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Peripubertal Binge Ethanol, Age and Sex Modulate Microrna Expression in the Ventral and Dorsal Hippocampus of the Adolescent Rat

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PERIPUBERTAL BINGE ETHANOL, AGE AND SEX MODULATE

MICRORNA EXPRESSION IN THE VENTRAL AND DORSAL HIPPOCAMPUS

OF THE ADOLESCENT RAT

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To Peter, Stephen and Matthew
For value, curiosity and balance

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ABSTRACT

Adolescent binge ethanol (EtOH) abuse induces long-term changes in gene expression, resulting in an increased risk for the development of adult mood disorders. microRNAs (microRNAs) are small, noncoding RNAs that regulate gene expression by translational repression. microRNAs are implicated as important contributors to the neural mechanisms underlying alcohol addiction and are also altered in response to EtOH in the developing brain and can be expressed in sexually dimorphic manners. The biogenesis of mature 22-24 nucleotide (nt), single-stranded microRNAs involves 1) transcription of a 100-1000 nt primary-microRNA (pri-microRNA) product, 2) cropping of the transcript by the enzyme Drosha into the preliminary-microRNA (pre-microRNA), 3) cleaving of the pre-microRNA by the enzyme Dicer and 4) loading of the mature microRNA onto the RNA induced silencing complex (RISC). The microRNA/RISC complex (miRISC) is guided to the microRNA target gene and induces either cleavage or translational repression of the messenger RNA (mRNA) transcript. Our lab has found that repeated binge EtOH exposure alters gene expression in the hypothalamus and dysregulates the Hypothalamic-Pituitary-Adrenal (HPA) Axis in a sexually dimorphic, long-term and gonadal hormone-dependent manner in Wistar rats, and that a subset of microRNAs targeting brain-derived neurotrophic factor (BDNF) and sirtuin 1 (SIRT1) are differentially expressed in the ventral hippocampus dependent on age and sex. We examine whether microRNA expression, microRNA biogenesis enzymes Drosha and Dicer, and microRNA target genes BDNF and SIRT1 are altered by peripubertal binge

EtOH in the ventral and dorsal hippocampus. We also document sex differences in the expression of microRNAs sensitive to EtOH and 17β-estradiol during pubertal development. Overall, we demonstrate : 1) peripubertal binge EtOH exposure induces long-term alterations in mature microRNA expression levels in the male rat hippocampus, and has the potential to modulate the expression of their downstream target genes, 2) expression profiles of EtOH-sensitive microRNAs, miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495, and their target genes, are dependent on sex and age in the pubertal rat hippocampus and therefore may contribute to sexually dimorphic hippocampus neurodevelopment, 3) expression profiles of E_2-responsive microRNAs, miR-7a, miR-9, miR-125a and miR-181a, are differentially dependent on sex and age throughout pubertal development, suggesting that they play distinct developmental roles during puberty and 4) EtOH-sensitive and E_2-responsive microRNAs have distinct expression profiles in the dorsal and ventral hippocampus throughout pubertal development, suggesting that their respective functions are region-specific. This research increases our understanding of how pubertal binge EtOH exposure affects microRNA expression, provides evidence that microRNA are expressed in sexually dimorphic patterns throughout pubertal development, and suggest that microRNAs play a role in normal pubertal hippocampus development as well as hippocampus dysfunction following adolescent alcohol abuse.

CHAPTER ONE

STATEMENT OF THE PROBLEM

Introduction

A division of the Department of Health and Human Services, the Substance

Abuse and Mental Health Services Administration (SAMHSA) has documented that

alcohol is the most highly abused drug amongst adolescents, that binge drinking begins

and peaks during puberty and that underage drinkers have the highest rates of alcohol

dependence (SAMHSA 2000, 2002, 2003, 2008, 2009). Such reports led the U.S.

Department of Health and Human Services Office of the Surgeon General to announce a

call to action aiming to prevent and reduce underage drinking. Despite these efforts, more

current reports from the Centers for Disease Control and Prevention (CDC) indicate that

binge drinking is an even bigger problem than previously thought and that it is

particularly serious and yet under-recognized among women and girls (CDC 2012, 2013).

Indeed, binge drinking during puberty puts adolescents at an increased risk for

neurological complications later in life [1,2,3,4,5,6,7]. For example, adolescent alcohol

abuse has long-term consequences on many neurological systems responsible for

learning, memory and the regulation of the stress response [8,9,10,11,12]. Indeed,

research demonstrates that the pubertal brain continues to develop well into the twenties

[13]. Adolescent brain development is characterized by a remodeling of neural networks

established in the perinatal period [14,15,16] and the generation of new connections,

which together, refine the neural control of behavior [17]. Yet, despite our increased understanding of how the adolescent brain develops and how alcohol abuse alters this development, many questions remain about what regulates alcohol abuse-induced brain damage, particularly with respect to gene expression regulation and possible sex differences.

Hypothesis and Specific Aims

Pubertal brain development is characterized, in part, by sexually dimorphic alterations in physiology and behavior and is predominantly thought to be regulated in a sex biased manner, yet, precise sex-specific mechanisms of gene regulation remain largely elusive. A major regulator of gene expression includes microRNAs (microRNAs), which are small, noncoding RNA molecules that target and bind, via imperfect base pairing, to protein-coding messenger RNA (mRNA) transcripts and lead to mRNA degradation or translational repression. Despite the relatively short time during which microRNAs have been researched (~12 years), they are currently recognized as regulators of at least 70% of fundamental biological processes [18,19]. Development of the pubertal brain is a dynamic biological process that involves sexually dimorphic anatomical changes in grey and white matter and cellular strengthening of relevant neuronal networks. Therefore, as master regulators of gene expression, microRNAs may play a role in orchestrating the complex molecular adaptations taking place during adolescent brain development. In this way, microRNAs may regulate the cellular, anatomical and behavioral transformations manifested throughout this significant stage of brain development.

Typical adolescent behaviors are often "high-risk," such as binge drinking, and moreover, can manifest in a sex-specific manner. Alcohol abuse induces particular morphological, cellular and molecular damage to the hippocampus, which plays a pivotal role in brain development, as it is the primary source of adult neurogenesis, spatial and emotional memory, and is a major contributor to the regulation of mood. Importantly, microRNAs have been linked to alcohol-induced neurological afflictions including addiction and fetal alcohol spectrum disorder (FASD) and in addition, demonstrate age, cell type and sex-specific expression profiles in multiple species. However, the normal developmental expression profiles of microRNAs during puberty and how peri-pubertal alcohol abuse alters these profiles, remains undocumented. Using multiple target prediction algorithms, four microRNAs (miR-10a-5p, miR-26a, miR-103 and miR-495) were identified to target the 3'UTR of two genes important for hippocampus function and development, *BDNF* and *SIRT1*. Importantly, miR-26a and miR-103 are in the top 15 most highly expressed microRNAs in the rodent hippocampus, miR-10a is important for developmental processes as it regulates homeobox (Hox) gene expression [20],[21] and miR-495 regulates the neuroprotective effects of mood stabilizing drugs [22]. Therefore, I generated the following hypothesis that *adolescent hippocampal microRNA expression throughout pubertal development is sex biased, dependent on age, and altered by peri-pubertal binge EtOH exposure*. The following specific aims were developed to test this hypothesis:

AIM 1: Determine whether peripubertal binge EtOH alters expression of microRNA, microRNA processing enzymes and microRNA target genes throughout pubertal development.

Pubertal binge drinking induces long-term changes in the expression of genes that regulate the stress response. An altered stress response often underlies depression and anxiety-related disorders. Importantly, these conditions are commonly experienced by over 50% of alcohol-dependent patients and present in a sexually-dimorphic fashion. Sex biased mood and memory impairments are often present in tandem with alcohol abuse and neuropsychiatric illnesses that emerge post-puberty. Disruption of mature microRNA expression and/or function has been linked to alcohol-induced neurological afflictions including addiction and FASD.

The following questions were answered in this aim:

1) Is the normal expression profile of miR-10a-5p, miR-26a, miR-103 and miR-495 during pubertal development altered in response to peri-pubertal binge EtOH in an immediate manner?

2) What is the long-term effect of peri-pubertal binge EtOH exposure on the expression profile of miR-10a-5p, miR-26a, miR-103 and miR-495?

3) What are the expression profiles of microRNA biochemical processing enzymes and the downstream target genes of miR-10a-5p, miR-26a, miR-103 and miR-495 (BDNF and SIRT1) throughout pubertal development immediately following mid/peri-pubertal binge EtOH exposure as well as 30 days post EtOH?

In order to answer these questions, males were chosen for the binge studies in order to avoid potential experimental design and data analysis complications due to the effects that cycling hormone levels in females would have on the results. Male Wistar rats were distributed into 3 groups: early pubertal (PND 30), peripubertal (PND 37), and

late pubertal (PND 73). The early, peripubertal and late pubertal age animals each had an untreated group (N=10/age group) which were sacrificed at PND 30, PND 44 and PND 73, respectively. In addition to the untreated groups at each age, there were 2 groups administered treatments at peripubertal age (N= 20/treatment group; total of 40 animals): peripubertal water (control) and peripubertal binge EtOH treated. The water and EtOH groups were handled for 5 minutes once/day beginning at PND 30 to eliminate non-specific effects of handling stress. Peripubertal water or EtOH treatments were treated with water or the following repeated binge alcohol (EtOH) paradigm via oral gavage beginning at PND 37 (3g/kg; 1x/day/3days EtOH, +1x/day/2days water + 1x/day/3days EtOH). Half of the peripubertal water/EtOH treated animals were sacrificed 60 minutes following the last EtOH/water treatment at PND 44 (N = 10 water + N = 10 EtOH). The remaining animals (N = 10 water + N = 10 EtOH) were left undisturbed following the last EtOH treatment in their home cage until sacrificed at late puberty (PND 73). Brains were sectioned at 200 μm on a freezing microtome and ventral and dorsal hippocampi were microdissected using a 0.75 mm Palkovit's brainpunch tool. Total RNA was isolated and cDNA was made using Invitrogen's NCode microRNA Frist-strand cDNA synthesis kit.

In summary, the data from this aim demonstrate that the expression of each microRNA tested (miR-10a-5p, miR-26a, miR-103 and miR-495) is dynamic across pubertal development and that the developmental profiles for each microRNA are distinct between the dorsal and ventral hippocampus. Moreover, peripubertal binge EtOH exposure altered normal pubertal development expression patterns of miR-10a-5p, miR-26a, miR-103, miR-495, Dicer, Drosha, BDNF and SIRT1 in an age- and brain region-

dependent manner. Most striking, our results showed that peripubertal binge EtOH exposure had significant long lasting effects on several microRNAs studied, as well as their processing enzymes and target genes. These effects were evident for as long as one-month following the last EtOH exposure, suggesting that EtOH could have lasting consequences on gene expression profiles in the male rat hippocampus through long-term regulation of microRNA expression patterns.

AIM 2: Determine whether there are sex and brain region differences in EtOH-sensitive microRNAs, microRNA processing enzymes and microRNA target gene expression throughout pubertal development

Pubertal binge EtOH changes in expression of genes regulating the stress response in a sexually dimorphic manner. microRNAs regulate gene expression by targeting RNA transcripts and have recently been recognized as critical mediators of nearly all basic cellular processes. microRNAs are small, non-protein coding RNAs which are sequentially processed to their mature form by the enzymes Drosha and Dicer, which allows them to bind their complementary mRNA sequences and lead to the prevention of gene translation. microRNAs regulate neuronal development during embryogenesis, postnatal neuronal maintenance and survival, and hippocampal neurogenesis throughout life. Preliminary data generated using an RT-microRNA-PCR array identified EtOH-sensitive hippocampal microRNAs and five microRNAs were chosen for further analysis: miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495. Importantly, these microRNAs target mutual genes—BDNF and SIRT1—which regulate synaptic plasticity [23,24,25,26,27] an essential element of hippocampus-dependent memory and mood processing. Moreover, pubertal EtOH abuse can alter the expression

of genes important for mood regulation in a sexually dimorphic manner, and some brain microRNAs are expressed differentially between males and females, but the fundamental sex differences in microRNA expression throughout puberty for EtOH-sensitive microRNAs is unknown.

The following questions were answered in this aim:

1) Are miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495 expressed in sex, age and brain region-dependent manners?

2) In the dorsal and ventral hippocampus, are Drosha and Dicer expressed in sex, age, and brain region-dependent manners and do their expression profiles suggest that they regulate miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495 expression profiles throughout pubertal development?

3) Are BDNF and SIRT1 expressed in sex, age, and brain region-dependent manners and do their expression profiles suggest that miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495 regulate their expression profiles throughout pubertal development?

In order to answer these questions male and female Wistar rats at PND 30, PND 44 and PND 73 (N=10/group) were sacrificed by decapitation, brains were rapidly frozen and sectioned at 200 μm, and the ventral and dorsal hippocampus were microdissected using a 0.75 mm Palkovit's brainpunch tool. RNA was isolated using TriZol according to manufacturers' instructions, and used to reverse transcribe microRNA (NCode™ microRNA First-Strand) and mRNA (SuperScript® VILO™) into cDNA. microRNA and mRNA expression levels were quantified using qRT-PCR with Fast Start Universal

SYBR Green Master Mix (Roche) and primers designed for the respective genes of interest.

In summary, data from this aim demonstrate that miR-10a-5p and miR-103 expression in females and miR-26a and miR-495 expression in males was dynamic across pubertal development in the dorsal hippocampus. In the dorsal hippocampus, the normal expression profile throughout pubertal development was significantly different between males and females at early puberty for miR-10a-5p and at each time point for miR-26a. miR-10a-5p, miR-26a, miR-103 and miR-495 expression profiles were dynamic in males as well as sexually dimorphic throughout pubertal development in the ventral hippocampus. Expression of BDNF and SIRT1 in males and SIRT1 in females was dynamic across pubertal development. Expression of BDNF and SIRT1 is significantly increased at peripuberty in males as well as sexually dimorphic in the ventral hippocampus.

AIM 3: Determine whether there are sex and brain region differences in expression of estrogen-responsive microRNAs throughout pubertal development.

Circulating levels of gonadal hormones increase rapidly throughout pubertal development, particularly estradiol (E_2) in females and play a role in the development of sexually dimorphic behaviors. Interestingly, microRNA expression has been demonstrated to be sensitive to E_2 in many systems. For example, Rao et al. demonstrated that the expression of five microRNAs, including let-7i, miR-9, miR-125a and miR-181a, are dependent on age and E_2 in female rats [28]. Furthermore, Morgan et al. demonstrated that blocking E_2 synthesis can alter microRNA expression in neonates [29] and suggest

that microRNAs may play an important role in initiating brain sex differences during fetal development. Overall, E_2 may modulate neuronal target genes using microRNAs as a fine tuning mechanism and it is important to determine pubertal sex differences in the expression of microRNAs sensitive to E_2, as such information may yield insight into whether microRNAs play a role in brain sex differences arising throughout pubertal development.

The following questions were answered in this aim:

1) Are let-7i, miR-7a, miR-9, miR-125a and miR-181a expressed in sex, age and brain region-dependent manners?

2) In the ventral hippocampus, specifically, how are let-7i, miR-7a, miR-9, miR-125a, miR-181a expressed in males and females throughout and is the expression of each microRNA dynamic throughout pubertal development?

3) In the dorsal hippocampus, specifically, how are let-7i, miR-7a, miR-9, miR-125a, miR-181a expressed in males and females throughout puberty and is the expression of each microRNA dynamic througout pubertal development?

In order to answer these questions, male and female Wistar rats at PND 30, PND 44 and PND 73 (N=10/group) were sacrificed by decapitation, brains were rapidly frozen and sectioned at 200 μm, and the ventral and dorsal hippocampus were microdissected using a 0.75 mm Palkovit's brainpunch tool. RNA was isolated using Trizol according to manufacturers' instructions, and used to reverse transcribe microRNA (NCode™ microRNA First-Strand) into cDNA. qRT-PCR was performed with Fast Start Universal

SYBR Green Master Mix (Roche), using primers designed for microRNAs of interest, to quantify microRNA expression levels.

In summary, data from this aim demonstrate that miR-7a, miR-9, miR-125a and miR-181a are expressed in sex, age and brain region-dependent manners. In the ventral hippocampus, expression of miR-7a, miR-9, miR-125a, miR-181a are sexually dimorphic throughout puberty, and miR-9 expression is dynamic throughout pubertal development. In the dorsal hippocampus, expression of miR-7a, miR-9, miR-125a, miR-181a are sexually dimorphic throughout puberty and expression of miR-7a, miR-9, miR-125a and miR-181a in female and miR-7a in males is dynamic throughout pubertal development.

CHAPTER TWO

LITERATURE REVIEW

Adolescence

Propensity for Binge Drinking

Adolescence in humans comprises, on average, the years between 10-25 years of age [30,31]. During this period, the brain is in an exceptionally plastic state, such that morphological changes occur in gray matter, white matter and ventricle volume [32,33] as well as in neural networks as due to highly active molecular events taking place involving neurogenesis [15] and synaptogenesis [34,35] as well as apoptosis [36] and synapse elimination [37,38]. This combination of complex molecular events gives rise to the adolescents' brain being extremely sensitive to environmental stimuli, yet, impulse control and inhibitory drive are some of the last behaviors to develop [39]. Together, these characteristics lend adolescents to engage in increased risky behaviors such as binge drinking [17]. Binge drinking is defined as raising the blood alcohol concentration (BAC) greater than the legal limit (>0.08 g/100 g) within a two-hour period, typically 3-4 servings of alcohol for a woman and 4-5 drinks for a man [40]. In 2007, SAMSA documented that on average, binge drinking begins around age 13 and peaks between ages 18-22 and that adolescents' drink 2 times more EtOH per occasion than adults (SAMSA, 2007). Using rat models of adolescent binge drinking, studies from our lab and others have demonstrated that peripubertal binge drinking induces long-lasting alterations

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in hypothalamus and hippocampus gene expression, suggesting that this behavior impairs normal brain development [8,10,11,12,41].

Alcohol Alters Neurodevelopment

Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis

Alcohol is a potent physiological stressor, as it activates the neuroendocrine stress response controlled by the hypothalamic-pituitary-adrenal (HPA) axis [42]. HPA axis activation can be induced by acute psychological or physical stressors and entails hypothalamic release of corticotrophin-releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and, in turn, causes the release of adrenal glucocorticoids. Further activation of the HPA axis is controlled by a negative feedback mechanism wherein the increased glucocorticoid (cortisol (CORT) in humans and corticosterone (CORT) in rodents) that is released from the adrenals inhibits further activation of the axis. Importantly, the development of HPA axis and other brain circuits is incomplete during puberty [43], making these immature neural networks at great risk for limited maturation and/or flawed development due to environmental insults that affect the brain, such as binge drinking. Indeed, the peripubertal rat is responsive acute EtOH, such that acute exposure significantly increases plasma CORT levels. However, following repeated exposure to high doses of EtOH (a.k.a. binge EtOH exposure) during puberty, HPA axis activation becomes desensitized, demonstrated by significantly lower plasma CORT levels compared to rats treated with a single high dose of EtOH [44]. Furthermore, EtOH abuse during puberty alters the expression of CRH in both an immediate and long-term fashion [12,44,45]. Moreover,

adult male rats exposed to peripubertal binge EtOH exhibit different responses to acute EtOH exposure compared to adult rats never previously exposed to binge EtOH, as indicated by different plasma CORT levels following a single dose of EtOH [12]. These data provide physiologic evidence that peripubertal binge EtOH induces long-lasting dysregulation of the neuroendocrine stress response, and suggest that peripubertal binge EtOH can have long-lasting consequences on an individuals' ability to respond to stress.

Interestingly, the effects of peripubertal alcohol abuse on the HPA axis are sexually dimorphic [42,44,46] and dependent on gonadal hormones [47]. Moreover, a dysregulated HPA axis is associated with mood disorders that present differentially in males and females and are often comorbid with alcohol abuse [48,49,50,51,52,53,54]. In females, fluctuating levels of circulating steroid hormones are associated with HPA axis activation [55] and the dysregulation of the HPA axis observed in patients with major depressive disorder (MDD) is associated with abnormal steroid hormone levels in women compared to healthy individuals [56]. Overall, EtOH consumption alters the adolescent stress response in a long-term manner which likely contributes to the development of anxiety and related mood disorders, however the molecular mechanisms underlying these events remain unclear. It is, therefore, possible that EtOH's effects on other brain regions such as the hippocampus, contribute to EtOH-induced HPA axis dysregulation, as the hippocampus has major neural connections to the hypothalamus.

Hippocampus Malfunctioning: Ventral and Dorsal Perspectives

The hippocampus is a functionally and structurally complex brain region. It traverses both the dorsal and ventral planes and has various connections to cortical and

limbic areas, allowing the hippocampus to contribute to numerous neural networks regulating a range of behaviors. The information flow into the hippocampus enters through a restricted area, yet the monosynaptic outputs connect to a large variety of cortical and subcortical regions and contribute to the complex network of hippocampus circuit. Anatomical and genomic evidence supports the hippocampus playing a role in regulating both memory and cognitive processing, as well as stress, emotion, sensory–motor integration and goal-directed activity via its dorsal and ventral regions, respectively [57,58,59]. Indeed, afferent and efferent connections to parahippocampal regions gradually shift along the dorsal and ventral axis and gene expression profiles differ between the two regions [60,61].

Importantly, alcohol abuse imparts particularly detrimental effects on both memory and mood functions supported by the hippocampus. For example, hippocampus-dependent memory capacity has long been evident in adults with a history of chronic heavy drinking [62] and long-term impairments in executive functioning, motor control and emotional memory capabilities following EtOH abuse are attributed to long-term alterations in neurocircuitry [63,64] and brain morphology [65,66]. Moreover, alcohol abusers often suffer from anxiety and other mood disorders [67,68,69,70,71,72], and both alcohol abuse and mood disorders are associated with a dysregulated stress response [73,74,75,76,77,78]. Therefore the effects of peripubertal binge drinking on the neuroendocrine stress response may be controlled by upstream mechanisms initiated within the hippocampus. Indeed, it is becoming more evident that heavy drinking during adolescence also damages hippocampus morphology, gene expression and behavior. For instance, adolescent rats previously exposed to EtOH perform worse on Morris water

maze, indicating that EtOH impairs spatial learning in adolescents to a greater extent than it does in adults [79]. Moreover, alcohol abuse by adolescents is also associated with a reduction in hippocampus volume [3] and EtOH abuse alters gene expression differentially in the ventral and dorsal hippocampus [8], but the mechanisms remain unclear.

Many studies have identified genes essential for hippocampus development including brain-derived neurotrophic factor (BDNF) which regulates dendritic branching, spine density, learning and memory, and neuronal survival and sirtuin 1 (SIRT1), a class III nicotinamide-adenine dinucleotide (NAD^+)-dependent histone deacetylase recently implicated in the regulation of hippocampal synaptic plasticity, neurogenesis and anxiety. Importantly, both BDNF and SIRT1 expression levels are sensitive to EtOH, but what regulates this sensitivity is less understood.

Hippocampal Brain-Derived Neurotrophic Factor Role in Development

BDNF is a member of the neurotrophin family and is essential for synaptic plasticity—the alteration of the strength between two neurons—which involves functional and structural changes at the synapse [80,81,82,83,84,85]. Synaptic plasticity is required for the consolidation of hippocampal-dependent learning [24,26,27]. Indeed, heterozygous *BDNF* knockout mice (+/-) have impaired learning and memory processing which can be rescued with recombinant BDNF in the hippocampus [24,26,27]. The hippocampus expresses an abundance of BDNF, which is particularly essential to neuronal survival processes. BDNF is expressed in the astrocytes, fibroblast and various types of neurons found within the hippocampus, hypothalamus, amygdala and neocortex.

The highest expression of BDNF mRNA in the brain is found in the granule neurons of the dentate gyrus and the pyramidal neurons of areas CA3 and CA2 in the hippocampus, indicating that BDNF plays a critical role in hippocampus functions [86]. Indeed, hippocampus-dependent memory can be disrupted by EtOH [87,88,89]. For example, early postnatal pups exposed to EtOH vapor inhalation for 2 hours and 40 mins each day on PND 4-10 had significantly increased BDNF protein expression [90]. However, other studies suggest that the adolescent brain is particularly sensitive to EtOH-mediated changes in the molecular mechanism underlying learning and memory [4,5,91]. Importantly, alterations in hippocampal BDNF expression lead to reduced hippocampal volume and poor performance on declarative memory tasks and impaired fear extinction [92]. Moreover, because BDNF expression is critical for memory formation and EtOH increases BDNF levels while disrupting memory function, it is important to understand how BDNF may play a role in the mechanisms underlying EtOH-mediated effects on memory function. Importantly, EtOH's effects on various brain functions are likely mediated by multiple proteins with gene regulatory capacity, and interesting research indicates the chromatin modifying enzyme sirtuin1 (SIRT1) in the regulation of gene expression following EtOH abuse [93].

Hippocampal Sirtuin 1 Role in Development

SIRT1 is a NAD+ - dependent protein deacetylase and therefore, has the potential to regulate a large variety of biological functions by silencing the expression of genes coding for a wide range of cellular processes. Indeed, sirtuins are involved in neuronal development, neuroprotection, dendritic branching and neurogenesis [94,95,96,97,98,99,100] as well as hippocampus-specific functions including memory

performance and mood regulation. For example, SIRT1 expression in the hippocampus is

positively correlated with adult hippocampal-dependent learning and memory

[23,25,101], however, it is also mediates anxiety in mice [102]. These data suggest that

SIRT1 may play an important role in both dorsal and ventral hippocampus functions.

Moreover, SIRT1 gene variants are associated with a risk for anxiety [102], which is

often comorbid with alcohol abuse [67,68,69,70,71,72]. Indeed, adolescent binge

drinking in rats differentially alters SIRT1 expression levels in the male rat dorsal and

ventral hippocampus [8] and SIRT1 expression is altered in the hypothalamus of male

mice exposed to alcohol in utero [93]. Interestingly, many of the SIRT1 functions are

associated with changes in expression levels of microRNA (microRNA) [8,23], small

RNA molecules with gene regulatory potential. Indeed, the importance of SIRT1 in

mediating hippocampal development and functions, as well as its potential to do so via

microRNAs, requires future research to elucidate these mechanisms.

microRNA

What are microRNAs?

Whole-genome sequencing data from a variety of species have underscored the

importance of post-transcriptional and post-translational modifications needed to achieve

extensive phenotypic diversity. microRNAs (microRNAs) are small, non-protein coding

RNAs (~22-nt long) and contribute to the complexity of the regulation of gene

expression by mediating downstream target gene expression via translational repression

and/or degradation. In this fashion, they can regulate virtually all biological processes

[18,19]. 50% of microRNA genes are located in intergenic regions of the genome, 40%

within gene introns, and 10% are situated in exons, and thus, some microRNA expression mimics that of their host gene(s), however, much remains to be discovered regarding how microRNA expression is regulated. Evolutionarily, the emergence and conservation of microRNA genes localized within host protein-coding genes suggests that this localization emerged in response to environmental pressures that required tight control of gene expression and coordination of specific cell functions [103]. Importantly, microRNAs expression profiles are region-specific in the brain and [8,104,105,106,107,108,109,110] play critical roles in neuronal growth and synaptic plasticity [111,112,113,114,115,116] and their dysregulation has been implicated as a causative factor in a variety of neuropathologies [117,118,119,120,121,122]. Therefore, it is important to determine the mechanisms of microRNA biogenesis, regulation and dysregulation in both healthy and pathological tissues in order to better understand the mechanisms of microRNA-based regulation of gene expression and the consequences on biological phenotypes [123,124].

microRNA Biogenesis

The biogenesis of most mature 22-24 nucleotide (nt) single stranded microRNAs involves 4 processes: 1) transcription, 2) cropping, 3) dicing and 4) loading. microRNAs are transcribed in an RNA Polymerase II-dependent fashion which produces an intergenic pri-microRNA, about 100-1000 nt in length which forms a stem-loop structure [125,126]. Cropping of microRNA is performed by the microprocessor complex, composed of Di George Syndrome critical component 8 (Dgcr8), which recognizes the stem-loop structure, and the RNase III enzyme Drosha, which contains the catalytic component important for cleaving the double stranded stem, removing the loop, and generating the

pre-microRNA product [127,128]. The pre-microRNA is ~ 70-100 nt in average length and are exported from the nucleus. In the cytosol, the dicing step occurs via the RNase III enzyme Dicer, which catalytically cleaves the pre-microRNA into a double-stranded ~22 nt-long product, one strand of which is the mature microRNA. In the loading step, the single-stranded mature microRNA is transferred to the RNA-induced silencing complex (RISC), which comprises the microRNA-RISC complex (miRISC) which allows microRNAs to locate specific messenger RNA transcripts target genes and this leads to either translation repression or mRNA degradation. Interestingly, microRNAs imperfectly bind small (~6 nt) seed sequences on the 3' UTR of their gene targets, permitting promiscuity to microRNAs, as a single microRNA can target multiple genes [129]. Likewise, one gene can be targeted by multiple microRNAs. Such features enable microRNAs to mediate quick, post-transcriptional gene silencing which would be particularly useful in the regulation of synaptic plasticity.

microRNAs Regulate Neurodevelopment

The central nervous system (CNS) development requires a precise temporal orchestration of events that is uniquely suited for the fine-tuning attributes of microRNAs. The importance of microRNAs in embryonic nervous system development was originally demonstrated using transgenic animal models that manipulated microRNA biosynthetic processing enzymes, including Dicer. There is a single gene that encodes Dicer in C. elegans, mice, and humans, and depletion of Dicer results in severe developmental consequences. Studies in Dicer-mutant zebrafish showed that they had disrupted embryonic morphogenesis and neural differentiation [130]. More specifically, the brains lacked ventricles, neuronal positioning was disrupted suggesting migration

defects, and many neurons had defasciculated axons [130]. Strikingly, injections of microRNAs from the miR-430 family (miR-430a/b/c) reversed many of the brain morphogenic defects that resulted from Dicer deletion in the zebrafish, revealing a direct connection between mature microRNAs and Dicer during development. The partial rescue of neuronal defects in this study also provided some of the first evidence for tissue-specific effects of microRNAs [130,131]. Global Dicer deletion in mice is embryonic lethal [132] prompting the generation of tissue-specific conditional Dicer-null mouse models. In the developing neocortex, the absence of Dicer resulted in a smaller cortex, improper cortical layering, increased apoptosis, as well as an overall reduction in neural progenitor cells and oligodendrocytes [133,134,135].

microRNAs and their biogenesis enzymes are found enriched in the synapto-dendritic compartments of neurons [113,136,137,138] indicating their potential role in regulating synaptic plasticity. Indeed, rapid changes in local protein synthesis triggered by synaptic activation [139,140,141] may rely on the function of microRNAs [142]. It has been demonstrated that expression of the important synaptic plasticity molecule BDNF is regulated by miR-26a which targets the conserved sequences of the BDNF 3'UTR [143]. Moreover, miR-26a and BDNF expression have been implicated in the vulnerability and onset of schizophrenia, alcohol abuse and mood disorders in both human patients and rodent models [143,144,145,146,147,148]. In the brain, it has been demonstrated that microRNAs play a role in synaptic plasticity [113,136,137,138,149] are expressed in a sexually dimorphic manner [150] and that their expression is dependent on steroid hormones [28]. Lamina-specific expression of miR-495 was observed to be complementary to the BDNF expression in human prefrontal cortex [151].

Furthermore, miR-495 has been associated with the mechanism whereby the mood stabilizers valproate and lithium exert their neuroprotective effects [152]. Overall, microRNAs have the capacity to regulate neuronal development and maintenance—essential processes for a functioning nervous system and they also demonstrate sensitivity to mood altering substances, including EtOH.

Alcohol Effects on microRNA

The effects of alcohol on brain microRNA remains an ongoing investigation, however many recent developments have pointed towards the sensitivity of microRNA expression in response to EtOH. In early life development, fetal brain microRNA expression levels are altered by high levels of alcohol consumption by the pregnant mother, and importantly, these changes in microRNA expression following maternal EtOH abuse have been implicated in the teratology of EtOH-induced malformations in fetal brain development [153,154]. Furthermore, microRNA expression levels are known to exhibit differential expression profiles in fetal vs. postnatal brain, suggesting that microRNAs are fundamentally involved in regulating developmental processes in the brain [80,105,155,156] and suggest that alterations in microRNA expression in response to EtOH can impart damage to brain development. microRNA expression levels in the adolescent rat brain are also altered following peripubertal binge drinking [8] and in the rat prefrontal cortex in alcohol dependent rats [157]. Importantly, peripubertal or adult alcohol abuse in rats does not impart global changes in microRNA expression levels, but rather alters the expression of specific microRNAs in distinct regions of the brain. This specificity suggests that EtOH alters specific microRNA expression levels in order to damage specific brain functions. microRNA expression profiles are also distinct in

human alcoholic prefrontal cortex, providing further evidence that EtOH mediates brain damage by altering microRNA expression [158]. Identifying the effector functions of the target genes of EtOH-sensitive microRNAs is indeed a more challenging task, however, in both rat prefrontal cortex and hippocampus, BDNF has been identified as a target gene of EtOH-sensitive microRNA [157], suggesting the importance of the microRNA-mediated regulation of BDNF in the brain, and the potential for alcohol abuse to disrupt this regulation. How microRNA expression levels throughout the lifespan are expressed and what regulates their expression still remains unclear, however much evidence suggests the importance of gonadal hormones in this process. Indeed, gonadal hormones levels themselves are dynamic throughout different developmental stages of life as well as responsive to changes in the environment.

Gonadal Hormone Effects on microRNA

Steroid hormone regulation of microRNAs has been documented in a variety of sex-specific cancer models such as breast, prostate and endometrial cancer [159,160,161,162,163], however, less is known about steroid hormone regulation of microRNA expression in brain tissue. Nonetheless, studies examining the effects of sex steroid-induced microRNA regulation using in vivo models have indicated that sex-steroid regulation of microRNA expression in the brain differs across the lifespan and support the theory that sex steroid functions in the brain evolve with age. For example, blocking synthesis of E_2 in the neonate brain induces sexually dimorphic patterns of microRNA expression, such that males and female microRNA expression profiles become indistinguishable [29]. In the aging female brain multiple microRNAs were identified as E_2-responsive in the hippocampus, central amygdala and the paraventricular

nucleus, some of which were differentially regulated by E_2 dependent on age [28]. Despite these informative studies demonstrating steroid-mediated microRNA expression regulation in the brain, the mechanism remains unresolved.

microRNA expression changes in response to differential hormone environments indicate that hormones may mediate changes in microRNA expression via their nuclear receptors. Steroid hormone receptors (i.e. nuclear receptors (NRs) classically function as transcription factors, by binding to promoter elements and recruiting coactivators and corepressors, together, induce gene activation or suppression. Because microRNA genes often lie within the protein-coding regions of genes, NRs can thereby regulate microRNAs in this way. Alternatively, downstream target genes regulated by NRs may, themselves, influence subsequent microRNA gene expression in an indirect fashion. Indeed, many NR target genes are transcriptionally regulated via secondary effects [164]. Another indirect manner in which microRNA expression can be regulated is via the proteins required for microRNA biogenesis and RNA interference (RNAi) activity, including the RNase III enzymes Drosha and Dicer which are responsible for the sequence-specific cleavage of complementary RNA targets. Indeed, E_2 has been demonstrated to mediate expression of these proteins [165], however, no such regulation has been documented in the brain.

Summary

Peripubertal neurodevelopment is an important period of brain development wherein increased synaptic plasticity lends environmental exposures to play a large role in shaping how the neural circuitry develops. A common environmental exposure in the

United States is engagement in binge EtOH drinking behavior, which has been demonstrated to have sexually dimorphic, long-lasting neurological consequences in hippocampus-mediated functions. Neurological consequences of EtOH abuse have been recently demonstrated to alter microRNA expression, which have also recently been discovered to play vital regulatory roles in neuronal maintenance. Therefore, we conclude the introduction with the following hypothesis and aims to investigate microRNA expression in male and female pubertal hippocampus development as well as investigate the effects of peripubertal binge EtOH.

CHAPTER THREE

LONG-TERM EFFECTS OF PERIPUBERTAL BINGE ETOH EXPOSURE ON
HIPPOCAMPAL MICRORNA EXPRESSION IN THE RAT

Introduction

Heavy episodic alcohol consumption (i.e. binge drinking) has been steadily

increasing among adolescents in recent decades [40,166]. Indeed, data from the

Department of Health and Human Services: Substance Abuse and Mental Health Services

Administration (SAMHSA 2005) showed that an alarming 90% of the alcohol consumed

by youth occurs in a binge-like pattern, defined as raising the blood alcohol concentration

(BAC) above the legal driving limit (0.08%) within a 2 hour time period (NIAAA, 2012).

Extensive remodeling of the brain occurs during adolescence, which includes changes in

cortical gray and white matter, synaptic connectivity, and increased neurogenesis

[167,168,169,170,171] and alcohol exposure during this critical time can have severe

detrimental effects on brain function [44,172,173,174,175,176]. Studies from our

laboratory and others have demonstrated that adolescent binge-pattern alcohol exposure

results in long-term dysregulation of the neuroendocrine stress response, memory

impairments and behavioral deficits [12,67,177,178,179].An altered stress response often

underlies depression- and anxiety-related disorders and importantly, these conditions are

commonly experienced by over 50% of alcohol-dependent patients [67,180,181]. Indeed,

mood and memory impairments are often present in tandem with alcohol abuse and

neuropsychiatric illnesses that emerge post-puberty [182,183].The hippocampus is an important brain region that mediates learning, memory, and mood and it has been well established that hippocampus structure and function is impaired by EtOH abuse [10,11,184,185,186,187,188]. Notably, pubertal EtOH abuse inhibits adult neurogenesis, impairs learning and memory in adulthood, and impairs information retention. The precise molecular mechanisms mediating the long-term effects of adolescent binge EtOH exposure are poorly understood, however short non-coding regulatory RNAs are sensitive to EtOH and have recently been recognized as critical mediators of nearly every basic cellular process [154,189]. In particular, microRNAs (microRNAs, ~22 nucleotide single-stranded non-coding RNA) regulate the translation of proteins important for neuronal development during embryogenesis, postnatal neuronal maintenance and survival, and hippocampal neurogenesis throughout life [112,113,190,191,192,193]. Moreover, disruption of mature microRNA expression and/or function has been linked to alcohol-induced neurological afflictions including addiction and fetal alcohol spectrum disorder (FASD) [194,195]. In this study we used a Wistar rat model to identify EtOH-sensitive microRNAs that target genes involved in regulating hippocampal processes, such as memory and mood. Using multiple target prediction algorithms (Targetscan: www.targetscan.org; microRNA database: www.miRDB.org) [129,196,197], we identified brain-derived neurotrophic factor (BDNF) as a target gene of miR-10a-5p, miR-26a, miR-103, and miR-495, synapsin-2 (SYN2) as a target gene of miR-32, and sirtuin 1 (SIRT1) as a target gene of miR-26a, miR-103 and miR-495. BDNF and SYN2 are both critical regulators of synaptic plasticity. For example, BDNF is a neurotrophic factor instrumental in neurodevelopment and alterations in its expression are evidenced in

numerous psychiatric disorders, SYN2 is a synaptic vesicle phosphoprotein that modulates neurotransmitter release onto the post-synaptic membrane and its dysregulation disrupts the delicate balance of excitatory and inhibitory neurotransmission throughout the brain whereas SIRT1 is a class III protein deacetylase that has been recently associated with anxiety behavior [102,180,181,198,199].

We tested the hypothesis that mid/peripubertal binge EtOH exposure alters hippocampal microRNA expression and that this leads to changes in the expression of their target genes. Importantly, the normal expression profile of these particular microRNAs during pubertal development has not been reported in any species studied to date. Therefore, we quantified the normal developmental expression profile of miR-10a-5p, miR-26a, miR-103 and miR-495 in the rat hippocampus at three time points in pubertal development (early, peri, and late). Next, we determined how peri-pubertal binge EtOH exposure altered those normal expression levels immediately following EtOH exposure, as well as 30 days after the last EtOH dose using a binge pattern that is reliable for testing the effects of typical adolescent alcohol abuse [44,200]. Gene expression levels of microRNA processing enzymes, Drosha and Dicer, were also quantified at each time point in order to determine a possible molecular mechanism for binge EtOH effects. Lastly, to determine the downstream physiological effects of alterations in microRNA target gene transcription, we quantified the gene expression levels of putative microRNA target genes, BDNF and SIRT1 as well as their protein expression levels. Overall, our data provide evidence that peripubertal binge EtOH exposure induces long-term alterations in mature microRNA expression levels in the rat

hippocampus, which has the potential to modulate the expression of their downstream target genes.

Approach

Figures 2 and 3: Male Wistar were handled daily beginning at PND 30 and given one of the following treatment paradigms starting on PND 37 (n=6/group): 1) saline control (1x/day/8days), 2) acute EtOH (saline 1x/day/7days + 1x/day/1day EtOH) or 3) binge EtOH (1x/day/3days EtOH + 1x/day/2days saline + 1x/day/3days EtOH) via intraperitoneal injection. One hour after the last dose, brains were collected, rapidly frozen, sectioned at 200 μm on a freezing microtome and the whole hippocampus was microdissected using a 0.75 mm Palkovit's brainpunch tool. The array was performed using SABiosciences RT^2 miRNA PCR Array. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to quantify the expression levels of six microRNAs: (miR-10a-5p, miR-26a, miR-32, miR-103, mmiR-423 and miR-495) as well as BDNF and SIRT1 mRNA.

Figures 4-13: The dorsal and ventral hippocampus were microdissected from male Wistar rats that were either untreated or treated with an EtOH paradigm administered via oral gavage beginning at PND 37 (3g/kg; 1x/day/3days EtOH, +1x/day/2days water + 1x/day/3days EtOH) . Brains collected at three different ages during pubertal development (early puberty, PND 30; mid/peripuberty, PND 44; late puberty, PND 71; N=10/group). Importantly, peripuberty in rats is considered to be ~ PND 30-45 [201,202]. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to quantify the expression levels of a total of five microRNAs: (miR-10a-5p, miR-

26a, miR-32, miR-103 and miR-495) and four genes (Drosha, Dicer, BDNF and SIRT1). Western Blot was used to detect protein expression of pro-BDNF, BDNF and SIRT1.

Results

Blood alcohol concentrations following peripubertal binge EtOH exposure

In order to model typical pubertal binge alcohol drinking behavior, we utilized an 8-day binge EtOH exposure paradigm that has been used previously to mimic the pattern of alcohol consumption commonly observed in human adolescents (Fig. 1A) [8,44]. Experimentally, this paradigm does not affect body weight/growth curves during pubertal development in male rats [8,44,200]. Blood alcohol concentrations (BAC) were measured on the final day of treatment (day 8) 60 minutes following the last dose. The BAC in EtOH treated animals was 190 ± 21 mg/dl which was consistent with our previous studies using this peripubertal binge EtOH paradigm (Fig. 1B) [8,44,200]. Overall, our observed BAC levels fall within the defined BAC threshold of binge drinking.

miR-10a-5p, miR-26a, miR-103, miR-32 and miR-495 expression levels are sensitive to peripubertal binge-pattern EtOH exposure in the rodent hippocampus.

In the brain, microRNAs play an important role in neurodevelopment and neurogenesis, two processes that continue to occur throughout adolescent development [203] and microRNAs are implicated in the teratology of EtOH-induced malformations infetal brain development [153,154]. Importantly, our understanding of microRNAs in the brain during adolescent development is severely limited and there is little to no data
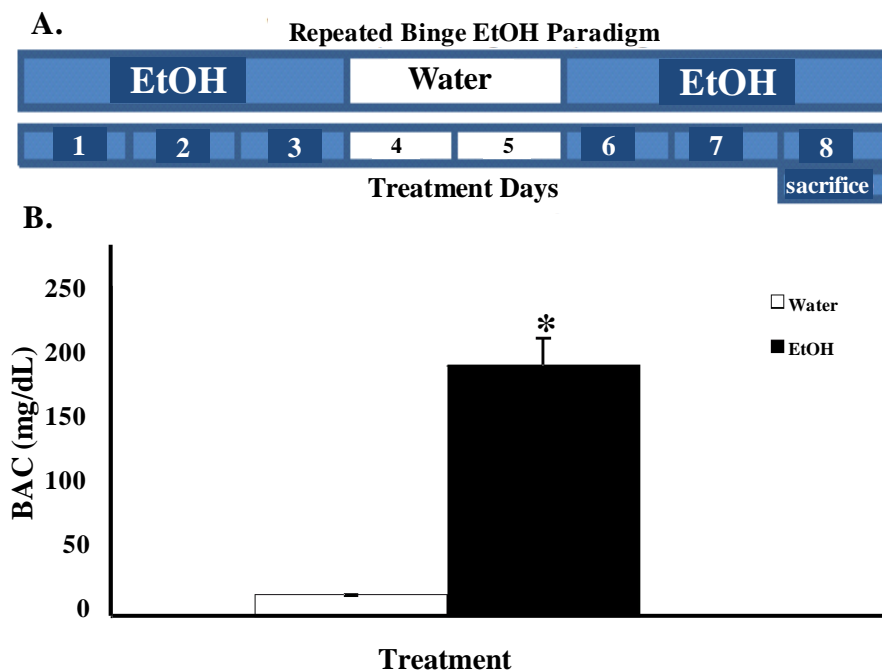
Figure 1: Peripubertal repeated binge EtOH paradigm alters blood

alcohol concentration. On PND 37, male Wistar rats are given 3g/kg

EtOH (20% v/v in water), or water alone, via oral gavage at 10AM

and this process is repeated according to the following schedule: 3d

EtOH, 2d water, 3d EtOH (A). Blood alcohol concentrations (BAC)

1.0 h following the last dose of water (control) or peripubertal binge

EtOH (B). Data expressed as mean BAC (mg/dl). *Statistically

significant difference via Student's T-test N=6 (p<0.05).

describing how EtOH might affect microRNAs during this critical developmental period.

microRNA expression was measured in the hippocampus of male rats treated with an 8-

day binge EtOH paradigm (see Fig. 1A) using a microRNA RT-PCR profiling array

containing a genome-wide panel of the 88 most well-researched rat microRNAs (as

annotated by the Sanger miRBase Release 14). We identified 2 novel EtOH-sensitive

microRNAs (miR-10a-5p and miR-423) which demonstrated altered expression levels

following an 8-day peripubertal binge-pattern EtOH exposure. Specifically, miR-10a-5p

and miR-423 expression levels were increased >10-fold in the hippocampus of binge-

EtOH treated rats (Fig. 2). We next designed our own primers to validate the array-

generated results with qRT-PCR and when using these primers, the expression level of

miR-10a-5p increased in the binge-treated animals as it did in the array (Fig. 3A).

However, miR-423 expression levels did not change when using the new primers, and

therefore, miR-423 was not further studied. To add depth to the study, we also quantified

the expression levels of additional microRNAs, miR-26a, miR-32, miR-103 and miR-495

that were predicted to target genes which regulate hippocampus functions. These data

revealed that peripubertal binge-pattern EtOH exposure increased miR-32, miR-103 and

miR-495, and decreased miR-26a expression levels in the male rat whole hippocampus

(Fig. 3A). Using the target prediction algorithms available via Targetscan:

www.TargetScan.org and microRNA database: www.miRDB.org [129,196,197], we

identified BDNF and SIRT1 as a putative target genes of miR-10a-5p, miR-26a, miR-103

and miR-495. These computer algorithms identify predicted microRNA target genes
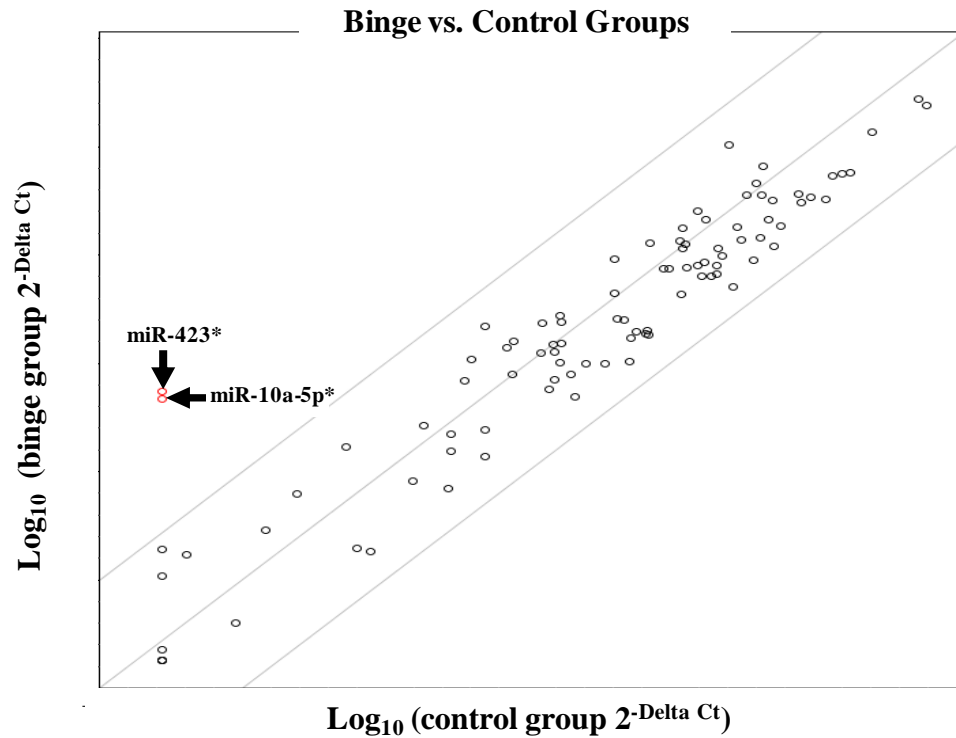
Figure 2. Peripubertal binge EtOH increases miR-10a-5p and miR-423

expression in the male rat whole hippocampus. Scatter plot data represents

microRNA expression levels in binge EtOH treated rats relative to controls

(open circles). miR-423 and miR-10a-5p (red) expression levels increased >

10-fold in binge EtOH-treated rats compared to control (one-way ANOVA,

*p<0.05).
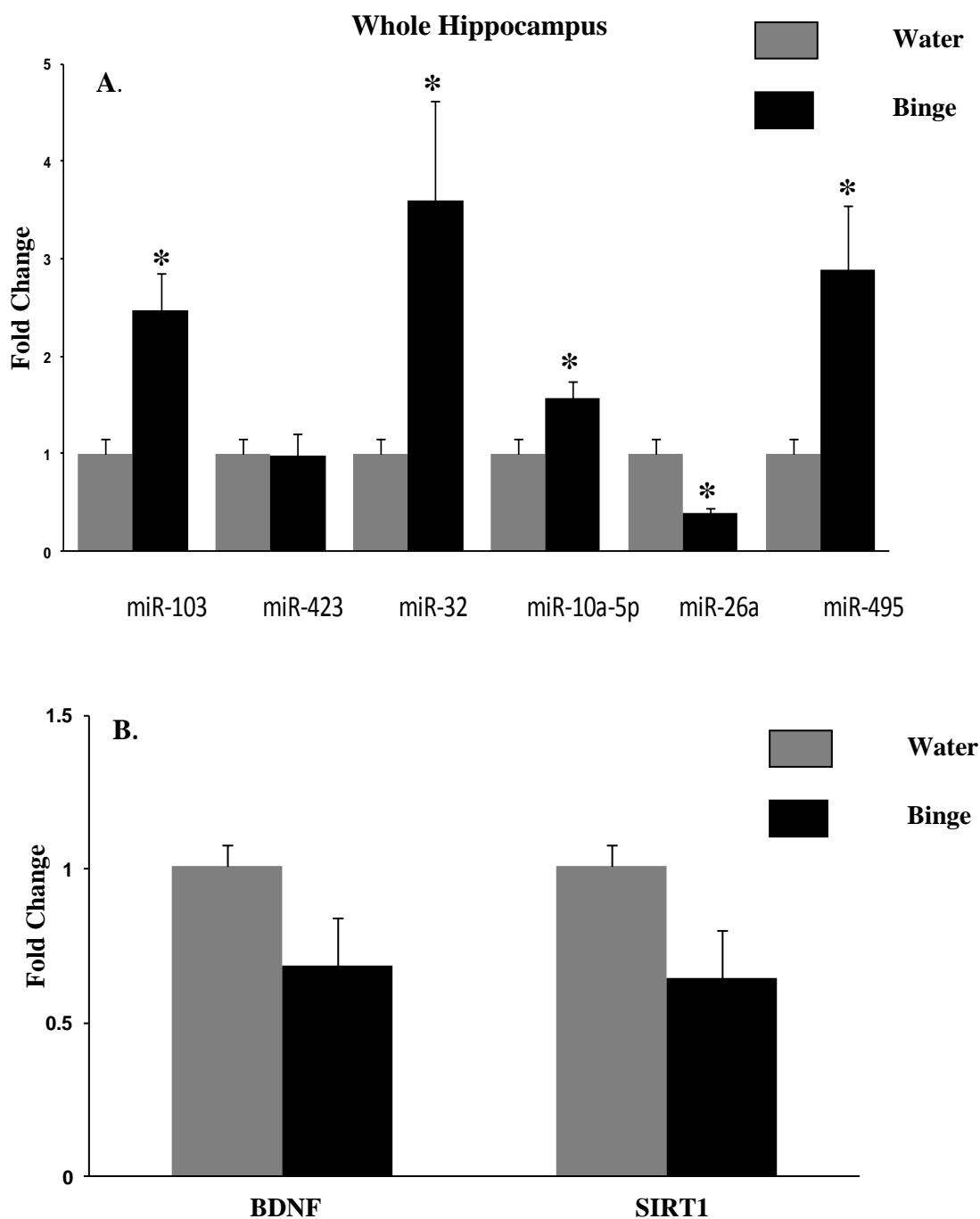
Figure 3: Peripubertal binge-pattern EtOH exposure alters microRNA and target gene expression in male rat hippocampus. microRNA (A) and target gene (B) expression levels 1.0 h following the last dose of water or EtOH. Data represent mean fold change ± SEM as compared control. * indicates significance between groups (one-way ANOVA, $p < 0.05$). target gene. Analysis of the potential target genes for these EtOH-sensitive microRNAs,

we identified one reported binding site for miR-10a-5p, miR-26a and miR-103 within the 3'UTR of BDNF and four for miR-495. One binding site was reported within the 3'UTR of SIRT1 for miR-26a, mir-103 and miR-495. Importantly, the number of predicted binding sites is indicative of the relative specificity of the microRNA for that gene.

We next quantified the expression levels of the EtOH-sensitive microRNA gene targets, BDNF and SIRT1, which displayed strong decreasing trends in the binge EtOH-treated animals, but did not reach not statistical significance (Fig. 3B). Importantly, the male rat brain displays both age-, cell type- and region-specific microRNA expression profiles and the hippocampus consists of functionally distinct ventral and dorsal regions [57,58,105,204,205]. Therefore, we next aimed to determine the potential for peripubertal binge EtOH to induce region-specific alterations in the ventral and dorsal hippocampus.

Mature miR-10a-5p, miR-26a, and miR-495 expression levels in the dorsal hippocampus of untreated male rats are age dependent.

Expression levels of these specific mature microRNAs in the brain during pubertal development have not been previously described. Therefore, we first determined the normal developmental profile of mature miR-10a-5p, miR-26a, miR-103 and miR-495 expression in the dorsal hippocampus using untreated male Wistar rats. miR-32 expression levels were not significantly altered in the dorsal or ventral hippocampus by age or EtOH, and therefore, it was not further studied. Mature microRNA expression
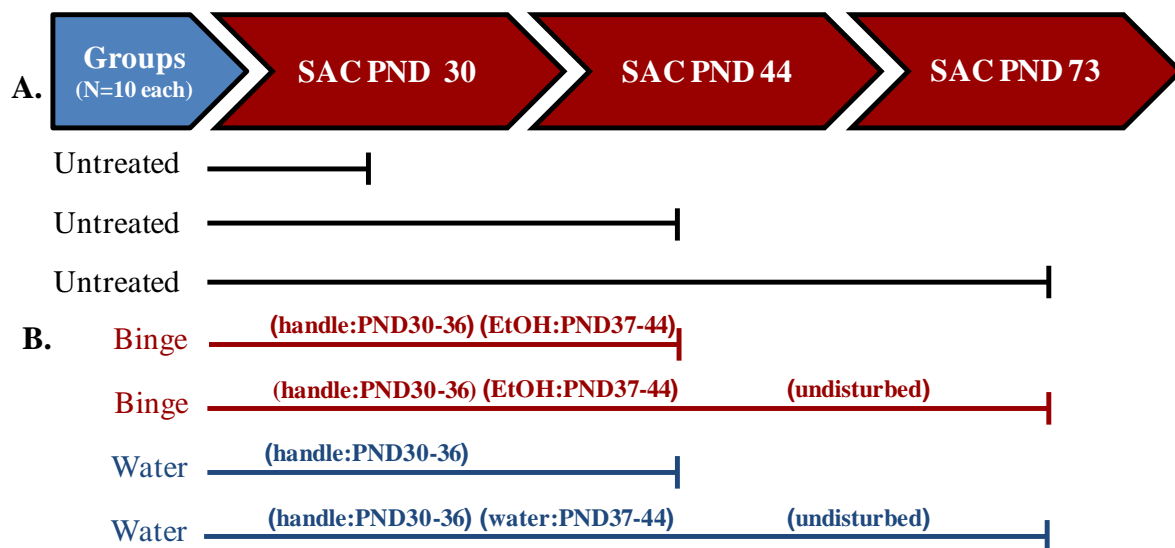
Figure 4. Diagram of experimental paradigm. Diagram depicting the age of sacrifice and specific treatment paradigms for each group of male Wistar rats. 3 groups received no treatments (A). 4 groups received water or binge EtOH treatments at peripuberty, for a total of 2 control and 2 EtOH groups (B). N=10/group.

Table 1. Statistical Analysis of Gene Expression Levels in the Dorsal Hippocampus

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF TREATMENT	INTERACTION: AGE X TREATMENT
miR-10a-5p	Yes: $F(2,33) = 12.293$ $p < 0.001$	No	No
miR-26a	Yes: $F(2,44) = 3.222$ $p < 0.049$	Yes: $F(1,44) = 5.212$ $p < 0.027$	Yes: $p < 0.001$
miR-103	No	No	No
miR-495	Yes: $F(2,37) = 4.923$ $p < 0.013$	Yes: $F(1,37) = 59.23$ $p < 0.001$	Yes: $p < 0.001$
GENE	**MAIN EFFECT OF AGE**	**MAIN EFFECT OF TREATMENT**	**INTERACTION: AGE X TREATMENT**
Drosha	Yes: $F(2,54) = 24.906$ $p < 0.001$	No	Yes: $p = 0.008$
Dicer	Yes: $F(2,54) = 18.725$ $p < 0.001$	No	Yes: $p < 0.001$
BDNF	Yes: $F(2,52) = 12.845$ $p < 0.001$	Yes: $F(1,52) = 6.574$ $p < 0.013$	No
SIRT1	No	Yes: $F(1,54) = 110.941$ $p < 0.001$	Yes: $p < 0.001$
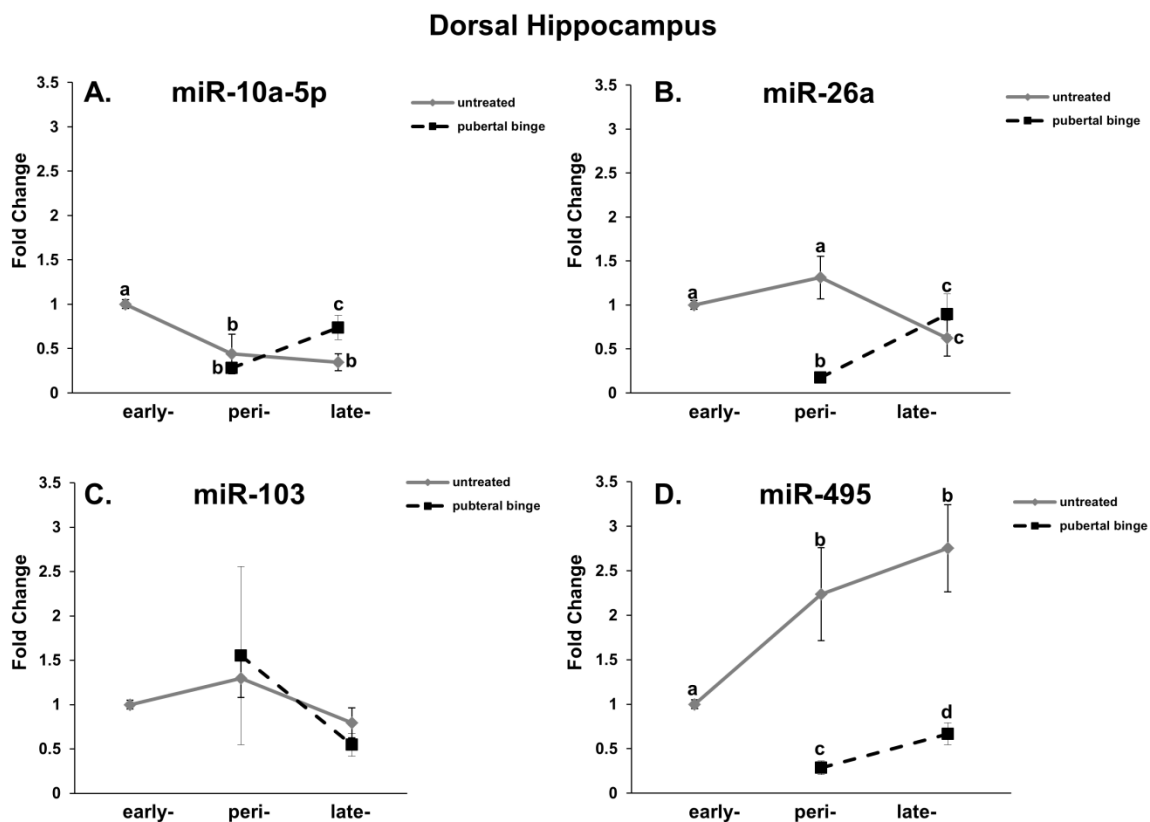
Figure 5. Peripubertal binge EtOH exposure alters microRNA expression during pubertal development in the dorsal hippocampus. miR-10a-5p (A), miR-26a (B), miR-103 (C), and miR-495 (D) expression levels in untreated (solid line) and EtOH-treated (dashed line) pubertal male rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 animals. Dissimilar letters indicate a statistically significant difference between groups (2-way ANOVA, $p < 0.05$).

levels were measured using qRT-PCR at three time points throughout pubertal development(early = 30 d, peri = 44 d, late = 73 d) (Fig. 4A). In the dorsal hippocampus, a two-way ANOVA revealed a main effect of age on the expression levels of all microRNAs tested, except miR-103 (Table 1). Each of the three microRNAs that showed a significant effect of age in the dorsal hippocampus had a distinct developmental pattern. For instance, miR-10a-5p expression decreased significantly between early and peripuberty, and remained lower than early puberty levels until late puberty (Fig. 5A, solid line). By contrast, miR-26a expression did not change between early and peripuberty, but significantly decreased at late puberty (Fig. 5B, solid line). Finally, a significant increase was observed in miR-495 expression between early and peripuberty and these levels remained high until late puberty (Fig. 5D, solid line).

Binge EtOH exposure during peripuberty significantly alters normal developmental profile of microRNAs in the dorsal hippocampus.

Next, we determined the effects of repeated binge EtOH exposure during peripuberty on these microRNA levels in the brain. Rats were administered our repeated binge-pattern EtOH exposure paradigm (Fig. 1A) and dorsal hippocampal microRNA expression of miR-10a-5p, miR-26a, miR-103 and miR-495 was compared with untreated rats/water-treated rats immediately following binge EtOH exposure and one-month post-EtOH exposure. Our results demonstrated a significant main effect of EtOH treatment on the expression of miR-26a and miR-495 and there was also a significant interaction between age and treatment, demonstrating that the effects of EtOH were age dependent (Table 1). Although there was no main effect of EtOH treatment on the expression of miR-10a-5p, and there was no immediate change following EtOH exposure at

peripuberty, by late puberty its expression was significantly increased compared to untreated rats/water-treated rats (Fig. 5A). miR-26a expression levels significantly decreased immediately following binge EtOH exposure at peripuberty, however this difference did not persist and was equivalent to those of untreated animals by late puberty (Fig. 5B). miR-103 was not significantly altered by peripubertal EtOH treatment in the dorsal hippocampus, similar to the results observed with age alone (Fig. 5C). Most striking were the results of EtOH exposure on miR-495. Similar to miR-26a, miR-495 was significantly decreased as a result of binge EtOH exposure at peripuberty. Notably however, expression levels remained significantly below normal even one-month post EtOH exposure (Fig. 5D), suggesting a potential long-term effect of pubertal binge EtOH exposure on miR-495 in the dorsal hippocampus.

Mature miR-10a-5p, miR-26a, miR-103, and miR-495 expression levels in the ventral hippocampus of untreated male rats are age-dependent

Distinct region and age-dependent expression of microRNAs has been demonstrated in the brain of a variety of species [105,206,207]. Therefore, we quantified the expression of miR-10a-5p, miR-26a, miR-103, and miR-495 in the ventral hippocampus across pubertal development in untreated rats to determine if there were region specific microRNA expression patterns in the hippocampus. In the ventral hippocampus, there was a significant main effect of age in the untreated animals on all four microRNAs tested, including miR-103, which previously did not show a significant change across pubertal development in the dorsal hippocampus (Table 2). Specifically,

Table 2. Statistical Analysis of microRNA Expression Levels in the Ventral

Hippocampus

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF TREATMENT	INTERACTION: AGE X TREATMENT
miR-10a-5p	Yes: $F(2,42) = 17.492$ $p < 0.001$	Yes: $F(1,42) = 8.847$ $p = 0.005$	No
miR-26a	Yes: $F(2,37) = 5.064$ $p = 0.011$	No	No
miR-103	Yes: $F(2,54) = 4.582$ $p = 0.015$	Yes: $F(1,54) = 5.739$ $p = 0.02$	Yes: $p < 0.001$
miR-495	Yes: $F(2,42) = 8.359$ $p < 0.001$	Yes: $F(1,42) = 5.998$ $p = 0.019$	Yes: $p < 0.001$
GENE	**MAIN EFFECT OF AGE**	**MAIN EFFECT OF TREATMENT**	**INTERACTION: AGE X TREATMENT**
Drosha	Yes: $F(2,54) = 4.650$ $p = 0.014$	No	Yes: $p < 0.001$
Dicer	Yes: $F(2,54) = 10.746$ $p < 0.001$	No	Yes: $p < 0.001$
BDNF	Yes: $F(2,41) = 13.622$ $p < 0.001$	Yes: $F(1,41) = 9.109$ $p = 0.004$	Yes: $p < 0.001$
SIRT1	Yes: $F(2,43) = 16.484$ $p < 0.001$	Yes: $F(1,43) = 14.759$ $p < 0.001$	Yes: $p < 0.001$

Ventral Hippocampus
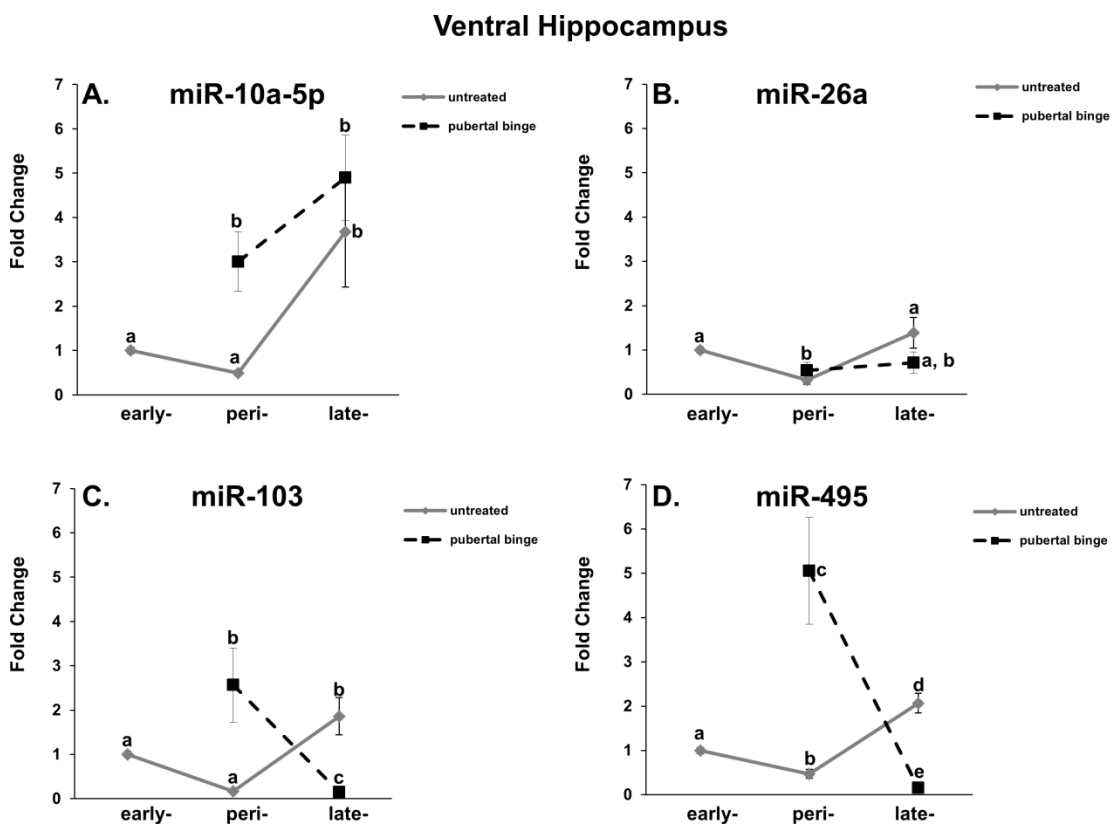
Figure 6. Peripubertal binge EtOH alters microRNA expression during pubertal development in the ventral hippocampus. miR-10a-5p (A), miR-26a (B), miR-103 (C), and miR-495 (D) expression levels in untreated (solid line) and EtOH-treated (dashed line) pubertal male rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 animals. Dissimilar letters indicate a statistically significant difference between groups (2-way ANOVA, $p < 0.05$).

miR-10a-5p showed no change between early and peripuberty, but significantly increased by late puberty in the ventral hippocampus (Fig. 6A, solid line). Also, in contrast to the dorsal hippocampus miR-26a significantly decreased at peripuberty, but this change did not persist and was equivalent to early pubertal levels by late puberty (Fig. 6B, solid line). The ventral hippocampus levels of miR-103 and miR-495 had a similar profile. Both had a statistically significant decrease, or a strong trend towards decreasing, at peripuberty compared to early pubertal levels, but then the levels increased significantly above that of early pubertal levels by late puberty (Fig. 6C, D, solid line). Notably, miR-495 expression in the ventral hippocampus demonstrated the most dynamic expression profile throughout pubertal development, as it had distinct expression levels at each time point measured.

Repeated adolescent binge EtOH exposure differentially alters expression of miR-10a-5p, miR-26a, miR-103 and miR-495 in the ventral hippocampus

We predicted that peripubertal binge EtOH exposure would alter the normal developmental profile of microRNA expression in the ventral hippocampus, based on the evidence obtained from the dorsal hippocampus. Indeed, there was a significant main effect of treatment and a significant interaction between age/treatment for miR-10a-5p, miR-103, and miR-495 in the ventral hippocampus (Table 2). Interestingly, the magnitude of changes in microRNA expression was overall much higher in the ventral hippocampus compared to the dorsal, with some microRNAs changing by as much as 5-fold (Fig. 6). One example of a large fold change was observed with miR-10a-5p. The mature expression levels of miR-10a-5p were significantly increased by an average of 3-5

Table 3. Summary of peripubertal EtOH exposure on microRNA and mRNA gene expression. Arrows indicate a statistically significant effect (increase/decrease) of EtOH compared to age-matched water-treated controls.

microRNA	Dorsal Hipp immediate ETOH effect	Dorsal Hipp long-term ETOH effect	Ventral Hipp immediate ETOH effect	Ventral Hipp long-term ETOH effect
10a-5p	−	↑	↑	−
26a	↓	−	−	↓
103	−	−	↑	↓
495	↓	↓	↑	↓
Gene	**Dorsal Hipp immediate ETOH effect**	**Dorsal Hipp long-term ETOH effect**	**Ventral Hipp immediate ETOH effect**	**Ventral Hipp long-term ETOH effect**
Drosha	↑	↑	↑	↓
Dicer	↑	↓	↑	↓
BDNF	↑	↑	↓	−
SIRT1	↑	↑	↓	−

fold following peripubertal binge EtOH exposure (Fig. 6A, dashed line). Further, the expression levels continued to show an increase at late puberty, paralleling the untreated group at that same age (Fig. 6A). There was no observed statistical effect of peripubertal EtOH treatment on miR-26a. Nevertheless, the expression levels in the EtOH-treated group did not appear to follow the normal age-dependent increase observed by late puberty (Fig. 6B). The most striking effects of peripubertal binge EtOH exposure in the ventral hippocampus were observed in miR-103 and miR-495 expression, as their normal developmental expression levels at both peripuberty and late puberty were opposite following peripubertal binge EtOH exposure. Overall, our results demonstrate both immediate and long-term effects of peripubertal binge EtOH exposure in the rat hippocampus and these effects were distinct between the dorsal and ventral regions (Table 3).

Mature microRNA biosynthetic processing enzymes are altered by peripubertal binge EtOH exposure in the dorsal and ventral hippocampus

Primary microRNA transcripts are transcribed from the genome in a RNA polymerase II dependent manner and sequentially cleaved by the nuclear enzyme Drosha and the cytoplasmic enzyme Dicer to form the functionally mature single-stranded form of the microRNA [127,208,209]. We next measured mRNA levels of both Drosha and Dicer in our dorsal and ventral hippocampal tissue samples to better understand the molecular basis of altered mature microRNA expression levels. Drosha and Dicer mRNA expression levels were measured using qRT-PCR in the untreated animals at early, mid and late puberty and those levels were compared to animals that were administered our

Enzymes in D. Hipp and V. Hipp
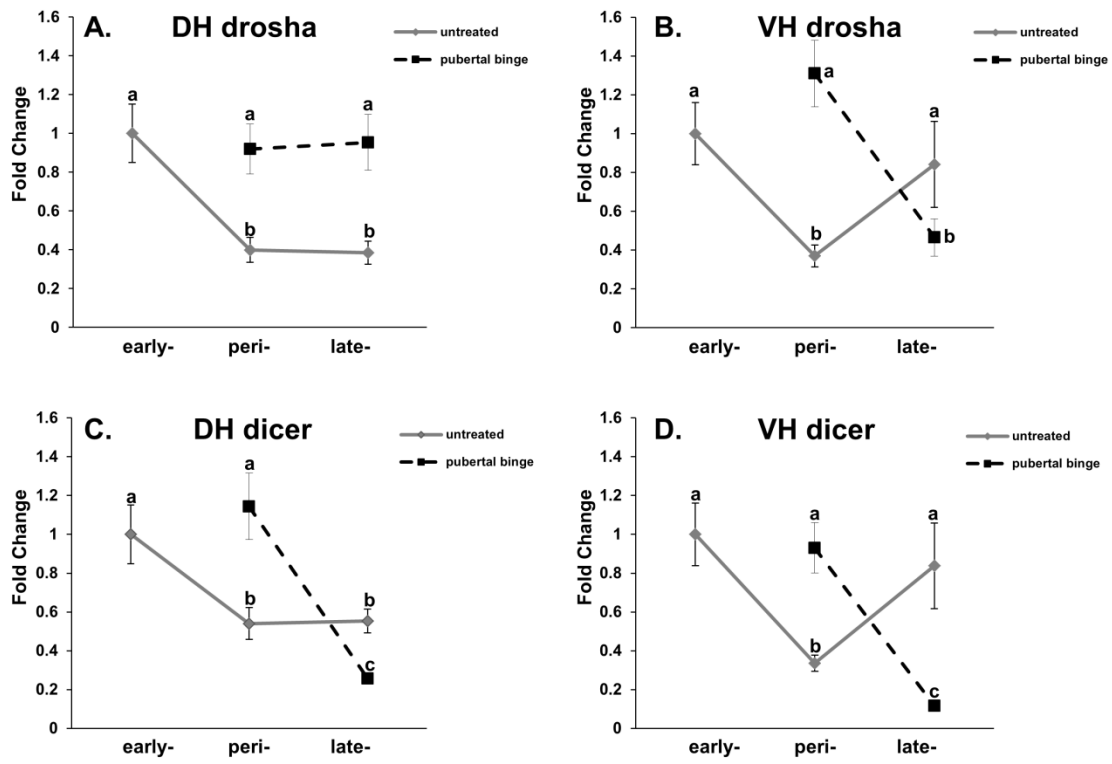
Figure 7. Peripubertal binge EtOH exposure differentially alters microRNA biosynthetic processing enzymes in the dorsal and ventral hippocampus. Drosha (A, B) and Dicer (C, D) mRNA levels in the dorsal and ventral hippocampus in untreated (solid line) and EtOH-treated (dashed line) pubertal male rats. N=10/group. Data represent mean fold change $\pm$ SEM as compared to untreated PND 30 animals. Dissimilar letters indicate a statistically significant difference between groups (2-way ANOVA, $p<0.05$).

binge EtOH treatment paradigm at peripuberty. Our results showed a main effect of age

on Drosha in both the dorsal and ventral hippocampus (Tables 1, 2; Fig. 7A, B, solid

line). Specifically, Drosha mRNA levels were significantly decreased between early and

peripuberty in both regions of the hippocampus. The gene expression levels remained

low until late puberty in the dorsal hippocampus but returned to early pubertal levels in

the ventral hippocampus (Fig. 7A, B, solid line), thereby demonstrating region-specific

regulation. Further, there was a significant interaction between age and EtOH on Drosha

mRNA expression in both hippocampal regions and an overall main effect of EtOH

treatment in the ventral hippocampus (Table 1, 2; Fig. 7A, B, dashed line). In both

regions, peripubertal binge EtOH exposure significantly elevated Drosha mRNA levels

immediately following EtOH exposure, suggesting a potential for increased microRNA

biosynthetic processing. These increased levels persisted for up to one month (late

puberty, Fig. 7A) following EtOH exposure in the dorsal hippocampus, but were

significantly decreased at that same age in the ventral hippocampus (Fig. 7B). Dicer

mRNA expression followed the same pattern as Drosha in untreated animals for both

regions of the hippocampus and there was a statistically significant overall main effect of

age (Table 1, 2; Fig. 7C, D). Dicer mRNA levels decreased between early and

peripuberty in both regions and the levels remained low until late puberty in the dorsal

hippocampus (Fig. 7C). By contrast, at late puberty Dicer mRNA levels were no longer

significantly different from those in early puberty in the ventral hippocampus (Fig. 7D).

Binge EtOH exposure in peripuberty had the same effect on Dicer mRNA expression

levels in both regions of the hippocampus, with a statistically significant overall main

effect of treatment and a significant interaction between age and treatment (Table 1, 2;

Fig. 7C, D). Dicer mRNA levels were immediately increased compared to untreated animals following EtOH exposure at peripuberty, but then by late puberty had decreased significantly to levels even lower than that of untreated animals at early puberty (Fig. 7C, D). In sum, these results demonstrate that both Dicer and Drosha change dynamically throughout pubertal development in the hippocampus and that these levels can be dramatically altered by peripubertal binge EtOH exposure (Table 3).

Peripubertal Binge EtOH exposure alters putative target gene mRNA of EtOH-sensitive microRNA in the dorsal and ventral hippocampus.

The most well understood mechanism of microRNA action is through microRNA complementary binding to the 3' untranslated region (UTR) of a primary gene transcript and its subsequent facilitation of mRNA degradation and/or inhibition of mRNA translation [210,211]. This miR-mediated degradation of mRNA target genes is attributable to observed downstream phenotypic changes. We identified two genes that were putative targets of all 4 EtOH-sensitive microRNAs in the dorsal and ventral hippocampus using publically available software target prediction programs (Targetscan: www.targetscan.org; miR database: www.miRDB.org) [129,196,197]. Our analysis of potential targets for each EtOH-sensitive miR identified a single putative binding site in the 3'UTR of BDNF for each miR-10a-5p, miR-26a, and miR-103 (Fig. 8). Also, there were four possible binding sites on BDNF for miR-495, suggesting miR-495 might have a stronger regulatory effect on BDNF than the other microRNAs. There were no potential binding sites in the 3'UTR of SIRT1 for miR-10a-5p, but there was a single potential

A. **Rat BDNF 3' UTR: 2842 bp**

B. **Rat SIRT1 3' UTR: 1607 bp**
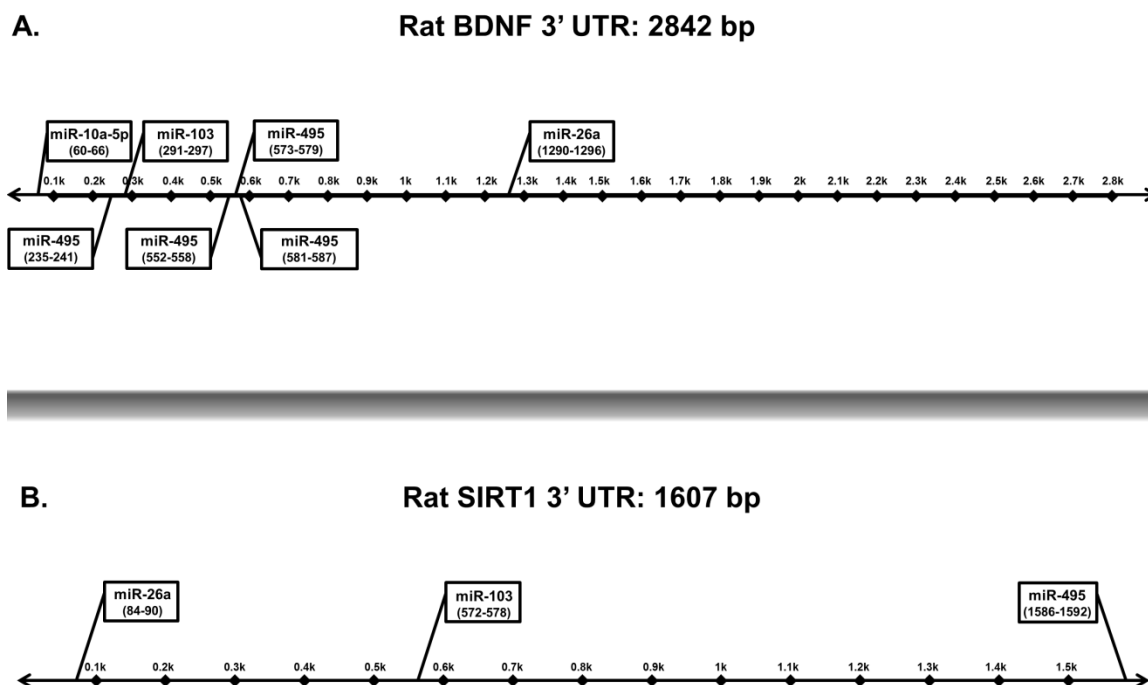
Figure 8. Diagram depicting predicted microRNA binding sites for BDNF and SIRT1. Schematic diagram of the 3'UTR of (A) BDNF – 2,842 bp and (B) SIRT1- 1,607 bp. The putative binding sites for each microRNA were predicted using Targetscan (www.targetscan.org) and miRDB (www.miRDB.org) computer algorithm programs. The binding sites were predicted based on the presence of an 8-mer or 7-mer conserved microRNA seed sequence. Precise seed sequence positions are shown in parentheses.
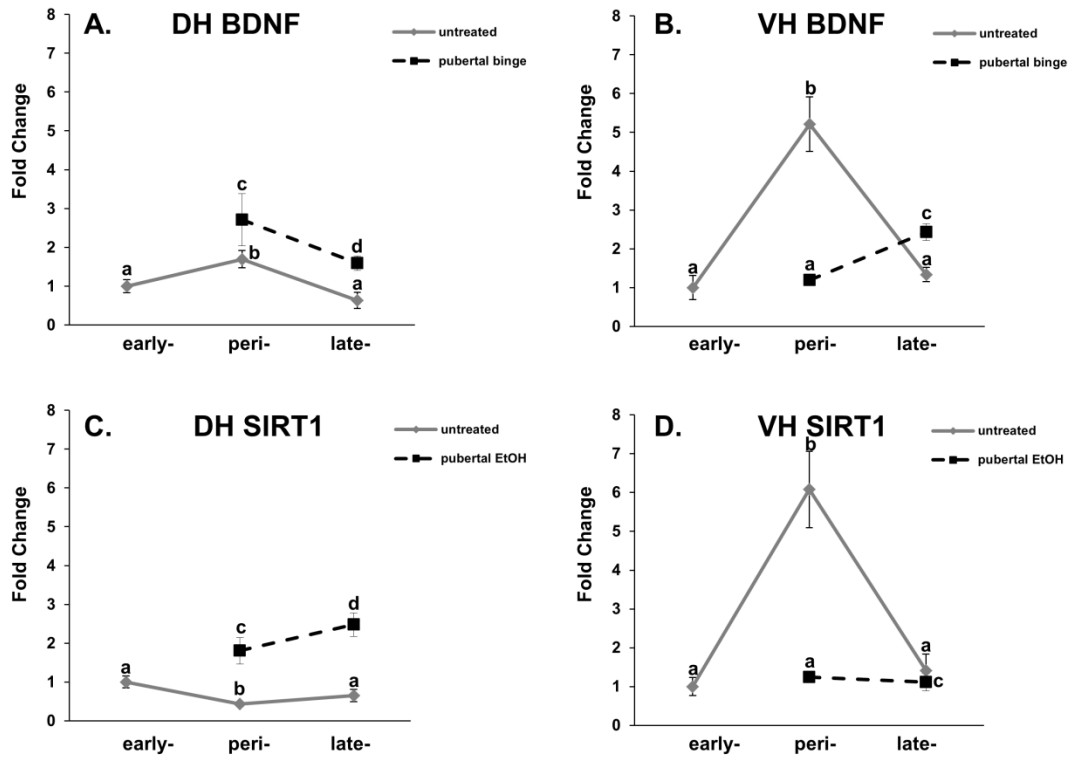
Figure 9. Peripubertal binge EtOH exposure differentially alters miR target genes, BDNF

and SIRT1, in the dorsal and ventral hippocampus. BDNF (A, B) and SIRT1 (C, D)

mRNA levels in the dorsal and ventral hippocampus in untreated (solid line) and EtOH-

treated (dashed line) pubertal male rats. N=10/group. Data represent mean fold change $\pm$

SEM as compared to untreated PND 30 animals. Dissimilar letters indicate a statistically

significant difference between groups (2-way ANOVA, $p < 0.05$).

binding site for each of the other microRNAs tested (miR-26a, miR-103 and miR-495; Fig. 8).

To determine whether peripubertal binge EtOH exposure altered the normal expression levels of BDNF and SIRT1 mRNA, we compared mRNA levels in untreated animals at each age (early, mid, and late puberty) to animals that had been treated with binge EtOH at peripuberty in both the dorsal and ventral hippocampus. Overall, there was a significant main effect of age on BDNF mRNA expression in both regions of the hippocampus (Tables 1, 2; Fig. 9A, B, solid line). By contrast, there was a significant main effect of age on SIRT1 mRNA expression in the ventral, but not dorsal, hippocampus (Tables 1, 2; Fig. 9C, D, solid line). There was also a significant main effect of EtOH treatment on BDNF and SIRT1 mRNA expression in both hippocampal regions (Tables 1, 2). A statistically significant interaction between age and EtOH treatment was observed for SIRT1 in the dorsal hippocampus (Table 1) and BDNF and SIRT1 in the ventral hippocampus (Table 2), demonstrating that the effects of binge EtOH exposure on BDNF and SIRT1 mRNA expression was dependent on age. Interestingly, EtOH exposure significantly increased BDNF and SIRT1 mRNA levels compared to untreated animals at peripuberty in the dorsal hippocampus (Fig. 9A, C, dashed line), while the opposite was observed in the ventral hippocampus (Fig. 9B, D, dashed line). The effects of EtOH persisted for up to one-month post-EtOH exposure for BDNF and SIRT1 in the dorsal hippocampus (Fig. 9A, C) and for BDNF in the ventral hippocampus (Fig. 9A). Although the ventral hippocampus mRNA levels of SIRT1 were not statistically different from untreated controls one-month following binge EtOH exposure, the data suggest that the normal developmental profile of SIRT1 gene

expression was retarded at a pre-pubertal phenotype as a result of peripubertal EtOH exposure (Table 3).

We next investigated the effects of peripubertal binge EtOH exposure on BDNF and SIRT1 protein expression. We quantified the dorsal and ventral hippocampus pro-BDNF (the higher molecular weight precursor of mature BDNF), mature BDNF and SIRT1 protein levels in peripubertal animals treated with water or binge EtOH one hour following the last treatment.. In the ventral hippocampus, binge EtOH-treated animals demonstrated a strong decreasing trend in expression of pro-BDNF, with a p-value = 0.05 (Fig. 10B).

Binge EtOH exposure did not alter circulating testosterone levels.

Increased gonadal steroid hormones during pubertal development can potentially modulate microRNA and/or Drosha, Dicer, BDNF and SIRT1 mRNA levels. The animals in this study were kept gonad-intact throughout puberty, however previous studies have demonstrated that EtOH can alter gonadal steroid hormones [44]. To determine the effects of binge EtOH exposure during puberty on circulating gonadal steroid hormone levels in our system, plasma testosterone (T) was measured in each age group on the day of sacrifice. As expected, circulating T levels continued to increase with age in all animals (Fig. 11), demonstrating a normal progression through pubertal development. Peripubertal EtOH exposure tended to decrease circulating T levels, but the differences between EtOH-treated animals and controls of the same age group were not statistically
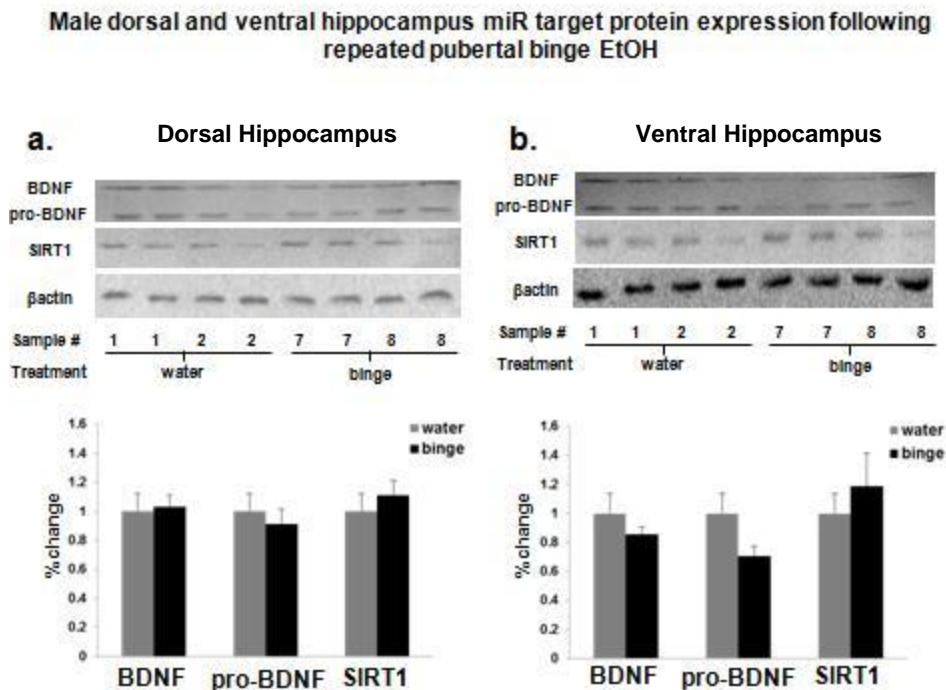
Figure 10. Assessment BDNF and SIRT1 protein expression levels following

peripubertal binge EtOH. Representative immunoblot for BNDF, pro-BDNF, SIRT1 and

β-actin expression in the dorsal hippocampus and the % change in BNDF, pro-BDNF and

SIRT1 expression normalized to β-actin (A). Representative immunoblot for BNDF, pro-

BDNF, SIRT1 and β-actin expression in the ventral hippocampus and the % change in

BNDF, pro-BDNF and SIRT1 expression normalized to β-actin (B). Quantification of

densitometric analysis of protein expression calculated from at least 3 independent

experiments (N=6). No significant difference between groups was observed (2-way

ANOVA, p<0.05).

Plasma Testosterone
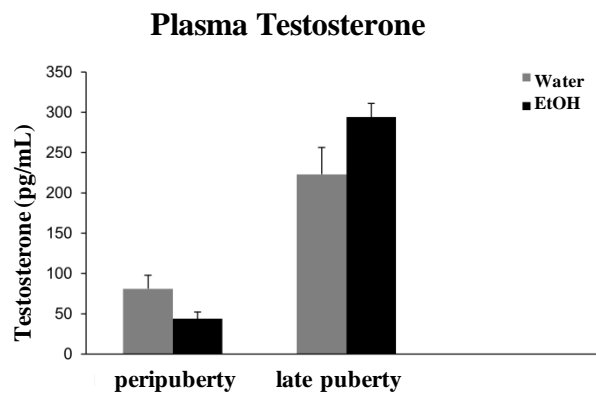
Figure 11. Peri-pubertal binge EtOH exposure did not affect circulating testosterone levels. Plasma concentrations of testosterone (T) 60 min. after the last treatment. Data expressed as mean ± SEM T pg/ml. No statistically significant differences were observed.

significant (Fig. 11). Moreover, there were no apparent long-lasting effects of peripubertal EtOH treatment on circulating T levels measured at one month post EtOH treatment (Fig. 11).

Discussion

Adolescent alcohol abuse has been shown to exert long-lasting detrimental effects on brain function, neuronal gene expression and behaviors, yet the precise molecular targets of EtOH remain poorly understood. Indeed, previous studies by our laboratory and others have demonstrated both immediate and long-term effects of repeated peripubertal binge EtOH exposure on genes that regulate the physiological stress response [12,44,67,212,213]. Therefore, the goals of this study were to provide a potential mechanistic explanation for EtOH-induced effects on gene expression by quantifying the expression of EtOH sensitive microRNAs (miR-10a-5p, miR-26a, miR-103 and miR-495) during normal pubertal development in the male rat hippocampus, and then elucidate how peripubertal binge EtOH exposure alters the expression of those microRNAs. Importantly, microRNAs have emerged as highly conserved critical regulators of downstream gene expression in nearly all physiological systems. Genome-wide microRNA expression profiles revealed that the microRNAs investigated in this study, miR-103 and miR-26a, are among the top 15 most abundantly expressed microRNAs in the rodent hippocampus [104]. Taken together our data revealed that the expression of miR-10a-5p, miR-26a, miR-103 and miR-495 are dynamic across pubertal development and that the developmental profiles for each microRNA are distinct between the dorsal and ventral hippocampus. Moreover, peripubertal binge EtOH exposure altered normal pubertal development expression patterns of miR-10a-5p, miR-26a, miR-103,

miR-495, Dicer, Drosha, BDNF and SIRT1 in an age and brain region-dependent manner. Most striking, our results showed that peripubertal binge EtOH exposure had significant long lasting effects on several microRNAs studied, as well as their processing enzymes and target genes. These effects were evident for as long as one-month following the last EtOH exposure, suggesting that EtOH could have lasting consequences on gene expression profiles in the male rat hippocampus through long-term regulation of microRNA expression patterns.

Quantifying EtOH-induced changes in Drosha and Dicer mRNA levels can yield insight into the mechanistic actions of EtOH by revealing specific points of EtOH-mediated perturbations along the microRNA biosynthetic pathway. The biogenesis of mature 22-24 nucleotide (nt), single-stranded microRNAs involves the following sequential processes: 1) transcription of a 100-1000 nt primary-microRNA (pri-microRNA) product, 2) cropping of the transcript by the nuclear Rnase III enzyme Drosha into the preliminary-microRNA (pre-microRNA), 3) cleavage of the pre-microRNA by the cytoplasmic RNAse III enzyme Dicer and 4) loading of the mature microRNA onto the RNA-induced silencing complex (RISC), which guides it to its mRNA target for degradation or translational inhibition [210,211]. Notably, the expression of microRNA biogenesis genes (i.e. Drosha, Dicer) was shown to be significantly correlated with addiction-related phenotypes [214]. Our studies revealed the interesting observation that both Drosha and Dicer mRNA significantly decreased between early and peripuberty, although these results do not necessarily reflect changes in enzyme catalytic activity. Regardless, decreased mRNA levels of microRNA biosynthetic enzymes would theoretically result in reduced mature microRNA levels

leading to increased translation of gene targets, consistent with the idea that there are global changes in overall gene expression during adolescent development. The effects of peripubertal binge EtOH exposure on Drosha and Dicer mRNA levels continued to persist for as long as one-month after the last EtOH exposure, which raises the possibility of a potential long-term EtOH-induced dysregulation of microRNA biosynthetic processing.

One of the biggest challenges since the discovery of microRNAs has been the identification of their target genes. In mammals, their imperfect base pair hybridization with mRNA targets results in promiscuous binding, such that a single microRNA can have multiple mRNA gene targets. For instance, miR-495 is predicted to target as many as 754 genes in the rat genome (miRDB). Similarly, a single mRNA transcript can be regulated by several different microRNAs and whether multiple microRNAs must act in concert to regulate a specific target gene remains unresolved. Indeed, BDNF is predicted to be targeted by 51 microRNAs (miRDB), therefore the results shown herein are not exhaustive of all potential regulators of BDNF. Nevertheless, our data demonstrating differential expression of the same microRNA in functionally distinct hippocampal regions strongly implied that the targets of these microRNAs may also be differentially expressed. Therefore, we identified putative target genes of miR-10a-5p, miR-26a, miR-103 and miR-495 that were relevant to known dorsal and ventral hippocampus functions using target prediction software programs, Targetscan and MirDB. For instance, BDNF was identified as a putative target gene for miR-10a-5p, miR-26a, miR-103 and miR-495. BDNF plays a fundamental role in guiding neurodevelopment as well as in the fine-tuning of synaptic plasticity, a critical event during adolescent brain development.

Previous studies have demonstrated that regulation of BDNF expression is mediated by miR-26a targeting the conserved BDNF 3'UTR sequence [143] and our data demonstrate inverse relationships between miR-26a and BDNF expression in the dorsal hippocampus (Fig. 5B, Fig. 9A). Together, these data suggest a role for miR-26a in the regulation of BDNF expression levels in the pubertal dorsal hippocampus. Importantly, both BDNF gene variants and miR-26a expression have been implicated in the vulnerability and onset of schizophrenia, alcohol abuse and mood disorders in both human patients and rodent models [143,144,145,146,147,148]. Moreover, these data serve as an important reminder that the computer algorithm-predicted microRNA target genes do not always translate into actual microRNA targets and that correlated changes in microRNA and target gene mRNA expression levels do not always indicate causality. Taken together, peripubertal disruption of miR-26a, miR-10a-5p and miR-495 expression following binge EtOH exposure could result in altered BDNF expression in the pubertal hippocampus.

The histone deacetylase sirtuin 1 (SIRT1) was also predicted by computer algorithms to be a putative gene target of miR-26a, mir-103 and miR-495 (Fig. 7B). Immediate responses to peripubertal binge EtOH resulted in increased SIRT1 expression in the dorsal hippocampus, and decreased SIRT1 expression in the ventral hippocmapus. These data suggest that SIRT1 mRNA expression may be regulated by distinct microRNAs in the dorsal and ventral hippocampus, a hypothesis consistent with studies demonstrating that these two hippocampus regions are functionally distinct, as they have dissimilar neuronal projection patterns [57,58,205]. Moreover, the ventral and dorsal hippocampus may utilize different cellular mechanisms to mediate their respective responses to EtOH. Our data further demonstrated that peripubertal binge EtOH exposure

had long-lasting effects on SIRT1 gene expression, showing significantly increased

mRNA levels lasting until late puberty in the dorsal hippocampus. Similar to BDNF,

SIRT1 has recently been implicated as critical for mediating synaptic plasticity, one

mechanism underlying memory formation in rodent and human cell models [23,25].

Moreover, many studies have demonstrated that EtOH exposure has long-lasting

consequences on gene expression, which may be regulated, in part, by microRNAs

targeting chromatin-modifying enzymes, such as SIRT1.

Regulation of gene expression mediated by microRNAs is theoretically carried

out via the alterations in the microRNA target gene effector functions, i.e. the function of

the protein encoded by the microRNA target gene. Effector function is best understood to

occur following sequestration or degradation of the microRNA target gene mRNA.

However, a single gene can be targeted and, therefore, regulated by multiple microRNAs

suggesting that binding of multiple microRNAs to a single target gene's 3'UTR could be

necessary for alterations in protein expression to mimic changes in microRNA target

gene mRNA expression. Indeed, our data make evident that miR-10a-5p, miR-495, miR-

103 and miR-26a expression are sensitive to the presence or absence of EtOH depending

on age and brain region and that consequential alterations in BDNF and SIRT1 target

gene expression are significantly correlated with observed EtOH-induced alterations in

the expression of some of the microRNAs that target them in an immediate manner. The

near significance values of BDNF and SIRT1 protein data suggest many different

possibilities. One interpretation is that the protein levels of BDNF and SIRT1 following

peripubertal binge EtOH may be altered to differing degrees amongst individuals. Indeed,

unlike other drugs such as cocaine, alcohol alters cells membranes, ion channels,

enzymes, and receptors of neurons globally throughout the brain [215], and likely

contribute to evidenced individual, sex and racial variations in alcohol metabolism and

alcohol-induced neuroplastic changes [216,217] [218,219,220,221]. Importantly, the six

rats whose tissue was collected for protein analysis were not from the same cohort of

animals collected for RNA analysis. Furthermore, the groups of animals collected for

RNA analysis had an N=10, whereas the groups collected for protein analysis had N=6. It

is therefore, possible that there was not enough power to detect changes in protein

expression levels, especially considering that protein levels change at a kinetically slower

rate than mRNA. Alternatively, because any given miR targets multiple microRNAs, it is

possible that target genes other than BDNF and SIRT1 may be altered by peripubertal

binge EtOH exposure. For instance, miR-10a plays a role in regulating the homeobox

developmental genes [21], and has similar expression patterns to *HOXB4* in the mouse

embryo suggesting that it may have similar important functional roles in development

[20,222], and therefore may have pubertal brain developmental functions. Furthermore, a

member of the Nkx family of homeobox genes, thyroid transcription factor 1 (TTF1), is

expressed in the postnatal hypothalamus and this expression is required for rodent

pubertal onset [222]. Therefore, it is possible that miR-10a-5p targets Hox gene

transcription factors expressed during pubertal development, and alterations of target

genes important for morphogenesis may contribute to disruption of brain development

following peripubertal binge EtOH abuse. Lastly, Overall, the molecular mechanisms

underlying differences in microRNA responses to pubertal binge EtOH warrant further

studies and as it is possible that changes in microRNA expression may lead to

complications in downstream target gene translation in some but not all individuals

engaging in binge drinking behavior during adolescence. Therefore, given the strong supporting evidence that changes it the expression of EtOH sensitive microRNAs significantly correlate with changes in target gene mRNA, and that corresponding changes in the protein expression reach near significance, it is important that further experimentation explore the effects of peripubertal binge EtOH exposure on protein expression changes in BDNF and SIRT1.

Overall, our data reveal novel findings about the age and brain-region specific expression of miR-10a-5p, miR-26a, miR-103, and miR-495 during pubertal development in male rats. Further, we showed that mid/peripubertal binge EtOH exposure significantly alters the normal expression profile of these microRNAs, their biosynthetic processing enzymes, and two of their putative target genes, BDNF and SIRT1. However, it is important to note that these results are not necessarily predictive of microRNAs in females or other species, as several studies have demonstrated both species and sex-specific expression profiles for microRNAs [150,223,224]. Moreover, the precise molecular targets of EtOH in the biogenesis of microRNAs remain unclear and require further investigation. An important next step is the identification of specific cell types (i.e. neurons vs. glia) in which microRNAs are affected by binge EtOH exposure, as our study was limited to whole hippocampal tissue homogenates. Taken together, our data raise the possibility that EtOH modulation of these fours microRNAs is a potential cellular mechanism underlying long-term changes in gene expression induced by adolescent EtOH abuse.

CHAPTER FOUR

SEXUALLY DIMORPHIC GENE EXPRESSION OF HIPPOCAMPUS ETOH-
SENSITIVE MICRORNA, MICRORNA TARGET GENES AND MICRORNA
PROCESSSING ENZYMES DURING PUBERTAL DEVELOPMENT

Introduction

Adolescent binge drinking is a major public health issue [40,166] that incurs

sexually dimorphic consequences on brain function and development

[44,225,226,227,228,229,230]. Both male and female adolescents engage in underage

drinking, and the Substance Abuse and Mental Health Services Administration

(SAMHSA) has reported that males begin drinking earlier, drink more frequently and

have a higher prevalence of binge drinking than their female counterparts (SAMHSA

2006) [231,232]. However, more recently the Center for Disease Control and Prevention

(CDC) reported that binge drinking among females--including young girls--is also a

serious, yet unrecognized, problem (CDC 2012). Moreover, studies have demonstrated

that females who abuse alcohol are more prone to developing alcohol addiction,

alcoholism and anxiety than men [68,72]. Sex hormones have been demonstrated to

mediate, in part, sexually dimorphic responses to EtOH [233,234,235,236,237], however,

a clear mechanism remains unknown.

Sex hormones can also influence microRNA expression in the brain [28,150].

microRNAs are small (~20 nt) non-coding RNA molecules encoded in the genome. Their

synthesis begins in the nucleus where RNA Polymerase II or III transcribes a primary-

microRNA (pri-microRNA). The pri-microRNA is then processed by the nuclear enzyme Drosha into the precursor-microRNA (pre-microRNA) which can exit the nucleus. Once in the cytoplasm, the pre-microRNA is cleaved by the endoribonuclease Dicer into a small duplex. One strand of this duplex is the mature microRNA that then associates with the RNA-induced silencing complex (RISC) which guides the microRNA to the 3' untranslated region (UTR) of a target gene to ultimately induce gene silencing by either translational repression or mRNA cleavage. In the brain, microRNAs have been shown to regulate neuronal differentiation [238], neuronal survival [112] and synaptic plasticity [113,136]. Interestingly, both sex hormones and microRNAs have been demonstrated to govern synaptic plasticity and the development of sexually dimorphic psychiatric disorders during puberty [113,151,239,240]. These data further suggest that microRNAs, in addition to sex hormones, may work in concert to contribute to pubertal brain development.

microRNA expression is sensitive to EtOH in a variety of species [157,158,241,242], neural cultures [195,243,244], and at developmental stages [193,245,246]. We previously identified 5 microRNAs as having age and brain region-dependent expression profiles in the male rodent hippocampus that was also altered by peripubertal binge EtOH [247]. Other studies have also demonstrated that hypothalamic microRNAs are important regulators of pubertal onset in rats, and that this is true for both males and females [248]. In order to better understand how microRNA-based regulation of gene expression may play a role in the sexual dimorphic effects of EtOH abuse on brain development and behavior, it is essential to first establish the normative expression profile of microRNAs during healthy pubertal development in both sexes. In this pursuit,

the current study tested the hypothesis that during pubertal development, microRNA expression profiles of EtOH-sensitive microRNAs are sexually dimorphic. We further hypothesized that these pubertal microRNA expression profiles will be different in the ventral and dorsal hippocampus. In order to test our hypotheses, we quantified the expression levels of five EtOH-sensitive microRNAs, miR-10a-5p, miR-26a, miR-103 and miR-495 [247], two target genes of the investigated microRNAs, neurotrophin brain-derived neurotrophic factor (BDNF) and the histone deacetylase sirtuin1 (SIRT1), as well as the major microRNA biosynthetic processing enzymes, RNAse III-type endonucleases Drosha and Dicer. Overall, we identified sex differences at multiple stages of normal pubertal development and in multiple brain regions in the gene expression of EtOH-sensitive microRNAs, their target genes and microRNA processing enzymes. We conclude that throughout puberty, the hippocampal miRNome of EtOH-sensitive microRNAs is dependent on sex and age, and suggest that both short and long-term developments within the hippocampus during pubertal development may rely on changes of microRNA expression over this period. Additionally, we determined that the microRNA expression profiles in both the male and female throughout pubertal development are distinct between the ventral and dorsal hippocampus.

Approach

The dorsal and ventral hippocampus were microdissected from untreated male and female Wistar rats and collected at three different ages during pubertal development (early puberty, PND 30; mid/peripuberty, PND 44; late puberty, PND 71; N=10/sex/group). Importantly, peripuberty in rats is considered to be ~ PND 30-45 [201,202]. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to

quantify the expression levels of a total of five microRNAs (miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495), and four genes (Drosha, Dicer, BDNF and SIRT1). microRNAs were previously identified to be EtOH-sensitive in pubertal male Wistar rats [247], and in Chapter 2 preliminary data.

Results

Dorsal hippocampus expression levels of EtOH-sensitive miR-10a-5p, miR-26a, and miR-495 in untreated rats are age and sex dependent.

In the dorsal hippocampus, a 2-way analysis of variance (ANOVA) revealed significant main effects of age or sex on the expression of miR-10a-5p, miR-26a and miR-495 (Table 4). There was also a statistically significant interaction between age and sex for these 3 microRNAs, indicating that the sex differences are dependent on age (Table4). On the other hand, there was a main effect of age alone on miR-32 expression (Table 4). miR-10a-5p and miR-32 expression levels demonstrated differences across puberty in females but not males (Fig. 12A, C). Specifically, early puberty expression levels of miR-10a-5p were significantly higher in females compared to males (Fig, 12A). By mid-puberty, the female levels decreased to match the expression levels of miR-10a-5p observed in males, which stayed constant from early to late puberty (Fig. 12A). Expression of miR-32 in females remained constant between early and mid-puberty, after which it increased significantly at late puberty (Fig. 12C). Interestingly, the expression of

Table 4. Statistical Analysis of Dorsal Hippocampus Gene Expression

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF SEX	INTERACTION: AGE X SEX
miR-10a-5p	Yes: $F(2,39) = 12.892$ $p < 0.001$	Yes: $F(2,39) = 21.589$ $p < 0.001$	Yes: $p = 0.005$
miR-26a	Yes: $F(2,48) = 16.703$ $p < 0.001$	Yes: $F(1,48) = 138.623$ $p < 0.001$	Yes: $p < 0.001$
miR-103	No	No	No
miR-32	Yes: $F(2,53) = 3.379$ $p = 0.042$	No	No
miR-495	Yes: $F(2,44) = 11.972$ $p < 0.001$	Yes: $F(1,44) = 174.639$ $p < 0.001$	Yes: $p < 0.001$
GENE	**MAIN EFFECT OF AGE**	**MAIN EFFECT OF SEX**	**INTERACTION: AGE X SEX**
Drosha	Yes: $F(2,54) = 14.973.$ $p < 0.001$	Yes: $F(2,54) = 45.914 p < 0.001$	Yes: $p < 0.001$
Dicer	Yes: $F(2,54) = 10.769$ $p < 0.001$	Yes: $F(2,54) = 145.010$ $p < 0.001$	Yes: $p < 0.001$
BDNF	Yes: $F(2,54) = 10.779$ $p < 0.001$	Yes: $F(1,54) = 90.143$ $p < 0.001$	Yes: $p < 0.001$
SIRT1	Yes: $F(2,54) = 92.432$ $p < 0.001$	Yes: $F(1,54) = 94.246$ $p < 0.001$	Yes: $p < 0.001$
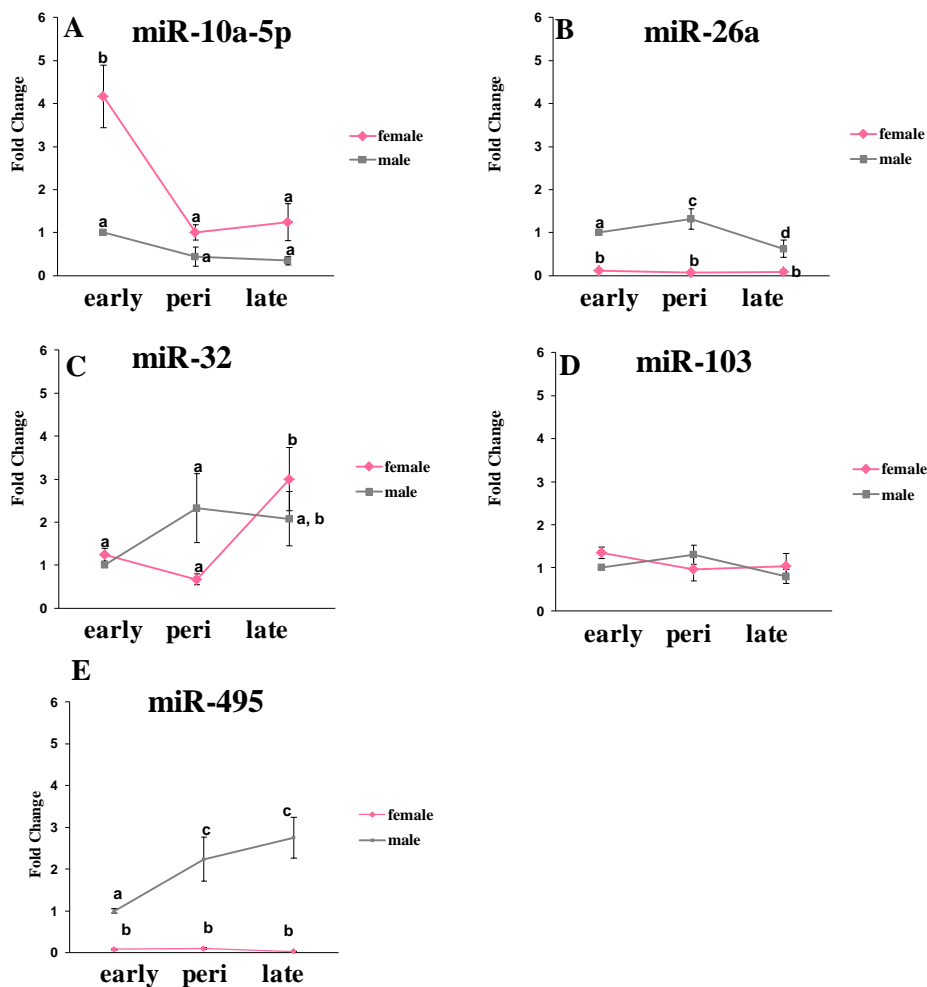
Figure 12. Sexually dimorphic microRNA expression is evident during pubertal development in the dorsal hippocampus. miR-10a-5p (A), miR-26a (B), miR-32 (C), miR-103 (D) and miR-495 (E) expression levels in untreated male (grey line) and untreated female (pink line) pubertal rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 male animals. * Dissimilar letters indicate a statistically significant difference between groups, two-way ANOVA (p<0.05).

miR-26a and miR-495 in males was significantly higher than in females throughout the entire duration of pubertal development (Fig. 12B, E). Moreover, relative to male at much lower levels (Fig. 12B, E). In males, both miR-26a and miR-495 expression levels increased between early and mid puberty, and by late puberty, miR-26a decreased significantly to reach an expression level lower than the early puberty level (Fig. 12B).However, by late puberty, miR-495 continued to increase with age in the males (Fig. 12E). miR-103 expression levels were not significantly different between the sexes or between stages of pubertal development in the dorsal hippocampus (Fig. 12D).

Ventral hippocampus expression levels of EtOH-sensitive miR-10a-5p, miR-26a, and miR-495 in untreated rats are age and sex dependent.

We next tested whether the expression levels of the five EtOH-sensitive microRNAs were sexually dimorphic in the ventral hippocampus. RT-PCR analysis followed by a 2-way ANOVA revealed that EtOH-sensitive microRNA expression patterns were sexually dimorphic throughout pubertal development in the ventral hippocampus. Furthermore, the sex difference patterns observed in the ventral hippocampus of these microRNAs throughout puberty differed from those observed in the dorsal hippocampus. For example, there was a significant main effect of age and sex on expression levels of miR-26a, miR-103 and miR-495, and a significant interaction between age and sex in miR-10a-5p, miR-26a, miR-103 and miR-495 (Table 5). Specifically, miR-10a-5p expression levels did not change statistically over the course of pubertal development in females, but miR-10a-5p expression did significantly increase between mid and late puberty in males (Fig. 13A). Furthermore, no other EtOH-sensitive microRNA demonstrated any change in the female ventral hippocampus over time and,

similar to the dorsal hippocampus, female expression levels of miR-26a and miR-495 were very lowly expressed compared to the levels in males (Fig. 13B, E). Male expression levels of miR-26a and miR-495 both decreased significantly between early to mid pubertal levels, followed by a stabilization of miR-26a expression at late puberty (Fig. 13B) and a significant increase in miR-495 expression between mid and late puberty, with levels exceeding those expressed at early puberty (Fig. 13E). In contrast, miR-103 expression levels in males remained stable between early to mid-puberty, followed by a significant increase at late puberty (Fig. 13D).

Mature microRNA biosynthetic processing enzymes are altered by pubertal development in a sexually dimorphic manner in the dorsal and ventral hippocampus.

We next assessed the expression of Drosha and Dicer in male and female dorsal and ventral hippocampus at early, mid and late puberty. We hypothesized that mature microRNA biogenesis enzyme expression levels are sexually dimorphic during puberty. Importantly, previous reports indicate that the expression of microRNA processing enzymes may be regulated, in part, by sex hormones or their nuclear receptors. For example, breast cancers can be distinguished by their estrogen receptor (ER) status (i.e. can either be ER positive (ER+) or negative (ER-) and indeed, ERα breast cancer cell lines and clinical mechanisms remains unknown [249]. Therefore, it is possible that Drosha and Dicer levels correlate with pubertal changes in gonadal hormone levels. We identified a significant main effect of both sex and age on Drosha and Dicer expression

Table 5. Statistical Analysis of Gene Expression Levels in the ventral hippocampus

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF SEX	INTERACTION: AGE X SEX
miR-10a-5p	No	No	Yes: $p = 0.004$
miR-26a	Yes: $F(2,41) = 9.600$ $p < 0.001$	Yes: $F(2,41) = 59.915$ $p < 0.001$	Yes: $p = 0.002$
miR-32	No	No	No
miR-103	Yes: $F(2,53) = 13.038$ $p < 0.001$	Yes: $F(1,53) = 8.244$ $p = 0.006$	Yes: $p = 0.002$
miR-495	Yes: $F(2,41) = 22.776$ $p < 0.001$	Yes: $F(1,41) = 136.974$ $p < 0.001$	Yes: $p < 0.001$
GENE	**MAIN EFFECT OF AGE**	**MAIN EFFECT OF SEX**	**INTERACTION: AGE X SEX**
Drosha	Yes: $F(2,54) = 4.401$ $p = 0.017$	Yes: $F(2,54) = 21.607$ $p < 0.001$	Yes: $p = 0.006$
Dicer	Yes: $F(2,54) = 5.547$ $p = 0.006$	Yes: $F(2,54) = 53.759$ $p < 0.001$	Yes: $p = 0.003$
BDNF	Yes: $F(2,45) = 4.310$ $p = 0.019$	Yes: $F(1,45) = 14.607$ $p < 0.001$	Yes: $p = 0.009$
SIRT1	Yes: $F(2,47) = 28.346$ $p < 0.001$	Yes: $F(1,47) = 53.143$ $p < 0.001$	Yes: $p < 0.001$
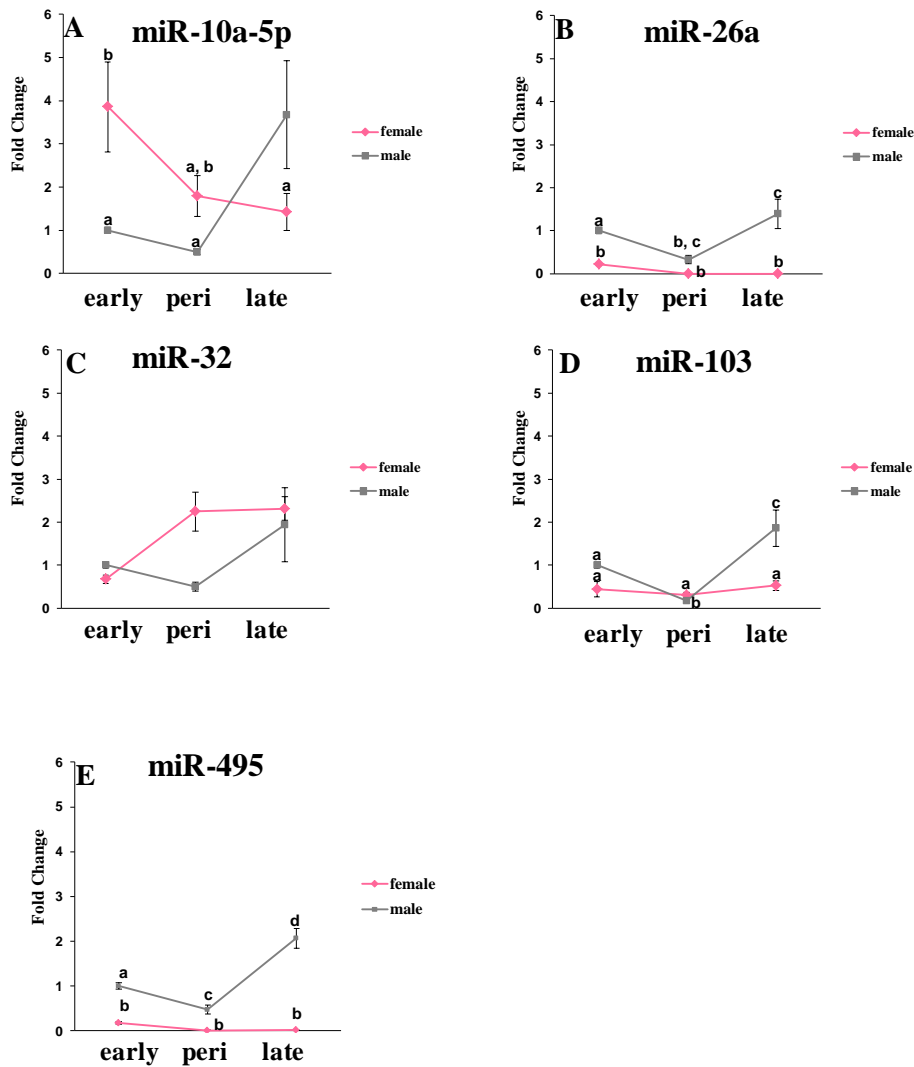
Figure 13. Sexually dimorphic microRNA expression is evident during pubertal development in the ventral hippocampus. miR-10a-5p (A), miR-26a (B), miR-32 (C), miR-103 (D) and miR-495 (E) expression levels in untreated male (grey line) and untreated female (pink line) pubertal rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 male animals. Dissimilar letters indicate a statistically significant difference between groups (p<0.05).

levels, as well as a significant interaction between age and sex in both the dorsal and ventral hippocampus (Table 4, Table 5). Interestingly, female expression levels of Drosha and Dicer in the dorsal and ventral hippocampus were lower than the expression levels in males (Fig. 14), except for one stage of pubertal development in the ventral hippocampus wherein male and females had similar Drosha expression levels (Fig. 14B). Furthermore, significant differences across age were apparent in male Drosha and Dicer expression levels in both hippocampus regions. Between early and mid-puberty, the male Drosha and Dicer expression levels decreased and remained decreased throughout late puberty in the dorsal hippocampus (Fig. 164, C) and significantly increased between mid puberty to late puberty in the ventral hippocampus (Fig. 14B, D). In stark contrast, the female microRNA biogenesis enzymes varied very little throughout pubertal development, such that only in the ventral hippocampus, did Drosha expression levels significantly decrease between early and peripuberty, followed by a further decrease below early pubertal levels at late puberty (Fig. 14B).

Expression levels of EtOH-sensitive microRNA target genes are differentially dependent on age and sex in the dorsal and ventral hippocampus.

The neurotrophic factor BDNF and the histone deacetylase SIRT1 are both neuroprotective. BDNF mediates synaptic plasticity and hippocampus-dependent memory by supporting neurotransmitter release and inducing long-term potentiation [84,85,253]. SIRT1 also contributes to hippocampus-dependent learning and memory as well as regulates anxiety [23,25,102,254]. Importantly, memory and mood are partially controlled by the dorsal and ventral hippocampus, respectively. We have previously
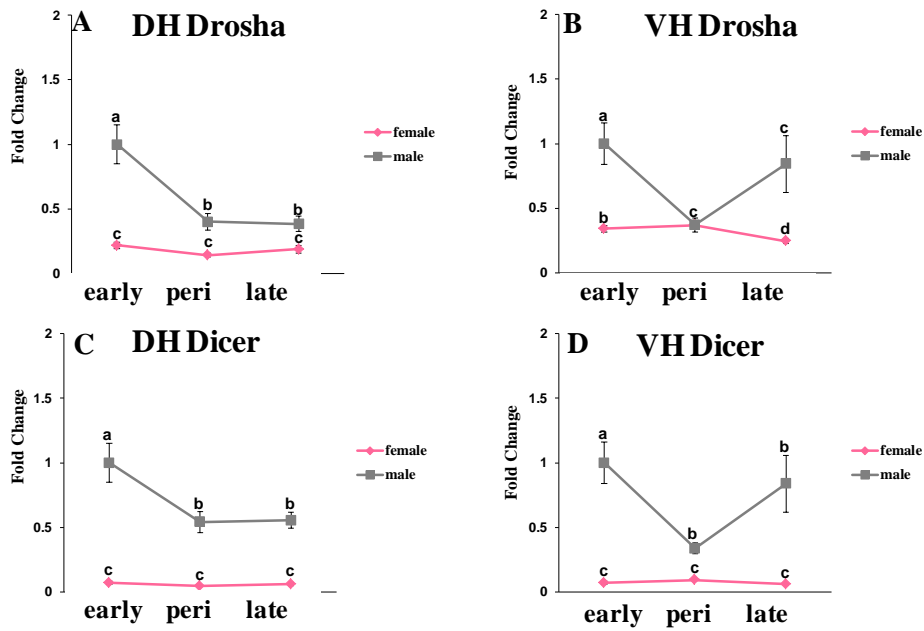
Figure 14. Sexually dimorphic microRNA processing enzyme expression is evident during pubertal development in the dorsal and ventral hippocampus. Drosha (A, C) and Dicer (B, D) expression levels in untreated male (grey line) and untreated female (pink line) pubertal rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 male animals. Dissimilar letters indicate a statistically significant difference between groups (p<0.05).

demonstrated that alterations in the expression of microRNAs following peripubertal

binge EtOH exposure in the male hippocampus significantly correlated with alterations in

the expression levels of the microRNA target genes BDNF and SIRT1 [247]. The

regulation of memory and mood are impaired by adolescent EtOH abuse [255,256] and

such effects can also present in a sexually dimorphic manner [228,257] yet the

mechanisms governing such processes remain unclear. Therefore, we hypothesized that

the EtOH-sensitive microRNA expression previously reported in males would be

differentially expressed in females across pubertal development and that the ventral and

dorsal hippocampus would display region-specific sex differences. Our results indicate a

statistically significant main effect of both age and sex on the expression levels of SIRT1

and BDNF in both the dorsal and ventral hippocampus, as well as a statistically

significant interaction between age and sex (Table 4 and Table 5). In the female dorsal

hippocampus, SIRT1 expression was dynamic across puberty such that it decreased

between early and mid puberty and remained lowly expressed into late puberty (Fig.

15C). The same pattern throughout puberty was also observed for male ventral

hippocampus SIRT1 expression, however, the level of SIRT1 was greater in males than

in females at each time point (Fig. 15C). Overall, females expressed these EtOH-sensitive

microRNA target genes at much lower levels relative to males and furthermore,

expression levels in males demonstrated a great deal of variation over the course of

pubertal development (Fig. 15). Interestingly, the expression patterns of BDNF in the

ventral and dorsal hippocampus and of SIRT1 in the ventral hippocampus over puberty

were similar, such that between early and mid puberty there was an increased in

expression, which then lowered back to early pubertal levels at late puberty (Fig. 15A, B,
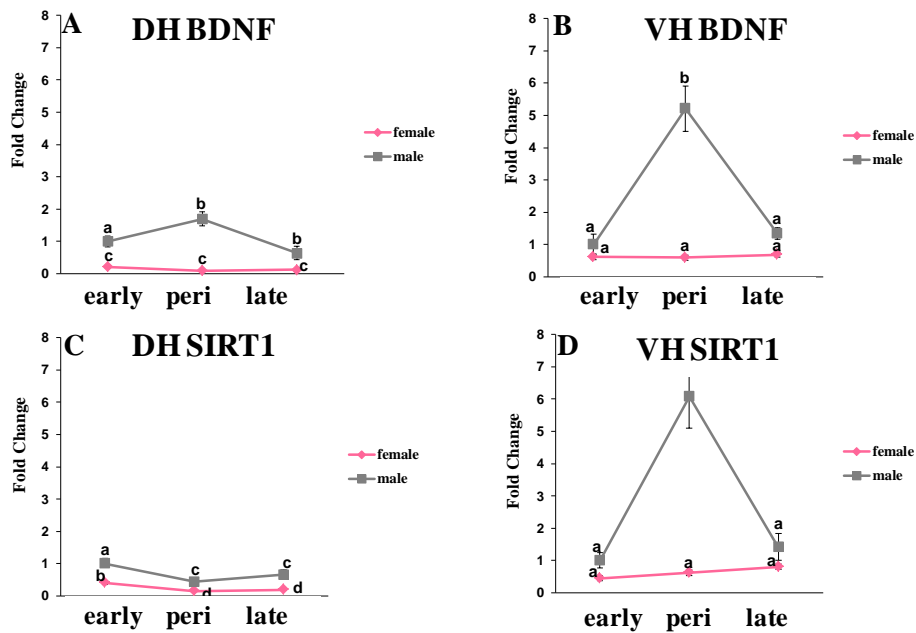
Figure 15. Sexually dimorphic microRNA target gene expression is evident during pubertal development in the dorsal and ventral hippocampus. BDNF (A, B) and SIRT1 (C, D) mRNA levels in the dorsal and ventral hippocampus in untreated male (grey line) and untreated female (pink line) pubertal rats. N=10/group. Data represent mean fold change ± SEM as compared to untreated PND 30 male animals. Dissimilar letters indicate a statistically significant difference between groups (p<0.05).

D). This similar pattern suggests that mid puberty is a possible critical window of time during which high SIRT1 and BDNF expression is critical for developmental processes in the male hippocampus.

Discussion

In the current study we tested the hypothesis that EtOH-sensitive microRNAs exhibit sexually dimorphic expression profiles in the ventral and dorsal hippocampus during pubertal development. The dorsal hippocampus primarily operates learning and memory while the ventral portion regulates emotional memory and mood [57,58,258]. The continuous development of neuronal circuitry throughout pubertal development [43,259] is partially regulated by gonadal hormones [47,233,236,260,261]. Gonadal hormone signaling via nuclear receptors has also been demonstrated to influence the expression of microRNAs [165,262,263,264,265,266]. Therefore, gonadal hormone exposure may mediate hippocampal maturation during puberty via downstream microRNA effector molecules. Furthermore, the rise in sex steroids during pubertal development may contribute to a microRNA-mediated regulation of the sexually dimorphic development of memory capacities and emotional responses during this period. Bioinformatic analysis predicted that miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495 bind to the 3' UTRs of genes regulating synaptic plasticity, including SIRT1, BDNF and synapsin II (SYN2). Four of these five microRNAs have recently been identified to demonstrate dynamic expression patterns throughout healthy pubertal development in the male rat hippocampus and furthermore, their normal developmental expression profile is disrupted following peripubertal binge EtOH, a typical adolescent behavior [247].

The main goal of this study was to quantify sex differences in the expression levels of miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495, the microRNA target genes SIRT1 and BDNF and the major microRNA biosynthetic processing enzymes Drosha and Dicer in the pubertal dorsal and ventral hippocampus. Overall, this study demonstrates that miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495 are expressed in an age and sex-specific manner during intact pubertal development and that these sex differences are distinct between the dorsal and ventral hippocampus. Furthermore, we quantified, to our knowledge for the first time, sex differences in the expression levels of BDNF, SIRT1, Drosha and Dicer mRNA in the dorsal and ventral hippocampus at three stages of pubertal development, and identified significant sex differences in expression levels throughout puberty and between the ventral and dorsal hippocampus. Taken together, sexually dimorphic expression levels of EtOH-sensitive microRNAs, their gene targets and microRNA processing enzymes during pubertal development suggests that the expression of these microRNAs may be influenced by sex hormones. Furthermore, the sex, age and hippocampus region-dependent fluctuations in EtOH-sensitive microRNAs and their target genes suggest that sex differences in pubertal microRNA expression may underlie the sexual dimorphisms observed in response to stress and alcohol abuse, suggesting that they may influence the development of anxiety and depression-related disorders during this period of life.

The hippocampus plays a major role in regulating sexually dimorphic behaviors, such as learning, memory and the stress response. For instance, males perform better than females on hippocampus-dependent spatial memory acquisition tests despite the well-known positive effects that E_2 imparts on hippocampus-dependent function in females.

Indeed, it has been proposed that E_2 and T differentially influence memory functions in

males and females [267,268]. It is likely that the hormonal processes of sexual

differentiation during development creates organizational differences in neural substrates

and that these organizational differences underlie the sexually dimorphic effects of E_2 and

T on cognitive tasks in males and females as well as contribute to the different strategies

males and females use to solve similar tasks [269]. However, it is difficult to ascertain

clear sexually dimorphic effects that gonadal hormones have on hippocampus-mediated

cognition in males and females because hormonal influences can differ with task type,

task aspect and the degree to which the task relies on one or more brain region. In

contrast, clear responses to gonadal hormone treatment can be observed when

investigating morphological, anatomical and cellular aspects of hippocampus-mediated

functions. For instance, synaptic plasticity in males and females relies on gonadal

hormones. Gonadectomy (GDX) in males reduces spine density (structures that receive

excitatory inputs and therefore serve as a measurement of synaptic plasticity and

memory) and while E_2 treatment fails to restore this loss [270], androgen receptor

activation increases spine density via up-regulation of N-methyl-D-aspartate receptors

(NMDARs) [270,271,272]. In contrast, female exposure to androgens and endogenous

and exogenous E_2 leads to increased spine density [270,272,273,274] and the

electrophysiological measurement of learning and memory, long-term potentiation (LTP)

[275,276]. These data demonstrate that differential mechanisms are initiated by AR and

ER activation to mediate synaptic plasticity in males and females. Therefore, it is not

unusual that we observed sex differences in the expression of hippocampal microRNAs

(Fig. 12 and Fig. 13), as they may play a role in mediating signaling pathways

responsible for normal sexually dimorphic brain development during puberty as well as in the sexually dimorphic responses to peripubertal binge EtOH [44].

Our current understanding of how peripubertal binge EtOH affects the male and female developing brain differently is incomplete, and therefore requires further investigation into the potential molecular targets of binge-pattern of EtOH abuse. In our study we observed relatively low expression levels in the female hippocampus of the microRNAs sensitive to pubertal binge EtOH compared to males (Fig. 12 and Fig. 13). Indeed, it is possible that different microRNAs are sensitive to EtOH in males than in females. Alternatively, microRNAs may not be involved in mediating the effects of binge EtOH on the stress response or memory capacity in females, a theory in line with previous findings that binge EtOH exposure-mediated alterations in genes regulating the stress response were observed in pubertal males but not in females [44,47]. It is possible that sex differences in microRNA expression results from differences in the rates by which males and females reach sexual maturity, regarding reproductive function. Indeed, females typically develop reproductive capacity earlier than males [17,277]. While the rates of maturation for adult-like memory processing and stress responsiveness in males and females are not clearly delineated, some studies support the notion that observed sex differences in the pubertal stress response [278] are indeed established in the brain prior to pubertal onset in females [279]. These data suggest that microRNAs, microRNA biogenesis enzymes, BDNF and SIRT1 expression levels are sexually dimorphic during pubertal development and distinct between the dorsal and ventral hippocampus. Because the ventral hippocampus regulates the stress response, it is possible that the sexually dimorphic expression levels of microRNAs may play a role in sex differences in mood

and emotional memory regulation. A more completely developed female stress response and/or synaptic plasticity at pubertal onset may also underlie observed sex differences in the mechanisms employed by AR and ER activation-induced synaptic plasticity in the male and female adolescent brain, respectively. Taken together, these data underscore the need to better understand whether the observed sex differences in hippocampal microRNA expression may contribute to the sexually dimorphic adolescent responses to stressors such as binge alcohol exposure.

The biogenesis of mature microRNAs relies on the RNAse III enzymes, Drosha and Dicer which generate the pre-microRNA and microRNA, respectively. In cancer cell models, E_2 interferes with microRNA biogenesis by acting via estrogen receptors α and β (ERα, ERβ), transcription factors of the nuclear receptor family of homeostatic regulators [161,264]. For instance, E_2-induced ERβ signaling in an E_2-responsive cancer cell line lead to an accumulation of pre-microRNA transcripts, possibly due to an ERβ-mediated release of Drosha from sequestration in an inhibitory chromatin complex [161]. Castellano et al. also observed alterations in pri-microRNA precursors more often than in the mature microRNAs derived from it following E_2-induced nuclear receptor signaling in breast cancer cell lines, which suggests that the regulation of microRNA expression occurs at the level of microRNA biogenesis [264]. The details of how particular microRNAs are altered by nuclear receptor-mediated regulation of Drosha and/or Dicer activity remains unclear, yet these data set precedence for further investigation into the mechanisms by which mature microRNA expression can be regulated. Importantly, estrogen receptor-mediated regulation of microRNA expression has not been confirmed in the brain. However, it is possible that sex differences in hippocampal microRNA

expression throughout rat pubertal development are influenced by the regulation of the microRNA biogenesis. We demonstrate here that expression levels of Drosha and Dicer mRNA in males was overall much greater when compared to females (Fig. 14). Furthermore, the male expression levels of these enzymes fluctuated over pubertal development while the female expression levels of Drosha and Dicer had little to no changes over time (Fig. 14). The fluctuations in expression levels of Drosha and Dicer, or the lack thereof, in male and females respectively, do not correlate with the patterns of microRNA expression level changes during pubertal development in either sex. However, recent studies have repeatedly demonstrated in [280,281] multiple tissues, that models of Drosha and Dicer knockouts, knockdowns or mutants alters the expression of only subset of microRNAs [130,282,283]. For instance, homozygous Dicer mutant zebrafish demonstrate arrested growth and early death, yet ablation of Dicer does not induce complete abolishment of mature microRNA expression and the authors conclude that a maternal contribution of Dicer may maintain the biogenesis mature microRNAs [284]. An additional study examined a Dicer exon 5-deficient colorectal cell line which demonstrated once again, that only 57% of known microRNAs were downregulated in their expression in the mutant cells compared to wild type [280]. In addition, Kuehbacher et al. demonstrate that Drosha and Dicer siRNA downregulates expression of only two microRNAs (let-7f and miR-27b) out of many (members of the let-7 family, miR-21, miR-126, miR-221 and miR-222) that are highly expressed in endothelial cells [282]. Therefore, although it is surprising that Drosha and Dicer expression levels don't correlate with the observed changes in microRNA expression throughout pubertal development in our study, it is becoming clear that a lack these biogenesis enzymes may

not necessarily dictate a reduction in mature microRNA expression. Furthermore, because the *in vivo* function of Drosha remains unknown and because Drosha belongs to the same class of endonucleases as Dicer, it is not completely surprising that decreases in Drosha expression also do not correlate with decreases in microRNA expression.

microRNAs are considered master regulators of the cellular transcriptome and have been repeatedly demonstrated to play critical regulatory roles in synaptic plasticity. Indeed, the highly plastic nature of the adolescent brain underlies the substantial brain maturation that takes place at this time and plays a particular role in the formation and consolidation of spatial and emotional memory. The evolutionary advantage to a microRNA-mediated regulation of synaptic plasticity is that it enables a neuron to quickly fine-tune its gene expression and protein composition in order to adapt to different environmental stimuli. We not only focused our study on identifying the expression pattern of microRNAs that are sensitive to EtOH exposure in the pubertal hippocampus, but also on the microRNA target genes important for hippocampus functions. Indeed, the two microRNA target genes of interest, BDNF and SIRT1, are also both important mediators of synaptic plasticity. In the male ventral hippocampus we demonstrated that the significantly dynamic expression patterns of BDNF and SIRT1 correlated with the significant changes in expression levels of miR-26a, miR103 and miR-495 throughout pubertal development (Fig. 13B, D, E and Fig. 15B, D). Importantly, using target prediction software programs, Targetscan and MirDB, we identified BDNF and SIRT1 are targets of miR26a, miR-103 and miR-495. Moreover, in the male dorsal hippocampus, decreased SIRT1 expression levels during pubertal development correlated with increased miR-495 expression levels (Fig. 13E and Fig.

15C). These data suggest that mir-26a, miR-103 and miR-495 mediate important developmental roles in the pubertal male ventral and dorsal hippocampus, possibly by regulating the expression levels of BDNF and SIRT1. Consistent with this interpretation, previous studies have experimentally confirmed that miR-26a binds the 3'UTR of BDNF [143]. Also a separate study, by using massive parallel sequencing demonstrated that miR-26a is abundantly expressed in the mouse hippocampus and by using integrated genomics demonstrated that miR-26a shares a seed sequence with other microRNAs targeting genes important for hippocampus function [285]. Overall, female expression levels of EtOH-sensitive microRNAs, BDNF and SIRT1 did not correlate with one another in this study (Fig. 12, 13 and 15). Moreover, the expression levels of SIRT1 and BDNF were extremely low and nearly unchanging in the female hippocampus compared to males throughout pubertal development (Fig. 15). These "negative" data most likely do not reflect a lack of dynamic synaptic plasticity, but rather suggest that SIRT1 and BDNF are not the primary mediators of synaptic plasticity in the female pubertal hippocampus. Indeed, the female hippocampus may utilize different mechanisms responsible for synaptic plasticity. This interpretation is supported by previous work identifying that AR and ER in males and females, respectively, initiate different mechanisms to regulate synaptic plasticity [270,272,273,274,275,276]. Indeed, BDNF is well known to be regulated by gonadal hormones and it is possible that the differences we detect in pubertal male and female hippocampus BNDF expression is a reflection of respective male and female gonadal hormones partially directing different mechanisms of synaptic plasticity between the sexes. Importantly, gonadal hormone levels were not directly tested because pubertal gonadal hormone levels have been well characterized

[286,287,288]. Overall, this chapter determined that EtOH-sensitive microRNAs are expressed in different developmental and regional patterns within the male and female pubertal hippocampus, and suggests that these microRNAs may differentially regulate their gene targets in the pubertal male and female brain.

CHAPTER FIVE

SEX DIFFERENCES IN HIPPOCAMPUS 17β-ESTRADIOL-RESPONSIVE
MICRORNA EXPRESSION DURING PUBERTAL DEVELOPMENT OF THE
WISTAR RAT

Introduction

Adolescence is a dynamic period of life during which the brain undergoes
countless physiological and psychological developments. Pubertal maturation is the most
clear physiological process that transpires during adolescence, one hallmark of which is
the rapid rise in the gonadal hormones 17β-estradiol (E_2) and testosterone (T) in females
and males, respectively. Puberty is initiated, in part, by activity of the neuroendocrine
hypothalamo-pituitary-gonadal (HPG) axis. In turn, gonadal hormones induce
physiological changes in brain structure and gene expression, which are ultimately
reflected in the functional development of adultlike behaviors. Indeed, differences in
circulating gonadal hormones between males and females has long been supported to
underlie sexually dimorphic brain morphology [289,290,291,292], neurochemistry
[293,294,295,296,297,298], and function [294,299] in most species.

Classical gonadal hormone action throughout the body and brain involves the
binding of gonadal hormones to nuclear receptors which, upon activation, can regulate
gene transcription. More recently, E_2 has been of particular interest regarding its
influence on the transcription of microRNAs [19,223]. Recently, Rao et al. demonstrated
that microRNA expression is altered by E_2 in an age-dependent manner in the

hippocampus of young and aged female rats [28], which suggests that E_2 can influence microRNA expression levels differentially throughout the lifespan. Indeed, experimental manipulation of puberty at different ages also alters microRNA expression in the male rodent [248]. Furthermore, alterations in brain microRNA expression are associated with sexually dimorphic psychiatric disorders that develop during adolescence [182]. For example, miR-30b expression is lower in female schizophrenic brains compared to males and this expression is associated with mutations in the human estrogen receptor gene, *Esr1* [151]. Moreover, miR-30b sensitivity to E_2 in the brain suggests that E_2-sensitive microRNA may play a role in the sexual dimorphic etiology of psychiatric disorders. The adolescent alterations in neurocircuitry and neurochemistry which contribute to the development of adultlike behaviors undoubtedly require a high degree of finely orchestrated synaptic plasticity and regulation of gene expression during this important developmental period. It is possible that the remarkable neuronal plasticity observed in the adolescent brain is mediated, in part, due to gonadal hormone-signaling via microRNAs.

Gonadal hormones and microRNAs have both been observed to influence synaptic plasticity, however, whether they do so during adolescence is not known. In the hippocampus, E_2 alters synapse formation by increasing expression of pre- and post-synaptic proteins and thereby increasing dendritic spine formation in vivo [300,301,302,303,304]. In addition, miR-132 promotes dendrite growth in response to neuronal stimulation, regulating synaptic plasticity in a p250GAP-dependent mechanism [305]. That microRNAs regulate synaptic plasticity and are differentially expressed in psychiatric disorders suggests that they are active players in adolescent brain

development. Moreover, differences in gonadal hormone levels between males and females may reflect differences in microRNA expression.

Overall, the roles that microRNAs play in postnatal brain development and maturation still requires a great deal of investigation. Given that the hippocampus plays a critical role in synaptic plasticity, a process required for pubertal neuronal rewiring and maturation of adultlike behaviors, it is possible that the expression of microRNAs in the hippocampus are appropriately poised to play a crucial role in regulating the biological processes involved in pubertal neuronal development. To explore this theory, we hypothesized that hippocampal expression of E_2-sensitive microRNAs is sexually dimorphic throughout pubertal development and that this expression is distinct between the dorsal and ventral hippocampus. To test this hypothesis, we quantified the ventral and dorsal hippocampus expression levels of 5 microRNAs discovered by Rao et al. to be altered by E_2 dependent on age in the female brain: let-7i, miR-7a, miR-9, miR-125a and miR-181a. The ventral and dorsal hippocampus were examined separately because the dorsal hippocampus' primary function is that of spatial memory acquisition, distinguishing it from the ventral hippocampus which regulates emotional memory and affective processing via its many projections to amygdalar nuclei [57,58,205]. Importantly, the normal developmental expression patterns these specific microRNAs have not been documented in either sex at any stage of puberty, nor between distinct functional regions of the hippocampus.

Approach

The dorsal and ventral hippocampus were microdissected from untreated male and female Wistar rats and collected at three different ages during pubertal development

(early puberty, PND 30; mid/peripuberty, PND 44; late puberty, PND 71;

N=10/sex/group). Importantly, peripuberty in rats is considered to be ~ PND 30-45

[201,202]. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to

quantify the expression levels of a total of five microRNAs: (let-7i, miR-7a, miR-9, miR-

125a, and miR-181a). microRNAs were previously identified to be 17β-estradiol-

sensitive in female rats by Rao et al. [247].

Results

let-7i, miR-7a, miR-9, miR-125a, and miR-181a expression levels throughout

pubertal development in the dorsal hippocampus are dependent on age and sex.

microRNA expression can be regulated by E_2 in whole body homogenates [306]

as well as a variety of other tissues [165,307,308]. In the brain, expression levels of

mature let-7i, miR-7a, miR-9, miR-125a, and miR-181a are altered by E_2 dependent on

age in the adult female rat [28], however, the normal physiological expression profile of

these microRNAs throughout puberty, when circulating E_2 levels increase dramatically in

females, is unknown. Therefore, we measured the expression levels of 5 E_2-responsive

microRNAs in the dorsal hippocampus using qRT-PCR at three time points throughout

pubertal development (early = 30 d, peri = 44 d, late = 73 d) in untreated male and female

rats (Fig. 16). In the dorsal hippocampus, a two-way ANOVA revealed a main effect of

sex in 4 out of the 5 microRNAs tested including miR-7a, miR-9, miR-125a, and miR-
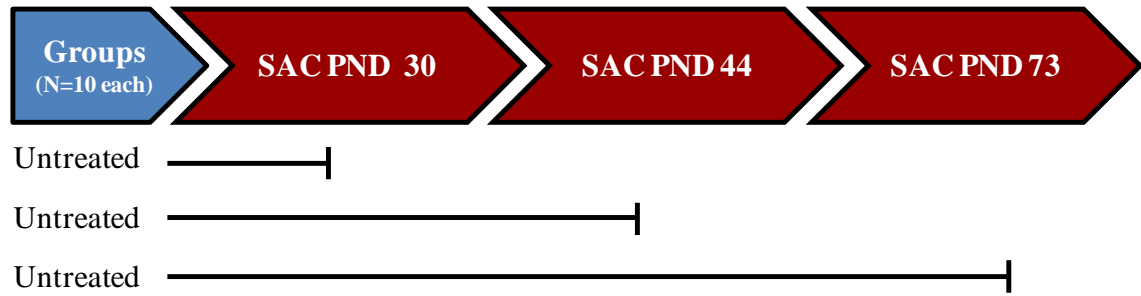
Figure 16. Diagram of experimental paradigm. Diagram depicting the age of sacrifice for each group of male and female Wistar rats. N=10/group.

Table 6. Statistical Analysis of microRNA Expression Levels in the Dorsal Hippocampus

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF SEX	INTERACTION: AGE X SEX
let-7i	No	No	No
miR-7a	No	Yes: $F(1,46) = 10.138$ $p = 0.003$	Yes $p = 0.015$
miR-9	Yes: $F(2,48) = 20.038$ $p < 0.001$	Yes: $F(1,48) = 13.933$ $p < 0.001$	Yes $p = 0.003$
miR-125a	Yes: $F(2,48) = 4.984$ $p = 0.011$	Yes: $F(1,48) = 10.476$ $p = 0.002$	Yes $p = 0.004$
miR-181a	Yes: $F(2,45) = 10.076$ $p < 0.001$	Yes: $F(1,54) = 8.206$ $p\ p = 0.006$	Yes $p < 0.001$

181a, and 3 of these microRNAs, (miR-9, miR-125a and miR-181a) demonstrated a main effect of age (Table 6). A statistically significant interaction between sex and age was observed for miR-7a, miR-9, miR-125a, and miR-181a expression in the dorsal hippocampus (Table 6), demonstrating that the sex differences in microRNA expression is dependent on age. Interestingly, the only E_2-responsive microRNA that was expressed in a manner singly dependent on sex, miR-7a, demonstrated an expression pattern throughout puberty that was different from the other 3 sex-dependent E_2-responsive microRNAs. Specifically, the expression levels of miR-9, miR-125a and miR-181a significantly decreased in females between early and peripuberty, and remain decreased until late puberty (Fig. 17C, D, E). In contrast, male expression levels of miR-9, miR-181a and miR-125a remained constant. On the other hand, miR-7a expression did not change between early and peripuberty, but significantly increased and decreased between peripuberty and late puberty in females and males, respectively (Fig. 17B). These data suggest that miR-9, miR-125a, miR-181a may play important roles in dorsal hippocampus sexual dimorphisms during early puberty and that miR-7a may be important for mediating sex differences in brain function during late puberty. Overall, dorsal hippocampus microRNA expression levels that are dependent on sex and age decrease over the course of puberty whereas the microRNAs dependent on sex alone demonstrate divergent expression patterns between males and females.

Sexually dimorphic expression patterns of E_2-responsive microRNAs throughout pubertal development in the ventral hippocampus are distinct from the dorsal hippocampus.
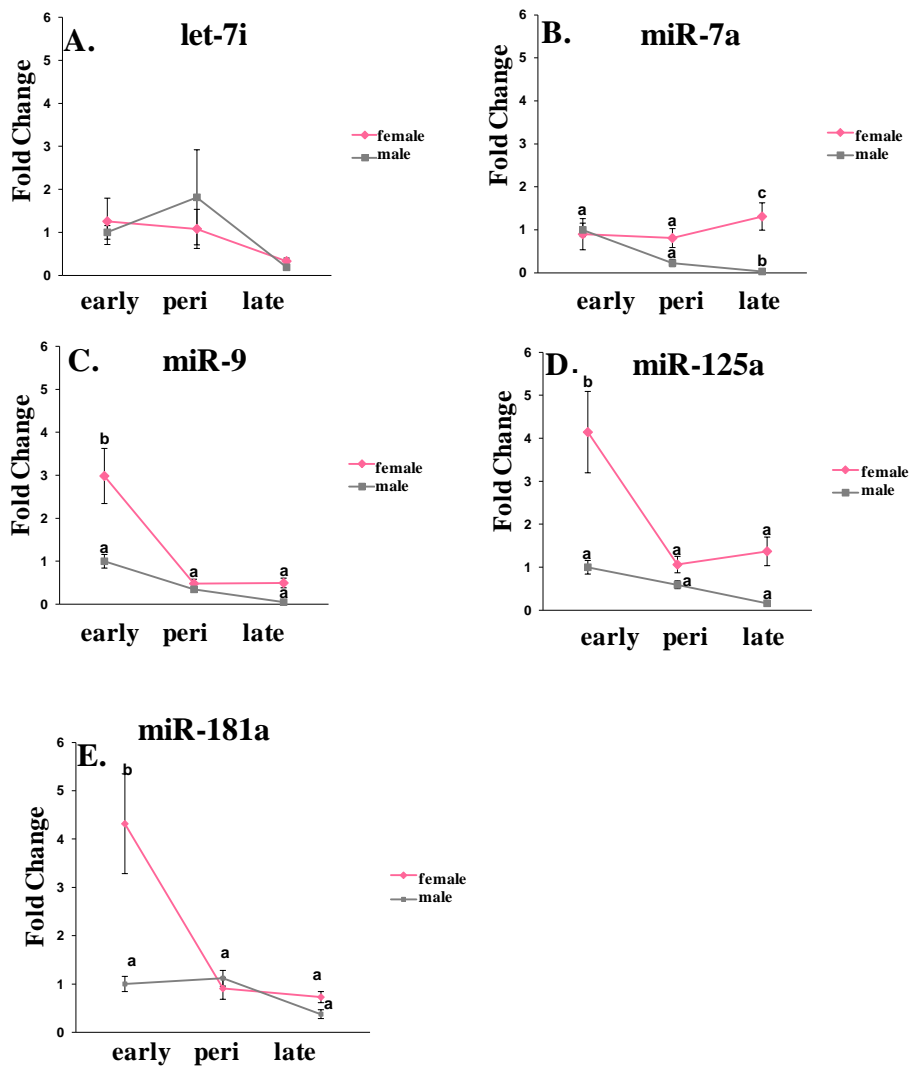
Figure 17. Sexually dimorphic microRNA expression is evident during

pubertal development in the dorsal hippocampus. let-7i (A), miR-7a (B),

miR-9 (C), miR-125a (D) and miR-181a (E) expression levels in untreated

male (grey line) and untreated female (pink line) pubertal rats. N=10/group.

Data represent mean fold change ± SEM as compared to PND 30 male

animals. Dissimilar letters indicate a statistically significant difference

between groups based on a 2-way ANOVA (p<0.05).

microRNA expression levels in the brain can be dependent on age, sex and hormone levels [309,310,311]. However, sex differences in microRNA expression during puberty have not been compared between the anatomically and functionally distinct dorsal and ventral hippocampus regions [57,58,205]. Therefore, we next quantified the expression levels of the E_2-responsive microRNAs let-7i, miR-7a, miR-9, miR-125a, and miR-181 [28] in the ventral hippocampus to determine if they are differentially expressed from the dorsal hippocampus during pubertal development. In the ventral hippocampus there was a significant main effect of sex, independent of age, in 4 out of the 5 microRNAs, miR-7a, miR-9, miR-125a and miR-181a (Table 7). This is in stark contrast to the alterations in E_2-responsive microRNAs in the dorsal hippocampus, in which these microRNAs were altered by sex dependent on age (Table 6). Interestingly, miR-7a, miR-125a and miR-181a expression levels remained constant throughout pubertal development in both males and females, yet female microRNA expression levels were significantly greater than males throughout this time period (Fig. 18B, D, E). The expression level of miR-9 however, was dynamic across pubertal development in males and not in females. Specifically, during early puberty, miR-9 expression levels were significantly greater in males compared to females, after which miR-9 significantly decreased below female levels at peripuberty and remained lowered until late puberty (Fig. 18C). Finally, no interaction was detected between age and sex for the E_2-responsive microRNAs in the ventral hippocampus which demonstrated sexually dimorphic expression levels, suggesting that these observed sex differences are not dependent on E_2.

Discussion

Table 7. Statistical Analysis of Gene Expression Levels in the Ventral Hippocampus

microRNA	MAIN EFFECT OF AGE	MAIN EFFECT OF SEX	INTERACTION: AGE X SEX
let-7i	No	No	No
miR-7a	No	Yes: $F(2,53) = 5.319$ $p = 0.008$	No
miR-9	No	Yes: $F(1,53) = 25.571$ $p < 0.001$	No
miR-125a	No	Yes: $F(1,47) = 71.900$ $p < 0.001$	No
miR-181a	No	Yes: $F(1,45) = 20.266$ $p < 0.001$	No
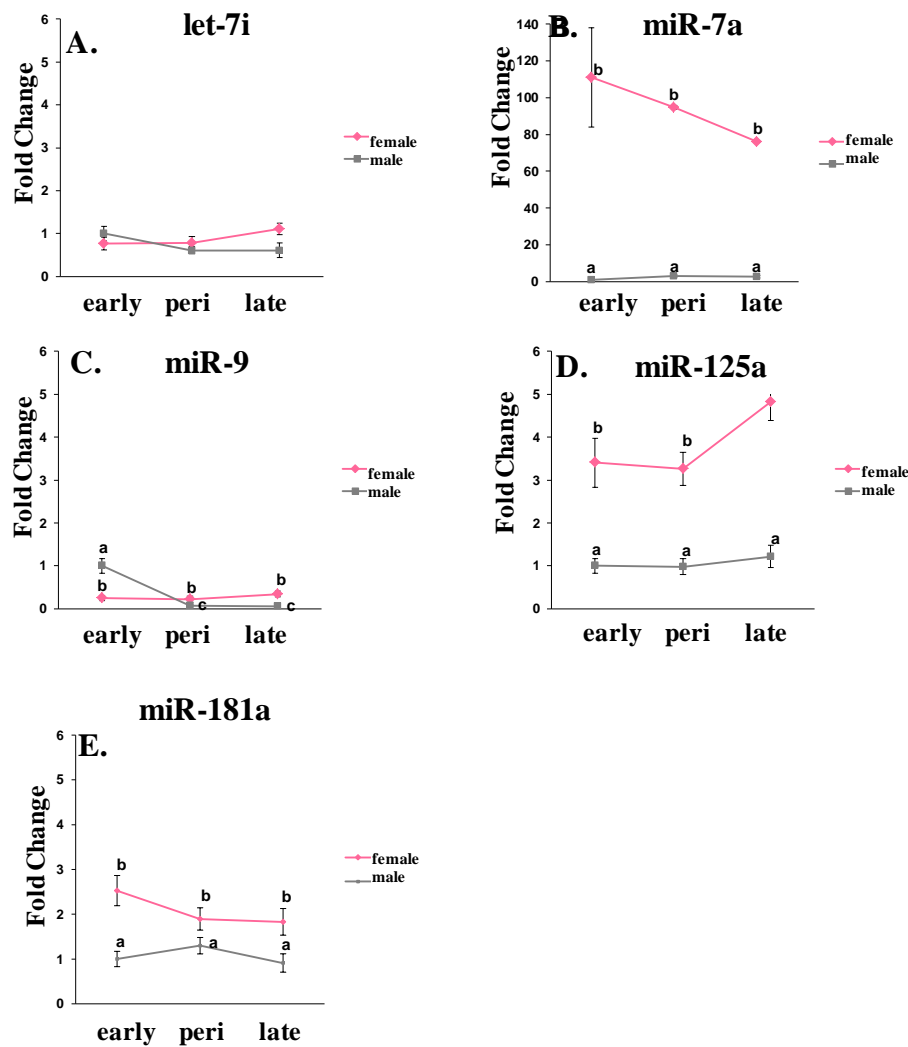
Figure 18. Sexually dimorphic microRNA expression is evident during
pubertal development in the ventral hippocampus. let-7i (A), miR-7a (B),
miR-9 (C), miR-125a (D) and miR-181a (E) expression levels in untreated
male (grey line) and untreated female (pink line) pubertal rats. N=10/group.
Data represent mean fold change ± SEM as compared to PND 30 male
animals. Dissimilar letters indicate a statistically significant difference
between groups based on a 2-way ANOVA (p<0.05).

Two novel findings emerged from these studies. First, 4 E_2-responsive microRNAs, miR-7a, miR-9, miR-125a and miR-181a, are differentially expressed between males and females throughout pubertal development. Second, the degree to which microRNA expression levels vary by sex differs between brain regions. To our knowledge, these are the first studies to identify sexually dimorphic microRNA expression within the ventral and dorsal hippocampus. Moreover, the regional expression patterns of microRNAs in the hippocampus suggest that these microRNAs have distinct roles in the ventral and dorsal hippocampus. Our study extends these findings by determining that miR-7a, miR-9, miR-125a and miR-181a expression levels are dependent on sex and age during pubertal development. Overall, we determined that E_2-sensitive microRNAs expression levels are distinct between three stages of pubertal development and that these expression profiles are sexually dimorphic and brain region-dependent.

Our data support the concept that microRNA expression levels are dynamic during periods of significant hormone fluctuations (such as puberty), although more experiments would be required to ascertain whether gonadal hormones directly regulate these microRNA expression profiles. Importantly, E_2 and T levels are well characterized, such that they increase during pubertal development [286,287,288] and recent studies also indicate that periods of shifting gonadal hormone levels are associated with shifts in microRNA profiles in the brain [28,29,312]. For example, perinatal exposure to T associated with brain masculinization dramatically changes microRNA expression patterns [29] and E_2 treatment in the aged female brain leads to different patterns of brain microRNA expression in the aged compared to young female rats [28]. A recent miR RT-

PCR array also revealed distinct microRNA expression profiles in the prefrontal cortex of pre-pubertal (PND 28) male and female mice, providing further evidence that sexually dimorphic gonadal hormones and/or sex can influence miR expression patterns in the brain [312]. Therefore, our results which revealed that miR-7a, miR-9, miR-125a and miR-181a are differentially expressed between males and females throughout pubertal development suggests that the expression of these microRNAs may be regulated by gonadal hormones.

The primary purpose of this study was to determine the effect of pubertal development (i.e. age) on the expression levels of E_2-responsive microRNAs [28] in male and female rats, yet our data reveal that microRNA expression levels in the ventral hippocampus are dependent on sex alone throughout pubertal development. This is in contrast to the dorsal hippocampus, where none of the E_2-responsive microRNAs were dependent on sex alone. Therefore, these data reveal that sex is a critical factor in determining the microRNA expression profile in the pubertal rat ventral hippocampus, suggesting that these microRNAs may regulate the development of the ventral hippocampus and the sexually dimorphic mood and memory behaviors that it mediates.

Our data also demonstrate regional differences in the expression levels of sexually dimorphic E_2-responsive microRNAs during puberty. For instance, in the dorsal hippocampus, the expression levels of 3 microRNAs (miR-9, miR-125a and miR-181a) are altered by sex dependent on age, whereas only one microRNA (miR-7a) is dependent on age alone. These data indicate that the expression levels of miR-9, miR-125a and miR-181a are likely to regulate key sexually dimorphic developmental processes in the dorsal hippocampus occurring at distinct stages of puberty. This further suggests pubertal

microRNA expression levels can be sensitive to alterations in hormonal signaling and synaptic activation, two constantly evolving processes that shape the developing adolescent brain [313]. Interestingly, the expression level of let-7i is not dependent on sex or age in either the ventral and dorsal hippocampus, despite previous findings indicating its E_2-responsiveness in the hippocampus of older rats [28]. This discrepancy underscores the concept that microRNAs can have very specific functions at different stages of development and/or different stages of hormonal environments in the brain and suggest that regional differences in ventral and dorsal hippocampus neurodevelopment during puberty may be mediated, in part, by differential E_2-responsive microRNA expression

Currently, there is not much information regarding the functions of the microRNAs measured in this study, particularly in the brain. Indeed, many efforts in the microRNA field are focused on identifying the potential target genes of microRNAs in different biological systems in order to elucidate their functions. However, most of these studies rely largely on imperfect computer algorithms to predict gene targets, making it difficult to draw compelling conclusions from such experimentally-derived data. For example, current microRNA target prediction programs cannot determine cell-type specificity nor indicate whether multiple microRNAs—as opposed to a singly identifiable microRNA—may be required to act in concert to regulate the expression of a given target gene, and indeed, complete mechanisms regarding microRNAs' regulatory potential remain to be elucidated. Of the 5 microRNAs Rao et al. found to be E_2-responsive, miR-9 is by far the most widely researched regarding its neuronal functions including neurogenesis and neuronal outgrowth. For instance, undifferentiated human [314] and

mouse-derived [315] stem cells rely on miR-9 expression during neural progenitor cell differentiation and miR-9 can also convert human fibroblasts into neurons [316,317]. miR-9 plays an instructive role in mediating neuronal cell fate by targeting transcription factors[318,319], suggesting that miR-9 may influence hippocampal neurogenesis during puberty. Moreover, miR-9 contributes to neuronal communication by regulating dendritic branching [320]. Together, these studies reveal that miR-9 regulates the generation and architecture of neuronal networks, linking miR-9 function to synaptic plasticity. However, our limited understanding of microRNAs lends functional studies to appear far-reaching. Therefore, documenting the developmental profiles of microRNAs in all biological systems is a logical starting point towards the goal of identifying functional capacity. Towards this aim, the current study identified both temporal and sex-specific expression patterns of E_2-responsive microRNA in the adolescent brain, and provided insight into their potential functions as being mediators of sexually dimorphic pubertal brain development. Taken together, the data presented here establish fundamental rationale for future studies aiming to classify the effector (i.e target gene) functions of these microRNAs.

CHAPTER SIX

GENERAL DISCUSSION

Summary

The previous chapters have described the following four main findings: 1) peripubertal binge EtOH exposure induces long-term alterations in mature microRNA expression levels in the male rat hippocampus, and has the potential to modulate the expression of their downstream target genes, 2) expression profiles of EtOH-sensitive microRNAs, miR-10a-5p, miR-26a, miR-32, miR-103 and miR-495, and their target genes, are dependent on sex and age in the pubertal rat hippocampus and therefore may contribute to sexually dimorphic hippocampus neurodevelopment, 3) expression profiles of E_2-responsive microRNAs, miR-7a, miR-9, miR-125a and miR-181a, are differentially dependent on sex and age throughout pubertal development, suggesting that they play distinct developmental roles during puberty and 4) EtOH-sensitive and E_2-responsive microRNAs have distinct expression profiles in the dorsal and ventral hippocampus throughout pubertal development, suggesting that their respective functions are region-specific. Taken together, these data chapters significantly contribute to the scientific literature that the expression levels of microRNAs can vary throughout pubertal development in brain region and sex-specific manner, and that distinct microRNAs are sensitive to peripubertal binge EtOH. Global analysis of our findings suggest the following two overarching possibilities that 1) peripubertal binge EtOH-mediated alteration of microRNA expression and/or processing underlies long-term alcohol abuse-

induced central nervous system impairments, and 2) that microRNAs may play a regulatory role in sexually dimorphism of adolescents. Chapters 3-5 have delivered data generating a framework from which one can further study the above two theories/hypotheses.

Key Findings

- **Chapter III:**

    A) Characterization of EtOH-sensitive microRNAs:

    ➢ dorsal hippocampus miR-10a-5p expression decreases between early and mid puberty, remaining decreased until late puberty

    ➢ dorsal hippocampus miR-26a expression levels decrease between mid and late puberty

    ➢ dorsal hippocampus miR-103 expression levels do not change throughout pubertal development

    ➢ dorsal hippocampus miR-495 expression levels increase between early and mid puberty, remaining increased until late puberty

    ➢ peripubertal binge EtOH increases miR-10a-5p expression levels between mid and late puberty in the dorsal hippocampus

    ➢ peripubertal binge EtOH decreases miR-26a expression levels immediately following the last treatment at mid puberty in the dorsal hippocampus

    ➢ peripubertal binge EtOH does not alter miR-103 expression levels during puberty in the dorsal hippocampus

- ➢ peripubertal binge EtOH decreases miR-495 expression levels immediately following binge, remaining decreased until late puberty in the dorsal hippocampus

- ➢ ventral hippocampus miR-10a-5p expression levels increase between mid and late puberty

- ➢ ventral hippocampus miR-26a expression levels decrease between early and mid puberty, and increase again by late puberty

- ➢ ventral hippocampus miR-103 expression levels increase between mid and late puberty

- ➢ ventral hippocampus miR-495 expression levels decrease between early and mid puberty and increase beyond early puberty levels by late puberty

- ➢ peripubertal binge EtOH increases miR-10a-5p expression levels immediately following the last treatment in the ventral hippocampus

- ➢ peripubertal binge EtOH does not alter miR-26a expression levels in the ventral hippocampus

- ➢ peripubertal binge EtOH increases miR-103 and miR-495 expression levels immediately following the last treatment, and the levels are decreased by late puberty in the ventral hippocampus

B) Characterization of microRNA biogenesis enzymes:

➢ dorsal hippocampus Drosha and Dicer expression levels decrease between early and mid puberty, remaining decreased until late puberty

➢ ventral hippocampus Drosha and Dicer expression levels decrease between early and mid puberty, and increase by late puberty

➢ peripubertal binge EtOH increases Drosha expression levels in an immediate and long-term manner in the dorsal hippocampus

➢ peripubertal binge EtOH increases dorsal hippocampus Dicer and ventral hippocampus Drosha and Dicer expression levels immediately following treatment and these levels are decreased beyond untreated levels by late puberty

C) Characterization of microRNA target genes BDNF and SIRT1:

➢ dorsal hippocampus BDNF expression levels increase between early and mid puberty, then decrease by late puberty

➢ dorsal hippocampus SIRT1 expression levels decrease between early and mid puberty, and increase by late puberty

➢ ventral hippocampus BDNF and SIRT1 expression levels increase between early and mid puberty then decrease by late puberty

➢ peripubertal binge EtOH increases BDNF and SIRT1 expression levels in immediate and long-term manners in the dorsal hippocampus

> peripubertal binge EtOH decreases BDNF expression levels between early and mid puberty, and levels increase beyond untreated levels by late puberty in the dorsal hippocampus

> peripubertal binge EtOH decreases SIRT1 expression levels in an immediate and long-term manner in the ventral hippocampus

- **Chapter IV:**

  A) **Characterization of sex differences in EtOH-sensitive microRNAs:**

  > dorsal hippocampus miR-10a-5p expression levels are increased at early puberty in the female relative to male

  > dorsal hippocampus miR-26a and miR-495 expression levels are decreased throughout pubertal development in the female relative to male

  > dorsal hippocampus miR-32 and miR-103 expression levels are not sexually dimorphic

  > ventral hippocampus miR-10a-5p expression levels are increased at early puberty, equivalent at mid puberty and decreased at late puberty in the female relative to male

  > ventral hippocampus miR-26a and miR-495 expression levels are decreased throughout pubertal development in the female relative to male

  > ventral hippocampus miR-103 expression levels are increased at mid puberty and decreased at late puberty in the female relative to male

➢ ventral hippocampus miR-32 expression levels are not sexually dimorphic

B) Characterization of sex differences in miR biogenesis enzymes and EtOH-sensitive miR target gene expression:

➢ Dorsal hippocampus Drosha and Dicer and ventral hippocampus Dicer expression levels are decreased in the females relative to males throughout pubertal development

➢ Ventral hippocampus Drosha expression levels are decreased at early and late puberty, but equivalent at mid puberty in females relative to males

➢ Dorsal hippocampus BDNF and SIRT1 expression levels are decreased throughout pubertal development in females relative to males

➢ Ventral hippocampus BDNF and SIRT1 expression levels are equivalent at early and late puberty but decreased at mid puberty in females relative to males

▪ **Chapter V:**

A) Characterization of sex differences in E$_2$-sensitive microRNAs:

➢ Dorsal and ventral hippocampus let-7i expression levels are not sexually dimorphic, nor do they change, throughout pubertal development

➢ Dorsal hippocampus miR-7a expression levels increase and decrease in females and males, respectively, between mid and late puberty

➢ Dorsal hippocampus miR-9, miR-125a and mir-181a expression levels are increased at early puberty in females relative to males; male expression levels do not change throughout puberty

➢ Ventral hippocampus miR-7a, miR-125a and miR-181a expression levels are not dynamic throughout puberty in males or females, however, females expression higher levels relative to males throughout pubertal development

➢ Ventral hippocampus miR-9 expression levels in females is increased at early puberty and decreased at mid and late puberty relative to males

Final Remarks

Selection of Model Organism

Rats demonstrate markers unique to an adolescent growth spurt such as hyperphagia (developmental overeating) and accelerated growth [321], making them a valuable model organism of choice for this study. Memory processes are more adversely affected in adolescent rodents abusing alcohol than in the adults abusing the same dose [5,79,322], highlighting the important need to study how alcohol alters the adolescent brain, and the hippocampus in particular. Importantly, our lab has demonstrated that no differences in body weight results from administering our binge EtOH paradigm in adolescent Wistar rats and that the blood alcohol concentration does not significantly

differ when administering EtOH via gavage or intraperitoneal injection [8,12,44]. Moreover, our binge EtOH exposure paradigm has been shown to be a reliable model for administering amounts of EtOH typically consumed amongst adolescents [200].

microRNA Functions in the Brain: Relevance to Neuronal Development

The discrete biological functions of individual microRNAs during neuronal development are not well understood. Lsy-6 was the first specific microRNA recognized to have a role in nervous system development *in vivo*, where it was shown to regulate left/right asymmetrical patterning of the taste receptor neurons in *C. elegans* [323]. Two other microRNAs, miR-9 and miR-10, are highly expressed in the brain and have been shown to play important roles in the brain development of many species including humans, rodents, zebrafish, and drosophila, demonstrating a high degree of evolutionary conservation among these microRNAs. Specifically, miR-9 promotes migration and proliferation in human neural progenitor cells by targeting stathmin, a gene required for microtuble assembly [314] and peripheral nervous system sensory organ development in drosophila [324]. Further, miR-9 is significantly reduced in the presenilin-1 null mouse model, which exhibits severe CNS developmental defects, during specific stages of development compared with wild-type mice [325]. Although our microRNA RT-PCR array experiment did not identify miR-9 as EtOH sensitive following peripubertal binge EtOH, it is possible that by examining the whole hippocampus, we were unable to delineate ventral vs. dorsal hippocampus EtOH-mediated alteration in miR-9 expression. This is further supported by our experiments identifying ventral vs. dorsal differences in miR-9 expression levels in untreated peripubertal rats. Another important microRNA during development is miR-10, which targets members of the HOX gene family, a highly

conserved group of transcription factors that coordinate anterior-posterior body axis alignment in zebrafish and other species during development [326]. These studies have revealed that specific microRNAs are critical for proper gene expression and brain function throughout development, however, the role of microRNAs beyond early developmental periods is just beginning to be investigated. Mature microRNA expression is age-dependent, and accordingly, microRNAs regulate both early developmental gene expression changes as well as those that occur throughout the lifespan in various species [43, 46, 47, 111]. In our studies, miR-10a-5p was sensitive to EtOH in both a short and long-term manner in the ventral and dorsal hippocampus, respectively. Moreover, as microRNAs can regulate the expression of numerous target genes, it is extremely likely that targets change as a function of age. Indeed, microRNAs have been demonstrated as important regulators neuronal stem cell fate determination such as the SOX family of transcription factors which control NSC differentiation and gliogenesis [327,328].

The origin of oligodendroglial fate determination has been historically controversial in the literature, and interesting recent studies implicate microRNAs in this process. For example, in a mouse model of Dicer1 KO specific to oligodendrocytes, miR-219 and miR-338 expression is absent, whereas they are abundantly expressed in wildtype oligidendrocytes [329,330]. Moreover, manipulation of these microRNAs in oligodendrocyte precursor cells demonstrates that they are essential for the differentiation of oligodendrocyte precursors into myelinating oligodendrocytes. Myelination of neurons by oligodendrocytes is essential for the process of salutatory nerve conduction and many studies have implicated reduced myelination as a mediator of neurological insults during

adolescence. Therefore, the control of oligodendrocyte maturation via microRNAs highlights yet another important role of microRNAs in brain development.

How microRNA expression changes over time is another active area of research in the microRNA field. A deep sequencing study recently highlighted 75 microRNAs that were differentially expressed in the brain with age [206]. Interestingly, let-7 microRNAs are inhibited by the RNA binding protein Lin28 in the reproductive areas of the neonate rat brain, yet, upon progression towards puberty, this expression pattern is reversed with increasing and decreasing expression of let-7 and Lin28, respectively, in the rat hypothalamus [248]. These data suggest a role for microRNA in the regulation of pubertal onset and highlight the potential for microRNAs to regulate pubertal brain development. Moreover, our data demonstrating that in the healthy adolescent brain, microRNA expression is dynamic at 3 distinct time points during pubertal development suggests that microRNAs are important for brain development at this time. Much still remains to be discovered regarding the role of microRNAs in regulating brain development, however, compelling data suggests that negative effects of alcohol on the developing brain may be mediated by alterations in brain microRNA expression and/or the mechanisms of their transcriptional regulation.

The Role of microRNAs in Ethanol Neurotoxicity During Development

Neurological consequences of alcohol abuse (repetitive and heavy drinking) occur throughout the lifespan, and an emerging role for microRNAs in mediated alcohol's effects on addiction, toxicity and teratology is becoming evident. The long-term consequences of alcohol exposure during important neurodevelopmental time periods has

recently become widely discussed, particularly with regards to prenatal EtOH exposure leading to fetal alcohol spectrum disorder (FASD). FASD describes a wide range of effects that a mother drinking alcohol during pregnancy can inflict upon the developing fetus and symptoms of FASD range from physical abnormalities, identified as fetal alcohol syndrome (FAS), to neurobehavioral alterations including depression, hyperactivity, learning disabilities and psychosis [331,332,333]. microRNAs have been implicated in the development of FASD. For example, a murine miR-9 (KO) model results in FASD phenotypes and growth retardation presumably by inhibiting the miR-9-mediated inhibition of Foxg1, a gene that promotes proliferation and prevents differentiation and whose mutation also leads to fetal growth retardation, microcephaly and mental retardation [318,334].This miR-9 KO leads to increased Foxg1 expression and inhibits maturation of neuronal populations. Interestingly, a subset of EtOH-sensitive microRNAs that play a role in cell and tissue maturation, miR-10a/b, miR-21 and miR-335, demonstrate developmental stage-specificity in their sensitivity to EtOH in neuronal stem cells (NSCs) [193]. This finding provides support to our observation that expression of a subset of microRNAs is dependent on age, sex and EtOH. As mentioned previously, miR-9 was not altered by peripubertal binge EtOH in males however it is possible that in females, miR-9 may be sensitive to EtOH. For instance, we observe sex differences in steady-state miR-9 expression in our untreated peripubertal rats and it is possible that miR-9 operates the peripubertal neuronal differentiation (i.e. peripubertal neuronal growth) in female rats. If so, the sex-differences observed in binge EtOH-exposed rats may be explained by a sensitivity of miR-9 to EtOH in females and not males. Future studies can investigate this possibility further with binge-EtOH-exposed female rodents.

Interestingly, Sathyan et al. demonstrated that miR-10a demonstrated senisitiviy to EtOH in NSC's as we did in peripubertal male rats. However, different target genes are implicated in these two EtOH studies. This difference may be due to EtOH-sensitive microRNAs identified in these two studies being responsible for regulating unique sets of developmental genes, supported by the fact that 5p- and 3p-microRNAs are expected to be complementary to different target genes.

The neurochemical mechanisms responsible for such EtOH-induced anatomical and behavioral alterations remain unclear. It is compelling to suggest that the mechanism of binge EtOH effects on the pubertal brain—such as an increased risk of adult alcohol abuse [335], spatial learning impairments [79], reduced hippocampus volume [3]—are carried out by microRNA-mediated regulation of gene expression. Our studies demonstrate that peripubertal binge EtOH leads to long-term alteration in microRNA and BDNF expression, suggesting a potential long-term effect of peripubertal binge drinking on microRNA-mediated regulation of synaptic plasticity (of which BDNF is a primary mediator). Moreover, differential effect of EtOH on microRNAs and target gene BDNF expression between the ventral and dorsal hippocampus in the peripubertal male rat suggest that EtOH may disrupt both ventral and dorsal hippocampus functions. In support of this possibility, others have demonstrated that EtOH alters mood and memory in response to peripubertal binge EtOH and have also described conflicting data regarding expression levels of BDNF mRNA and protein expression following EtOH. For instance, one study investigated early postnatal pups exposed to EtOH vapor inhalation for 2 hours and 40 mins each day on PND 4-10 and reported a significant increased BDNF protein expression [90]. Other studies also describe that BDNF increases in its expression in the

hippocampus following EtOH exposure and suggest that these effects lead to changes in synaptic plasticity leading to behavioral responses to EtOH [147]. Indeed, ethanol has been shown to increase neuronal synaptic adaptation [336] but also decrease hippocampal LTP [4,91,337] and CA3 BDNF mRNA expression [338]. Based on our data, such inconsistencies in the literature may be due to different responses of the ventral and dorsal hippocampus to EtOH, however, factors including experimental design, model organisms, EtOH paradigm, age and sex of test subjects cannot be ruled out. Controversy also exists regarding whether an ethanol-induced increase or decrease in hippocampal BDNF expression during developmental periods is neuroprotective or neurotoxic. Our data also indicate that this may also depend on whether the ventral or dorsal region is under consideration, as our data indicate that EtOH increases and decreases BDNF expression in a long-term manner differently in the dorsal and ventral hippocampus, respectively. Although we do not know from our studies whether these results directly result in reduced hippocampal functional capacity, it is compelling to suggest that our observed increases in the dorsal hippocampus BDNF and decreases in the ventral may correlate with the known impairment in mood and memory following EtOH abuse. It is important to continue to tease out the mechanisms behind EtOH-mediated effects and the potential role of BDNF expression in the hippocampus.

It is also possible that microRNA processing is broadly influenced by EtOH. Our data demonstrate that that Drohsa and Dicer expression are altered in both short and long-term fashions following binge EtOH. It has also been previously demonstrated in zebrafish embryos that pre-miR-9-3 transcripts accumulate in response to EtOH [194]. In our study, it is possible that the pri- and pre-microRNA transcripts are differentially

altered by binge EtOH and leads to altered mature microRNA expression. However, because binge EtOH doesn't alter global microRNA expression, it remains unclear whether microRNA processing is a mechanism by which EtOH alters expression of mature microRNAs.

Chapter 3 data introduces the novel finding that pubertal miR expression profiles for miR 10a-5p, miR-26a, miR-103 and miR-495 are altered by peripubertal binge EtOH exposure differentially in the dorsal and ventral hippocampus. The hippocampus is involved in learning and in consolidation of explicit memories from short-term to long-term memory storage in the cortex. Importantly, stress impairs hippocampal-dependent memory in both human and rats [339,340,341]. Its sensitivity to stress is thought to be due to its high expression of glucocorticoid receptors through which it participates in the termination of stress responses via the glucocorticoid-mediated negative feedback of the hypothalamo-pituitary-adrenal (HPA) axis [342,343]. Interestingly, studies have demonstrated a dissociation of stress-induced increases in corticosterone levels and the ability to induce hippocampal long-term potentiation (LTP) (the cellular process underlying memory), suggesting stress-induced effects on the hippocampus may extend beyond cellular LTP. Indeed, other studies have implicated that peripubertal binge EtOH imparts dysregulation of the neuroendocrine stress axis in a long-term manner [12]. The adolescent stress response is still maturing, evidenced by enhanced stress reactivity [344](Dahl RE and Gunnar, 2009, [345,346,347], suggesting that an altered development of the stress response following adolescent alcohol abuse may increase the risk of developing mood disorders commonly associated with alcohol abuse. Our studies suggest that the physiological stress of adolescent binge EtOH may incur long-lasting

stress-induced molecular alterations via changes in the expression levels of microRNAs that target genes important for synaptic plasticity. However, it has long been noted that changes in long-term potentiation LTP and/or dendritic spine density in the hippocampus are believed to demonstrate stress-induced hippocampal effects at the anatomical and cellular level [348,349,350,351]. Taken together, these studies demonstrate that high levels of alcohol consumption during adolescence lead to permanent adolescent-like brain function, such that the brain does not reach full maturity or that its development is altered.

EtOH-Induced Changes in Hippocampal microRNA Correlate with Altered BDNF and SIRT1 mRNA: Proposed Role of Ethanol Metabolism

The hippocampus is particularly sensitive to the negative effects of EtOH [62,352,353] and oxidative stress has been implicated in mediating EtOH's neurotoxicity [354], however, the complete neurobiological mechanism remains unclear. In this study we observe binge EtOH-mediated changes in hippocampal microRNA expression, and therefore, it is possible that this may be due to increased oxidative stress of hippocampal mitochondria. For example, ethanol metabolism begins with its conversion into acetaldehyde, which in the brain of binge drinkers, is accomplished largely by microsomal enzyme cytochrome (CYP2E1) [355]. This is because alcohol dehydrogenase (ALDH) becomes saturated under conditions of heavy alcohol consumption. CYP2E1, in turn, generates superoxide and hydrogen peroxide reactive oxygen species (ROS) leading to EtOH-induced oxidative DNA damage [356,357]. Mitochondrial oxidative stress disrupts the permeability of the inner and outer mitochondrial membrane, which leads to an increase in cytochrome c release and overproduction of ROS, which together, increase

DNA damage and programmed cell death or apoptosis [358]. It is unlikely that our paradigm of binge drinking increases programmed cell death because we do not see global decreases in microRNA expression and moreover, one would also expect that the control gene, U6, would also be decreased under conditions of global apoptosis. Based on evidence that EtOH induces mitochondrial oxidative stress response in the brain [359], it is possible that EtOH-mediated oxidative stress may be a mechanism through which microRNAs regulate expression of SIRT1 and BDNF in our study. For example, it has been demonstrated that microRNAs can be primarily expressed in the mitochondrial genome [360], that mature microRNAs can even localize to the mitochondria [361] and that these mitochondrial microRNAs may play a role in apoptosis. Under such circumstances, EtOH-mediated oxidative stress in neuronal mitochondria could release mature microRNAs into the cytoplasm where they can regulate the expression of target genes. Our observation that microRNAs increase following binge EtOH in the ventral hippocampus may do so via mitochondrial pore formation following an oxidative stress response, allowing microRNAs to leak into the cytosol where they can associate with RISC and regulation expression of BDNF and SIRT1 mRNA. In support of this theory, mitochondrial disruption has previously been reported to mediate translational repression, one of the mechanisms through which microRNAs postranscriptionally regulate gene expression [362]. Overall, our data identifying EtOH-sensitive microRNAs in the hippocampus and correlative alterations in BDNF and SIRT1 expression may be specific to repeated heavy (binge) EtOH consumption leading to an oxidative metabolic response. To further investigate this theory, one could fractionate cell lysate and run expression analysis on mitochondrial RNA. In summary, EtOH metabolism disrupting mitochondrial

membrane permeability demonstrates a targeted approach in which heavy doses of EtOH can disrupt gene regulation of a variety of cellular processes, as it may unleash the mitochondrial genome-containing epigenetic regulatory agents such as microRNAs.

However, it remains necessary to identify whether our observed EtOH-mediated microRNA changes directly alter the expression of BDNF and SIRT1. One way to test this is to assay for direct binding of these microRNAs to the target gene 3'UTR following EtOH exposure, as successfully demonstrated previously by Caputo et al. using luciferase reporter assays for the binding of miR-26a to the BDNF 3' UTR [143]. If direct microRNA-3'UTR binding is indicated, then to take this research one step further and test the functional effect of altered BDNF and SIRT1 mRNA in response to peripubertal binge EtOH. For example, performing behavioral assays which measure learning, anxiety and memory behavior may indicate whether CNS impairments occur as an extension of with EtOH-induced alterations in microRNA and BDNF and SIRT1 expression in our peripubertal binge EtOH paradigm.

Finally, given the developmental nature of the peripubertal period and the promiscuous nature of microRNAs, it is plausible that EtOH-sensitive microRNAs regulate the expression of a host of additional developmental genes. On that order, a microarray can efficiently identify potential additional target genes. However, an alternate hypothesis to EtOH-induced alterations in microRNA expression regulating the correlative changes in BDNF and SIRT1 mRNA is that these microRNAs are performing unconventional regulatory functions. For instance, it is becoming clear in the literature that microRNA functions are not restricted to mRNA degradation and translational repression but also orchestrate alternate gene regulatory mechanisms. For example,

epigenetic gene regulation by microRNAs include binding to the 5'UTR of mRNA targets to enhance translation [363], tethering Argonaute proteins to promoter regions to enhance or inactivate gene transcription [364,365], secretion into plasma to alter target cell behavior [366,367] which suggest microRNAs as potential endocrine factors. These additional functions of microRNAs demonstrate that microRNAs are not limited to regulating gene expression in the typical method of 3'UTR binding and therefore future experiments testing for BDNF and SIRT1 3'UTR binding will not exhaust all of the possible ways in which EtOH-sensitive microRNAs may regulate these gene targets.

Possible Role for Gonadal Hormones in Mediating Sexually Dimorphic Pubertal miR Expression

Puberty is a period of life associated with an increased risk of developing psychiatric disorders, most likely due to the incomplete maturation state of the brain in addition to increased risky behaviors common amongst adolescents. We have identified that the expression levels of 10 microRNAs (let-7i, miR-7a, miR-9, miR-10a-5p, miR-26a, miR-32, miR-103, miR-125a, miR-181a and miR-495) are specific to sex, stage of puberty and brain region, indicating that they play specific roles in the brain development of males and females during puberty. Moreover, normal fluctuations of brain microRNAs during puberty and their apparent sensitivity to EtOH, targets them as mediums through which noxious environments or genome may disrupt gene expression. To that end, it is possible that the regulation of miR expression levels may not only play a large role in normative brain maturation, but may also underlie the molecular mechanisms leading to psychiatric disorders. In agreement with this theory, we have recently shown that peripubertal exposure to repeated episodes of binge EtOH alters the normal

developmental profiles of microRNAs in the hippocampus [247], a brain region

susceptible to alcohol-abuse and suggested to be damaged in a host of neuropathologies

[11,338,358,368,369,370]. Furthermore, many studies in rodents and humans

demonstrate that substance abuse during puberty leads to altered adult gene expression,

memory dysfunction and continued substance abuse and dependence

[4,5,10,11,12,79,247,371]. Therefore, microRNAs altered by EtOH may have a variety of

negative (or positive) neurological consequences including impaired memory and stress

response regulation, however the mechanisms remain unclear.

Sexually dimorphic expression levels of microRNAs may be due to differences in

circulating gonadal hormones between males and females. It has been recently reported

that gonadal hormone-mediated generation of sexually dimorphic physiology is, in part,

due to their ability to influence miR expression. microRNAs are important regulators of

the genome, but what controls their regulation is not well understood. Studies using both

in vivo and *in vitro* systems have demonstrated that miR expression profiles (miRNomes)

can be altered by T, E_2 and their respective nuclear receptors (androgen receptor and

estrogen receptor) in healthy and cancerous tissues, as well as during different stages of

development [28,150,151,161,165,264,372,373]. These studies suggest that microRNAs

may contribute to the development of typical adolescent sexual dimorphisms.

Gonadal steroids can also influence the miR target genes we tested in our studies.

For instance, female levels of circulating E_2 is much higher in females than in males and

E_2 replacement following GDX restores levels of BDNF mRNA and protein in the

postnatal developing rat hippocampus, indicating that E_2 regulates the expression of

BDNF factor mRNA and protein in the rat hippocampus [374]. How E_2 regulates the

expression of BDNF is not known, but it is possible that E_2 regulates peripubertal BDNF expression in a sexually dimorphic manner. For instance, in the second postnatal week male rodents express higher levels of aromatase, the enzyme that converts T to E_2, as well as ERα/β in the hippocampus, suggesting a greater effect of E_2 signaling in males than in females at this developmental period [375]. Indeed, BNDF mRNA in the hippocampus is increased by E_2 [376], and peripubertal males may have greater local E_2 synthesis in hippocampal neurons and this may increase the levels of BDNF during the mid-phase of pubertal development. Furthermore, BDNF mRNA is known to fluctuate across the estrous cycle, in correspondence with the changing levels of E_2 [377] and it is possible that the lack of BDNF mRNA changes observed in our females compared to males may have occurred due to the females being in diestrus stage of their menstrual cycle when E_2 levels are lower. The consequences of differences in circulating E_2 levels, is that both E_2 and BDNF positively affect synaptic plasticity, and therefore, the dynamic expression of microRNAs throughout pubertal development in males may correspond with alterations in the BDNF and/or SIRT1 target genes and ultimately play a role in mediating the formation of long-term alterations in brain development during puberty.

The role of gonadal hormones and their influence on growth-promoting signals may underlie the necessity of peripubertal exposure to E_2 and T for development of adultlike mating, social and cognitive behaviors. For instance, ovariectomized (OVX) reduces mRNA and protein expression of (BDNF) and this is reversed with E_2 replacement [378,379]. The hippocampus is also a rare site wherein *de novo* synthesis of E_2 occurs outside of the gonads, and its expression is implicated in regulating local synaptic plasticity in dendritic spines [380]. Indeed, our data demonstrate clear sex

differences in the miR expression throughout pubertal development in multiple brain regions. In stark contrast to E_2 effects on BNDF and synaptic plasticity, gonadectomy (GDX) in adult male rats increases BDNF protein expression and synaptic plasticity in hippocampus mossy fibers [381] and the same pattern was observed in PND 90 male rats that gonadectomized at early puberty (PND 30) [86], suggesting that T represses hippocampal BDNF protein during puberty. Indeed, the author concludes the effects of gonadectomy are independent of pubertal status, however, because the immediate effects of PND 30 GDX on BNDF expression was not measured, it is impossible to know whether levels of BDNF were increased during peripuberty. Given that GDX incurs a reduction in BDNF protein measured at 4, 7 and 10 days post-GDX [374], it is clear that GDX at different developmental ages can have differential effects on BDNF protein expression. Furthermore, our data reveal that BDNF mRNA in male dorsal and ventral hippocampus is significantly increased at peripuberty compared to early puberty, suggesting that the rising levels of T correlate with rising BDNF mRNA. Our observed correlation in levels of T and BDNF are in contrast to experiments demonstrating that GDX increases BDNF protein levels. However, Solum and Handa have previously observed that neonatal GDX decreases BDNF mRNA yet increases BDNF protein levels in the same animals [374], demonstrating that BDNF mRNA levels do not always correspond with protein levels. Variable measurements of BDNF expression may also reflect differences in expression within the whole hippocampus vs. the isolated hippocampal subregions, the ventral and dorsal hippocampus. Indeed, differences in brain region expression levels of BDNF are to be expected, evidenced by T levels in the pelvic ganglia being correlated with BDNF protein expression following adult male GDX, such

that GDX decreased the number of BDNF-immunoreactive neurons [382]. In addition, T has been shown to contribute to the anxiogenic effect of exposure to a novel environment observed in male rodents as well as contribute to their social behavior [383,384], whereas in male Syrian hamsters, pubertal exposure to T contributes to aggressive, sexual and communication behaviors [385]. Lastly, T exposure during puberty in humans is necessary for the sex differences observed in spatial memory, performance of which is primarily controlled by the dorsal hippocampus [386,387]. Overall, the hippocampus plays a clear role in contributing to the gonadal hormone-mediated alterations in synaptic plasticity throughout pubertal development and as microRNAs can regulate gonadal hormone receptors, it is possible that microRNAs help to orchestrate the role that gonadal hormones play in the development of the adolescent brain.

Overall, female expression levels of EtOH-sensitive microRNAs and their target genes, BDNF and SIRT1, did not correlate with one another in this study. Moreover, the expression levels of SIRT1 and BDNF were extremely low and nearly unchanging in the female hippocampus compared males throughout pubertal development. These data most likely do not reflect a lack of dynamic synaptic plasticity. Indeed, the female expression levels of EtOH-sensitive microRNAs, BDNF and SIRT1 demonstrating no correlation whereas they did in the males (Chapter 4, Fig. 12, 153and 14), it is important to point out that these EtOH-sensitive microRNAs were identified in male specimens in (see Chapter 3, Fig. 3). Therefore, it is possible that future studies using the same approach which was used in Chapter 3 males (aka the microRNA array) in females, would indeed discover a set of different EtOH-sensitive microRNAs that is specific to females. Moreover, due to

the promiscuous nature by which microRNAs bind their mRNA target genes, it is very likely that potential female-specific EtOH-sensitive would also target BDNF and SIRT1.

Gonadal hormones also influence behavioral systems required for learning and memory. For instance, it has been demonstrated in multiple species that T acts via NMDAR circuits to alter synaptic plasticity and learning [388,389,390]. Specifically in a rodent model, males that are not exposed to androgen receptor (AR) activation during puberty exhibit altered hippocampal CA1 synaptic plasticity, as measured by social recognition memory [388]. Together these data suggest that organizational events mediated by T activation of ARs during puberty are required for the development of adultlike social behaviors and effective hippocampal-dependent learning and memory processes.

Adolescent EtOH abuse-induced disruption of neurodevelopment occurs in both males and females [391,392,393], although often times to different magnitudes [46], as well as in one sex and not the other. These studies implicate a role for the involvement of circulating gonadal steroids, yet the causality of these sexually dimorphic effects remain unclear. Evidence from both rodent and human studies supports the theory that gonadal hormones play a role in mediating the sex differences observed in EtOH consumption, a typical adolescent behavior. Indeed, T moderates EtOH intake in male rodents, such that pre- or post-pubertal castration increases adult EtOH intake and dihydrotestosterone (DHT) or T replacement following castration sufficiently increases EtOH intake back to intact, GDX, and sham-GDX male and female controls [236,394]. Furthermore, this increased EtOH consumption in adult male rats that were GDX either pre- or post-puberty, is not due a weakened aversion to the taste of EtOH over time as conditioned

taste aversion (CTA) experiments demonstrated an increased CTA in males GDX in pre-puberty compared to post-puberty [395], whereas GDX at either time point resulted in a similar increase in EtOH intake [236,260]. Interestingly, in adult male rodents bred selectively for high EtOH preference compared to those bred to not prefer EtOH, high T levels are strongly correlated with a preference for EtOH throughout the day when not intoxicated [396]. In human male twin studies, following individual analysis and adjustment for pubertal stage, a higher T levels correlated with a greater number of alcohol symptoms as well as diagnosis for alcohol dependence [397]. Indeed, T is positively correlated with EtOH consumption not only in males but also in females. In self-report studies, higher levels of T and E_2 in male and female adolescents, was positively associated with alcohol use [398]. Furthermore, female rodent consumption also varies across the estrous cycle, such that during proestrus (slowly rising E_2) and estrus (high E_2) there is a decrease in total alcohol consumption [399,400]. Potential neural mechanisms underlying sex differences in alcohol drinking behavior involves dopamine release, which is greater in the female rat nucleus accumbens [401]. These data underlie the importance of distinguishing sex differences regarding substance abuse.Although steroid hormones are likely not an exclusive factor, an important next step is to determine whether E_2 and/or T are responsible for mediating the sex-specific differences in EtOH-sensitive miR expression.

It is clear that gonadal hormones contribute to the exceedingly plastic nature of the adolescent brain as they contribute to neurite outgrowth, increases in neurogenesis and alterations in steroid receptor expression [240]. Moreover, the effects of sex hormones on synaptic plasticity are sexually dimorphic [402], yet the mechanism remains

unclear. It is possible that microRNA-mediated regulation of gene expression has evolved

to adapt to changing hormonal environments during puberty (and menstrual cycle),

however, contemporary lifestyles of adolescents including experimentation with binge

drinking and the use of oral contraceptives may have detrimental effects on microRNA

expression that is critical for adolescent brain development. Chapter 5 data demonstrate

that there are clear sex differences in the expression of 5 E_2-responsive microRNAs, let-

7i, miR-7a, miR-9, miR-125a and miR-181a, throughout pubertal development. We also

demonstrated that their expression is distinct between the functionally dimorphic

hippocampal regions, the ventral and dorsal hippocampus. In conclusion, we have

identified that males and females express E_2-responsive microRNAs in an age and sex

dependent manner throughout pubertal development and that the expression patterns are

unique between the dorsal and ventral hippocampus. However, it remains unclear

whether the pubertal expression levels of E_2-responsive microRNAs are correlated with

gonadal hormone levels in a sexually dimorphic manner. Therefore, it will be important

to determine how pre-pubertal removal of endogenous hormones and subsequent

exogenous treatment with E_2 alters the expression of these microRNAs in the male and

female dorsal and ventral hippocampus in future studies. It will be important to monitor

the stage of the estrus cycle in female rats and the analysis of expression levels of

microRNAs should be separate for females in different estrous cycle stages. It is

important to note that symptoms of depression, schizophrenia and anxiety fluctuate

across the menstrual cycle [403,404,405,406,407,408,409], and puberty is period of life

wherein there is an increased risk for developing anxiety and other related mood

disorders. Moreover, the course and prevalence of mood disorders are sexually

dimorphic, and it is possible that microRNAs expressed differentially between males and females in the mood-regulating ventral hippocampus target genes important for regulating mood. In addition, gonadectomy (GDX) followed by T treatment would be important to determine T effects on microRNA expression levels as gonadal androgen exposure during puberty is required for male-typical behaviors associated with the stress response such as anxiety-related decrease in social behavior in an unfamiliar environment [384]. Lastly, target genes of these microRNAs, such as GABA, SIRT1, BDNF and GR, could be measured in male and female hippocampi to identify potential mood-regulating target genes that these microRNAs may control the expression of during pubertal development. Indeed, puberty is a period of continuous sexual differentiation in the brain [410]and pubertal development is controlled by functionally connected networks coordinated by epigenetic mechanisms [411], indicating that miR expression levels can influence the establishment of neural networks during puberty which can have long-lasting consequences on adult brain function.

The dorsal hippocampus reduction of microRNA expression in females contrasts rising levels of circulating E_2 in females at this time. Furthermore, the dorsal hippocampus microRNA expression in males remains stable and indeed, males exhibit an increase in T throughout puberty but not E_2. These data would suggest that the decrease in these female microRNAs during pubertal development is E_2-dependent, however, one would also expect the early puberty levels of male microRNAs to be similar to the female early puberty levels. Overall, the lack of change in microRNA expression levels in males mimics their lack of exposure to changes in circulating E_2 throughout puberty. Of note, the early pubertal expression level of these 3 microRNAs is significantly increased in

females, but this may be due to the fact that it is not typical for males and females to exhibit similar levels of circulating E_2 at this time age [286,287,288]. Indeed, activation of the HPG axis in females can occur before males, and could explain the differences observed in E_2-responsive miR expression at early puberty between males and females. However, reduced miR expression over time as E_2 levels rise in females does not agree with the sex differences observed at early puberty such that female miR expression is high as their E_2 levels begin to rise. It is possible that ligand-independent effects can be regulating miR expression levels before the rise in gonadal hormones at pubertal onset, such that the estrogen receptor (ER) acts on the miR promoters to increase their transcription. Indeed, ERβ has been shown to have transcriptional activity in rodent neuronal cell lines [412,413] and protein-protein interactions in the female rodent ventral hippocampus [414] that are altered in the presence of E_2, suggesting that ERβ has ligand-independent functions in the female brain. Overall, it is possible that E_2 regulation of microRNAs can be dynamic throughout pubertal development, however, more experiments a required to adequately address this theory.

Take Home Message

In summary, adolescent alcohol abuse is strongly associated with multiple other health risk behaviors including alcohol-related motor-vehicle accidents, alcohol-related sexual assault, suicide and weakened learning and memory capacities [415,416,417,418] supporting the role that adolescence is a sensitive period with regards to brain organization. The mechanisms regulating EtOH abuse-induced brain changes remains unclear. These studies provide important data that peripubertal binge EtOH alters microRNA expression in both an immediate and long-term fashion. Our data also suggest

that BDNF and SIRT1 are potential effector molecules of microRNAs altered by EtOH.

However, the mechanisms regarding miR biogenesis as well as their regulation of target

genes expression remains controversial [223,419], and thus, the mechanisms underlying

EtOH-mediated regulation of miR expression as well as whether changes in miR target

gene expression plays a role in altering mood or memory function requires more studies.

Future studies are important in order to increase our understanding of the molecular

events leading to the maturation of an organized and functional brain and therefore,

increase the capacity for human livelihood.

Future Directions

One of the most interesting findings regarding microRNAs gene regulatory

capacity is that their mechanisms of gene regulation and even their biogenesis can me

mediated by different mechanisms. In particular, the potential for microRNAs to function

as endocrine molecules is an exciting new prospect. In this respect, circulating

microRNAs are suggested to mediate cell to cell communication in a long-distance

fashion to various cells and organs throughout the body. Moreover, the potential for

microRNAs to be able to function as biomarkers and as tools for disease prevention. Just

in this past year, studies have highlighted microRNAs as biomarkers for various

neurological impairments including but not limited to autism [420], glioblastoma [421],

neurodegeneration [422], stroke [423,424] and neurotoxicity [425]. Therefore,

microRNAs provide promising opportunities for better understanding and potentially

treating or preventing ETOH abuse-mediated neurological impairments.

CHAPTER SEVEN

GENERAL METHODS

Animals

All Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (postnatal day (PND) 23)) and allowed to acclimate for 7 days, after which they were randomly distributed into 3 groups: early pubertal (PND 30), peripubertal (PND 37), and late pubertal (PND 73). For Chapter 2 and 3 experiments there were *male and female* early, peripubertal and late pubertal grouped animals which were left untreated (N=10/age group) until euthanized at PND 30, PND 44 and PND 73, respectively. For Chapter 3 *whole hippocampus* experiments, there were 2 male groups, one administered treatments of peripubertal water (control) at peripubertal age and the other administered treatments of peripubertal binge EtOH (see methods below) (N= 6/treatment group; total of 12 animals). These 2 groups of animals were euthenized 1 hr. following the last treatment.For Chapter 3 *dorsal and ventral hippocampus experiments*, there were 3 male groups left untreated and euthenized at early (PND 30), mid (PND 44) and late (PND 73) puberty. In addition, there were 2 male groups administered treatments of peripubertal water (control) at peripubertal age (N= 10/treatment group; total of 20 water-treated animals) and 2 male groups administered treatments of peripubertal binge EtOH (N= 10/treatment group; total of 20 EtOH-treated animals) (Fig. 19B). The water and EtOH groups were handled for 5 minutes once/day beginning at PND 30 to eliminate non-specific effects of stress associated with handling. Peripubertal water or EtOH

treatments (see methods below) began on PND 37, an age which has been previously defined as peripuberty based on circulating gonadotropin levels and stages of spermatogenesis [286,287,288]. One group each from of the mid/peri-pubertal water and EtOH treated animals were sacrificed 60 min. following the last water and EtOH treatment, respectively, at PND 44 (N= 10 water + N= 10 EtOH). The remaining 2 groups (N= 10 water + N= 10 EtOH) were left undisturbed following the last EtOH treatment in their home cage until sacrificed at late puberty (PND 73). All animals were pair-housed on a 12:12 light/dark cycle with lights on at 07.00 h. Food and water were available ad libitum.

Ethics Statement

All animal protocols were approved by the Institutional Animal Care and Use Committee at Loyola University Chicago permit #2011002. All measures were taken to minimize animal numbers and suffering.

Binge Exposure Paradigm and Treatment Design

For Chapter 3 *whole hippocampus* experiments, peripubertal (PND 37) male animals were randomly assigned to either 1) peripubertal water (N = 6) or 2) peri-pubertal binge EtOH (N = 6) treatment groups, such that a total of 12 animals were used in these experiments. For Chapter 3 *dorsal and ventral* hippocampus experiments, peripubertal (PND 37) male animals were randomly assigned to either 1) peripubertal water (N = 20) or 2) peripubertal binge EtOH (N = 20) treatment groups. These animals were compared to the untreated groups (N=10) at each age, such that a total of 70 animals were used in these studies.

All animals in the binge EtOH group received the following 8-day peripubertal repeated binge-pattern EtOH paradigm: on PND 37, animals are given 3g/kg EtOH (20% v/v in water), or water alone, via oral gavage at 10.00 hr. This process is repeated according to the following schedule: 3d EtOH, 2d water, 3d EtOH (i.e. a total of 6 EtOH treatments over the course of 8 days). This binge exposure paradigm has been used previously to mimic the pattern of binge alcohol consumption typically observed in adolescents [44,200]. Moreover, our previous studies have shown that this repeated binge-pattern EtOH paradigm did not affect body weight/growth curves during pubertal development and consistently resulted in similar blood alcohol concentrations (BAC) [8,44,200]. The water group was administered room temperature tap water via oral gavage once/day for 8 consecutive days. The animals were anesthetized with inhalation of isoflorane then euthanized by rapid decapitation 60 minutes following EtOH treatment (PND 44, "immediate EtOH effects") or [in the Chapter 3 *ventral and dorsal hippocampus* experiments] 30 days following the last day of treatment at late puberty (PND 73, "long-term EtOH effects"). The blood alcohol concentration in EtOH treated animals was 190 ± 21 mg/dl, which is consistent with our previous reports using this peripubertal binge EtOH paradigm [12]. It is important to note that the untreated and the water-treated mid/peri- and late-pubertal groups were not statistically different for any parameter measured and were therefore, combined into one group for further statistical analyses.

Tissue Collection

Trunk blood and brains were collected immediately following decapitation. Trunk blood was collected on ice into heparinized glass tubes, centrifuged at 4000 rpm for 10

minutes, plasma separated and stored at -20°C until processed for testosterone levels

using enzyme immunoassay (EIA, see below). Brains were rapidly dissected, flash-frozen

in isopentane (-35°C) on dry ice, and stored at -80°C until further processing. Frozen

brains were sectioned at 200 μm on a freezing microtome, mounted onto glass slides, and

the ventral and dorsal hippocampi were microdissected using a 0.75 mm Palkovit's brain

punch tool (Stoelting Co., Wood Dale, IL). The coordinates of the microdissected regions

was determined using The Rat Brain in Stereotaxic Coordinates, Fourth Edition Atlas (G.

Paxinos and C. Watson). The ventral hippocampus was defined as being located between

1.8 mm to 3.8 mm posterior to Bregma, 3 mm below the top of the brain and 6.6 mm

from the bottom of the brain. The dorsal hippocampus was defined as being located

between 4.16 mm and 6.05 mm posterior to Bregma, 3 mm below the top of the brain and

2 mm above the bottom of the brain. Brain tissue punch samples were collected on ice

into microcentrifuge tubes containing 1 ml of TriZol reagent (Invitrogen, Inc., Carlsbad,

CA). Tissue samples were sonicated on ice prior to total RNA isolation.

RNA Isolation

Total RNA was isolated from micropunched tissue samples using Trizol reagent

(Invitrogen Inc., Carlsbad, CA) according to the manufacturer's directions. All RNA

samples were analyzed for quality by Nanodrop spectrophotometry and by visualization

of the RNA on a 1.5% agarose gel.

Rat RT^2 microRNA PCR Array

The Rat RT^2 microRNA PCR array (SABiosciences) was performed according to

manufacturer's instructions. Briefly, the cDNA was combined with RT^2 SYBR Green

qPCR Master Mix, RT^2 microRNA universal primer and water. Next, the mixture was aliquoted across the 96-well RT^2 microRNA PCR Array platform containing a panel of primers for 88 well-researched microRNAs in the rat genome followed by quantitative reverse transcription PCR quantification of gene expression using the ΔΔCt method [426]. Data analysis was performed using the free Web and Excel based microRNA PCR Array Data Analysis Software. (Importantly, the RNA population used for this experiment was a microRNA population isolated from total RNA using Qiagen's MiRNeasy Mini Kit and cDNA synthesis was performed using SABiosciences RT^2 microRNA First Strand Kit (MA-03) according to the manufacturer's instructions).

Quantitative Reverse Transcription PCR (qRT-PCR)

Following RNA isolation, 1.0 μg total RNA was reverse transcribed using the First Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Inc., Carlsbad, CA) for mRNA quantification, and 1.0 μg total RNA was used for NCode microRNA First-Strand cDNA Synthesis Kit for microRNA quantification. microRNA and mRNA qRT-PCR was performed with Fast Start Universal SYBR Green Master Mix (Roche) on an Eppendorf Realplex4 with a silver block. Forward primers for specific microRNAs were designed as described in the Ncode™ microRNA First-Strand cDNA synthesis kit handbook (Invitrogen, Inc., Carlsbad, CA) and using miRBase 18 as a sequence reference. The small RNA, U6 and housekeeping gene hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) were used as a loading control and to normalize the data for microRNA and mRNA analysis, respectively, as neither were altered by EtOH treatment [44]. The following thermocycler program was used for mRNA target genes: 1) 95°C for 10 minutes, 2) 95°C for 30 seconds, 3) 59°C for 30 seconds, 4) 72°C for 30 seconds, and

melting curve analysis. The following thermocycler program was used for microRNA: 1) 95°C for 10 minutes, 2) 95°C for 30 seconds, 3) 65.3°C for 20 seconds, 4) 72°C for 12 seconds. Quantification of the gene expression was achieved using the ΔΔCt method [426]. Importantly, all miR and mRNA fold change values were generated using the normalized expression levels of untreated male PND 30 rats as a baseline value. The following intron-spanning primers were used for analysis of selected microRNA target genes and for microRNA processing enzymes:

SIRT1:5'GCGGCCGCGGATAGGTCCATA, 3'TCCCACAGGAGACAGAAACCCCA, BDNF: 5'AGCCTCCTCTGCTCTTTCTGCTGGA, 3'CTTTTGTCTATGCCCCTGCAGCCTT,

Drosha: 5'GAAGTCACCGTGGAGCTGAGTA, 3'ATCATTGCATGCTGACAGACATC,

Dicer: 5'GGGAAAGTCTGCAGAACAAAC AND 3'GGCTGTCTGAGCTCTTAGTTC.

The following forward primers were used for analysis of selected mature microRNA along with a universal reverse primer provided in the NCode microRNA First-Strand cDNA Synthesis Kit:

miR-10a-5p: 5'CGCTACCCTGTAGATCCGAATTTGTG,

miR-26a: 5'CCGGGTTCAAGTAATCCAGGATAGGC,

miR-103: 5'GGAGCAGCATTGTACAGGGCTATGA,

miR-495: 5'CGCGAAACAAACATGGTGCACTTCTT.

let-7i: 5'CGCGTGAGGTAGTAGTTTGTGCTGTT

miR-7a: 5'GCGCTGGAAGACTAGTGATTTTGTTCT

miR-9: 5' CGCGTCTTTGGTTATCTAGCTGTATG

miR-125a: 5'CGTCCCTGAGACCCTTTAACCTGTGA

miR-181a: 5'CGAACATTCAACGCTGTCGGTGAGT

Hormone Measurements

Plasma levels of testosterone were measured using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. The range of detection was between 3.9 and 500pg/ml and the intra-assay CVs was 2.2. Briefly, blood samples were collected in heparinized tubes and centrifuged at 3,000 rpm for 10 min. at 4°C, and plasma was stored at −20°C. Plasma samples were combined with a testosterone-acetylcholinesterase (AChE) conjugate (testosterone tracer) as well as testosterone EIA antiserum and incubated in a 96-well IgG-coated plate for 2.0 hr. at room temperature. Samples were washed 5 times with provided wash buffer then combined with Ellman's reagent containing the substrate for AChE, and the plate was developed for 60 minutes, shaking and covered, at room temperature. Absorbance was read at 412 nm on a multimode Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT).

Blood Alcohol Concentration Assay

Trunk blood samples were collected into heparinized tubes, centrifuged at 3000 rpm for 10 min. at 4°C; and plasma stored at −20°C. Blood alcohol levels were

determined by measuring the change in absorbance at 340 nm following enzymatic

oxidation of EtOH to acetylaldehyde (Point Scientific Alcohol Reagent Kit). Assay range

is 0 to 400 mg/dl and intra and interassay CV = 6.4% and 7.9%, respectively.

Western Blot

Total protein is isolated using Tissue Protein Extraction Reagent (Thermo

Scientific, Waltham, MA) supplemented with protease inhibitor cocktail (Roche

catalogue #04693159001; 7x stock solution), according to manufacturer's instructions

and boiled at 95^0 C for 5 mins. 10 µg of protein was loaded onto 10% SDS-PAGE gel

and then transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane

was blocked with 5% bovine serum albumin (BSA) for 0.5 hr. and incubated with the

following primary antibodies: SIRT1 (Santa Cruz Biotechnology, H-300) at 1:200 or

BDNF (Santa Cruz Biotechnology, N-20) at 1:200 in TBST for 2 hr. at 4°C. Following

primary antibody incubation, the membrane was washed three times for 10 min. each in

10 ml TBST (Tris Base Solution containing 0.1% Tween 20), incubated in secondary

antibody (HRP conjugated goat anti rabbit IgG, Santa Cruz Biotechnology) at 1:5000

concentration in TBST for 1.5 hr., and washed three times for 10 min. each in TBST.

Stripping blots was performed with 6 M GnHCl, 0.2% Triton 100, 20mM Tris-HCl pH8,

two times at RT for 5 min. each followed by two 10 min. washes with TBSTr. In order to

control loading efficiency, blots are stripped as described above, re-blocked with 5%

BSA and incubated in primary rabbit β-actin antibody (Cell signaling, 4970S) at 1:3000

dilution in TBST for 1.0 hr., washed three times for 10 min. each in TBST, incubated in

HRP conjugated goat α rabbit IgG (Santa Cruz Biotechnology, sc-2004) at 1:5000

dilution in TBST for 1.5 hr., and washed three times for 10 min. each in TBST.

Following antibody applications and washes, each blot was imaged on the Biorad

Chemidoc XRS+ imager using ECL Chemiluminescent substrate (Pierce).

Statistical Analysis

Statistical analyses were performed by the Biostatistics Core Facility at Loyola

University Stritch School of Medicine in consultation with Dr. James Sinacore. Data

obtained from qRT-PCR or blood alcohol and hormone concentration assays were

analyzed by a one-way Analysis of Variance (ANOVA) when EtOH was a single

variable factor or by a two-way ANOVA when age and EtOH or age and sex were the

two variable factors followed by Tukey's posthoc test for all pair-wise comparisons when

there was a significant main effect and interaction. The above tests were performed using

SigmaStat Statistical Analysis Software. A p-value of less than 0.05 was designated as

significant. Exclusion criteria comprised outliers greater than or equal to 2 times the

standard deviation of the mean. Please note that all qRT-PCR age-dependent miR and

mRNA fold change values were generated using the normalized expression levels of

untreated male PND 30 rats as a baseline value. Importantly, the untreated and the water-

treated peri and late pubertal groups were not statistically different for any parameter

measured and were therefore, combined into one group for statistical analyses.

Data obtained from western blotting were subject to densitometry analysis using

ImageLab software. Lanes were detected manually and bands were detected using the

'high sensitivity' detection limit and lane-based background subtraction was applied.

Statistical significance was analyzed by a two-way ANOVA followed by Tukey's post-

hoc test using an average of 3 independent blots containing samples from 6 different

animals per treatment group (N=3, p<0.05). Western blotting data analysis tests were performed using SigmaStat Statistical Analysis Software. A p-value of less than 0.05 was designated as significant. Exclusion criteria comprised outliers greater than or equal to 3 times the standard deviation of the mean.

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VITA

Sarah A. Prins was born in Riverside, CA on July 23rd, 1986 to Peter and Enid Prins as the youngest of six children. In high school she developed a curiosity for neuroscience and earned a governor's scholarship for high academic achievement. Upon graduating in 2004, she moved to Santa Cruz, CA to major in Neuroscience and Behavior at University of California, Santa Cruz (UCSC). While at UCSC, she engaged in academic research in the laboratory of Dr. Yi Zuo, studying the morphological dynamics of dendritic spines during learning and memory formation. In 2009, Sarah earned her Bachelor of Science degree and moved to Chicago, IL to join the graduate program in Biomedical Sciences at Loyola University Chicago, Stritch School of Medicine.

In 2010, Sarah joined Dr. Toni Pak's laboratory in the Department of Cell and Molecular Physiology to study the effects of adolescent binge drinking on the developing brain. During her training, Sarah studied how hippocampal microRNAs are altered throughout pubertal development and following repeated adolescent binge drinking. She was awarded three years of support for her research with a National Institute on Alcohol Abuse and Alcoholism fellowship from Loyola's Alcohol Research Program and two years of support to attend the annual Society for Neuroscience meeting with Charles Robert Schuster travel awards from Loyola's Neuroscience Research Institute. At Loyola she also served with the Graduate Student Council and conducted assistant teaching for the Medical Neuroscience course.

Sarah actively offered neuroscientific services by teaching at Pritzker Elementary School's annual science night, reviewing manuscript submissions for the journal of Central Nervous System Agents in Medicinal Chemistry and volunteering with the International Drug Abuse Research Society. On April 15th, 2014, she and Jonathan Kulpit had their first child, Raymond. She plans to continue studying and communicating neuroscience research in her career.