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- Kumar, RS, Ijiri, S, **Kight, K**, Swanson, P, Dittman, A, Alok, D, Zohar, Y and Trant, JM (2000). "Cloning and functional expression of a thyrotropin receptor from the gonads of a vertebrate (bony fish): potential thyroid-independent role for thyrotropin in reproduction." *Mol Cell Endocrinol* 167(1-2): 1-9.
- Steven, C, Lehnen, N, **Kight, K**, Ijiri, S, Klenke, U, Harris, WA and Zohar, Y (2003). "Molecular characterization of the GnRH system in zebrafish (*Danio rerio*): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II." *Gen Comp Endocrinol* 133(1): 27-37.
- Goto-Kazeto, R, **Kight, KE**, Zohar, Y, Place, AR and Trant, JM (2004). "Localization and expression of aromatase mRNA in adult zebrafish." *Gen Comp Endocrinol* 139(1): 72-84 DOI: 10.1016/j.ygcen.2004.07.003.
- Wong, TT, Gothilf, Y, Zmora, N, **Kight, KE**, Meiri, I, Elizur, A and Zohar, Y (2004). "Developmental expression of three forms of gonadotropin-releasing hormone and ontogeny of the hypothalamic-pituitary-gonadal axis in gilthead seabream (*Sparus aurata*)." *Biol Reprod* 71(3): 1026-1035 DOI:biolreprod.104.028019.
- Palevitch, O, **Kight, K**, Abraham, E, Wray, S, Zohar, Y and Gothilf, Y (2007). "Ontogeny of the GnRH systems in zebrafish brain: in situ hybridization and promoter-reporter expression analyses in intact animals." *Cell Tissue Res* 327(2): 313-322 DOI:10.1007/s00441-0060279-0.
- Guzman, JM, Rubio, M, Ortiz-Delgado, JB, Klenke, U, **Kight, K**, Cross, I, Sanchez-Ramos, I, Riaza, A, Rebordinos, L, Sarasquete, C, Zohar, Y and Mananos, EL (2009). "Comparative gene expression of gonadotropins (FSH and LH) and peptide levels of gonadotropin-releasing hormones (GnRHs) in the pituitary of wild and cultured Senegalese sole (*Solea senegalensis*) broodstocks." *Comp Biochem Physiol A Mol Integr Physiol* 153(3): 266-277 DOI: 10.1016/j.cbpa.2009.02.032.
- Guzman, JM, Cal, R, Garcia-Lopez, A, Chereguini, O, **Kight, K**, Olmedo, M, Sarasquete, C, Mylonas, CC, Peleteiro, JB, Zohar, Y and Mananos, EL (2011). "Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRH_a) on the reproductive axis of Senegalese sole (*Solea senegalensis*)." *Comp Biochem Physiol A Mol Integr Physiol* 158(2): 235-245 DOI: 10.1016/j.cbpa.2010.11.016.

Kight, KE and McCarthy, MM (2014). "Using sex differences in the developing brain to identify nodes of influence for seizure susceptibility and epileptogenesis." *Neurobiol Dis* 72 Pt B: 136-143 DOI: 10.1016/j.nbd.2014.05.027.

McCarthy, MM, Pickett, LA, VanRyzin, JW and **Kight, KE** (2015). "Surprising origins of sex differences in the brain." *Horm Behav* 76: 3-10 DOI: 10.1016/j.yhbeh.2015.04.013.

Kight, KE and McCarthy, MM (2016). "Sex differences and estrogen regulation of BDNF gene expression, but not propeptide content, in the developing hippocampus." *J Neurosci Res* 95(1-2): 345-354 DOI: 10.1002/jnr.23920.

Conference Abstracts

Kight, KE and McCarthy, MM. "Expression of brain-derived neurotrophic factor (BDNF) in the early postnatal hippocampus of the rat differs between the sexes and exhibits region-specific regulation by estradiol." Society for Neuroscience Annual Meeting, San Diego, CA. November, 2013.

Kight, KE and McCarthy, MM. "Sex difference in brain-derived neurotrophic factor (BDNF) expression in the early postnatal hippocampus of the rat." Organization for the Study of Sex Differences Annual Meeting, Weehawken, NJ. April, 2014.

Kight, KE and McCarthy, MM. "Sex difference in brain-derived neurotrophic factor (BDNF) expression in the early postnatal hippocampus of the rat." Organization for the Study of Sex Differences Annual Meeting, Weehawken, NJ. April, 2014.

Kight, KE, Bowers, JM, and McCarthy, MM. "MicroRNA expression in the early postnatal hippocampus of the rat differs between sexes and is regulated by estradiol and DNA methylation." Society for Neuroscience Annual Meeting, Washington, DC. November, 2014.

Kight, KE and McCarthy, MM. "Sex difference in microRNA-124 in the neonatal hippocampus and impact on NKCC1." Society for Neuroscience Annual Meeting, Chicago, IL. October, 2015.

Kight, KE and McCarthy, MM. "The microRNA miR-124 is higher in the neonatal female hippocampus and impacts parameters mediating neuronal excitation." Organization for the Study of Sex Differences Annual Meeting, Philadelphia, PA. May, 2016.

Abstract

Defining the Mechanisms That Mediate Sexual Differentiation of the Developing Hippocampus

Katherine Elizabeth Kight, Doctor of Philosophy, 2019

Dissertation Directed by Margaret M. McCarthy, James and Carolyn Frenkil Dean's Professor and Chair, Department of Pharmacology

Studying the processes by which male and female brains develop differently is not only a rich source for understanding the contrasting mechanisms of brain development that enable an organism to respond appropriately as an adult to intrinsic and extrinsic factors, it is also important for understanding the etiology of the numerous neurodevelopmental disorders that exhibit a sex bias in prevalence or presentation. The hippocampus is an area of the brain responsible for context-dependent memory and regulation of the stress axis, and as such is implicated in many sex-biased neurodevelopmental disorders. There are two striking sex differences in the hippocampus of neonatal rats which may fundamentally shape the circuitry of this region of the brain differently between males and females. First, roughly twice as many proliferating cells are present in the hippocampus during the first week of life in males, compared to females, and second, the timing of the developmental shift in which GABA signaling switches from depolarizing to hyperpolarizing occurs later in males. This thesis sought to determine the mechanisms that promote the sex difference in depolarizing GABA in the neonatal hippocampus of rats, and whether there is a causal relationship between depolarizing GABA and cell genesis in this context. One set of experiments tested the role of the neurotrophin BDNF. Analyses of *Bdnf* gene expression patterns revealed a baseline sex difference that mirrored the sex difference in cell proliferation. However, *Bdnf* content in response to

steroid hormone signaling in the neonatal hippocampus showed subregion-specific expression patterns that did not correlate with cell proliferation, indicating cell-type specificity of BDNF function in the developing hippocampus. A second set of experiments found female-biased expression in the neonatal hippocampus of several microRNAs known to regulate cell proliferation and neurogenesis. One of these microRNAs, mir124, was tested for its potential role in regulating cell proliferation and the depolarizing response to GABA, using a combination of *in vitro* and *in vivo* approaches. Functional studies also tested the role of miR124 in regulating the expression of NKCC1, a key chloride channel involved in regulating depolarizing GABA and proliferation.

Defining the Mechanisms That Mediate Sexual Differentiation of the
Developing Hippocampus

by
Katherine Elizabeth Kight

Dissertation submitted to the Faculty of the Graduate School of the
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The effort represented by these pages is
dedicated to

my grandmother
Eleanor Jane Duffield Kight
resilient, adaptable, strong and unflappable
always young at heart
you probably never knew how much you inspire me

and to

Nathan and Teagan
child of my heart and child of my soul

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List of Abbreviations

ANOVA	analysis of variance
ASD	autism spectrum disorder
BDNF	brain-derived neurotrophic factor
BrdU	bromo-deoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
CREB	cyclic-AMP response element binding protein
DIV	days in vitro
E2	estradiol
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
Form	Formestane
GABA	gamma aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
icv	intracerebroventricular
ip	intraperitoneal
KCC2	potassium-chloride cotransporter 2
kDa	kilodalton(s)

miRNA	microRNA
miRs	microRNAs
mRNA	messenger RNA
NKCC1	sodium-potassium-chloride cotransporter 1
NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PN	postnatal
PTSD	post-traumatic stress disorder
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RT	reverse transcription
SOX9	SRY-box 9
subcu	subcutaneous
Tam	tamoxifen
TBS	Tris-buffered saline
TrkB	tropomyosin receptor kinase B/tyrosine kinase receptor B
UTR	untranslated region

Chapter 1: General Introduction

Sexual Differentiation of the Developing Brain

The brain begins as a largely bipotential organ that will acquire a masculine or feminine architecture in response to external and intrinsic factors during early development. The prime mover in this process is exposure to gonadal steroid hormones, which act on the developing brain during a critical perinatal period to organize the neural architecture and delimit the sex-specific influence of gonadal steroids later in life. This is the organizational-activational hypothesis of hormone action on the developing brain, and was first articulated in a seminal study by Phoenix and colleagues which demonstrated that treatment of pregnant Guinea pigs with testosterone masculinized the sexual behavior of their female offspring as adults (Phoenix et al., 1959). Although originally codified to explain sexual differentiation of the brain in terms of reproductive behavior, the organizational/activational hypothesis still provides a framework for understanding the developmental programming of other endpoints of brain physiology that differ between males and females (Arnold, 2009). The critical period for hormonal modulation of brain development in mammals is initiated by the perinatal surge in circulating testosterone that occurs with testicular development in male fetuses. In rats, the perinatal testosterone surge begins late in gestation, around embryonic day 18, and declines a few hours after birth (Weisz and Ward, 1980). Circulating testosterone enters the brain in developing male rats and is enzymatically converted to estradiol via aromatization (McEwen et al., 1977; Naftolin et al., 1975). Expression of aromatase in the brain varies among regions and additionally, there is evidence for *de novo* steroidogenesis in some regions of the developing brain (Amateau et al., 2004; Tsutsui, 2012). For these reasons,

androgen and estradiol content of the developing brain varies according to region and sex (Amateau et al., 2004; Konkle and McCarthy, 2011). During gestation and the early postnatal period, the developing brain is shielded from the effects of maternal estradiol by α -fetoprotein present in the neonatal circulation (Bakker et al., 2006). The sequestering effects of this steroid binding globulin can be overcome with a relatively high dose of exogenous estradiol, and this has been titrated to achieve effects in females that mimic endogenous estradiol in males (Amateau et al., 2004).

Estradiol signaling in the developing rodent brain induces various sex differences in cellular morphology and physiology that are the hallmarks of masculinization of specific brain areas. Among these are differences in cell death and survival, neuro- and glial genesis, activity-induced synaptogenesis, dendritic branching and changes in astrocyte and microglial morphology. The precise timing of a particular process in relation to the window of steroid action in brain masculinization varies according to brain region, but in general the critical period for sexual differentiation in the rat closes during the second week of life, around postnatal day 10 (reviewed in McCarthy, 2008; Lenz et al., 2012).

Although estrogen is required for sexual differentiation of many phenotypic endpoints in the rodent brain, this appears not to be the case for humans. Direct evidence testing the aromatization hypothesis is obviously precluded in humans, but correlations between circulating fetal testosterone and sex-specific volumes of brain nuclei, as well as studies in non-human primates, indicate that testosterone is the main organizing hormone driving sexual differentiation in the fetal brain (Wallen, 2005; Lombardo et al., 2012), although the presence of aromatase activity during late gestation and early postnatal life

indicates there may be a role for estrogen in sex-specific development in some brain areas (Naftolin et al., 1971; MacLusky et al., 1987). Masculinization occurs prenatally, and fetal testosterone production begins late in the first trimester and peaks around gestational week 16 (Abramovich and Rowe, 1973; Reyes et al., 1974). Circulating testosterone then falls to levels equivalent to what is found in female fetuses until it rises again at birth, declining around 24 hours later (Forest and Cathiard, 1975; Tapanainen 1983; Corbier et al., 1990). The magnitude of the perinatal testosterone surge in males is highly variable, but in general circulating testosterone in males at this time is 2-3 times higher than in females (Corbier et al., 1990). A second postnatal surge in testosterone occurs in male infants at 1 to 3 months of age (Forest and Cathiard, 1975; Winter et al., 1976) (Figure 1.1). One of the difficulties in modeling sex differences in the developing brain arises when trying to extrapolate the protracted gestational timeline of humans to that of experimental animals such as rodents, and a number of studies have sought to do this by correlating developmental milestones such as formation of neuroanatomical structures, region-specific neurogenesis and myelination, and synaptic pruning. From such studies it is generally considered that the perinatal period in rodents is roughly equivalent to the third trimester in terms of human brain development, and at about 12-13 days of life the rat brain is developmentally similar to that of a full-term infant on the day of birth (Clancy et al., 2007; Semple et al., 2013).

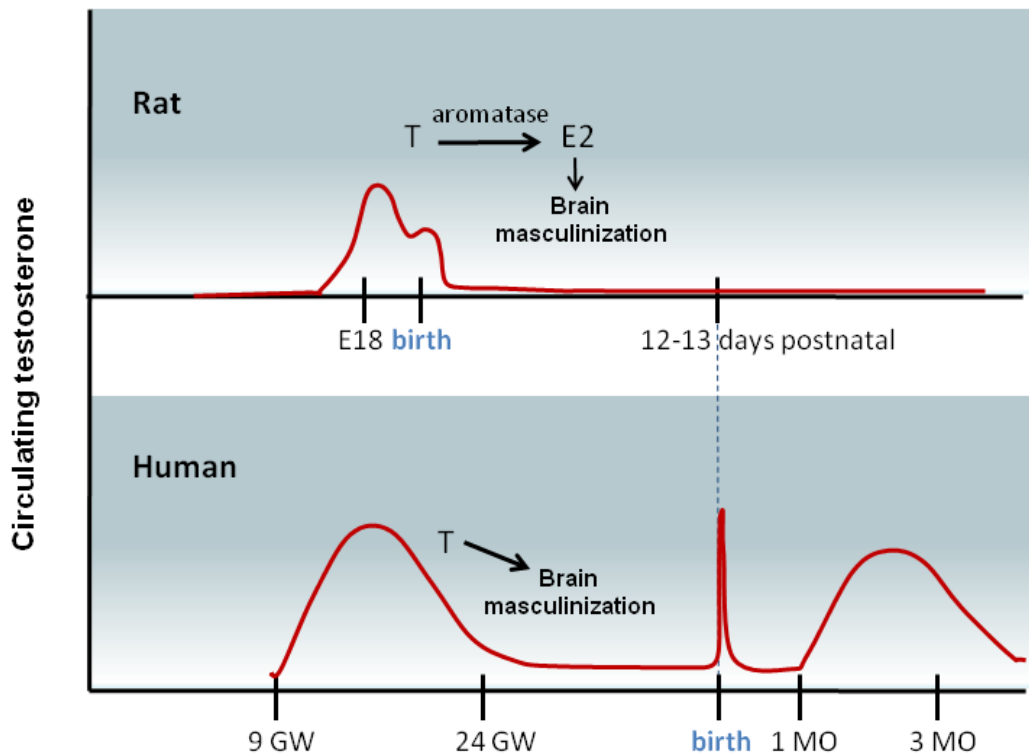


Figure 1.1: The critical period of brain sexual differentiation in relation to circulating steroid levels in humans and rat. Serum testosterone derived from the fetal testis peaks in male rats shortly before birth around embryonic day 18 (E18), and again on the day of birth. The aromatization of testosterone (T) to estradiol (E2) is required for the organization of a masculine brain phenotype. In humans, prenatal testosterone production from the fetal testes is elevated from gestational weeks 9 (GW9) to 24 (GW24), with a peak around GW16. Circulating testosterone is elevated on the day of birth, and again peaks in newborns between 1 and 3 months old (MO). During the third trimester, human brain development resembles that of the rat during the period of brain masculinization in the perinatal period and first week of life. The brain of a term infant is developmentally equivalent to that of a 12 to 13-day-old rat (dashed vertical line). From Kight and McCarthy, 2014.

The Hippocampus- Function and Dysfunction

The hippocampus is critically involved in learning and stress responding, and as such plays a vital role in both complex and innate behaviors (Fanselow and Dong, 2010; Sweatt, 2004). Since the groundbreaking clinical case of Henry Molaison, who suffered severe anterograde amnesia after excision of large portions of his temporal lobes to treat intractable seizures (Scoville and Milner, 1957), numerous clinical and animal studies have demonstrated that hippocampal function is required for episodic memory (for review see Squire, 1992), as well as spatial memory (Burgess et al., 2002), and context-dependent learning (Fanselow, 2000; Gerwitz et al., 2000; Sanders et al., 2003; Bengasser and Shors, 2007). The hippocampus is also a crucial brain region for regulation of the stress response (Jacobson and Sapolsky, 1991; McEwen et al., 2016). In both humans and animal models, aspects of contextual learning and stress responding differ in males and females across the lifespan, and in many cases hippocampal function has a demonstrated role in mediating these sex differences (Shors et al., 2001; Bengasser and Shors, 2007; Schoenfeld and Gould, 2012; Mahmoud et al., 2016).

Hippocampal dysfunction is associated with many neurological and neurodevelopmental disorders which differ between the sexes in terms of prevalence and/or presentation, including epilepsy, schizophrenia, ASD, depression and anxiety (see Harrison, 2004; McLean et al., 2011; Hill and Fitch, 2012; Schoenfeld and Cameron, 2015 and references therein). Several of these disorders are associated with prenatal and childhood risk factors which are also correlated with altered hippocampal structure. For example, childhood stress and trauma, well-established risk factors for depression and anxiety later in life, are associated with decreased hippocampal volume in adults (Vythilingham et al., 2002; Frodl et al., 2010; Paquola et al., 2016). Increased

hippocampal volume is seen in adolescents with anxiety and PTSD who have experienced early life trauma (Tupler et al., 2006). And smaller hippocampal volume is seen in schizophrenia patients with a history of obstetric complications, compared to patients who did not experience obstetric complications (van Erp et al., 2002; Ebner et al., 2008). Numerous animal studies have revealed molecular and cellular effects of developmental influences on the hippocampus which manifest in sex-specific ways and persist into adulthood. These include altered neurogenesis in response to gestational stress or maternal separation (Kawamura et al., 2006; Lemaire et al., 2006; Oomen et al., 2010; Korosi et al., 2012; Lajud and Torner, 2015), altered expression of neuroendocrine markers associated with anxiety-like behavior (Brunton and Russell, 2010; Hill et al., 2014; Huang, 2014), differences in adult spine density and synaptic plasticity in response to early life stress (Biala et al., 2011; Bock et al., 2011; Liao et al., 2014), and alterations in interneuron subtypes associated with electrophysiological changes and anxiety-like behavior in adults (Murthy et al., 2019). The wealth of data linking the hippocampus to sex-specific brain function in health and disease makes clear that understanding how the hippocampus differs between males and females during early development is crucial for understanding the etiology of many neurodevelopmental disorders.

Structure and Development of the Hippocampus

The hippocampus is a telencephalic structure located in the medial temporal lobe, and as an element of Papez' Circuit is considered part of the limbic system. Its structure is highly layered, and consists of two discrete substructures. Ammon's horn, composed of excitatory principle neurons and inhibitory interneurons in a stratified arrangement, can be generally subdivided into CA1 and CA3 subregions, based on afferent and efferent

connections and resident cell types. Ammon's horn is folded around the dentate gyrus subregion, which consists of a distinct population of interneurons and excitatory granule neurons, and which is a major site of neurogenesis throughout the lifespan. In general, two broad functional divisions are assigned to the hippocampal formation along its longitudinal axis, based on neuroanatomical connectivity, ablation studies, and molecular profiling. The dorsal or septal half of the hippocampus is involved in processing of spatial information and memory, and sends output projections to the anterior cingulate cortex, mammillary nuclei and anterior thalamus. The ventral or temporal portion of the hippocampal formation, which is highly connected with the olfactory system, prefrontal cortex, amygdala and hypothalamus, preferentially mediates stress responding (Moser et al., 1993; Moser and Moser, 1998; Fanselow and Dong, 2010; Bienkowski et al., 2018).

Anatomical and imaging studies have shown that the human hippocampus is primarily formed during the 2nd trimester, and by 18 to 20 weeks gestation is fully formed and resembles that of the adult, with Ammon's horn and the dentate gyrus structurally assembled (Kier et al., 1997), although the dorsal and ventral portions of the hippocampus continue to differentiate structurally well into childhood (Gogtay et al., 2006). Detailed cellular birthdating studies in rodents also demonstrate a protracted period of embryonic and postnatal development of the hippocampal formation involving distinct waves of cellular proliferation and migration (Bayer, 1980; Altman and Bayer, 1990a,b,c). Ammon's horn is largely formed by birth, and cells that populate this subregion of the hippocampal formation migrate from a germinal center in the medial region of the telencephalon around embryonic day 16. The dentate gyrus, however, which will remain a site for neurogenesis in the adult brain, arises from a distinct germinal zone

during the perinatal period and continues to form well into the second week of life. Proliferating neuroblasts migrate tangentially and radially over several days to form a third germinal zone which will give rise to the dentate gyrus. Cellular proliferation continues here until approximately 1 month of age, when a single layer of proliferating cells is present in the dentate as the subgranular zone. The protracted development of the hippocampal formation during the late gestational and early postnatal period indicate that the hippocampus is potentially vulnerable to intrinsic and environmental influences that may alter normal function later in life, and as discussed above, this is largely supported by the clinical and preclinical data. The fact that hippocampal development also occurs during the period of brain sexual differentiation raises the possibility that perturbations in this process may result in different functional outcomes between males and females, and sets an etiological framework for examining sex differences in the developing hippocampus.

Sex Differences in the Developing Hippocampus

In the adult hippocampus, numerous cellular and molecular differences between male and females have been described in rodent models, particularly in relation to steroid hormone responsiveness (reviewed in Galea et al., 2013), and stress-induced effects on cellular architecture (reviewed in McEwen et al., 2016). While many sex differences are known in the adult hippocampus, very few have been described in the hippocampus of neonatal or juvenile animals. Recent work by Nelson et al., (2017) demonstrates a difference in microglial phagocytosis in the neonatal hippocampus of rats, and minor differences in synaptic proteins have also been shown (Bian et al., 2012). In addition, however, there are two well-established sex differences in the neonatal hippocampus that

have the potential to fundamentally organize this brain region differently between males and females.

Sex Differences in the Developing Hippocampus- Neonatal Cell Genesis

There is a robust and striking sex difference in the neonatal hippocampus of rats within the neurogenesis pathway that has been most thoroughly characterized by the McCarthy lab. As shown by systemic BrdU labeling of dividing cells in newborn pups, males have roughly twice as many proliferating cells in the dentate gyrus and CA1 (but not CA3) regions of the hippocampal formation, compared to females, during the first week of life (Zhang et al., 2008; Bowers et al., 2010; Waddell et al., 2013). The same relative sex difference in proliferating cells is seen within hours of, and also 21 days after BrdU administration; it is recapitulated with endogenous Ki67 detection, and is not mirrored by a difference in pyknotic cells, and thus is unlikely due to a difference in cell death between males and females. However, a sex difference in the rate of proliferation cannot be ruled out. Timecourse labeling with BrdU over the lifespan of developing rats indicates that this sex difference in proliferating cells is restricted to the first postnatal week, as the number of proliferating cells is equivalent in the dentate of males and females after 6 days of age (Bannerjee, Bowers and McCarthy; Stockman and McCarthy, unpublished data). Up to 80% of proliferating newborn cells in the CA1 subregion of males will survive and differentiate into neurons, as shown by expression of the nuclear marker NeuN, while only 40% will eventually express NeuN in females (Bowers et al., 2010).

The baseline difference in proliferating neuronal precursors in the hippocampus of male and female neonatal rats is robustly modulated by estradiol signaling in a sex-

dependent manner. Estradiol administration to neonatal females increases the number of proliferating cells in the dentate and CA1 to levels seen in males, but does not alter cell genesis in males. Conversely, disruption of estrogen signaling by administering either the pan-estrogen receptor antagonist tamoxifen or the aromatase inhibitor Formestane decreases cell proliferation in neonatal males to that of females, but has no effect in females (Bowers et al., 2010; see Figure 1.2). Thus, in terms of the effects of estradiol, there appears to be a tightly controlled range of cellular proliferation in the neonatal hippocampus, the limits of which are expressed as a ceiling in males and a floor in females.

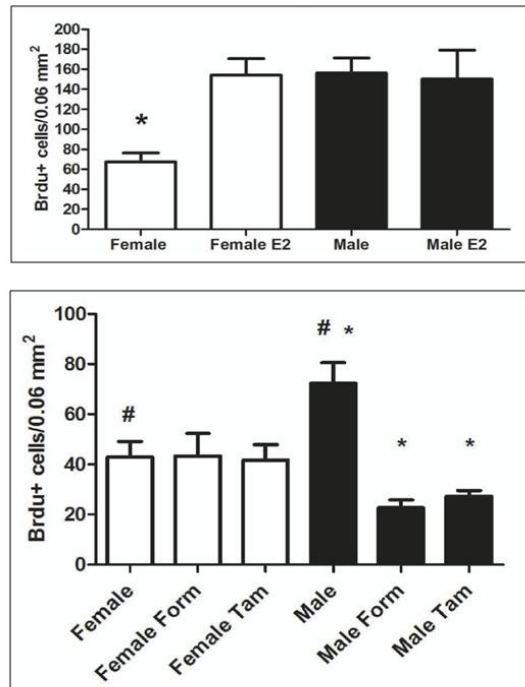


Figure 1.2: The sex difference in proliferation in the dentate gyrus of neonatal male and female rats, as shown via BrdU labeling. Top panel: males have twice as many BrdU-labeled cells as females. When pups are treated with a masculinizing dose of estradiol (E2), the number of proliferating cells increases in females, but not in males. Bottom panel: blocking estradiol signaling with Formestane (Form) or Tamoxifen (Tam) decreases proliferation in the dentate gyrus of males but has no effect in females. From Bowers et al., 2010.

The effects of estradiol on developmental cell genesis in the hippocampus are rapid, occurring within hours, and yet also persistent, as indicated by the number of proliferating cells that survive and differentiate (Bowers et al., 2010; Zhang et al., 2008). In addition, the fraction of NeuN+ cells surviving from the newborn proliferating population in estradiol-treated females is equivalent to males. Thus, in the neonatal rat hippocampus, estradiol promotes both proliferation and differentiation of neuronal precursors in the dentate gyrus and CA1, and thereby has an important role in organizing the hippocampal circuitry in a sex-specific manner. Estradiol also has an important modulatory role in the second major sex difference in the developing hippocampus, the depolarizing response to GABA.

Sex Differences in the Developing Hippocampus- Depolarizing GABA

In mature neurons, the GABA_A receptor mediates fast synaptic inhibition by gating the influx of chloride ions, thereby hyperpolarizing the cell and reducing action potential firing. Thus, in the adult central nervous system, GABA is the main inhibitory neurotransmitter. During development, however, the chloride gradient in immature neural cells is reversed, and activation of GABA_A receptors causes efflux of intracellular chloride ions and membrane depolarization (see reviews by Ben-Ari, 2002; 2007; 2014 for everything you could possibly want to know about depolarizing GABA). The timing of the developmental switch when GABA signaling moves from depolarizing to hyperpolarizing is cell autonomous, yet varies among brain regions, and is dictated by a shift in the driving force for chloride, which in turn is determined by the relative expression of chloride transporters on the cell membrane. The transporters most relevant for the developmental shift in chloride gradient are the sodium-potassium-chloride

cotransporter 1 (NKCC1), which facilitates electroneutral transport of chloride into the cell, and the potassium-chloride cotransporter 2 (KCC2), which pumps chloride ions out of the cell. Expression of these transporters is developmentally regulated within the cell. NKCC1 expression is high and KCC2 expression is essentially absent in neural precursors and immature neurons, but as the cell matures KCC2 expression is upregulated while NKCC1 expression falls, and the relative shift in chloride transport reverses the chloride gradient. This process is a hallmark of neuronal maturation as proliferating neural precursors and neuroblasts exit the cell cycle and differentiate (Rivera et al., 1999, 2005; Wang et al., 2002; see Figure 1.3).

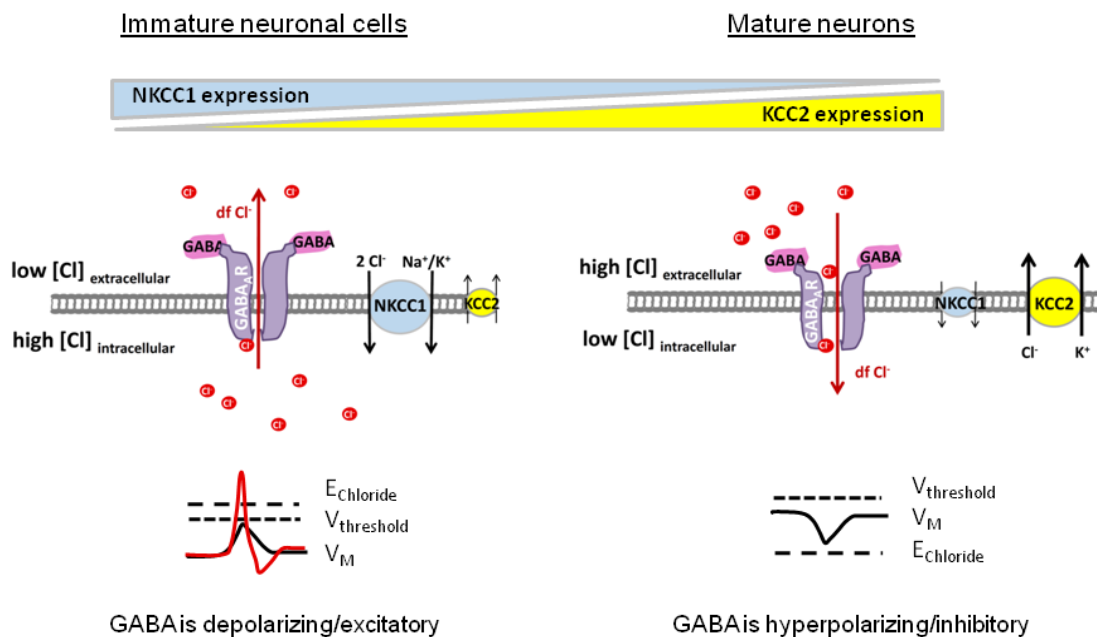


Figure 1.3: The developmental shift from depolarizing to hyperpolarizing GABA. In immature neural precursors and neuroblasts a high relative intracellular chloride concentration dictates an outward driving force for chloride ($dfCl$), and the reversal potential for chloride ($E_{chloride}$) is positive relative to the resting membrane potential (V_M). Activation of GABA_A receptors moves the membrane potential toward the threshold potential ($V_{threshold}$), depolarizing the cell and potentially generating an action potential. In mature and postmitotic neurons, intracellular chloride content is low. Activation of GABA_A receptors shifts the membrane potential in a negative direction and hyperpolarizes the cell. The driving force for chloride, and thus whether GABA is depolarizing or hyperpolarizing, is determined by the relative expression of the chloride channels NKCC1 (blue) and KCC2 (yellow). From Kight and McCarthy, 2014.

Although several factors that upregulate KCC2 expression during this process are known (Yeo et al., 2009, 2013), direct mechanisms responsible for downregulation of NKCC1 expression are not known. In the neonatal hippocampus of rats, a fundamental sex difference exists in the timing of the shift from depolarizing to hyperpolarizing GABA, wherein males make this shift later in development. Females respond to GABA agonists with hyperpolarizing currents before the end of the first postnatal week, but in males the response remains depolarizing until the second week of life (Nuñez and McCarthy, 2007; Galanopoulou, 2008). This corresponds with sex differences in the developmentally-regulated chloride transporters, where an earlier rise in KCC2 expression in females has been demonstrated (ibid). A sex difference is also evident in the magnitude of the response to depolarizing GABA. *In vitro* calcium influx in response to the GABA_A receptor agonist muscimol demonstrates a greater initial rise in calcium transients in hippocampal neurons derived from neonatal females, but a shorter decay time compared to male-derived neurons. Repeated muscimol administration attenuates this response in females, but not in males. Estradiol signaling during the perinatal period potentiates the response to depolarizing GABA in cultured hippocampal neurons (Nuñez et al., 2005; 2007; 2008; Nuñez and McCarthy, 2009). This is most likely due to upregulated NKCC1 expression in the presence of estradiol (Nuñez et al., 2005), and also indirect enhancement of NKCC1 activity by estradiol via the kinases OSR1 and SPAK (Nugent et al., 2012). In addition, phosphorylation of the transcription factor CREB in response to GABA_A receptor activation with muscimol increases in the CA1 region of the hippocampus in males, but is decreased in females (Auger et al., 2001). Because CREB activation promotes expression of immediate early genes, this indicates that

downstream activation of gene expression in response to depolarizing GABA differs in the neonatal hippocampus of males and females, and divergent neuronal physiology may result.

The depolarizing action of GABA in immature neurons can generate action potentials by activating voltage-gated sodium channels, promote glutamate signaling by removing the magnesium block of NMDA receptors, and increase intracellular Ca^{2+} by activating voltage-gated calcium channels. Through these mechanisms depolarizing GABA promotes synchronous network oscillations and modulates activity-dependent processes such as neuronal maturation and synaptogenesis (Cancedda et al., 2007; Ben-Ari et al., 2007; Pfeffer et al., 2009). Tonic activation of GABA_A receptors on immature neuronal cells stimulates their migration during development and in the adult subventricular zone (Behar et al., 1996; Bolteus and Bordey, 2004; Manent et al., 2005; Hsieh and Puche, 2015). The depolarizing action of GABA also regulates proliferation of cycling neuronal progenitors and neuroblasts, although whether this promotes or inhibits proliferation seems to depend upon the location and neurogenic stage of the cycling cells. Abrogating the depolarizing action of GABA in the adult ventricular zone by knocking down NKCC1 expression reduces proliferation of migrating neuroblasts (Young et al., 2012; Magalhaes and Rivera, 2016). In neural stem cells from the subventricular zone, however, depolarizing GABA has an inhibitory effect on proliferation (LoTurco et al., 1995; Liu et al., 2005). The depolarizing action of GABA thus plays critical roles in shaping the architecture of the developing brain, and likely does so in a sexually dimorphic manner. Given the role of depolarizing GABA in regulating cellular proliferation and neurogenesis, there may be a causal relationship between the sex

difference in depolarizing GABA and the greater cell proliferation in the hippocampus of neonatal male rats, as these two sex differences overlap developmentally.

The overarching goal of this thesis was to identify factors which may promote the sex difference in proliferation in the neonatal hippocampus of rats, motivated by the desire to add to the fascinating and growing body of knowledge that informs our understanding of how the brain is differentially sculpted between males and females. Although critically involved in sexually differentiated behaviors and sex-biased disorders of the brain, little is known about the mechanisms which regulate the developmental differentiation of the hippocampus between males and females. This pursuit is thus justified from both a basic science standpoint and in relation to points of influence or vulnerability for sex-biased behaviors and neurodevelopmental disorders, and is described in the chapters that follow.

Chapter 2: General Materials and Methods

Experimental animals

All procedures involving experimental animals were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Sprague-Dawley rat pups were obtained from dams mated in the University of Maryland School of Medicine Animal Care Facility and housed in a 12-hour light/dark cycle. The day of birth was designated postnatal day 0 (PN0). Pups were removed from their home cage and taken to the laboratory for experimental treatments. Care was taken to ensure pups were away from the dam for not more than 1 hour during each manipulation. Each cohort of pups remained with the dam until collection of brain tissue.

Subcutaneous injections

Estradiol benzoate (Sigma) or tamoxifen (Sigma) were administered at 100 µg in 0.1 ml sesame oil vehicle subcutaneously on PN0 and PN1. Subcu. injections were administered on the back between the shoulder blades using a 28-gauge beveled needle. An equivalent volume of sesame oil was similarly administered as vehicle control. This dose of estradiol and tamoxifen has been titrated to overcome circulating steroid hormone binding globulins present in the neonatal rat, and has previously been shown to modulate aspects of sexual differentiation of the rodent brain, including sex-specific effects in the developing hippocampus (Amateau et al., 2004; Bowers et al., 2010; Mong et al., 1999).

Intracerebroventricular injections

Intracerebroventricular (icv) injections were performed on neonatal rat pups under cryoanesthesia on PN0 and PN1. At this age the skull is thin and uncalcified, and midline and Bregma fissures of the brain are visible through the skull and hairless skin. Pups were

sedated by placing them on a foil-covered bed of ice in a humidified refrigerator at 4°C just until they were unresponsive to touch. During injections, pups rested belly-down on cloth-covered freezer packs. 1µl volumes were injected through the skull into each hemisphere using a Hamilton #7001 23-gauge syringe attached to a stereotaxic frame. The syringe needle was inserted 0.6 mm anterior to Bregma, 0.5 mm lateral to the midline, and 3.0 mm ventral to the top of the skull in order to target the lateral ventricle without damaging the hippocampus. The syringe was withdrawn dorsally to 2.5 mm ventral to the top of the skull, and the syringe contents were slowly injected over a period of 30 sec. After 1 min. the syringe was slowly withdrawn and the process repeated for the other hemisphere. Pups were placed in a 37°C incubator until they recovered, typically 5-7 minutes.

Dissection of hippocampal tissue

To collect individual subregions of the hippocampus, pups were decapitated with heavy scissors and brains quickly removed from the skull cavity. Brains were bisected sagittally in ice-cold saline and thalamic tissue removed to expose the ventricular surface of the hippocampus. The entire dentate gyrus was removed as a discrete structure by inserting a fine-gauge needle along the length of the hippocampal fissure. Fiber projections along the dentate axis proximal to CA3 were removed with fine forceps. Ammon's horn was dissected from the neocortical tissue and meninges removed using fine forceps. CA1 and CA3 areas were separated along the length of the hippocampus. For each hippocampal subregion, the entire tissue along the longitudinal axis was collected together, consisting of both dorsal and ventral portions. For each subregion,

tissue from both hemispheres of the brain of an individual pup were combined, frozen on dry ice and stored at -80 °C until subsequent processing.

Isolation of hippocampal protein

Ice-cold lysis buffer consisting of 10 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and containing protease and phosphatase inhibitors (Sigma) was added to frozen hippocampal tissues at a ratio of approximately 500 µl lysis buffer per 10 mg of tissue. An equal tissue volume of silica beads was added to the lysis buffer and mechanical homogenization performed for 2 minutes using a Bullet Blender (NextAdvance). Tissue homogenates were centrifuged at 20 X g at 4°C for 5 min, transferred to a clean tube and stored at -80°C until subsequent analysis. Protein content of tissue homogenates was determined by interpolation to a standard curve of purified BSA using the Bradford protein assay (BioRad).

Western immunoblotting

Protein from tissue homogenates was resolved on gradient polyacrylamide gels (NuPage, LifeTechnologies) under reducing and denaturing conditions, and transferred to PVDF membrane. Membranes were blocked with Odyssey Blocking Buffer (LiCor Biosciences) diluted 1:1 with Tris-buffered saline (pH 7.4, TBS). Membranes were incubated with primary antibodies diluted in a 1:1 solution of Blocking Buffer:TBS-0.05% Tween. Membranes were washed 3 times in TBS-Tween (0.05%) and incubated in Blocking Buffer diluted 1:1 in TBS-Tween/0.02% SDS containing secondary antibodies (IRDye®800 goat-anti-rabbit and IRDye®680 goat-anti-mouse IgG, LiCor Biosciences; both diluted 1:5000). After washing, membranes were imaged on an Odyssey CLx Infrared Imaging System (LiCor). Fluorescence intensity for each band of the protein of

interest was normalized to fluorescence intensity of the corresponding normalization protein band to obtain an integrated fluorescence value.

BDNF ELISA

Frozen tissues were homogenized in lysis buffer containing protease and phosphatase inhibitors, as described above. Total free BDNF propeptide was measured using the BDNF Emax Immunoassay System (Promega), according to the manufacturer's instructions. Tissue homogenates were diluted 1:5 in PBS (pH 7.4), acid treated at pH 3.5 for 15 minutes by the addition of 1/5 volume 2N HCl, and neutralized with NaOH prior to dilution in Block and Sample Buffer. According to the manufacturer, this ELISA has a sensitivity of 15.6 pg/ml and exhibits less than 3% cross-reactivity to other neurotrophins. The sensitivity and specificity of this ELISA were not independently confirmed for these studies.

Antibody characterization

Table 1 below lists the antibodies used in these studies. The BDNF antiserum used (RRID:AB 10644597) recognized a single band of approximately 27.5 KDa on Western immunoblots of neonatal rat hippocampal tissue homogenate. The NKCC1 antiserum used recognized bands of approximately 130 KDa and 200 KDa on Western immunoblots of neonatal rat hippocampal tissue homogenate. The SOX9 antiserum recognized a single band of approximately 65 KDa on Western immunoblots of neonatal rat hippocampal tissue homogenate. The TUJ1 antiserum recognized a single band of approximately 55 KDa on Western immunoblots of neonatal rat hippocampal tissue homogenate. The anti-Gapdh monoclonal antibody recognized a single band of approximately 35 KDa on Western immunoblots of rat hippocampal tissue homogenate.

Table 1: List of primary antibodies used

antigen	antibody source; host species; RRID	immunogen	working concentration used
BDNF	Aviva Systems Biology, Cat# ARP41970; rabbit polyclonal; RRID:AB_10644597	synthetic peptide corresponding to the middle portion of human BDNF	1.25 µg/ml
NKCC1	EMD Millipore, Cat# AB3560P; rabbit polyclonal; RRID:AB_91514	synthetic peptide corresponding to a 22 aa sequence within the C-terminus of rat NKCC1	1.25 µg/ml
SOX9	EMD Millipore, Cat# AB5535; rabbit polyclonal; RRID:AB_2239761	synthetic peptide corresponding to the C-terminus of human SOX9	1.67 µg/ml
neuronal class III β-Tubulin (TUJ1)	Covance Research Products, Cat# MMS-435P; mouse monoclonal; RRID:AB_2313773	purified microtubules from rat brain	1.0 µg/ml
GAPDH	Abcam, Cat# ab9484; mouse monoclonal; RRID:AB_307274	native human GAPDH	0.2 µg/ml

Isolation of RNA

Total RNA was isolated from homogenates of dissected hippocampal tissue or harvested hippocampal neurons *in vitro* using a modified phenol extraction method, according to the manufacturer's instructions (TriReagent RT, MRC Inc.). Genomic DNA was removed by digestion of the aqueous phase of tissue homogenates with 2 IU of RNase-free DNase I and subsequent purification through silica-based size-exclusion columns (RNAeasy Miniprep Kit, Promega). The resulting total RNA was resuspended in RNase-free water, and RNA yield and purity were determined using a NanoDrop 2000 spectrophotometer (ThermoFisher). All total RNA used for these experiments exhibited an A260/A280 ratio of 1.8-2.0.

For experiments involving microRNA quantitation, total RNA was isolated from dissected hippocampal tissue or harvested hippocampal neurons using a similar method, modified to retain RNA species smaller than 100 bp. After DNase digestion of the TriReagent-extracted tissue homogenate, the aqueous phase was combined with 1/5 volume high-salt buffer (Buffer RLT, Promega) and an equal volume of 100% EtOH. Total RNA, including small RNAs, was subsequently obtained from the samples using silica-based size-exclusion columns (RNAeasy Miniprep Kit, Promega). The resulting total RNA was resuspended in RNase-free water, and RNA yield and purity were determined using a NanoDrop 2000 spectrophotometer (ThermoFisher). RNA used for these experiments exhibited an A260/A280 ratio of 1.8-2.0.

Quantitative PCR

For quantification of mRNA transcripts, cDNA was transcribed from 0.5 or 1 μg total RNA using a High Capacity cDNA Reverse Transcription Kit and random hexamers (LifeTechnologies). For each batch of cDNA synthesis, every 5th RNA sample was also used as template in reaction containing no reverse transcriptase (no RT), to check for genomic DNA contamination of the samples. A portion of every cDNA synthesis reaction product (2 μl) was removed and pooled among samples. This pooled cDNA was serially diluted and used to generate standard curves to test each target primer set, and to test the quality of each batch of cDNA synthesis. The remainder of each cDNA synthesis product was diluted 1:10 and 5 μl was used for fluorescence-based, real-time PCR in a reaction mixture containing SYBR[®] Green PCR Master Mix (LifeTechnologies) and 250nM primers, except where otherwise noted. PCR reactions were performed for 40 cycles on a ViiA[™]7 Real-Time PCR System (ABI). All samples and standards were run in triplicate, and negative controls containing water as template and no RT cDNA were run for each target. Efficiency of each target primer pair was determined from standard curves of pooled cDNA run on each plate. Expression among experimental groups were calculated using the $\Delta\Delta C_t$ method (Pfaffl, 2001; Schmittgen and Livak, 2008). Data were expressed as relative mean fold expression +/- standard error of mean fold expression. Mean C_t values for the normalization gene (*Gapdh*) differed by less than 0.2 C_t between males and females or among all 3 hippocampal regions. Primer sequences for the qPCR assays used to measure mRNA targets are detailed in Table 2 below.

Table 2: List of qPCR primers used in these studies

primer	target region	spans intron/exon junction?	sequence
allBDNFfwd	ExonIX	no	5'-GCGGCAGATAAAAAGACTGC-3'
allBDNFrev	ExonIX	no	5'-CAGTTGGCCTTTTGATACCG-3'
GAPDHfwd	Exon 9-10	yes	5'-TGGTGAAGGTCGGTGTGAACGG -3'
GAPDHrev	Exon 10	no	5'- TAGATCGGTACTACAATTTCGA-3'
NKCC1fwd	Exon 2	no	5'- AGTCCATAAGCTAATCGATACG-3'
NKCC1rev	Exon 2-3	yes	5'-TCGTAAGCTCTAGCAATCGCTCCT-3'
SOX9fwd	Exon 3-4	yes	5'- CTAAGCTACCGTCCTAAGCTAC-3'
SOX9rev	Exon 4	no	5'- CTAAGCTTACCGCGCATCAT-3'

Because the Bdnf primers must target a single exon in order to detect all transcripts, it was not possible to design primers that span an intron-exon boundary. In addition to DNase digestion of total RNA samples, melting curve analysis was done on standards and samples for all primers in order to confirm a single peak melting temperature, indicating a single target species and the absence of genomic DNA amplification.

For quantification of microRNAs, reverse transcriptase reactions were performed with 0.5 or 1.0 µg total RNA using a TaqMan[®] MicroRNA Reverse Transcription Kit (Life Technologies) and a custom pool of primers targeting microRNAs of interest. qPCR was carried out on 1µl of undiluted cDNA product in a reaction mix containing TaqMan[®] Universal Master Mix II, No AmpErase UNG (Life Technologies) and TaqMan[®] primer/probe combinations specific for each targeted microRNA (Life Technologies). PCR reactions were performed for 40 cycles on a ViiA[™]7 Real-Time PCR System (ABI). All samples and standards were run in triplicate, and negative controls containing water as template and no RT cDNA were run for each target. The small nucleolar RNA U6 was used as a normalization standard. Expression among experimental groups were

calculated using the $\Delta\Delta C_t$ method (Pfaffl, 2001; Schmittgen and Livak, 2008). The number of biological replicates are indicated in the descriptions for each experiments in subsequent chapters. Data were expressed as relative mean fold expression +/- standard error of mean fold expression.

BrdU immunohistochemistry

To obtain brain tissue for BrdU immunohistochemistry, rat pups were deeply anaesthetized with an i.p. injection of sodium pentobarbital solution (Fatal-Plus, Vortech Pharmaceuticals), and brain tissue was fixed *in situ* via transcardial perfusion of 10 mM PBS (pH 7.4), followed by 4% paraformaldehyde in PBS (pH 7.4). Whole brains were dissected out and immersion fixed overnight in 4% paraformaldehyde/PBS (pH 7.4), followed by 3-5 days of immersion in 30% sucrose in PBS (pH 7.4) until saturation. Brains were frozen and sectioned on a cryostat at 45 μ m thickness through the rostral-caudal axis of the hippocampal formation. Every 6th section within the dorsal half of the hippocampus were mounted onto charged slides (SuperFrost Plus, Invitrogen) and allowed to air dry.

For immunohistochemical detection of BrdU, slide-mounted tissue sections were rinsed in 3 changes of PBS (pH 7.4), and antigen retrieval for BrdU was carried out with a 10-minute incubation in 0.1% trypsin/0.1% CaCl₂ in Tris-buffered saline (TBS), followed by 3 washes in TBS and a 30-minute incubation in 2N HCl. Slides were then washed in PBS, incubated in 50% MeOH/0.03% H₂O₂, washed again in PBS, and incubated overnight at room temperature in PBS containing 0.4% Triton-X-100/0.5% BSA and 1:250 dilution of mouse-anti-BrdU monoclonal antibody (Beckton-Dickinson). After washing with PBS, incubation with secondary antibody incubation was carried out

at room temperature for 1 hour using biotinylated goat-anti-mouse antibody (Sigma) at a 1:500 dilution in 0.4% PBS-T/5% BSA. Streptavidin-based amplification of secondary antibody binding was carried out using a 1:500 dilution of ABC solution (Vector Labs), and chemogenic detection of antibody binding to tissue was achieved using diaminobenzidine (Fast DAB, Sigma).

In vitro cell culture

For the *in vitro* experiments described in these studies, neurons were isolated from developing hippocampi of Sprague Dawley rat pups on the day of birth using routine methods (Nuñez et al., 2005). Single-sex cultures were obtained from pooled tissues of 4-7 male or female rat pups. Whole hippocampi were dissected from both hemispheres into cold Hanks Balanced Salt Solution (Sigma) containing 100 mM HEPES (pH 7.3) and 1/10 dilution of Antibiotic/Antimycotic solution (Sigma) (HBSS+). After washing in HBSS+, hippocampal tissue was dissociated using a papain enzyme mix from a Neural Tissue Dissociation Kit (Miltenyi Biotech), according to the manufacturer's instructions. Single-cell neuronal suspensions were obtained by triturating the tissue with fire-polished glass pipettes and filtering the tissue homogenate through a 70 μ m nylon strainer. After washing with HBSS, pelleted neuronal cells were resuspended in plating media, consisting of Neurobasal-A (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum. Cells were plated at a density of approximately 25 cells/mm² onto 6, 12, or 24-well plates coated with poly-D-lysine. Several hours after plating, cells were fed with 1 X volume of maintenance media, consisting of Neurobasal-A supplemented with B-27 and 0.5 mM Glutamax (Invitrogen). One-half the volume of culture media was

replaced with fresh maintenance media every 3 days throughout the course of the experiments.

Statistical analyses

Six to nine animals of each sex and treatment were used for all experiments, as indicated. Although no *a priori* power analysis was used to determine these numbers, this is based on 26 years of data from the McCarthy lab demonstrating moderate anticipated effect sizes of Cohen's $D=0.5$, magnitude changes of mean values between 1 and 2-fold, and standard deviations that generally do not exceed 10% of the mean, using similar molecular and cellular endpoints in neonatal rat pups. All data were first analyzed using Kolmogorov-Smirnov test for normalcy and Bartlett's test for homogeneity of variances. Non-parametric post-hoc statistical analyses were utilized where tests for normalcy and homogeneity of variance were not met, as indicated. Specific statistical tests and post-hoc analyses for each experiment are indicated in subsequent chapters. Significance was set at $p<0.05$ for all analyses.

Chapter 3: Sex Differences in BDNF Expression in the Neonatal Hippocampus in Relation to Cell Proliferation

Introduction

The downstream cellular mechanisms that mediate the effects of estradiol on neurogenesis in the developing hippocampus are largely unknown, although BDNF is a likely candidate. Many of the effects of estradiol in the adult hippocampus are mirrored by BDNF or have a demonstrated requirement for activation of the cognate BDNF receptor, TrKB (Scharfman and MacLusky, 2006). In addition, numerous studies indicate a role for BDNF in regulating neurogenesis in adult rodents. For example, direct administration of BDNF to the hippocampus increases cell proliferation (Scharfman et al., 2005), while knockdown of BDNF or conditional deletion of TrKB reduces proliferation (Lee et al., 2009; Taliáz et al., 2010). BDNF also negatively impacts cell survival via p75^{NTR} receptor signaling (Catts et al., 2008).

Based on studies demonstrating a role for BDNF in regulating cell proliferation in the adult hippocampus, as well as the expression of BDNF and TrKB receptors in the neonatal hippocampus of rats (Solum and Handa, 2002), one can speculate that the mechanism through which estradiol modulates proliferation in the developing hippocampus is via BDNF signaling. In addition, BDNF expression and secretion are promoted by depolarizing GABA (Berninger et al., 1995; Obrietan et al., 2002; Deidda et al., 2015), and BDNF in turn positively regulates GABA release and expression and retention of GABA_A receptors on immature neurons (Roberts et al., 2006; Porcher et al., 2011; Riffault et al., 2018), making BDNF a likely candidate mediating the downstream effects of depolarizing GABA in the developing brain. Thus, in the neonatal

hippocampus of rodents, where the depolarizing actions of GABA are more pronounced in males and estradiol signaling modulates the depolarizing response to GABA, BDNF may contribute to the sex difference in neuronal cell proliferation. The experiments described in this chapter, while largely descriptive, were carried out to test the role of BDNF in regulating neonatal hippocampal cell genesis.

Experiments and Results

Quantification of Bdnf gene expression in hippocampal subregions of male and female rats during the first two weeks of life.

The CA1, CA3 and dentate gyrus subregions of the hippocampal formation were dissected from 7 male and 7 female rats on postnatal day 4 (PN4) and PN15, and total RNA extracted from these tissues as detailed in Chapter 2. *Bdnf* transcripts were quantitated using a SybrGreen-based real-time qPCR assay. Because the rat *Bdnf* gene is expressed from multiple promoters and alternatively spliced exons that result in at least 12 distinct transcripts (Aid et al., 2007), primers for this qPCR assay were designed to target the coding region of Exon IX, which is common to all transcripts. Thus, the total population of *Bdnf* transcripts from all promoters was measured in the 3 hippocampal subregions at PN4 and PN15. Mean Ct values for *Bdnf* were normalized to *Gapdh* and expressed as mean fold expression +/- standard error of mean fold expression, relative to female dentate. Data were analyzed by 2-factor ANOVA with sex and region as independent variables, followed by pairwise *post-hoc* comparisons using Bonferroni correction.

On PN4, 2-factor ANOVA indicated a significant main effect of sex on *Bdnf* gene expression [F(1,36)=7.664, n=7, p=0.0088]. *Post-hoc* analysis confirmed that males have more *Bdnf* transcripts in the dentate gyrus (P <0.01) and CA1 region of Ammon's horn (P <0.05), compared to females (Figure 3.1, A). A significant main effect of hippocampal subregion on *Bdnf* expression was also indicated [F(2,36)=39.49, n=7, p<0.0001], as well as an interaction of sex and region [F(2,36)=5.303, n=7, p=0.0096], driven by higher expression in the dentate and CA1 of males. A significant regional effect on *Bdnf* expression was also seen at postnatal day 15 [F(2,36)=40.65, n=7, p<0.0001], where mean relative expression levels were greatest in the dentate gyrus for both sexes (Figure 3.1, B). No effect of sex on *Bdnf* expression was indicated among the 3 regions at this age [F(1,36)=2.619, n=7, p=0.1143].

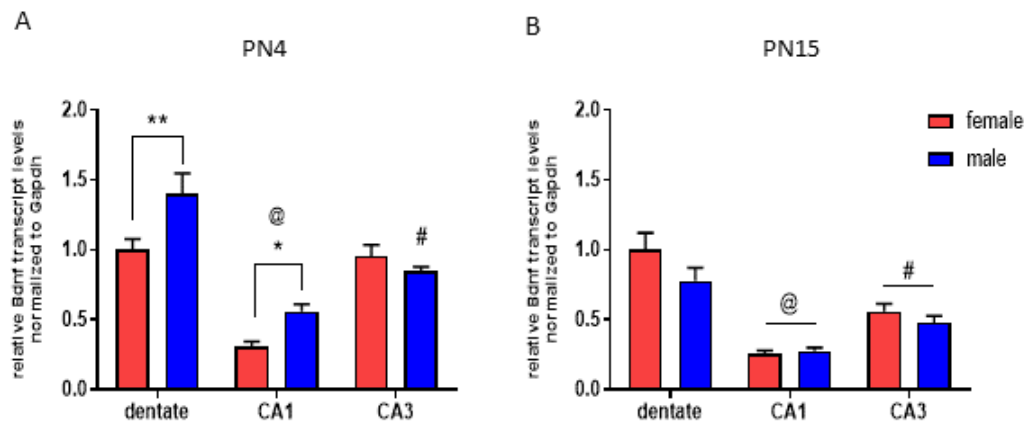


Figure 3.1: Relative abundance of *Bdnf* transcripts in the dentate gyrus, CA1, and CA3 regions of the hippocampal formation of rats on PN4 (A) and PN15 (B) as determined by real-time qPCR. Transcript levels are normalized to *Gapdh* transcripts and expressed as mean transcript abundance +/- s.e.m., relative to female dentate gyrus. A) On PN4, males have higher levels of *Bdnf* transcripts in dentate gyrus and CA1, compared to females (ANOVA, **p<0.01, *p<0.05). Regional differences in *Bdnf* expression were also seen at PN4 (ANOVA, #p<0.01, males only compared to male dentate; @p<0.0001, both sexes, compared to dentate). B) No sex differences in *Bdnf* transcripts seen at PN15. Greatest regional expression in *Bdnf* was seen in dentate gyrus (ANOVA, #p<0.01, @p<0.0001, compared to dentate). n= 7 animals per sex.

Determination of Bdnf expression in hippocampal subregions of neonatal male and female rats in response to estradiol signaling.

To determine if estrogen signaling influences *Bdnf* expression in the developing hippocampus, a second cohort of animals was treated systemically with estradiol benzoate, tamoxifen, or sesame oil vehicle on the day of birth and 24 hours later, as described in Chapter 2, and *Bdnf* transcripts quantified in dentate and CA1 regions on PN4 via qPCR. Within the dentate, 2-factor ANOVA for sex and treatment indicated a significant main effect of treatment [$F(2,31)=13.42$, $n=5-6$, $p<0.0001$]. *Post-hoc* pairwise comparisons confirmed that *Bdnf* expression was significantly decreased by estradiol treatment in both males ($P<0.01$) and females ($P<0.05$), compared to vehicle controls (Figure 3.2, A). Surprisingly, tamoxifen treatment also decreased *Bdnf* expression in the dentate of males ($P<0.05$), to the same degree as estradiol, but had no effect in females ($P>0.05$). A significant interaction between sex and treatment in the dentate was indicated [$F(2,31)=6.870$, $n=5-6$, $p=0.040$], however a main effect of sex was not [$F(1,31)=3.478$, $n=5-6$, $p=0.0735$], most likely because mean values for estradiol and tamoxifen treatments did not differ between males and females ($P>0.05$). *Bdnf* expression was significantly higher in vehicle treated males versus females ($P<0.01$), recapitulating the sex difference observed in untreated animals.

In CA1, a significant main effect of treatment was also evident [$F(2,30)=69.32$, $n=5-6$, $p<0.0001$], although here estradiol treatment greatly increased *Bdnf* expression in both males and females ($P<0.0001$ for both), while tamoxifen treatment had no effect in either sex ($P>0.05$) (Figure 3.2, B). A main effect of sex was indicated [$F(1,31)=11.79$, $n=5-6$, $p=0.0020$], due to higher *Bdnf* expression in vehicle treated males compared to

females ($P < 0.05$). No interaction between sex and treatment was indicated [$F(2,31) = 0.7136$, $n = 5-6$, $p = 0.4992$].

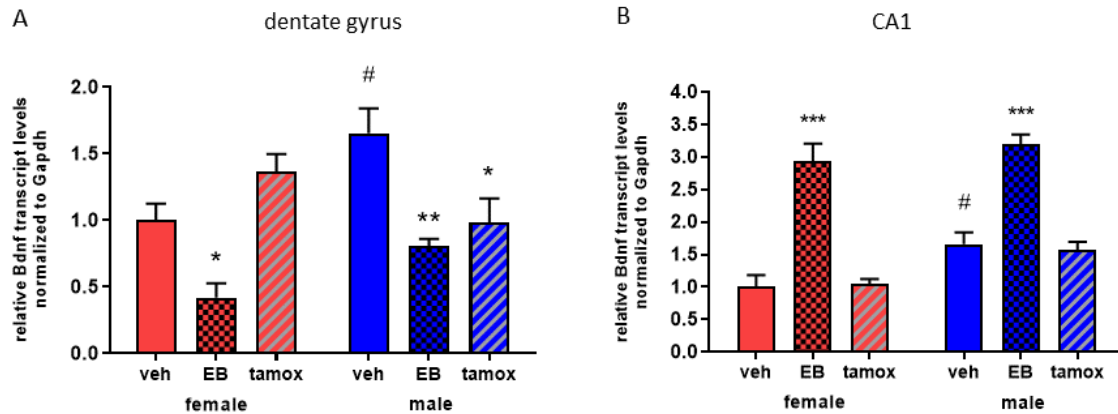


Figure 3.2: Regional effects of estradiol and tamoxifen on Bdnf gene expression in PN4 hippocampus, as determined by real-time qPCR. Transcript levels were normalized to Gapdh and expressed relative to vehicle treated females within each region. A) In dentate, estradiol decreases Bdnf transcripts in both sexes, while tamoxifen decreases Bdnf transcripts in males only (ANOVA, * $p < 0.05$, ** $p < 0.01$ compared to same sex vehicle control). B) In CA1, estradiol increases Bdnf gene expression in both sexes, while tamoxifen has no effect. (ANOVA, *** $p < 0.0001$, compared to same sex vehicle control). Bdnf expression was higher in vehicle treated males compared to vehicle treated females in both regions (ANOVA, # $p < 0.05$). $n = 5-6$ animals per treatment group for each sex. Veh: vehicle; EB: estradiol benzoate; tamox: tamoxifen.

Determination of BDNF propeptide content in the hippocampus during the first postnatal week in response to estradiol signaling.

To examine the effect of estradiol on BDNF peptide levels, a third cohort of animals was treated on PN0 and PN1 with estradiol benzoate (female pups only), tamoxifen (males only), or vehicle. Because we have found that hippocampal cell genesis is not altered by estrogen treatment in males or tamoxifen treatment in females, these two groups were not included in this cohort, in an effort to limit the number of experimental animals. BDNF peptide levels were determined in hippocampal subregions collected on

PN4 using a commercially-available ELISA and reported as pg of BDNF peptide per mg of total protein. Bartlett's test indicated inhomogeneity of variance among treatment groups within the dentate, so means were compared within the three hippocampal subregions using non-parametric ANOVA. No differences among groups within the dentate ($p=0.0508$; Figure 3.3, A), CA1 ($p=0.1463$, Figure 3.3, B) or CA3 ($p=0.1598$; Figure 3.3, C) regions were indicated using the Kruskal-Wallis test by ranks.

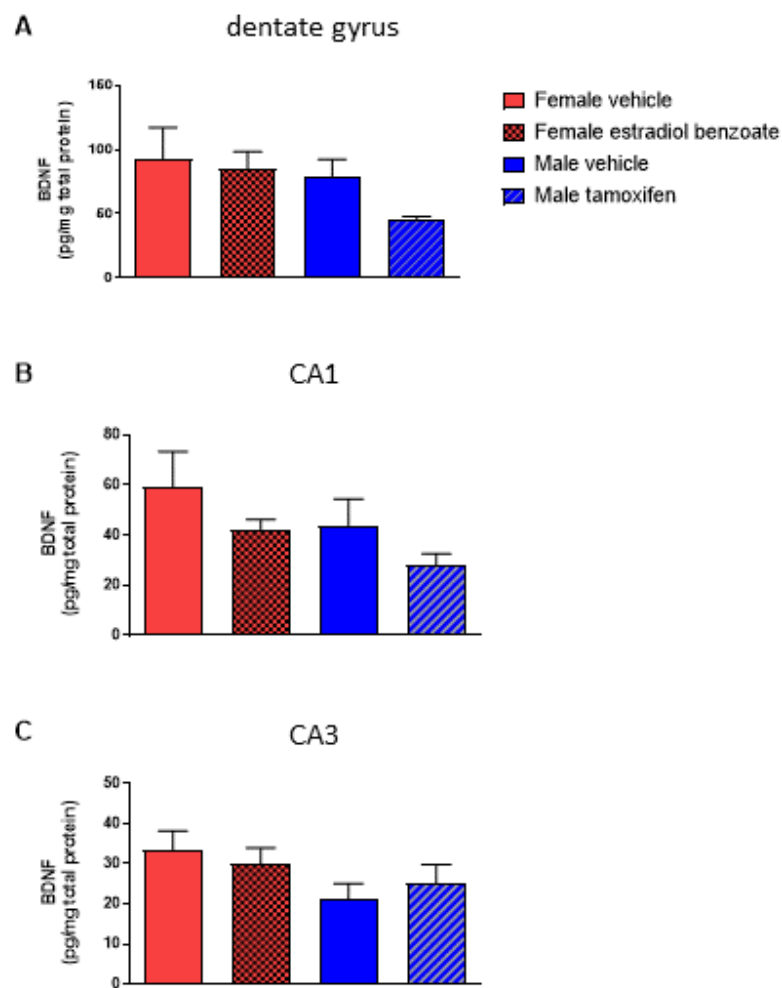


Figure 3.3: Levels of BDNF peptide in the hippocampus of PN4 animals treated with estradiol or tamoxifen, as determined by ELISA. No differences in BDNF content were detected among vehicle control or drug treated animals in the dentate gyrus (A) (non-parametric ANOVA, $p=0.0508$), CA1 (B) (non-parametric ANOVA, $p=0.1463$) or CA3 (C) (non-parametric ANOVA, $p=0.1598$). $n=5-6$ animals per group for each sex

Results from the ELISAs were confirmed by comparing relative amounts of BDNF peptide in the same sample homogenates using Western immunoblotting. A single band at 28 KDa corresponding to the predicted molecular weight of the BDNF propeptide was obtained with the anti-BDNF antibody (Aviva Systems Biology, RRID:AB10644597; Figure 3.4, C, D). As with the ELISA results, one-way ANOVA indicated no differences among groups within the dentate or CA1 regions on PN4 ($p=0.7858$, $p=0.4975$; Figure 3.4, A and B, respectively). BDNF propeptide content in CA3 was not assessed by Western immunoblotting.

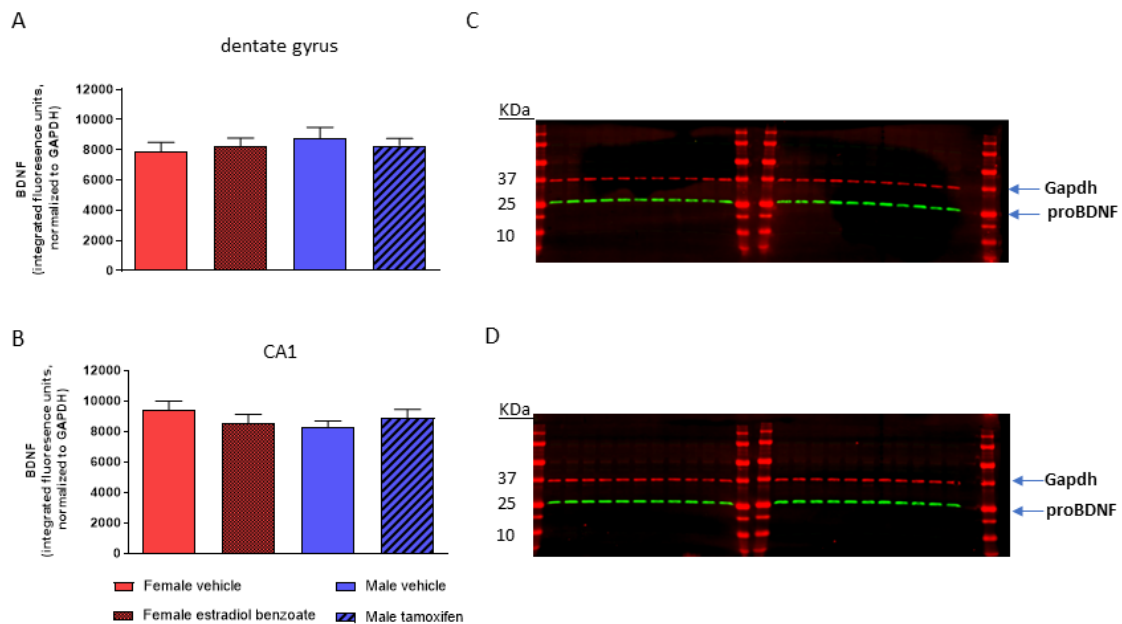


Figure 3.4: Levels of BDNF propeptide in hippocampus of PN4 rats treated with estradiol or tamoxifen, as determined by Western immunoblotting. No differences in BDNF levels were detected among vehicle, estradiol, or tamoxifen treated animals in the dentate gyrus (A) (ANOVA, $p=0.7858$) or CA1 (B) (ANOVA, $p=0.4975$). C, D) Western blots demonstrating a single band at 28 KDa was detected with the BDNF antibody in the dentate (C) and CA1 (D). $n=5-6$ animals per treatment for each sex.

The sex difference in Bdnf gene expression during the first postnatal week is not reflected in BDNF prohormone content.

Western immunoblotting was used to test for baseline sex differences in BDNF peptide content in hippocampal subregions during the first postnatal week using the same cohort of untreated PN4 animals used to demonstrate a sex difference in *Bdnf* gene expression. As with the hippocampal homogenates from animals treated with estradiol and tamoxifen, a single band corresponding to the 28 KDa BDNF propeptide was detected. Two-factor ANOVA confirmed no effect of sex on BDNF propeptide content among the three hippocampal subregions at postnatal day 4 ($p=0.9091$), nor were there regional differences in propeptide content ($p=0.9389$; Figure 3.5, A). No differences in BDNF propeptide content were seen between males and females (2-factor ANOVA, $p=0.5086$), or among the three hippocampal subregions (2-factor ANOVA, $p=0.0551$) from untreated animals at postnatal day 15 (Figure 3.5, B).

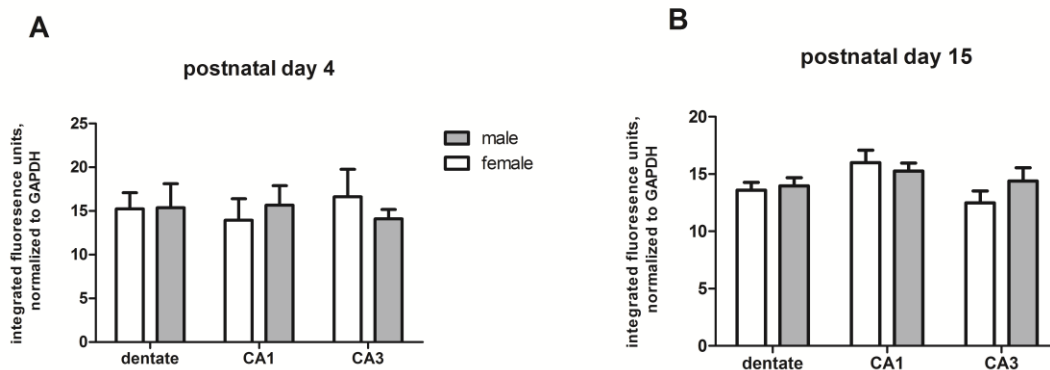


Figure 3.5: Levels of BDNF propeptide in dentate and CA1 regions of untreated animals at PN4 (A) and PN15 (B), as determined by Western immunoblotting. No sex differences were noted among hippocampal subregions at PN4 (ANOVA, $p=0.9091$) or PN15 (ANOVA, $p=0.5086$). Regional differences in BDNF propeptide content were also not indicated at either PN4 (ANOVA, $p=0.9689$) or PN15 (ANOVA, $p=0.0551$).

To confirm the results from Western immunoblotting, BDNF peptide content in dentate and CA1 was additionally assessed by ELISA in the same samples. Two-factor ANOVA confirmed there was no effect of sex on BDNF peptide content in either region ($p=0.7120$; Figure 3.6). A significant main effect of hippocampal subregion on BDNF peptide levels was indicated [$F(1,26)=58.45$, $n=7$, $p<0.0001$]. BDNF peptide was higher in the dentate of both sexes, compared to CA1, recapitulating the relative regional abundances for *Bdnf* transcript (Figure 3.1, A).

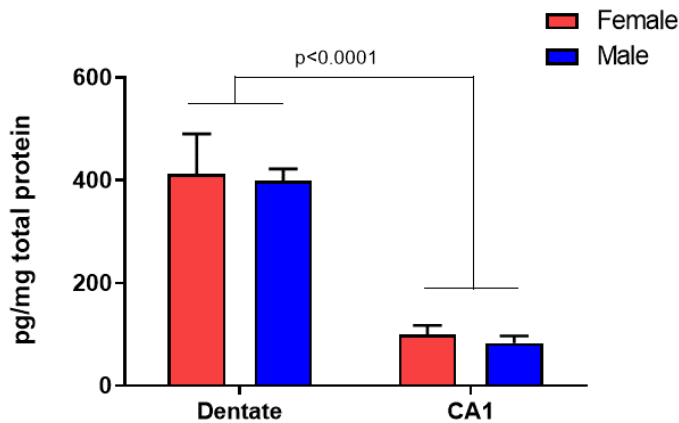


Figure 3.6: Levels of BDNF propeptide in dentate and CA1 regions of untreated animals at PN4, as determined by ELISA. More BDNF was measured in dentate, compared to CA1. No difference between males and females was detected in either dentate or CA1 (ANOVA, $p=0.7120$). $n=7$ animals per sex.

Discussion

This study quantified *Bdnf* transcripts in the dentate gyrus, CA1 and CA3 regions of the hippocampal formation in male and female rats during the first and second postnatal weeks. It is important to note that while this study captured total transcripts resulting from gene expression at all *Bdnf* promoters, this can only serve as an imperfect proxy for gene expression. Static measurements of transcript levels may indeed directly relate to primary transcriptional output, but also reflect transcript turnover. Cellular and

physiological differences or exogenous manipulations may have effects on transcriptional output, transcript stability, or both which alter the amount of transcript measured using PCR. Nevertheless, a baseline sex difference in *Bdnf* gene expression was found, where males had roughly 50% more *Bdnf* transcripts in the dentate gyrus and CA1, but not CA3, regions of the hippocampus during the first postnatal week. There was no sex difference in *Bdnf* gene expression in the hippocampal formation on PN15. Intriguingly, this pattern of expression mirrored the relative numbers of proliferating cells in the hippocampus at these two timepoints, where males produce roughly twice as many new cells in the dentate and CA1, compared to females, at postnatal day 4, but not at postnatal day 15 (Zhang et al., 2008; Bowers et al., 2010; Lima et al., 2014). However, the relationship between BDNF and cell genesis in the neonatal hippocampus is likely to be complex and region-specific, as estradiol positively regulates cell proliferation in both the dentate and CA1 (Zhang et al., 2008; Bowers et al., 2010), but had opposite effects on *Bdnf* expression between these two regions in the present study.

In contrast to long-standing interest in the role of BDNF in hippocampal plasticity and neurogenesis in adults, particularly with regard to the effects of estrogen, far fewer studies have examined BDNF expression in the developing hippocampus. Damborsky and Winzer-Serhan (2012) reported neonatal male rats have higher hippocampal *Bdnf* gene expression in dentate, CA1 and CA3 subregions at PN5, but not PN8. This is largely consistent with the observations of this study, with the exception of CA3, where a sex difference was not seen. This discrepancy may be due to methodological differences, as Damborsky and Winzer-Serhan utilized *in situ* hybridization autoradiography to

determine relative *Bdnf* transcript levels, and the present study measured transcripts via qPCR in whole, dissected subregions of the hippocampus.

One of the first studies to examine the ontogeny of BDNF expression in the developing hippocampus determined transcript and peptide levels in neonatal male rats in relation to gonadal steroids. Castration on the day of birth decreases *Bdnf* gene expression for the first two postnatal weeks in CA1 and CA3, yet a single dose of estradiol on the day of birth in castrated animals restores *Bdnf* transcript levels (Solum and Handa 2002). This demonstrates that not only does estradiol upregulate *Bdnf* expression in Ammon's horn of the developing hippocampus, but the duration of the effect of estradiol suggests that *Bdnf* expression during the first two weeks can be primed by the perinatal surge in gonadal steroids. In the present study, estradiol signaling was manipulated in neonatal rats using a paradigm of systemic administration of estradiol benzoate or the estrogen receptor antagonist tamoxifen. In the presence of a masculinizing dose of estradiol, total *Bdnf* transcripts in CA1 were upregulated in male and female neonates, similar to what was seen in males by Solum and Handa (2002). Interestingly, in the dentate of both sexes the opposite effect of estradiol was observed in this study, where *Bdnf* gene expression decreased. These opposing effects of estradiol between the two regions of the hippocampus may be explained by regional differences in estrogen receptor subtype and cellular localization. In rats, ER α mRNA and protein are upregulated in CA1 during the first two weeks postnatally, and immunocytochemistry localizes ER α to nuclei of pyramidal neurons (Ivanova and Beyer, 2000; O'Keefe and Handa, 1990; Solum and Handa, 2001). *Bdnf* is expressed in pyramidal neurons of the CA1, its synthesis and secretion is promoted by estradiol in parallel with CREB activation (Zhou et al., 2005),

and a functional estrogen response element is found in the *Bdnf* gene (Sohrabji et al., 1995). Together this suggests the observed upregulation of *Bdnf* gene expression in pyramidal neurons of neonatal CA1 occurs via classical genomic action of estrogen receptors. In the neonatal dentate gyrus, however, extranuclear ER β is localized to the plasma membrane of immature granule neurons and also glia (Herrick et al., 2006), raising the possibility that estradiol may have nongenomic effects on *Bdnf* expression from these cell types in the dentate.

Although exogenous estradiol elicited opposite effects on *Bdnf* expression in dentate and CA1, these effects were nevertheless the same between neonatal males and females. In contrast, blocking endogenous estradiol signaling at ER α and ER β with the pan-receptor antagonist tamoxifen appeared to have a sex-specific effect in the dentate gyrus, but not CA1. tamoxifen had no effect on *Bdnf* transcript levels in female dentate, but in males tamoxifen decreased *Bdnf* gene expression to the same level as in females. Because the 4-hydroxy metabolite of tamoxifen has significantly more affinity for the estrogen receptor than the drug itself (Fabian et al., 1981), the possibility is raised that the differential effects of tamoxifen in neonatal males and females may be due to sex differences in tamoxifen metabolism. However, the enzymes of the cytochrome P450 system which metabolize tamoxifen are present from birth in the rodent liver, and although low during the neonatal period, show a similar pattern of expression in males and females (Cui et al., 2012; Hart et al., 2009). In addition, the effects of exogenous estradiol administration in the brains of female neonatal rodents is blocked by systemic administration of tamoxifen (Gonzalez et al., 2012; Hilton et al., 2004). Together these indicate that tamoxifen is able to antagonize estrogen receptors similarly in neonatal

males and females, and the effects observed in this study on *Bdnf* expression in the dentate are sex specific. At first glance this suggests that the baseline sex difference in *Bdnf* expression in the dentate is promoted by estradiol. However, since estradiol and androgen content in the hippocampus at this age is equivalent in males and females (Amateau et al., 2004; Konkle and McCarthy, 2011), there must be sex-specific mechanisms downstream of estradiol signaling that mediate this difference.

One possible mechanism is the depolarizing action of GABA. The trophic effects of depolarizing GABA in the developing brain are proximally mediated by an influx of Ca^{2+} , which results in activation of the transcription factor CREB, a positive regulator of *Bdnf* expression (Finkbeiner et al., 1997; Conti et al., 2002). Depolarizing GABA upregulates *Bdnf* gene expression and peptide content in developing neurons in a CREB-dependent manner (Berninger et al., 1995; Obrietan et al., 2002; Shieh et al., 1998). As noted above, sex differences in CREB content and the depolarizing actions of GABA are found in the neonatal hippocampus of rats, where males respond to GABA_A receptor activation with a longer duration of calcium influx in a greater percentage of the neuronal population (Galanopoulou, 2008; Nuñez and McCarthy, 2007; 2009), and also have more activated CREB compared to females (Auger et al., 2001; Perrot-Sinal et al., 2003). Moreover, both the depolarizing actions of GABA and activated CREB content are positively regulated by estradiol (Nugent et al., 2012; Nuñez et al., 2005; Nuñez and McCarthy, 2009). The baseline sex differences in depolarizing GABA and CREB suggest a mechanism through which greater *Bdnf* expression might be achieved in the neonatal hippocampus of males in spite of equivalent estradiol content between the sexes.

An interesting, yet unsurprising, outcome of this study was the discordance between *Bdnf* transcript and peptide levels. Although the ELISA captured relative differences between dentate and CA1 regions that mirrored the results from qPCR, it did not detect any differences in peptide content between males and females, or in response to estradiol, and this was confirmed with Western immunoblotting. BDNF is synthesized and secreted as a glycosylated precursor propeptide of approximately 28 KDa, which is proteolytically cleaved to yield a mature peptide of 14 KDa (Mowla et al., 2001). Both the ELISA and the anti-BDNF antibody used for Western immunoblotting are able to detect the 28 KDa propeptide and the mature form of BDNF, and were intended to measure total translational output, although only one band corresponding to the 28 KDa propeptide was detected in Western immunoblots. The abundance of BDNF precursor peptide relative to the mature form is greatest in the hippocampus of neonatal and juvenile animals, due to developmentally regulated expression of tissue plasminogen activator, which is essential for zymogen activation of the protease which cleaves proBDNF to the mature peptide (Pang et al., 2004; Yang et al., 2009). The amount of mature BDNF found in the hippocampus of mice during the first 15 days postnatally is extremely low (Yang et al., 2014), and therefore the inability to detect mature BDNF in this study is either due to insufficient sensitivity of the Western immunoblots, or the absence of mature BDNF peptide at this developmental stage. Previous studies have noted differences between *Bdnf* transcript levels and peptide content. For example, Gibbs (1999) found that estradiol treatment of ovariectomized female rats increased *Bdnf* transcripts but had no effect on peptide levels in the hippocampus. In the neonatal male rat, castration induces a significant increase in BDNF mature peptide, while transcripts

decreased (Solum and Handa, 2002). More recently, Hill et al (2014) found that adolescent stressed male rats that had experienced maternal deprivation have lower levels of hippocampal *Bdnf* gene expression compared to unstressed animals, but higher levels of the mature BDNF peptide, while no changes are observed in the BDNF propeptide. Females undergoing maternal separation and adolescent stress also exhibit lower levels of mature BDNF peptide in the hippocampus, but no changes in propeptide or transcript levels. The BDNF propeptide and mature form can have opposing effects on several aspects of neuronal development, including cell proliferation (Hempstead, 2006). While BDNF increases cell genesis, as noted above, BDNF propeptide can promote apoptosis through preferential activation of p75^{NTR}, rather than TrKB receptors, thereby decreasing cell genesis (Teng et al., 2005). Mature BDNF was not measured in this study, but given the potential for sex-specific regulation of proBDNF cleavage, and the opposing effects of proBDNF and mature BDNF on cell genesis, this is worth future study. An alternative mechanism that may account for transcript levels that don't result in the same relative levels of peptide is posttranscriptional regulation via microRNAs. In the classical model of their mode of action, microRNAs regulate peptide levels by directly binding coding mRNAs and promoting their degradation or sequestration away from the translational machinery. As discussed in the following chapter, microRNAs are also important regulators of neurogenesis. An exploratory experiment was thus carried out to determine if sex differences in microRNA expression could account for the male-biased sex difference in neurogenesis.

Chapter 4: Sex Differences in microRNA Expression in the Neonatal Hippocampus in Relation to Cell Proliferation

Introduction

It is estimated that upwards of 60% of mammalian genes are regulated by microRNAs (miRNAs), a class of small, evolutionarily conserved, 21-22 nucleotide non-coding RNAs that modulate gene expression post-transcriptionally. They are transcribed from RNA polymerase II-type promoters, producing a primary transcript of up to several kilobases that is processed to form a 70 bp hairpin structure which is then exported from the nucleus (see Bartel, 2009; Krol et al., 2010; Ha and Kim, 2014 for review). This precursor miRNA is further cleaved in the cytoplasm by the Dicer complex to form the mature miRNA, which is loaded onto the RNA-induced silencing complex (RISC). The mature microRNAs/RISC typically functions by binding to conserved sequences within messenger RNAs and acting as guide molecules to recruit protein effectors that facilitate degradation or translational repression of the target transcript, although increased translation as a result of miRNA activity has been reported. The particular RISC proteins that associate with a mature miRNA dictate whether the targeted mRNA is shuttled into pathways for either degradation or translational repression, and this is highly context dependent, varying across phylogeny, cell type, and physiological process (Krol et al., 2010; Nowakowski et al., 2018).

A given protein-coding transcript may be targeted by more than one species of RISC-associated miRNA, which can act synergistically to regulate its product. The microRNA target sequence within a given transcript is typically found within the 3' untranslated region, although target sites can be found anywhere within the transcript.

The canonical binding site for a given miRNA is determined by a 6-8 nucleotide “seed” sequence of complementarity, surrounded by 14-16 nucleotides of contextual sequence of imperfect homology. For this reason, a single miRNA will target transcripts from many, even hundreds, of different genes (Selbach et al., 2008; Hausser and Zavolan, 2014; Malmevik et al., 2015). Deep sequencing of mRNA transcripts targeted by specific miRNAs have revealed that a single miRNA or family of miRNAs may directly regulate the expression of suites of genes involved in a single regulatory network, and often their targets encode transcription factors, making them master regulators of developmental and physiological processes (Shibata et al., 2011; Hausser and Zavolan, 2014; Nowakowski et al., 2018; Kim et al., 2019). In addition, primary transcripts encoding miRNAs are often encoded within the same gene as or under transcriptional control of the protein-coding genes they regulate and they are often involved in feed-forward regulatory loops (Baskerville and Bartel, 2005; Tsang et al., 2007). This, coupled with observations that miRNA activity typically results in a dampening rather than a complete abrogation of protein expression, while also thresholding low gene expression, support a model in which miRNAs function to limit extreme fluctuations in gene expression and thus fine-tune the regulation of cellular processes (Baek et al., 2008; Mukherji et al., 2011). This makes them particularly attractive as potential regulators of processes where sex differences occur, in which it’s critical to achieve differences between males and females while still maintaining physiological and cellular endpoints within a tightly limited, or canalized, range of normal (Posada and Carthew, 2014; McCarthy, 2016).

The brain is particularly rich in microRNA expression, and approximately 70% of all known miRNAs are found in the mammalian central nervous system (Chen et al.,

2015). In higher vertebrates, many miRNAs are found only in the central nervous system, and the greatest number of brain-specific miRNAs are present in primates, supporting the idea that they play critical roles in the complex regulatory networks during brain development and in response to the environment that are necessary for higher cognitive functions (Miska et al., 2004; Berezikov et al., 2006; Hu et al., 2011; Chen et al., 2015). Dysregulated microRNA expression or function is associated with numerous neurological and neurodevelopmental disorders that exhibit a sex bias (Qureshi and Mehler, 2012), including epilepsy (Henshall, 2014), schizophrenia (Beveridge and Cairns, 2012; Lett et al., 2013; Lai et al., 2016), autism (Abu-Elneel et al., 2008; Wu et al., 2016), Alzheimer's disease (Moradifard et al., 2018), traumatic brain injury (Pan et al., 2017), anxiety and panic disorder (Kohen et al., 2014), and depression (Lopez et al., 2014a,b). Animal studies have shown that miRNAs are particularly important in the developing brain, and have demonstrated roles in regulating dendritic outgrowth and branching (Fiore et al., 2009; Christensen et al., 2010; Xu et al., 2013; Lippi et al., 2016), cell motility (Shibata et al., 2011; Han et al., 2016; Wu et al., 2018), synaptogenesis and synaptic plasticity (Schratt et al., 2006; Jasinska et al., 2016; Lippi et al., 2016), directing neuro- and glial-genesis (La Torre et al., 2013; Patterson et al., 2014; Selvi et al., 2015; Tsuyama et al., 2015), and proliferation (Liu et al., 2010; Zhao et al., 2010; see also Table 3). The role of miRNAs in neuronal proliferation has been particularly well studied, primarily as a result of their dysregulated function in glioma, but also in the context of the neurogenic niche. Several microRNAs are known to be critical for regulating the balance between proliferation and differentiation of neural stem cells in both the adult and developing brain, and their expression patterns are tightly regulated in relation to developmental

context and cell cycle status (Sempere et al., 2004; Nishino et al., 2008; Sun et al., 2011; Akerblom et al., 2012; Kim et al., 2015; Zhang et al., 2016). The proximal regulatory factors that control miRNA expression during neurogenesis are largely unknown. Outside the brain however, steroid hormones are known to directly influence miRNA activity in relation to cell proliferation. Signaling through glucocorticoid, progesterone, androgen and estrogen receptors have all been shown to mediate steroid-dependent proliferation of cancer cells through direct regulation of miRNA expression (see Yang and Wang, 2011, for review). Estrogen in particular has a variety of effects on miRNAs involved in breast cancer, including transcriptional control of miRNA expression via estrogen response elements (Klinge, 2012; Paris et al., 2012), regulation of miRNA processing (Paris et al., 2012), and non-genomic effects on miRNA expression (Kondo et al., 2008). The potential for steroid hormone-mediated regulation of miRNA activity in the brain, along with the demonstrated importance of miRNAs in regulating cell proliferation and their function as precise modulators of developmental processes, prompted the consideration of miRNAs as potential regulatory agents promoting the sex difference in developmental cell genesis in the rat hippocampus.

Experiments and Results

Quantification of several microRNAs that regulate proliferation in the neonatal hippocampus of male and female rats using qPCR.

As a first step toward testing whether microRNAs regulate cell proliferation in the neonatal dentate gyrus, a targeted qPCR approach was used to determine the relative abundances of 14 microRNAs known to have roles in regulating cell proliferation, neurogenesis, or BDNF expression (see Table 3). Each of these 14 miRs is expressed in the rat hippocampus, as confirmed using search algorithms of the microrna.org web

portal (Computational Biology Center of Memorial Sloan-Kettering Cancer Center) to access tissue-specific expression data compiled by Landgraf et al (2007).

Table 3: microRNAs targeted in the neonatal rat hippocampus using qPCR.

let-7b	Inhibits NSC proliferation in adult SVZ	Nishino et al., 2008 Zhao et al., 2010
miR-9	Inhibits NSC proliferation in adult SVZ Enhances proliferation and migration in embryonic SGZ Upregulated in maturing neurons	Zhao et al., 2009 Delaloy et al., 2010 Uchida, N. et al., 2010
miR-16	Inversely correlated with <i>Bdnf</i> expression in prefrontal cortex after maternal deprivation Targets cell cycle regulators	Bai et al., 2012 Linsley et al., 2007
miR-22	Targets c-myc pathway to lengthen cell cycle in cerebellar granule neuron precursors	Berenguer et al., 2013
miR-26a	Represses <i>Bdnf</i> expression in striatum; dysregulated in schizophrenia	Caputo et al., 2011
miR-29a	Upregulated in maturing neurons; increased in prefrontal cortex after maternal separation	Uchida, S. et al., 2010
miR-29b	Expression induced by estradiol; downregulates DNMTs in germ cells Overexpression promotes apoptosis in glioblastoma	Meunier et al., 2012 Shin et al., 2017
miR-29c	Downregulated in glioma; promotes cell cycle arrest Regulates <i>Bdnf</i> expression and proliferation via targeting DNMT3	Fan et al., 2013 Yang et al., 2015
miR-124a	Abundant in maturing neurons; low expression in neural progenitors and neuroblastoma Overexpression in neural progenitors promotes cell cycle exit and differentiation	Fowler et al., 2010 Akerblom et al., 2012 Makeyev et al., 2007
miR-132	Promotes neurite outgrowth; downregulates <i>Bdnf</i> expression via MeCP2 Upregulated in maturing neurons; increased in prefrontal cortex after maternal separation	Klein et al., 2007 Uchida, S. et al., 2010
miR-134	Inhibits <i>Bdnf</i> expression and cell death via CREB-dependent feedback loop in hippocampal neurons	Huang et al., 2015
miR-137	Promotes proliferation, inhibits differentiation in adult SGZ Inhibits <i>Bdnf</i> expression in human neural progenitors	Szulwach et al., 2010 Hill et al., 2014
miR-184	Promotes proliferation in adult neural stem cells	Liu et al., 2010
miR-195	Inversely correlated with <i>Bdnf</i> expression in human prefrontal cortex; Altered expression associated with schizophrenia Promotes proliferation in adult neural stem cells	Mellios, 2009 Liu et al., 2013

Total RNA, including small RNAs, was isolated from CA1, CA3 and dentate gyrus subregions of the hippocampal formation from male and female rats on PN4 and used to quantitate mature microRNAs, according to methods detailed in Chapter 2. Single-strand cDNA was generated using a pool of primers targeting each of the 14 microRNAs and the small nucleolar RNA U6. Because mature microRNAs are only 20-22 bases in length, the primers used in the reverse transcriptase reaction target the 3' end of the mature microRNA and contain additional sequence that forms a stem-loop structure during the cDNA synthesis. This prevents the RT primer from annealing to the longer primary microRNA transcripts and provides sufficient target sequence for subsequent annealing of qPCR primers (figure 4.1).

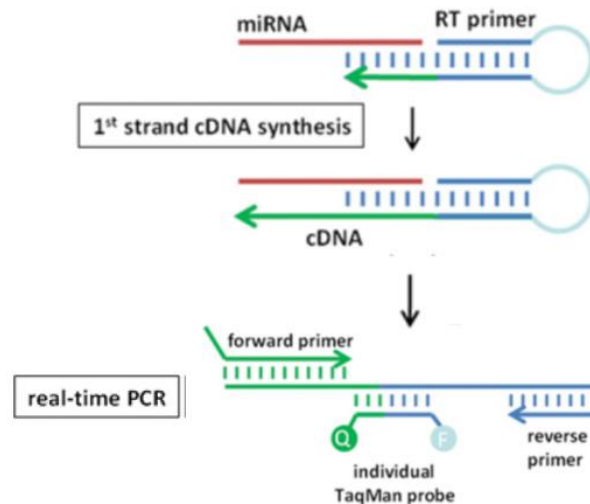
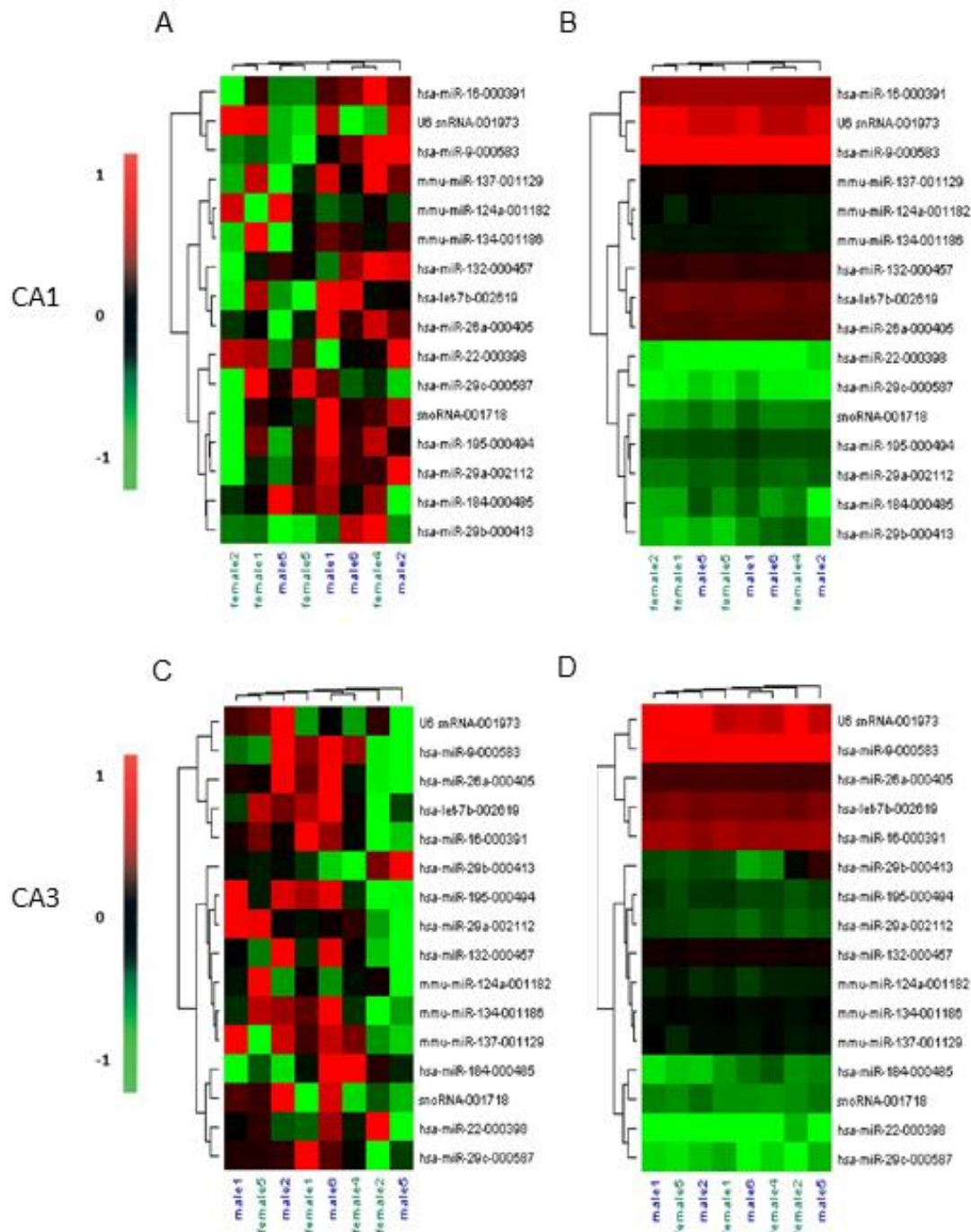


Figure 4.1: Schematic representation of mature microRNA quantitation via qPCR. cDNA copies of mature, fully processed microRNA are made using primers containing microRNA-specific sequence incorporated into the 3' end of a 22-base primer that forms a hairpin structure. During qPCR, the stem-loop portion of the cDNA provides complementary sequence for one amplification primer, while the other amplification primer is specific to the mature microRNA sequence. A fluorescently-tagged oligonucleotide probe (TaqMan) spans the junction between the 3' end of the target microRNA and the stem-loop sequence. Adapted from Jung *et al.*, 2013.

The resulting cDNA was applied to custom TaqMan MicroRNA Array cards (ABI, ThermoFisher) which contain TaqMan qPCR reagents for individual target microRNAs in a 384-well format. All 14 microRNA targets, U6 normalization target and background controls were included on a single card, configured to test 8 samples in triplicate. Thus, cDNA from one of the three hippocampal subregions from 4 males and 4 females was amplified on each array card. For each microRNA target, mean Ct values of technical replicates from each sample were normalized to mean Ct values of U6 according to the $\Delta\Delta\text{Ct}$ method (Schmittgen and Livak, 2008).

As a first pass toward discerning any sex-specific expression patterns, hierarchical clustering analysis was applied to normalized mean Ct values of miRNA targets for each sample. Application of Euclidian distance measurement to agglomerative clustering algorithms revealed no discernable pattern differentiating males and females in the CA1 or CA3 regions of the hippocampal formation at PN4 (figure 4.2; A,C). In addition, the relative expression of each microRNA among all 14 microRNAs quantified was the same among all samples in both these regions of Ammon's horn, as seen when hierarchical clustering was centered on global average expression levels of individual microRNAs (4.2; B,D). miR9 and miR16 were the most abundant of these 14 microRNAs, and miR22, miR184 and miR29c were the least abundant. The majority of the remaining microRNAs quantified were expressed within a 0.5-fold range of the aggregate average expression level for all 14 microRNAs tested.

Figure 4.2: Hierarchical clustering of mean normalized expression of 14 microRNAs expressed in CA1 (A,B) and CA3 (C,D) regions of the hippocampal formation of male and female rats at PN4. Euclidean distance measure algorithms were applied in agglomerative bootstrap analyses using ExpressionSuite Software (ABI, ThermoFisher). A, C: Clustering of individual microRNA expression among all samples. B,D: Within-sample clustering of all targeted microRNAs. Scale indicates \log_2 -fold change in expression from aggregate mean expression of all microRNAs.



In the dentate gyrus, however, a distinct pattern of expression between males and females was seen. As shown in Figure 4.3; E, females generally expressed higher levels of 13 of the 14 microRNAs, compared to males, although this pattern was reversed for miR29b. As with the hippocampal subregions of Ammon’s horn at this age, there was no sex difference in the relative abundances among all microRNAs measured (Figure 4.3;F). miR9 was the most abundant of these 14 microRNAs in the dentate at this age.

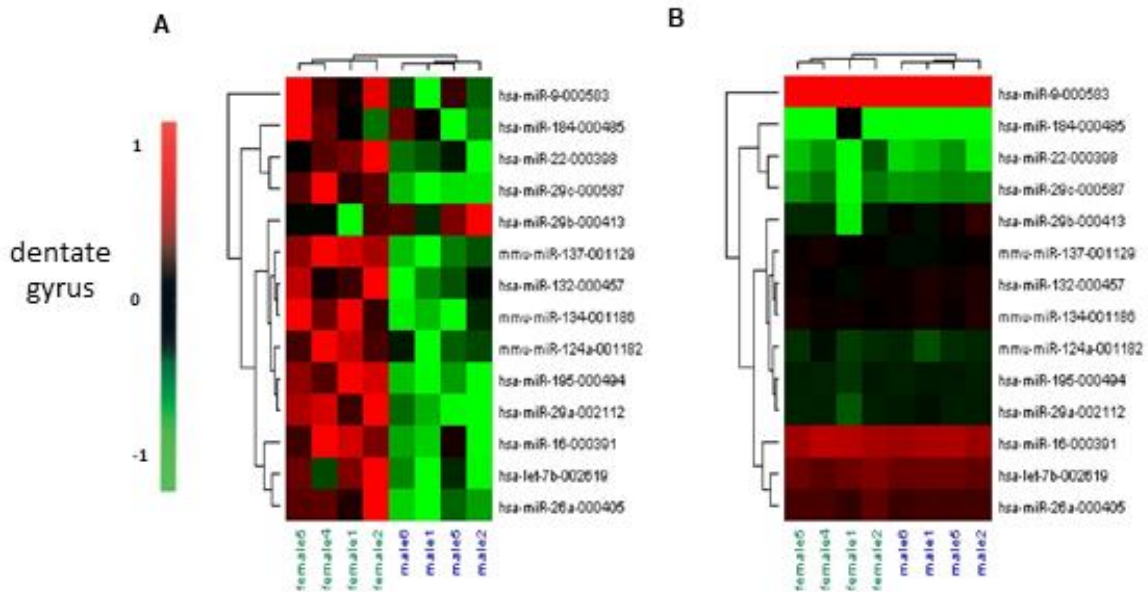


Figure 4.3: Hierarchical clustering of mean normalized expression of 14 microRNAs expressed in the dentate gyrus of male and female rat pups at PN4. Euclidean distance measure algorithms were applied in agglomerative bootstrap analyses using ExpressionSuite Software (ABI, ThermoFisher). A: Clustering of individual microRNA expression among all samples. B: Within-sample clustering of all targeted microRNAs. Scale indicates log₂-fold change in expression from aggregate mean expression of all microRNAs.

Mean normalized Ct values for each target microRNA in the dentate gyrus on PN4 were expressed as fold-change values relative to males, which are tabulated below in Table 4 and shown in Figure 4.4. The combined error associated with technical and biological replicates for each miRNA target is represented by 95% confidence intervals of the mean. With the exception of miR29b, which showed a non-significant trend to lower abundance in females, most microRNAs tested in this qPCR array were 1.5-2.5-fold more abundant in the female dentate gyrus, compared to males. Mean relative values of miR26a and miR184 were not significantly different between males and females, although both seemed to trend toward greater abundance in females.

Table 4: Relative expression of 14 microRNAs in the dentate gyrus of neonatal rats as determined by qPCR.

microRNA	Male		Female	
	Mean $\Delta\Delta Ct$	95% CI	Mean $\Delta\Delta Ct$	95% CI
let-7b	1.00	0.83 – 1.19	1.82	1.30 – 2.54
miR9	1.00	0.79 – 1.25	1.42	1.23 – 1.63
miR16	1.00	0.78 – 1.27	1.84	1.57 – 2.16
miR22	1.00	0.54 – 1.83	4.32	2.24 – 8.34
miR26a	1.00	0.88 – 1.13	2.00	1.51 – 2.67
miR29a	1.00	0.93 – 1.07	1.48	1.35 – 1.62
miR29b	1.00	0.60 – 1.65	0.49	0.21 – 1.14
miR29c	1.00	0.96 – 1.03	1.89	1.48 – 2.41
miR124a	1.00	0.68 – 1.45	2.30	1.78 – 2.96
miR132	1.00	0.87 – 1.15	1.38	1.21 – 1.58
miR134	1.00	0.86 – 1.15	1.68	1.44 – 1.97
miR137	1.00	0.82 – 1.22	2.41	2.16 – 2.69
miR184	1.00	0.68 – 1.47	1.43	0.91 – 2.26
miR195	1.00	0.94 – 1.06	1.76	1.56 – 2.00

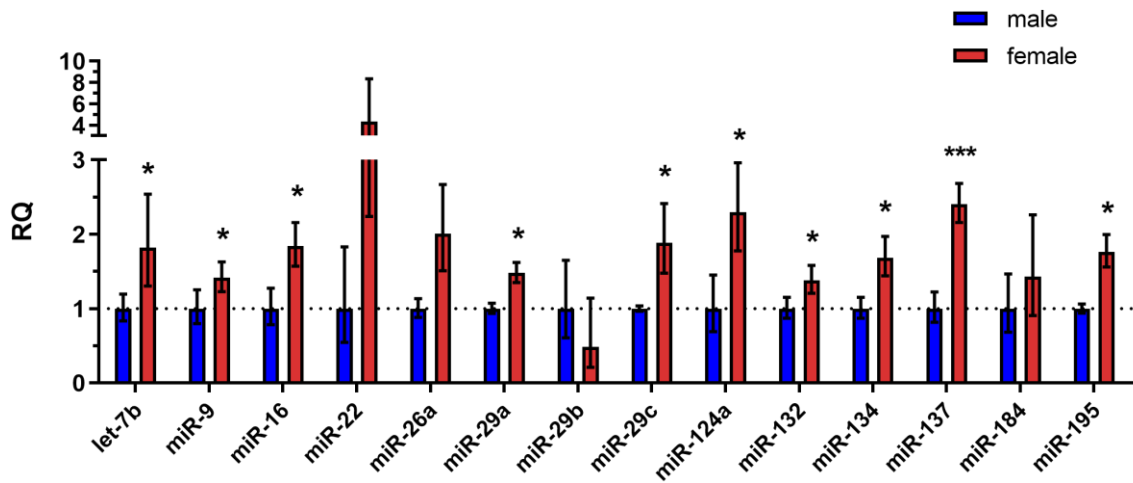


Figure 4.4: Relative abundances of 14 mature microRNAs in the dentate gyrus of male and female rats on PN4, as determined using custom TaqMan qPCR arrays. Ct values were calculated for each microRNA, normalized to U6, and mean Δ Ct values were expressed relative to males. These $\Delta\Delta$ Ct values are indicated as relative quantification (RQ). Error bars indicate 95% confidence intervals of the transformed data. n= 4 of each sex. * p<0.05; *** p<0.001.

Quantification of all known rat microRNAs in the dentate gyrus of neonatal males and females using NanoString panels.

In order to confirm the sex difference in microRNA expression in the dentate gyrus using an alternative methodology and expand upon the limited dataset obtained with the TaqMan arrays, a comprehensive determination of the microRNA population in the neonatal dentate was done using nCounter[®] miRNA Expression Panels (NanoString Technologies). The nCounter[®] technology utilizes fluorescently-barcoded probes to detect individual microRNA sequences in a highly multiplexed, solution hybridization reaction, providing a direct readout of absolute molecule numbers of each microRNA species in a sample of total RNA. For these studies, the Rat v1.5 miRNA Assay was used (NanoString Technologies), which contains 453 annotated rat mature microRNA sequences in a 12-assay format. Total RNA was isolated from the dentate gyrus of 6 male

and 6 female rat pups on PN4, as described in Chapter 2. 100 ng of total RNA from each sample was used in solution hybridization with the barcode probe set. The nCounter® miRNA assay panels were loaded and processed at the Johns Hopkins Medical Institute Transcriptomics and Deep Sequencing Core Facility (Baltimore, Maryland). Raw counts were screened for failed hybridization or probe detection using the internal quality controls contained on each panel. The mean aggregate signal from on-panel background controls was subtracted from each sample probe output. A global normalization strategy was employed for each background-subtracted sample using the arithmetic mean of counts for the top 80 most abundant microRNAs. Individual t-tests were performed on mean counts for each target microRNA between males and females, using a Benjamini-Hochberg discovery approach with a false discovery rate of 5%.

Relative abundances of all 453 microRNAs included in the NanoStrings rat microRNA panel are visualized in Figure 4.5 by regressing mean average counts for each miRNA relative to males. As seen in panel A, microRNAs that are present at low abundance, 32 molecules per 100 ng total RNA or less, are more highly expressed in females, compared to males. When only the 63 X-chromosome-encoded microRNAs are plotted, only 14 of these account for the low abundance, female-biased microRNAs (Figure 4.5B). Relative abundances of rat X-chromosome-encoded microRNAs mirror the overall expression pattern of all microRNAs.

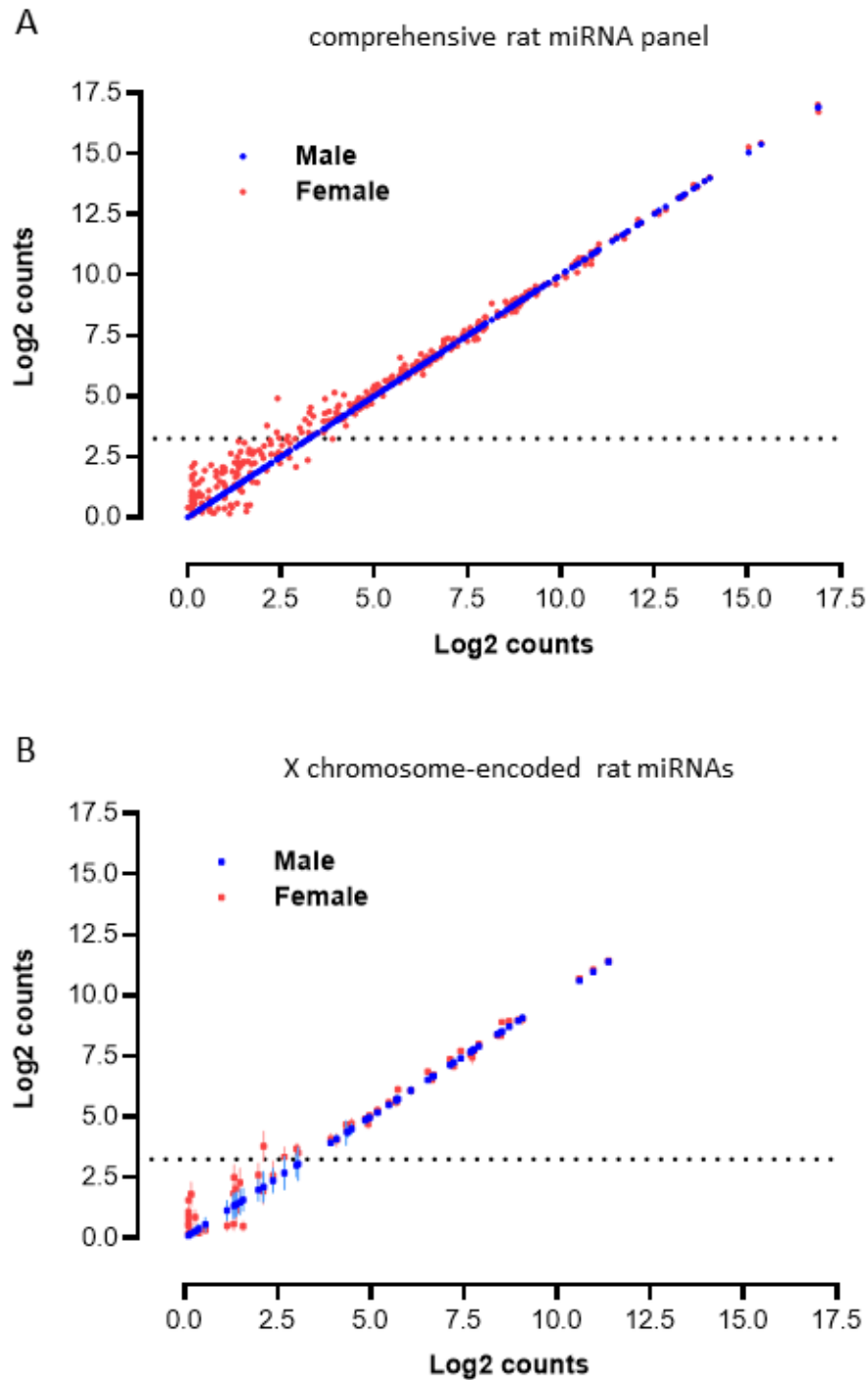


Figure 4.5: Abundances of microRNA sequences in the dentate gyrus of male and female rats on PN4, as determined by NanoString rat microRNA arrays. Each point represents mean counts for a single microRNA sequence determined from 6 females (red) or 6 males (blue) regressed against mean counts for males. A) All 453 microRNAs included in the Nanostring array. B) X chromosome-encoded microRNAs included in the NanoString array. Dotted line indicates 10 counts per 100 ng total RNA, which is 2 standard deviations above background counts.

Of the entire panel of 453 microRNAs, 243 were detected at more than 10 average counts/100 ng total RNA in either sex. Of these only 18 exhibited a sex difference, 7 of which were more abundant in females (see Table 5). Surprisingly, miR9 is the only one of these that exhibited a difference in abundance between males and females using the NanoString arrays (123658.6 +/-9444.3 mean counts in females, vs 107441.9 +/- 6981.8 mean counts in males; p=0.0113) that was previously found to be sex-biased using the targeted qPCR approach.

Table 5: Amounts of microRNA per 100 ng total RNA in the rat dentate gyrus on PN4 which exhibit a sex difference, as determined via NanoString panels. miRNAs in red are more abundant in females.

microRNA	Male Avg. counts +/- SD	Female Avg. counts +/- SD	P value
miR9	107442 +/- 4642	123658 +/- 3412	0.0113
let7c	39570 +/- 1061	33746 +/- 2327	0.0482
let7e	4956 +/- 94	4324 +/- 193	0.0338
miR135a	1401 +/- 136	1516 +/- 91	0.0357
miR340	1383 +/- 65	1602 +/- 75	0.0411
miR338	1098 +/- 32	1393 +/- 74	0.0071
miR191 #	504 +/- 15	437 +/- 24	0.0448
miR361#	476 +/-19	367 +/-34	0.0161
miR199a	542 +/- 50	285 +/-46	0.0413
miR301a	390 +/- 11	441 +/-19	0.0248
miR328a	309 +/- 12	252 +/- 16	0.0378
miR148b	229 +/- 7	254 +/- 6	0.0268
miR376b	153 +/- 7	181 +/- 7	0.0123
miR199a	95 +/- 8	52 +/- 16	0.0332
miR152	35 +/- 3	15 +/- 4	0.0042
miR301b	33 +/- 7	18 +/- 8	0.0094
miR219-5p	16 +/- 3	9 +/- 5	0.0224
miR741-3p#	14 +/- 8	4 +/- 4	0.0376

Mean numbers of microRNA molecules determined via NanoString arrays for each of the 14 mature microRNA sequences previously quantified via qPCR are shown in Table 6. As was the case with the qPCR arrays, miR9 is also the most abundant of these 14 microRNAs. miR184 was detected at the theoretical detection limit of approximately 1 molecule in both males and females. This is consistent with high Ct values for this microRNA in the qPCR assays (mean Ct value of 35.99 +/- 0.199 for males, mean Ct value of 34.49 +/- 0.079 for females).

Table 6: Amounts of 14 microRNAs previously quantified in the dentate gyrus of rats on PN4 using qPCR arrays, as determined via NanoString panels.

microRNA	Male Avg. counts +/- SD	Female Avg. counts +/- SD	P value
let-7b	44100 +/- 7535	42527 +/- 6956	0.7272
miR9	107442 +/- 4642	123658 +/- 3412	0.0113
miR16	4389 +/- 842	4246 +/- 734	0.7699
miR22	2888 +/- 483	3347 +/- 377	0.1103
miR26a	133 +/- 14	118 +/- 12	0.0921
miR29a	797 +/- 67	814 +/- 43	0.6243
miR29b	50 +/- 13	50 +/- 8	0.9631
miR29c	430 +/- 82	486 +/- 67	0.2429
miR124a	9445 +/- 886	9970 +/- 1394	0.4862
miR132	1978 +/- 281	1843 +/- 246	0.4191
miR134	626 +/- 81	570 +/- 85	0.3004
miR137	1364 +/- 417	1338 +/- 272	0.9032
miR184	1 +/- 0.9	1 +/- 0.2	0.3554
miR195	52 +/- 15	43 +/- 8	0.2430

Discussion

These data profile miRNA expression in the developing dentate gyrus of male and female rats at a timepoint when a sex difference in neuronal precursor proliferation is present. Although numerous studies have examined comprehensive microRNA expression in the adult brain in the context of psychiatric disorders or neurological pathologies, there are few studies which profile microRNA expression in the developing brain, particularly in relation to sex. The first report to link miRNAs to sexual differentiation of the brain was provided by Morgan and Bale (2011), using a mouse model of gestational exposure to maternal stress. This study assessed the expression of hundreds of microRNAs in whole brain tissue of postnatal day 1 mice and showed that normally developing pups exhibit a sex-specific pattern of miRNA expression that is driven by perinatal aromatization of testosterone in males. In human postmortem samples, comprehensive miRNA profiling by Ziats and Rennert (2014) demonstrated differential miRNA expression between males and females in the prefrontal cortex across various stages of development, from infancy through adolescence. Interestingly, of the female-biased miRNAs found in this study, most are upregulated during adolescence, suggesting a role for these in sex-specific programming of the brain during puberty. And in the developing rat cortex, roughly 2 dozen microRNAs are more highly expressed in one sex over the other on the day of birth (Murphy et al., 2014). Several of these sex-biased miRNAs undergo a developmental switch during the period of brain sex differentiation, as they are found to be more abundant in cortical tissue of the opposite sex, or equal in males and females, when measured at PN7 and in adults (ibid). The data presented here are limited to a single timepoint, and it would have been useful to compare male and female miRNA expression in the dentate at earlier or later ages. A similar but

time-shifted expression pattern for proliferation-associated miRNAs between males and females may give insight into whether the female dentate is more mature in its neurogenic trajectory and more cells have exited proliferation, compared to males.

Like the work reported by Murphy et al., this study used 2 distinct approaches to quantify miRNAs: a qPCR-based custom TaqMan array, and direct counting of each miRNA using NanoString molecular barcodes. Roughly 7% of miRNAs detected above background were more abundant in one sex versus the other, and 7 out of these 18 were more abundant in females. Regardless of sex, the difference in abundance for any of these miRNAs was between 1.2 and 1.5-fold. The sensitivity of this assay was able to detect 1 molecule above background counts, and numerous miRNAs were quantified at less than 100 counts, or a log₂ value of 6.5. As illustrated in panel A of Figure 4.5, most of these very low-expressed miRNAs trend toward more abundance in females, however, their expression is likely not actively regulated, at least in the context of cellular proliferation. Assuming a total RNA content of 10-25 pg per cell, the amount of material used for each sample represents 4000-10,000 cells. If just 1% of these cells from isolated dentate gyrus are relevant for the sex difference in proliferation, a cutoff of 100 mean miRNA counts is justified in determining which miRNAs are likely to be of biological interest. The amount of a particular miRNA varies depending on cell type, but generally, less than 1000 copies per cell is considered low abundance expression, while more typical miRNA expression results in several thousand or even tens of thousands of copies per cell (Song et al., 2017). One possibility for the apparent female bias in very low abundance miRNAs is escape from X-inactivation. In mammals, the X chromosome is particularly dense with miRNA-encoding genes. Fifty-nine miRNAs are encoded on the rat X chromosome,

roughly twice as many as encoded by most autosomes (www.mirbase.org, release 22). Assuming X-linked genes for miRNAs escape inactivation to the same degree as other X-linked genes, roughly 15-25% of these will be expressed from both X chromosomes in females (Carrel and Willard, 2005). Only 3 of the 59 X-encoded miRNAs, miR191, miR361 and miR741, were found to be sex-biased in this assay, fewer than expected to escape X-inactivation, and these were more abundant in males. In addition, most of the low-abundance miRNAs that trend toward greater expression in females are encoded on the autosomes, suggesting “leaky” or unregulated, stochastic expression.

In contrast to the NanoString data, quantification of a small number of miRNAs using TaqMan-based qPCR arrays showed that almost all miRNAs tested were more abundant in the dentate gyrus of females. For those miRNAs that showed a statistically significant difference, mean fold increases in females ranged from 1.4 to 2.5-fold over males. Surprisingly, only one of the miRNAs included in the qPCR array, miR9, also showed a sex difference in the NanoString panel, and was shown by both assays to be elevated in females. A survey of the literature makes clear that this is not unusual, and is most likely due to the different quantification platforms (Wang et al., 2011; Meyer et al., 2012; Pritchard et al., 2012; Mestdagh et al., 2014; Wan et al., 2014). Although the detection of unique miRNAs among different profiling methods typically has a high degree of concordance, the quantification of differential expression between samples can vary greatly depending on the assay. Cross-platform, comprehensive profiling of miRNAs from standardized samples have found as few as a single miRNA in common across platforms out of dozens detected at differential levels between control and disease tissues (Wang et al., 2011). In a careful examination of this issue, Mestdagh and

colleagues (2014) profiled standardized samples among 4 different miRNA platforms, including direct sequencing, hybridization microarrays, NanoString and TaqMan-based qPCR arrays, and found the average concordance between any two of these platforms in terms of detecting expression differences was only 54%. When taken among all four platforms, there was only 3% overlap. The detection of differential expression is dependent upon normalization strategy, and this can have a profound effect on concordance across platforms (Meyer et al., 2012). In the NanoString arrays, absolute counts of individual miRNAs were normalized to the geometric mean of total counts for the 80 most abundant miRNA. While this global normalization strategy is generally considered the best method when profiling large numbers of targets, detection of differential expression can be skewed if there are differences in many highly-abundant miRNAs. Individual T-tests of background-subtracted miRNA counts, prior to normalization, indicated this was likely not the case for this study, although perhaps more rigorous statistical tests are needed to be sure. The qPCR array data in this study was normalized to U6, a highly abundant small nucleolar RNA intended to serve as a proxy for the total amount of small RNA loaded for each sample. Average Ct values for U6 were not significantly different between males and females for this experiment, indicating equivalent amount of starting material for males and females and no differences in terms of amplification efficiency among samples. Thus, the lack of concordance between these two platforms may be due to the inherent differences in normalization strategy.

The logical next step in assigning functional significance to miRNAs that are differentially expressed in the male and female dentate would be to focus on miR9, which was found to be differentially expressed in both the qPCR and NanoString arrays.

Unfortunately, the NanoString data was obtained 2 years after hypothesis testing had begun based on the qPCR array data, so subsequent experiments used the qPCR array data as a starting point for functional analyses.

It should be noted that several of the differentially-expressed miRNAs included in the qPCR array are known to target *Bdnf* transcripts or are involved in regulating BDNF expression. As noted in Chapter 3, one possible explanation for the disconnect between *Bdnf* transcripts and peptide levels may be translational inhibition of *Bdnf* transcripts by miRNAs in the hippocampus of males. None of the miRNAs known to regulate BDNF expression were more abundant in males in either the qPCR or NanoString arrays, which would be predicted if translational inhibition of BDNF occurred. The fact that almost all miRNAs targeted in the qPCR array are more abundant in females is striking, especially since many of them promote or inhibit different aspects of proliferation and neuronal maturation in various cellular contexts. It's also notable that differential expression of these miRNAs was found only in the dentate, where dividing cells make up a significant portion of the population, and not in CA1 or CA3 regions of Ammon's horn, where the number of proliferating cells is extremely low. The apparent sex difference in expression of these neurogenesis-related miRNAs may be directly related to cellular expression patterns in proliferating versus non-proliferating cells, or it may simply be reflective of the number of cells in the dentate of males versus females. Subsequent experiments focused on the function of a single miRNA that exhibited a sex difference in the neonatal dentate gyrus, as determined by the qPCR array, in an effort to determine which of these two possibilities is correct.

Chapter 5: Testing the Role of miR124 in Regulating NKCC1 Expression and Cell Proliferation in the Neonatal Hippocampus.

Introduction

One of the most well-studied miRNAs, in the contexts of both normal cellular function and pathology, is miR124. miR124 is an evolutionarily-conserved microRNA found in many tissues but highly enriched in the central nervous system (Sempere et al., 2004; Landgraf et al., 2007; Guo et al., 2009). It is the most highly expressed miRNA in the brain (Olsen et al., 2009; Shao et al., 2010; Hu et al., 2011). It's preferentially expressed in neurons, although also reportedly found in microglia, where it modulates activation of these cells and promotes a less reactive state (Ponomarev et al., 2011). Among the functional roles for miR124 in neurons are axon outgrowth (Sanuki et al., 2011) and regulation of CREB-dependent synaptic plasticity (Rajasehupathy et al., 2009), but the most well-established role for miR124 in the brain is as a master regulator of neurogenesis. miR124 expression is extremely low or absent in neuroblasts and proliferating progenitors, but highly upregulated in differentiating and mature neurons (Wienholds et al., 2005; Makeyev et al., 2005, 2007; Cao et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009; Maiorano and Mallamaci, 2009; Fowler et al., 2010; Akerblom et al., 2012). Upregulated miR124 expression promotes the transition of neuroblasts to exit the cell cycle and undergo maturation into a differentiated neuron, and has been shown to be both necessary and sufficient for this process. Knockdown of endogenous miR124 in purified stem cells from the subventricular zone maintains them as dividing precursors, while miR124 overexpression increases neuron maturation in this context (Makeyev et al., 2007; Cheng et al., 2009; Akerblom et al., 2012). In HeLa cells, ectopic

miR124 expression halts proliferation and induces expression of a suite of neuron-specific genes and a cellular neuronal phenotype (Lim et al., 2005).

miR124's role as a repressor of proliferation is perhaps most powerfully illustrated by its dysregulated expression in a variety of cancers in the brain and other tissues, where it inhibits proliferation and invasion (Lang et al., 2012; Shi et al., 2012; Lv and Yang, 2013; Wang et al., 2014; Zhou et al., 2016; Wang et al., 2016; Zhang et al., 2018). For this reason, miR124 is often classified as a tumor suppressor, and many of the known downstream targets of miR124 have been found studying its role in cancers. In neurogenesis, miR124 is known to directly interact with transcripts encoding key regulators of the transition from neuronal precursor to differentiated neuron. One of these is SRY-homeobox 9, or SOX9, which is required for maintenance of stem-like characteristics in transit amplifying cells of the SVZ and is downregulated by miR124 as these cells transition into neuroblasts (Cheng et al., 2009). miR124 also directs the degradation of transcripts encoding PTBP1, a repressor of alternative splicing of primary RNAs in progenitor cells, and in so doing promotes a shift toward neuron-specific patterns of RNA splicing (Makeyev et al., 2007; Chen et al., 2011). In addition, miR124 expression is repressed by the RE-1 silencing factor/Neuron Restrictive Silencing Factor, a transcription factor whose activity is required for preventing expression of a neuronal phenotype in cells. Dissociation of REST/NRSF from the miR124 promoter enables miR124 expression, which results in downregulation of non-neuronal transcripts and promotion of a neuron phenotype (Conaco et al., 2006). Transcriptome analyses have identified hundreds of transcripts downregulated as a result of miR124 activity (Lim et

al., 2005; Hendrickson et al., 2009) as well as directly targeted by miR124 (Chi et al., 2009), but few of these have been validated functionally.

In addition to its functional importance in regulating neurogenesis and the cellular transition from a proliferative to a quiescent phenotype, two observations suggest miR124 may be involved in promoting the sex difference in cell genesis in the neonatal hippocampus. The first, as described in Chapter 4, is the inverse sex difference between the amount of miR124 and cell proliferation in the dentate gyrus, where males have more proliferating cells but less miR124, compared to females. This is consistent with a role for upregulated miR124 expression in promoting neuronal maturation and cell cycle exit earlier in females, although it does not suggest any direct mechanism. A potential mechanism is suggested by a second observation, a putative target site for miR124 in the mRNA transcript encoding NKCC1, the chloride transporter whose expression is crucial for conferring a depolarizing response to GABA in neuroblasts and neural progenitors. A search of potential targets for miR124 in the rat genome using target prediction algorithms (Griffiths-Jones, 2006) yielded a number of hits, one of which was *Slc12a2*, the transcript encoding NKCC1. Subsequent *in silico* analysis of rat *Slc12a2* confirmed a miR124 target sequence of perfect homology and with strong contextual sequence in the 3'UTR. This result was confirmed using TargetScan, MirTarget and Miranda algorithms, and is highly conserved across species (Figure 5.1, A). This result suggests a potential mechanism and role for differential miR124 expression in the dentate gyrus of neonatal male and female rats, illustrated in Figure 5.1, B. As discussed in Chapter 1, the sex difference in depolarizing GABA in the neonatal rat hippocampus is characterized as an earlier shift to a hyperpolarizing response in females, during the first week of life, and

this is dependent on downregulated NKCC1 expression. The hypothesized role of miR124 in this context is to promote this shift by targeting *Slc12a2*. A corollary to this is a subsequent decrease in proliferation in females, although whether depolarizing GABA does indeed promote proliferation in the neonatal hippocampus is inferred from the literature and has not been shown. The experiments described in this chapter were designed to directly test the role, if any, of miR124 in regulating NKCC1 expression in the neonatal hippocampus.

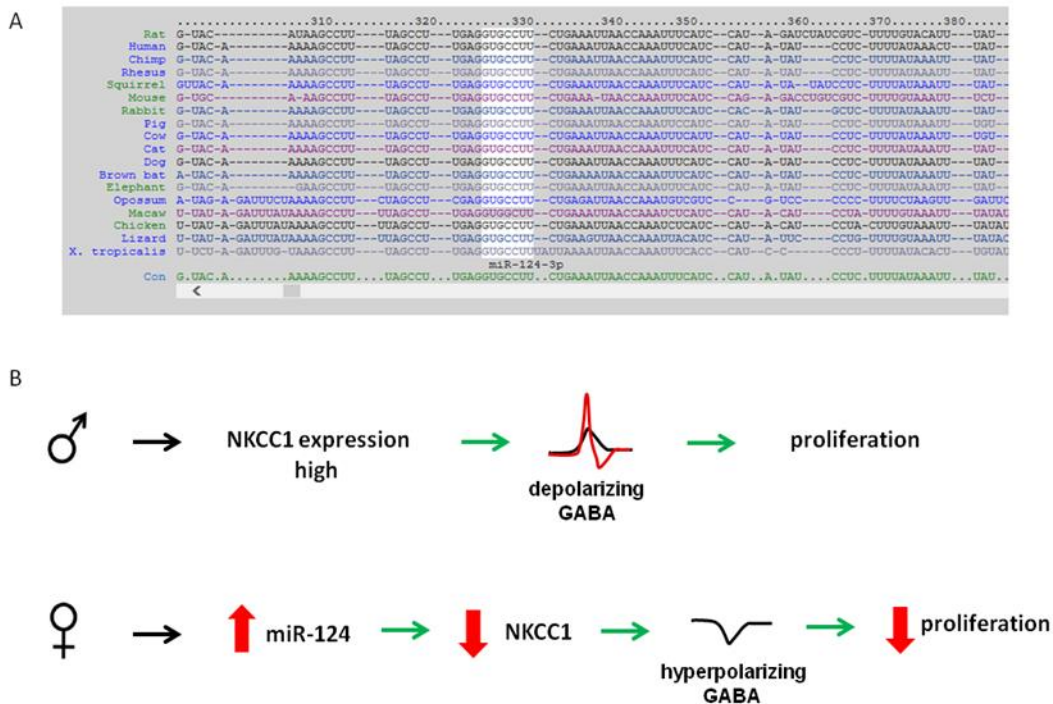


Figure 5.1: The hypothesized role of miR124 in regulating NKCC1 expression in the neonatal hippocampus of rats. A: Putative miR124 target site in the 3'UTR of *Slc12a2*, conserved across species. B: Schematized hypothesis for miR124 role in regulating NKCC1 expression and promoting the sex difference in cell proliferation in the dentate gyrus of neonatal rats. In males, high expression of NKCC1 in immature neuronal cells dictates a depolarizing response to GABA, maintaining the proliferative state of precursors. In females, a rise in miR124 downregulates NKCC1 expression, shifting neuronal cells to a hyperpolarizing response to GABA, and subsequently promoting exit from the proliferative state and neuronal maturation.

Experiments and Results

Quantification of miR124 in the hippocampus of males and females in relation to cell proliferation and NKCC1 expression.

The sex difference in miR124 expression seen in the neonatal dentate gyrus using qPCR was confirmed using a new cohort of animals. The effect of estradiol signaling on miR124 expression was also examined in this experiment. Rat pups were treated on the day of birth and 24 hours later with subcutaneous injections of estradiol benzoate (females only), tamoxifen (males only), or vehicle, as described in Chapter 2. On PN4, whole dentate gyrus was microdissected from the hippocampal formation of pups. miR124 was quantified from total RNA extracted from these tissues using a TaqMan-based qPCR assay specific for mature rat miR124 (ABI, LifeTech). Mean Ct values for miR124 were normalized to the small nucleolar RNA U6, and expressed as mean fold expression +/- standard error of mean fold expression, relative to vehicle treated males. Data were analyzed by one-way ANOVA with, followed by pairwise *post-hoc* comparisons using Tukey's multiple comparisons test. As shown in Figure 5.2, mean miR124 expression differed among vehicle-treated males and females and in response to estradiol manipulation [$F(3,12)=8.648$, $n=4$, $P=0.0025$]. The sex difference in dentate gyrus miR124 content previously seen in untreated males and females at this age was recapitulated in vehicle-treated pups. An approximately 2.2-fold elevation of miR124 was seen in vehicle-treated females, relative to vehicle-treated males ($p = 0.0017$). Administration of exogenous estradiol to females decreased miR124 content 0.76% ($p = 0.0381$), and antagonism of estradiol signaling in males increased miR124 content 75% to levels equivalent to vehicle-treated females ($p = 0.0406$). Thus, the effects of altered

estradiol signaling on miR124 content in the dentate were opposite the proliferative response to estradiol signaling.

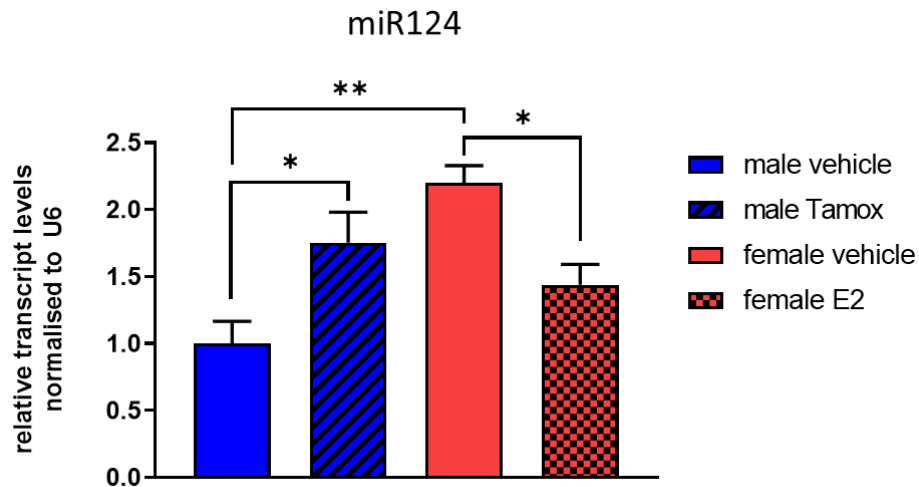


Figure 5.2: Relative transcript levels of mature miR124 in the dentate gyrus of PN4 male and female pups in response to altered estradiol signaling. Mean transcript levels of miR124 are normalized to U6 and expressed relative to vehicle-treated males. N= 4 animals per group.

After establishing a sex difference in dentate gyrus miR124 content during the first postnatal week using qPCR, the relationship between miR124 and NKCC1 expression in the hippocampus was first characterized by quantifying their relative abundances over the first two weeks of life, spanning the time when a sex difference in cell genesis comes and goes and the developmental shift in depolarizing GABA takes place. Dentate gyrus and CA1 regions of the hippocampus were microdissected from 6 male and 6 female pups at 2, 4, 7 and 15 days postnatally, and total protein and total RNA were extracted from the same tissue homogenates, as described in Chapter 2. NKCC1 was quantified in protein homogenates via Western blot, and values were expressed as mean arbitrary units normalized to Gapdh. NKCC1 gene expression was

quantified from purified total RNA by measuring *Slc12a2* transcripts with qPCR, as described in Chapter 2. Mean transcript levels were normalized to *Gapdh* expression and expressed relative to males to PN2. Mature miR124 was measured in purified total RNA using a TaqMan-based qPCR assay (ABI, LifeTechnologies), normalized to U6 content, and expressed relative to males at PN2. Two-tailed Student's T was used to determine differences between mean values for males and females at each postnatal timepoint. In the dentate gyrus, males had more NKCC1 content compared to females at postnatal days 2 and 4, but not at postnatal days 7 or 15 (Figure 5.3 A). However, there was no sex difference in *Slc12a2* transcripts in the dentate across the 4 postnatal ages (Figure 5.3, B). miR124 content in the dentate was the same between males and females on PN2, significantly higher in females compared to males on PN4, as seen in Chapter 4, and showed no sex difference thereafter (Figure 5.3, C). In the CA1 subregion, there were no differences in levels of NKCC1 protein, *Slc12a2* transcripts, or miR124 content between males and females at any of the ages sampled during the first two weeks (Figure 5.3, D through F).

Figure 5.3 (Following page): Timecourse of NKCC1 protein (A, D), *Slc12a2* transcript (B, C) and mature miR124 (C, E) content in the dentate gyrus (A-C) and CA1 (D-F) hippocampal subregions of male and female rats, during the first 2 weeks of life. NKCC1 protein is expressed as arbitrary units, normalized to *Gapdh* content. *Slc12a2* transcripts are normalized to *Gapdh* transcripts and expressed relative to males at PN2. miR124 content is normalized to U6 content and expressed relative to males at PN2. N=6 males and 6 females at each timepoint. * $p < 0.05$ by 2-tailed Student's T test, mean values each sex at each timepoint.

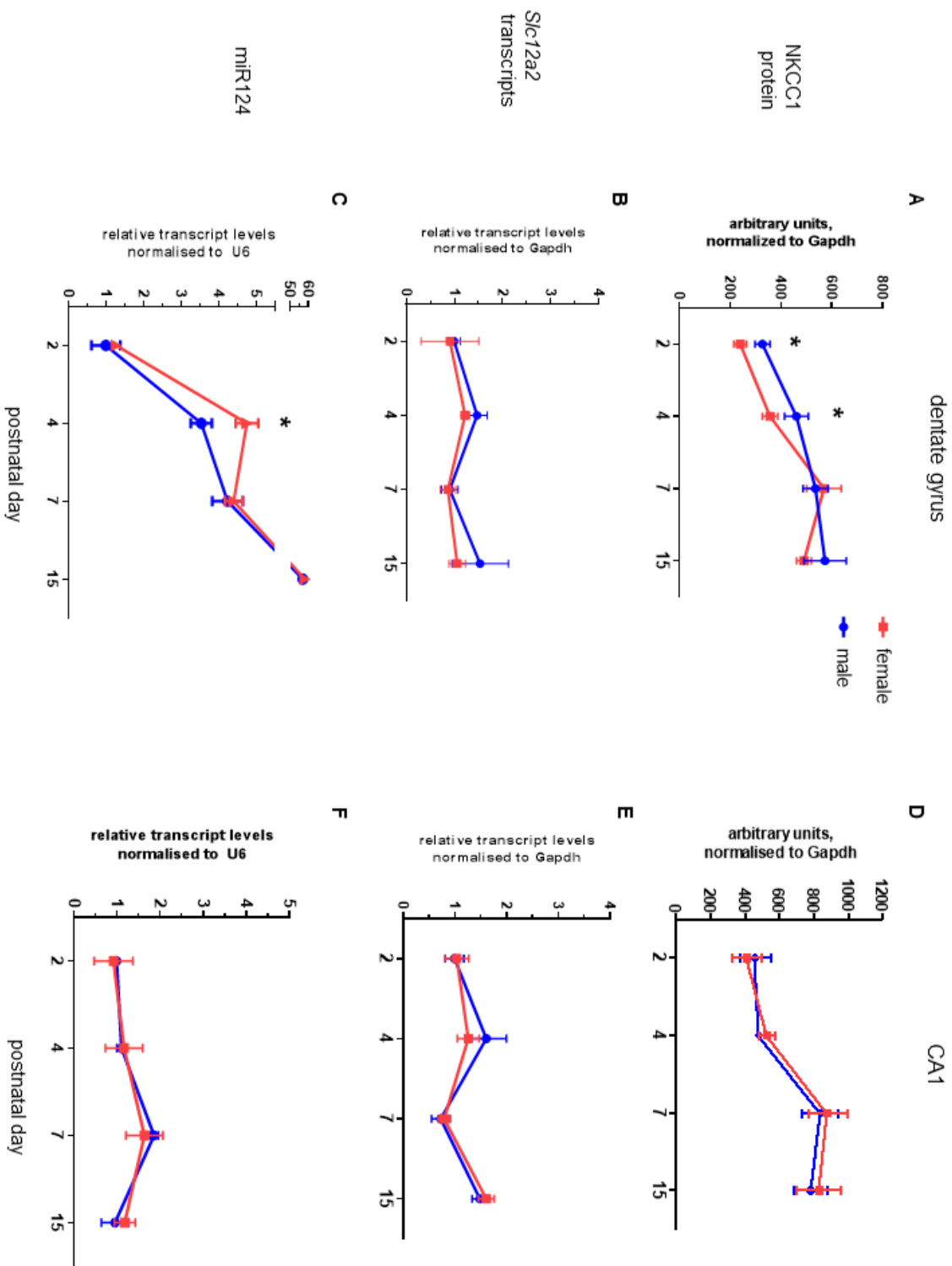


Figure 5.3 (Legend previous page)

In vivo antagonism of miR124 in the hippocampus of males and females and effects on NKCC1 expression.

The descriptive data obtained from the timecourse of NKCC1 expression and miR124 content in the neonatal hippocampus supported a hypothesized role for miR124 in downregulating NKCC1 expression and subsequent modulation of cell genesis. Based on the inverse relationship between NKCC1 and miR124 in males and females, one would predict that blocking miR124 activity would result in increased NKCC1 protein. A pilot study was done to test the feasibility of antagonizing miR124 activity *in vivo* using a locked-nucleic-acid (LNA) modified oligonucleotide with complementary sequence homology to mature miR124 designed to bind and specifically inhibit miR124. Such LNA-modified oligonucleotides are extremely stable, and this “antagomir” was further modified with a cholesterol moiety on the 3’ end in order to facilitate uptake by neural cells (miRCURY LNA Inhibitor, Exiqon). Female rat pups (n=3) were treated with bilateral icv injections of 120, 250 or 500 pmol miR124 antagomir, or saline, on PN0. Whole hippocampi were removed 24 hours and 4 days after treatment (PN1 and PN4) and NKCC1 protein content determined via Western blot. Protein content of SOX9 was also determined, as this is a validated target of miR124 in neuroblasts of the adult subventricular zone (Cheng et al., 2009), and other proliferative cells (Farrell et al., 2011; Wang et al., 2016). Target protein content was normalized to GAPDH. This experiment was designed to titrate the dose and timecourse of action of the antagomir, and blocking miR124 action was expected to increase levels of SOX9 as a technical control. Results are shown in Figure 5.4.

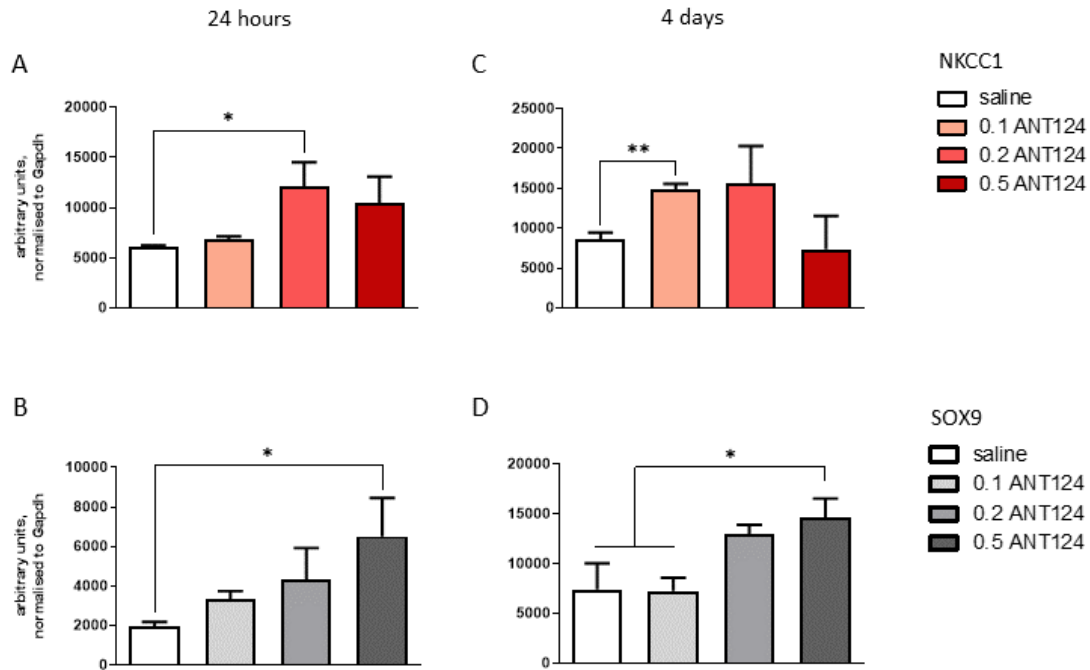


Figure 5.4: Antagonism of miR124 using LNA-modified oligonucleotide in female rat pups. A,B: Hippocampal content of NKCC1 (A) and SOX9 (B) measured 24 hrs. post treatment via Western blot. C,D: Hippocampal content of NKCC1 (C) and SOX9 (D) measured 4 days post treatment. N=3 females per group. *p<0.05; **p<0.001 Fisher's LSD

Because this experiment was underpowered, one-way ANOVA did not indicate significant differences among treatment groups for hippocampal NKCC1 content at 24 hours post injection [$F(3,8)=2.65$; $p=0.1198$], although multiple comparisons using Fisher's LSD indicated increased NKCC1 content in response to 250 pmol antagomir, compared to saline-injected animals (Figure 5.4, A). A similar response in NKCC1 was seen at 4 days post injection (Figure 5.4, B), where no effect was seen among group means [$F(3,8)=1.79$; $p=0.2256$], but treatment with 250 pmol antagomir raised NKCC1 protein content compared to controls ($p=0.0067$, Fisher's LSD). Hippocampal SOX9 content did not differ among treatments at 24 hours post treatment [$F(3,8)=2.25$; $p=0.1597$], although multiple comparisons using Fisher's LSD indicated increased SOX9

protein in animals treated with 500 pmol antagomir, compared to saline-treated controls ($p=0.0358$) (Figure 5.3, C). SOX9 content among treatment groups differed at 4 days post injection [$F(3,8)=4.342$; $p=0.0430$], largely driven by an increase when 500pmol of antagomir was administered, compared to animals treated with saline ($p=0.0229$) or 120 pmol antagomir ($p=0.0210$) (Figure 5.4, D).

Based on the mildly encouraging results of this pilot, a fully-powered experiment was done to antagonize miR124 activity in both males and females. Eight pups of each sex were treated via bilateral icv injections on PNO with 300 pmol of the same miR124 antagomir used in the pilot experiment, or a similarly-modified LNA-oligonucleotide, with no sequence homology to any known microRNA, as control (Exiqon, Qiagen). Four days post injection (PN4), the dentate gyrus and CA1 hippocampal subregions were individually removed and processed for determination of NKCC1 and SOX9 content via Western blot. Due to technical issues with the GAPDH antibody, target protein content was normalized to β -tubulin. As shown in Figure 5.5, there was no effect of antagomir administration on NKCC1 content in either the dentate gyrus [$F(1,19)=0.09147$; $p=0.7656$] or CA [$F(1,19)=4.342$; $p=0.0430$] ((Figure 5.5, A,C). Interestingly, both the dentate gyrus and CA1 exhibited strong, though opposite, sex differences in NKCC1 content. In the dentate, females had approximately twice as much NKCC1 protein as males [$F(1,19)=8.934$; $p=0.0075$], while males had greater NKCC1 content in CA1 [$F(1,19)=7.7731$; $p=0.0112$]. There was no sex difference in SOX9 content in either the dentate [$F(1,19)=0.6526$; $p=0.4287$] or CA [$F(1,20)=1.118$; $p=0.3024$] (Figure 5.5, B,D). Most significantly, however, neither region showed an effect on SOX9 expression with

miR124 antagonism [F(1,20)=0.03621; p=0.8510 for dentate gyrus], [F(1,20)=0.0156; p=0.9016 for CA1] (Figure 5.5, B,D).

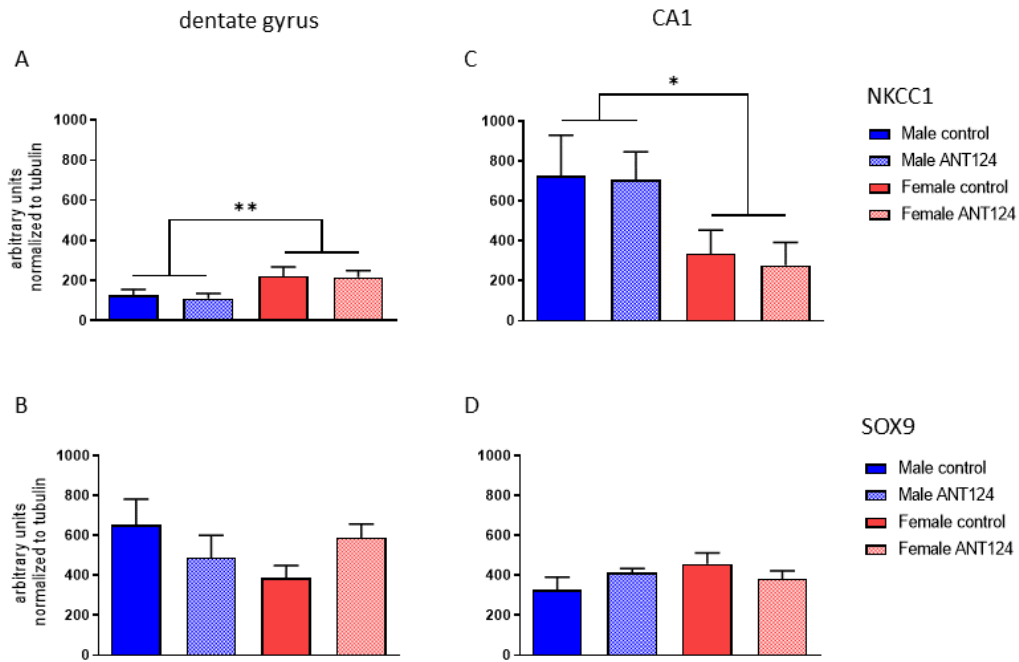


Figure 5.5: In vivo antagonism of miR124 activity using LNA-modified oligonucleotides in male and female rat pups. 300 pmol miR124 antagonist (ANT124) or control oligomer (control) was administered on P0, and hippocampal subregions removed on P4 for determination of NKCC1 (A,C) and SOX9 (B,D) content. A main effect of sex was seen for NKCC1 content in both the dentate (A) and CA1 (C), but not for SOX9 (B,D). N=8 pups each sex and treatment. * p=0.0112, ** p=0.0075 2-way ANOVA with Bonferroni post-hoc.

In vivo antagonism of miR124 in the hippocampus of neonatal males and females and effects on cell proliferation.

A separate cohort of animals was used to test the effect of miR124 antagonism on cell proliferation in the neonatal dentate gyrus. Male and female pups were treated on the day of birth with 300 pmol miR124 antagomir, or control oligonucleotide, via icv injection as described above. Pups were also injected ip with 100 mg/kg BrdU in saline 5 hours after icv injections. On PN4, pups were euthanized, perfused transcardially, and whole brains removed for subsequent immunohistological analysis of BrdU labeling in the dentate gyrus, as described in Chapter 2. Stereological quantification of BrdU-labeled cells was done for both the right and left hemisphere dentate in all animals, as shown in Figure 5.6. There was no effect of sex or treatment with the miR124 antagomir in either the left [$F(1,19)=1.299$; $p=0.2686$]; or right [$F(1,19)=1.567$; $p=0.2275$] dentate gyrus. Surprisingly, contrary to what has been found in previous studies, there were not greater numbers of proliferating cells in either the right or left dentate gyrus of males, compared to females, as no sex difference in BrdU labeling was seen among any of the groups [$F(1,19)=0.06483$; $p=0.8081$ left dentate], [$F(1,19)=0.01287$; $p=0.9110$ right dentate].

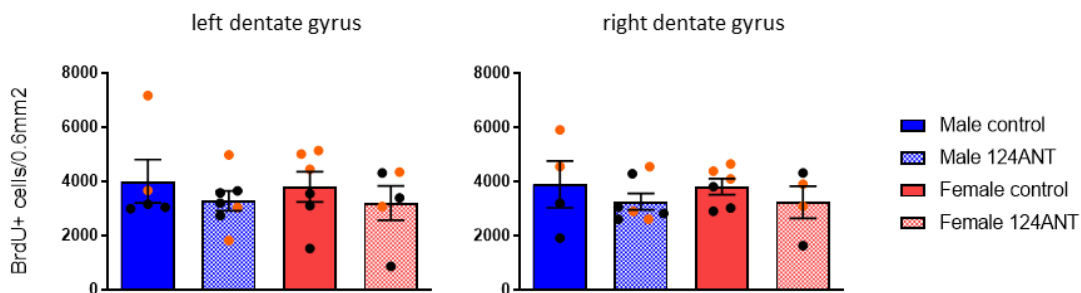


Figure 5.6: BrdU-labeled cells in the left and right dentate gyrus of PN4 male and female rats, as determined by stereological analysis. Pups were injected icv with miR124 antagomir (124ANT) or control oligomer on PN0. N= 5-7 animals per group; individual data points are colored according to litter. Two-factor ANOVA was used to determine effects of sex and treatment.

In vitro antagonism of miR124 in neonatal rat hippocampal neurons and effects on NKCC1 expression.

A complementary *in vitro* approach was used to test the function of miR124 using primary cultures of hippocampal neurons from neonatal rat pups. In spite of the inconclusive results from the *in vivo* functional experiments, this approach is justified for two reasons. First, a simpler and more direct system was needed in order to eliminate possible technical barriers that may have obscured a clear outcome with the *in vivo* treatments. And second, contrasting results from *in vivo* and *in vitro* experiments would yield insight into whether miR124 functions cell-autonomously within neuronal cells. Whole hippocampi were isolated from male and female rats on P0 and neuronal cells isolated from pooled tissues and plated as sex-specific cultures into 24-well plates, as detailed in Chapter 2. On the second day after plating (DIV2), after primary neuronal cultures were established, the adherent cells were transfected with a LNA-modified miR124 antagomir that was labeled with fluorescein (Exiqon), or a LNA-modified oligonucleotide of the same length and composition that lacked any homology to known miRNA sequences as control, using a lipid-based transfection reagent (RNAiMax, Invitrogen). Pilot experiments were first carried out to determine the best ratio of transfection reagent to oligomer, and transfection efficiency was estimated by counting fluorescein-expressing cells out of a total population of cells stained with DAPI. Transfection efficiencies were typically 20-50% (data not shown). Five days after transfection (DIV7), cells were rinsed with PBS, and collected by scraping. NKCC1 and SOX9 protein content were determined in the resulting cell homogenates from primary neurons transfected with control oligomer, 2 nM, 5 nM or 10 nM miR124 antagomir, or exposed to transfection reagent without any DNA (mock). It was predicted that

transfection of miR124 antagomir would increase NKCC1 content in homogenates of primary neurons if miR124 does indeed regulate expression of this protein in a cell-autonomous fashion. As before, SOX9 was expected to serve as a positive control.

There was no effect of sex on SOX9 protein content, so results for males and female-derived neurons were collapsed and an effect of treatment was shown using 2-way ANOVA [$F(4,30)=4.349$; $p=0.0068$] (Figure 5.7, A). Dunnett's post-hoc tests indicated that transfection with 2nM and 5nM miR124 antagomir resulted in approximately 33% ($p=0.0400$) and 44% ($p=0.0042$) increases in SOX9 protein, respectively, compared to neurons mock transfected with no DNA. Treatment with miR124 antagomir did not, however, have a significant effect on SOX9 protein content when compared to neurons transfected with the control antagomir ($p=0.9978$ control vs 2nM; $p=0.7398$ control vs 5nM; $p=0.8651$ control vs 10nM), nor was the control antagomir group significantly different in SOX9 content from mock transfected neurons (mean difference of 0.3037; $p=0.1146$).

In terms of NKCC1 protein content, there was no difference among any treatment or control groups for male-derived neurons [$F(4,15)=0.1702$; $p=0.9503$], however there was an effect of treatment in female-derived neurons [$F(4,15)=3.3903$; $p=0.0248$] (Figure 5.7, B). Both 2nM and 5nM miR124 antagomir resulted in significant increases in NKCC1 content compared to treatment with control antagomir (6.8%, $p=0.04113$ for 2nM; 18%, $p=0.0077$ for 5nM via Dunnett's post-hoc).

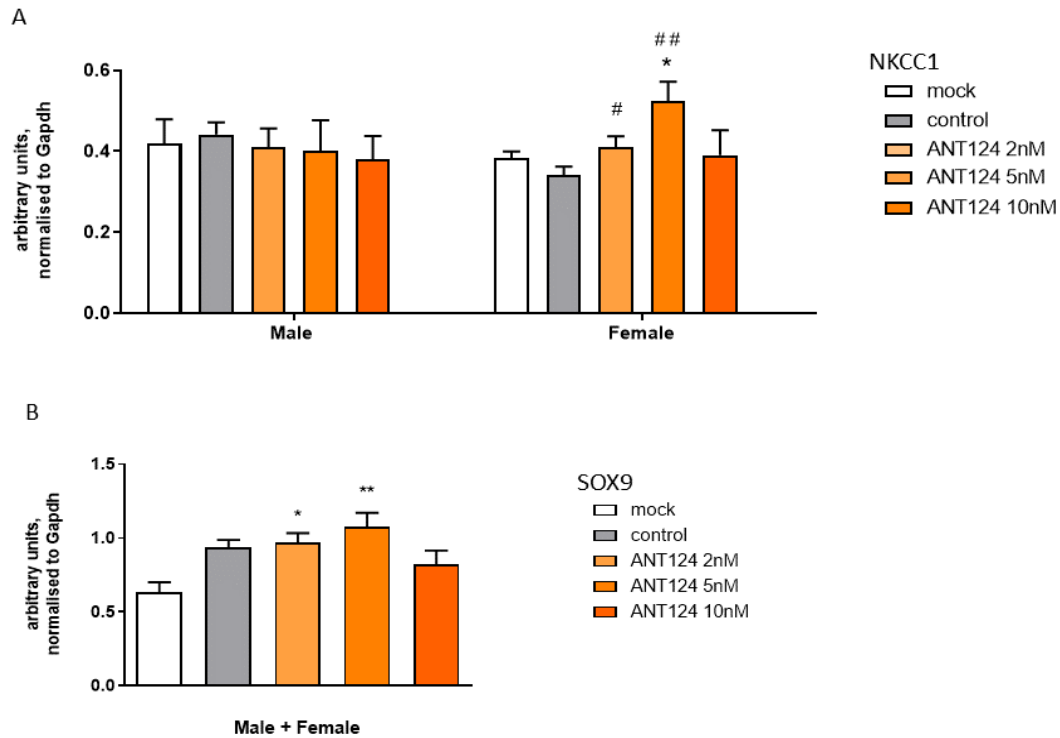


Figure 5.7: Effect of antagonizing miR124 activity on NKCC1 (A) and SOX9 (B) expression in primary hippocampal neurons derived from male and female neonatal rats, as determined by Western blot. A) Treatment with 2nM and 5nM miR124 antagonism increased NKCC1 content in female-derived neurons only. * $p=0.0121$ compared to mock transfected; # $p=0.04113$, ## $p=0.0077$ compared to control antagonism. B) * $p=0.0400$, ** $p=0.0042$ compared to mock transfected. No effect of sex was seen in SOX9 protein content.

The effects of miR124 antagonism using the LNA-modified oligonucleotide in cultured hippocampal neurons appeared to increase NKCC1 protein, as would be predicted if miR124 downregulates miR124 expression. Intriguingly, this was shown only in female neurons, although this only occurred at one specific dose. In addition, the predicted effect on SOX9 was seen, but the effect size was relatively small and only seen in comparison with mock transfected neurons, not in relation to transfection with control antagonism. An alternative in vitro approach, utilizing a vector-based expression construct

containing miR124 antagomir sequence, was employed to confirm an effect of miR124 antagonism in cultured neurons. The miRZIP lentivector (Systems BioSciences) is a plasmid DNA expressing a short hairpin RNA that is processed by the endogenous cellular machinery to produce antisense miRNA directed against the desired miRNA target. A reporter protein, in this case GFP, is also expressed from the vector under control of a CMV promoter. When packaged in lentiviral particles, expression of the antisense miRNA from this vector is robust, stable and can be achieved in a high percentage of cells due to viral transduction. In this experiment, however, naked vector DNA was transfected into hippocampal neuronal cultures using Nucleofector™ reagents (Lonza) and electroporation parameters optimized by the vendor for rat primary hippocampal neurons. Transfection efficiencies using Nucleofection greatly exceed those using lipid-based transfection reagents, and typically 60-80% transfection efficiencies can be achieved with primary cells. Primary hippocampal cultures were prepared from female rat pups on the day of birth, as described earlier. Prior to plating in 24-well plates, suspensions of isolated cells were transfected with empty miRZIP vector expressing only GFP, miRZIP vector containing GFP and control hairpin sequence having no homology to any known microRNAs, or 3 different doses of miRZIP vector expressing GFP and antisense hairpin targeting miR124. Cells were also plated that did not undergo any transfection manipulations. Cells were collected at 2 and 4 days in vitro (DIV) after plating, and SOX9 and NKCC1 protein content determined via Western blot as previously described.

At 2 DIV, one-way ANOVA showed differences among treatment groups [F(5,16)=3.260; p=0.0322], and Dunnett's post-hoc tests were performed to compare all groups to control antagomir vector treatment (Figure 5.8). Compared to neurons transfected with the antagomir control vector, untreated neurons had roughly 61% more NKCC1 protein content (p=0.0345). Transfection with 10ug miR124 antagomir construct increased NKCC1 content roughly 61%, compared to neurons treated with the control construct (p=0.0353). There was no difference in NKCC1 protein among neurons treated with the lower two doses of miR124 antagomir, the GFP-expressing control vector, or the control antagomir vector. At 4 DIV, any effect of transfection with antagomir or control DNA had disappeared, as there was no difference in NKCC1 protein content among any of the groups [F(5,16)=1.161; p=0.3700]. Taken together, this set of data seems to show a short-term, generalized effect of nucleofection on NKCC1 content, and an effect of antagonizing miR124 activity only at the 10 μ g dose.

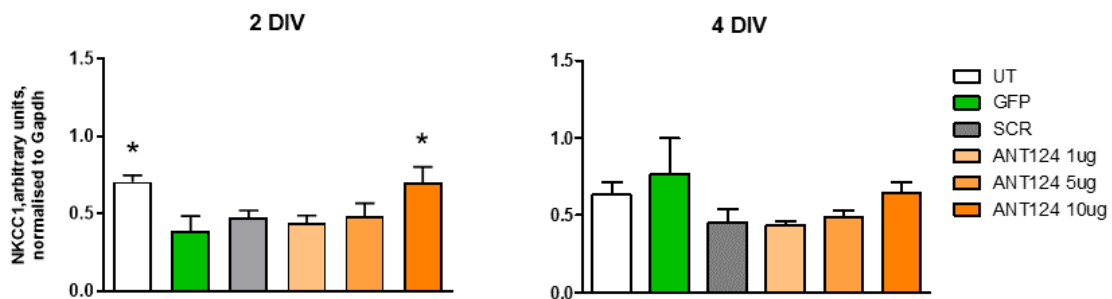


Figure 5.8: Effect of miR124 antagonism on NKCC1 expression in cultured hippocampal neurons from female neonatal rats, via nucleofection of vector-based DNA constructs. NKCC1 protein was quantified using Western blot and expressed as arbitrary units normalized to Gapdh content. Left panel: untransfected (UT) neurons or neurons transfected with miRZIP vector containing GFP sequence (GFP), a scrambled sequence as antagomir control (SCR), or 3 doses of miR124 antagomir sequence (ANT124) collected 2 days after nucleofection and plating (2DIV). Right panel, separate transfections of the same treatments, collected 4 days after nucleofection and plating (4DIV). N=4 wells each treatment/timepoint. *p<0.05 compared to the antagomir control (SCR).

Finally, in order to better understand the relationship between endogenous NKCC1 and miR124 expression in the context of the in vitro experimental setting, NKCC1 and miR124 content were measured in sex-specific cultures of primary hippocampal neurons, established from pooled hippocampi of several P0 rat pups as described previously. Primary hippocampal neurons were collected from 6 wells each of 24-well plates at 2, 4, 6 and 8 DIV, and NKCC1 and miR124 content were quantified using Western blot and TaqMan-based qPCR assays.

Over the eight days in culture, there was no difference in NKCC1 protein between male and female primary neurons in culture [$F(1,35)=0.1304$; $p=0.7202$] (Figure 5.9, A). As expected with maturing of the cell population, NKCC1 decreased over time in culture [$F(3,35)=6.699$; $p=0.0011$], dropping approximately 79% in males ($p=0.0180$) and 81% in females ($p=0.0463$) from 2DIV to 8DIV. Two-way ANOVA found an effect of time in culture for miR124 expression as well [$F(3,40)=6.0012$; $p=0.0018$], although the amount of miR124 dropped by half after 2 DIV and remained steady thereafter (Figure 5.9, B). Although there was no difference between miR124 content at 4, 6 and 8 DIV, there was also no statistical difference between miR124 expression at 2DIV and 6 DIV ($p=0.2274$) and 8 DIV ($p=0.1557$), indicating that this experiment was underpowered. Two-way ANOVA also found a main effect of sex on miR124 expression [$F(1,40)=6.005$; $p=0.0187$], although post-hoc tests comparing mean values for males and females at each timepoint could not find significant differences, probably due to insufficient powering of this experiment.

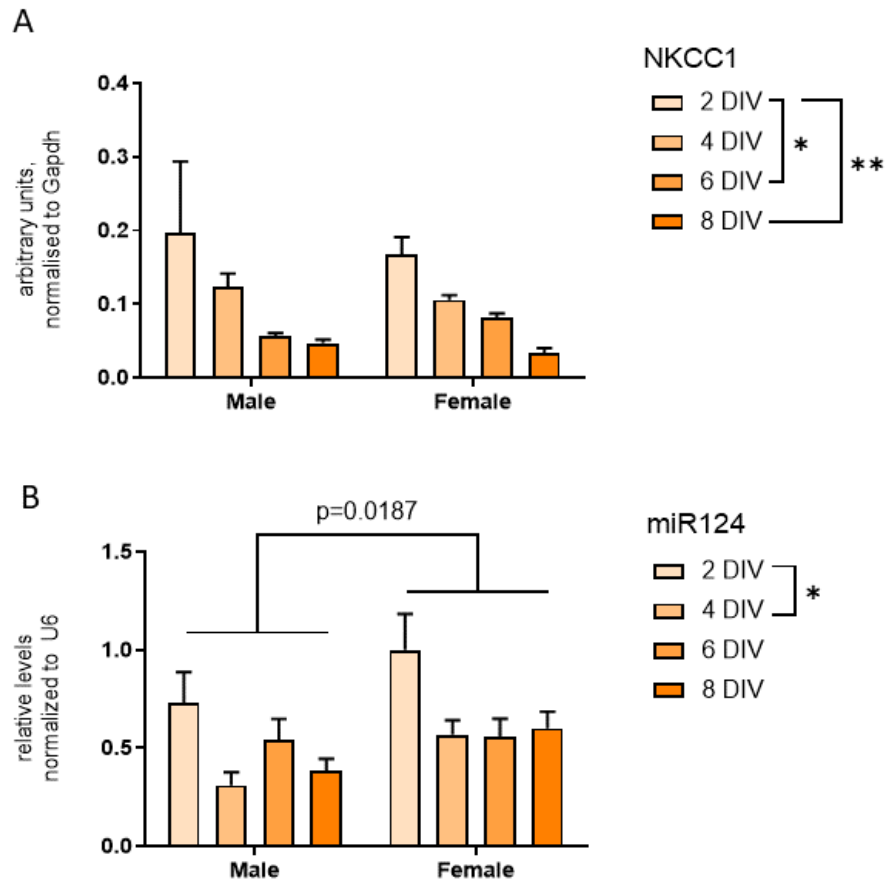


Figure 5.9: Expression of NKCC1 and miR124 in primary cultures of hippocampal neurons over 8 days in vitro (DIV). Cultures were derived from pooled whole hippocampi of 5-7 male or female pups on P0. NKCC1 protein levels determined via Western blot and expressed as arbitrary units normalized to Gapdh. miR124 levels normalized to U6 and expressed relative to female cultures at 2DIV. N=6 wells per sex at each timepoint. * $p < 0.05$, ** $p < 0.01$ via 2-way ANOVA with sex and time in culture as main factors.

Discussion

The experiments described in this chapter were designed to test a hypothesized role for miR124 in regulating NKCC1 expression in the neonatal hippocampus of male and female rats. Such a role would be consistent with miR124's function as a master regulator of neurogenesis and neuroblast proliferation, as downregulation of NKCC1 expression is essential for transitioning immature neuroblasts and proliferating precursors to a mature neuron with a hyperpolarizing response to GABA. This would also be a novel addition to the validated functional targets of miR124. It is fair to say that the data presented do not support a role for miR124 in regulating NKCC1 expression, and the hypothesis is rejected. Importantly, however, much of the data here is inconclusive, and therefore it should be emphasized that the hypothesis was also not properly tested. Much of the foundation for the hypothesis is descriptive data, and at first blush is consistent with a functional role for miR124 in regulating NKCC1 expression. A highly-conserved miR124 target sequence is found in the 3'UTR of *Slc12a2*, the transcript encoding NKCC1. The male-biased sex difference in hippocampal NKCC1 content is inverse to a sex difference in miR124, which is higher in females. A timecourse quantification of NKCC1 protein, *Slc12a2* transcript, and miR124 content in the dentate gyrus over the first two postnatal weeks confirmed that the sex difference in NKCC1 is restricted to the first week, when there is a sex difference in the depolarizing response to GABA. The sex difference in NKCC1 was not mirrored by *Slc12a2*, which was equivalent in males and females throughout the first two weeks, and this would be consistent with a microRNA-mediated post-transcriptional regulation of NKCC1 in females. However, although miR124 levels are higher in the female dentate at PN4, they are equivalent to males at PN2, and this is inconsistent with a role for miR124 in promoting the sex difference in

NKCC1 at this timepoint. The rise in miR124 in females at PN4, compared to males, may indicate that other processes involving this microRNA are switched on at this time, the downstream effect of which may promote cellular maturation and the decrease in NKCC1 content. On the other hand, changes in the fraction of cycling progenitors to mature neurons may alter the expression of Gapdh per cell, and as this was used to normalize NKCC1 and Slc12a2 quantification, may not give a true picture of actual cellular output of these endpoints over time. Mean differences in in Gapdh protein or transcripts did not appear to differ significantly between males and females, or per unit of total mRNA across time.

Two types of functional experiments were carried out, both of which yielded inconclusive results. An *in vivo* pilot experiment antagonizing miR124 activity in female rat pups suggested that miR124 does dampen NKCC1 expression, although this data set was small. When a sufficiently-powered experiment was done, in both sexes and also measuring the effect in both the dentate gyrus and CA1, no effects were seen with miR124 antagonism on NKCC1 content. In addition, however, the predicted increase in SOX9 protein with miR124 antagonism was also not seen, pointing to two possibilities. The first is technical issues, and the most likely source for this would be the LNA-modified anti-miR124 oligomer, although these reagents are known to be extremely stable and effective in knocking down microRNA activity (Kruzfeldt et al., 2005; Jimenez-Mateos et al., 2012). In general, LNA-modified oligonucleotides are quite effective in reaching their biochemical targets in the brain when administered icv (McCarthy et al., 1994a,b; Nugent et al., 2012). Nevertheless, this experiment was repeated using fresh miR124 antagomir and the same results were obtained. The second

possibility is that SOX9 is not a miR124 target in the neonatal hippocampus. This seems unlikely, given that miR124 directly targets SOX9 in proliferating cells outside the brain (Real et al., 2013; Wang, X. et al., 2016), but could be tested by choosing another validated miR124 target as a positive control.

A second set of functional experiments were performed *in vitro*, using isolated hippocampal neurons in primary culture. Moving to an *in vitro* system to test the effect of antagonizing miR124 activity serves two purposes. Experimentally, it provides a simpler system which eliminates potential technical barriers inherent in an *in vivo* setting, such as delivery of the antagomir to relevant cells in the brain. The simplicity of the primary culture also enables a more precise interpretation of functional activity when compared with the *in vivo* setting, enabling one to determine whether effects are cell-autonomous, or likely due to effects on afferent input or other tissue context. Here again however, while results from a pilot experiment seemed promising, fully-powered experiments were largely inconclusive. Small effects in the expected direction were seen in primary neurons transfected with the anti-miR124 oligonucleotide (Figure 5.7), and interestingly, the effect on NKCC1 expression seemed only to occur in primary cultures derived from females. Lipid-based transfection methods are notoriously inefficient in neuronal cultures, and in an effort to achieve more robust expression of miR124 antagomir, an *in vitro* knockdown approach using nucleofection of a vector-based miR124 “sponge” was utilized (Figure 5.8). A small increase in NKCC1 was seen at the highest dose of miR124 antagonism in female neurons, but lower doses of anti-miR124 vector and even control vector significantly decreased NKCC1 content compared to untreated cultures, indicating

a confounding effect of the nucleofection process, perhaps on the overall physiology of the cells.

In the *in vitro* setting, isolation and plating of the hippocampal neurons may itself alter endogenous miR124 content, which dropped significantly in neurons from both sexes from 2-4 DIV in untreated cultures and did not recover (Figure 5.9). Endogenous NKCC1 content decreased steadily over time in culture, which would be expected as plated cells mature and differentiate. The fact that an inverse correlation in changes in NKCC1 and miR124 content is not seen over time in culture is the strongest indication in all of these data that direct interaction between Slc12a2 and miR124 is not promoting decreased NKCC1 expression. As with the timecourse determination of miR124 and NKCC1 expression *in vivo*, this may be illustrative of decreased NKCC1 expression as an indirect, downstream effect of miR124 action, which peaks just prior to a drop in NKCC1 protein content. It is also possible that the pattern of miR124 and NKCC1 content in primary hippocampal neurons over several days *in vitro* reflects a switch in proliferative or maturational status of the neurons which occurs very soon after plating. After 2 days the cultured neurons may have sufficient miR124 to maintain the neuronal phenotype after initiating a switch from immature, proliferating precursors, while NKCC1 levels continue to fall. One way to test this would be to isolate neural stem cells or neuronal precursors from the dentate gyrus and maintain them in a proliferative, undifferentiated state using growth media supplemented with EGF and FGF-2. Manipulation of miR124 activity in proliferating precursors, and subsequent assay for NKCC1 expression, along with the natural or induced maturation of these cells *in vitro*, would remove confounds of

timing of miR124 activity in relation to the progressive maturational state of cultured cells.

One experiment was performed in this study that was intended to test the corollary hypothesis that miR124 promotes the sex difference in cell proliferation in the neonatal hippocampus, prompted by the changes in miR124 that are elicited by altered estradiol signaling, which mirror the effects on proliferation. Central administration of miR124 antagonist to neonatal rats had no effect on cell proliferation in the dentate, which may be real, but which is more likely due to technical issues, as reflected in the inconclusive results when SOX9 and NKCC1 were tested as biochemical readouts of miR124 antagonism in a similar experiment. More striking, however, is the lack of a sex difference in proliferation seen in control animals. More striking, however, is the lack of a sex difference in hippocampal cell proliferation in control animals, which is an otherwise well-established phenomenon in this laboratory. This suggests a latent factor altering the balance of proliferation in either males or females that may have obscured the effect of miR124 activity. Fortunately, potential effects of miR124 on proliferation and NKCC1 may be interrelated but are not necessarily interdependent, and functional regulation of NKCC1 expression by miR124, if any, would likely hold true in the absence of sex differences. Thus while concerning, a failure to recapitulate the original sex difference in proliferation should not effect the outcome of the biochemical or *in vitro* experiments. Or, maybe it does.

It should be noted that the functional experiments described in this chapter, which all antagonized miR124 activity, would only have tested the necessity of miR124 activity for whatever role it has in regulating NKCC1 expression, or cell proliferation. In order to

test whether NKCC1 is in fact sufficient to alter these endpoints, similar experiments would need to be conducted in which miR124 activity is increased, particularly in males, using synthetic microRNA mimics. Given the frustratingly inconclusive outcome of the miR124 antagonism experiments, follow-up experiments augmenting miR124 activity were not pursued, although perhaps these would have had a more interpretable outcome. Nearly all the data reported in this study do not support the hypothesis, or are inconclusive due to a lack of response from positive controls. In some experiments, technical difficulties surely had an impact on data interpretation. The real value of this study lies not in the data, but in the template they provide for a structured, systematic testing of miRNA function in the developing brain. This is briefly discussed in the concluding chapter that follows.

Chapter 6: Conclusion

Summary of Results

The work described in this thesis began as an attempt to identify factors underlying the sex difference in proliferation in the neonatal hippocampus of rats. This sex difference is male-biased, reflected as a two-fold greater number of proliferating cells in the dentate gyrus of males, and is restricted to the first week of life. Intriguingly, the sex bias and timeline of hippocampal cell proliferation corresponds to another fundamental sex difference in this region of the brain, the depolarizing response to GABA_A receptor activation. Both cellular proliferation and the depolarizing response to GABA in the neonatal hippocampus are modulated by estradiol, and in the case of cellular proliferation, perturbations of estradiol signaling reveal that this particular sex difference is likely a canalized process- the response to estradiol signaling is different in males and females in order to maintain the number of proliferating cells within a tightly controlled range. Because depolarizing GABA is known to regulate cell proliferation and maturation in both developmental and adult neurogenic contexts, it seems reasonable to hypothesize that the sex difference in cell genesis in the neonatal hippocampus is driven by the greater depolarizing response to GABA and the delayed transition to a hyperpolarizing GABA response seen in males, compared to females. Efforts to directly probe the role of depolarizing GABA in this context, by pharmacologically blocking the activity of NKCC1 and reversing the depolarizing response to GABA, have been undertaken by this author and other members of the McCarthy lab, but have proven to be technically challenging. So the experiments described in this thesis tested the involvement of two other factors which regulate proliferation and neurogenesis, the

neurotrophin BDNF and the microRNA miR124. Although not directly indicative of functional involvement, the response of each of these factors to estradiol signaling in the neonatal hippocampus was used as an initial test to determine whether they were reasonable candidates for mediating the sex difference in cellular proliferation.

Bdnf in relation to cellular proliferation

As discussed in Chapter 3, several things make BDNF an attractive candidate for mediating the sex difference in cell genesis in the developing hippocampus: 1) BDNF has a demonstrated role in regulating proliferation and neuronal maturation, during development and also in the neurogenic niche of adult hippocampus, 2) depolarizing GABA promotes expression and secretion of BDNF, which in turn functions in a positive feedback loop to promote GABA_A-dependent signaling, and 3) BDNF synthesis and secretion is promoted by estradiol. In an attempt to test whether it contributes to the baseline sex difference in proliferation, BDNF expression was quantified at both the transcript and peptide level, in the three main subregions of the neonatal hippocampus. Interestingly, total *Bdnf* transcript levels mirrored the sex difference in proliferation in terms of both sex bias and regional distribution- *Bdnf* transcripts were roughly 1.5 times more abundant in males compared to females in both the dentate gyrus and CA1 subregions, but were equivalent in CA3. In response to estradiol signaling, however, *Bdnf* expression did not exhibit a sex difference in CA1 and diverged from the proliferative response in a region-specific manner. Estradiol signaling had a positive effect on *Bdnf* transcript content of CA1 in both males and females, whereas exogenous estradiol promoted proliferation only in females. In the dentate, total *Bdnf* transcripts were downregulated by estradiol in both males and females, opposite to the proliferative

response, which is seen only in females. An interesting sex-specific response to tamoxifen was seen in the dentate, where *Bdnf* transcript content decreased in response to ER antagonism only in males, reminiscent of the divergent proliferative response to estradiol. While the upregulation of *Bdnf* expression in response to estradiol in CA1 has been reported in adult animals, the differential response between CA1 and the dentate gyrus in neonatal animals is novel, and intriguing, suggesting perhaps different signaling mechanisms for estradiol in these two hippocampal subregions. However, what makes this mechanistic question not worthy of pursuit in the context of cellular proliferation is the demonstration of equivalent amounts of BDNF propeptide, between males and females and regardless of whether estradiol signaling is augmented or antagonized. This was found in CA1, CA3 and the dentate, although BDNF content did vary among the three regions. Estradiol-induced effects on neuronal physiology may differ mechanistically among the developing hippocampal subregions, but if BDNF is involved in these pathways a clear hypothesis for this role is not apparent. Because there is no baseline difference in BDNF prohormone between the sexes, or in response to estradiol, BDNF was rejected as a proximal mechanism for promoting the sex difference in cell genesis or the proliferative response to estradiol.

miR124 (and other microRNAs) in relation to cellular proliferation

The fact that the proliferative response to altered estradiol signaling is sexually differentiated in a way that maintains cell genesis with a tightly defined range suggests that canalizing agents may be regulating cell proliferation in the neonatal hippocampus, and prompted an examination of microRNA function in this context. Two approaches were used to identify miRNA candidates associated with the sex difference in

hippocampal cell proliferation: a TaqMan[®]-based qPCR array, and quantification of individual miRNAs using NanoStrings arrays. As discussed in Chapter 4, each of these methods are routinely used in the field, and because they employ different biochemical methods for quantification they are complementary. Although a perfect concordance between the two methods in terms of differentially expressed miRNAs was not expected, it was somewhat surprising that of the 14 miRNAs quantified using qPCR, 10 of which exhibited a significant content difference between males and females, only 1 of these was also found to be differentially expressed using the NanoStrings panel, and in this case an opposite sex difference was found. These two data sets give distinctly different pictures of miRNA expression patterns in relation to the sex difference in proliferation found in the dentate gyrus. The qPCR dataset indicated elevated expression of proliferation-related miRNAs generally in the female dentate gyrus, and was the impetus for further functional testing of one specific miRNA. This may indicate a functional upregulation of these miRNAs in females, or it may simply be a reflection of the number of proliferating cells in each sex. Regarding this point, however, it should be noted that the amount of each miRNA in the qPCR array was normalized to a highly abundant nucleolar RNA standing as a proxy for cell number. Using the NanoStrings panels, no sex differences were seen for almost all of the known neurogenesis-related miRNAs, and a generally female-biased expression of extremely low-abundance miRNAs was found. These very different pictures of miRNA content may be due to the different normalization strategies employed between the two assays, or it may be an artifact of using different sample collections for each of these data sets. This is an outstanding question which might be resolved experimentally using targeted qPCR assays to quantify those miRNAs identified as

differentially expressed in the NanoStrings data set. Comparison with NanoStrings data from the dentate gyrus with additional data obtained from the CA1 and CA3 subregions of males and females would also be valuable in this regard. Perhaps the most compelling indication that the sex differences in miRNA expression seen with the qPCR data is truly related to proliferation is that these differences were seen in the dentate gyrus, but not in the CA1 or CA3 subregions, where the population of proliferating cells is roughly ten-fold smaller.

Based on the well-established role of miR124 in promoting cell-cycle exit and downregulating proliferation, particularly during neurogenesis, and its elevated levels in the female dentate, miR124 was chosen as a focus for functional testing in relation to hippocampal cell genesis. This was initially supported by the changes in miR124 content in the dentate when estradiol signaling was manipulated, and the timecourse of the miR124 sex difference, which only occurred during the first postnatal week. However, direct testing of the involvement of miR124 in regulating proliferation by antagonizing miR124 activity *in vivo* had no effect on proliferation in either males or females. Unfortunately this result was confounded by the lack of a baseline sex difference in proliferation in control animals, and the lack of an effect of miR124 antagonism on the expression of a validated miR124 target (SOX9), as seen in separate experiments.

Does miR124 regulate NKCC1 expression?

This author's inability to replicate the sex difference in proliferation that has been well-established in the neonatal hippocampus by others was unfortunately not discovered until hypothesis testing for miR124 function was well underway. However, although the hypothesized roles for miR124 in regulating proliferation and NKCC1 expression are

interrelated (given the overlapping sex differences in proliferation and depolarizing GABA), they are not interdependent. In the context of the same cell type and developmental timing, it's reasonable to assume that the biochemical functions of miR124 should remain the same regardless of differences in neurophysiological outcomes. The lack of a sex difference in cell genesis may occlude any relative effects on proliferation, but a more reductionist hypothesis is still quite testable.

This study found a male-biased sex difference in NKCC1 protein content in the dentate gyrus during the first postnatal week that was not matched by transcript levels, which were equivalent in males and females. This is consistent with post-transcriptional regulation of NKCC1 expression, as might occur with miRNA targeting, and miR124 was found to be greater in the dentate of females during this time. This, along with a putative miR124 target site in the NKCC1 transcript, prompted functional experiments which antagonized miR124 activity in neonatal animals and also in cultured hippocampal neurons. Pilot experiments for both *in vivo* and *in vitro* experiments suggested that inhibition of miR124 activity increases NKCC1 expression, but these results did not hold up in larger experiments. Because of the lack of predicted effect on the SOX9 control, both the *in vivo* and *in vitro* functional experiments were inconclusive, and it is apparent that technical hurdles have stymied the proper testing of the hypothesized role of miR124 in regulating NKCC1 expression or proliferation. Although a role for miR124 in regulating NKCC1 expression cannot be confidently rejected, based on recent literature there is likely not a direct interaction between miR124 and NKCC1 transcripts. Because miR124 is an important regulator of cell proliferation and onco- and neurogenesis, it's targets of direct interaction have been determined in several studies, either in the interest

of discovery or as a means of validating new methodologies for determining miRNA-mRNA interactions. A few reports have comprehensively examined the direct targets of miR124, and transcripts encoding NKCC1 have not been reported in these studies (Chi et al., 2009; Cambronne et al., 2102).

A Template for Testing microRNA Function in the Developing Brain.

Although the experiments described in this thesis did not fully test the hypothesized role of miR124 in regulating NKCC1 expression, they nevertheless outline a set of experiments that can be used to test the function of any microRNA in the developing brain. Once a miRNA of interest has been identified, either through a targeted approach focusing on a single miRNA, or via discovery-based approaches such as bulk sequencing, microarray or NanoString analyses, functional testing would follow a set of experiments outlined as follows:

1) *Determination of expression patterns of the miRNA of interest.* In an ideal scenario, conditions are identified where the miRNA of interest varies in abundance, for example in a pathological state relative to normal physiology, at different developmental stages, or between the sexes. If a putative target transcript is identified, both the mRNA and the target protein should be quantified. Localization of the miRNA of interest to specific cell types can be done using *in situ* hybridization. While the information gained from this is largely descriptive, an outcome wherein content of the miRNA and putative target protein change in opposition to each other is supportive of a role for the miRNA in regulating the protein of interest.

2) *Determination of the effect of altered miRNA function in vivo.* A variety of reagents are available for either reducing or augmenting miRNA function, and these two

approaches will yield distinct information when applied *in vivo*. Functional inhibition will show whether a particular miRNA is necessary in the regulatory pathway of a particular protein of interest or physiological endpoint, and can be achieved using modified oligonucleotides that are complementary to the target miRNA, or vector-based constructs that contain several of the miRNA target sites and will generate a population of miRNA “sponge” transcripts. Sufficiency of a particular miRNA for producing a regulatory effect on target expression or tissue physiology is shown by augmenting activity, and here again either highly stable miRNA hybrid oligonucleotides or vector-based systems which express precursor miRNA hairpin sequences can be used. Such expression constructs are can be packaged and delivered to the brain in lentiviral or adeno-associated viral vectors, which confer robust and stable expression, typically within 24 hours of injection. Careful titering of the viral dose can result in expression of the miRNA-targeting reagent that is sparse and mosaic or more ubiquitous, and judicious choice of viral serotypes may also confer a degree of cell-type specificity in the brain (Kim et al., 2013; 2016).

3) *Determination of the effect of altered miRNA function in vitro*. If the cell or tissue type of interest can be cultured, blocking or augmenting function *in vitro* enables a more direct approach to determining the cell-autonomous functions of a miRNA on aspects of neuronal development such as proliferation, migration, neurite outgrowth and synaptogenesis.

4) *Determination of direct interaction of the miRNA of interest with putative target transcripts*. Changes in abundance of the protein of interest after manipulating miRNA activity are suggestive but not firm evidence that the miRNA directly targets the protein-coding transcript. Biochemical evidence that the miRNA directly targets a

particular transcript can be obtained using immunoprecipitation assays of cultured cells or tissues isolated *ex vivo*. Sequencing or microarray analysis of pools of RISC-associated transcripts isolated using antibodies to RISC protein components are compared under conditions where the miRNA of interest is present and has been depleted (Karginov et al., 2007; Cambronne et al., 2012). While this is a powerful technique that can be used to identify an entire population of direct downstream targets of a particular miRNA, confirmation of direct interaction between the miRNA and a target transcript is typically obtained using transcription reporter systems *in vitro* (Vasudevan, 2012; Turk et al., 2018). Most commonly, this is based on an expression vector encoding a hybrid cDNA coding for a biochemical reporter such as Luciferase fused to a portion of the putative target transcript containing the miRNA target sequence. Direct interaction of the miRNA of interest can be demonstrated *in vitro* in mammalian cells cotransfected with the target reporter vector and an expression vector encoding the miRNA of interest, where presence of the miRNA sequence will decrease Luciferase expression. Careful controls are needed with these experiments to discern off-target effects, foremost of these is the use of a hybrid reporter-target transcript construct in which the miRNA target sequence has been mutated.

Future Directions and Concluding Thoughts.

Although the experiments outlined here and attempted in this set of studies can be technically challenging, the literature indicates that they are certainly possible, and may be useful for pursuing alternative hypotheses centered around the sex difference in proliferation or other cellular endpoints in the developing hippocampus. The experiments described in this thesis did not provide compelling evidence that BDNF is a primary

driver of the baseline sex difference in proliferation, nor does the data support a direct role for BDNF in mediating the unique sex-specific proliferative response to estradiol signaling in the neonatal hippocampus. These data also do not provide evidence that miR124 regulates the sex difference in depolarizing GABA through direct regulation of NKCC1 expression. However, there are some details in the data that may be worth pursuing. The robust sex difference in miR9, which was found to be more abundant in females using both methods of quantitation, may be a good candidate for follow-up according to the generic scheme outlined above. miR9 is highly abundant and has critical roles in promoting maturational processes in post-mitotic neurons, and a sex difference in this microRNA may be indicative of a more advanced timeline of cell-cycle exit and neuronal maturation in females, rather than an overall smaller pool of neuronal precursors. Also intriguing is the lack of mature, fully-spliced BDNF in the hippocampus during the first postnatal week, as seen in these studies. As noted earlier, expression of the endoproteolytic machinery that produces the mature peptide is developmentally regulated, and the mature and prohormone forms of BDNF can have opposing effects on neurophysiology. A timecourse determination of BDNF proteolytic cleavage during development, particularly in the context of the sex difference and shift in depolarizing GABA, might yield an interesting new avenue for exploration. And while the sex difference in neurogenesis in the neonatal hippocampus has been well-characterized in terms of developmental timing and the acute and long-term proliferative response to estradiol and other physiological manipulations (see thesis work of Sara Stockman), very little is known regarding the downstream consequences of this sex difference on the neurophysiology of the hippocampus or the animal as a whole. An important place to

start with this would be to characterize the relative abundances of mature, differentiated cell types that result from the perinatal pool of proliferating precursors. Although initially descriptive, any sex differences found here would open up a significant new line of inquiry where the functional relevance of this phenomenon can begin to be addressed.

The true value of these studies lies in the process and not in the data, and several important points are aptly illustrated when these experiments are considered as a whole: First, it is important to replicate foundational data. Hypothesis-driven experimentation is necessarily built on previous findings, which may or may not be reproducible, and which likely are important for framing predictions and results. Second, reserve judgement on pilot data. Pilot data is important and exciting, but as was illustrated throughout much of this thesis, it can break your heart. And finally, while a testable hypothesis is ultimately the goal, an overly broad or rigid hypothesis can be useless in terms of advancing the understanding of a complex system. Is it reasonable and testable to hypothesize that more BDNF in males will translate into more proliferation in the hippocampus? Certainly, yes. But such an oversimplified model leaves little room for nuance and is easily rejected when broad predictions fail. Contrasting the data that fits with the data that doesn't gives us the ability to refine and restructure a hypothesis, and can mean the difference between judicious application of Occam's razor to reject a hypothesis, and the wholesale dismissal of a line of inquiry because the complexities of a system can mask effects¹. This is likely to be especially true for the developing brain.

¹ The term "Occam's Lobotomy" was coined for this idea by I. J. Good, and is discussed in "The Two-Edged Sword of Skepticism: Occam's Razor and Occam's Lobotomy" H.H Bauer, 2006. *Journal of Scientific Exploration* 20: 421-27. The idea is also captured in a quote attributed to Einstein in the January 8, 1950 edition of the New York Times: "Everything should be made as simple as possible, but no simpler."

References

- Åkerblom, M., R. Sachdeva, I. Barde, S. Verp, B. Gentner, D. Trono and J. Jakobsson (2012). "MicroRNA-124 is a subventricular zone neuronal fate determinant." J Neurosci **32**(26): 8879-8889.
- Abramovich, D. and P. Rowe (1973). "Foetal plasma testosterone levels at mid-pregnancy and at term: relationship to foetal sex." Journal of Endocrinology **56**: 621-622.
- Abu-Elneel, K., T. Liu, F. S. Gazzaniga, Y. Nishimura, D. P. Wall, D. H. Geschwind, K. Lao and K. S. Kosik (2008). "Heterogeneous dysregulation of microRNAs across the autism spectrum." Neurogenetics **9**(3): 153-161.
- Aid, T., A. Kazantseva, M. Piirsoo, K. Palm and T. Timmusk (2007). "Mouse and rat BDNF gene structure and expression revisited." J Neurosci Res **85**(3): 525-535.
- Altman, J. and S. A. Bayer (1990(a)). "Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods." J Comp Neurol **301**(3): 365-381.
- Altman, J. and S. A. Bayer (1990(b)). "Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale." J Comp Neurol **301**(3): 343-364.
- Altman, J. and S. A. Bayer (1990(c)). "Mosaic organization of the hippocampal neuroepithelium and the multiple germinal sources of dentate granule cells." J Comp Neurol **301**(3): 325-342.
- Amateau, S. K., J. J. Alt, C. L. Stamps and M. M. McCarthy (2004). "Brain estradiol content in newborn rats: sex differences, regional heterogeneity, and possible de novo synthesis by the female telencephalon." Endocrinology **145**(6): 2906-2917.
- Auger, A., D. Hexter and M. McCarthy (2001). "Sex difference in the phosphorylation of cAMP response element binding protein (CREB) in neonatal rat brain." Brain Research **890**: 110-117.
- Baek, D., J. Villén, C. Shin, F. D. Camargo, S. P. Gygi and D. P. Bartel (2008). "The impact of microRNAs on protein output." Nature **455**(7209): 64-71.
- Bai, M., X. Zhu, Y. Zhang, S. Zhang, L. Zhang, L. Xue, J. Yi, S. Yao and X. Zhang (2012). "Abnormal hippocampal BDNF and miR-16 expression is associated with depression-like behaviors induced by stress during early life." PLoS One **7**(10): e46921.

- Bartel, D. P. (2009). "MicroRNAs: target recognition and regulatory functions." Cell **136**(2): 215-233.
- Baskerville, S. and D. P. Bartel (2005). "Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes." RNA (New York, N.Y.) **11**(3): 241-247.
- Bauer, H. H. (2006). "The two-edged sword of skepticism: Occam's razor and Occam's lobotomy." Journal of Scientific Exploration **20**: 421-427.
- Behar, T., Y. Li, H. Tran, W. Ma, V. Dunlap, C. Scott and J. Barker (1996). "GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms." Journal of Neuroscience **16**: 1808-1818.
- Ben-Ari, Y. (2002). "Excitatory actions of gaba during development: the nature of the nurture." Nat Rev Neurosci **3**.
- Ben-Ari, Y. (2014). "The GABA excitatory/inhibitory developmental sequence: a personal journey." Neuroscience **279**: 187-219.
- Ben-Ari, Y., J. Gaiarsa, R. Tyzio and R. Khazipov (2007). "GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations." Physiological Reviews **87**: 1215-1284.
- Berenguer, J., A. Herrera, L. Vuolo, B. Torroba, F. Llorens, L. Sumoy and S. Pons (2013). "MicroRNA 22 regulates cell cycle length in cerebellar granular neuron precursors." Mol Cell Biol **33**(14): 2706-2717.
- Berezikov, E., F. Thummler, L. W. van Laake, I. Kondova, R. Bontrop, E. Cuppen and R. H. Plasterk (2006). "Diversity of microRNAs in human and chimpanzee brain." Nat Genet **38**(12): 1375-1377.
- Berninger, B., S. Marty, F. Zafra, M. da Penha Berzaghi, H. Thoenen and D. Lindholm (1995). "GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro." Development **121**(8): 2327-2335.
- Beveridge, N. J. and M. J. Cairns (2012). "MicroRNA dysregulation in schizophrenia." Neurobiol Dis **46**(2): 263-271.
- Biala, Y. a. N., Y. Bogoch, C. Bejar, M. Linial and M. Weinstock (2011). "Prenatal stress diminishes gender differences in behavior and in expression of hippocampal synaptic genes and proteins in rats." Hippocampus **21**(10): 1114-1125.

- Bian, C., K. Zhu, Q. Guo, Y. Xiong, W. Cai and J. Zhang (2012). "Sex differences and synchronous development of steroid receptor coactivator-1 and synaptic proteins in the hippocampus of postnatal female and male C57BL/6 mice." Steroids **77**(1): 149-156.
- Bienkowski, M. S., I. Bowman, M. Y. Song, L. Gou, T. Ard, K. Cotter, M. Zhu, N. L. Benavidez, S. Yamashita, J. Abu-Jaber, S. Azam, D. Lo, N. N. Foster, H. Hintiryan and H.-W. Dong (2018). "Integration of gene expression and brain-wide connectivity reveals the multiscale organization of mouse hippocampal networks." Nature Neuroscience **21**(11): 1628-1643.
- Bock, J., M. S. Murmu, Y. Biala, M. Weinstock and K. Braun (2011). "Prenatal stress and neonatal handling induce sex-specific changes in dendritic complexity and dendritic spine density in hippocampal subregions of prepubertal rats." Neuroscience **193**: 34-43.
- Bolteus, A. J. and A. Bordey (2004). "GABA release and uptake regulate neuronal precursor migration in the postnatal subventricular zone." J Neurosci **24**.
- Bowers, J. M., J. Waddell and M. M. McCarthy (2010). "A developmental sex difference in hippocampal neurogenesis is mediated by endogenous oestradiol." Biol Sex Differ **1**(1): 8.
- Brunton, P. J. and J. A. Russell (2010). "Prenatal social stress in the rat programmes neuroendocrine and behavioural responses to stress in the adult offspring: sex-specific effects." J Neuroendocrinol **22**(4): 258-271.
- Cambronne, X. A., R. Shen, P. L. Auer and R. H. Goodman (2012). "Capturing microRNA targets using an RNA-induced silencing complex (RISC)-trap approach." Proceedings of the National Academy of Sciences **109**(50): 20473-20478.
- Cancedda, L., H. Fiumelli, K. Chen and M. M. Poo (2007). "Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo." J Neurosci **27**.
- Cao, X., S. L. Pfaff and F. H. Gage (2007). "A functional study of miR-124 in the developing neural tube." Genes & Development **21**(5): 531-536.
- Caputo, V., L. Sinibaldi, A. Fiorentino, C. Parisi, C. Catalanotto, A. Pasini, C. Cogoni and A. Pizzuti (2011). "Brain derived neurotrophic factor (BDNF) expression is regulated by microRNAs miR-26a and miR-26b allele-specific binding." PLoS One **6**(12): e28656.
- Carrel, L. and H. F. Willard (2005). "X-inactivation profile reveals extensive variability in X-linked gene expression in females." Nature **434**(7031): 400-404.

- Catts, V. S., N. Al-Menhali, T. H. Burne, M. J. Colditz and E. J. Coulson (2008). "The p75 neurotrophin receptor regulates hippocampal neurogenesis and related behaviours." Eur J Neurosci **28**(5): 883-892.
- Chen, J. S., M. S. Pedro and R. W. Zeller (2011). "miR-124 function during *Ciona intestinalis* neuronal development includes extensive interaction with the Notch signaling pathway." Development **138**(22): 4943-4953.
- Chen, W., Q. Chang, Y. Lin, A. Meissner, A. West, E. Griffith, R. Jaenisch and M. Greenberg (2003). "Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2." Science **302**: 885-889.
- Chen, W. and C. Qin (2015). "General hallmarks of microRNAs in brain evolution and development." RNA Biol **12**(7): 701-708.
- Cheng, L. C., E. Pastrana, M. Tavazoie and F. Doetsch (2009). "MiR-124 regulates adult neurogenesis in the subventricular zone stem cell niche." Nature Neuroscience **12**(4): 399-408.
- Chi, S. W., J. B. Zang, A. Mele and R. B. Darnell (2009). "Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps." Nature **460**(7254): 479-486.
- Christensen, M., L. A. Larsen, S. Kauppinen and G. Schratt (2010). "Recombinant Adeno-Associated Virus-Mediated microRNA Delivery into the Postnatal Mouse Brain Reveals a Role for miR-134 in Dendritogenesis in Vivo." Front Neural Circuits **3**: 16.
- Clancy, B., B. Finlay, R. Darlington and K. Anand (2007). "Extrapolating brain development from experimental species to humans." Neurotoxicology **28**.
- Conaco, C., S. Otto, J.-J. Han and G. Mandel (2006). "Reciprocal actions of REST and a microRNA promote neuronal identity." Proceedings of the National Academy of Sciences **103**(7): 2422.
- Conti, A. C., J. F. Cryan, A. Dalvi, I. Lucki and J. A. Blendy (2002). "cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs." J Neurosci **22**(8): 3262-3268.
- Corbier, P., L. Dehennin, M. Castanier, A. Mebazaa, D. Edwards and H. Roffi (1990). "Sex differences in serum luteinizing hormone and testosterone in the human neonate during the first few hours after birth." Journal of Clinical Endocrinology and Metabolism **71**: 1344-1348.

- Cui, J. Y., H. J. Renaud and C. D. Klaassen (2012). "Ontogeny of Novel Cytochrome P450 Gene Isoforms during Postnatal Liver Maturation in Mice." Drug Metabolism and Disposition **40**(6): 1226-1237.
- Damborsky, J. C. and U. H. Winzer-Serhan (2012). "Effects of sex and chronic neonatal nicotine treatment on Na²(+)/K⁽⁺⁾/Cl⁽⁻⁾ co-transporter 1, K⁽⁺⁾/Cl⁽⁻⁾ co-transporter 2, brain-derived neurotrophic factor, NMDA receptor subunit 2A and NMDA receptor subunit 2B mRNA expression in the postnatal rat hippocampus." Neuroscience **225**: 105-117.
- Delaloy, C., L. Liu, J. A. Lee, H. Su, F. Shen, G. Y. Yang, W. L. Young, K. N. Ivey and F. B. Gao (2010). "MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors." Cell Stem Cell **6**(4): 323-335.
- Ebner, F., R. Tepest, I. Dani, U. Pfeiffer, T. G. Schulze, M. Rietschel, W. Maier, F. Träber, W. Block, H. H. Schild, M. Wagner, H. Steinmetz, W. Gaebel, W. G. Honer, T. Schneider-Axmann and P. Falkai (2008). "The hippocampus in families with schizophrenia in relation to obstetric complications." Schizophrenia Research **104**(1): 71-78.
- Fabian, C., L. Tilzer and L. Sternson (1981). "Comparative binding affinities of tamoxifen, 4-hydroxytamoxifen, and desmethyltamoxifen for estrogen receptors isolated from human breast carcinoma: correlation with blood levels in patients with metastatic breast cancer." Biopharm Drug Dispos **2**: 381-390.
- Fan, Y. C., P. J. Mei, C. Chen, F. A. Miao, H. Zhang and Z. L. Li (2013). "MiR-29c inhibits glioma cell proliferation, migration, invasion and angiogenesis." J Neurooncol **115**(2): 179-188.
- Farrell, B. C., E. M. Power and K. W. Mc Dermott (2011). "Developmentally regulated expression of Sox9 and microRNAs 124, 128 and 23 in neuroepithelial stem cells in the developing spinal cord." Int J Dev Neurosci **29**(1): 31-36.
- Finkbeiner, S., S. F. Tavazoie, A. Maloratsky, K. M. Jacobs, K. M. Harris and M. E. Greenberg (1997). "CREB: a major mediator of neuronal neurotrophin responses." Neuron **19**(5): 1031-1047.
- Fiore, R., S. Khudayberdiev, M. Christensen, G. Siegel, S. W. Flavell, T. K. Kim, M. E. Greenberg and G. Schratt (2009). "Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels." EMBO J **28**(6): 697-710.
- Forest, M. and A. Cathiard (1975). "Pattern of plasma testosterone and delta4-androstenedione in normal newborns: evidence for testicular activity at birth." Journal of Clinical Endocrinology and Metabolism **41**: 977-980.

- Fowler, A., D. Thomson, K. Giles, S. Maleki, E. Mreich, H. Wheeler, P. Leedman, M. Biggs, R. Cook, N. Little, B. Robinson and K. McDonald (2011). "miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion." Eur J Cancer **47**(6): 953-963.
- Frodl, T., E. Reinhold, N. Koutsouleris, M. Reiser and E. M. Meisenzahl (2010). "Interaction of childhood stress with hippocampus and prefrontal cortex volume reduction in major depression." Journal of Psychiatric Research **44**(13): 799-807.
- Galanopoulou, A. (2008). "Dissociated gender-specific effects of recurrent seizures on GABA signaling in CA1 pyramidal neurons: role of GABA(A) receptors." Journal of Neuroscience **28**: 1557-1567.
- Galea, L. A. M., S. R. Wainwright, M. M. Roes, P. Duarte-Guterman, C. Chow and D. K. Hamson (2013). "Sex, Hormones and Neurogenesis in the Hippocampus: Hormonal Modulation of Neurogenesis and Potential Functional Implications." Journal of Neuroendocrinology **25**(11): 1039-1061.
- Gibbs, R. (1999). "Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult brain." Brain Research **844**: 20-27.
- Gogtay, N., T. F. Nugent Iii, D. H. Herman, A. Ordonez, D. Greenstein, K. M. Hayashi, L. Clasen, A. W. Toga, J. N. Giedd, J. L. Rapoport and P. M. Thompson (2006). "Dynamic mapping of normal human hippocampal development." Hippocampus **16**(8): 664-672.
- Gonzales, K. L., P. Quadros-Mennella, M. J. Tetel and C. K. Wagner (2012). "Anatomically-specific actions of oestrogen receptor in the developing female rat brain: effects of oestradiol and selective oestrogen receptor modulators on progesterin receptor expression." J Neuroendocrinol **24**(2): 285-291.
- Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman and A. J. Enright (2006). "miRBase: microRNA sequences, targets and gene nomenclature." Nucleic Acids Res **34**(Database issue): D140-144.
- Guo, L., B. Sun, F. Sang, W. Wang and Z. Lu (2009). "Haplotype Distribution and Evolutionary Pattern of miR-17 and miR-124 Families Based on Population Analysis." PLOS ONE **4**(11): e7944.
- Ha, M. and V. N. Kim (2014). "Regulation of microRNA biogenesis." Nat Rev Mol Cell Biol **15**(8): 509-524.

- Han, J., Hyung J. Kim, Simon T. Schafer, A. Paquola, Gregory D. Clemenson, T. Toda, J. Oh, Aimee R. Pankonin, Bo S. Lee, Stephen T. Johnston, A. Sarkar, Ahmet M. Denli and Fred H. Gage (2016). "Functional Implications of miR-19 in the Migration of Newborn Neurons in the Adult Brain." Neuron **91**(1): 79-89.
- Harrison, P. J. (2004). "The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications." Psychopharmacology **174**(1): 151-162.
- Hart, S. N., Y. Cui, C. D. Klaassen and X.-b. Zhong (2009). "Three Patterns of Cytochrome P450 Gene Expression during Liver Maturation in Mice." Drug Metabolism and Disposition **37**(1): 116-121.
- Hausser, J. and M. Zavolan (2014). "Identification and consequences of miRNA-target interactions--beyond repression of gene expression." Nat Rev Genet **15**(9): 599-612.
- Hempstead, B. (2006). "Dissecting the Diverse Actions of Pro- and Mature Neurotrophins." Current Alzheimer Research **3**(1): 19-24.
- Hendrickson, D. G., D. J. Hogan, H. L. McCullough, J. W. Myers, D. Herschlag, J. E. Ferrell and P. O. Brown (2009). "Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA." PLoS Biol **7**(11): e1000238.
- Henshall, D. C. (2014). "MicroRNA and epilepsy: profiling, functions and potential clinical applications." Curr Opin Neurol **27**(2): 199-205.
- Herrick, S. P., E. M. Waters, C. T. Drake, B. S. McEwen and T. A. Milner (2006). "Extranuclear estrogen receptor beta immunoreactivity is on doublecortin-containing cells in the adult and neonatal rat dentate gyrus." Brain Res **1121**(1): 46-58.
- Hill, M. J., J. G. Donocik, R. A. Nuamah, C. A. Mein, R. Sainz-Fuertes and N. J. Bray (2014). "Transcriptional consequences of schizophrenia candidate miR-137 manipulation in human neural progenitor cells." Schizophr Res **153**(1-3): 225-230.
- Hill, R. A., M. Klug, S. Kiss Von Soly, M. D. Binder, A. J. Hannan and M. van den Buuse (2014). "Sex-specific disruptions in spatial memory and anhedonia in a "two hit" rat model correspond with alterations in hippocampal brain-derived neurotrophic factor expression and signaling." Hippocampus **24**(10): 1197-1211.
- Hilton, G. D., A. N. Ndubuizu and M. M. McCarthy (2004). "Neuroprotective effects of estradiol in newborn female rat hippocampus." Developmental Brain Research **150**(2): 191-198.

- Hsieh, Y. C. and A. C. Puche (2015). "GABA modulation of SVZ-derived progenitor ventral cell migration." Dev Neurobiol **75**(8): 791-804.
- Huang, W., X. Liu, J. Cao, F. Meng, M. Li, B. Chen and J. Zhang (2015). "miR-134 regulates ischemia/reperfusion injury-induced neuronal cell death by regulating CREB signaling." J Mol Neurosci **55**(4): 821-829.
- Ivanova, T. and C. Beyer (2000). "Ontogenetic expression and sex differences of aromatase and estrogen receptor-alpha/beta mRNA in the mouse hippocampus." Cell Tissue Res **300**(2): 231-237.
- Jacobson, L. and R. Sapolsky (1991). "The Role of the Hippocampus in Feedback Regulation of the Hypothalamic-Pituitary-Adrenocortical Axis*." Endocrine Reviews **12**(2): 118-134.
- Jasińska, M., J. Miłek, I. A. Cymerman, S. Łęski, L. Kaczmarek and M. Dziembowska (2016). "miR-132 Regulates Dendritic Spine Structure by Direct Targeting of Matrix Metalloproteinase 9 mRNA." Mol Neurobiol **53**(7): 4701-4712.
- Jimenez-Mateos, E. M., T. Engel, P. Merino-Serrais, R. C. McKiernan, K. Tanaka, G. Mouri, T. Sano, C. O'Tuathaigh, J. L. Waddington, S. Prenter, N. Delanty, M. A. Farrell, D. F. O'Brien, R. M. Conroy, R. L. Stallings, J. DeFelipe and D. C. Henshall (2012). "Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects." Nature Medicine **18**: 1087.
- Kawamura, T., J. Chen, T. Takahashi, Y. Ichitani and D. Nakahara (2006). "Prenatal stress suppresses cell proliferation in the early developing brain." Neuroreport **17**(14): 1515-1518.
- Kier, E. L., J. H. Kim, R. K. Fulbright and R. A. Bronen (1997). "Embryology of the human fetal hippocampus: MR imaging, anatomy, and histology." American Journal of Neuroradiology **18**(3): 525.
- Kight, K. E. and M. M. McCarthy (2017). "Sex differences and estrogen regulation of BDNF gene expression, but not propeptide content, in the developing hippocampus." J Neurosci Res **95**(1-2): 345-354.
- Kim, D. Y., I. Hwang, F. L. Muller and J. H. Paik (2015). "Functional regulation of FoxO1 in neural stem cell differentiation." Cell Death Differ **22**(12): 2034-2045.
- Kim, J. Y., R. T. Ash, C. Ceballos-Diaz, Y. Levites, T. E. Golde, S. M. Smirnakis and J. L. Jankowsky (2013). "Viral transduction of the neonatal brain delivers controllable genetic mosaicism for visualising and manipulating neuronal circuits in vivo." Eur J Neurosci **37**(8): 1203-1220.

- Kim, J. Y., S. D. Grunke and J. L. Jankowsky (2016). "Widespread Neuronal Transduction of the Rodent CNS via Neonatal Viral Injection." Methods Mol Biol **1382**: 239-250.
- Klein, M. E., D. T. Lioy, L. Ma, S. Impey, G. Mandel and R. H. Goodman (2007). "Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA." Nat Neurosci **10**(12): 1513-1514.
- Klinge, C. M. (2012). "miRNAs and estrogen action." Trends Endocrinol Metab **23**(5): 223-233.
- Kohen, R., A. Dobra, J. H. Tracy and E. Haugen (2014). "Transcriptome profiling of human hippocampus dentate gyrus granule cells in mental illness." Transl Psychiatry **4**: e366.
- Kondo, N., T. Toyama, H. Sugiura, Y. Fujii and H. Yamashita (2008). "miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer." Cancer Res **68**(13): 5004-5008.
- Konkle, A. and M. McCarthy (2011). "Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain." Endocrinology **152**: 223-235.
- Korosi, A., E. F. G. Naninck, C. A. Oomen, M. Schouten, H. Krugers, C. Fitzsimons and P. J. Lucassen (2012). "Early-life stress mediated modulation of adult neurogenesis and behavior." Behavioural Brain Research **227**(2): 400-409.
- Krol, J., I. Loedige and W. Filipowicz (2010). "The widespread regulation of microRNA biogenesis, function and decay." Nat Rev Genet **11**(9): 597-610.
- Krützfeldt, J., N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan and M. Stoffel (2005). "Silencing of microRNAs in vivo with 'antagomirs'." Nature **438**(7068): 685-689.
- La Torre, A., S. Georgi and T. A. Reh (2013). "Conserved microRNA pathway regulates developmental timing of retinal neurogenesis." Proc Natl Acad Sci U S A **110**(26): E2362-2370.
- Lai, C. Y., S. Y. Lee, E. Scarr, Y. H. Yu, Y. T. Lin, C. M. Liu, T. J. Hwang, M. H. Hsieh, C. C. Liu, Y. L. Chien, M. Udawela, A. S. Gibbons, I. P. Everall, H. G. Hwu, B. Dean and W. J. Chen (2016). "Aberrant expression of microRNAs as biomarker for schizophrenia: from acute state to partial remission, and from peripheral blood to cortical tissue." Transl Psychiatry **6**: e717.

- Lajud, N. and L. Torner (2015). "Early life stress and hippocampal neurogenesis in the neonate: sexual dimorphism, long term consequences and possible mediators." Frontiers in Molecular Neuroscience **8**: 3.
- Landgraf, P., M. Rusu, R. Sheridan, A. Sewer, N. Iovino, A. Aravin, S. Pfeffer, A. Rice, A. O. Kamphorst, M. Landthaler, C. Lin, N. D. Socci, L. Hermida, V. Fulci, S. Chiaretti, R. Foà, J. Schliwka, U. Fuchs, A. Novosel, R. U. Müller, B. Schermer, U. Bissels, J. Inman, Q. Phan, M. Chien, D. B. Weir, R. Choksi, G. De Vita, D. Frezzetti, H. I. Trompeter, V. Hornung, G. Teng, G. Hartmann, M. Palkovits, R. Di Lauro, P. Wernet, G. Macino, C. E. Rogler, J. W. Nagle, J. Ju, F. N. Papavasiliou, T. Benzing, P. Lichter, W. Tam, M. J. Brownstein, A. Bosio, A. Borkhardt, J. J. Russo, C. Sander, M. Zavolan and T. Tuschl (2007). "A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing." Cell **129**(7): 1401-1414.
- Lang, Q. and C. Ling (2012). "MiR-124 suppresses cell proliferation in hepatocellular carcinoma by targeting PIK3CA." Biochemical and Biophysical Research Communications **426**(2): 247-252.
- Lee, E. and H. Son (2009). "Adult hippocampal neurogenesis and related neurotrophic factors." BMB Rep **42**(5): 239-244.
- Lemaire, V., S. Lamarque, M. Le Moal, P. V. Piazza and D. N. Abrous (2006). "Postnatal stimulation of the pups counteracts prenatal stress-induced deficits in hippocampal neurogenesis." Biol Psychiatry **59**(9): 786-792.
- Lenz, K., B. Nugent and M. McCarthy (2012). "Sexual differentiation of the rodent brain: dogma and beyond." Frontiers in Neuroscience **6**.
- Lett, T. A., M. M. Chakravarty, M. M. Chakavarty, D. Felsky, E. J. Brandl, A. K. Tiwari, V. F. Gonçalves, T. K. Rajji, Z. J. Daskalakis, H. Y. Meltzer, J. A. Lieberman, J. P. Lerch, B. H. Mulsant, J. L. Kennedy and A. N. Voineskos (2013). "The genome-wide supported microRNA-137 variant predicts phenotypic heterogeneity within schizophrenia." Mol Psychiatry **18**(4): 443-450.
- Liao, X.-M., X.-D. Yang, J. Jia, J.-T. Li, X.-M. Xie, Y.-A. Su, M. V. Schmidt, T.-M. Si and X.-D. Wang (2014). "Blockade of corticotropin-releasing hormone receptor 1 attenuates early-life stress-induced synaptic abnormalities in the neonatal hippocampus." Hippocampus **24**(5): 528-540.
- Lim, L. P., N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley and J. M. Johnson (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature **433**(7027): 769-773.

- Lima, M., J. Malheiros, A. Negrigo, F. Tescarollo, M. Medeiros, D. Suchecki, A. Tannus, R. Guinsburg and L. Covolan (2014). "Sex-related long-term behavioral and hippocampal cellular alterations after nociceptive stimulation throughout postnatal development in rats." Neuropharmacology **77**: 268-276.
- Linsley, P. S., J. Schelter, J. Burchard, M. Kibukawa, M. M. Martin, S. R. Bartz, J. M. Johnson, J. M. Cummins, C. K. Raymond, H. Dai, N. Chau, M. Cleary, A. L. Jackson, M. Carleton and L. Lim (2007). "Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression." Molecular and cellular biology **27**(6): 2240-2252.
- Lippi, G., C. C. Fernandes, L. A. Ewell, D. John, B. Romoli, G. Curia, S. R. Taylor, E. P. Frady, A. B. Jensen, J. C. Liu, M. M. Chaabane, C. Belal, J. L. Nathanson, M. Zoli, J. K. Leutgeb, G. Biagini, G. W. Yeo and D. K. Berg (2016). "MicroRNA-101 Regulates Multiple Developmental Programs to Constrain Excitation in Adult Neural Networks." Neuron **92**(6): 1337-1351.
- Liu, C., Z.-Q. Teng, N. J. Santistevan, K. E. Szulwach, W. Guo, P. Jin and X. Zhao (2010). "Epigenetic Regulation of miR-184 by MBD1 Governs Neural Stem Cell Proliferation and Differentiation." Cell Stem Cell **6**(5): 433-444.
- Liu, C., Z. Q. Teng, A. L. McQuate, E. M. Jobe, C. C. Christ, S. J. von Hoyningen-Huene, M. D. Reyes, E. D. Polich, Y. Xing, Y. Li, W. Guo and X. Zhao (2013). "An epigenetic feedback regulatory loop involving microRNA-195 and MBD1 governs neural stem cell differentiation." PLoS One **8**(1): e51436.
- Liu, X., Q. Wang, T. F. Haydar and A. Bordey (2005). "Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors." Nat Neurosci **8**.
- Lombardo, M., E. Ashwin, B. Auyeung, B. Chakrabarti, K. Taylor, G. Hackett, E. Bullmore and S. Baron-Cohen (2012). "Fetal testosterone influences sexually dimorphic gray matter in the human brain." Journal of Neuroscience **32**: 674-680.
- Lopez, J. P., R. Lim, C. Cruceanu, L. Crapper, C. Fasano, B. Labonte, G. Maussion, J. P. Yang, V. Yerko, E. Vigneault, S. El Mestikawy, N. Mechawar, P. Pavlidis and G. Turecki (2014). "miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment." Nat Med **20**(7): 764-768.
- LoTurco, J. J., D. F. Owens, M. J. S. Heath, M. B. E. Davis and A. R. Kriegstein (1995). "GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis." Neuron **15**(6): 1287-1298.
- Lv, Z. and L. Yang (2013). "MiR-124 inhibits the growth of glioblastoma through the downregulation of SOS1." Mol Med Rep **8**(2): 345-349.

- MacLusky, N., A. Clark, F. Naftolin and P. Goldman-Rakic (1987). "Estrogen formation in the mammalian brain: possible role of aromatase in sexual differentiation of the hippocampus and neocortex." Steroids **50**: 459-474.
- Magalhães, A. C. and C. Rivera (2016). "NKCC1-Deficiency Results in Abnormal Proliferation of Neural Progenitor Cells of the Lateral Ganglionic Eminence." Front Cell Neurosci **10**: 200.
- Mahmoud, R., S. R. Wainwright and L. A. M. Galea (2016). "Sex hormones and adult hippocampal neurogenesis: Regulation, implications, and potential mechanisms." Frontiers in Neuroendocrinology.
- Maiorano, N. A. and A. Mallamaci (2009). "Promotion of embryonic cortico-cerebral neuronogenesis by miR-124." Neural Development **4**(1): 40.
- Makeyev, E. V., J. Zhang, M. A. Carrasco and T. Maniatis (2007). "The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing." Mol Cell **27**(3): 435-448.
- Manent, J., M. Demarque, I. Jorquera, C. Pellegrino, Y. Ben-Ari, L. Aniksztejn and A. Represa (2005). "A noncanonical release of GABA and glutamate modulates neuronal migration." Journal of Neuroscience **25**: 4755-4765.
- McCarthy, M. (2016). "Multifaceted origins of sex differences in the brain." Philosophical Transactions of the Royal Society B: Biological Sciences **371**: 20150106.
- McCarthy, M. M., S. P. Kleopoulos, C. V. Mobbs and D. W. Pfaff (1994). "Infusion of Antisense Oligo-deoxynucleotides to the Oxytocin Receptor in the Ventromedial Hypothalamus Reduces Estrogen-Induced Sexual Receptivity and Oxytocin Receptor Binding in the Female Rat." Neuroendocrinology **59**(5): 432-440.
- McCarthy, M. M., D. B. Masters, K. Rimvall, S. Schwartz-Giblin and D. W. Pfaff (1994). "Intracerebral administration of antisense oligodeoxynucleotides to GAD65 and GAD67 mRNAs modulate reproductive behavior in the female rat." Brain Research **636**(2): 209-220.
- McCarthy, M. M., L. A. Pickett, J. W. VanRyzin and K. E. Kight (2015). "Surprising origins of sex differences in the brain." Hormones and Behavior **76**: 3-10.
- McEwen, B. S., C. Nasca and J. D. Gray (2016). "Stress Effects on Neuronal Structure: Hippocampus, Amygdala, and Prefrontal Cortex."
- McLean, C. P., A. Asnaani, B. T. Litz and S. G. Hofmann (2011). "Gender differences in anxiety disorders: Prevalence, course of illness, comorbidity and burden of illness." Journal of Psychiatric Research **45**(8): 1027-1035.

- Mellios, N., H. S. Huang, S. P. Baker, M. Galdzicka, E. Ginns and S. Akbarian (2009). "Molecular determinants of dysregulated GABAergic gene expression in the prefrontal cortex of subjects with schizophrenia." Biol Psychiatry **65**(12): 1006-1014.
- Mestdagh, P., N. Hartmann, L. Baeriswyl, D. Andreasen, N. Bernard, C. Chen, D. Cheo, P. D'Andrade, M. DeMayo, L. Dennis, S. Derveaux, Y. Feng, S. Fulmer-Smentek, B. Gerstmayer, J. Gouffon, C. Grimley, E. Lader, K. Y. Lee, S. Luo, P. Mouritzen, A. Narayanan, S. Patel, S. Peiffer, S. Rüberg, G. Schroth, D. Schuster, J. M. Shaffer, E. J. Shelton, S. Silveria, U. Ulmanella, V. Veeramachaneni, F. Staedtler, T. Peters, T. Guettouche, L. Wong and J. Vandesompele (2014). "Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study." Nature Methods **11**: 809.
- Meunier, L., B. Siddeek, A. Vega, N. Lakhdari, L. Inoubli, R. P. Bellon, G. Lemaire, C. Mauduit and M. Benahmed (2012). "Perinatal programming of adult rat germ cell death after exposure to xenoestrogens: role of microRNA miR-29 family in the down-regulation of DNA methyltransferases and Mcl-1." Endocrinology **153**(4): 1936-1947.
- Meyer, S. U., S. Kaiser, C. Wagner, C. Thirion and M. W. Pfaffl (2012). "Profound effect of profiling platform and normalization strategy on detection of differentially expressed microRNAs--a comparative study." PLoS One **7**(6): e38946.
- Mong, J., E. Glaser and M. McCarthy (1999). "Gonadal steroids promote glial differentiation and alter neuronal morphology in the developing hypothalamus in a regionally specific manner." Journal of Neuroscience **19**: 1464-1472.
- Moradifard, S., M. Hoseinbeyki, S. M. Ganji and Z. Minuchehr (2018). "Analysis of microRNA and Gene Expression Profiles in Alzheimer's Disease: A Meta-Analysis Approach." Sci Rep **8**(1): 4767.
- Morgan, C. P. and T. L. Bale (2011). "Early prenatal stress epigenetically programs dysmasculinization in second-generation offspring via the paternal lineage." J Neurosci **31**(33): 11748-11755.
- Moser, E., M. Moser and P. Andersen (1993). "Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions." The Journal of Neuroscience **13**(9): 3916-3925.
- Moser, M. B. and E. I. Moser (1998). "Functional differentiation in the hippocampus." Hippocampus **8**(6): 608-619.
- Mowla, S. J., H. F. Farhadi, S. Pareek, J. K. Atwal, S. J. Morris, N. G. Seidah and R. A. Murphy (2001). "Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor." J Biol Chem **276**(16): 12660-12666.

- Murphy, S. J., T. A. Lusardi, J. I. Phillips and J. A. Saugstad (2014). "Sex differences in microRNA expression during development in rat cortex." Neurochemistry International **77**: 24-32.
- Murthy, S., G. A. Kane, N. J. Katchur, P. S. Lara Mejia, G. Obiofuma, T. J. Buschman, B. S. McEwen and E. Gould (2019). "Perineuronal Nets, Inhibitory Interneurons, and Anxiety-Related Ventral Hippocampal Neuronal Oscillations Are Altered by Early Life Adversity." Biol Psychiatry **85**(12): 1011-1020.
- Naftolin, F., K. Ryan and Z. Petro (1971). "Aromatization of androstenedione by limbic system tissue from human fetuses." Journal of Endocrinology **51**: 795-796.
- Nelson, L. H., S. Warden and K. M. Lenz (2017). "Sex differences in microglial phagocytosis in the neonatal hippocampus." Brain, Behavior, and Immunity **64**: 11-22.
- Neo, W. H., K. Yap, S. H. Lee, L. S. Looi, P. Khandelia, S. X. Neo, E. V. Makeyev and I. h. Su (2014). "MicroRNA miR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-tuning Ezh2 Expression." Journal of Biological Chemistry **289**(30): 20788-20801.
- Nishino, J., I. Kim, K. Chada and S. J. Morrison (2008). "Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression." Cell **135**(2): 227-239.
- Nowakowski, T. J., N. Rani, M. Golkaram, H. R. Zhou, B. Alvarado, K. Huch, J. A. West, A. Leyrat, A. A. Pollen, A. R. Kriegstein, L. R. Petzold and K. S. Kosik (2018). "Regulation of cell-type-specific transcriptomes by microRNA networks during human brain development." Nat Neurosci **21**(12): 1784-1792.
- Nugent, B., C. Valenzuela, T. Simons and M. McCarthy (2012). "Kinases SPAK and OSR1 are upregulated by estradiol and activate NKCC1 in the developing hypothalamus." Journal of Neuroscience **32**: 593-598.
- Nunez, J., G. Aberdeen, E. Albrecht and M. McCarthy (2008). "Impact of estradiol on gamma-aminobutyric acid and glutamate-mediated calcium responses of fetal baboon (*Papio anubis*) hippocampal and cortical neurons." Endocrinology **149**: 6433-6443.
- Nunez, J., L. Bambrick, B. Krueger and M. McCarthy (2005). "Prolongation and enhancement of gamma-aminobutyric acid receptor mediated excitation by chronic treatment with estradiol in developing hippocampal neurons." European Journal of Neuroscience **21**: 3251-3261.

- Nunez, J. and M. McCarthy (2007). "Evidence for an extended duration of GABA-mediated excitation in the developing male versus female hippocampus." Developmental Neurobiology **67**: 1879-1890.
- Nunez, J. and M. McCarthy (2009). "Resting intracellular calcium concentration, depolarizing Gamma-Aminobutyric Acid and possible role of local estradiol synthesis in the developing male and female hippocampus." Neuroscience **158**: 623-634.
- O'Keefe, J. A. and R. J. Handa (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus." Brain Res Dev Brain Res **57**(1): 119-127.
- Obrietan, K., X. B. Gao and A. N. Van Den Pol (2002). "Excitatory actions of GABA increase BDNF expression via a MAPK-CREB-dependent mechanism--a positive feedback circuit in developing neurons." J Neurophysiol **88**(2): 1005-1015.
- Olsen, L., M. Klausen, L. Helboe, F. C. Nielsen and T. Werge (2009). "MicroRNAs Show Mutually Exclusive Expression Patterns in the Brain of Adult Male Rats." PLOS ONE **4**(10): e7225.
- Oomen, C. A., H. Soeters, N. Audureau, L. Vermunt, F. N. van Hasselt, E. M. Manders, M. Joëls, P. J. Lucassen and H. Krugers (2010). "Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood." J Neurosci **30**(19): 6635-6645.
- Pan, Y. B., Z. L. Sun and D. F. Feng (2017). "The Role of MicroRNA in Traumatic Brain Injury." Neuroscience **367**: 189-199.
- Pang, P. T., H. K. Teng, E. Zaitsev, N. T. Woo, K. Sakata, S. Zhen, K. K. Teng, W. H. Yung, B. L. Hempstead and B. Lu (2004). "Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity." Science **306**(5695): 487-491.
- Paquola, C., M. R. Bennett and J. Lagopoulos (2016). "Understanding heterogeneity in grey matter research of adults with childhood maltreatment—A meta-analysis and review." Neuroscience and Biobehavioral Reviews **69**: 299-312.
- Paris, O., L. Ferraro, O. M. Grober, M. Ravo, M. R. De Filippo, G. Giurato, G. Nassa, R. Tarallo, C. Cantarella, F. Rizzo, A. Di Benedetto, M. Mottolese, V. Benes, C. Ambrosino, E. Nola and A. Weisz (2012). "Direct regulation of microRNA biogenesis and expression by estrogen receptor beta in hormone-responsive breast cancer." Oncogene **31**(38): 4196-4206.

- Patterson, M., X. Gaeta, K. Loo, M. Edwards, S. Smale, J. Cinkornpumin, Y. Xie, J. Listgarten, S. Azghadi, S. M. Douglass, M. Pellegrini and W. E. Lowry (2014). "let-7 miRNAs can act through notch to regulate human gliogenesis." Stem Cell Reports **3**(5): 758-773.
- Perrot-Sinal, T. S., A. P. Auger and M. M. McCarthy (2003). "Excitatory actions of GABA in developing brain are mediated by l-type Ca²⁺ channels and dependent on age, sex, and brain region." Neuroscience **116**(4): 995-1003.
- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.
- Pfeffer, C. K., V. Stein, D. J. Keating, H. Maier, I. Rinke, Y. Rudhard, M. Hentschke, G. M. Rune, T. J. Jentsch and C. A. Hübner (2009). "NKCC1-dependent GABAergic excitation drives synaptic network maturation during early hippocampal development." J Neurosci **29**(11): 3419-3430.
- Ponomarev, E. D., T. Veremeyko, N. Barteneva, A. M. Krichevsky and H. L. Weiner (2010). "MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP- α -PU.1 pathway." Nature Medicine **17**: 64.
- Posadas, D. M. and R. W. Carthew (2014). "MicroRNAs and their roles in developmental canalization." Current Opinion in Genetics & Development **27**: 1-6.
- Pritchard, C. C., H. H. Cheng and M. Tewari (2012). "MicroRNA profiling: approaches and considerations." Nat Rev Genet **13**(5): 358-369.
- Qureshi, I. A. and M. F. Mehler (2012). "Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease." Nat Rev Neurosci **13**(8): 528-541.
- Rajasethupathy, P., F. Fiumara, R. Sheridan, D. Betel, S. V. Puthanveetil, J. J. Russo, C. Sander, T. Tuschl and E. Kandel (2009). "Characterization of Small RNAs in Aplysia Reveals a Role for miR-124 in Constraining Synaptic Plasticity through CREB." Neuron **63**(6): 803-817.
- Rajman, M. and G. Schratt (2017). "MicroRNAs in neural development: from master regulators to fine-tuners." Development **144**(13): 2310.
- Real, F. M., R. Sekido, D. G. Lupiáñez, R. Lovell-Badge, R. Jiménez and M. Burgos (2013). "A MicroRNA (mmu-miR-124) Prevents Sox9 Expression in Developing Mouse Ovarian Cells1." Biology of Reproduction **89**(4).

- Reyes, F., R. Boroditsky, J. Winter and C. Faiman (1974). "Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations." Journal of Clinical Endocrinology and Metabolism **38**: 612-617.
- Riffault, B., N. Kourdougli, C. Dumon, N. Ferrand, E. Buhler, F. Schaller, C. Chambon, C. Rivera, J.-L. Gaiarsa and C. Porcher (2018). "Pro-Brain-Derived Neurotrophic Factor (proBDNF)-Mediated p75NTR Activation Promotes Depolarizing Actions of GABA and Increases Susceptibility to Epileptic Seizures." Cerebral Cortex **28**(2): 510-527.
- Rivera, C., J. Voipio and K. Kaila (2005). "Two developmental switches in GABAergic signalling: the K⁺-Cl⁻ cotransporter KCC2 and carbonic anhydrase CAVII." Journal of Physiology **562**: 27-36.
- Rivera, C., J. Voipio, J. Payne, E. Ruusuvuori, H. Lahtinen, K. Lamsa, U. Pirvola, M. Saarna and K. Kaila (1999). "The K⁺/Cl⁻ co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation." Nature **397**: 251-255.
- Sanuki, R., A. Onishi, C. Koike, R. Muramatsu, S. Watanabe, Y. Muranishi, S. Irie, S. Uneo, T. Koyasu, R. Matsui, Y. Chérasse, Y. Urade, D. Watanabe, M. Kondo, T. Yamashita and T. Furukawa (2011). "miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression." Nat Neurosci **14**(9): 1125-1134.
- Sasidharan, V., S. Marepally, S. A. Elliott, S. Baid, V. Lakshmanan, N. Nayyar, D. Bansal, A. Sánchez Alvarado, P. K. Vemula and D. Palakodeti (2017). "The *miR-124* family of microRNAs is crucial for regeneration of the brain and visual system in the planarian *Schmidtea mediterranea*." Development **144**(18): 3211.
- Scharfman, H., J. Goodman, A. Macleod, S. Phani, C. Antonelli and S. Croll (2005). "Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats." Exp Neurol **192**(2): 348-356.
- Scharfman, H. E. and N. J. MacLusky (2006). "Estrogen and brain-derived neurotrophic factor (BDNF) in hippocampus: complexity of steroid hormone-growth factor interactions in the adult CNS." Front Neuroendocrinol **27**(4): 415-435.
- Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.
- Schoenfeld, T. J. and H. A. Cameron (2015). "Adult neurogenesis and mental illness." Neuropsychopharmacology **40**(1): 113-128.
- Schoenfeld, T. J. and E. Gould (2012). "Stress, stress hormones, and adult neurogenesis." Experimental Neurology **233**(1): 12-21.

- Schratt, G. M., F. Tuebing, E. A. Nigh, C. G. Kane, M. E. Sabatini, M. Kiebler and M. E. Greenberg (2006). "A brain-specific microRNA regulates dendritic spine development." Nature **439**(7074): 283-289.
- Selbach, M., B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin and N. Rajewsky (2008). "Widespread changes in protein synthesis induced by microRNAs." Nature **455**(7209): 58-63.
- Selvi, B. R., A. Swaminathan, U. Maheshwari, A. Nagabhushana, R. K. Mishra and T. K. Kundu (2015). "CARM1 regulates astroglial lineage through transcriptional regulation of Nanog and posttranscriptional regulation by miR92a." Mol Biol Cell **26**(2): 316-326.
- Sempere, L. F., S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky and V. Ambros (2004). "Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation." Genome biology **5**(3).
- Semple, B., K. Blomgren, K. Gimlin, D. Ferriero and L. Noble-Haeusslein (2013). "Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species." Progress in Neurobiology **106-107**: 1-16.
- Shao, N.-Y., H. Y. Hu, Z. Yan, Y. Xu, H. Hu, C. Menzel, N. Li, W. Chen and P. Khaitovich (2010). "Comprehensive survey of human brain microRNA by deep sequencing." BMC Genomics **11**(1): 409.
- Sharma, S. and M. Eghbali (2014). "Influence of sex differences on microRNA gene regulation in disease." Biology of Sex Differences **5**(1): 3.
- Shi, X.-B., L. Xue, C. G. Tepper and R. W. d. White (2012). "Abstract 2292: Tumor suppressive *miR-124* targets androgen receptor and inhibits proliferation of prostate cancer cells." Cancer Research **72**(8 Supplement): 2292.
- Shibata, M., H. Nakao, H. Kiyonari, T. Abe and S. Aizawa (2011). "MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors." J Neurosci **31**(9): 3407-3422.
- Shieh, P. B., S. C. Hu, K. Bobb, T. Timmusk and A. Ghosh (1998). "Identification of a signaling pathway involved in calcium regulation of BDNF expression." Neuron **20**(4): 727-740.
- Shin, J., H. G. Shim, T. Hwang, H. Kim, S. H. Kang, Y. S. Dho, S. H. Park, S. J. Kim and C. K. Park (2017). "Restoration of miR-29b exerts anti-cancer effects on glioblastoma." Cancer Cell Int **17**: 104.

- Shors, T. J., C. Chua and J. Falduto (2001). "Sex Differences and Opposite Effects of Stress on Dendritic Spine Density in the Male Versus Female Hippocampus." The Journal of Neuroscience **21**(16): 6292.
- Sohrabji, F., R. C. Miranda and C. D. Toran-Allerand (1995). "Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor." Proc Natl Acad Sci U S A **92**(24): 11110-11114.
- Solum, D. T. and R. J. Handa (2001). "Localization of estrogen receptor alpha (ER alpha) in pyramidal neurons of the developing rat hippocampus." Brain Res Dev Brain Res **128**(2): 165-175.
- Solum, D. T. and R. J. Handa (2002). "Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus." J Neurosci **22**(7): 2650-2659.
- Song, Y., D. Kilburn, J. H. Song, Y. Cheng, C. T. Saeui, D. G. Cheung, C. M. Croce, K. J. Yarema, S. J. Meltzer, K. J. Liu and T.-H. Wang (2017). "Determination of absolute expression profiles using multiplexed miRNA analysis." PLOS ONE **12**(7): e0180988.
- Sun, Z., Q. Wei, Y. Zhang, X. He, W. Ji and B. Su (2011). "MicroRNA profiling of rhesus macaque embryonic stem cells." BMC Genomics **12**(1): 276.
- Szulwach, K. E., X. Li, R. D. Smrt, Y. Li, Y. Luo, L. Lin, N. J. Santistevan, W. Li, X. Zhao and P. Jin (2010). "Cross talk between microRNA and epigenetic regulation in adult neurogenesis." J Cell Biol **189**(1): 127-141.
- Taliaz, D., N. Stall, D. E. Dar and A. Zangen (2010). "Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis." Mol Psychiatry **15**(1): 80-92.
- Tapanainen, J. (1983). "Hormonal changes during the perinatal period: serum testosterone, some of its precursors, and FSH and prolactin in preterm and fullterm male infant cord blood during the first week of life." Journal of Steroid Biochemistry **18**: 13-18.
- Teng, H. K., K. K. Teng, R. Lee, S. Wright, S. Tevar, R. D. Almeida, P. Kermani, R. Torkin, Z. Y. Chen, F. S. Lee, R. T. Kraemer, A. Nykjaer and B. L. Hempstead (2005). "ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin." J Neurosci **25**(22): 5455-5463.
- Tsang, J., J. Zhu and A. van Oudenaarden (2007). "MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals." Mol Cell **26**(5): 753-767.

- Tsuyama, J., J. Bunt, L. J. Richards, H. Iwanari, Y. Mochizuki, T. Hamakubo, T. Shimazaki and H. Okano (2015). "MicroRNA-153 Regulates the Acquisition of Gliogenic Competence by Neural Stem Cells." Stem Cell Reports **5**(3): 365-377.
- Tupler, L. A. and M. D. De Bellis (2006). "Segmented Hippocampal Volume in Children and Adolescents with Posttraumatic Stress Disorder." Biological Psychiatry **59**(6): 523-529.
- Uchida, N. (2010). "MicroRNA-9 controls a migratory mechanism in human neural progenitor cells." Cell Stem Cell **6**(4): 294-296.
- Uchida, S., K. Hara, A. Kobayashi, H. Funato, T. Hobara, K. Otsuki, H. Yamagata, B. S. McEwen and Y. Watanabe (2010). "Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents." J Neurosci **30**(45): 15007-15018.
- van Erp, T. G. M., P. A. Saleh, I. M. Rosso, M. Huttunen, J. Lönnqvist, T. Pirkola, O. Salonen, L. Valanne, V.-P. Poutanen, C.-G. Standertskjöld-Nordenstam and T. D. Cannon (2002). "Contributions of Genetic Risk and Fetal Hypoxia to Hippocampal Volume in Patients With Schizophrenia or Schizoaffective Disorder, Their Unaffected Siblings, and Healthy Unrelated Volunteers." American Journal of Psychiatry **159**(9): 1514-1520.
- Vasudevan, S. (2012). "Functional validation of microRNA-target RNA interactions." Methods **58**(2): 126-134.
- Visvanathan, J., S. Lee, B. Lee, J. W. Lee and S. K. Lee (2007). "The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development." Genes and Development **21**(7): 744-749.
- Vythilingam, M., C. Heim, J. Newport, A. H. Miller, E. Anderson, R. Bronen, M. Brummer, L. Staib, E. Vermetten, D. S. Charney, C. B. Nemeroff and J. Douglas Bremner (2002). "Childhood trauma associated with smaller hippocampal volume in women with major depression." American Journal of Psychiatry **159**(12): 2072-2080.
- Waddell, J., J. Bowers, N. Edwards, C. Jordan and M. McCarthy (2013). "Dysregulation of neonatal hippocampal cell genesis in the androgen insensitive Tfm rat." Hormones and Behavior(64): 144-152.
- Wallen, K. (2005). "Hormonal influences on sexually differentiated behavior in nonhuman primates." Frontiers in Neuroendocrinology **26**: 7-26.

- Wang, B., P. Howel, S. Bruheim, J. Ju, L. B. Owen, O. Fodstad and Y. Xi (2011). "Systematic Evaluation of Three microRNA Profiling Platforms: Microarray, Beads Array, and Quantitative Real-Time PCR Array." PLOS ONE **6**(2): e17167.
- Wang, C., C. Shimizu-Okabe, K. Watanabe, A. Okabe, H. Matsuzaki, T. Ogawa, N. Mori, A. Fukuda and K. Sato (2002). "Developmental changes in KCC1, KCC2, NKCC1 mRNA expressions in the rat brain." Brain Research **139**: 59-66.
- Wang, P., L. Chen, J. Zhang, H. Chen, J. Fan, K. Wang, J. Luo, Z. Chen, Z. Meng and L. Liu (2014). "Methylation-mediated silencing of the miR-124 genes facilitates pancreatic cancer progression and metastasis by targeting Rac1." Oncogene **33**(4): 514-524.
- Wang, X., Y. Liu, X. Liu, J. Yang, G. Teng, L. Zhang and C. Zhou (2016). "miR-124 inhibits cell proliferation, migration and invasion by directly targeting SOX9 in lung adenocarcinoma." Oncology Reports **35**: 3115-3121.
- Wang, Y., L. Chen, Z. Wu, M. Wang, F. Jin, N. Wang, X. Hu, Z. Liu, C.-Y. Zhang, K. Zen, J. Chen, H. Liang, Y. Zhang and X. Chen (2016). "miR-124-3p functions as a tumor suppressor in breast cancer by targeting CBL." BMC Cancer **16**(1): 826.
- Wienholds, E., W. P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H. R. Horvitz, S. Kauppinen and R. H. A. Plasterk (2005). "MicroRNA Expression in Zebrafish Embryonic Development." Science **309**(5732): 310.
- Winter, J., I. Hughes, F. Reyes and C. Faiman (1976). "Pituitary-gonadal relations in infancy: 2. Patterns of serum gonadal steroid concentrations in man from birth to two years of age." Journal of Clinical Endocrinology and Metabolism **42**: 679-686.
- Wu, Q., L. Xu, C. Wang, W. Fan, H. Yan and Q. Li (2018). "MicroRNA-124-3p represses cell growth and cell motility by targeting EphA2 in glioma." Biochemical and Biophysical Research Communications **503**(4): 2436-2442.
- Wu, Y. E., N. N. Parikshak, T. G. Belgard and D. H. Geschwind (2016). "Genome-wide, integrative analysis implicates microRNA dysregulation in autism spectrum disorder." Nat Neurosci **19**(11): 1463-1476.
- Yang, G., Y. Song, X. Zhou, Y. Deng, T. Liu, G. Weng, D. Yu and S. Pan (2015). "DNA methyltransferase 3, a target of microRNA-29c, contributes to neuronal proliferation by regulating the expression of brain-derived neurotrophic factor." Mol Med Rep **12**(1): 1435-1442.

- Yang, J., Lauren C. Harte-Hargrove, C.-J. Siao, T. Marinic, R. Clarke, Q. Ma, D. Jing, John J. LaFrancois, Kevin G. Bath, W. Mark, D. Ballon, Francis S. Lee, Helen E. Scharfman and Barbara L. Hempstead (2014). "proBDNF Negatively Regulates Neuronal Remodeling, Synaptic Transmission, and Synaptic Plasticity in Hippocampus." Cell Reports **7**(3): 796-806.
- Yang, J., C. J. Siao, G. Nagappan, T. Marinic, D. Jing, K. McGrath, Z. Y. Chen, W. Mark, L. Tessarollo, F. S. Lee, B. Lu and B. L. Hempstead (2009). "Neuronal release of proBDNF." Nat Neurosci **12**(2): 113-115.
- Yang, Z. and L. Wang (2011). "Regulation of microRNA expression and function by nuclear receptor signaling." Cell & Bioscience **1**(1): 31.
- Yeo, M., K. Berglund, G. Augustine and W. Liedtke (2009). "Novel repression of Kcc2 transcription by REST-RE-1 controls developmental switch in neuronal chloride." J Neurosci **29**(46): 14652-14662.
- Yeo, M., K. Berglund, M. Hanna, J. U. Guo, J. Kittur, M. D. Torres, J. Abramowitz, J. Busciglio, Y. Gao, L. Birnbaumer and W. B. Liedtke (2013). "Bisphenol A delays the perinatal chloride shift in cortical neurons by epigenetic effects on the Kcc2 promoter." Proc Natl Acad Sci U S A **110**(11): 4315-4320.
- Zhang, J., A. Konkle, S. Zup and M. McCarthy (2008). "Impact of sex and hormones on new cells in the developing rat hippocampus: a novel source of sex dimorphism?" European Journal of Neuroscience **27**: 791-800.
- Zhang, L., X. Chen, B. Liu and J. Han (2018). "MicroRNA-124-3p directly targets PDCD6 to inhibit metastasis in breast cancer." Oncol Lett **15**(1): 984-990.
- Zhang, W., P. J. Kim, Z. Chen, H. Lokman, L. Qiu, K. Zhang, S. G. Rozen, E. K. Tan, H. S. Je and L. Zeng (2016). "MiRNA-128 regulates the proliferation and neurogenesis of neural precursors by targeting PCM1 in the developing cortex." Elife **5**.
- Zhao, C., G. Sun, S. Li, M. F. Lang, S. Yang, W. Li and Y. Shi (2010). "MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling." Proc Natl Acad Sci U S A **107**(5): 1876-1881.
- Zhao, C., G. Sun, S. Li and Y. Shi (2009). "A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination." Nat Struct Mol Biol **16**(4): 365-371.
- Zhou, J., H. Zhang, R. S. Cohen and S. C. Pandey (2005). "Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures." Neuroendocrinology **81**(5): 294-310.

- Zhou, L., Z. Xu, X. Ren, K. Chen and S. Xin (2016). "MicroRNA-124 (MiR-124) Inhibits Cell Proliferation, Metastasis and Invasion in Colorectal Cancer by Downregulating Rho-Associated Protein Kinase 1(ROCK1)." Cellular Physiology and Biochemistry **38**(5): 1785-1795.
- Zhou, Z., E. Hong, S. Cohen, W. Zhao, H. Ho, L. Schmidt, W. Chen, Y. Lin, E. Savner, E. Griffith, L. Hu, J. Steven, C. Weitz and M. Greenberg (2006). "Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation." Neuron **52**: 255-269.
- Ziats, M. N. and O. M. Rennert (2014). "Identification of differentially expressed microRNAs across the developing human brain." Mol Psychiatry **19**(7): 848-852.