s Health Initiative (WHI)

The Women’s Health Initiative (WHI) was the first ever large scale clinical trial to test the safety and effectiveness of HT for the purpose of preventing chronic disease. In the U.S. about 40% of women were using HT before WHI findings were published publicly.

The trial enrolled 27,347 post-menopausal women, with an age range between 50-79 years old, between 1993 and 1998. Women that had not undergone prior hysterectomy (i.e. uterus intact; N =16,608) were given oral CEE (0.625 mg/d) with MPA (2.5 mg/d), while another group of 10,739 women with a prior hysterectomy were given CEE alone [16]. The women were followed throughout their treatment and assessed periodically.

The results shocked the clinical and basic science communities. Contrary to previous data the WHI studies revealed that HT was in fact detrimental to postmenopausal women’s health [16]. A sub-study of the WHI, the Women’s Health Initiative Memory Study (WHIMS), showed that HT actually increased the risk for mild cognitive impairment (MCI) and dementia [5,6,7]. The WHIMS study also showed a significant association
between MCI, dementia, and depressive symptoms [5,7]. Overall the WHI studies concluded that HT was not appropriate treatment for chronic cognitive disorders in post-menopausal women. The results caused the physicians across the nation to reject hormone therapy.

The Timing Hypothesis

The overall conclusion of the WHI data seemed to indicate that HT was not an effective treatment. However, detailed analysis of the data revealed that there was a time gap in the effectiveness of HT. Women who received HT just as they were starting the progression of menopause showed a decreased risk for AD, however women who initiated HT 10 years post-menopause had an increased risk for AD [4]. Other studies have also demonstrated that the early administration of HT reduced the risk of developing MCI [2]. Collectively the findings indicated that there is a “critical window” for HT to be effective. As of this writing there are two clinical trials, ELITE (Early versus Late Intervention Trail with Estradiol) and KEEPS (Kronos Early Estrogen Prevention Study), currently in progress, which are aimed at specifically testing the “Timing Hypothesis”. Importantly both of these studies will also test the efficacy of synthetic bioidentical human E2 compared to CEE [17]. One of main criticisms of the WHI study was the use of CEE because it was not a chemically pure E2, which might have contributed to the lack of effectiveness.

The Timing Hypothesis raises the possibility that there is an alteration in the molecular mechanisms that mediate HT effects on cognition during the period when a woman has
low circulating E2 levels. In animal models there has been some evidence for a “critical window” for estrogen effects on memory [18,19]. However, currently there is no molecular mechanism described to account for the “Timing Hypothesis”. Estradiol primarily regulates the transcription of genes through the ligand activated transcription factor the estrogen receptor. There are two isoforms of the estrogen receptor, ERα and ERβ, both of which have distinct expression patterns in the brain. Aging results in the altered expression of estradiol responsive genes in brain, however it is not known what is causing the change. Both of these receptors have been shown to be decreased in expression with aging in animal models, however it is brain region dependent and not consistent. In the early 2000’s a group of non-coding RNAs called microRNAs (miRNAs) came to prominence. These small RNAs were shown to have a powerful regulatory role in the biological processes by repressing gene expression at the post-transcriptional level. miRNAs were quickly shown to be regulated by estradiol in cancers, however no published data had explored if estradiol could regulate miRNA expression in the brain.

MicroRNAs

Documented evidence through most of the twentieth century showed that RNA played a passive role in the flow of genetic information. However, in the 1980s evidence emerged that RNAs consisting of fewer than 100 nucleotides could regulate the translation of genes in E. coli [20]. This was followed by work from Victor Ambros and David Ruvkun demonstrating that small RNAs were responsible for regulating key developmental steps
in C. elegans [21,22]. However, no mechanism had been ascribed to these small RNAs, until Craig Mello and Andrew Fire demonstrated that short RNA duplexes could induce the degradation of a complementary messenger (m)RNA at the post-transcriptional level, a mechanism that was soon named RNA interference (RNAi) [23,24,25].

Mello and Fire’s seminal manuscript was soon followed by the discovery of endogenous small RNAs with RNAi capabilities; these small RNAs were named microRNAs (miRNAs) [26,27,28,29]. miRNAs had been described in lower organisms such as C. elegans and drosophila, yet it was immediately noticed that these small RNA sequences were evolutionary conserved with other higher organisms such as humans [27,28,29]. It wasn’t long before miRNAs were being profiled with deep sequencing experiments in a variety of mammals including rodents, non-human primates, and humans. As of this writing there are 2588 and 765 identified miRNA sequences in human and rat genomes, respectively (miRBase 21) [30].

miRNAs are encoded from either intergenic (between protein coding sequences) or intragenic (lying within the intron of protein coding genes) regions of the genome [31,32,33,34,35,36,37]. The majority of miRNAs are dependent on RNA polymerase II for their transcription, however there are about third of miRNAs that may be RNA polymerase III dependent [35,36,37]. RNA pol III dependent miRNAs lie within ALU repeat elements in the DNA [36]. Genome-wide association studies have shown that intergenic miRNAs have similar core promoter elements as protein coding genes [35,38,39,40,41]. The core promoters of these small RNAs lie within ~500bp of the
miRNA sequence and contain TATA box motifs along with CpG islands [35,41]. Transcription factor binding motifs are also abundant amongst the majority of intergenic miRNAs [40]. Intragenic or intronic (also known as mirtrons) miRNAs make up a small percentage of miRNA sequences in mammals, but are highly abundant in lower organisms [35,38,42]. Mirtrons were thought to be dependent on the promoter of the gene they lie within, however there is recent evidence that they have their own promoters from which they can be independently transcribed [38,42]. Taken together the experimental evidence has demonstrated that miRNAs are under the same complex regulatory mechanisms that control protein-coding genes. Additionally, miRNAs are also subjected to extensive posttranscriptional enzymatic processing before they become a function unit in the cell.

MicroRNA Biogenesis

The transcription of a miRNA gene leads to the production of a long RNA transcript termed a primary miRNA (pri-miRNA; Fig. 1) [32,43,44]. This pri-miRNA transcript contains hairpin secondary structures, which is where the mature miRNA resides. The hairpin structure is cleaved from the pri-miRNA, the freed hairpin RNA is then called a precursor miRNA (pre-miRNA). The cleavage event that converts the pri-miRNA to the pre-miRNA is catalyzed by the RNase III enzyme drosha [45]. Importantly, RNase III enzymes can only cleave double stranded RNA molecules. Drosha is accompanied by the RNA binding protein DGCR8 (di-George syndrome critical region 8), and together they form the microprocessor complex [46,47]. Drosha is capable of cleaving the pri-miRNA
to pre-miRNA without DGCR8, or other accessory proteins; however the efficiency of
the reaction is very low [46]. DGCR8 binds to the hairpin RNA and acts as a “molecular
ruler” for drosha, so that the RNase III enzyme can cleave at the correct 5’ and 3’ ends of
the hairpin [46]. The cleavage event results in the 3’ end that is two bases longer than the
5’ end.
Figure 1. MicroRNA biogenesis pathway.

There is some evidence that cleavage of the pri-miRNA to pre-miRNA is a co-
transcriptional event [32,44]. For instance, Drosha and DGCR8 have been co-
immunoprecipitated with RNA pol II, demonstrating co-transcriptional occupancy, and 
transcriptional inhibition using actinomycin D halts microprocessor activity [32].

The resulting pre-miRNA is then exported out of the nucleus and into the cytoplasm via 
the double-stranded RNA binding protein, exportin-5 [48,49]. Once in the cytoplasm, the 
pre-miRNA is then cleaved by another RNase III enzyme, dicer. Dicer cleaves the hairpin 
pre-miRNA to form a RNA duplex [50,51]. Dicer forms a herterodimeric complex with 
another RNA binding protein, Tar RNA binding protein (TRBP), which regulates the 
activity of dicer [52,53,54]. Unlike drosha, dicer can efficiently cleave RNAs without its 
binding partners. Notably, mirtrons are the exception to this paradigm, in that they do not 
require the microprocessor complex 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 [33].

Once the miRNA duplex has been formed, the leading strand (also called the 
Guide) is selected for use in targeting mRNAs, while the lagging strand (LAO called the 
Passenger) is typically degraded [55]. However, this is not always the case. For example, 
the lagging strand has been shown to repress mRNAs as well, which suggests that a 
miRNA duplex could have dual roles with each strand repressing distinct mRNA pools 
[55,56,57,58]. The mature 22 nucleotide miRNA is then loaded on to an agronaute 
protein for mRNA targeting. The argonaute proteins form the core of the RNA-induced-
silencing complex (RISC), which constitutes a multimetric protein complex responsible for binding to mRNA and repressing mRNA translation [59,60,61]. There are multiple argonaute proteins that associate with miRNAs. In humans, there are four currently identified argonaute proteins (AGO1, AGO2, AGO3, and AGO4), with AGO2 being the only one that can cleave mRNAs [62]. AGO2 also been shown to be important for the processing of the pre-miRNA to the RNA duplex by associating with dicer [52]. However, there has been a description of an RNase III independent biogenesis of miRNAs, where pre-miRNA cleavage is accomplished by the actions of AGO2 [63].

**MicroRNA Regulation of Gene Expression**

miRNAs associated with the RISC complex bind to a complementary sequence on the 3’ untranslated region (UTR) of a mRNA to repress the translation and/or degrade the mRNA transcript [64]. 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 [64]. Unlike in plants, animal miRNAs do not share perfect complimentary binding with their target mRNA sequences. Thus, this loose base pairing makes miRNAs highly promiscuous in their ability to regulate multiple target mRNAs. Indeed, experimental evidence has shown that overexpression of a single miRNA can result in the repression of hundreds of genes [61,64]. Based on sequence complementarily, miRNAs could potentially bind to sites other than the 3’UTR of the target mRNA. Specifically, miRNA binding to the coding region of an mRNA transcript has also been shown to repress
translation [70]. These data suggest that translational repression and degradation can be predicted based on the location of miRNA binding to the mRNA transcript.

Translational repression has been shown to precede mRNA degradation by recruiting eIF6 to inhibit the 80s ribosomal subunit assembly, thereby preventing 43s ribosomal scanning of the mRNA transcript [65,66,67,68]. Once translation has been stalled, the RISC complex recruits the Carbon Catabolite Repressor protein 4-Negative On TATA (CCR4-NOT) de-adenylation complex to remove the poly-A tail of the mRNA, which signals exoribonulcease-mediated degradation [69]. The effect of miRNAs on protein expression has been shown to be modest, which is likely due to the many other regulatory factors that can impact a particular protein [70,71,72]. Therefore, it is hypothesized that the main role for miRNAs is to “fine tune” protein expression; a subtle change that may be necessary to maintain homeostasis in the face of changing cellular conditions [71]. 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 [70]. 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 [70].

It is also important to note that the physiological effects of miRNA target gene repression depend on tissue type. As each type of tissue has its own unique gene expression profile,
they also have their own specific set of miRNAs to regulate the post-transcriptional fate of those genes.

MicroRNAs and the Brain

The discovery of miRNAs led quickly to many high-throughput studies examining their unique profiles in multiple species and tissue types. Results from these early profiling studies recognized that miRNAs, just like mRNAs, can be tissue specific giving rise to the idea these specific miRNAs might play an important role for development or function of those specific tissues [73]. The initial studies identified miRNAs that were enriched in the brain, though they did not differentiate between brain regions or cell types. One example is miR-124, which was identified as a brain-specific miRNA since it was highly expressed in the brain and not in other tissues [73,74,75,76,77]. Subsequent profiling studies began to dissect the brain into discrete anatomical regions, and the results showed that miRNAs are indeed expressed in region-specific areas within the brain [78,79,80,81,82].

MicroRNAs and Brain Development

MicroRNAs were first discovered as regulators of embryonic development and further shown as necessary factors of tissue specific development [21,22,83]. One of the first studies generated a transgenic dicer-null zebrafish model [84]. These animals had failed brain development, however axis formation was not disturbed in the embryo [84]. Importantly, the authors were able to rescue normal phenotypic brain development by introducing miR-430, providing strong evidence that miRNAs are required for normal
brain maturation [84]. Mice lacking either dicer or DGCR8 (proteins critical for miRNA biogenesis) were also generated and both knockout mice resulted in an embryonic lethal phenotype [85]. However, these mice also displayed several abnormal brain morphologies: a deformed hippocampus, reduced size in the cortex, and increased neuronal cell death [85].

Profiling studies examining miRNA expression across various developmental time points have all demonstrated a shift in miRNA expression as the brain develops in multiple species including zebrafish, mouse, non-human primates, and humans [86,87,88,89,90]. The alterations in miRNA expression coincide with mRNA and protein expression profiles as well, providing further evidence for a miRNA regulatory role in brain development [87].

**Neuron Specific MicroRNAs**

Profiling studies also identified specific miRNAs that are markers of developing neurons. For instance, miR-124 was shown to be a marker of mature neurons and miR-9 a marker of differentiating neurons [88]. It is important to note that miR-124 was also shown to decrease the expression of non-neuronal genes [91,92]. Analysis of miRNA expression in primary neuronal cell cultures and use of fluorescent in-situ hybridization (FISH) demonstrated a group of miRNAs that are specific to neurons: miR-124, miR-9, miR-128, miR-29, let-7, and miR-26 [93,94,95]. 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 [95,96]. While miRNAs were found to be equally distributed between the soma and dendrites, there were some miRNAs that were preferentially localized to the dendrites [95]. In support of these studies, argonaute proteins were also found to be 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 found to be localized to the dendrites, and pre-miRNAs were found to be in the synaptic fractions [96,97]. Together, these results provide evidence that the pre-miRNA structures are actively trafficked to the dendrites where they are further processed to their mature forms through dicer cleavage.

Neurons have the specialized capability of depolarizing their membrane and generating an electrical action potential. Neuronal communication occurs when the action potential reaches the synapse of a neuron to cause the release of neurotransmitters to depolarize the membrane of the adjacent neuron. The proteins involved in neurotransmission are highly dynamic, which suggests that miRNAs may play a role in their regulation. One of the first studies to demonstrate that miRNAs maybe be involved in neurotransmission showed that miRNA ribonucleic protein complexes (miRNP) and processing bodies (P-bodies) re-localized to distant sites in the dendrites of neurons during neuronal activation [98,99,100]. These results were followed by observations that miRNA expression could be regulated by light in retinal neurons. Using tetrodotoxin (TTX) to block sodium channels, the authors demonstrated that miRNAs degrade much faster in retinal neurons; an observation which held for primary neuronal cultures isolated from the hippocampus
and cortex [101]. Another study demonstrated that depolarization of neurons increased miRNA expression of specific miRNAs in electroshocked mice [102]. Together these studies demonstrate that miRNAs in brain are sensitive to perturbations in the cellular environment and are likely involved in regulation of neuronal communication.

MicroRNAs and Synaptic Plasticity

Most early studies focused on the role of miRNAs during embryonic development, therefore the role of miRNAs in adult brain function was not clear. One of the first studies to explore the role of miRNAs in the adult brain used an inducible dicer knockout transgenic mouse model. Konopka et al. demonstrated that loss of dicer expression in the brain of adult mice resulted in increased learning and memory scores on the morris water maze [103]. The study also showed increased expression of BDNF (brain-derived neurotrophic factor) and MMP-9 (matrix metallopeptidase 9), critical regulators of synaptic plasticity, as a result of miRNA loss [103].

The hippocampus is a region of the brain responsible for learning and memory, and has been the focus for the study of the regulation of synaptic plasticity. Two miRNAs, miR-132 and miR-134, are highly expressed in the hippocampus [78]. miR-132 has been shown to be regulated by BDNF via CREB activation [104]. Overexpression of miR-132 increases the likelihood of synaptic transmission in mouse hippocampal neurons while the knockout of miR-132 results in decreased dendritic arborization [104,105]. Together these studies show that miR-132 is a key regulator of synaptic plasticity. In addition, miR-134 is localized to the synapse in rat hippocampal neurons where it negatively
regulates the size of dendritic spines through the inhibition of a protein kinase, Limk1 [106]. MiR-134 is regulated by the protein deacetylase, SIRT1. SIRT1 knock down results in increased miR-134 expression, decreased CREB and BDNF, and decreased synaptic plasticity [107]. These data demonstrate the power of miRNAs in regulating neuronal communication and brain physiology.

MicroRNAs and Brain Aging

Aging results in the dysregulation of normal processes resulting in a lower threshold for pathogenic process to occur. In particular, gene expression changes are significantly altered in advanced age, suggesting that there is dysregulation of gene expression. The first studies to look at miRNA expression during aging were done using C. elegans as a model [108,109]. Profiling miRNA expression during the span of the nematode’s life showed that nearly half of the 114 miRNAs analyzed were altered during the adult phase, suggesting a powerful regulatory role for miRNAs in aging [108]. Another study took the observation one step further and questioned whether miRNAs can directly regulate lifespan. The authors used C. elegans that were daf-2 (Insulin-like growth factor-1) mutants, which result in an extended lifespan, and identified which miRNAs were altered in expression as compared to wildtype nematodes [110]. Mutation of specific miRNAs identified in their screen resulted in alteration in the lifespan of the nematodes, suggesting that miRNAs can either positively or negatively regulate aging [110].

Aging studies were further extended to analyze miRNA expression in the aged brain. Expression profiles of miRNAs in the brains young and old mice identified three
miRNAs (let-7, miR-124, and miR-34) that were altered as a function of age [111,112]. A similar study identified miR-34a as a potential marker of brain aging in mice by demonstrating that its expression was increased in the brains of aged mice and also in the blood, suggesting that miRNAs could be used a convenient biomarker of brain aging in humans [113]. miRNA, mRNA, and protein expression were analyzed during embryonic development and aging in human and macaque brains. Somel et al. showed that the changes in miRNA/mRNA/protein that occurred during development were either reversed or extended into aging, suggesting that aging is a form of dysregulated development [114]. In particular, the study identified miR-124, miR-125a, miR-9, miR-181a, and miR-29 as being altered with aging in the brain [114]. Age related changes in miRNA expression could also be identified in the blood of aged humans [115]. Taken together, these studies of gene and miRNA expression in the brains of non-human primates and humans during aging further cemented the universality of these age related changes in miRNA expression, and show that they can be used as potential biomarkers for the pathological conditions associated with the aging brain.

**Estradiol regulation of MicroRNA expression**

Many different extracellular signals regulate miRNA expression and the sex steroid hormone E2 is one of them. Estradiol regulation of miRNAs has been demonstrated in MCF-7 breast cancer cells, where E2 both enhances and represses miRNA expression depending on the specific miRNA [116,117,118,119,120]. Estradiol mediates its effects through two nuclear receptors called estrogen receptor α (ERα) and β (ERβ) [117,121].
Both receptors are able to bind to the promoter regions upstream of a miRNA and induce transcription [117,121]. Importantly, breast cancer cell proliferation is mediated in part by E2-induced miRNA expression, which provides a molecular mechanism explaining the differences between ER negative and ER positive breast cancer phenotypes [122]. Interestingly, E2 regulation of miRNA expression in non-cancer tissues has not been extensively studied. The lack of research is surprising since E2 is an important regulator of many neurobiological processes. It is likely that regulation of miRNA expression is a key regulatory mechanism used by E2 to exert its pleiotropic effects on cognitive processes.

In summary, the data to date strongly suggest that miRNAs as a plausible molecular mechanism mediating E2 effects on brain function. Further, the data suggest that aging could alter those effects on brain function, thereby providing a mechanistic explanation for reduced efficacy of E2 in postmenopausal women. The overall goal of this research project was to determine the effect of aging on E2-mediated regulation of miRNA expression and determine the mechanisms by which E2 regulates miRNA expression in the brain. Chapter 3 describes differences in E2 mediated regulation of miRNAs in young vs. aged brains and demonstrates both age- and brain-region specific effects of E2 on miRNA expression. Chapter 4 directly tests the timing hypothesis using an aged model of with varying lengths of ovarian hormone deprivation as well as defining the points in the miRNA biogenesis pathway regulated by E2 and aging. Chapter 5 directly describes the effect of aging on mature miRNA expression in non-ovariectomized female rats. Finally, chapter 6 attempts to replicate the in-vivo findings in an in-vitro setting. Taken together
the findings point to altered regulation of miRNAs with aging as an underlying factor in the reduced effectiveness of E2 in postmenopausal women.
CHAPTER III

MICRORNAS IN THE AGING FEMALE BRAIN: A PUNITATIVE MECHANISM FOR AGE SPECIFIC ESTROGEN EFFECTS

Introduction

Advanced age is accompanied by global changes in neuronal gene expression. Recent studies have demonstrated that microRNA (miRNA) expression also changes with age, an intriguing correlation suggesting that age-related miRNA fluctuations could be responsible for modulating the overall change in global gene expression [108,109,111,112,114,123]. miRNAs are noncoding regulatory RNAs transcribed from intergenic or intragenic regions of the genome in a RNA polymerase II dependent manner [31,124]. Primary miRNA (pri-miRNA) transcripts are sequentially cleaved by the nuclear enzyme drosha, and the cytoplasmic enzyme dicer, to form the functionally mature single-stranded form of the miRNA [45,50,125]. Complementary binding of the mature miRNA to the 3’ untranslated region (UTR) of a target messenger RNA (mRNA),
and its subsequent association with the RNA-induced silencing complex (RISC), leads to mRNA translational repression and/or degradation [64,126,127]. Current efforts are focused on elucidating potential target genes that are regulated by miRNAs, however less is known about the upstream pathways that regulate miRNA expression, processing, and temporal activity in the brain.

The gonadal steroid hormone 17β-Estradiol (E2) is one factor that has been shown to regulate miRNA expression and biochemical processing in some systems, such as cancer cell models [117,118,121,128,129,130]. Furthermore, E2 has been shown to be neuroprotective by decreasing the recovery time after stroke and improving learning and memory in aged rodents, primates, and humans, [131,132]; however, the beneficial effects observed in older women are largely dependent on the length of time between the onset of menopause and the administration of hormone therapy [4,133]. Consequently, the “timing hypothesis” suggests that the molecular mechanisms regulating E2 action are fundamentally changed during an extended hypo-estrogenic period [134,135,136], with aging being a further layer of complexity that alters these postmenopausal mechanisms.

Numerous studies clearly show that aging has detrimental consequences for the hippocampus, a brain region that is required for learning and memory, as well as for mediating the physiological responses to perceived stressors. Multiple excitatory signaling pathways, growth factors, and steroid hormones govern hippocampal neuronal
functions [137,138]. This brain structure has been extensively studied for its importance in memory formation, however hippocampal neurons also interact broadly with other brain regions to mediate a variety of more complex behaviors [139]. The hippocampus can be functionally divided into ventral and dorsal sub-divisions, which primarily mediate emotion/stress and memory/cognition, respectively [140]. In addition, these two divisions have distinct gene expression profiles, which are thought to delineate their specific functions [141,142]. Similarly, region-specific expression of miRNAs has been observed in the brain, suggesting that they have unique and specific neurological functions [74,75,78,79,80,81,131]. For instance, miRNAs have been shown to play a critical role in neuronal development, survival, and synaptic plasticity [91,103,104,105,106,107,143,144,145]. Consequently, disruption of miRNA activity has been associated with several neuropathological conditions such as Alzheimer’s disease, schizophrenia, and general mood disorders [146,147,148,149,150,151].

In our studies, we hypothesized that E2 differentially regulates miRNA expression in the aged female hippocampus leading to altered gene expression important for neuronal function. To test our hypothesis, we compared the hippocampal miRNA expression profile of young (3 month) and aged (18 month) female rats that underwent surgically induced menopause (i.e., ovariectomy). Following a 7-day period of E2 withdrawal, animals were then administered with E2 or vehicle (control) for 3 days. Global changes in miRNA expression in the ventral hippocampus were evaluated using a rat miRNA microarray. The analysis of global miRNAs confirmed the presence of brain region-specific miRNA composition. More importantly, our results also showed miRNA
changes to be E2 and/or aging-dependent. Furthermore, we identified potential miRNA target genes that are for: cognition/memory (i.e., sirtuin1 (SIRT1), brain-derived neurotrophic factor (BDNF)), stress (i.e., glucocorticoid receptor (GR)), and synaptic function (i.e., gamma-aminobutyric acid (GABA) A receptor 1 (GABRA1)). Collectively, our results showed that the loss of circulating levels of E2 results in a fundamental shift in the composition of miRNA species in the aged brain, which may be one molecular mechanism that explains the “timing hypothesis”.

Results

E2 differentially regulated miRNA expression in the ventral hippocampus of aged female rats.

Our first objective was to determine whether steady state miRNA expression in the ventral hippocampus (vHIPP) was altered by age (3 vs. 18 mo) and/or E2 bioavailability. For this initial scan, we used a microarray platform that analyzed a total of 723 previously identified rat miRNAs of which 421 (58.2%) reached detectable levels. Figure 2A shows all of the miRNAs that reached a high threshold of detection and were significantly changed across all groups. Examination of the relative expression levels of the miRNAs expressed in the vHIPP revealed that the majority (288 miRNAs) are expressed at low (<500 SI) levels (Fig. 2A, Supporting Table 1). We refined our analysis to include only those miRNAs that were expressed at moderate to very high expression levels (133 miRNAs >500 SI). Many of the miRNAs that had high or very high signal intensity in our array, such as miR-9, have previously been shown to be highly expressed
in the brain and regulate important neuronal functions [78]. Interestingly, some passenger strand miRNAs (formerly denoted as miRNA*) were amongst the highest expressed miRNAs, supporting previous studies indicating a functional role for passenger strand miRNAs [56]. Notably, 58 additional miRNAs reached statistical significance, however the signal intensity was low (< 500 SI) and these miRNAs were excluded from further analysis. Of the subset that reached statistical significance and had a high signal intensity, 34 were significantly altered by E2 treatment regardless of age, 21 were significantly altered by age alone, and 9 were altered by E2 dependent upon age (Fig. 3B). These 9 miRNAs were chosen for further validation using qRT-PCR. Ventral hippocampal (vHIPP) samples taken from the right hemisphere (contralateral the side used for the microarray) were used to validate the 9 miRNAs that were differentially regulated by E2 dependent upon age, as identified from the microarray. Of the miRNAs chosen for validation, miR-154 and miR-218 failed to reach statistical significance using qRT-PCR and were not pursued further.

A significant interaction between treatment and age was observed for let-7i and miR-495 (Table 1), but not the other 5 miRNAs tested. However, a significant main effect of treatment was seen in 6 of the 7 miRNAs tested and a main effect of age was observed in 2 of 7 miRNAs (see Table 1). E2 significantly increased (p<0.05, let-7i, miR-7a, miR-9-3p, miR-125a, miR-181a) or decreased (miR-495) steady state expression levels of all of the 7 tested miRNAs in the old animals, whereas only miR-9-3p was increased in the young (Fig. 4A). Notably, the only miRNA that was decreased due to E2 treatment in the vHIPP of either age group was miR-495 (Fig. 4A). Moreover, unique to
the vHIPP there were no significant changes in any of the seven E2-regulated miRNAs tested due to age alone (i.e. vehicle-treated).
Figure 217β-estradiol (E$_2$) differentially regulates miRNA expression in the ventral hippocampus of young and old female rats.

Heat maps depicting significantly regulated (p<0.05) miRNAs in the ventral hippocampus of A) 3 and 18 month old, B) 3 month old, and C) 18 month old female rats. The expression of an individual miRNA in each sample is shown using a color code where red and green represent high and low fold change respectively. Samples O1-O5 and Y1-Y5 represent 18 and 3 month vehicle treated animals respectively. Samples O6-O10 and Y6-Y10 represent 18 and 3 month E$_2$ treated animals respectively.
Figure 3. Summary of expression levels and E₂/Age regulated miRNAs in the ventral hippocampus of female rats.

A) Pie chart depicting the number of miRNAs with very high (>20,000), high (10,000-20,000), moderate (500-10,000), and low (<500) signal in the ventral hippocampus of 3 and 18 month old ovariectomized female rats. B) A Venn diagram depicting the number of miRNAs regulated E₂, age, and E₂/age together by miRNA microarray analysis.

E₂ regulation of miRNA expression is brain region specific.
Similar to protein coding RNA species (i.e. mRNA), some studies have shown that miRNAs are expressed in a brain region-specific manner suggesting they might play an important role in the function of that particular brain region [75,78,81]. To determine whether E2 regulates the same miRNAs from the vHIPP in other brain regions, we examined three additional brain regions that are anatomically and functionally connected to the vHIPP: the dorsal hippocampus (dHIPP), the paraventricular nucleus of the hypothalamus (PVN), and the central amygdala (CeA). Across all brain regions E2 significantly altered the expression levels of the greatest number of miRNAs tested in the vHIPP (2 in young animals, and 7 in old animals, Fig. 4A), whereas only 3-4 miRNAs were significantly changed following E2 treatment in each of the other 3 brain regions (Figs. 4B, 5A,B). Although we observed the greatest number of miRNA changes in the vHIPP, the largest magnitude (>12 fold increase) of E2-induced changes were in the dHIPP and CeA (Figs. 4B, 5B). Taken together, miR-7a was the only miRNA that E2 consistently regulated in all 4 brain regions.

Dorsal Hippocampus. Despite the anatomical proximity of the ventral and dorsal hippocampus, E2 effects in the dHIPP were substantively different from the vHIPP (Fig. 4B). A significant interaction between age and treatment was observed for miR-7a (Supporting Table 2), but not the other 7 miRNAs tested. A significant main effect of treatment was observed for 2 out of 7, and a main effect of age for 5 out of 7 miRNAs tested (Supporting Table 2).
Figure 4. E₂ regulation of miRNA expression in the ventral and dorsal hippocampus.
Real time qPCR validation of miRNAs in the A) ventral hippocampus (vHIPP) and B) dorsal hippocampus (dHIPP) of 3 and 18 month old ovariectomized rats treated with E₂ or vehicle control. Results shown are mean fold change ± SEM as compared young vehicle treated animals. Data were analyzed by two-way ANOVA with age and treatments as factors. Dissimilar symbols indicate a statistically significant difference between groups (p<0.05).
Table 1. 2 way-ANOVA analysis of miRNA expression in the vHIPP

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Main Effect: Treatment</th>
<th>Main Effect: Age</th>
<th>Interaction: Treatment x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7i</td>
<td>Yes ((F(1,15)=38.563, p&lt;0.001))</td>
<td>Yes ((F(1,15)=7.950, p=0.013))</td>
<td>Yes ((F(1,15)=12.258, p=0.003))</td>
</tr>
<tr>
<td>miR-7a</td>
<td>Yes ((F(1,16)=7.528, p=0.014))</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>miR-9</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>miR-9-3p</td>
<td>Yes ((F(1,14)=11.249, p=0.005))</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>miR-125a</td>
<td>Yes ((F(1,16)=14.574, p=0.002))</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>miR-181a</td>
<td>Yes ((F(1,16)=5.109, p=0.038))</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>miR-495</td>
<td>No</td>
<td>Yes ((F(1,16)=6.677, p=0.020))</td>
<td>Yes ((F(1,16)=11.472, p=0.004))</td>
</tr>
</tbody>
</table>
Interestingly, 3 of the tested miRNAs showed a significant age-dependent increase in the absence of E2 (miR-7a, miR-9, miR-181a, p<0.05, Fig. 4B), with miR-7a being increased by more than 12 fold by age alone. Notably, this observed age-related increase was completely abolished by E2 treatment in the old animals (Fig. 4B). Unlike in the vHIPP, miR-9 was significantly decreased by age alone (p<0.05), and E2 treatment did not further potentiate this effect (Fig. 4B). Similarly, miR-181a was significantly increased with age alone (p<0.01) and E2 had no additional effects (Fig. 4B).

Paraventricular Nucleus (PVN). In the PVN, there was an significant interaction between treatment and age for miR-7a only, similar to what we observed in the dHIPP (Supporting Table 3). A significant main effect of treatment was observed for 4 out of 7, and a main effect of age for 4 out of 7 miRNAs tested (Supporting Table 3). Age alone induced significant increases in let-7i and miR-7a, both of which were either potentiated by E2 treatment (i.e. let-7i) or reversed to levels significantly below those observed in the young animals (i.e. miR-7a) (Fig. 5A). Similarly, E2 significantly increased miR-9 in the old animals as compared to the young (Fig. 5A). miR-125a was not altered due to age alone, however treatment with E2 significantly increased miR-125a in the young, but did not increase it by the same magnitude in the old PVN (Fig. 5A).

Central Amygdala (CeA). In the CeA there was a significant interaction between treatment and age in 3 of the 7 miRNAs (Supporting Table 4). Main effects of treatment and age were seen in 1 of 7, and 3 of 7, miRNAs, respectively (Supporting Table 4).
Figure 5. E$_2$ regulation of miRNA expression in the PVN and CeA.
Real time qPCR validation of miRNAs in the A) paraventricular nucleus of the hypothalamus (PVN) and B) central amygdala (CeA) of 3 and 18 month old ovariectomized rats treated with E$_2$ or vehicle control. Results shown are mean fold change ± SEM as compared young vehicle treated animals. Data were analyzed by two-way ANOVA with age and treatments as factors. Dissimilar symbols indicate a statistically significant difference between groups (p<0.05).
Post hoc analysis showed that E2 significantly altered 1 miRNA in the young animals, and 3 miRNAs in the old animals (Fig. 5B). The analysis revealed a significant decrease in miR-7a due to age alone and this “old” phenotype was mirrored in the young animals following E2 treatment (p<0.01, Fig. 5B.) Notably, the CeA is the only brain region where we observed a significant E2-mediated effect (decrease) on miR-7a in the young animals (Fig. 5B). Both miR-9 and miR-9-3p were significantly increased by E2 in the old animals but not in the young (Fig. 5B). Finally, there was a significant age-related increase in miR-495 (p<0.001), which was significantly potentiated following E2 treatment (Fig. 5B).

E2 had no effect on miRNA processing in the ventral hippocampus

To begin to define the molecular mechanisms for E2 regulation of miRNA in the ventral hippocampus, we measured three components of miRNA processing and regulation pathways: drosha, dicer, and argonaute 2 (AGO2) in our young and old animals. Our results showed that E2 treatment had no statistically significant effect on the steady-state expression levels of any of these genes in the ventral hippocampus in either age group (Fig. 6), although there was a strong trend for an E2-mediated increased dicer expression in the young animals.

E2 regulated miRNA predicted target genes important for neuronal function.

In order to understand the physiological impact of these E2 regulated miRNAs, we analyzed potential mRNA targets for each miRNA using four different target prediction programs (DIANA-mirpath, Targetscan, microRNA.org, and MicroCosm)
The predicted gene targets were represented in several cellular pathways including neuronal specific pathways, such as long term potentiation and axon guidance (Fig 7). Putative targets were chosen for testing in our ventral hippocampal tissue samples based on their known role in neuronal function and their algorithm score, as determined by the computer modeling programs (Fig. 8A). Degradation of mRNA transcripts is one mechanism whereby miRNAs decrease the protein expression of their target genes [64,126] and previous studies have demonstrated an inverse correlation of miRNA expression with their target mRNA expression [114]. To determine if there was an inverse correlation between the E2 regulated miRNAs in the ventral hippocampus and their predicted targets, we measured SIRT1, GR, BDNF, and GABRA1 mRNA expression. Consistent with our expected outcomes, SIRT-1 mRNA expression was significantly decreased in the ventral hippocampus of young animals (Fig. 8B) which inversely corresponded to SIRT1 predicted miRNAs, miR-9 (Figs. 4A, Fig. 8B). Moreover, E2 significantly increased GR mRNA levels in the young animals, although miRNA-181a was unaffected by E2 in that same age group, suggesting that multiple miRNAs could target GR. E2 had no effect on GABRA1 or BDNF mRNA expression at either age (Fig. 8B), although there was a strong trend for an E2-mediated increase in BDNF in the young animals.
Figure 6. miRNA processing enzymes are not affected by E$_2$ treatment. Real time qRT-PCR analysis of drosha, dicer, and argonaute 2 in the A) 3 month and B) 18 month old ventral hippocampus. Results shown are mean fold change ± SEM as compared young vehicle treated animals. Data were analyzed by one-way ANOVA with treatment as a factor. No significant differences were noted (p>0.05)
Figure 7. Predicted cellular pathways of potential target genes of identified miRNAs.
DIANA-mirpath was used to predict KEGG cellular pathways impacted by potential target genes of significantly regulated miRNAs in the ventral hippocampus of female rats. Results are depicted as the number of genes targeted by miRNAs (y-axis) in each cellular pathway (x-axis).
Figure 8. Predicted gene targets of identified miRNAs are regulated by E₂.
A) Table of predicted miRNA gene targets determined based on algorithm scores of three computer prediction programs: Targetscan, microRNA.org, and MicroCosm; B) real time qPCR for sirtuin 1 (SIRT1), glucocorticoid receptor (GR), gamma-aminobutyric acid receptor A1 (GABRA1), and brain-derived neurotrophic factor (BDNF) in the 3 month, and C) 18 month old ventral hippocampus. Results shown are mean fold change ± SEM as compared young vehicle treated animals. Data were analyzed by one-way ANOVA with treatment as a factor. * indicates statistically significant difference (p< 0.05).
Summary

Two novel findings emerged from these studies. First, we demonstrated that E2 is a critical regulator of mature miRNA expression levels in the brain and that the magnitude of E2 action differs according to specific brain regions. Second, we showed that even within a specific brain region, the effects of E2 on miRNA levels are temporally distinct. To our knowledge this is the first evidence demonstrating E2 regulation of miRNA expression in the brain and it suggests that this may be an important mechanistic pathway for E2 modulation of neuronal target genes resulting in a “fine tuning” of gene expression. This concept of “fine tuning” has gained critical recognition as an important determinant of cellular function and is especially vital in the brain, as communication between neurons requires rapid modulation of gene expression at the synapses where local translation of proteins is crucial.

Our data underscore the concept that E2 can have completely different actions in an aged system compared to a young, although more time points are needed to ascertain the linearity of these changes. Overall, we observed significant interactions between age and E2 treatment for 5 specific miRNAs: Let-7i, miR-7a, miR-9, miR-9-3p, and miR-495. These results highlight a potential molecular mechanism to explain the fundamental tenet of the timing hypothesis, namely that E2 has differential physiological effects dependent on age. Interestingly, most of the brain regions had unique miRNAs that were significantly affected by both age and treatment with the notable exception of miR-7a, which was altered in 3 of the 4 brain regions analyzed. Putative gene targets for miR-7a include SIRT1, which is critically important for mediating anxiety and memory, among
other things [107,157]. The timing hypothesis also posits that the length of time between menopause and subsequent hormone therapy dictates the nature of E2 action. In our study, we measured a relatively short time frame between surgically-induced menopause and hormone replacement (i.e. 7 days) and the changes we observed might vary greatly under conditions with longer periods of E2 withdrawal. Longer periods of E2 withdrawal can alter multiple factors including estrogen receptor (ER) levels. ERalpha has been shown to physically associate with drosha and participate in the maturational processing of several miRNAs [12]. Therefore, the length of E2 deprivation could have a significant impact on the expression of mature miRNAs leading to differential gene target expression profiles. Another important observation from these data was the revelation that large-scale global changes in miRNA expression levels were not observed, as only a small subset of the total 723 miRNAs were altered by age and hormone treatment. These results suggest that most miRNAs are likely key regulators of fundamental cellular processes which could be detrimental to have under the control of fluctuating steroid hormones.

The primary focus of our study was to determine the effects of E2 on miRNA expression between young and old animals, however the data revealed several miRNAs that were regulated by age alone. Importantly, our data show for the first time that age-dependent miRNA expression varied across brain regions, suggesting that the brain does not age uniformly. For instance, none of the miRNAs that we tested were significantly increased by age alone in the vHIPP. This observation was sharply contrasted by the results from the other 3 brain regions that each had 2 or 3 miRNAs that were regulated by age alone. Perhaps the most striking of these were the results of miR-7a, which had a 12-
fold increase in the old dHIPP compared to that of young animals. Interestingly, this magnitude of effect was not observed for miR-7a in any of the other brain regions, suggesting that miR-7a could have significant biological consequences for dHIPP function in old animals. The majority of studies that have investigated age dependent changes in mRNA and/or miRNA have been analyzed in only one brain region (prefrontal cortex) or in the entire brain as a whole [158,159,160,161,162,163]. While these studies have provided important observations of age-dependent gene expression changes in the brain, the conclusions relating to brain region-specific functions and/or pathologies are limited by the brain samples used.

One of the more intriguing findings from our study was the observation that E2 differentially regulated miR-9 and miR-9-3p. These two functionally mature miRNAs are derived from the same primary miRNA transcript (pri-miRNA), yet typically one strand of the cleaved duplex is preferentially recruited to the RISC complex and becomes the biological effector, while the other strand undergoes degradation. Since the two strands are inverse complements, it is likely that they have vastly discrepant target genes and several studies have demonstrated biologically functional roles for selected miRNA passenger strands [164,165]. In our studies, E2 decreased miR-9 expression in the dorsal hippocampus of 3 month old animals, while simultaneously increasing the expression of miR-9-3p. Conversely, by 18 months of age miR-9-3p was no longer significantly regulated by E2, yet the E2-induced reduction of miR-9 remained the same. The differential regulation of miR-9 and miR-9-3p suggest that a separate cohort of target genes may be affected in the aged ventral hippocampus, which could have a significant
impact on the hippocampal-mediated control of emotion/stress. Since miR-9/9-3p sequences are on the same primary miRNA transcript, it stands to reason that E2 regulates the processing of the pri-miRNA to favor the retention of miR-9-3p sequence over the miR-9 in the young brain. The miR-9-3p strand selection likely occurs after the enzyme dicer has cleaved the precursor miRNA (pre-miRNA) to form the mature miRNA duplex since the mature miRNA duplex still contains both miR-9/9-3p sequences. Therefore, one possibility is that E2 facilitates the retention of miR-9-3p by the RISC complex longer than miR-9. Alternatively, E2 could mediate the active degradation of miR-9, but not miR-9-3p however, the mechanisms by which miRNAs are targeted for degradation are still largely unresolved.

The pleotropic nature of E2 action suggests that the mechanisms underlying those actions are equally diverse. Conventional views dictate that a majority of E2 effects are due to direct transcriptional regulation of target genes. Consequently, the precise molecular mechanisms that mediate E2 actions on miRNA expression levels remain unresolved, especially in the in vivo system. One possibility is that E2 regulates miRNA processing, through modulation of key enzymes such as drosha or dicer, or by altering components of the RISC complex, such as argonaute 2. In our studies, E2 did not causes significant changes in any of these parameters. These observations led us to hypothesize that that E2 might act directly at the promoter level to modulate pri-miRNA transcription. E2 actions are mediated primarily by two high-affinity nuclear receptors, estrogen receptor alpha and beta (ERα, ERβ), and both subtypes are expressed in the ventral hippocampus, although ERβ is expressed at higher levels and in different hippocampal
divisions compared to ERα [166,167,168]. Moreover, the temporal expression of ER subtypes in the brain has been well documented [164,169] and it is exciting to postulate that some of the differential E2-mediated effects on miRNA expression are due to age-related changes in ER subtype expression. Interestingly, previous studies have shown that E2 can act through both estrogen receptor subtypes to regulate miRNA expression in cancer cell models [121,130]. In these cell models, E2 has been shown to not only regulate miRNA transcription, but also to play a role in the processing of the miRNAs via direct ERα interaction with components of the drosha processing complex [130].

The functional relevance of miRNAs is the focus of intensive investigation and, as such, defining the specific targets for these miRNAs is of paramount importance. We identified miRNAs uniquely regulated by E2 and then used computer prediction algorithms to pinpoint putative target genes. Sirtuin 1 (SIRT1), which is a protein deacetylase that first gained recognition for its effects on longevity [170,171,172] was identified as such a target gene. Recent research also showed that SIRT1 to be important for energy balance, memory, anxiety, and neuroprotection in the brain [107,157]. Our data demonstrated an inverse correlation between miR-9 and SIRT1 expression at 3 months, which is consistent with a recent report revealing a complementary region on the SIRT1 3’ UTR with the miR-9 seed sequence [173]. Two other putative miRNAs that target SIRT1 is miR-7a and miR-495. Compared to any miRNA tested our data showed that miR-7a and miR-495 had the largest magnitudes of age-dependent change (i.e., greater than 12 fold increase). Strikingly, E2 treatment completely abolished the age-related effect on miR-7a. This observation is in line with recent evidence in steroid
hormone-regulated cancer models suggest that SIRT1 interacts with the estrogen receptor to regulate transcriptional activity [165]. Overall, our observations raise the possibility that E2 activated estrogen receptors may be another pathway whereby SIRT1 activity is regulated in the brain. Other predicted gene targets in this study, such as GR and GABRA1, did not show a direct correlation between their expression level, and the miRNA expression level. This observation may indicate that the E2-regulated miRNAs induced translational repression, as opposed to degradation of their target mRNA transcripts. The mechanism that leads to miRNA-mediated mRNA degradation or translational repression is not well understood, though it is clear that both processes are equally viable mechanisms of miRNA action on their target genes [12,174,175]. Taken together, our data clearly indicate that E2-regulated miRNA expression in the brain is age-dependent and the predicted targets of these miRNAs have important implications for neuronal function. Further validation of these and other potential targets are necessary, as predicative algorithms are not well developed enough to accurately predict true targets of miRNAs.

While our data show that E2-regulated miRNA expression is altered in the aged brain as opposed to the young brain, it is not certain at this time whether this is dysregulation that would result in a net increase in adverse effects or protective mechanisms. The in vivo paradigm presented here offers a broad picture of the impact E2 has on miRNA expression in the brain and how those effects might be altered by aging. There are inherent limitations using an in vivo paradigm, many of which preclude elucidating the precise molecular pathways of E2 action. Overall our data reveal a novel
temporal and neuroanatomical-dependent regulation of miRNAs by E2, thereby furthering our understanding of the complex mechanisms for E2 in regulating neuronal functions.
CHAPTER IV

PROLONGED ESTROGEN DEPRIVATION ALTERS THE EFFECTS OF 17β-ESTRADIOL ON MATURE MICRORNA EXPRESSION IN THE AGED FEMALE RAT HYPOTHALAMUS

Introduction

The average life expectancy has increased over the past century due to better living conditions and medical advancements [1]. Women in particular are living longer, yet the age of menopause has remained constant at approximately 50 years of age. This results in most women living nearly a third of their lives without high levels of circulating estrogens. Risk factors for many diseases, such as cognitive and mood disorders, increase in women following menopause [5,6,176] and these risk factors have been correlated with the sharp decline in circulating levels of estrogens. Hormone therapy (HT) is often prescribed for women to mitigate many of the adverse symptoms associated with menopause, based primarily on evidence from animal models demonstrating that estrogens are anxiolytic and increase cognitive function [12,13,131,132,177,178]. Data from studies in postmenopausal women, however, are less convincing that HT is beneficial. For instance, results from the large-scale clinical trial (Women’s Health Initiative (WHI)) showed that there was a temporal discrepancy in the beneficial efficacy of HT [3,4,5,6,7,16,133,179,180]. The beneficial effects were largely dependent on the
length of time following menopause onset that women received HT, with younger women realizing the greatest benefits. Follow up studies have since revealed that timing of HT is critical, both for maximum benefits, as well as minimal detrimental effects [3,7,16,133,179,180]. However, the molecular mechanisms underlying these age-dependent effects of estrogens are unknown.

Estrogens regulate gene transcription primarily through binding to estrogen receptors (ERs), which then act as transcription factors on gene promoters. However, estrogens have also been shown to regulate gene expression at the post-transcriptional level through microRNAs (miRNAs). miRNAs are a class of small non-coding RNAs that regulate gene expression via translational repression of target mRNAs [64,127], thereby decreasing the protein products of those genes. The miRNA biogenesis pathway reveals several potential regulatory points for estrogens. First, miRNAs are transcribed from the genome raising the possibility of ER-mediated regulation of miRNA transcription. Second, they are highly regulated post-transcriptionally by a series of RNase III enzymes, which sequentially cleave the primary transcript (pri-miRNA) into shorter fragments, and these enzymes act in conjunction with several cofactor proteins, some of which are regulated by estrogens [31,43,45,46,52,53,129,181]. Finally, the mature 22 nt miRNA duplex dissociates such that one strand (guide strand) is loaded onto an argonaute (AGO) protein, thereby forming the RNA-induced silencing complex (miRISC). The guide strand is largely protected from degradation due to its association with target mRNA and AGO, yet the passenger strand is typically degraded. Therefore,
miRNA degradation is another potential point for estrogens regulation of miRNAs. The half-life of miRNAs can vary from minutes to days [151,182,183], yet the precise mechanisms regulating their stability are unclear. Several studies have shown that miRNAs are subject to post-transcriptional modifications, which can affect their stability. For instance, poly(A) polymerase associated domain 4 (PAPD4) is a nucleotidyltransferase that adenylates the 3’ end of miRNAs [184,185]; this modification to the miRNA can increase its stability and alter its ability to bind to the RISC complex [184,185]. Ultimately, miRNAs are degraded by at least one known exonuclease, 5’-3’ exoribonuclease 2 (XRN2) [186]. To our knowledge, there have been no studies to date demonstrating whether estrogens regulate any proteins that are involved in miRNA stabilization pathways.

In the brain, miRNAs display both an age-and brain region-specific expression pattern, suggesting that they play critical roles in normal brain function [74,75,78,81,82,111,123]. Indeed previous studies have demonstrated that miRNAs are crucial for neuronal development and synaptic plasticity, and are also implicated in regulating affective behaviors [104,105,146,147,150,187,188,189]. Previously, our laboratory demonstrated that the major circulating estrogen, 17β-estradiol (E2), regulates miRNAs in an age- and brain region-dependent manner in female rats, suggesting that there would be differential mRNA translation in those brain regions at varying ages due to E2 dependent miRNA regulation [190]. E2 exerts its effects through two estrogen receptors, ERα and ERβ. ERα and ERβ display brain region specific expression patterns, and also have dichotomous effects on brain function [191,192]. Additionally, both
receptors have been shown to regulate miRNA expression in in-vitro conditions, however it is not known whether one or both receptors are required for mediating E2-induced miRNA expression in the brain [117,121].

We propose that there is a biological switch in estrogens’ actions that occurs coincident with age and length of time after ovarian hormone depletion, and we hypothesize that age dependent regulation of miRNAs could be the molecular basis for that switch. Our previous work showed that E2 differentially regulated miRNA expression in the brain of young (3 mo.) compared to aged (18 mo.) female rats. In this current study we extended those observations by investigating if longer periods of E2 deprivation in aged female rats altered the E2-dependent regulation of miRNAs we previously observed in the brain. Female Fisher 344 rats (18 mo. old) were ovariectomized (OVX) and then given an acute E2 treatment at 1, 4, 8, or 12 weeks post-OVX. We analyzed the expression of our previously identified E2 regulated mature miRNAs, as well as their intermediary biosynthetic products (pri-miRNA, pre-miRNA) in the hypothalamus. In addition, we quantified the expression of several miRNA processing proteins including drosha, DiGeorge syndrome critical region 8 (DGCR8), exportin-5 (XPO5), dicer, AGO2, XRN2, and PAPD4. Finally, we determined which ER mediates the E2-induced increase in miRNA expression observed at 1 wk. post-OVX in the aged female brain. Collectively our results show that extended deprivation of ovarian hormones markedly alters E2 regulation of mature miRNAs in the aged female hypothalamus, suggesting that there is a shift in how the brain responds to the re-introduction of E2 after prolonged periods of ovarian hormone deprivation.
Results

Treatment with E2 fails to regulate mature miRNA expression following longer periods of ovarian hormone deprivation.

We developed an animal paradigm to directly test the timing hypothesis in aged female rats. Aged rats (18 mo., equivalent to 55 years old in human) were ovariectomized (OVX) to model surgically induced menopause and eliminate the source of all ovarian hormones. Acute administration of E2, or vehicle control, was given following varying lengths of time post-OVX: 1, 4, 8, or 12 weeks (Fig. 9A). Animals were euthanized 24 hours after the last injection, brains rapidly removed, and total RNA isolated from the hypothalamus. Next, we measured the expression levels of mature let-7i, miR-7a, miR-9, miR-9-3p, miR-125, miR-181a, and miR-495 using qRT-PCR (Fig 10A-G). We had previously identified these 7 miRs as being regulated by E2 in an age dependent manner [190]. A two-way ANOVA analysis determined that there was no statistically significant interaction between treatment and length of ovarian hormone deprivation, indicating that the two factors were independent. However, analysis of treatment within each time point demonstrated that E2 significantly increased the expression of mature let-7i, miR-7a, miR-9, miR-9-3p, and miR-181a at 1 week post-OVX, (consistent with our previous published data, [190] but not any other time point (Fig. 10A-D, F, gray line, denoted by *). Interestingly, E2 treatment significantly decreased miR-495 at 12 weeks post-OVX, which was the only miRNA tested that showed an E2-mediated decreased expression (Fig. 10G).
One-way ANOVA analyses across the deprivation time points showed that six of the seven miRNAs (let-7i, miR-9, miR-9-3p, miR-125a, miR-181a, and miR-495) were significantly increased due to age alone (Fig. 10, black line, denoted by #). Specifically, a post-hoc analysis showed that let-7i expression was increased at 12 weeks post OVX (Fig. 10a black line, #) in vehicle treated animals. Similarly, miR-9, miR-9-3p, miR-125a, miR-181a, and miR-495 were significantly increased in both vehicle and E2 treated animals at 8 and 12 weeks post OVX (Fig. 10C-G, black line, #).
Figure 9. Ovarian hormone deprivation paradigm.
a) Schematic representation of the animal experimental paradigm. Fischer 344 female rats were obtained at 18 months of age and ovariectomized. Rats were given a subcutaneous injection once/day for 3 days 1, 4, 8, or 12 weeks post-OVX (N=6/age/treatment). b) E2 plasma concentrations assayed by ELISA from blood samples taken 24 hours after the last injection of E2. Data displayed as mean ± SEM pg/mL.
Figure 10. E$_2$ regulation of mature miRNA expression in the hypothalamus after increasing lengths of ovarian hormone deprivation.

a-g) Mature miRNA expression was analyzed by real time qRT-PCR and displayed as mean ± SEM fold change as compared to 1 week vehicle treated animals (N= 6/age/treatment). An * denotes a statistically significant effect of treatment within a time point. Different symbols (#,%) denote a statistically significant difference across time points.
Mature miRNA expression levels are not paralleled by the expression of their pri- and precursor forms.

Effects of E2 treatment on primary miRNA (pri-miR) expression levels in the hypothalamus.

The data from the initial experiments were consistent with our previously published findings and demonstrated that E2 regulates mature miRNA expression following short-term, but not long-term, OVX. We next analyzed the expression of their primary and precursor transcripts using qRT-PCR to determine the level of the biosynthetic pathway that E2 acts to regulate these mature miRNAs. Similar to the results of the mature miRNAs, a two-way ANOVA analysis revealed that there was no significant interaction between treatment and age for the pri- or precursor forms of these miRNAs. Moreover, analysis of treatment within a single time point showed that E2, in general, had no effect on the transcription of most of the pri-miRNAs at any time point (Fig. 11). However, there were a few exceptions. Specifically, comparison of treatment within a time point revealed that E2 significantly increased expression of the primary miRNA transcript of let-7i one-week post OVX, but not at any other time point (Fig. 11A, gray line, *). Also, it is important to note that two of the mature miRNAs, miR-7a and miR-9, are transcribed from multiple chromosomes, allowing for unique regulation of biogenesis at each locus. Therefore, we designed primers corresponding to distinct primary sequences on each chromosome for miR-7a and miR-9, in order to account for possible differences between mature miRNA products derived from the different chromosomes. Interestingly, these data revealed a switch in E2 regulation of each chromosome depending on the age post-OVX. Specifically, E2 increased pri-miR-7a transcribed from chromosome 1 at 4 weeks post-OVX, but not any other time points (Fig. 11B, pri-miR-7a-1, gray line, *). Meanwhile, E2 significantly increased pri-miR-7a transcribed from chromosome 17 at 1 week post-OVX (Fig. 11C, pri-miR-7a-2, gray line, *). These data suggest that the mature miR-7a product was derived from different chromosomes.
depending on the time point. Interestingly, however, our data only showed a significant increase in mature miR-7a at the one-week time point, which points to chromosome 17 as potentially more important than chromosome 1 for E2-regulated mature miR-7a expression.

Figure 11D-F depicts results from pri-miR-9. It is important to note that miR-9 (guide strand) and miR-9-3p (passenger strand) are derived from the same primary and precursor transcripts which are located on two different loci on chromosome 1 (pri-miR-9-1, pri-miR-9-2) and one locus on chromosome 2 (pri-miR-9-3). Our previously published data showed that E2 significantly regulated both the guide and passenger strands of mature miR-9 [190]. Analysis of the primary miR-9 expression showed that E2 treatment significantly increased pri-mir-9-1 and pri-mir-9-2 at 1 week post OVX, as well as pri-mir-9-2 at 4 weeks post OVX (Fig. 11D,E, gray line, *). By sharp contrast, E2 did not affect the expression levels of pri-mir-9-3 at any time point (Fig. 3F, gray line, *). Finally, E2 had no effect on pri-miR-181a or pri-miR-495 at any time point (Fig. 3H, I).
Figure 11. E₂ regulation of the primary miRNA expression in the hypothalamus after increasing lengths of ovarian hormone deprivation.

A-1) Primary miRNA expression was analyzed by real time qPCR and displayed as fold change as compared to 1 week vehicle treatment animals (N= 6/age/treatment). (*) denotes a significant effect of E₂ as compared vehicle treated animals with in that time point (Student’s t-test). Different symbols (#,%) denote a significant difference between each time point (One-way ANOVA).
Effects of aging on primary miRNA (pri-miR) expression levels in the hypothalamus.

One way ANOVA analyses across the deprivation time points showed that pri-mir-7a-2, pri-mir-9-1, pri-mir-125a, pri-mir-181a, and pri-mir-495 were all significantly altered by age alone, and in general, they were all decreased with age (Fig. 11, black line, #). Specifically, expression levels of pri-mir-9-1 (Fig. 11D, black line, #) were significantly decreased at 8 weeks post OVX, while pri-mir-125a expression was decreased at 12 weeks post OVX (Fig. 11G, black line, #). Further, both primary transcripts for miR-181a and miR-495 were significantly decreased at 4, 8, and 12 weeks post OVX (Fig. 11H-I, black line, #). The only exception was for pri-mir-7a-2, which was significantly decreased at 4 weeks, but then increased at 8 weeks post OVX (Fig. 11C, black line, #).

Effects of E2 treatment and aging on precursor miRNA (pre-miR) expression levels in the hypothalamus.

A two-factor ANOVA analysis revealed that there was no significant interaction between age and treatment for any of the precursor miRNAs, again suggesting that the two factors are independent. Next, statistical analyses within each separate time point demonstrated that E2 treatment significantly altered the expression levels of 3 out of 7 pre-miRNAs tested when compared to vehicle-treated controls (Fig. 13, gray line, *). Interestingly, E2 treatment significantly increased the expression of pre-miR-7a-2 at both 1 and 12 weeks post-OVX, but had no effect on pre-miR-7a-1 at any time point (Fig. 13B,C, gray line, *).
Figure 12. E2 regulation of the precursor miRNA expression in the hypothalamus after increasing lengths of ovarian hormone deprivation.
A-G) Precursor miRNA expression was analyzed by real time qPCR and displayed as fold change as compared to 1 week vehicle treatment animals (N= 6/age/treatment). (*) denotes a significant effect of E2 as compared vehicle treated animals with in that time point (Student’s t-test). Different symbols (#,%) denote a significant difference between each time point (One-way ANOVA).
These results sharply contrast what was observed with the primary transcripts of these same miRNAs (see Fig. 12B,C), suggesting that E2 acts differently at multiple levels of the miRNA biogenesis pathway. Similarly, the effects of E2 treatment were strikingly differently for pre-miR-9 (Fig. 13D) compared to its effects on the primary form (pri-miR-9; see Fig. 12D-F). Specifically, E2 significantly decreased pre-miR-9 expression at 1 week post OVX, but then significantly increased its expression at 12 weeks post OVX (Fig. 13D, gray line, *). While the primary transcript of miR-9 is transcribed from three different loci (hence, pri-miR-9-1, pri-miR-9-2, and pri-miR-9-3), the precursor hairpins generated from each primary transcript are too similar to be able to differentiate them using qRT-PCR. Therefore, it is possible that there were offsetting changes in each precursor form that might have obscured the final results. Interestingly, E2 completely abolished the steep age-related increase in pre-miR-181a observed at 4 weeks post-OVX (Fig. 13F, gray line, *). These results were unexpected given the E2-induced increase in both mature and pri-miR-181a expression levels at varying time points (see Figs. 11F and 4h). Finally, there were no effects of aging alone on the expression levels for any of the precursor miRNAs tested (Fig. 13, black lines).

E2 and age-related changes in mature miRNA expression are not explained by concomitant changes in the mRNA or protein levels of key components involved in miRNA biogenesis.

Our data show that E2 regulates miRNA expression at various levels along the miRNA biosynthetic pathway, raising the possibility that E2 could regulate the transcription (mRNA) and/or protein levels of critical components required for miRNA biosynthesis. Therefore, we analyzed the mRNA expression of the nuclear proteins
drosha and DGCR8 (key components of the microprocessor complex), as well as cytoplasmic proteins dicer, AGO2, XPO5, XRN2, and PAPD4 in the hypothalamus of animals subjected to our ovarian hormone deprivation paradigm (Fig. 14). The statistical analysis revealed that there was a significant interaction of treatment and age on the mRNA expression levels of drosha, but not any of the other genes tested (F(3,38)=3.762, p=0.019). Specifically, the post hoc analysis revealed that E2 treatment increased drosha mRNA expression at 8 weeks post OVX (Fig. 14A). Statistical analyses within each time point revealed that E2 treatment also increased DGCR8, dicer, and AGO2 mRNA expression levels at 8 weeks post OVX (Fig. 14B-D). Conversely, E2 treatment significantly decreased XPO5 at 1 week post OVX (Fig. 14E). We did not observe any significant changes in the mRNA expression XRN2 and PAPD4 between treatment groups at any time point (Fig. 14F, G, gray line). Moreover, we did not observe changes due to age alone in the mRNA expression of these genes (black lines).

Next, we compared the mRNA levels of drosha, DGCR8, dicer, AGO2, XPO5, XRN2, and PAPD4 with their translated protein levels from the same animals (Figs. 15-16). Interestingly, there was no statistically significant effect of treatment or age on the protein levels of any of these genes, despite our observed statistically significant changes in mRNA levels. These data suggest that E2-mediated changes in mature miRNA expression levels cannot be explained by mRNA/protein changes in these key miRNA biosynthetic components.
Figure 13. Regulation of miRNA biogenesis genes by E₂ and ovarian deprivation. A-G) mRNA expression of miRNA biogenesis genes was analyzed by real time RT-PCR (N=6 treatment/age group). Data is displayed as fold change as compared to 1 week vehicle treated animals.
Figure 14. Western blot analysis of miRNA biogenesis gene expression.
A-D) 60µg of protein was run on a 10% PAGE. Images are representative blots (N=3/treatment group). B-actin expression was used as the loading control.
Figure 15. Estradiol and ovarian hormone deprivation does not regulate miRNA biogenesis protein expression.

A-D) Densitometry analysis of western blot images (Fig. 17) using BioRad Image lab software. Data are displayed at percent change as compared to vehicle treated animals (N=6 /treatment/age group).
ERβ mediates E2 effects on mature miRNA expression in the Hypothalamus

The effects of E2 are mediated primarily by two estrogen receptors, ERα and ERβ, and both have been shown to regulate miRNA expression in cancer cell lines [116,117,121]. To determine which receptor mediates the regulation of miRNA expression in the aging female brain, we first examined the expression levels of ERα and ERβ mRNA in our ovarian hormone deprivation paradigm. A two-way ANOVA analysis revealed that there was a statistically significant interaction between treatment and deprivation period for ERβ (F(3,38)=3.762, p=0.014), but not ERα. ERβ mRNA expression was significantly decreased at 4, 8, and 12 weeks post-OVX in the vehicle treated samples (Fig. 17B, black line, #). However, E2 significantly increased ERβ mRNA expression at 4 and 12 weeks post-OVX (Fig. 17B, gray line, *).

Next, we ovariectomized aged (18 month old) Fischer 344 female rats and 1 week later administered either E2, or an ERα (PPT) or ERβ (DPN) selective agonist. We then measured the primary, precursor, and mature miR expression levels for each of our previously identified E2 regulated miRNAs using qRT-PCR (Fig. 18). First, we analyzed the expression levels of the primary transcripts, which demonstrated that both PPT and DPN treatment significantly decreased the expression of pri-miR-7a, pri-miR-125a, pri-miR-181a, and pri-miR-495 compared to either vehicle or E2 treated animals (Fig. 18A). These results were surprising given that no effect was observed with E2 treatment alone. Interestingly, let-7i primary transcript expression was the only miRNA to be significantly increased with DPN and PPT treatment (Fig. 18A). By contrast, PPT significantly
increased the expression of the precursor form of miR-181a (pre-miR-181a), but had no effect on the precursor forms for any of the other miRNAs (Fig. 18B). Similarly, DPN significantly increased the precursor form of miR-495 (pre-miR-495), but not any other precursor miRNA (Fig. 18B). Finally, DPN, but not PPT, mimicked the effects of E2 on the expression levels of mature let-7i, and miR-7a, which was consistent with our earlier observations after 1 week of E2 deprivation (Fig. 18C).

The effects of PPT and DPN on miR-9 were particularly interesting. Our previously published observations, as well as replicated data herein, demonstrated that E2 significantly increased both the guide (miR-9) and passenger (miR-9-3p) strands of mature miR-9 after 1 week of E2 deprivation in aged animals. However, both PPT and DPN significantly decreased the pri-miR-9 and pre-miR-9 forms of this miRNA (Fig. 18D, E). Notably, E2 tended to increase pri-miR-9, and decrease pre-miR-9, but neither effect was statistically significant. Finally, E2, PPT, and DPN all significantly increased mature miR-9 and miR-9-3p, but DPN increased each to a greater degree (Fig. 18F, G).
Figure 16. Estrogen receptor isoform expression in the hypothalamus. A-B) Expression of ERα (A) and ERβ (B) mRNA in the hypothalamus analyzed by real time RT-PCR (N=6 treatment/age group).
Figure 17. ERβ regulates mature miRNA expression in the hypothalamus.

A-G) Primary (A,D), precursor (B,E), and mature (C,F-G) miRNA expression in the hypothalamus of vehicle, E₂, DPN, and PPT treated female rats 1 week post-OVX. Data is displayed as fold change as compared to 1 week vehicle treated animals. Statistical significance was analyzed using one-way ANOVA with Tukey’s post-hoc test.
Summary

Our primary objective in these studies was to test the efficacy of acute E2 treatment, administered at different time points following the complete loss (via OVX) of ovarian hormones, on miRNA expression in the aged female brain. The results from this study demonstrated several novel findings. First, E2 treatment altered mature miRNA expression in the brain of aged animals after 1 week of ovarian hormone deprivation, but this effect was lost following longer periods of hormone deprivation. These results are consistent with that of the Timing Hypothesis, which suggests that the efficacy of E2 action changes with advanced age and length of time postmenopause. Second, our results showed that E2 treatment selectively altered the expression of the primary and precursor transcripts of these same miRNAs, however those expression levels often did not correspond to the levels of their mature miRNA counterparts. Our interpretation of these data is that E2 can act at multiple levels along the miRNA biosynthesis pathway, and that E2 perhaps plays a role in the stabilization of mature miRNAs. Third, we showed that aging alone (i.e. vehicle treatment) also had a significant effect on mature miRNA expression in many cases, and these are the first data to describe miRNA expression levels in the brains of animals at several time points of advanced age. Finally, using ER selective agonists we demonstrated that the effects of E2 on mature miRNA expression in the hypothalamus are likely mediated primarily through ERβ. Taken together these results extend our previous findings by suggesting a possible mechanism for the lack of therapeutic effectiveness for HT in post-menopausal women.
Our previous work showed that E2 differentially regulates the expression of mature miRNAs in the brains of young, compared with aged, female rats [190]. This current study extends those findings by focusing only on aged animals that were given E2 replacement at varying times post-OVX. Consistent with our earlier findings, E2 treatment significantly increased 5 out of 7 previously identified mature miRNAs after only a brief period of ovarian hormone deprivation (i.e. one week) in aged animals. It is important to note that these current studies examined mature miRNA expression only in the hypothalamus, whereas our previous findings were mostly derived from the hippocampus. Nevertheless, these data remained consistent with our earlier results in that there appears to be brain-region specific effects of E2 on miRNA expression. Notably, we observed no differences in mature miRNA expression between E2- and vehicle-treated animals following longer periods of ovarian hormone deprivation (i.e. 4, 8, and 12 weeks). These results are in-line with the concept of the Timing Hypothesis, which predicts a lack of E2 efficacy following longer periods of ovarian hormone deprivation.

miRNAs are important regulators of mRNA translation and are predicted to impact the expression of more than 60% of all protein-coding genes [152]. The implication of these results suggests that a loss of E2-regulated miRNAs postmenopause could negatively impact multiple downstream target genes, although it remains unclear which specific downstream target genes these particular miRNAs regulate.

Despite their critical role, little is known about the tissue-specific regulation of miRNA biosynthesis and processing. In general, global miRNA biogenesis follows a well-defined pathway beginning with the generation of a long primary transcript (pri-
miR) and ending with a single-stranded 22 nt mature miRNA product [43,45,46,52,54]. There are several steps along this pathway that are potential points where unique tissue- and age-specific expression of individual miRNAs could occur. Estrogen receptors act primarily as transcription factors on various gene promoters, therefore, the most likely site of E2 regulation is at the transcriptional level of the primary miRNA form. Indeed, others have demonstrated that E2 regulates several pri-miRs through canonical estrogen response elements (EREs) in breast cancer cell models [117,121,193]. Therefore, we next measured the expression levels of the primary (pri-miR) and precursor (pre-miR) forms of our identified E2-regulated mature miRNAs at varying times post-OVX. Our prediction was that expression levels of these earlier transcripts would match that of the mature miRNA effector, however this was not what we observed. In fact, there was considerable discordance between the pri-, pre-, and mature forms for most of the miRNAs tested. The discordance observed could be the result of altered processing of these miRNAs. Post-transcriptional modifications to RNAs, including miRNAs, have been shown to be common and important regulatory mechanisms, which can either promote or inhibit their processing [181,184,185,194]. Additionally some of these modifications have been shown to be altered with aging [195]. These studies suggest that not only expression, but sequence changes to the miRNA should be analyzed to more completely understand their regulation and function. There is little known about the temporal regulation of miRNA biosynthetic events, but these results suggest that E2 could regulate different steps along the pathway at different times. It also demonstrates a limitation of our studies in that each miRNA form was measured at just one snapshot in
time following E2 treatment (24 hours following the last injection). Nevertheless, the fact that E2 regulated all of the miRNAs forms at some time point indicates that E2 might also regulate some of the important key proteins involved in miRNA biogenesis and/or stabilization.

Mature single-stranded miRNA molecules in association with an AGO protein comprise the two core components of the RNA-induced silencing complex [62]. The mature miRNA molecule is formed after the cytoplasmic RNase III enzyme dicer cleaves the pre-miR to form a small miRNA duplex structure [52]. Only one strand of this duplex (leading strand) associates with AGO; the other strand (passenger strand) remains free, either to bind to RNAs and other proteins or be degraded [56,57,58]. In contrast to that description of miRNA formation, our data showed that E2 treatment not only increased the expression of mature miR-9 in aged females, but it also increased the expression of the miR-9a passenger strand, miR-9-3p (current study, and [190]). Because both strands of the miR-9 duplex are derived after dicer processing of the same precursor molecule, these results provide strong evidence that E2 can regulate miRNA expression at the level of molecular stabilization. Therefore, to determine if E2 regulates key components of miRNA biogenesis or stabilization we examined their mRNA and protein levels in our ovarian hormone deprivation paradigm. We observed that E2 treatment significantly increased drosha and dicer mRNA at 8 weeks post-OVX, however these changes were not mirrored by changes in protein levels at that same time point. One possibility is that we missed the window of detection, given that we measured both mRNA and protein at only time point following E2 treatment (24 hours after the last injection). Moreover, the
levels of mRNA and protein for these enzymes do not necessarily reflect changes in enzyme activity. Importantly, the enzymatic activities are acutely sensitive to rapid changes in co-factor binding and posttranslational modifications [52,53,54,181,196], all of which are potential targets for E2 regulation. At this time, we are unaware of any enzymatic assay that has been developed for the detection of dicer or drosha activity either in cell line models or tissue, making this a very important future direction for research efforts. Overall, there were no significant changes due to age or treatment on the protein expression for any of the other key miRNA biogenesis or stabilization components tested. These results are consistent with our findings, and that of others, showing that E2 and age only regulate a very small subset of miRNAs [116,190]. Therefore, E2 regulation of a major protein component in the miRNA biogenesis pathway is unanticipated, as that would predict a broad impact on a much greater number of miRNAs.

The results of the WHI studies prompted both basic and clinical scientists to reevaluate the role of E2 in the aging brain. Changes in the composition, number, or structure of estrogen receptors (ERα or ERβ) would be an obvious explanation for reduced E2 efficacy with age. Indeed, previously published studies have shown that both ERα and ERβ expression are altered with aging in specific nuclei of the hypothalamus, although the reported literature are somewhat contradictory with respect to ERβ [197,198,199]. Our data showed that there was a significant decrease in ERβ mRNA expression at 4, 8, and 12 weeks post-OVX (equivalent to 19, 20, and 21 months old, respectively) in both the vehicle and E2-treated animals. However, treatment with the
ERβ specific agonist DPN was equally effective as E2, and there were no changes observed in ERα expression, suggesting that decreased ER availability is not a primary factor for the lack of E2 efficacy in the aged female brain. However, one important consideration to take into account when evaluating receptor expression is that there are multiple splice variants of ERβ. Our experiments were designed to detect the full-length wild-type ERβ, which would also be inclusive of all known ERβ splice variants. In particular, ERβ2 has lower E2 binding affinity, different transcriptional efficacy, increases with age, and was correlated with an increase in depressive behaviors in aged Sprague-Dawley female rats [14,200,201]. Therefore, the ERβ mRNA expression levels that we observed could also represent a large cohort of ERβ2, which would not respond to E2 as well as wild-type ERβ. Another important consideration is that proper ER signaling requires the successful recruitment of a variety of co-regulator proteins. We have previously demonstrated that the protein complexes associated with ERβ in the brain are altered by E2 in an age-dependent manner, despite no age-related changes in the expression of the cofactor proteins themselves [202]. These data indicate that altered ER and/or cofactor gene expression might not be good markers to assess the efficacy of E2 signaling.

Although the actions of E2 are mediated primarily through its two classical nuclear receptors, ERα and ERβ, recent evidence has emerged implicating E2 regulation of cell function through a variety of other intracellular signaling pathways, some of which are mediated by membrane-bound ER or G protein-coupled receptors. In these studies, we used a straightforward approach to try to identify whether the classical ERs were
mediating the E2-induced regulation of mature miRNA expression in the aged brains following a brief period (1 week) of ovarian hormone deprivation. To that end, we took advantage of the recent development of the specific ERα and ERβ agonists, PPT (propylpyrazole-triol) and DPN (diarylpropionitrile), respectively. These agonists have been shown to be highly selective for their respective form of the receptor when used at the doses we administered [203,204]. In general, our results indicated that the E2-induced regulation of mature miRNAs after one week of OVX was mediated primarily by ERβ. Interestingly, there were some instances, most notably at the primary (pri-miR) level, where PPT and DPN were equally efficacious and E2 had no apparent effect (see Fig. 18). Previous studies have demonstrated that ERβ can act as a dominant negative to ERα and our results suggest that both receptors can separately mediate transcription of the primary transcripts, but they negate each other’s effects when activated in concert. An important future study will be to determine if PPT or DPN effects change following longer periods of ovarian hormone deprivation. Although we showed no effect of E2 at later time points, it is possible that each receptor alone would have time-specific effects, making these ER selective agonists a potentially useful therapeutic tool for postmenopausal women.
CHAPTER V

THE EFFECT OF AGING ON MATURE MICRORNA EXPRESSION IN THE HYPOTHALAMUS OF OVARY INTACT RATS

INTRODUCTION

Advanced aging increases the risk for developing chronic neurological disorders such as dementia and Alzheimer’s disease. These risks could be the result of a progressive dysregulation of developmental expression patterns of key genes. miRNAs are critical for the development of the brain and have been shown to show altered expression patterns with advanced aging [71,83,84,108,109,111,112,113,123]. In addition, miRNA expression patterns mirror changes in both gene mRNA and protein expression patterns with advanced aging, suggesting that they are critical regulators of age-related processes.

The previous chapters have shown alterations in the expression of miRNAs regulated by E2 with advanced aging. However, those animals had undergone removal of their ovaries and were then given an E2 treatment. It is possible that the age-related expression pattern of these miRNAs could be different if they had undergone removal of their ovaries. An understanding of the normal age-related expression changes of the
Figure 18. miRNA expression is altered with advanced aging in the hypothalamus of intact female rats.
A-F) Real time RT-PCR analysis of miRNA expression in the hypothalamus of 18, 19, 20, and 21 month ovary intact female Fischer 344 rats (N=6/age group). One-way ANOVA with tukey’s post-hoc was performed for statistical analysis.
miRNAs could help to understand what other factors are important for regulating these miRNAs.

**Results**

Fischer 344 female rats were obtained at 18 months of age and then allowed to age until 21 months of age. Animals were sacrificed at 18, 19, 20, and 21 months of age. The right side of the hypothalamus was isolated and processed for RNA isolation. Real-time RT-PCR analysis of the mature miRNA expression of the seven identified miRNAs was performed. One-way ANOVA statistical analysis identified a significant effect of aging with all seven miRNAs. 5 of the 7 miRNAs (let-7i, miR-7a, miR-9, miR-9-3p, and miR-495) were significantly increased in expression at 20 and 21 months of age. Only miR-125a and miR-181a showed a decrease in expression. miR-125a was decreased at 19 months of age, but then increased in expression at 20 and 21 months. miR-181a was significantly decreased at 19, 20, and 21 months of age.

miR-9 and miR-9-3p are derived from the same precursor transcript and therefore are transcribed from the same gene. Usually, the lagging strand of the miRNA duplex (miR-9-3p) is degraded. Interestingly miR-9-3p expression was significantly increased with aging, while miR-9 was also increased but not to same extent. These data suggest that there is differential strand selection occurring with aging.

**Summary**

The results herein have demonstrated that the previously identified seven miRNAs are significantly regulated with aging. These data are line with other previous
studies have demonstrated. The data also demonstrate, in the case of miR-9/miR-9-3p, there is an alteration in the selection of the lagging strand of the precursor miRNA with aging. Since the both the mature miRNA s are derived from the same primary and precursor miRNA transcripts, these data suggest a post-transcriptional mechanism. Currently it is not understood how the mature miRNA strand selection occurs, though modifications to miRNAs themselves is a possibility.

Importantly these expression patterns are not what were seen the vehicle treated animals in the previous Chapter IV. These data preliminary suggest that ovariectomy greatly altered the expression pattern of the miRNAs. It is possible that these miRNAs could be regulated by another steroid hormone secreted by the ovaries, such as progesterone. Further study is required to understand what other regulatory factors are important for regulating the miRNAs.
CHAPTER VI

17β-ESTRADIOL REGULATES THE EXPRESSION OF MIR-7 IN NEURONAL CELL LINES

Introduction

The previous chapters have demonstrated E2 regulation of miRNAs in-vivo. However, there are experimental limitations to what can be accomplished in-vivo to understand the precise E2 molecular mechanisms which are regulating miRNA expression. Therefore an in-vitro model is needed to be able to precisely manipulate the actions of E2 on miRNA expression. Previously studies on E2 regulation of miRNA expression have been performed in-vitro using breast and prostate cancer cell lines [116,117,121]; as of this writing there is no published work demonstrating the regulation of miRNAs in a neuronal cell line.

Previously, I have demonstrated the regulation of miRNAs by E2 in both the hippocampus and the hypothalamus. Therefore, in this chapter I will use two different neuronal cell lines derived from hippocampus and the PVN of the hypothalamus brain regions to examine the regulation of miRNA expression by E2. The IVB cell line is derived from the PVN of the rat hypothalamus and expresses only ERβ [205]. The HT22
cell line is a sub-line cloned from the HT4 cell line derived from the mouse hippocampus[206]. The HT22 cells do not express the either estrogen receptor and therefore provide a unique opportunity to study regulation of miRNA expression of estrogen receptors individually by ectopic expression. miR-7 was regulated by E2 in both the hippocampus and hypothalamus; therefore it was used to determine the effect of E2 on miRNA expression in-vitro. The results herein demonstrate that E2 is able to regulate miRNA expression in the both the IVB and HT22 neuronal cell lines, however that identified miRNAs are expressed at lower levels compared to what was seen in-vivo.

Results

Estradiol regulates miR-7 expression in the PVN derived cell line IVB through ERβ

The IVB cells were maintained in phenol-free media with charcoal dextran treated fetal bovine serum (FBS) to ensure that there were no estrogens in the media. The cells were then treated with 10nM E2 overnight (16 hours). To determine if the action of E2 was indeed through the estrogen receptor the cells were also treated with an estrogen receptor antagonist, ICI-182,780, alone and in combination with E2. RNA was isolated from the cells after the treatment period and miR-7 expression was analyzed by qRT-PCR. One-way ANOVA analysis demonstrated that there was an effect of treatment on miR-7 expression. Post-hoc pair wise comparisons show that miR-7 expression was significantly increased with E2 treatment as compared to vehicle treated cells (Fig. 20). Interestingly, the antagonist ICI-182,780 also significantly increased miR-7 expression.
and did not inhibit E2 induced miR-7 expression when administered in combination (Fig. 20).

The IVB cells express ERβ only; therefore the effect of E2 must be through that receptor. However to conclusively determine that ERβ regulates miR-7 expression, IVB cells were treated with 100nM 5α-androstane-3β, 17β-diol (3β-diol); a high affinity agonist for ERβ. miR-7 expression was also significantly increased in the IVB cells in response to treatment with 3β-diol (Fig. 21).
Figure 19. miR-7 expression in IVB cells after treatment with 17β-estradiol. IVB cells were maintained DMEM phenol-free media with 10% charcoal dextran treat FBS 24 hours prior to treatment. Cells were treated with 10nM E$_2$, 100nM ICI-182,780, or E$_2$+ICI for 16 hours (N=3 repeated experiments). RNA was isolated from cells and miR-7 expression was analyzed using real time RT-PCR. Data are displayed at fold change compared to vehicle treated cells. One-way ANOVA was performed with Tukey’s post-hoc test.
Figure 20. ERβ regulates miR-7 expression in IVB cells.
IVB cells were maintained DMEM phenol-free media with 10% charcoal dextran treated FBS 24 hours prior to treatment. Cells were treated with 100nM 3β-diol for 16 hours (N=3 repeated experiments). RNA was isolated from cells and miR-7 expression was analyzed using real time RT-PCR. Data are displayed at fold change compared to vehicle treated cells. * indicates p-value < 0.05 (student’s t-test).
Estradiol does not regulate miR-7 expression in hippocampal cell line HT22

Estradiol was able to regulate miR-7 expression in the IVB cell line; however we also wanted to see if this effect would also be seen in the hippocampal cell line HT22. HT22 cells were maintained in phenol-free media with dextran charcoal treated FBS. Cells were transfected with either pcDNA vector or a vector containing rat ERβ. 24 hours later the cells were treated with either E2, an estrogen receptor antagonist tamoxifen (OHT), or both in combination. Analysis of miR-7 expression by qRT-PCR showed that there was no effect of treatment with either pcDNA or ERβ transfection (Fig. 22). However, ERβ transfection alone significantly decreased miR-7 expression. In order to see if the effect of ERβ transfection was unique to miR-7 or was actively repressing other miRNAs, miR-9 expression was analyzed by qRT-PCR. Expression of miR-9 was not repressed by ERβ transfection (Fig. 22). Estradiol increased miR-9 expression modestly in ERβ transfected cells in comparison to pcDNA transfected cells treated with E2 (Fig. 22). However, OHT treatment also increased miR-9 expression in both pcDNA and ERβ transfected cells (Fig. 22).

The previous results are the first to demonstrate E2 regulation of miRNA expression in a neuronal cell line. The effects of E2 on miR-7 and miR-9 are modest; therefore we wanted to determine if we could see an effect of E2 on a well-established miRNA, miR-21. miR-21 has been shown to be regulated by E2 in different cancer cell lines [116,117]. HT22 cells were transfected with pcDNA, ERα, or ERβ for 24 hours and then treated with 10nM E2 for 16 hours. Expression of miR-21 was significantly
increased with E2 in ERα transfected cells, but not in pcDNA and ERβ transfected cells (Fig. 23).

miR-7 expression is lower in-vitro than in-vivo

The effects of E2 on miR-7 expression in in-vitro have been modest in comparison to in-vivo. One possible explanation is that since these neuronal cells are immortalized they may not express miR-7 at the same level as a neuron would. To determine if was true, miR-7 expression was compared across ventral hippocampus, dorsal hippocampus, hypothalamus, HT-22 cells, and IVB cells. miR-7 expression in both the HT22 and IVB cells were much more lowly expressed than in the ventral hippocampus, dorsal hippocampus, and hypothalamus (Fig. 24).
Figure 21. ERβ transfection in to HT22 cells represses miR-7a expression.
HT22 cells were transfected with either pcDNA (control) or rat ERβ1 plasmid for 24 hours and then treated with vehicle, 10nM E₂, 100nM OHT, or E₂+OHT for 16 hours (N=3 repeated experiments). miR-7a (A) and miR-9 (B) expression are displayed as fold change as compared to pcDNA vehicle cells.
Figure 22. miR-21 is regulated by E₂ in HT22 cells transfected with the estrogen receptors.
HT22 cells were transfected with pcDNA, ERα, or ERβ1 for 12 hours and then treated with either vehicle or 10nM E₂ for 16 hours (N=3 repeated experiments). miR-21 expression is displayed as fold change compared to pcDNA vehicle samples.
Figure 23. Relative expression of miR-7 across brain regions and neuronal cell lines.
Fold change in miR-7 expression in 18 month old OVX vehicle treated ventral hippocampus (VHipp), dorsal hippocampus (DHipp), hypothalamus, hippocampal neuronal cell line HT22, and PVN derived cell line IVB.
In the present chapter I have demonstrated that E2 can increase miR-7 expression in the PVN derived cell line IVB, but not in the HT22 cell line. Interestingly, miR-7 expression was greatly reduced upon transfection of ERβ in to the HT22 cell line; this effect of ERβ transfection was not observed on miR-9 or miR-21 expression. miR-7 was also shown to be much more lowly expressed in the both the IVB and HT22 cell lines as compared to hippocampal and hypothalamus tissue.

It is possible that the immortalization process has induced some unknown factors to repress miR-7 expression. Detection of the primary and precursor transcripts of miR-7 was attempted, but they were not able be detected; these observations further suggest a repression of the transcription of miR-7 in both the IVB and HT22 cell lines. Taken together these data suggest that an in-vitro approach using neuronal cell lines is not recommended as the relative levels of miR-7 are much lower than what is seen in-vivo.
CHAPTER VII

DISCUSSION

Summary of Key Findings

The WHI clinical studies identified that HT was no longer beneficial for ameliorating the risks of chronic cognitive disorders in women who were more than 10 years post-menopause. I hypothesized that this loss of E2 effectiveness could be due to age related alterations in the E2 regulation of miRNAs, which would ultimately result in altered expression of protein coding genes. In support of my hypothesis I have identified a sub-set of miRNAs (let-7i, miR-7a, miR-9, miR-9-3p, miR-125a, miR-181a, and miR-495) that are regulated by E2 in an age dependent manner in the ventral hippocampus. These same miRNAs are differentially regulated by E2 in other anatomically connected brain regions (dHipp, PVN, and CeA). While aging is key component in altering E2 regulation of miRNA expression, I have also demonstrated that extended hormone deprivation results in the loss of E2 regulation of miRNA expression. Furthermore, I used selective estrogen receptor agonists to determine that ERβ is primarily responsible for mediating the effects of E2 on miRNA expression. I have also analyzed the expression of the primary and precursor transcript of these identified E2-regulated miRNAs and
determined that E2 can alter the transcription of the primary miRNA transcript. The results presented suggest that E2 regulation of miRNAs in brain could play a key role in the loss of effectiveness of HT in post-menopausal women.

The Impact of miRNAs for Women’s Health

The modern age has increased the life span of humans greatly and as such women are living almost a third of their lives without circulating E2. The WHI studies were successful in determining that generally women who were in the peri-menopausal time period could be see a beneficial effect of HT. However there is no clinical test for patients to determine whether they may actually see any benefit from HT. miRNAs are uniquely positioned to be excellent biomarkers to judge the effectiveness of HT. For instance, miRNAs have been shown to be circulating in the blood of human patients and animals, which allow them to be assayed using a minimally invasive procedure [113]. Previous studies have also demonstrated that circulating miRNAs are actively secreted in the bloodstream and can act as signaling molecules [207]. It is possible that the seven miRNAs that I have identified could also be detected in the bloodstream or CSF (cerebral spinal fluid) of human patients. Specifically, members of the let-7 family of miRNAs have identified in the CSF of AD patients [207]. Therefore, an increased circulating level of let-7i in the CSF following HT might be a good indication that HT is not a good treatment option for a patient, since I demonstrated that E2 regulation of let-7i is age dependent.
Biomarkers are a good way for identifying if a woman can use HT without ill effect, however those women for which HT is not option still need a therapy to relieve the negative symptoms associated with menopause and decrease their risk for chronic diseases. Targeting specific miRNAs can be used as a therapeutic agent to repress pathogenic pathways in the brain. Inhibition of specific miRNAs, by the use of antagonist miRNAs (antagomiRs), would inhibit the effects of those miRNAs. Alternatively, overexpressing a miRNA identified to be neuroprotective might also help in reducing a post-menopausal women’s risk of developing chronic neurological disorders. Taken together, the data presented herein, along with that of others, strongly suggest that miRNAs have clear clinical applications for the assessment and advancement of women’s health.

Estradiol regulation of miRNA expression

The microarray for miRNAs in the ventral hippocampus detected 421 miRNAs out of 723 miRNAs on the array. Of the 423 miRNAs detected, 133 miRNAs displayed moderate to high expression. The seven miRNAs that were chosen were among those that were highly expressed. Yet there were 288 miRNAs that displayed low expression. While I did not focus on these miRNAs for further consideration, these miRNAs still may play a crucial role in neurobiology of the brain. Indeed there have been two studies that have demonstrated that altered expression of a miRNA determined from total RNA isolated does not necessary reflect a functional impact [208]. Low expression of a miRNA has been shown to correlate to increased occupancy to the RISC complex, whereas high
expression of miRNA correlates to a low occupancy [208]. These studies suggest that in order to gain insight to whether a miRNA is truly functional, analysis of its occupancy in the RISC complex is crucial since it’s through the RISC complex that miRNA exerts its actions.

**Brain region specific miRNA expression**

It is very possible that our initial microarray study, which was performed on ventral hippocampus samples, biased our focus on a particular group of miRNAs, since miRNAs have a brain region specific distribution. E2 probably regulates more and/or different miRNAs in other brain regions. Furthermore, our study did not separate out specific brain cell types, such as glia versus neurons, which means we cannot say for certain where E2 is acting to regulate these particular miRNAs. However, previous studies using in-situ hybridization and specific cell type analysis on miRNA expression in the brain showed that the let-7 family members, miR-9, miR-7a, and miR-125a are all specific to neurons [94]. Therefore, the altered regulation of miRNA expression by E2 is most likely impacting neuronal processes.

**Non-validated miRNAs from the microarray**

My studies focused on seven miRNAs that were regulated by E2 in an age-dependent manner. Yet, the microarray identified 34 miRNAs that were regulated by E2 independent of age. Some of these miRNAs, such as miR-21, were already identified as E2 regulated miRNAs; however those previous studies were all done in breast cancer cell lines [116,117,121]. If E2 can regulate the same miRNA in multiple different tissues, it
may suggest that the miRNA may have the same gene target across all the tissues. For instance, miR-29a, identified in our study as being E2 regulated, has also been shown to be a sexually dimorphic miRNA; suggesting that its regulation by E2 may be key in regulating sex specific functions. Further, miR-26a is a miRNA that was previously identified to be localized to the dendrites of neurons and is also regulated by E2. This observation suggests that E2 might regulate synaptic plasticity through miR-26a. These miRNAs and others await further exploration in their roles in E2 regulation of brain function.

Transcriptional regulation of miRNA expression by E2

E2 has previously been shown to regulate miRNAs in a transcriptional dependent process [121]. These studies demonstrated that E2 was able regulate both the pri- and pre-miRNA expression correlating with that of the mature miRNA expression level. My analysis of the primary and precursor transcript expression levels demonstrated that E2 can regulate the transcription of miRNAs, however the precursor miRNAs were generally unchanged across the board following E2 treatment. The discrepancy between the three levels of miRNAs could be due the enzymatic activity of the miRNA processing enzymes. The pre-miRNA could be efficiently cleaved to the mature miRNA form thereby not allowing for the accumulation that would detected by qRT-PCR. Interestingly, the mature miR-181a was increased at 1 week post OVX by E2, yet there was no regulation of the primary transcript at this time point. These results suggest that
E2 may be able to regulate the mature miRNA expression through other non-transcriptional mechanisms.

Non-transcriptional effects of E2 on miRNA biogenesis

The discrepancy between miR-181a mature and primary transcript levels could be explained by E2 preventing the degradation of the miRNA. There are multiple factors that can regulate the stability of the miRNA transcript. miRNAs associated with AGO have been shown to have very long half-lives, on the order of days to weeks, presumably due to protection from exoribonuclease degradation enzymes [209]. However, the majority of these studies have been done in immortalized cells, where in-vivo analysis has shown that some miRNAs can be rapidly degraded [101]. The precise mechanism of how miRNAs are unloaded from the RISC complex and then degraded is not completely understood. The exonuclease, XRN2, is one known enzyme responsible for degrading mature miRNAs in animals. How XRN2 gains access to the mature miRNA is not yet clear, however it has been shown that the target mRNA binding induces rapid degradation of the miRNA. miRNAs can also be post-transcriptionally modified, the results of which can regulate their stability and loading on to the RISC complex. Uridylation of miRNAs is another mechanism that has been shown to target miRNAs for degradation [181]. However, different post-transcriptional modifications can have opposing effects on miRNA stability. Methylation, on the one hand, stabilizes miRNA expression by preventing uridylation of the miRNA [210]. Additionally, previous studies have shown that the abundance of mRNA transcripts helps to determine the stability of a
miRNA by binding to it when in the RISC complex, thereby preventing its degradation [182]. Therefore, regulation of miR-181a could be indirectly due to E2 increasing the transcription of a specific mRNA target gene of miR-181a, resulting in retention of miR-181 in the RISC complex and consequent prevention of its degradation (Fig. 24).

The Estrogen Receptors in the aging female brain

The estrogen receptors are the primary mediators of all of E2 actions within the cell. I had determined that four of these miRNAs (let-7i, miR-7a, miR-9, and miR-9-3p) were regulated by ERβ in the hypothalamus, although ERα was able to also regulate miR-9 and miR-9-3p.
Figure 24. mRNA transcript abundance prevents miRNA degradation
Since these miRNAs were not regulated by E2 at 4, 8, and 12 weeks post-OVX, it suggests that the estrogen receptors themselves might be altered during this time period. Therefore, I analyzed the mRNA expression of both ERα and ERβ, of which only ERβ expression significantly increased at 8 and 12 weeks post OVX. However, neither receptor was regulated by E2 treatment, but rather was regulated by age alone. Previous studies of estrogen receptor expression in the brain of middle age rats have shown mixed results of the effect of E2. One study by Yamaguchi et al. demonstrated that ERβ was decreased in several nuclei of the hypothalamus with E2 [211]. Another study by Chakraborty et al. showed that there was no effect of E2 on ERβ expression in the hypothalamus; however, they did show that ERβ expression was decreased in specific nuclei of the hypothalamus as a result of aging [198]. ERα expression has been shown be increased with aging in the hypothalamus [197]; however I did not see any change in the mRNA expression of ERα.

Another important consideration is that estrogen receptors do not act alone, and require assistance by a group of co-regulatory proteins. A study by Mott et al. demonstrated that ERβ’s interaction with co-regulatory proteins is altered with aging in the hippocampus [202]. In particular, Mott et al. identified HNRNP H as differentially associated with ERβ with aging. The HNRNP proteins, including HNRNP H, have been previously demonstrated to be key regulators of RNA processing as well as microRNA processing. The data from this study suggests that ERβ may alter miRNA expression by regulating the processing of miRNAs by interacting with HNRNP H. Additionally, the study found that altered interactions with these proteins could affect ERβ transcriptional
effects. For example gelsolin, which was increased in its association with ERβ in 3 month old animals with E2 but not in 18 month old animals, inhibited ERβ’s ability to increase transcription of an AP-1 luciferase reporter. The inhibition was relieved when gelsolin was knocked down by siRNA. These data suggest that co-regulatory associations can be altered with aged which can change how ERβ is able to regulate transcription.

Age dependent alterations in miRNA expression

Aging not only altered the regulation of these miRNAs by E2 but also altered their basal expression alone. Previous studies have also shown that miRNAs are altered with aging in the brain and that their expression pattern is often a reversal or extension of the developmental period [90,111,114]. These data suggest that there are other developmental factors that become dysregulated with aging resulting in the altered miRNA expression pattern. Some of the miRNAs I have identified in this study, such as let-7i and miR-9, are known to be important for neurodevelopment. For example, miR-9 has been previously been shown to be regulated at the transcriptional level by REST, a transcription factor key for suppressing neuronal genes in non-neuronal cells [150]. Similarly, the fragile X mental retardation (FMRP) is a key protein in neurodevelopment and its mutation results in mental retardation. Dicer can interact with FMRP and regulates the processing of pre-miR-9 cleavage to the mature miR-9, thereby altering miR-9 expression [212]. It is possible that both of these neurodevelopmental proteins, REST and FMRP are altered with aging and they also may interact with, directly or indirectly, ERβ to regulate miRNA expression. Identification of these other factors, developmental and
non-developmental, are critical for understanding how aging is altering the expression of these miRNAs.

MicroRNA Cellular Function

The main effect that miRNAs have on a cell is to repress the translation of protein coding genes by binding to the 3’ UTR. I used three different miRNA target gene prediction computer algorithms to identify potential candidates in order to understand the downstream effects of the miRNAs I identified. Out of the four likely gene targets identified, SIRT1 (sirtuin 1) was the only one shown to have decreased mRNA expression correlating with my observed increases in miRNA expression. It is important to note that I did not measure the protein levels of SIRT1 to see if they correspond to the mRNA expression. However, a previous study using 3’UTR luciferase reporter constructs demonstrated that SIRT1 is a genuine target for miR-9, increasing the likelihood that SIRT1 is being regulated an E2 regulated miRNA [173].

There are hundreds of possible gene targets for each miRNA due the fact that base pairing requirements are very loose, which makes target prediction algorithms somewhat unreliable since they are solely based on identifying complementary sequences between the miRNA and mRNA. There are multiple other factors besides the sequence that can affect targeting that can impact miRNA binding to a particular site, such as the secondary RNA structure of the 3’ UTR. Additionally, RNA binding proteins (RBP) not associated with the RISC complex can either promote or inhibit miRNA binding to an mRNA [174]. The precise mechanism of how a miRNA “chooses” a gene target is a key knowledge gap
that needs to examined if we are too be able understand the ultimate physiologically impacts of miRNAs. It is important to note, however, that there is a growing body of evidence suggesting that miRNAs are pleiotropic and can alter genes in a number of ways besides translational repression.

Non-canonical Functions of miRNAs

One of the first non-canonical functions discovered for miRNAs was the translational enhancement of ribosomal mRNAs by miR-10a [213]. The mechanism by which miR-10a enhanced the translation of these mRNAs was by binding to the 5’ UTR of the transcript, which would result in the recruitment of ribosomal components [213]. Since that publication, miRNA binding to the 5’UTR has also been demonstrated for miR-196b and miR-103a-3p [214,215]. These results underscore the importance of the location at which the miRNA binds on the transcript.

miRNA binding to an mRNA transcript was originally thought to occur only in the cytoplasm, and this is where the majority of studies have focused their attention. However there is recent evidence that miRNAs can be localized to the nucleus. The first study to demonstrate this showed that miRNAs could be localized to the nucleus based on the presence of a nuclear import sequence [216]. Additionally, argonaute proteins can also be localized to the nucleus where they are bound to miRNAs [217,218]. One of the proposed functions of nuclear miRNAs is to regulate gene transcription. For example, let-7i has been shown to bind to the TATA box promoter of interleukin 2, recruit RNA Pol II, and increase its transcription in CD4+ T lymphocytes [219]. Regulation of
transcription by miRNAs has also been shown to involve chromatin modifications via interactions with other non-coding RNAs, such as long non-coding RNAs (lncRNAs) [220,221].

Limitations of the Study

The experiments within this study have conclusively demonstrated that there is an age-dependency to E2 regulation of mature miRNA expression in the brain, and that ovarian hormone deprivation in aged female rats induces loss of E2 regulation of these same miRNAs. However, from these data it is not yet certain how E2 is regulating miRNA expression. The expression of the primary miRNA transcript sometimes correlates with mature miRNA, however there was not an observed correlative change in the precursor miRNA transcript for the majority of miRNAs tested. Additionally, E2 regulated miR-181a expression only at the mature miRNA level, which suggests a non-transcriptional mechanism for E2 action. The data obtained from these experiments were a snapshot of miRNA expression, obtained 24 hours after the last E2 injection was given. Therefore it is possible that any transcriptional effect of E2 could have occurred before 24 hours. Likewise, the precursor miRNA expression could have been processed well before the animals were sacrificed. Importantly, E2 has been shown to have rapid effects on neurons which can occur within minutes of treatment. The treatment paradigm used in the present studies did not allow for an understanding of the dynamics of miRNA expression, which can occur within minutes to hours of E2 treatment.
The method to analyze miRNA expression in the study was real time RT-qPCR; for which I used total RNA isolated from specific brain regions. The brain is composed of multiple cell types besides neurons, which the total RNA isolation would be pooling the transcriptome from all these cells. While previous studies have shown some of the miRNAs I identified to be restricted to a specific cell type (i.e. miR-7 and miR-9 are expressed only in neurons), there is still not much known about cell type specific miRNA expression in the brain. The functional significance of these miRNAs is therefore difficult to predict since the gene targets of a given miRNA depend on the cell type. Additionally, miRNAs can localize to specific areas of cell, which can alter function. The function of miRNAs is also dependent on other interacting proteins. For instance, studies have demonstrated that not all highly expressed miRNAs are associated with the RISC complex. Determination of the mechanisms governing and the roles of the identified miRNAs require more selective measurements and is an area for future research.

Future Studies

The results of the present study have laid a foundation upon which future experiments can be based in order to determine the precise regulatory mechanism used by E2 to alter miRNA expression and the ultimate function of these miRNAs. To this end, the treatment paradigm should include time points that are hours (1, 4, and 8 hours) after the last injection to determine when E2 is regulating miRNA expression.

Future experiments should also attempt to replicate the ovarian deprivation paradigm in younger (3 month) animals to determine if the effect of the deprivation is
independent of the age of the animal. Importantly, a group of animals that did not undergo OVX surgery are also needed to determine the effect aging coincident with naturally progressing reproductive senescence on miRNA expression.

The identification of specific miRNAs regulated by E2 in the brain allows for more in depth analysis of their regulation. To this end, cell specific expression and cellular localization should be assayed for the identified seven miRNAs by the use of in-situ hybridization. Concurrently, RNA-immunoprecipitations (RIP) should be performed to assay for changes miRNA association with the RISC complex with aging and E2 treatment.
CHAPTER VIII

MATERIALS AND METHODS

Ethics Statement

All animal protocols were approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, IACUC approval #2009018. Surgeries were performed under vaporized isoflurane anesthesia. Post-operation, animals were singly housed and provided with acetaminophen analgesic (122.7 mg/kg) in tap water for 3 days. All measures were taken to minimize pain and suffering.

Animals

Female Fischer 344 rats (N=20) were obtained from the National Institutes of Aging (NIA) colony (Taconic) at 3 and 18 months of age for the experiments in Chapter III. Female Fischer 344 rats were obtained from the National Institutes of Aging (NIA) colony (Charles River Laboratories, Wilmington, MA) at 18 months (N = 59) of age for the experiments in Chapter IV. The animals were allowed to acclimate to the housing facility for 7 days after arrival. Animals were housed two per cage and were allowed free access to food and water.

Surgery
One week after arrival, animals were deeply anesthetized with vaporized isoflurane and bilaterally ovariectomized (OVX). Briefly, the ovary and distal end of the uterine horn were pulled from the body cavity through a 1 cm incision made through the skin and body wall. The uterine horn was clamped with a hemostat and ligated proximal to the clamp. The entire ovary and distal uterine horn were then removed. Animals were singly housed and provided with acetaminophen analgesic (122.7 mg/kg) in their water for 3 days postoperative. During this time, animals were weighed once/day and their water intake measured. Following 3 days of analgesia the animals were double-housed with their previous cage mate for the duration of the experiment.

Treatment

Seven days following OVX animals in each age group were given a subcutaneous injection of either safflower oil (vehicle) (N=10/age group) or 2.5μg 17β-estradiol (E2, N=10/age group) dissolved in safflower oil once/day for 3 days in Chapter III experiments. In Chapter IV experiments, following OVX animals recovered for 1, 4, 8, or 12 weeks (N=11-12/age group). After the designated recovery time the animals were given a subcutaneous injection of either safflower oil (vehicle) (N=5-6/age group) or 2.5 μg/kg 17β-estradiol (E2, N=6/age group) dissolved in safflower oil once/day for 3 days.

We measured the E2 concentrations in both the E2 and vehicle treated animals by ELSIA (Enzo Life Sciences) after sacrifice. The animals were anesthetized and sacrificed by rapid decapitation 24 hours following the last E2 injection. Trunk blood was collected on
ice into heparinized 10 ml round bottom tubes, centrifuged at 4000 RPM for 8 min., and plasma stored at -20°C until further processing.

Experiments in Chapter III had the rat brains quickly removed, sagitally sectioned into left and right hemispheres, and the hippocampus was microdissected from the left side of the brain and then further separated into the ventral and dorsal sub-divisions. The ventral and dorsal hippocampi were placed in separate microcentrifuge tubes containing QIAzol lysis reagent (Qiagen) reagent for subsequent homogenization and RNA extraction. The right side of the brain was rapidly frozen in 2-methylbutane, sectioned at 200 µm on a freezing microtome, and the paraventricular nucleus (PVN, -1.40 to -2.12 mm relative to bregma), central amygdala (CeA, -2.12 to -2.80 mm relative to bregma), ventral hippocampus (vHIPP, 4.30 to 6.04 mm relative to bregma), and dorsal hippocampus (dHIPP, -2.30 to 4.16 mm relative to bregma) were microdissected using a Palkovit’s brain punch tool (0.75 mm, Stoelting, Inc., Wood Dale, IL).

In Chapter IV the brains were quickly removed, sagittal sectioned on ice into left and right hemispheres, and the whole hypothalamus was microdissected from the right side of the brain. The hypothalami were placed in separate microcentrifuge tubes containing QIAzol lysis reagent (Qiagen, Inc., Germantown, MD) reagent for subsequent homogenization and RNA extraction. The left side of the brain was rapidly frozen in 2-methylbutane, sectioned at 200 µm on a freezing microtome, and the whole hypothalamus (-0.26 to -4.52 relative of bregma), were microdissected using a Palkovit’s
brain punch tool (1.0 mm, Stoelting, Inc., Wood Dale, IL). Tissue punches were placed in a microcentrifuge tube on dry ice and stored at -80°C.

17β-estradiol plasma concentrations

The plasma samples first underwent a liquid-liquid extraction using diethyl ether. Briefly, 1.0mL of diethyl ether was added to 200ul of plasma sample in a microfuge tube and mixed (vortex) for 2 minutes. Organic phase was transferred to a glass tube containing 1.0mL of H2O and mixed (vortex) for 2 minutes. Phases were allowed to separate for 2 minutes. Organic phase was transferred to clean glass tube and dried using nitrogen gas. Samples were reconstituted using 17β-Estradiol high sensitivity ELISA kit (Enzo Life Sciences, #ADI-901-174) sample buffer. The kit protocol was then followed. ELISA plates were read on BioTek Synergy HT plate reader. The circulating plasma E2 concentrations of in the E2 and vehicle animals were 56.68 (+/- 20.29) and 36.10 (+/- 9.70) pg/mL respectively (Figure 1B); these E2 levels are consistent with physiological levels observed during late diestrous/early proestrous [222]. The intra- and inter-assay %CV was 4.23 and 6.85 respectively.

Cell Culture

IVB cells were generously donated from Dr. Kasckow. Both IVB and HT22 cells were maintained in DMEM with high glucose and L-glutamine with 10% FBS. Cells were seeded in to six well plates at 100,000 cells/well in DMEM phenol free media with charcoal dextran treated FBS. IVB cells were treated with vehicle (ethanol), 10nM E2, 100nM ICI-182,780, or E2+ICI for 16 hours. HT-22 cells were transfected with either 1.0µg pcDNA, rat ERβ1, or ERα with X-tremeGENE 9 DNA transfection reagent
24 hours after transfection, cells were then treated with vehicle (ethanol), 10nM E2, 100nM OHT, or E2+OHT for 16 hours.

RNA isolation

Total RNA was isolated from the whole right hypothalamus by the miRNeasy Mini Kit (Qiagen, Inc., Germantown, MD). All RNA samples were analyzed for quality by Nanodrop spectrophotometry and visualization of the RNA on 1.5% agarose gel.

miRNA microarray processing and analysis

5.0 μg of total RNA from the left side ventral hippocampus in each treatment (N = 10) and age group (N = 10) was sent to LC sciences for rat miRNA microarray processing (miRBase 18 probe content). Multiple redundant regions were included in each chip and each region comprised a miRNA probe region listed from Sanger miRBase Release 18.0. Multiple controls were also included in each chip to control for quality of chip production, sample labeling and assay condition. Analysis of the microarray data was initially performed by LC sciences and further validated in our hands. The raw data from the microarray was normalized using quantile normalization and the false discovery rate was set at 5%, which is considered highly stringent. miRNAs that were statistically significant between age and treatment group were validated in the ipsi- and contra-lateral sides of the brain using qRT-PCR. We also analyzed each of the normalization genes for evidence of E2 regulation and samples were normalized to housekeeping genes that were not significantly altered by E2 or age (i.e. RNU5G).

Precursor and mature miRNA cDNA synthesis
To assay for mature miRNA expression, 1.0 μg of total RNA was used to reverse transcribe miRNA using NCode™ VILO™ miRNA cDNA synthesis kit (Life Technologies, Carlsbad, CA). To assay for the primary and precursor miRNA, we utilized a miRNA specific reverse transcription method described previously [223]. Briefly, 2.0 μg of total RNA was added to 1.5 μL of primer cocktail containing 10 μM of antisense primers (Table 1). The reaction mixture was incubated at 80°C for 5 minutes, 60°C for 5 minutes, and then finally allowed to cool to room temperature. Reagents from the ThermoScript™ RT-PCR System (Life Technologies, Carlsbad, CA) were then added for a final volume of 20 μL. The reactions were incubated at 60°C for 45 minutes and then 85°C for 5 minutes. 1.0 μL of RNase H was added to each reaction and then incubated at 37°C for 20 minutes.

**mRNA cDNA synthesis**

2.0 μg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA).

**Quantitative Real-Time PCR**

miRNA and mRNA qRT-PCR was performed with Fast Start Universal SYBR Green Master Mix (Roche-Genentech, San Francisco, CA) on an Eppendorf Realplex4. Forward primers for specific miRNAs were designed as described in the Ncode™ miRNA First-Strand cDNA synthesis kit handbook (Life Technologies, Carlsbad, CA) and using miRBase 18 as a sequence reference. The small RNA, RNU5G, was used as a loading control and to normalize the data for analysis. The following program was used: 1) 95°C for 10 minutes, 2) 95°C for 20 seconds, 3) 59°C for 20 seconds, 4) 72°C for 10
sec, and melting curve analysis. miRNA expression was analyzed using the \( \Delta \Delta Ct \) method as described previously [224].

**Western Blots**

Protein lysate from hypothalamus tissue punches were isolated using T-PER reagent (Thermo Scientific). Briefly, tissue punches were homogenized in 500μl of T-PER reagent with 1x cOmplete mini EDTA-free protease inhibitor cocktail (Roche) with a motorized mortar. Tissue lysate was centrifuged at 10,000x g for 5 minutes and the supernatant was collected into a separate microfuge tube. Protein samples were then concentrated using a methanol-chloroform extraction as described previously (CITE). 60μg of total protein lysate was dissolved and boiled at 95°C for 5 minutes in a 1x reducing sample buffer (Thermo Scientific). Protein samples were ran on a 4-10% polyacrylamide gel at 90V for 20 minutes and then 120V for 60 minutes using Bio-Rad Mini-Protean 3 system. Separated proteins were then transferred on to PVDF Immoblion P membranes (Millipore) at 100V for two hours. Membranes were blocked with 5% BSA with 1x TBST (0.1% tween) for 1 hour. Membranes were then incubated with the indicated primary antibody (Table) overnight with shaking at 4°C. The primary antibody was removed and the membranes were rinsed three times with 1x TBST (0.1% tween). Membranes were then incubated with 1:5000 goat anti-rabbit HRP secondary antibody (Santa Cruz) in 5% BSA with 1x TBST (0.1% tween) for two hours with shaking at room temperature. Membranes were then rinsed with 1x TBST (0.1% tween) three times and then developed using Super Signal West Pico Chemiluminescent substrate (Thermo Scientific). Blots were visualized using the Bio-Rad Chemi-doc stations. Densitometry
analysis was performed using the Image Lab software (Bio-Rad). Membranes were then stripped using a mild stripping buffer (Abcam) and re-blotted with another primary antibody.

Statistics

Expression of miRNAs and mRNAs in the ovarian hormone deprivation paradigm were analyzed by two-way ANOVA with age and treatment as factors. A significant interaction between age and treatment was followed by a Tukey’s post hoc test to determine statistically significant differences (p<0.05) between groups. A separate Tukey’s post-hoc test was performed within groups that showed a statistically significant main effect of age and/or treatment. One-way ANOVA with treatment as the main factor, followed by Tukey’s posthoc test, was used to determine significant differences of E2, DPN, and PPT. All data are presented as mean ± SEM. Statistical significance was noted when p<0.05.
APPENDIX A

RAW VALUES FROM MICRORNA MICROARRAY
Table 2. Raw values from miRNA microarray

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<th>18 months</th>
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APPENDIX B

SUPPLEMENTAL 2-WAY ANOVA STATISTICAL VALUES
Table 3. DHipp 2-way ANOVA analysis

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Main Effect: Treatment</th>
<th>Main Effect: Age</th>
<th>Interaction: Treatment x Age</th>
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<tbody>
<tr>
<td>Let-7i</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( (F(1,16)=6.292, p=0.023) )</td>
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<tr>
<td>miR-7a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>( (F(1,15)=106.178, p&lt;0.001) )</td>
<td>( (F(1,15)=110.163, p&lt;0.001) )</td>
<td>( (F(1,15)=109.984, p&lt;0.001) )</td>
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<tr>
<td>miR-9</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
<tr>
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<td>( (F(1,15)=5.609, p=0.032) )</td>
<td>( (F(1,15)=4.726, p=0.046) )</td>
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<tr>
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<td>Yes</td>
<td>No</td>
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<tr>
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<td></td>
<td>( (F(1,15)=5.011, p=0.041) )</td>
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<tr>
<td>miR-125a</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>miR-181a</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
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<td>( (F(1,15)=32.893, p&lt;0.001) )</td>
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Table 4. PVN 2-way ANOVA analysis

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<th>Main Effect: Age</th>
<th>Interaction: Treatment x Age</th>
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<tbody>
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<td>Yes (F(1,14)=11.987, p=0.004)</td>
<td>Yes (F(1,14)=22.550, p&lt;0.001)</td>
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<tr>
<td>miR-7a</td>
<td>Yes (F(1,14)=4.810, p=0.046)</td>
<td>Yes (F(1,14)=6.065, p=0.027)</td>
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<tr>
<td>miR-9</td>
<td>Yes (F(1,12)=7.869, p=0.016)</td>
<td>Yes (F(1,12)=4.921, p=0.047)</td>
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<tr>
<td>miR-9-3p</td>
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<td>No</td>
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Table 5. CeA 2-way ANOVA analysis

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<tr>
<td>miR-7a</td>
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<td>miR-125a</td>
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REFERENCE LIST


17. Wharton W, Gleason CE, Miller VM, Asthana S (2013) Rationale and design of the Kronos Early Estrogen Prevention Study (KEEPS) and the KEEPS Cognitive and Affective sub study (KEEPS Cog). Brain Res 1514: 12-17.


VITA

The author, Yathindar (Yathi) S. Rao was born in Chicago, IL on February 26th, 1985 to Satya and Nagamani Rao. He attended and received a bachelor’s degree in Biology at Loyola University Chicago (Chicago, IL) in 2007.

In August of 2007, Yathi joined the master’s program of Molecular and Cellular Biochemistry at Loyola University Medical Center (Maywood, IL). Shortly thereafter, he joined the laboratory of Dr. Toni R. Pak, where he studied kisspeptin signaling in the hypothalamo-pituitary-adrenal axis. Yathi published his first paper on his master’s thesis and received his master’s degree in 2009. Yathi then joined the PhD program at Loyola and continued to work Dr. Pak’s lab studying the regulation of microRNAs in the aging female brain by 17β-estradiol. While at Loyola, Yathi received a travel award from the Endocrine Society, represented Loyola in the pre-doctoral oral presentation competition at the annual meeting of the Chicago chapter of Society for Neuroscience, and the Arthur J. Schmitt Dissertation Fellowship. Yathi married his fiancé, Georgina Giffin, in 2010. In October of 2014, Yathi and Georgina welcomed the birth of their first child Kailas Oliver Giffin-Rao.

After completing his Ph.D., Yathi will begin a post-doctoral position in Dr. Mary Jo LaDu’s laboratory at the University of Illinois at Chicago (Chicago, IL) where he will study apolipoprotein E (APOE) and Alzheimer’s disease.