University of New Mexico
UNM Digital Repository

Biomedical Sciences ETDs | Electronic Theses and Dissertations

12-1-2010

Epigenetic regulation of neuronal maturation : the effect of MeCP2 and MicroRNAs on the maturation of hippocampal neurons

Richard Smrt

Follow this and additional works at: https://digitalrepository.unm.edu/biom_etds

Recommended Citation
Smrt, Richard. "Epigenetic regulation of neuronal maturation : the effect of MeCP2 and MicroRNAs on the maturation of hippocampal neurons." (2010). https://digitalrepository.unm.edu/biom_etds/26

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biomedical Sciences ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.

Richard Smrt
Candidate

Dept. of Neurosciences
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

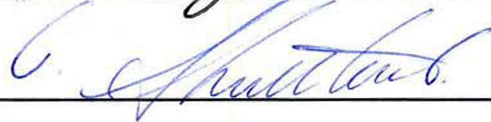
Approved by the Dissertation Committee:

Xinyu, Zhao, Chairperson

Lee Anna Cunningham

Michael Wilson

David Bear


Bill Shuttleworth

by

ABSTRACT OF DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

The University of New Mexico
Albuquerque, New Mexico

by

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

The University of New Mexico
Albuquerque, New Mexico

EPIGENETIC REGULATION OF NEURONAL MATURATION: The effect of MeCP2 and MicroRNAs on the maturation of hippocampal neurons

By

Richard D. Smrt

B.S., New Mexico Institute of Mining and Technology, Socorro, NM, 2004

Ph.D., University of New Mexico School of Medicine, Albuquerque, NM, 2010

ABSTRACT

Dendrites and the dendritic spines of neurons play key roles in the connectivity of the brain and have been recognized as the locus of long-term synaptic plasticity, which is correlated with learning and memory. The development of dendrites and spines in the mammalian central nervous system is a complex process that requires specific molecular events over a period of time. It has been shown that specific molecules are needed not only at the spine's point of contact, but also at a distance, providing signals that initiate a cascade of events leading to synapse formation. The specific molecules that act to signal neuronal differentiation, dendritic morphology, and synaptogenesis are tightly regulated by genetic and epigenetic programs. It has been shown that the dendritic spine structure and distribution are altered in many diseases, including many forms of mental retardation (MR) such as Rett syndrome, and can also be potentiated by neuronal activities and an enriched environment. Because dendritic spine

pathologies are found in many types of MR, it has been proposed that an inability to form normal spines leads to the cognitive and motor deficits that are characteristic of MR. Epigenetic mechanisms, including DNA methylation, chromatin remodeling, and the noncoding RNA-mediated process, have profound regulatory roles in mammalian gene expression. My dissertation research focused on two aspects of epigenetic mechanisms, Mecp2-DNA methylation pathway and noncoding microRNAs that regulate the development and maturation of dendrites and spines. It is well known that Rett Syndrome, a severe postnatal childhood neurological disorder is mostly caused by mutations in the *MECP2* gene. My studies focused on the role of MeCP2-mediated epigenetic regulation in postnatal brain development in a Mecp2-deficient mouse model. I found that, while Mecp2 was not critical for the production of immature neurons in the dentate gyrus (DG) of the hippocampus, the newly generated neurons exhibited profound deficits in neuronal maturation, including delayed transition into a more mature stage, altered expression of presynaptic proteins, and reduced dendritic spine density. Furthermore, I found that cultured neurons and brains lacking Mecp2 exhibited altered expression of microRNAs. My studies demonstrate that one brain-enriched microRNA, miR-137, has a significant role in regulating neuronal maturation by translational regulation of Mind bomb1. Despite extensive efforts to understand the molecular regulation of dendrite and spine development, epigenetic and non-coding RNA pathways have only recently been considered. In this thesis, I will first summarize the literature on epigenetic mechanisms that regulate the development and maturation of dendrites and spines, and discuss some general methodologies as well as recent technological advances in biology and neurosciences. I will then present my own data to show how epigenetic alterations could result in the morphological and phenotypic abnormalities that are a fundamental characteristic MR, such as Rett syndrome.

Table of Contents

EPIGENETIC REGULATION OF NEURONAL MATURATION: The effect of MeCP2 and MicroRNAs on the maturation of hippocampal neurons iv

ABSTRACT iv

Acknowledgements xi

CHAPTER 1: Neuronal Dendrites and dendritic spines in development and disease 1

1.1 Introduction to neuronal dendrites and dendritic spines 1

1.2 Activity-dependent modulation of gene expression controls dendrite and spine development 3

1.3 Dendritic spine pathology 5

CHAPTER 2: Epigenetic regulations are critical for neuronal dendritic development 9

2.1 Introduction to epigenetic regulation 9

2.2 DNA methylation 10

2.3 Histone code 13

2.4 Noncoding RNAs 16

2.5 Epigenetic diseases that affect dendritic spine development 19

2.6 Rett syndrome 20

2.7 Fragile X syndrome 22

CHAPTER 3: Methods for studying the epigenetic regulation of dendrite and spine development 25

3.1 Genetic and molecular methods 25

3.1.1 Drosophila genetics 25

3.1.2 Genetic mouse models 27

3.1.3 Isolation and RNA content analysis of synaptoneurosomes 30

3.2 Histological methods 30

3.2.1 The Golgi method 30

3.2.2 Lipophilic dye 31

3.2.3 The single-cell genetic approach 32

3.3 Conclusions 33

CHAPTER 4: Goal of my Thesis Dissertation 35

CHAPTER 5: Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons 39

5.1 ABSTRACT ... 40
5.2 Introduction ... 41
5.3 Materials and Methods ... 44
5.4 RESULTS ... 51
5.4.1 Early postnatal neurogenesis appears normal in MeCP2-deficient mice ... 51
5.4.2 Impaired maturation of MeCP2-deficient neurons ... 54
5.4.3 Impaired expression of developmentally regulated presynaptic proteins in MeCP2-deficient hippocampus ... 57
5.4.4 Altered dendritic spine distribution in MeCP2-deficient mutant neurons ... 59
5.4.5 Altered gene expression of synaptic proteins in MeCP2-deficient DG granule neurons ... 61
5.5 Discussion ... 63
5.6 Acknowledgements ... 68
5.7 Tables ... 68
CHAPTER 6: MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase Mind Bomb-1 ... 70
6.1 ABSTRACT ... 71
6.2 INTRODUCTION ... 72
6.3 MATERIALS AND METHODS ... 73
6.4 RESULTS ... 80
6.4.1 miR-137 is enriched in neurons ... 80
6.4.2 miR-137 regulates dendritic development and phenotypic maturation of new neurons in vivo ... 82
6.4.3 miR-137 regulates neuronal dendritic development in vitro ... 88
6.4.4 Mind Bomb-1 is a Translational Target of miR-137 ... 91
6.4.5 Expression of MIB1 rescues the miR-137-mediated reduction in dendritic complexity ... 94
6.5 DISCUSSION ... 96
6.6 SUMMARY ... 99
6.7 ACKNOWLEDGEMENTS ... 100
6.8 SUPPLIMENTAL FIGURES ... 101
CHAPTER 7: Summary and Critique ... 105
7.1 Summary ... 105

7.2 Critical analysis of my dissertation research 110
7.2.1 Limitations and concerns surrounding cultured neurons 110
7.2.2 Limitations and concerns surrounding in vivo retroviral targeting 112
7.3 Additional studies that could strengthen my dissertation research 113
7.3.1 Validation of MeCP2 target genes in neurons 113
7.3.2 How MeCP2 regulates miR-137 in neurons 114
7.3.3 Other potential targets of miR-137 117
7.3.4 Cell type-specific and subcellular localization of miRNA 121
7.3.5 Potential function of MIB1 in neuronal maturation 122
7.4 Future directions of this project 125
7.4.1 Activity-dependant epigenetic regulation (MeCP2) of miRNA 125
7.4.2 Synergistic effects of multiple miRNAs 127
7.4.3 How MeCP2 regulated miRNAs may regulate synaptic function 128
7.4.4 Studying in vivo miRNA functions using mouse genetics 130
APPENDIX A: SUPPLEMENTAL DATA 132
A.1. miR-137 is upregulated in MeCP2-deficient neurons 132
A.2. miR-137 is enriched in the dentate gyrus of the hippocampus 133
A.3. Characterization of *Mecp2*-siRNA 134
A.4. MeCP2 and mir137 rescue (morphology data) 135
A.5. MeCP2/mir-137 ChIP assay in adult brain and cultured post-mitotic neurons 136
A.6. Characterization of neuronal cultures 138
APPENDIX B: UNPUBLISHED MANUSCRIPT 140
Age-Dependent dynamic alteration of wild type neurons in a Novel *Mecp2* Heterozygous Mosaic Mouse Model 140
B.1. ABSTRACT 141
B.2. INTRODUCTION 142
B.3. MATERIALS AND METHODS 143
B.4. RESULTS 145
B.5. DISCUSSION 150
B.6. ACKNOWLEDGEMENTS 151
REFERENCES 152

List of Figures

CHAPTER 1: Neuronal Dendrites and dendritic spines in development and disease 1

Figure 1.1 Schematic diagram of a mature neuron and synapse 2

Figure 1.2 Common dendritic spine types in the CNS.. 3

Figure 1.3 The stages of dendrite morphological development 4

Figure 1.4 Pathological changes in spine density.. 6

CHAPTER 3: Methods for studying the epigenetic regulation of dendrite and spine development 25

Figure 3.1 Epigenetic regulation of biogenesis and function of miRNA in the neuron 34

CHAPTER 5: Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons 39

Figure 5.1 *Mecp2* KOs have normal neurogenesis, but abnormal Dcx morphology 53

Figure 5.2 Immature neurons have a delayed transitioning to mature neurons 56

Figure 5.3 *Mecp2* KO mice have altered presynaptic protein expression 58

Figure 5.4 *Mecp2* KO mice have reduced dendritic spine density in hippocampus 61

Figure 5.5 *Mecp2* KO mice have altered expression of synaptogenenic genes....................... 63

CHAPTER 6: MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase Mind Bomb-1 70

Figure 6.1 miR-137 is enriched in neurons and expressed in the dentate gyrus..................... 81

Figure 6.2 miR-137 regulates dendritic development of new neurons in vivo........................ 86

Figure 6.3 Overexpression of miR-137 leads to altered phenotypic maturation in vivo 87

Figure 6.4 miR-137 is important for dendritic development in vitro...................................... 90

Figure 6.5 MIB1 is a functional target of miR-137 93

Figure 6.6 MIB1 could rescue miR-137-mediated neuronal maturation deficits 95

Supplimental Figure 6.1 Adult hippocampal miRNA expression profile............................... 101

Supplimental Figure 6.2 Sample traces of *in vivo* virus-infected neurons............................ 102

Supplimental Figure 6.3 Dendritic spine morphology data 102

Supplimental Figure 6.4 Differentiation data for miR-137 infected progenators 103

Supplimental Figure 6.5 Sample traces of *in vitro* transfected neurons............................... 103

Supplimental Figure 6.6 Validation of *Mib1* shRNA using Western Blots............................. 104

CHAPTER 7: Summary and Critique 105

Figure 7.1. Simplified model for neuronal maturation 107

Figure 7.2. Complex overall model for neuronal maturation 109

APPENDIX A: SUPPLEMENTAL DATA 132

Appendix Figure A.1 miR-137 is upregulated in MeCP2-deficient neurons 132

Appendix Figure A.2 miR-137 is enriched in the dentate gyrus of the hippocampus 133

Appendix Figure A.3 Characterization of *Mecp2*-siRNA 134

Appendix Figure A.4 MeCP2 and miR-137 rescue 135

Appendix Figure A.5 MeCP2/miR-137 ChIP assay in brain and cultured neurons 136

Appendix Figure A.6 Characterization of cultured neurons 138

APPENDIX B: UNPUBLISHED MANUSCRIPT 140

Appendix Figure B.1 Breeding strategy and quantification of GFP+ cells 144

Appendix Figure B.2 Tissue-staining for astrocytic and neuronal markers 146

Appendix Figure B.3 Tissue-staining of MeCP2 and GFP in the dentate gyrus 147

Appendix Figure B.4 Quantification of GFP+ neurons in the hippocampus and cortex 149

Appendix Figure B.5 Camparison of *Mecp2* heterozygous mice 150

Acknowledgements

In this section I would like to acknowledge the individuals who made significant contributions to this dissertation work. I would also like to mention the specific contributions made by co-authors of my published works. I would like to give special thanks and extend my deepest appreciation to my mentor, Dr. Xinyu Zhao for her persistence and dedication to guiding my professional development as graduate student. I would also like to thank my dissertation committee for always engauging me in critical thinking to solve scientific concerns, and helping me maintin a focused dissertation research project.

Here is a list of co-authors from my primary publications, and a brief description of their contributions to my published works.

James B. Aimone (Salk Institute)**:** Performed gene expression analysis for LCM-captured mouse hippocampus in Smrt et al 2007 (Table 5.1).
Basam Z. Barkho: Performed qRT-PCR for Syndican and Prefoldin expression in Smrt et al 2007 (Fig 5.5). Helped Xinyu Zhao with initial analysis performed at the Salk Insitute for the Smrt 2007 paper.
Angelique Bordey (Yale University): Contributed expertise and guidance on FISH experiment in Smrt et al 2010.
Julialea Eaves-Egenes: Performed tissue sectioning and stereology for 4-wk and 8-wk in vivo neurogenesis data in Smrt et al 2007 (Fig 5.1). Contributed to staining for (Fig 5.2), and unpublished Mosaic project (Appendix Fig B.2 A-C).
Fred H. Gage (Salk Institute): Corresponder on neurogenesis and retroviral delivery to mouse hippocampus. First part of Smrt et al 2007 was conceptualized and partially performed by Xinyu Zhao and Basam Barkho in the Gage lab. Gage provided the *Mecp2* mutant mouse model.
Weixiang Guo: Contributed to a neuronal culture rescue experiment while I was away to a scientific meeting (Appendix Fig A.4).
Peng Jin (Emory University): Major collaborator. He was largely responsible for the conceptualization and design of the Smrt et al 2010 paper. He provided extensive molecular and genetics expertise.
Xuekun Li: Contributed intellectually to miRNA function in the brain. Performed a number of miRNA validation experiments (Table S6.1), and assisted in animal surgery for Smrt et al 2010. Provided most of the DNA plasmids needed for neuronal transfection and viral production. Produced the CAG-GFP-miR137 retrovirus used during surgery.
Yuping Luo: Designed and produced the CAG-GFP-miR137 retroviral vectors used in Smrt et al 2010 (Fig 6.4G).
Manavendra Pathania (Yale University):**:** Performed the miR-137 FISH experiment in Smrt et al 2010 (Fig 6.1E, S6.1C)
Junmin Peng (Emory University)**:** Gave us the MIB1 antibody, expression vector, and relevant information regarding MIB1 used in Smrt et al 2010.
Rebecca L. Pfeiffer: Performed a large number of neuronal traces using Neurolucida in Smrt et al 2010 (Fig 6.4C-F, 6.6A-C), and performed a large number to staining/quantification in unpublished mosaic project (Appendix Fig B.4A-G). Performed all staining, quantification, and analysis of *Mecp2* het mice comparison (Appendix B.5A,B). Rebecca also performed genotyping for the novel mosaic mouse model.
Nicholas J. Santistevan: Performed all genotyping of genetic mouse model, and quantification for Smrt et al 2007 (Fig 5.2).
Keith E. Szulwach (Emory University)**:** Major collaborator/contributor to Smrt et al 2010. Performed miRNA expression profiling and subsequent gene expression analysis (Fig 6.1A-C, S6.1A-B), MIB1 Western Blot (6.5G), and generated *Mib1* 3'UTR Lucifearse construct and mutation for luciferase activity assays (6.5A,B).
Zhao-Qian Teng: Performed lentivirus production and MIB1 western blots (Fig S6.6A)
Chunmei Zhao(Salk Institute)**:** Provided the retrovector, and method details on retroviral delivery to mouse hippocampus used in Smrt et al 2007.
Xinyu Zhao: PI for Smrt et al 2007, 2010. Xinyu is largely responsible for the conceptualization and design of the Smrt et al 2007 and 2010 papers. She performed all the in vitro NSC isolation and culture in Smrt et al 2007 (Fig 5.1B,D). The LCM isolation of hippocampus and subsequent gene array experiments (Fig 5.5A-C, Table 5.1) were performed by her at Salk Institute.

CHAPTER 1:
Neuronal Dendrites and dendritic spines in development and disease

(Chapters 1-3 were published in a recent review article (Smrt and Zhao, 2010))

1.1 Introduction to neuronal dendrites and dendritic spines

Santiago Ramon y Cajal, the founder of neuroscience and Nobel Prize winner, was the first to propose in the late 19th century that the nervous system is made up of individual neurons that are able to communicate with neighboring cells through long projecting axons and highly branched dendrites. He first described dendritic spines in 1891 as "the tips of charge or points of reception of impulses, their retraction would result in the individualization or disaggregation of neurons. The awake state would correspond to the swelling and lengthening of spines, while the resting state would correspond to the retraction of these appendages" (Cajal, 1891).

Cajal's observations were based on Golgi staining and a light microscope over 100 years ago. To date, significant advances in biology have confirmed that dendritic spines can undergo long-term modifications, such as changes in number and shape, in response to novel experiences, suggesting that dendritic spines are the locus of long-term synaptic plasticity associated with memory storage in the brain (Segal, 2005). In light of advances in genetic and molecular tools, scientists still ponder why dendrites and dendritic spines become grossly impaired in individuals faced with mental retardation (MR) and neuropathology.

The brain is composed of a complex network of neurons that communicate with each other through specialized cell junctions called synapses (Figure 1.1A,B). Most of these synaptic junctions are chemical synapses, in which a chemical neurotransmitter is released by the axon of the presynaptic neuron. The neurotransmitter can diffuse into the synaptic cleft (space between synaptic contact), where it can act on the corresponding neurotransmitter receptors on the postsynaptic neuron (Figure 1.1B). The synapses are typically found on the dendritic shaft, located on stubby spines and filipodia (long, thin dendritic protrusions), and are characterized by the clustering of certain proteins on the pre- and postsynaptic sites of contact. Dendritic spines have been classified into many types based on their shape. The most common types of spines in the central nervous system (CNS), and also the primary focus of pathological studies, are the following: mushroom-like spines with a bulbous head attached to the dendrite by a narrow neck,

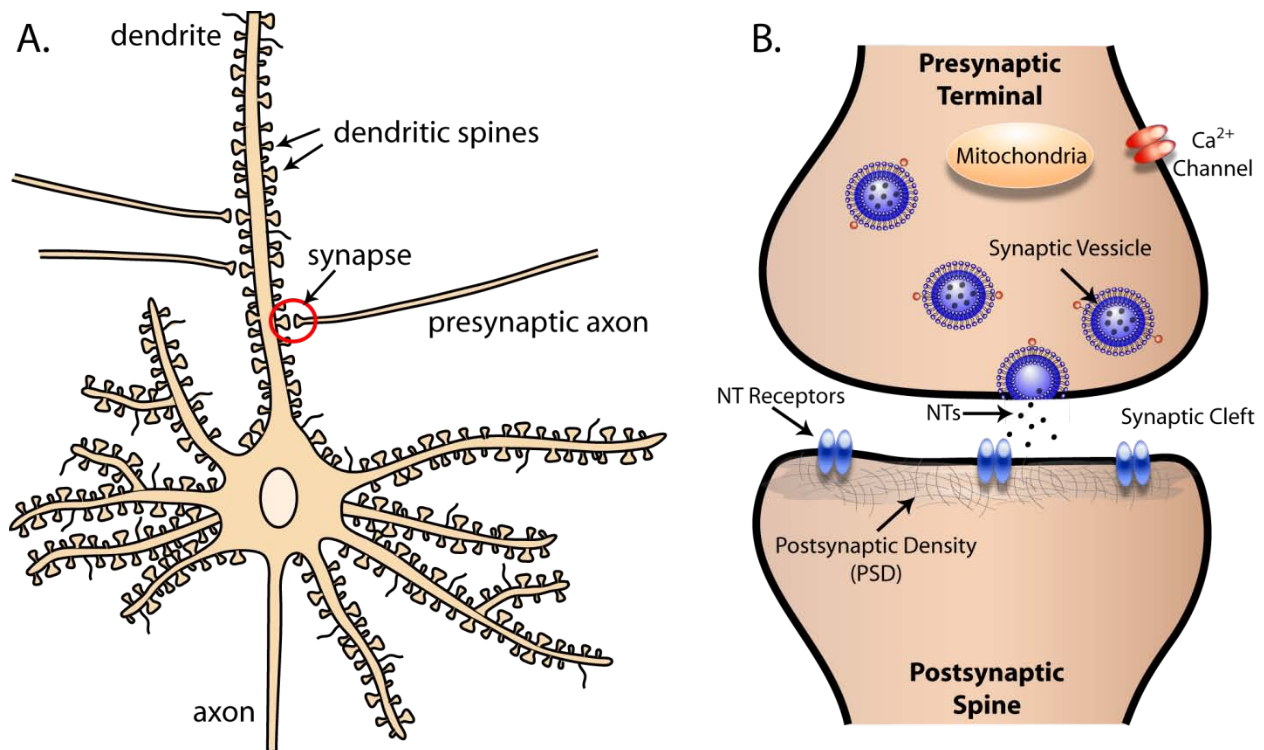


Figure 1.1: Schematic diagram of a mature neuron and synapse. A: A mature neuron has elaborate processes that are composed of multiple dendrites and one axon. Dendrites contain a large number of dendritic spines that form contacts, or synapses, with other neurons. The synapses are typically found on the dendritic shaft, located on stubby spines and filipodia. B: A neuronal synapse is the point of contact between two neurons, a presynaptic neuron and a postsynaptic neuron. It is characterized by specific synaptic proteins located on the pre and postsynaptic sites.

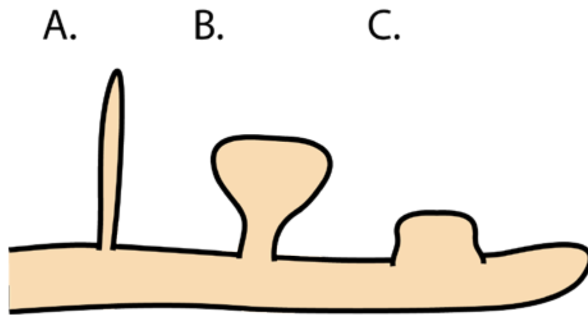


Figure 1.2: Common dendritic spine types in the CNS. Three types of dendritic spines are commonly found in the CNS. A: the long and thin filipodia spine with no head enlargement; B: the mushroom-like spine with a bulbous head attached to the dendrite by a narrow neck; and C: the short and stubby spine with no neck. It is believed that long, thin spines are mostly immature spines, and they develop into the more mature mushroom-like spines.

short and stubby spines with no neck, and long and thin spines with no head enlargement (Fiala et al., 2002). It is believed that long, thin spines are mostly immature spines, and they develop into the more mature mushroom-like spines (Figure 1.2). During development, dendrites start out with no spines, and during synaptogenesis and neuronal maturation, filipodia emerge from the dendrites and form nascent synapses with axons (Fiala et al., 1998; Fiala et al., 2002). Mature synapses are gradually formed on mushroom-like spines that have a well-defined head (Harris and Kater, 1994), and the density of mature spines increases along the dendrite as the neuron matures and forms functional connections with the surrounding brain circuitry (Zhao et al., 2006) (Figure 1.3). More about the specific molecular machinery found in the mature excitatory synapse will be discussed in the next section.

1.2 Activity-dependent modulation of gene expression controls dendrite and spine development

Glutamatergic neurons in the mammalian brain have elaborate dendrites covered with dendritic spines. These spines function as the primary sites of excitatory synaptic input for the neuron (Alvarez and Sabatini, 2007). The formation of an excitatory synapse in the CNS during

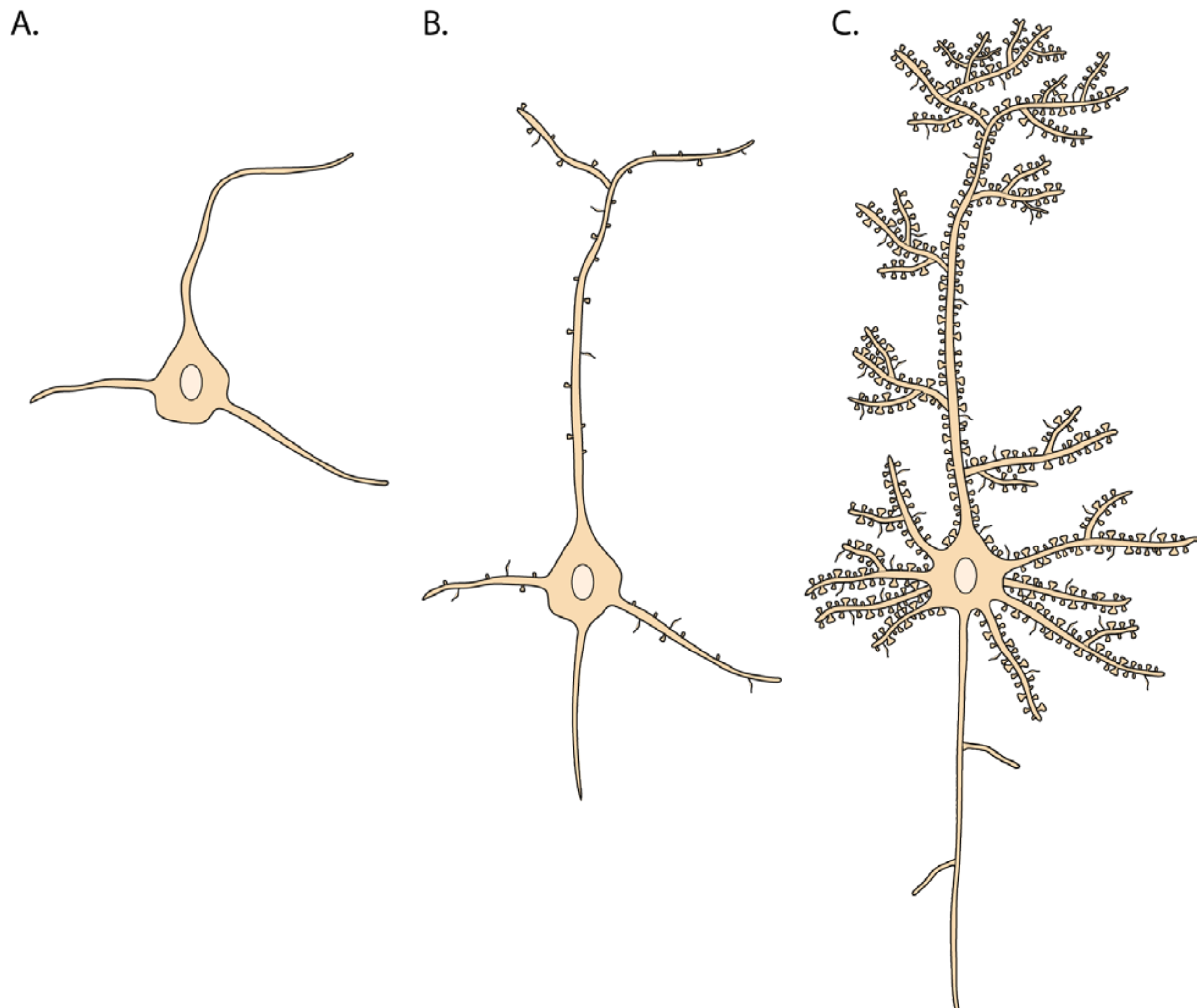


Figure 1.3: The stages of morphological development of dendrites and dendritic spines. **A**: Immature neurons have shorter dendrites, and these dendrites have no spines. **B**: During synaptogenesis and neuronal maturation, neurons develop a more complex dendritic arbor, and these dendrites begin to form spines. Some of these spines start to receive input from the axons of other neurons. **C**: Mature neurons have elaborate dendritic trees and a high density of mature spines. These neurons form functional connections with other neurons and participate in the brain circuitry.

development is initiated by the contact between the presynaptic axon and the postsynaptic dendrite, followed by the recruitment of pre- and postsynaptic proteins to the site of contact, and the stabilization of these interactions. Dendritic development and synapse formation are highly influenced by neuronal activities. Spine pruning occurs during early postnatal development, characterized by an experience-dependent loss of spines that selectively maintains spines of

active synapses resulting in the appropriate maturation of neuronal circuitry (Grutzendler et al., 2002).

A major area of research in neurodevelopment is how neuronal activity-dependent modulation of gene expression affects dendritic development and synapse formation. It has been shown that dendritic and spine morphogenesis depends on proper neuronal development and activation of glutamate receptors, which maintain appropriate connections between neurons (Parrish et al., 2007b). Activation at the synapse leads to calcium influx into the dendrites of the postsynaptic neuron, which regulates the dendritic outgrowth of postsynaptic neurons. Calcium not only functions locally at the site of entry, but also leads to changes in gene transcription in the nucleus. Calcium influx through the NMDA receptor or voltage-sensitive calcium channels (VSCCs) during development can activate many signaling pathways, such as the calcium-sensitive calcium/calmodulin kinases (CaMKs) known to be important for signal transduction in neurons. For example, activated CaMKII regulates the number of AMPA receptors at the synapse and the complexity of neuronal dendrites by influencing actin cytoskeleton (Dillon and Goda, 2005). In cultured neurons, CaMK activity initiates signaling to the nucleus, where activation of cAMP response element binding protein (CREB) leads to activity-dependent gene expression and subsequent dendritic morphological changes (Redmond et al., 2002; Wayman et al., 2006). Thus, intrinsic gene expression programs can be modified by neuronal activity to modulate spine morphogenesis and dendritic development (Cohen and Greenberg, 2008).

1.3 Dendritic spine pathology

Because spines are the key sites of synaptic input, altered spine morphology associated with pathological conditions may have a dramatic impact on the properties of the individual neuron, the neural networks, and mental function as a whole. In fact, dendritic spine distribution and structure is abnormal in many diseases and injuries, as well as many forms of MR (Figure 1.4). Thus, it has been proposed that the cognitive and motor deficits observed in MR may result from altered spine development and function. Understanding the effect of altered spines in pathological conditions will provide researchers with a better understanding of how spines contribute to normal synaptic conditions during development and learning in the adult brain.

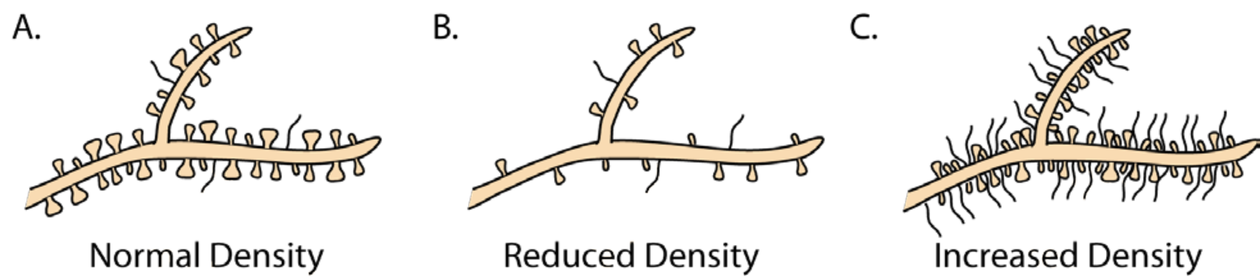


Figure 1.4: Pathological changes in spine density. Common pathological alterations in dendritic spines are altered spine density and abnormal spine shapes. **A**: Healthy neuronal dendrites contain spines with a typical variation of spine types. **B**: Reduced spine density is common in many cognitive and developmental disorders, such as Rett syndrome. **C**: Increased spine density with abnormally increased long and thin types of spines is commonly found in patients with fragile X syndrome.

As summarized in Table 1, morphological dendritic spine abnormalities are found in many types of pathological conditions, including MR such as is seen in autism spectrum disorder (Persico and Bourgeron, 2006), Rett syndrome (Zhou et al., 2006), and fragile X syndrome (Bagni and Greenough, 2005). Spine abnormalities are also found in schizophrenia (Lewis et al., 2003b), depression, stress (Pittenger and Duman, 2008), drug addiction (Robinson and Kolb, 2004), epilepsy, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease (Day et al., 2006), and Huntington's disease (Spires et al., 2004).

Table 1.1: Spine pathology related to genetic mutations and mental retardation.

Disorder	Genetic abnormality	Spine pathology	Reference
Rett syndrome	MECP2 mutation	Reduced spine density, reduced dendritic complexity and maturation	(Amir et al., 1999; Belichenko et al., 1994; Moretti et al., 2006; Smrt et al., 2007)
Fragile X syndrome	FMR1 protein deficiency	Increased spine density, abnormal spine morphology	(Comery et al., 1997; Hinton et al., 1991; Irwin et al., 2001; Wisniewski et al., 1991)
Angelman syndrome	Chromosome 15q11-q13, UBE3A	Reduced spine density, abnormal spines, small RNA pathway	(Ding et al., 2008; Lalande and Calciano, 2007; Miura et al., 2002)
Prader–Willi syndrome	Chromosome 15q11-q13	Autism, reduced cognitive ability	(Battaglia, 2005; Sahoo et al., 2008)
Down's syndrome	Chromosome 21 trisomy	Reduced spine density, reduced dendritic complexity	(Ferrer and Gullotta, 1990; Marin-Padilla, 1972; Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Takashima et al., 1981; Takashima et al., 1994)
Lafora disease	EPM2 mutation	Reduced spine density	(Busard et al., 1987)
Patau syndrome	Chromosome 13 trisomy	Abnormal spine morphology	(Marin-Padilla, 1972)
Tuberous sclerosis	Mutations of TSC1 or TCS2 genes	Reduced spine density, abnormal spine morphology	(Machado-Salas, 1984)
Niemann–Pick disease	Deficiency of sphingomyelinase	Reduced spine density, reduced dendritic complexity	(Higashi et al., 1993; Sarna et al., 2003; Walkley and Baker, 1984)
Potocki–Lupski syndrome	Duplications of 17p11.2	Autism	(Potocki et al., 2007)
Smith–Magenis syndrome	Chromosome 17p11.2, *RAI1* mutations	Autism, abnormal brain anatomy	(Elsea and Girirajan, 2008; Slager et al., 2003)
Williams–Beuren syndrome	7q11.23 (CYLN2, LIMK1, FZD9)	Reduced brain volume, altered spine morphology	(Berg et al., 2007; Hoogenraad et al., 2002; Lim et al., 2007; Meng et al., 2002; Zhao et al., 2005)
22q11.2 deletion syndrome and DiGeorge syndrome	Deletion of 22q11.2, DGCR8	miRNA pathway, autism, smaller spines, smaller dendrites	(Gothelf et al., 2007; Kobrynski and Sullivan, 2007; Lee and Lupski, 2006; Stark et al., 2008)

Aberrant dendritic spine development includes a broad range of changes in spine morphology and structure, such as increases or decreases in spine density, altered spine size or shape, dendritic beading with subsequent loss of spines, and ectopic spines in abnormal locations (Fiala et al., 2002). Since the shape and structure of a spine are closely associated with its function, the presence of abnormal spine morphology in many of these diseases suggests that the

resulting cognitive phenotype is a result of dysfunctional spines. Understanding the effect of altered spines in pathological conditions will provide researchers with a better understanding of how spines contribute to normal synaptic conditions during development and learning in the adult brain.

The spine pathogenesis among various types of MR is strikingly similar. These observations suggest that various genetic and epigenetic deficits related to MR may be the result of abnormal dendrites and spines leading to the disruption of neural homeostasis and the ability of the brain to return to a set point following perturbation (Ramocki and Zoghbi, 2008). Interestingly, many of the MR susceptibility genes encode proteins that regulate neuronal dendrite and spine development. This suggests that the molecular factors involved in behavioral and cognitive processes function in a tightly regulated homeostatic fashion. More specifically, single genes or noncoding RNAs do not encode specific cognitive processes, but instead encode biological processes. It is when a particular biological process, such as synaptic transmission, is disrupted during development that we can appreciate the resulting numerous neurological phenotypes.

Despite the fact that several protein pathways have been identified as critical players in spine development and pathology, the molecular pathogenesis of aberrant spine morphology in these diseases has yet to be clearly and comprehensively elucidated. It is apparent that complex programs of gene expression work to shape the developing nervous system. In this review, we will discuss the roles of epigenetic mechanisms in this important process. Revealing the roles of signaling factors and epigenetic gene regulators in dendritic spine pathologies will provide us with a better understanding of dendritic and spine disease and offer new approaches to treating neurodevelopmental disorders.

CHAPTER 2:
Epigenetic regulations are critical for neuronal dendritic development

2.1 Introduction to epigenetic regulation

In eukaryotic cells, genomic DNA exists in the form of chromatin and is tightly associated with histones and other chromatin proteins. Epigenetic regulation is defined as heritable changes in gene expression that are not coded within the DNA sequence itself. Epigenetic modulation of the genome involves three interacting systems, DNA methylation, histone modification (Egger et al., 2004; Li and Zhao, 2008), and noncoding RNA-mediated processes (Li and Zhao, 2008) (see Table 2). Recent literature has demonstrated that the phenotype of the cell is not only dependent on the genotype, but also the epigenotype. For example, DNA methylation within promoter regions (e.g., CpG islands) can result in heritable silencing of gene expression and has likely evolved as a host defense mechanism against viral sequences (Bestor and Tycko, 1996; Yoder et al., 1997). This type of epigenetic modulation can imprint dynamic environmental changes on a fixed genome, resulting in a stably transmitted alteration of phenotype, and has long been proposed to be an epigenetic silencing mechanism of fundamental importance (Holliday and Pugh, 1975; Riggs, 1975). The epigenotype shows far greater plasticity than the genotype during normal development, and disruption of these systems can lead to inappropriate expression or silencing of genes, resulting in "epigenetic diseases." This section will review the epigenetic mechanisms that regulate dendrite and spine development and related diseases.

Table 2.1: Summary of epigenetic modifications and their direct effects on gene expression.

Epigenetic modification	Target	Direct Effect on gene expression
DNA methylation	CpG dinucleotides	Repression
Histone methylation	H3 (K4,K36,K79)	Activation
	H3 (K9, K27), H4(K20)	Repression
	H3 (R17,R23), H4(R3)	Activation
Histone acetylation	H3 (K9,K14,K18,K56), H4 (K5,K8,K13,K16)	Activation
microRNA	mRNA	Repression

2.2 DNA methylation

DNA methylation involves covalent modification of cytosine at position C5 in CpG dinucleotides. Over 70% of CpG dinucleotides in the mammalian genome are methylated, and most DNA methylation occurs at CpG dinucleotides. The exception is CpG islands, defined as more than 500 base pairs of sequence composed of 55% GC content. CpG islands are found in the promoters and the first exon of about 40% of the mammalian genes, and they are normally kept free of DNA methylation by mechanisms that are still unclear (Takai and Jones, 2002). Methylation of CpG islands is associated with stable heritable transcriptional silencing (Jones and Baylin, 2002). The methylation of CpG dinucleotides is catalyzed by several DNA methyltransferases (DNMTs). The *de novo* establishment of DNA methylation relies on DNMT3a and 3b and cofactor DNMT3L, whereas the maintenance of DNA methylation depends on DNMT1, which specifically recognizes semi-methylated DNA and methylates the remaining strand (Bestor, 2000; Jaenisch and Bird, 2003; Robertson et al., 2000). Mammalian DNA methylation has been implicated in a diverse range of cellular functions, including tissue-specific

gene expression, cell differentiation, cell fate determination, genomic imprinting, and X chromosome inactivation (Bird, 2002b). Here we focus on the function of DNA methylation in neurodevelopment.

Deletion of DNMT1 in neural progenitor cells leads hypomethylation in neurons and subsequent abnormalities in synaptic maturation and function (Golshani et al., 2005; Hutnick et al., 2009). Although deletion of DNMT1 in neuronal precursors at E9-E10 leads to normal development through birth, it adversely affects neuronal survival after birth, resulting in death of the mice (Fan et al., 2001). Recently, Feng et al. have shown that DNMT1 and DNMT3a regulate synaptic function in neurons (Feng et al., 2010). Feng's group made use of conditional mutant mice that lacked Dnmt1, Dmnt3a, or both specifically in post-mitotic forebrain neurons of the postnatal mice. Their findings suggest that DNMT1 and DNMT3a are required for synaptic plasticity and learning and memory, likely due to their functions in maintaining DNA methylation and controlling gene expression in post-mitotic neurons. Although these studies provide sufficient evidence that DNMTs and DNA methylation are critical for neuronal development, the function of DNA methylation in post-mitotic neurons of the adult brain is still for the most part unknown. DNMT1 is highly expressed in neurons, and the deletion of DNMT1 from post-mitotic neurons of postnatal brains leads to neuronal death (Fan et al., 2001), suggesting that DNMT1 may have other roles in addition to its methyltransferase activity. In fact, DNMT1 has been shown to form a complex with HDACs and participates in transcriptional repression (Jaenisch and Bird, 2003; Robertson et al., 2000), suggesting that DNMTs may have complex roles in transcriptional regulation. Researchers have yet to identify specific genes affected by Dnmt deficiency, which impacts synaptic function, as well as learning and memory.

Despite the magnitude of literature on DNMTs and DNA methylation, much less is known about DNA demethylation. It has been proposed that the steady-state methylation of a particular gene is a dynamic equilibrium between methylase and demethylase activities. It has been found that MBD2 has DNA demethylase activity (Bhattacharya et al., 1999; Detich et al., 2002). It has also been proposed that Gadd45a erases DNA methylation marks by DNA repair-mediated DNA demethylation (Barreto et al., 2007). More recently, Gadd45b was found to be important for the activity-induced demethylation of promoters and the expression of corresponding genes critical for adult neurogenesis, such as *Bdnf* and *Fgf-1* (Ma et al., 2009). It was also shown that demethylation can take place in the absence of DNMT1 and DNMT3a *in vivo* (Feng et al., 2010). Although researchers have yet to elucidate the mechanisms of active DNA demethylation in the brain, it has been proposed that DNA oxidation and repair are possible mechanisms underlying this process.

DNA methylation represses gene transcription either through directly blocking the access of transcription factors to their binding sites or through indirectly recruiting methyl-CpG binding proteins (MBDs). The MBD protein family consists of a growing number of DNA-binding proteins with the ability to recognize methylated CpGs in the genome. The MBD family includes at least MBD1, MBD2, MBD3, MBD4, MECP2, and Kaiso (Klose and Bird, 2006). In a sense, MBDs translate genomic CpG methylation into gene expression changes; therefore, these proteins are the central components of the DNA methylation pathway. DNA methylation is important in mammalian brain development (Chahrour and Zoghbi, 2007). One of the best examples of this occurs in Rett syndrome (RTT), an X-linked dominant pervasive neurodevelopmental disorder caused by de novo mutations in methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999). Mecp2 is thought to be involved in the structural conformation of

chromatin. Once MeCP2 is bound to Methylated DNA, MeCP2 recruits a complex of chromatin-remolding enzymes that help to condense the DNA surrounding the MeCP2 binding site, and silence transcription (Bird, 2002b; Chahrour and Zoghbi, 2007). Mutations in MeCP2 lead to Rett Syndrome, a severe neurodevelopmental disorder. As discussed below, extensive evidence suggests that Mecp2 plays an important role in dendritic development and neuronal maturation (Smrt et al., 2007).

2.3 Histone code

Histones are abundant nuclear proteins. Eukaryotic genomic DNA wraps around histones, which form the basic unit of chromatin, the nucleosome that consists of ~147 base pairs of DNA wrapped around a core histone octamer (~1.65 turns). Each histone octomer includes two copies of H2A, H2B, H3, and H4 histones, and all of them can undergo different types of post-translational modifications, such as acetylation, methylation, and phosphorylation (Lachner et al., 2003). The types and sites of histone modifications, the so-called "histone code," have a significant impact on chromatin structure and gene expression.

Chromatin is present in two states: heterochromatin and euchromatin. Heterochromatin is in a condensed state that is repressive for gene transcription. When the chromatin is in an open state, called euchromatin, genes can be transcribed. Chromatin remodeling is a mechanism that alters the chromatin structure and functions to modulate DNA-protein interactions and gene activity without changing genomic DNA sequences. The chromatin structure and maintenance are not only critical for gene expression, but also for many cellular processes, such as

chromosome segregation during mitosis and X-chromosome inactivation (Grewal and Elgin, 2007).

Recent genome-wide studies have demonstrated that histone modifications, as well as recruitment of other chromatin proteins, can be used as markers for gene expression state. Among histone modifications, lysine acetylation and methylation are the most characterized markers. For example, methylation of a histone at lysine 4 (H3K4), H3K36, or H3K79 is correlated with open chromatin and generally active gene transcription, whereas methylation of H3K9, H3K27, or H4K20 is correlated with condensed chromatin and gene inactivation (Krogan et al., 2003; Santos-Rosa et al., 2002; Schubeler et al., 2004). Additionally, mono-, di-, and trimethylation at the same lysine residues lead to different levels of gene activation or repression and are involved in distinct cellular pathways (Barski et al., 2007). Histone acetylation leads to less condensed chromatin structure and can be used to mark transcriptionally active regions. On the other hand, histone hypoacetylation is associated with more condensed heterochromatin and is used to mark transcriptionally inactive regions (Grewal and Elgin, 2007). Additionally, histones are subject to a number of other modifications, including phosphorylation, ubiqutinylation, etc. (Lachner et al., 2003). Thus, post-translational modifications of histones demonstrate a high degree of diversity and complexity and reflect the importance of the "histone code" in gene expression regulation.

The enzymes that catalyze histone modifications are critical components of the epigenome and play important roles during neurodevelopment. For example, studies of histone acetylation have focused on two opposing enzymes, histone acetyltransferases (HAT) that catalyze acetylation, and histone deacetylases (HDAC) that catalyze deacetylation. Transcription activators can either recruit HATs or utilize their own internal HAT domains (for example CREB

binding protein, CBP) to catalyze histone acetylation to promote active chromatin structure. Conversely, transcription repressors can recruit HDACs that lead to histone deacetylation and gene repression. The opposing activities of HATs and HDACs are important for gene transcription regulation and therefore are tightly regulated during development. Alteration of these processes leads to developmental disorders, such as Rubinstein-Taybi syndrome, which results from heterozygote mutations of CBP. It is thus not surprising that HDAC inhibitors, such as VPA, have been developed and used to treat diseases, including psychiatric disorders (Phiel et al., 2001). VPA has been shown to increase neuronal differentiation in hippocampal neural progenitors (Hsieh et al., 2004). More recently, VPA has been shown to alter the morphology of motor cortex neurons in a rat model of autism (Snow et al., 2008) and affect neurite outgrowth in mouse neuroblastoma cells (Yamauchi et al., 2008; Yamauchi et al., 2009). Another HDAC inhibitor, trichostatin A (TSA), has also been shown to increase neuronal differentiation of neural stem cells (NSCs) and enhance the dendritic length and complexity of NSC-derived neurons (Balasubramaniyan et al., 2006). More recently, it was found that the neuronal abundant HDAC2 suppresses synapse formation and dendritic spine development of hippocampal neurons through its negative regulation of multiple neuronal genes. The authors demonstrated that HDAC2 functions to suppress synaptic plasticity and memory formation (Guan et al., 2009).

Another histone modification enzyme in the spotlight is enhancer of zeste homolog 2 (Ezh2), a H3-K27 histone methyltransferase and a part of the Polycomb group (PcG) protein complexes. PcG proteins and Ezh2 are important for neurogenesis and stem cell function in the brain (Lee et al., 2006; O'Carroll et al., 2001). PcG proteins are known in to function in maintaining the bivalent chromatin state in stem cells (Boyer et al., 2006; Lee et al., 2006). For example, genes in embryonic stem cells related to differentiation contain both activating

(methylated-H3K4) and repressing (trimethylated-H3K27) chromatin markers (Azuara et al., 2006; Bernstein et al., 2006). This bivalent chromatin state is believed to “prime” genes for expression, but “hold them in check” at the same time, therefore control the balance between proliferation and differentiation in embryonic stem cells (Bernstein et al., 2007). Recently PcG proteins have been shown to regulate dendritic arborization in *Drosophila* sensory neurons in a cell-autonomous manner (Parrish et al., 2007a; Parrish et al., 2007b). We have recently found that Ezh2 expression is controlled by the crosstalk between MeCP2 and microRNAs (Szulwach et al., 2010), suggesting that aberrant regulation of histone methyltransferase could be involved in the mammalian neuronal development and pathogenesis of neurodevelopmental disorders.

Together, these studies provide evidence that histone modification and chromatin structure modulation play significant roles in the regulation of neuronal development and the formation of dendrites and spines.

2.4 Noncoding RNAs

Of the human genome, only about 2% is comprised of coding genes, whereas more than 80% of the genome is transcribed into RNA. Mounting evidence points to important roles for noncoding RNAs in gene expression regulation and cell phenotype determination (Li and Zhao, 2008). MicroRNAs (miRNAs) are a class of small noncoding RNAs that are transcribed from the genome. Experimental evidence indicates that miRNAs function to modulate gene expression at the post-translational level by partial base-paring with the seed sequence located in the 3’UTR of the protein-coding mRNAs, leading to repression of translation efficiency or cleavage of the target mRNA (Filipowicz et al., 2008). miRNAs are expressed in many different tissues,

particularly in the brain. The expression levels and patterns of miRNAs in the brain are dynamically regulated, suggesting that they play important roles in neuronal development (Barbato et al., 2008; Bushati and Cohen, 2007). One of the most studied brain specific miRNAs is miR-9. This miRNA has been of particular interest because it plays an important role in neurogenesis (Krichevsky et al., 2006), zebrafish brain patterning (Leucht et al., 2008), and its expression levels increase as neuronal precursors differentiate into the neurons. It was shown that miR-9 and miR-124 repress BAF53a, a process important for neural stem cell (NSC) proliferation and post-mitotic dendritic outgrowth (Yoo et al., 2009). Additionally, miR-9 regulates the expression of orphan nuclear receptor (Tlx), a gene important for self renewal of NSCs (Shi et al., 2004). Additionally, Tlx also regulates expression of miR-9, demonstrating a regulatory loop that functions to regulate neurogenesis (Zhao et al., 2009). Tlx is also targeted by *Let-7b* (Zhao et al., 2010), a member of the *let-7* miRNA family. The let-7 miRNA family was one of the first families of miRNAs found to regulate stem cell function (Liu and Zhao, 2009a; Rybak et al., 2008; Schwamborn et al., 2009). Additionally, let-7 is known to affect self-renewal and differentiation and it has been shown to interact with the 3'UTR of high mobility group A2 (HMGA2), a known target of let-7. Together, let-7 and HMGA2 regulate the expression of p16Ink4a partially responsible for aged-dependent decline in self-renewal ability of brain NSCs (Nishino et al., 2008).

It has been shown previously that miRNAs are important for a number of cellular processes, such as differentiation, apoptosis, metabolism, and dendritic development (Brennecke et al., 2003; Chang et al., 2004; Chen et al., 2004; Johnston and Hobert, 2003; Poy et al., 2004). Loss of important components of the miRNA pathway, such as Dicer and DGCR8, can alter the proliferation and differentiation of stem cells (Kanellopoulou et al., 2005; Wang et al., 2007).

Additionally, miRNAs have been demonstrated to play a role in the modulation of proliferation and differentiation of different stem cell types (Ivey et al., 2008; Szulwach et al., 2010).

One of the most exciting recent discoveries in neuroscience is that miRNAs have a role in synaptic plasticity. Learning and memory in the brain requires the synapse to undergo long-term modifications. It is believed that these changes require the local translation of factors important for synaptic function (Sutton and Schuman, 2005). Local protein synthesis at the dendritic spine involves transport of mRNAs to the dendritic compartment; however, we know little about how translation at the synaptic compartment is regulated. In *Drosophila melanogaster*, it has been shown that mRNAs important for synaptic plasticity are targets of the miRNA pathway (Ashraf et al., 2006). The authors have analyzed the brains of RISC mutants and demonstrated that protein translation of neuronal CaMKII is increased in dicer-, armitage-, or aubergine-mutant brains. At the same time, Schratt et al. were able to directly link a specific miRNA to dendrite and spine development in mammalian neurons (Schratt et al., 2006). First, they showed that miR-134 is localized in the dendrites of cultured mouse hippocampal neurons and is in dendritic spines that are apposed to synapsin-positive presynaptic terminals. Next, they found that overexpression of miR-134 leads to decreased spine size, whereas inhibition of endogenous miR-134 leads to increased spine size. Therefore miR-134 may act as a negative regulator of dendritic spine maturation. Recently, the same group found that miR-138 is important for dendritic patterning and spine morphogenesis. miR-138 is highly enriched in the brain and is localized in the dendrites. The authors revealed that miR-138 negatively regulates dendritic spine size in rat hippocampal neurons by controlling the protein translation of APT1 (Siegel et al., 2009). In addition, brain-specific miR-124 is localized at presynaptic terminals of Aplysia and regulates synaptic plasticity by regulating the transcription factor CREB (Rajasethupathy et al., 2009).

Recently, a neuronal activity-dependent miRNA, miR-132, was shown to regulate dendritic development by targeting a Rho family GTPase-activating protein, p250GAP (Wayman et al., 2008). We have demonstrated that neuron-enriched miR-137 has a significant impact *in vivo* and *in vitro* on the maturation and dendritic morphogenesis of young hippocampal neurons by regulating the translation of *Mib1*, a ubiquitin ligase known to be important for neurogenesis and neurodevelopment (Smrt et al., 2010). Overall, these reports demonstrate that miRNAs have the capacity to modulate the expression of mRNAs important for dendrite and spine development (Figure 3.1). The identification of these specific miRNAs that are important for dendritic development and synaptic plasticity are critical for our understanding of miRNAs in the CNS. New methods for detecting and quantifying miRNA at dendritic spines and synapses will help untangle the role of miRNAs in dendritic spine development and synaptic plasticity (see review (Schratt, 2009)).

2.5 Epigenetic diseases that affect dendritic spine development

Cognitive functions like learning and memory are extremely complex and require a precise balance of epigenetic and regulatory mechanisms. Extensive data support the link between epigenetic dysregulation of gene expression and neurodevelopmental disorders (Table 1). In this section, we will focus on two of the best-characterized epigenetic disorders with known dendritic pathology, Rett syndrome and fragile X syndrome.

2.6 Rett syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder and an autism spectrum disorder. RTT is one of the most common forms of MR in young females. RTT patients develop normally until 6-18 months of age, and thereafter experience a myriad of neurological deficits, including seizures, ataxia, and stereotypical hand movements. RTT is caused by loss-of-function mutations in *MECP2*, an X-linked gene encoding the MeCP2 protein, one of the MBDs. MeCP2 is a central player in epigenetic regulation by binding methylated DNA and recruiting factors, such as histone deacetylases, leading to the repression of gene expression (Amir et al., 1999; Bird, 2002b) (Figure 3.1).

Extensive evidence supports the role of MeCP2 in neuronal maturation (Hagberg et al., 1983a; Smrt et al., 2007). The neurological symptoms of RTT appear after a seemingly normal embryonic development, suggesting that MeCP2 is not required for early developmental neurogenesis. However, MeCP2 expression coincides with a period of synaptogenesis (Akbarian et al., 2001a; Shahbazian and Zoghbi, 2002b; Zoghbi, 2003), suggesting that MeCP2-dependent epigenetic modulation of gene expression is important for the maturation and maintenance of neurons during brain development. Several mouse models have contributed to our understanding of the function of MeCP2 in neuronal and dendritic spine development (Bienvenu and Chelly, 2006; Chahrour and Zoghbi, 2007; Smrt et al., 2007). Expression studies in these animal models indicate that MeCP2 expression steadily increases during neuronal maturation, and is low or absent in immature neurons (Kishi and Macklis, 2004). Both RTT patients and *Mecp2* mutant mice have excess immature neurons in the olfactory epithelium, and reduced transition of immature neurons into mature neurons (Matarazzo et al., 2004; Smrt et al., 2007). Additionally,

human postmortem tissues show less complex dendritic arborization, smaller soma size, decreased dendritic spine density (Figure 1.4B), and lowered levels of the dendritic cytoskeletal protein MAP2 in RTT brains (Armstrong, 2002; Kaufmann and Moser, 2000a). On the other hand, exogenous Mecp2 expression leads to increased neurite complexity in cultured neurons (Jugloff et al., 2005; Smrt et al., 2007). Consistent with human pathology, adult *Mecp2* mutant mice have smaller soma sizes and less complex dendrites in layer II/III pyramidal neurons in the cortex (Kishi and Macklis, 2004). Using retroviral labeling of new cells, we have shown that *Mecp2*-deficient mice have reduced dendritic spine density and exhibit delayed maturation of newborn dentate granule neurons (Smrt et al., 2007). On the other hand, both we and others have shown that exogenous MeCP2 expression can lead to increased neurite complexity in cultured neurons (Jugloff et al., 2005; Smrt et al., 2010), further suggesting an important role for MeCP2 in dendritic development.

Extensive efforts have been devoted to the identification of downstream effectors of MeCP2, but only a handful of them have been verified, including *BDNF*, *DLX5*, and *DLX6* (Chahrour and Zoghbi, 2007; Tudor et al., 2002). Since these known targets cannot fully explain the deficits associated with MeCP2 deficiency, the identification of additional MeCP2 effector genes is needed to paint a full picture of the pathogenesis of RTT. Recently, epigenetic regulation of noncoding RNAs by MeCP2 has been suggested as a novel mechanism for understanding the pathogenesis of Rett syndrome. It was found that the miR-184 transcript is imprinted and exclusively expressed on the paternal allele and that MeCP2 binds upstream of miR-184 before neuronal depolarization, and releases after depolarization in an activity-dependent manner (Nomura et al., 2008). However, the miR-184 expression level is decreased in *Mecp2* KO brains, which contradicts the authors' finding. It is possible that miR-184 is indirectly

regulated by MeCP2. In fact, we have found that miR-184 is directly regulated by MBD1 (Liu et al., 2010). We have shown that MeCP2 can directly regulate the expression of a subset of miRNAs in primary neural progenitor cells isolated from postnatal brains. One of these miRNAs, miR-137, modulates NPC proliferation and differentiation by regulating protein translation of *Ezh2*, a histone H3K27 methyltransferease (Szulwach et al., 2010). In developing neurons, miR-137 represses dendrite and spine morphogenesis by targeting another neurodevelopmental factor, MIB1 (Smrt et al., 2010). Therefore, it is conceivable that MeCP2 regulates neuronal dendrite and spine development through noncoding RNAs. Functional crosstalk between the DNA methylation pathway and small regulatory RNA could be an important and novel mechanism regulating mammalian neurodevelopment (Figure 3.1).

2.7 Fragile X syndrome

Fragile X syndrome (FXS) is the most common cause of inherited MR and is attributed to mutations in the X-linked *FMR1* gene. Epigenetic mechanisms have been implicated in the molecular mechanisms of the disease (Graff and Mansuy, 2009). Typically, fragile X syndrome is caused by the expansion of a polymorphic CGG repeat in the 5' untranslated region (UTR) of the gene. If the CGG expansion reaches more than 200 repeats in female carriers, it is considered a full mutation. As a result, the repeat, the upstream CpG island, and the surrounding sequence become hypermethylated, and the gene is silenced (Oberle et al., 1991). One distinct characteristic of patients with FXS is that they have many more dendritic spines than normal individuals (Figure 1.4C), and their spines are longer and thinner, resembling immature spines (Hinton et al., 1991; Irwin et al., 2001). It has been suggested this is due to misregulated

development and elimination. This phenotype is also seen in the mouse model of FXS (Comery et al., 1997; Greenough et al., 2001; Hinton et al., 1991; Irwin et al., 2001; Nimchinsky et al., 2001). During dendritic spine development, synapses may be formed in excess numbers. Therefore, maturation and pruning are needed to establish the final synaptic pattern. In FXS, the activity-dependent events that lead to removal of excess or inappropriately placed synapses do not occur (Galvez et al., 2003).

Fragile X mental retardation protein (FMRP) is an RNA-binding protein, part of the heterogeneous nuclear ribonucleoproteins (hnRNPs), which function in many aspects of mRNA metabolism and biology, including nuclear export of mRNA and subcellular localization (Van de Bor and Davis, 2004). FMRP is part of a large protein complex that is involved in the transportation and translation of mRNA in neurons. Studies have shown that abnormal spines in FXS are associated with impaired neuronal plasticity; therefore, it has been suggested that FMRP may function to transport coding and noncoding RNA to the synapse and participate in local protein synthesis in dendrites (Figure 3.1). Local protein synthesis in dendrites modulated by FMRP can potentially influence biochemical pathways or signaling cascades involved with spine morphogenesis, such as Rac1, MAP1B, CamKII, calbindin, and cadherins (Grossman et al., 2006; Penagarikano et al., 2007). The mechanism by which FMRP regulates translation of synaptic factors at the dendritic spine is still under investigation. It has also been shown that the bidirectional transport of the FMRP-mRNA complex between the soma to the dendrites and spines is driven by neuronal activity (Antar et al., 2004; Antar et al., 2005; Ling et al., 2004), indicative of multiple roles for FMRP in the activity-dependent regulation of gene expression at the dendritic spine.

It has been suggested that FMRP can associate with miRNAs and regulate the expression of a subset of target synaptic mRNAs (Fiore et al., 2008; Jin et al., 2004; Penagarikano et al., 2007; Vanderklish and Edelman, 2005) (Figure 3.1). Recently, it was shown that a number of microRNAs, including miR125b and miR-132, can associate with FMRP in the mouse brain. While these miRNAs have opposing effects on dendritic spine morphology, knockdown of FMRP ameliorates the effects of overexpressing these microRNAs on spine morphology. The FMRP-associated miRNA 125b was found to target glutamate receptor subunit NR2A, suggesting FMRP-associated miRNAs may have a profound impact on synaptic plasticity, as well as on the pathophysiology of FXS (Edbauer et al., 2010). Although there is supporting evidence that FMRP collaborates with miRNAs to suppress the expression of genes important for dendritic spine morphology and synaptic plasticity, the details of how miRNA and FMRP interact are unclear. One proposed interaction is that miRNA and mRNA could act as the "kissing complex" RNA structure that has previously been proposed to bind the KH2 domain of FMRP (Bassell and Warren, 2008).

CHAPTER 3:
Methods for studying the epigenetic regulation of dendrite and spine development

3.1 Genetic and molecular methods

Significant advances in genetics and molecular biology have made a profound impact on our understanding of neurodevelopment and disease. Here we will summarize a few important methods.

3.1.1 Drosophila genetics

Drosophila neurogenetics was born in Seymour Benzer's lab at Caltech in the mid-1960s, when he proposed that genes can control behaviors in the fruit fly (Benzer, 1967). Benzer's trainees went on to isolate the shaker (sk) gene, which is a member of the transient receptor potential (trp) ion channel family (Papazian et al., 1987). A number of classical studies of brain functional genetics involved the use of loss-of-function approaches in *Drosophila* (Hotta and Benzer, 1970; Lin et al., 1998; Lush et al., 1998; Min and Benzer, 1999).

Many neurologic as well as other human diseases can be modeled using *Drosophila* to characterize genetic, epigenetic, and cellular pathways that lead to the disease state. *Drosophila* genetics are appealing to biologists because the tools of this trade are relatively simple and behavioral paradigms are well characterized, providing an extremely powerful method to go from mutant phenotypes to genotypes (forward genetics). Thus, the use of *Drosophila* models of human disease has grown rapidly and significantly contributed to our understanding of the

human pathogenesis. The molecular and cellular pathways in *Drosophila* are generally considered to be highly conserved with vertebrates; approximately 75% of human genes known to be associated with disease correspond to a *Drosophila* ortholog (Reiter et al., 2001). *Drosophila* can be used to conduct assays ranging from genetic screens to drug target validation. For example, genetic modifier screens can identify proteins and genes that interact with pathways associated with pathology (Egger et al., 2004). Additionally, flies are excellent subjects for high-throughput drug screening libraries and are responsible for the identification of mGluR5 as a drug target for treating fragile X syndrome (Chang et al., 2008; Marsh and Thompson, 2006).

Drosophila has been used to understand the genetic pathways implicated in fragile X syndrome. The *Drosophila* genome contains the gene *Drosophila* fragile X-related (*dfxr* or *dfmr1*), which is homologous to *FMR1* (Wan et al., 2000) and contains all the functional motifs related to FMRP. It was found that *Drosophila* genetics could be used to show dFXR plays a role in neuronal development, including synaptic formation, axonal growth, and dendritic development (Dockendorff et al., 2002; Lee et al., 2003; Michel et al., 2004; Morales et al., 2002; Zhang et al., 2001).

Drosophila has been used for studying epigenetic regulation related to histones and miRNAs. For example, dFMR1 is important in the microRNA (miRNA) pathway (Caudy et al., 2002; Ishizuka et al., 2002; Yang et al., 2007) and has been shown to modulate miR-124a levels, which are important for dendritic branching in *Drosophila* (Xu et al., 2008). However, CpG methylation is essentially absent in *Drosophila*, making it an invalid model for studying mammalian DNA methylation.

3.1.2 Genetic mouse models

Genetic manipulation of the mouse genome along with the characterization of mutant phenotypes has made a profound impact on our understanding of the pathophysiology of many human diseases. Mice and humans share 99% of their genes and have similar physiological and biochemical features, making genetic mouse models a powerful tool to study human disease (Rosenthal and Brown, 2007). This section will briefly describe the most common mouse models used to study Rett syndrome and fragile X syndrome, both implicated in abnormal spine development.

The MeCP2 mouse

To model Rett syndrome and related disorders in mice, three mouse models were generated with different *Mecp2* genetic alterations. One of these models is the *Mecp2* conditional KO mouse, which lacks either exon 3 or both exons 3 and 4 of the *Mecp2* gene (Chen et al., 2001a; Guy et al., 2001). These conditional knockout mice develop normally during the first 3-6 weeks of life, and thereafter develop motor dysfunction, hind limb clasping, and breathing irregularities. Mutant brains show reduced brain weight and more densely packed neurons, but do not show neuroanatomical abnormalities. The female mice, which are heterozygous for the *Mecp2* mutation (*Mecp2*+/-), show behavioral phenotypes that are less severe and with a later onset.

Another mouse model was generated by truncating *Mecp2* at amino acid 308. This truncation resulted in a hypomorphic allele that contains a truncated C-terminal region, reminiscent of Rett patients found with C-terminal deletions (Shahbazian et al., 2002a). These

mice have many similarities with the conditional knockout model described above, but have a less severe phenotype that leads to longer longevity.

The third mouse model involves a two-fold overexpression of human MeCP2 that leads to an initial increase in synaptic plasticity and contextual learning; however, by 20 weeks of age the mice develop progressive neurological phenotypes, including motor abnormalities, hind limb clasping, and seizures, and they die by 1 year of age (Collins et al., 2004). This is also seen in the clinic, where duplication of *MECP2* in human male patients causes severe MR. Moreover, selective overexpression of MeCP2 in post-mitotic neurons, under the tau promoter, lead to a progressive neurological phenotype in mice (Luikenhuis et al., 2004).

In yet another mouse model, deletion of *Mecp2* specifically in post-mitotic neurons using a CaMKII Cre transgene results in a phenotype resembling the *Mecp2*$^{-/y}$ knockout. This suggests that dysfunction of brain-specific MeCP2 leads to the neurological phenotype observed in Rett syndrome (Chen et al., 2001a; Gemelli et al., 2006). Additionally, when *Mecp2* is expressed in post-mitotic neurons under the tau promoter in null mice, the Rett phenotype is rescued, further supporting this idea (Luikenhuis et al., 2004). Taken together, these studies demonstrate that perturbations in the homeostatic balance of MeCP2 expression can result in aberrant neurological functioning.

The fragile X mouse

FMR1 is highly conserved between humans and mice (Ashley et al., 1993). The FMR1 knockout mouse was generated by disrupting exon 5 of the FMR1 gene by homologous recombination. This resulted in the absence of normal FMRP protein (Bakker et al., 1994). These mice show cognitive and anatomical impairments that are related to those seen in human

patients, making the fragile X knockout mouse an important model to study the function of FMRP. The fragile X knockout mouse displays distinct phenotypes, such as hyperactivity, special learning deficits, macroorchidism, and altered dendritic spines (Bakker et al., 1994; Kooy, 2003). Among the similarities between the human pathology and the mouse model is cognitive function. It has been found that FMR1 KO mice have special learning deficits when challenged with the Morris water maze and radial arm maze (Bakker et al., 1994; Kooy, 2003; Kooy et al., 1996; Mineur et al., 2002), two tests generally considered to reflect problems in hippocampal functioning (Logue et al., 1997; Morris et al., 1982). We found that FMR1 KO mice exhibit impaired hippocampal neurogenesis, which may contribute to the deficits in hippocampus-dependent learning (Luo et al., 2010). Among structural abnormalities in the mouse model, FMR1 KO mice show macroorchidism due to increased Sertoli cell proliferation (Slegtenhorst-Eegdeman et al., 1998). No gross neuroanatomical differences are observed in human patients or fragile X mouse brains (Bakker et al., 1994); however, it is well appreciated that patients and mice have long, thin immature spines with increased spine density in dendrites of the cortex (Comery et al., 1997; Irwin et al., 2001). FRM1 KO mice also show a propensity for epileptic seizures. Seizures occur in fragile X patients (Hull and Hagerman, 1993) and can be elicited by auditory stimuli in knockout mice (Chen and Toth, 2001). Epileptic seizures in fragile X are likely due to dendritic spine abnormalities in mice and patients. Fragile X mice have been used for developing therapeutic treatments, though the results are still controversial. Pharmalogical approaches have been proposed to compensate for the loss of FMRP (Kooy, 2003; Penagarikano et al., 2007). More work is required for a better understanding of the mechanism of how FMRP regulates mRNA translation, and how FMRP functions in activity-dependent local protein synthesis in the dendrite.

3.1.3 Isolation and RNA content analysis of synaptoneurosomes

Synaptoneurosomes are small vesicle structures that are prepared by subcellular fractionation of brain homogenate. These structures are known to be enriched in dendritic spines (Lugli et al., 2008). It has been shown that polyribosomes, as well as many mRNAs, are present in dendrites and are recruited into dendritic spines (Bourne et al., 2007; Ostroff et al., 2002). Gene chip analysis of PSD fraction-associated mRNAs shows that mRNAs encoding many postsynaptic proteins are highly concentrated in PSD fractions (Suzuki et al., 2007). In addition, synaptoneurosomes also contain a large number of miRNAs. To characterize microRNA expression at the synapse, Lugli and collaborators isolated synaptic fractions from the mouse forebrain and analyzed microRNA expression using microarrays; enriched microRNAs were subsequently confirmed by qRT-PCR (Lugli et al., 2008). Lugli et al. found that a number of microRNAs were highly enriched in synaptoneurosomes and are predominately associated with PSDs. Their study further supports the role of miRNAs in protein synthesis at the synapse. It is likely that synaptosomes contain other types of RNAs and perhaps even DNA. With the development of next-generation sequencing, more synaptosome RNAs will be revealed, providing a better picture of the regulatory network governing dendrite and spine development.

3.2 Histological methods

3.2.1 The Golgi method

The Golgi method was discovered by Camillo Golgi and published in 1873 (Golgi, 1873); however, it was popularized by Ramon e Cajal, who modified the method to study a myriad of species and tissues during many developmental periods, including the brain (De Carlos and Borrell, 2007). Since then, the application of the Golgi technique in neurosciences has been used to study morphological abnormalities in dendrites that are typical in diseases associated with MR. The pioneers of Golgi impregnations to study dendrites in MR were Huttenlocher (Huttenlocher, 1970; Huttenlocher, 1974) and Purpura (Purpura, 1975), who relied on post-mortem and biopsy material. Using the Golgi method, Parpura found cortical pyramidal neurons to have shorter and less complex dendritic branches in individuals with unclassified MR (Purpura, 1974). This dendritic "dysgenesis" was also observed in individuals with chromosomopathies and genetic disorders associated with MR (Ferrer et al., 1984; Marin-Padilla, 1972) (see Figure 1.4). Golgi impregnation in Rett syndrome post-mortem tissue showed a reduction in the dendritic arborization of cortical neurons throughout life in RTT patients (Armstrong et al., 1995). Similar results were obtained using this method in an animal model of RTT lacking MeCP2 (Kishi and Macklis, 2004). In fragile X syndrome, Golgi preparations showed long dendritic spines (Hinton et al., 1991), and the *FMR1* knockout mouse also shows spine dysgenesis (Comery et al., 1997).

3.2.2 Lipophilic dye

Lipophilic dyes are fluorescent substances that can be microinjected into a single cell and visualized using a confocal microscope to study neuronal morphology in both animals and humans (Belichenko et al., 1994). This method offers advantages over the Golgi method because that experimenters can select the neuron(s) that are to be infused with the dye and perform digital

3-D reconstruction of dendritic morphology with laser confocal imaging, instead of the traditional light microscope with Camera-Lucida method. Belichenco's group used the dye Lucifer Yellow and confocal microscopy to reconstruct the 3-D morphology of neurons in the prefrontal, motor, and temporal areas of RTT brain tissue. They showed reduced apical dendritic morphology and spine density, as well as dendritic segments lacking spines, which they called "naked dendrites," in pyramidal neurons of the frontal cortex (Belichenko et al., 1997a; Belichenko et al., 1994). Belichenko and his colleagues used this method to study the morphology of neurons in a number of diseases and injuries associated with spine pathology (Belichenko et al., 1997a; Belichenko et al., 2004; Belichenko et al., 1994; Johansson and Belichenko, 2002).

3.2.3 The single-cell genetic approach

The use of retrovirus-mediated gene delivery, also known as the single-cell genetic approach, has made it possible to study the morphology and functional properties of newborn neurons throughout their lifetime (Gage, 2002; van Praag et al., 2002). Newborn neurons in the adult hippocampus have been used as a model to study neuronal development, because they recapitulate many features of embryonic hippocampal development (Song et al., 2005). When using the retroviral approach, newborn cells in the hippocampus are infected with retrovirus, causing them to express a live reporter, such as green fluorescent protein (GFP), throughout the cell, including the dendritic processes spines. This allows researchers to conduct a temporal analysis of the dendrite and spine development of newborn neurons by direct visualization of living newborn cells (Zhao et al., 2006). We used the single-cell genetic approach in an animal model of RTT to investigate the role of Mecp2 in developing neurons of the adult hippocampus

(Smrt et al., 2007). We found that four-week-old new neurons in adult *Mecp2* knockout animals showed reduced dendritic spine density, a characteristic feature of immature neurons suggesting that newborn dentate granule neurons lacking Mecp2 have impaired maturation. Using a similar approach, we demonstrate that miR-137 overexpression inhibits dendritic morphogenesis (Smrt et al., 2010). These data are consistent with similar findings using other methods described in this section to study dendritic spines under pathological conditions and support the idea that abnormal dendrite and spine development is the common point of vulnerability leading to the neurological deficits in diseases associated with MR.

3.3 Conclusions

In first 3 chapters, I have discussed the role of epigenetics in dendritic and spine morphogenesis. Since Cajal's epic contributions to neuroscience in the late 19th and early 20th centuries, contemporary science and advances in genetic and molecular tools have enabled researchers to look deeper into the underlying mechanisms of dendritic and synaptic development. The advent of epigenetic research has provided much new knowledge and also opened up more questions regarding the role of gene expression regulation in neurodevelopment. My focus as a researcher has been to use our arsenal of tools and knowledge of molecular biology and genetics to understand how dendrites and dendritic spines become grossly impaired in individuals faced with MR and neuropathology.

Recent studies have shown that mutations in the epigenetic machinery lead to many forms of MR disorders. Synaptic plasticity, as well as learning and memory, are believed to depend on proper neuron-neuron communication, and identification of the genes involved in synaptic development is crucial to understanding these human disorders.

The important challenge for both scientists and clinicians is to identify the molecular basis for cognitive and behavioral symptoms in order to design adequate pharmacological treatments. A number of epigenetic-based therapeutic methods have been proposed as treatments for epigenetic-associated diseases. Many pharmacological agents have been discovered that alter methylation patterns on DNA and histones, many of which are being extensively tested in vitro and in clinical trials (Egger et al., 2004). However, epigenetic reagents, such as HDAC or DNA methylation inhibitors, have met with concerns about the specificity of the drug's action. MicroRNAs have recently been identified as promising candidates for the treatment of diseases, such as heart disease and cancers. The challenge is to identify the specific miRNA(s) that could be considered treatment targets and to deliver that miRNA or miRNA inhibitor precisely to the target cells with high efficiency and without side effects. The next chapter will state the goal of my dissertation research and how I will explore the link between epigenetics, miRNA, and disease such as Rett Syndrome.

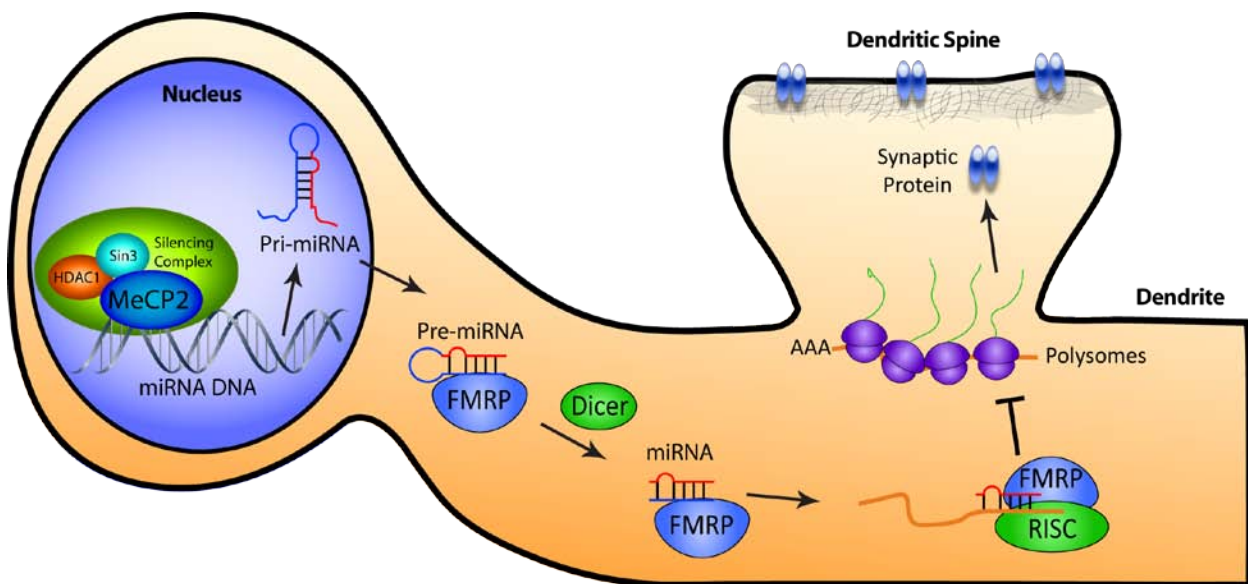


Figure 3.1. Epigenetic regulation of biogenesis and functions of miRNA in the neuron. Transcription of miRNA, which can be regulated by epigenetic machinery, leads to the production of miRNA transcripts (pri-miRNAs), which are cleaved in the nucleus and exported into the cytoplasm as pre-miRNAs. The pre-miRNA is processed by the RNase Dicer and is likely associated with other accessory proteins, such as FMRP, to form an intermediate miRNA duplex. The leading strand of the miRNA duplex can then associate with the miRNA-induced silencing complex (miRISC), and then pair with sequences located on the 3'UTR of target mRNAs. This leads to translational repression of the target mRNA. This example illustrates the effect of miRNA-mediated translational repression of proteins important for dendritic spine function.

CHAPTER 4:
Goal of my Thesis Dissertation

The goal of this thesis is to determine how epigenetic factors influence the key characteristics of neuronal maturation in developing neurons. Despite the fact that several protein pathways have been identified as critical players in spine development and pathology, the molecular pathogenesis of aberrant spine morphology in epigentic diseases such as Rett syndrome has yet to be clearly and comprehensively elucidated. The scientific studies I conducted focus on how epigenetic mechanisms that are known to regulate gene expression, including DNA methylation, chromatin remodeling, and noncoding RNA-mediated processes may play a role in regulating the development and maturation of dendrites and spines.

In Chapter 5, I investigated the role of MeCP2-mediated epigenetic regulation in neurogenesis and neuronal maturation of the hippocampus in postnatal brains (Smrt et al, 2007). Using the Mecp2-deficient mouse mutant, we found that MeCP2 was not critical for the production of immature neurons in the dentate gyrus (DG) of the hippocampus. However, the newly generated neurons exhibited pronounced deficits in neuronal maturation, including delayed transition into a more mature stage, altered expression of presynaptic proteins, and reduced dendritic spine density. Furthermore, analysis of gene expression profiles of isolated DG granule neurons revealed abnormal expression levels of a number of genes previously shown to be important for synaptogenesis. Our studies suggest that MeCP2 plays a central role in neuronal maturation, which might be mediated through epigenetic control of expression pathways that are instrumental in both dendritic development and synaptogenesis. The data collected in this study suggest that Mecp2 is not critical for the early stages of neurogenesis, but is important for neuronal maturation in the postnatal brain.

It has been shown that epigenetic factors such as MeCP2 have a profound effect on normal brain and dendritic development (Chahrour and Zoghbi, 2007); however, how epigenetic factors such as MeCP2 regulate brain development and neuronal maturation is unclear due to the challenges of identifying the downstream targets by classical gene expression analyses (Bienvenu and Chelly, 2006). Despite extensive effort to understand how MeCP2-deficiency leads to abnormal neuronal development, relatively few genes have been confirmed to be regulated by MeCP2, and the MeCP2-targeted gene(s) responsible for the pathogenesis of Rett syndrome have yet to be elucidated. To understand the function of MeCP2 in dendritic and dendritic spine development, I investigated non-coding miRNAs as the potential downstream effectors of MeCP2. In a paper I co-authored (Szulwach et al., 2010), we show that MeCP2 epigenetically regulates a number of specific miRNAs in adult brain-derived neural stem cells (NSCs) under both proliferating and neuronal differentiating conditions. One of the miRNAs is miR-137. MeCP2 was found to bind directly to the genomic region proximal to miR-137, and absence of MeCP2 binding to this region correlated with an altered chromatin state and enriched miR-137 expression (Szulwach et al., 2010). This study suggests that the crosstalk between MeCP2-mediated epigenetic regulation of gene expression and miRNA pathways can function to modulate adult neurogenesis. In separate experiments done in the adult mouse brain and primary neurons, we show that miR-137 is significantly upregulated in the absence of MeCP2 (APPENDIX A). Thus, because MeCP2 can alter expression of specific miRNAs, including miR-137, we proposed that altered expression of miRNAs may contribute to the dendrite and dendritic spine pathogenesis observed in Rett Syndrome.

In Chapter 6, I demonstrate that miR-137 is a brain-enriched microRNA that has a significant role in regulating neuronal maturation in vivo and in vitro (Smrt et al, 2010).

Overexpression of miR-137 inhibited dendritic morphogenesis, phenotypic maturation, and spine development both in brain and cultured primary neurons. Similarly, a reduction in miR-137 had opposite effects. Our research group produced a luciferase construct containing the 3'UTR of *Mib1*, which I used to show that miR-137 targets the MIB1 protein through the conserved target site located in the 3' untranslated region of *Mib1* mRNA. Exogenously expressed MIB1 partially rescued the phenotypes associated with miR-137 overexpression suggesting that a novel miRNA-mediated mechanism involving miR-137 and MIB1 can function to regulate neuronal maturation and dendritic morphogenesis during development. I used the "single-cell genetic approach" in newborn neurons of the adult hippocampus and found reduced dendritic complexity and spine density; however, since the single-cell genetic approach specifically targets proliferating cells prior to neuronal differentiation, I also confirmed that overexpression of miR-137 has the same effect on postmitotic cultured hippocampal neurons. Both overexpression and inhibition of miR-137 have significant but opposite effects on dendritic complexity. Therefore, my data indicate that proper expression of miR-137 is required for the normal dendritic development of hippocampal neurons.

Chapter 7 expands the discussion of the previous chapters. In Chapter 7, I summarize the significance of my thesis research, address specific concerns and limitations regarding my research, define specific steps that I have taken to address many of those concerns, and propose additional experimental procedures that may function to strengthen my previous findings. Finally, I discuss potential future directions of this project based on my current findings.

In summary, the results of my dissertation research demonstrate that epigenetic regulations, particularly those involving MeCP2 and epigenetically controlled noncoding microRNAs, are important modulators for normal development of neuronal dendrite and

dendritic spines. My data show how alterations in epigenetic and non-coding RNA-mediated processes may result in morphological and phenotypic abnormalities of neurons that are a fundamental characteristic for many forms of MR, such as fragile X, autism, and Rett syndrome.

CHAPTER 5:
Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons

Richard D Smrt[1], Julialea Eaves-Egenes [1], Basam Z. Barkho[1], Nicholas J. Santistevan[1], Chunmei Zhao[2], James B. Aimone[2], Fred H. Gage[2], and Xinyu Zhao[1]

[1] Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131

[2]The Salk Institute for Biological Studies, Laboratory of Genetics, La Jolla, CA 92037.

(Published in Neurobiology of Disease. 2007; 27: 77–89.)

5.1 ABSTRACT

It is well known that Rett Syndrome, a severe postnatal childhood neurological disorder is mostly caused by mutations in the human *MECP2* gene. However, how deficiencies in the MeCP2 protein contribute to the neurological dysfunction of Rett Syndrome is not clear. We aimed to resolve the role of MeCP2-mediated epigenetic regulation in postnatal brain development using a *Mecp2*-null mutant mouse model. We found that, while MeCP2 was not critical for the production of immature neurons in the dentate gyrus (DG) of the hippocampus, the newly generated neurons exhibited pronounced deficits in neuronal maturation, including delayed transition into a more mature stage, altered expression of presynaptic proteins, and reduced dendritic spine density. Furthermore, analysis of gene expression profiles of isolated DG granule neurons revealed abnormal expression levels of a number of genes previously shown to be important for synaptogenesis. Our studies suggest that MeCP2 plays a central role in neuronal maturation, which might be mediated through epigenetic control of expression pathways that are instrumental in both dendritic development and synaptogenesis.

5.2 Introduction

Rett Syndrome (RTT) is a neurodevelopmental disorder that affects one of every 15,000 female births. RTT patients develop normally until 6 to 18 months of age, but then regress rapidly experiencing a wide range of neurological defects, such as seizures, ataxia, and stereotypical hand movements. Individuals affected by RTT often survive into adulthood, and while some symptoms stabilize, others may worsen (Hagberg et al., 1983b; Hagberg and Witt-Engerstrom, 1986; Kriaucionis and Bird, 2003). In most cases, RTT can be linked to loss-of-function mutations in the X-linked human *MECP2* gene (Amir et al., 1999), which encodes a methylated-CpG binding protein that recruits additional factors such as histone deacetylase to repress transcription (Bird, 2002a). Several lines of *Mecp2* mutant mice (KO) have been generated and these mice develop similar symptoms to those seen in RTT patients and have been widely used to study the etiology of human RTT (Chen et al., 2001b; Guy et al., 2001; Pelka et al., 2006; Shahbazian et al., 2002a). Nevertheless, the neurodevelopmental pathways and specific genes targeted by the disruption of this epigenetic regulatory control have not been determined.

Recent experimental evidence indicates that MeCP2 may play a vital role in neuronal maturation (Bienvenu and Chelly, 2006). A critical step in the process of neuronal maturation is synaptogenesis, which coincides with the increased expression of MeCP2 in developing neurons (Akbarian et al., 2001b; Zoghbi, 2003) (Shahbazian and Zoghbi, 2002a), suggesting that epigenetic modulation of gene regulation during this period might be critical for brain development. In fact, postmortem analysis has demonstrated reduced numbers of axonal and dendritic processes, decreased dendritic spine density, and lowered levels of the dendritic cytoskeletal protein MAP2 in RTT brains (Armstrong, 2002; Kaufmann and Moser, 2000b). Consistent with human pathology, pyramidal neurons in the cortex of adult *Mecp2* null mutant

(KO) mice were found to have smaller soma and less complex dendrites, though the morphology and density of dendritic spines were not determined in this study (Kishi and Macklis, 2004). Exogenous MeCP2 expression could also lead to increased neurite complexity in cultured neurons (Jugloff et al., 2005), further suggest a role of MeCP2 in dendritic development. However, in another study, analyses of Golgi-stained cortical and subcortical neurons of *Mecp2* truncation mutant mice (*Mecp2*$^{y/308}$) did not reveal significant abnormalities in either dendritic arbor or spine density (Moretti et al., 2006). The discrepancy between these results could be due to differences in either the model systems analyzed or the methods used. Abnormalities in dendritic spines have been found in several developmental disorders [reviewed by (Fiala et al., 2002)]. Therefore it is critical to clarify whether *Mecp2* mutations affect spine development by monitoring the maturation of single neurons in a well defined cell population in order to understand the function of MeCP2 in neural development and the etiology of RTT.

Unlike most other brain regions, neurogenesis in the adult dentate gyrus (DG) persists throughout life. In adult mice, newborn DG neurons develop properties similar to mature granule neurons after approximately 4-8 weeks of differentiation. The properties of newborn neurons in the adult DG recapitulate embryonic hippocampal development (Song et al., 2005), providing a unique model system for studying the generation and maturation of neurons in postnatal brains (Gage, 2002). The hippocampus also provides a logical framework to study the pathogenesis of MeCP2 deficiency because the morphological maturation, functional properties, and molecular mechanisms of the hippocampus have been extensively characterized due to their potentially critical roles in learning and memory (Nicoll and Schmitz, 2005; Ziv and Garner, 2004), and because *Mecp2* KO mice have been shown to have impaired long-term potentiation and

depression, impaired excitatory neurotransmission, and altered expression of neurotransmitter receptors in hippocampal neurons (Asaka et al., 2006; Moretti et al., 2006; Nelson et al., 2006).

MeCP2 has been found to be expressed in neural stem cells (NSCs) (Jung et al., 2003; Namihira et al., 2004). While MeCP2 was shown to be involved in embryonic neurogenesis in Xenopus, studies have indicated that this is not the case in mice (Kishi and Macklis, 2004; Stancheva, 2003). Recent evidence has revealed that adult NSCs are different from embryonic NSCs in both the cellular environment they encounter and in their intrinsic genetic and epigenetic properties (Cheng et al., 2005; Zhao et al., 2003). Moreover, deletion of *Mecp2*-related Methyl-CpG binding protein 1 (*Mbd1*) specifically affects postnatal, but not embryonic, neurogenesis (Zhao et al., 2003), suggesting that postnatal neurogenesis may be particularly vulnerable to altered epigenetic regulation. Therefore, analyzing postnatal neurogenesis in the absence of MeCP2 will provide critical information for understanding the function of this protein.

In this study, we have determined that MeCP2 is not critical for the early stages of neurogenesis. In contrast, we show that immature neurons in the DG of KO mice exhibit deficits in their ability to transition into later mature stages of development. This deficit results in adult *Mecp2* KO mice retaining characteristic features of immature brains, suggesting a stalled maturation. At a single neuronal level in the postnatal hippocampus, MeCP2-deficient neurons exhibited a reduced number of dendritic spines. By analyzing gene expression profiles of a homogeneous population of DG neurons isolated from KO brains, we have found that the expression levels of several genes encoding proteins that are likely to be involved in synaptogenesis were altered. Together, these data suggest that MeCP2 is critical for the maturation of young neurons, possibly through regulating synaptogenic factors.

5.3 Materials and Methods

Animals: All animal procedures were performed according to protocols approved by the University of New Mexico Animal Care and Use Committee. The *Mecp2* KO mice (*Mecp2*$^{tm1.1Jae}$) used in this study were created by deleting exons 3 containing the MBD domain of *Mecp2* (Chen et al., 2001b). These mice have been bred over 40 generations on to ICR background. They start to show neurological symptoms between 5 and 7 weeks of age and die before 10 weeks of age. For histological analyses, mice were euthanized by intraperitoneal injection of sodium pentobarbital. Mice were then perfused with saline followed by 4% PFA. Brains were dissected out, post-fixed overnight in 4% PFA, and then equilibrated in 30% sucrose. Forty-µm brain sections were generated using a sliding microtone and were stored in -20°C freezer as floating sections in 96-well plates filled with cryoprotectant solution (glycerol, ethylene glycol, and 0.1M phosphate buffer, pH 7.4, 1:1:2 by volume).

Statistical analyses: All statistical analyses were performed using unpaired, two-tailed, Student's t-test and in all figures, the data bars and error bars indicate mean ± standard error (s.e.m).

Isolation and in vitro analyses of adult NSCs: Isolation of adult NSCs was performed based on the published method (Zhao et al., 2003). Briefly, forebrains without olfactory bulb and cerebellum (4 mice/ genotype, age- and sex-matched) were dissociated mechanically followed by enzymatic digestion using PPD (2.5 U/ml papain, 1U/ml DNAseI, and 200 mg/100 ml Dispase II) in DMEM high glucose (Cellgro, Herndon, VA). After filtering through a 70-µm cell strainer (BD Falcon, San Jose, CA), a single cell suspension was loaded onto 50% percoll. The NSCs were separated from other cells by ultracentrifugation at 127 krpm for 30 min at 20°C using a

SW41 rotor (Beckman, Fullerton, CA). The fraction containing NSCs (immediately above the red blood cell layer in the gradient) was collected, washed with PBS, and plated in N2 medium (DMEM/F12 1:1 containing N2 supplement (Invitrogen, Carlsbad, CA) supplemented with 20 ng/ml FGF-2 and 20 ng/ml EGF in a 5% CO_2 incubator. Cell proliferation analyses were performed as described (Lie et al., 2005). Briefly, BrdU was added to the culture medium at 5 μM for 16 hours, followed by fixation using 4% PFA. Cells were then stained with antibodies against BrdU (1:500, Accurate Chemicals, Westbury, NY) and Ki67 (1:1000, NovoCastra Laboratories, Newcastle upon Tyne, UK) and 10 μg/ml DAPI. The percentage of $BrdU^+$ cells or $Ki67^+$ over total $DAPI^+$ cells indicates the percentage of cells that are proliferating. For in vitro differentiation analysis, cells were incubated in N2 media containing 1 μM forskolin, 1 μM all-trans retinoic acid and 0.5% FBS for 7 days. Cells were then fixed by 4% PFA, followed by immunocytochemical analysis as described previously (Zhao et al., 2003). Primary antibodies used were: rabbit anti-type III β-tubulin (1:4000, Covance, Berkeley, CA), RIP (1:50, Hybridoma Bank, Iowa City, Iowa), s-100β (1:1000; Sigma-Aldrich, St Louis, MO), and all secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used in 1:250 dilution. Cell phenotypes were analyzed using an Olympus BX51 Research microscope equipped with epifluorescence, an optronics microfire digital color camera, and StereoInvestigator software (MicroBrightField). Cell counting was performed using an optical fractionator sampling design and formula (Gundersen et al., 1988). Four independent experiments (each had triplicates), using similar passages of cells, were performed for in vitro proliferation and differentiation assays.

In vivo neurogenesis analyses: *In vivo* neurogenesis analyses were performed essentially as described previously (Zhao et al., 2003). Briefly, in 8-week-old mice (11 WT and 8 KO), BrdU

(50mg/kg) was injected daily for 7 consecutive days to increase the amount of labeling. In 4-week-old mice (6 WT and 9 KO), BrdU was injected once daily for 4 consecutive days. Mice were then euthanized 1 day post-injection to assess proliferation (and early survival) of labeled cells. For cell survival analysis, mice injected at 4 weeks of age (6 WT and 9 KO, 1 injection/day for 4 days) were euthanized 4 weeks post-injection. For immunohistological analysis, 1-in-6 serial floating brain sections (240 μm apart) were performed based on the published method (Zhao et al., 2003). The primary antibodies used were: rat-anti-BrdU (1:500; Accurate Chemicals), mouse anti-NeuN (1:5000; Chemicon International, Temecula, CA), rabbit anti-S-100β (1:500; Sigma), and chicken anti-Mecp2 (1:5000, a generous gift from Dr. Janine LaSalle University of California, Davis). Fluorescent secondary antibodies were used at 1:250 dilutions (donkey, Jackson ImmunoResearch). After staining, sections were mounted, coverslipped, and maintained at 4°C in the dark until analysis. BrdU-positive cells in the granule layer were counted using unbiased stereology (StereoInvestigator, MicroBrightField) with a 5-μm guard zone as described elsewhere (Zhao et al., 2003). DG volume and cell density determinations were performed as described (Zhao et al., 2003). Phenotype analysis of BrdU$^+$ cells was performed as described previously (Zhao et al., 2003). Briefly, 50 BrdU$^+$ cells in the DG were randomly selected and their phenotypes (double labeling with either NeuN, S100β, or neither) were determined using a Zeiss LSM510 laser scanning confocal microscope. The data were analyzed using a Student's t-test (Graphpad software, www.graphpad.com).

Quantification of mature, immature, and "transitioning neurons" in the DG: This procedure was performed based on the published method (Brown et al., 2003). Briefly, 40-μm thick coronal tissue sections containing hippocampus were stained with antibodies against DCX (1:1000, goat,

Santa Cruz), NeuN, and DAPI. The immunofluorescence signals were captured using a spinning disk confocal microscope (Nikon Eclipse TE2000-U, 40x oil,1.2 NA). Quantification was done by a person who was blind to the genotypes of the mice. The numbers of $NeuN^-/Dcx^+$, $NeuN^+/DCX^+$, $NeuN^+/DCX^-$, and cells were quantified by examining z-stacks taken at 1-um intervals using MetaMorph imaging software (Molecular Devices Inc., Sunnyvale, CA). A total of 5 WT and 6 KO mice for the 4-week time point and 8 WT and 5 KO mice for the 8-week time point were used. Three z-stacks were taken from each animal and 6 image planes per optical stack were used for quantification.

Immunohistological analyses and quantification of synaptophysin: Staining and analyses of synaptophysin immunoreactivity were performed according to published method (Li et al., 2002). Briefly, 40-µm thick brain sections were incubated in primary antibody against synaptophysin (rabbit, 1:100; Zymed, San Francisco, CA), followed by biotinylated secondary antibody (donkey anti rabbit IgG; 1:250; Jackson ImmunoResearch), then incubated in ABC reagent (VECTASTAIN ABC Kit, Vector Laboratories) and detected by diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, CA). The sections were then thoroughly washed, mounted, air dried, and coverslipped with Permount (Biomedia Corp., Foster City, CA). Sections incubated with normal rabbit IgG instead of a primary antibody (Sigma-Aldrich) were used as negative controls. Optical density analysis of synaptophysin staining was performed by placing 10 circles in each region using Image-J software, as described elsewhere (Li et al., 2002). To categorize the clustered staining pattern, 63X (Zeiss Axioscope, NA = 1.4) images of brain sections were used. Images were captured at 1300 pixels x 1030 pixels using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Using Image-J (NIH) image analysis software, a

threshold value was determined for positive staining and remained the same throughout the data analysis. Large clusters were determined to be clusters greater than 300 $pixels^2$ and were quantified using the "Analyze Particles" function of Image-J. Sections with 0-5 "large clusters" in the molecular layer of the hippocampus were placed into category 1, and sections with greater than 5 "large clusters" were placed into category 2. The experimenter was blind to the genotypes of the sections. The number of large clusters in each category was counted for each genotype and age group and the data were used to create Figure 5.3e.

Retroviral grafting. Production of CAG-eGFP retrovirus and in vivo grafting into the DG of mice were performed as described elsewhere (Zhao et al., 2006). Briefly, CAG-eGFP plasmid was co-transfected with packaging plasmids pCMV-gag-pol and pCMV-Vsvg into HEK293T cells and the medium containing virus was collected, filtered, and concentrated using ultracentrifugation. For in vivo grafting, 4-week-old mice were anesthetized with isofluorane and virus (1.5 µl with titer greater than $5X10^5$/µl) was injected stereotaxically into the DG using the following coordinates relative to bregma: anteroposterior, -(1/2) x d mm; lateral, -1.8 mm (if d > 1.6) or -1.7 mm; ventral, -1.9 mm (from dura). Four weeks after injection, mice were deeply anesthetized with pentobarbital and perfused with saline followed by 4% PFA.

Immunohistochemistry and dendritic spine density analyses: 1-in-3 floating brain sections containing $eGFP^+$ cells (120 µm apart, approximately 8 brain sections) were used for immunohistological staining using a protocol described elsewhere (Zhao et al., 2003). The primary antibody used was rabbit anti-GFP (Invitrogen, Eugene, OR). Briefly, for spine quantification, a minimum of 12-15 images of dendritic fragments were taken at 25-100 µm from

the cell body of each $eGFP^+$ neuron using a confocal microscope with an oil immersion objective (100x; NA = 1.3; Zeiss). Z-stacks at 1 μm intervals were taken and merged for a maximum intensity projection. For quantification of dendritic spines, protrusions were counted along 10 μm long dendrite segments measured using Image-J software (NIH Image). The "dendritic spine density" result was calculated as number of spines per 10 μm length of dendrite. A minimum of 40 dendritic fragments (10-μm each) from a minimum of 4 $eGFP^+$ neurons were quantified from each animal. At least 3 animals from each genotype were analyzed and the final results were compared using a Student's t-test. The apposition of immunostained presynaptic boutons with eGFP-expressing postsynaptic spines was determined according to a published method (Belichenko et al., 2004). Briefly, the presynaptic terminal ($synaptophysin^+$) and postsynaptic spine ($eGFP^+$) were defined as apposed when there was an overlap between pre- and postsynaptic elements, or when these elements were separated by no more than one pixel (0.1 μm).

Laser Capture Microdissection (LCM) and gene expression analyses: LCM, amplification by in vitro transcription, and probe labeling were performed using a highly reproducible protocol that has been adapted and optimized to analyze gene expression profiles using as little as 5ng total RNA (Phillips and Eberwine, 1996) (Dr. FH Gage, unpublished). Briefly, 4 KO and 4 WT mice (8 weeks of age) were used. Brains were rapidly removed from the cranium and flash-frozen in OCT mounting medium (TissueTek, Sakura Finetek, Torrance, CA) in a dry ice-isopentane slurry and stored at –80°C. The day before LCM, 12 μm sections of brain were generated, stained with cresyl violate, and dehydrated. The granule cell layers of DG were captured using an Arcturus PixCelII LCM microscope (Arcturus Bioscience Inc., Mountain View, CA). An

example is shown in Figure 5.5. LCM was performed at a power level between 25 and 45 mV, between 2 and 20 ms duration, and at a median spot size setting. A minimum of 30 brain sections (less than half of the sections generated from each brain) were used to capture sufficient DG neurons for this study. Captured tissue was dissolved in cell lysis buffer in the MicroRNA kit (Stratagene, San Diego, CA) for 2 to 5 minutes immediately after capturing. Total RNA was isolated from cell lysate using a MicroRNA kit (Stratagene), and approximately 20 ng total RNA was obtained. Half of the sample was used to quantify RNA using a Ribo Green kit (Invitrogen). Finally, 10 ng total RNA was amplified using 3 rounds of a MessageAmep kit (Ambion, Austin, TX). During the last round, RNA was labeled with biotin (Enzo kit, Affymetrix, Santa Clara, CA), purified, and quantified. Biotin-labeled cRNA (30 μg) was fragmented and hybridized to Affymetrix U430 arrays (Affymetrix). Analysis of microarray data was performed using three distinct software programs as described in our previous publication (Barkho et al., 2006). Briefly, the data were pre-processed using the Affymetrix Microarray Analysis Suite (MAS) 5.0 and subsequently analyzed using dChip (Li and Wong, 2001), Drop Method (Aimone and Gage, 2004), and the algorithms of RMA (Irizarry et al., 2003; Tusher et al., 2001). The combination of these methods has been shown to reduce both false positives and false negatives (Aimone et al., 2004; Barkho et al., 2006). Specifically, for dChip, we selected genes that passed with 90% confidence and had a fold change greater than 1.2. For the Drop method (the PM-only, and PM-MM are considered as one method), we selected genes that passed with a confidence of 70%. For RMA, a fold change had to meet the criteria of being greater than 1.2 and have a false discovery rate (FDR) less than 30%. The FDR value used for RMA was based on the Significance for Analysis of Microarrays software (SAM) (Tusher et al., 2001). Only genes identified by all three software programs were included as differentially expressed genes and are listed in Table 5.1.

Real time quantitative PCR: Real time quantitative PCR was performed as described (Barkho et al., 2006; Zhao et al., 2001). Briefly, the cDNA was synthesized using MessageAmep kit, (Ambion) for LCM samples. PCR primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA) and ordered from IDT Inc. (CA). The primer sets were first evaluated by standard PCR to determine that single PCR products of the predicted size were generated. A typical Real Time PCR reaction mix contained 1X SYBR Green Master Mix (Applied Biosystems), 100 nM of each oligonucleotide primer and 10 ng cDNA in a total volume of 25µl. The reaction was carried out in an ABI 7700 System (Applied Biosystems). Each condition was acquired in at least triplicate, and data analysis was performed according to the protocol provided by Applied Biosystems. Standard curves were generated using a pre-made pool of mouse brain and spinal cord total RNA. The amount of mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, hypoxanthine phosphoribosyl transferase (HPRT).

5.4 RESULTS

5.4.1 Early postnatal neurogenesis appears normal in MeCP2-deficient mice

We have previously found that mice deficient for Mbd1, a MeCP2-related protein, exhibited reduced adult hippocampal neurogenesis both in vivo and in vitro (Zhao et al., 2003). Because RTT manifests at 6-18 months of age in patients, well after primary neurogenesis, we asked

whether a lack of functional MeCP2 causes deficits in postnatal neurogenesis that might be linked to neurological symptoms comparable to those seen in RTT patients. We compared neural stem/progenitor cells (NSCs) isolated from 6-week-old male KO and wild type (WT) mice. At this age, the majority of KO mice have developed characteristic neurological signs of disease as previously reported (Chen et al., 2001b; Guy et al., 2001). Using bromodeoxyuridine (BrdU) incorporation as a measure of proliferation index, we found that KO NSCs proliferated at a rate that was indistinguishable from WT control cells (Fig 1A and 1B, p=0.41). Moreover, KO and WT cultured NSCs also differentiated into similar numbers of neurons (Fig 5.1C and 1D, p=0.43) and astrocytes (data not shown), indicating that there was no marked impairment in the proliferation or differentiation potential of MeCP2-deficient NSCs in culture.

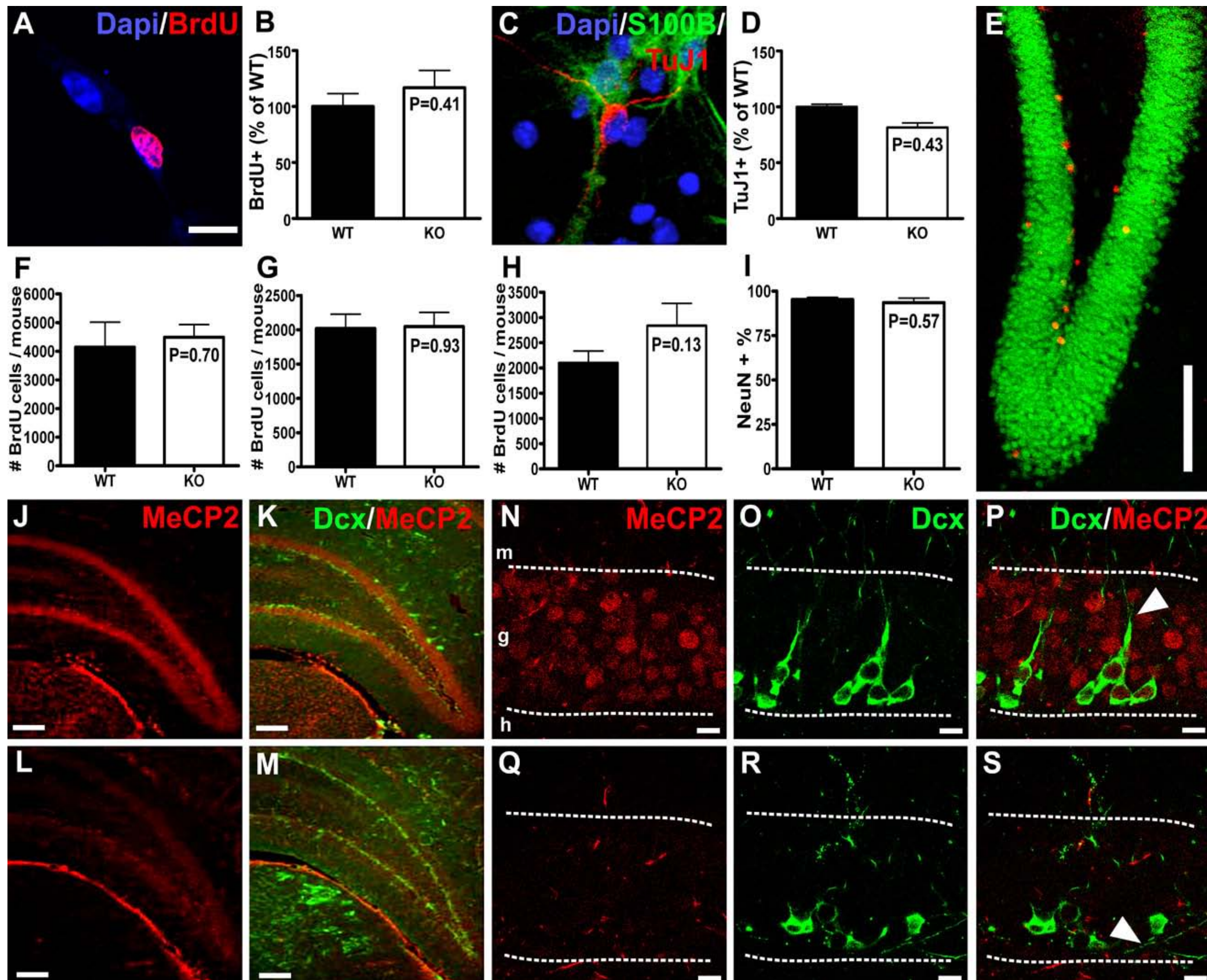


Figure 5.1. ***Mecp2*** **KO mice exhibit a normal early stage of postnatal neurogenesis even though immature neurons have an abnormal morphology.** (A) For cell proliferation analyses, NSCs isolated from 6-week-old KO and WT brains were cultured in the presence of BrdU to label dividing cells. BrdU-labeled cells were detected by immunocytochemistry (red, BrdU; blue: DAPI nuclear staining; scale bar=10 µm). (B) Quantitative analyses of BrdU-labeled cells indicated no significant difference in cell proliferation between KO and WT NSCs in vitro (p=0.41, n=3, t-test). (C) *Mecp2* KO NSCs can differentiate into neurons ($TuJI^+$, red) and astrocytes ($S100b^+$, green) (blue: DAPI nuclear staining); scale bar=10 µm. (D) There was no significant difference in neuronal differentiation between KO and WT NSCs in vitro (p=0.43, n=3 t-test). (E) Example of a brain section stained with antibodies to NeuN (green) and BrdU (red) for in vivo neurogenesis analyses. Neither 4-week-old (F) nor 8-week-old (G) KO mice exhibited significant deficits in the number of $BrdU^+$ cells at either 1 day post-BrdU injection (F and G) or 4 weeks post-BrdU injection (H). At 4 weeks post-labeling, $BrdU^+$ KO cells differentiated into similar numbers of new neurons (I) compared to WT mice. Low magnification (J-M, scale bar=100 µm) and high magnification (N-S, scale bar=10 µm) images of DG stained with antibodies against Mecp2 (red nuclear staining) and DCX (green). Note that Mecp2 staining is absent in KO brains (L, M, Q-S). (N-S) DCX^+ immature neurons in KO brains have disorganized morphologies compared to those in WT brains (arrowhead in P), with abnormal orientation of the processes of many DCX^+ neurons (arrowhead in S). The dotted lines in N-S indicate the boundary of the granule cell layer. m, molecular layer, g, granule cell layer, and h, hilar region.

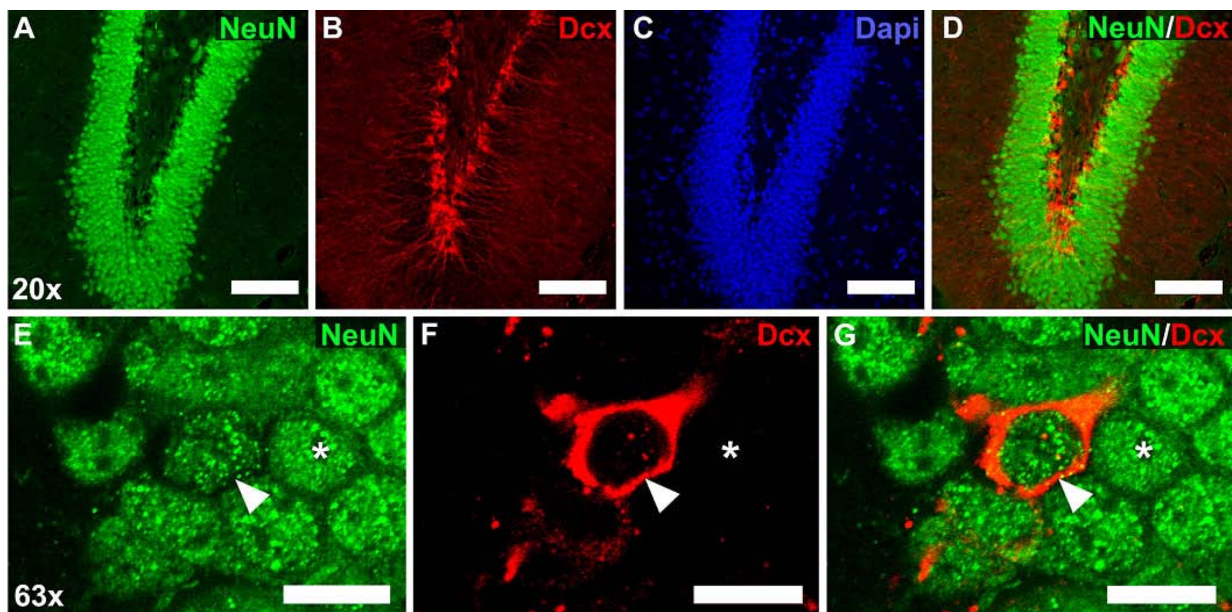
To examine postnatal neurogenesis in vivo, we assessed proliferation, survival, and differentiation of NSCs in the hippocampus of young mice. In the rodent hippocampus, granule cells of the DG develop postnatally, becoming morphologically mature at about 4 weeks of age, which corresponds roughly to the second year in humans (Seress et al., 2001; Seress and Mrzljak, 1992), when RTT symptoms first become apparent. Newborn cells were distinguished by incorporation of BrdU administered through intraperitoneal injections into either 4 week-old juvenile or 8-week-old young adult mice. Quantitative histological analysis at one day after the last BrdU injection showed no difference between KO and WT mice either at 4 or 8 weeks of age (Fig 5.1E-G). This finding suggested that, as found in cultured NSCs, the NSCs in KO mice proliferate normally in the DG of developing and mature mice. In a separate group of mice, long-term survival and differentiation of BrdU-labeled cells were examined by analyzing labeled cells 4 weeks after BrdU injections. We found that the numbers of BrdU-labeled cells that survived from 4 weeks to 8 weeks were also similar in both KO and WT mice (Fig 5.1H, p=0.13). Moreover, the percentage of BrdU-labeled cells co-labeled for either the neuronal marker NeuN (Fig 5.1I) or astrocyte marker GFAP (data not shown) did not differ significantly between KO

and WT mice. Furthermore, neither the cell density nor the volume of the DG of KO mice was significantly different from WT mice (data not shown). Finally, to investigate whether there was a difference in the population of transient migrating neuroblasts or immature neurons, we quantified the number of cells stained with doublecortin (DCX), a microtubule associated protein (Brown et al., 2003) Francis et al., 1999; Gleeson et al., 1999; Magavi et al., 2000). Again, we found no difference between the number of DCX-positive cells in the DG of KO and WT mice (Fig 5.1J-S). Taken together, these *in vitro* and *in vivo* results strongly suggest that the lack of MeCP2 does not impair the proliferation, survival, or differentiation of neural progenitors during this early stage of postnatal hippocampal neurogenesis.

5.4.2 Impaired maturation of MeCP2-deficient neurons

Despite the overall similar numbers of immature neurons in the DG of KO and WT mice, we noticed clear abnormalities in the neurite outgrowth from MeCP2-deficient immature neurons (Fig 5.1R and S). In the DG of WT mice, the cell bodies of the DCX^+ neurons were typically located in the subgranular zone (SGZ) of the DG adjacent to the hilar region, with their processes perpendicularly extended through the granule cell layer (GCL) and exited into the opposite molecular layer (Fig 5.1N-P, arrowhead) (Brown et al., 2003; Rao et al., 2005; Rao and Shetty, 2004). In contrast, the processes of most DCX^+ neurons in the DG of *Mecp2* KO mice were found to traverse nearly parallel along the hilar boundary of the DG (Fig 5.1Q-S, arrowhead). This morphological difference might reflect impaired maturation because since horizontal and short processes are characteristic features for new neurons at early stage of differentiation (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). Because the majority of the DG granule neurons are generated during postnatal development, we exploited the possibility that this deficit

was widespread in the developing DG. We therefore compared the ratio of NeuN$^+$ mature and DCX$^+$ immature neurons in the DG of *Mecp2* KO and WT mice at two different ages: when the DG had just past the peak of primary cell genesis (4 weeks of age) and when DG had reached the adult level of maturity (8 weeks of age) (Fig 5.2A-G) (Mullen et al., 1992). Furthermore, we quantified the number of cells positive for both NeuN and DCX to distinguish the cells that were transitioning from an immature to a mature phenotype and to provide an additional index of this developmental maturation process (Fig 5.2E-G).



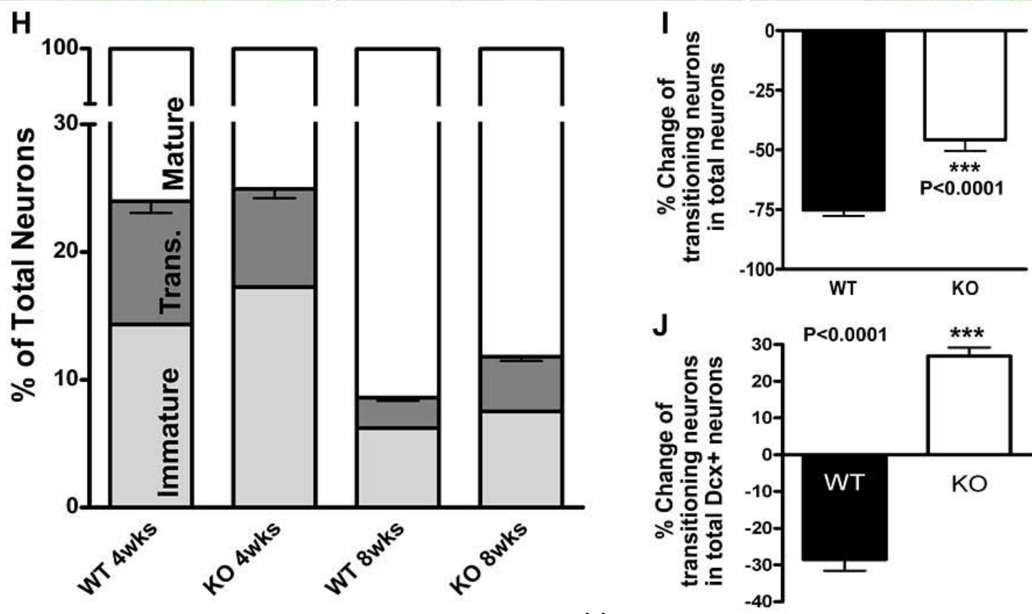


Figure 5.2. Immature neurons in the DG of *Mecp2* KO mice have delayed transitioning to mature stage. (A-D) Confocal images showing DG of the hippocampus labeled with antibodies against NeuN (A, green, mature neurons), DCX (B, red, immature neurons), and DAPI (C, blue, nuclear dye). Scale bars=100 µm. (D) Merged image of A-C. (E-G) Higher magnification images of granule neurons and examples of neurons that are $NeuN^+DCX^-$ (asterisk) and $NeuN^+DCX^+$ (arrowhead). Scale bars=10 µm. (H) Quantitative analyses indicate the percentage of mature neurons ($NeuN^+DCX^-$, white bar), immature neurons (DCX^+NeuN^-, light gray) and “transitioning neurons” ($NeuN^+DCX^+$, dark gray) in 4-week-old and 8-week-old mice. (I) Age-dependent changes in the proportion of “transitioning neurons” over total neurons are significantly different between KO and WT mice ($p<0.0001$), (J) The percentage of “transitioning neurons” among total DCX^+ neurons are also significantly different between WT and KO mice at both 4 and 8-weeks-old. While WT mice exhibited an age-dependent decrease in the proportion of “transitioning neurons” over total DCX^+ neurons, KO mice displayed an increase ($p<0.0001$), suggesting more neurons are stalled at the transitioning stage in the KO brains.

Quantitative analysis of confocal images was used to determine the percentage of neurons in each maturation stage (Fig 5.2A-G, $DCX^+/NeuN^-$; $DCX^+/NeuN^+$; $DCX^-/NeuN^+$) in both the KO and WT DG. The results summarized in Figure 5.2H show that whereas neither 4- nor 8-week-old *Mecp2* KO mice displayed significant differences in the percentage of mature or immature neurons compared to WT mice, the DG of 8-week-old *Mecp2* KO mice had a significantly higher percentage of “transitioning neurons” ($p<0.001$). In fact, comparison of the number of “transitioning neurons” ($DCX^+/NeuN^+$) among total neurons showed that maturation from juvenile (4 weeks) to young adult (8 weeks) was accompanied by a 75 % decrease in the percentage of “transitioning neurons” in normal WT brain but only a 44% reduction in the KO brains (Fig 5.2I, $p<0.0001$). In addition, comparison of the number of “transitioning neurons” ($DCX^+/NeuN^+$) and total immature neurons (both $DCX^+/NeuN^-$ and $DCX^+/NeuN^+$) showed that maturation from a juvenile (4 weeks) stage to a young adult (8 weeks) stage was accompanied, in the normal WT brain, by a 28.5% decrease in the percentage of “transitioning neurons” among the total population of DCX^+ neurons (Fig 5.2J). However, the age-dependent reduction of this subpopulation of double positive “transitioning neurons” among total immature neurons did not occur in MeCP2-deficient mutants; but rather there was a 26.9% increase in the percentage of these neurons during this developmental time period (Fig 5.2J, $p<0.0001$). This finding may

indicate that the deficiency in MeCP2 may have led to $DCX^+/NeuN^+$ double positive "transitioning neurons" being held up and failing to differentiate into more mature $DCX^-/NeuN^+$ neurons.

5.4.3 Impaired expression of developmentally regulated presynaptic proteins in MeCP2-deficient hippocampus

Because synaptogenesis is a crucial step for the maturation and integration of newborn neurons into the preexisting circuitry of the hippocampus, we investigated whether KO mice have deficits in synapse formation. For this purpose, we used an antibody against synaptophysin, a synaptic vesicle protein whose expression is known to reflect the distribution and density of presynaptic terminals (Li et al., 2002). We focused on the molecular layer of the hippocampus, where DG granule neuron dendrites receive input from perforant path axons from the entorhinal cortex (Henze et al., 2000). Overall, no consistent difference was found in optical density measurements of synaptophysin immunostaining between 4- and 8-week-old WT and KO mice suggesting that the level of expression, and thus the number of nerve terminals, was not affected by the lack of MeCP2. However, we did observe two patterns of synaptophysin immunoreactivity in both WT and KO mice: highly distributed small positive spots (<300 pixels^2, Fig 5.3C, arrowheads), and "large clusters" (>300 pixels^2, Fig 5.3C and 3D, arrows) that appeared to vary in density in different brain regions. To quantify the distribution of these large clusters and determine whether they might differentiate between the synaptic density of WT and KO hippocampus, we analyzed the number of "large clusters" (determined by Image-J quantitative software) found in the molecular layer, where dendrites of granule neurons form synapses. We found no significant difference in the number of large clusters in either 4-week-old

or 8-week-old KO mice compared to their age-matched WT littermates (Fig 5.3E). However, when WT mice mature from 4 to 8 week of age, there was an 82.6% decrease in the number of large synaptic clusters in the molecular layer of the hippocampus (4-week, 18.29 ± 4.74, n = 14 mice; 8-week, 3.19 ± 1.10, n = 13 mice; $P < 0.01$), but such an age-dependent change was absent in KO mice ($P = 0.48$), suggesting a failure in dispersing these large clusters into a more uniform distribution of presynaptic terminals in the absence of functional MeCP2.

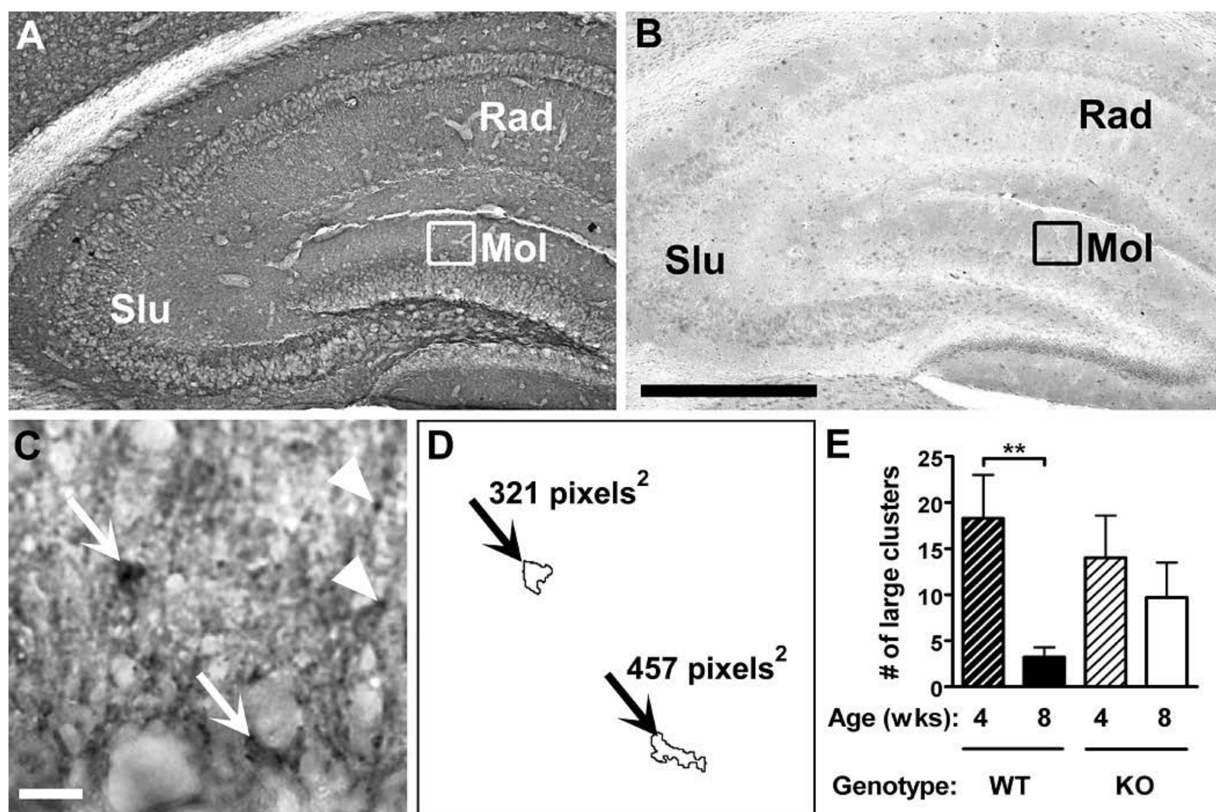


Figure 5.3. ***Mecp2*** **KO mice have altered presynaptic protein expression pattern.** (A-C) Digitized bright-field micrographs show synaptophysin immunoreactivity in the adult and young mice. Scale bar=100 µm. (A) The box indicates the region that is enlarged in C and D. (B) Sections were incubated with normal rabbit IgG, instead of synaptophysin antibody, as a negative control. (C) Example of a brain section with small synaptophysin positive spots (arrowheads) and two large clusters (arrows). scale bar=10 µm. (D) The output of particle analysis of (C) produced by Image-J showing the two large clusters (arrows). (E) Number of large clusters in 4- and 8-week-old WT mice (**$P < 0.01$) compared to 4- and 8-week-old KO mice. Note that WT animals showed a clear age-dependent reduction in the density of large synapse clusters, whereas KO failed to show this developmental change.

5.4.4 Altered dendritic spine distribution in MeCP2-deficient mutant neurons

As neurons mature the density of dendritic spines increases (Ge et al., 2006; Zhao et al., 2006). Consequently, reduced dendritic density is a common characteristic of the abnormal synaptic development seen in a variety of neurological disorders (Fiala et al., 2002). To clarify the current discrepancy in the literature and determine whether *Mecp2* mutations affect dendritic spine development, we decided to analyze the morphology of individual MeCP2-deficient neurons in vivo. Because the persistent, albeit low level, of postnatal neurogenesis in the DG allowed us to trace the maturation of single new neurons, we performed detailed morphological analyses to investigate the maturation of these newly generated neurons in the MeCP2-deficient mice. Recombinant retroviruses, which are only capable of infecting dividing cells, have been previously used to label and follow the differentiation of NSCs in postnatal DG (Ge et al., 2006; van Praag et al., 2002; Zhao et al., 2006). We therefore injected recombinant retrovirus expressing enhanced green fluorescence protein (eGFP) under a chicken actin promoter (CAG-eGFP) (Zhao et al., 2006) into 4-week-old KO and WT mice and analyzed the morphology of new neurons after 4 weeks, a time when labeled new neurons would be expected to develop the dendritic morphology of fully mature neurons (Ge et al., 2006; van Praag et al., 2002; Zhao et al., 2006) (Fig 5.4A). As shown in Figure 5.4B-E, whereas most of the eGFP$^+$ neurons expressed the mature neuronal marker NeuN, a few of them also expressed DCX, indicating NSCs that had not yet reached a fully matured state. To quantify the density of synapses, we counted the number of spines within each 10-μm segment of dendrites imaged by high-resolution confocal microscopy. Quantitative analyses indicated that the spine density of eGFP$^+$ neurons in the DG of *Mecp2* KO mice was significantly reduced compared to WT mice (Fig 5.4J, WT= 13.46 ± 0.31, KO= 11.41 ± 0.11; $P < 0.005$).

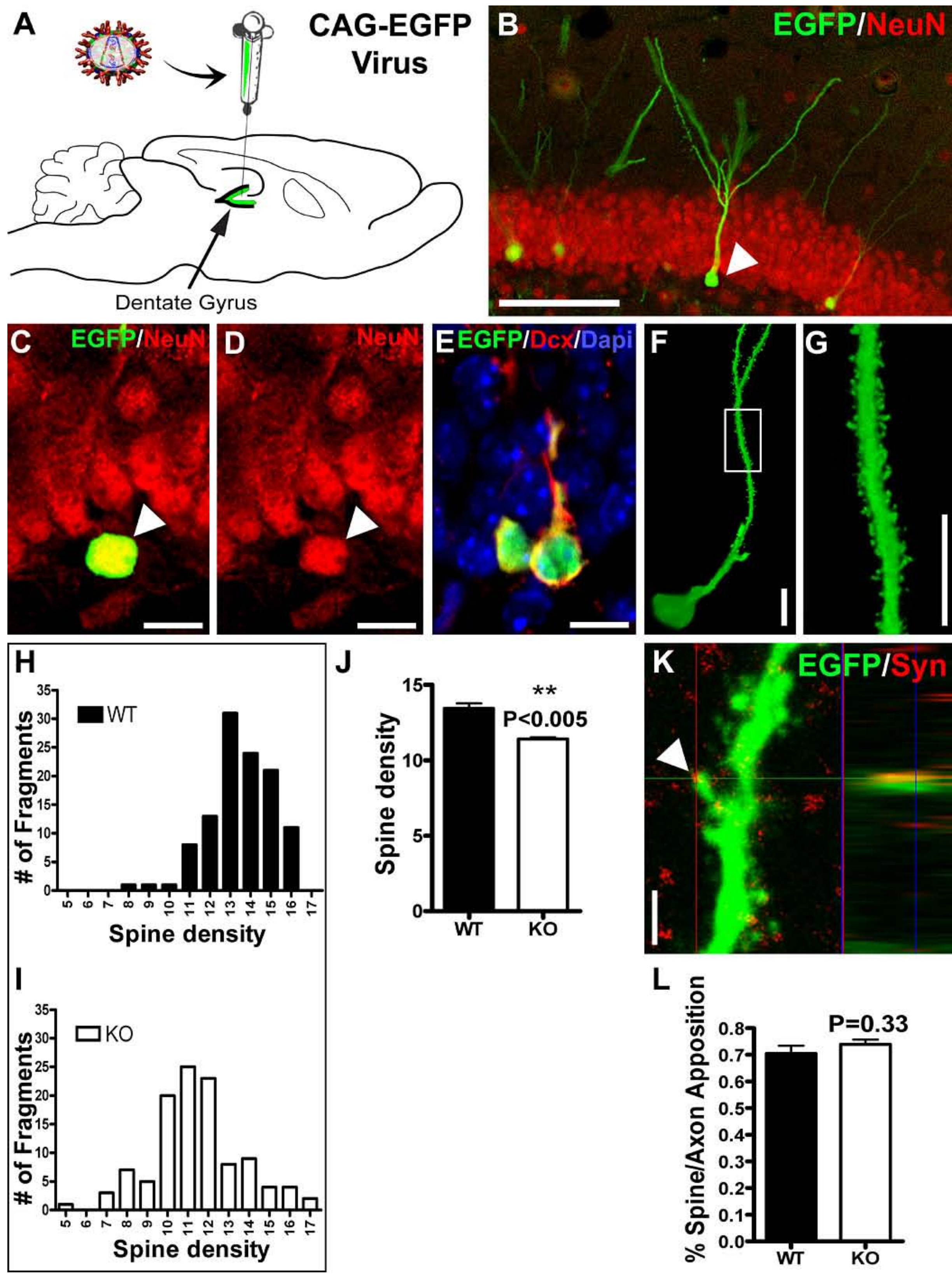

A
CAG-EGFP Virus
Dentate Gyrus
B
EGFP/NeuN
C
EGFP/NeuN
D
NeuN
E
EGFP/Dcx/Dapi
F
G
H
WT
# of Fragments
Spine density
I
KO
# of Fragments
Spine density
J
Spine density
**
P<0.005
WT
KO
K
EGFP/Syn
L
% Spine/Axon Apposition
P=0.33
WT
KO

Figure 5.4. Newly matured neurons in *Mecp2* KO hippocampus have reduced dendritic spine density and abnormal distribution. (A) Schematic diagram demonstrating stereotaxic grafting of CAG-eGFP retrovirus into the DG of 4-week-old KO and WT mice to label dividing neuroprogenitors in the germinal zone of the DG. At 4 weeks post grafting, many eGFP$^+$ cells had differentiated into NeuN$^+$ (B, C, D, arrowheads) and/or DCX$^+$ (E) new neurons. (F, G) High resolution image of dendrites of eGFP$^+$ neurons were used to quantify the density of dendritic spines (number of spines/10 µm dendrites) to generate the data in (H-J). (G) High magnification view of the box in F. Scale bars in F and G=10 µm. (J) New neurons in KO brains had reduced dendritic spine density (P<0.005, n=3 t-test). (H, I) Frequency distribution data indicate higher variation in spine density in KO mice (I) than in WT mice (H), indicating an uneven distribution of spine density. (K) Z-stack confocal image showing apposition of presynaptic terminal marker synaptophysin (red, arrow) with eGFP$^+$ spines of new neurons. (L) Quantitative analyses indicating that similar percentages of eGFP$^+$ spines were apposed to presynaptic terminals in both WT and *Mecp2* KO mice (p=0.33, n=3, t-test)

Because the formation of functional synapses requires apposition of presynaptic terminals and postsynaptic spines, we asked whether the postsynaptic spines of eGFP$^+$ neurons in the DG of KO mice were adjacent to synaptophysin-positive presynaptic terminals. As shown in Figure 5.4K and L, similar percentages of eGFP$^+$ spines in WT and *Mecp2* KO mice were apposed to synaptophysin–positive presynaptic terminals. These data indicate that, at the single neuron level, while newly matured neurons in MeCP2-deficient mice are able to form synaptic contacts, the number of these synapses is greatly diminished.

5.4.5 Altered gene expression of synaptic proteins in MeCP2-deficient DG granule neurons

To investigate the molecular mechanisms that might underlie the deficits in dendritic spine development in the DG of MeCP2-deficient mice, we investigated differential gene expression in the granule cells of the DG isolated by LCM from 8-week-old *Mecp2* KO and WT mice (Fig 5.5A-C). Relative levels of gene expression were determined by hybridization to mouse U430 gene array (Affymetrix) and the data were analyzed using three independent software packages based on distinct algorithms (see Materials and Methods). This bioinformatic strategy of using a combination of different analyses reduces the selection of both false positives and negatives as candidate genes (Barkho et al., 2006). Only genes that were identified by all

three algorithms, as differentially expressed genes, were listed in Table 5.1. We found that the expression of 13 genes was significantly changed in the *Mecp2* KO DG granule neurons compared to WT controls. Consistent with the function of MeCP2 as a transcription repressor, 12 of the 13 differentially expressed genes displayed increased expression in the KO neurons. The *Mecp2* gene was not on the list because a truncated mRNA corresponding to the 3' coding region of *Mecp2*, where Affymetrix probe sets hybridize, was expressed in KO neurons. Among the identified differentially expressed genes, Prefoldin 5 is involved in actin and tubulin folding and cytoskeleton formation (Hartl and Hayer-Hartl, 2002; Nolasco et al., 2005), and Syndecan 2 has been shown to be critical for synaptogenesis (Ethell et al., 2001; Ethell and Yamaguchi, 1999). We have confirmed that the expression levels of both Syndecan 2 and Prefoldin 5 mRNA were higher in KO neurons using Real time PCR analyses (Fig 5.5D-E). Alteration of either of these proteins could potentially affect dendritic development and neuronal morphology.

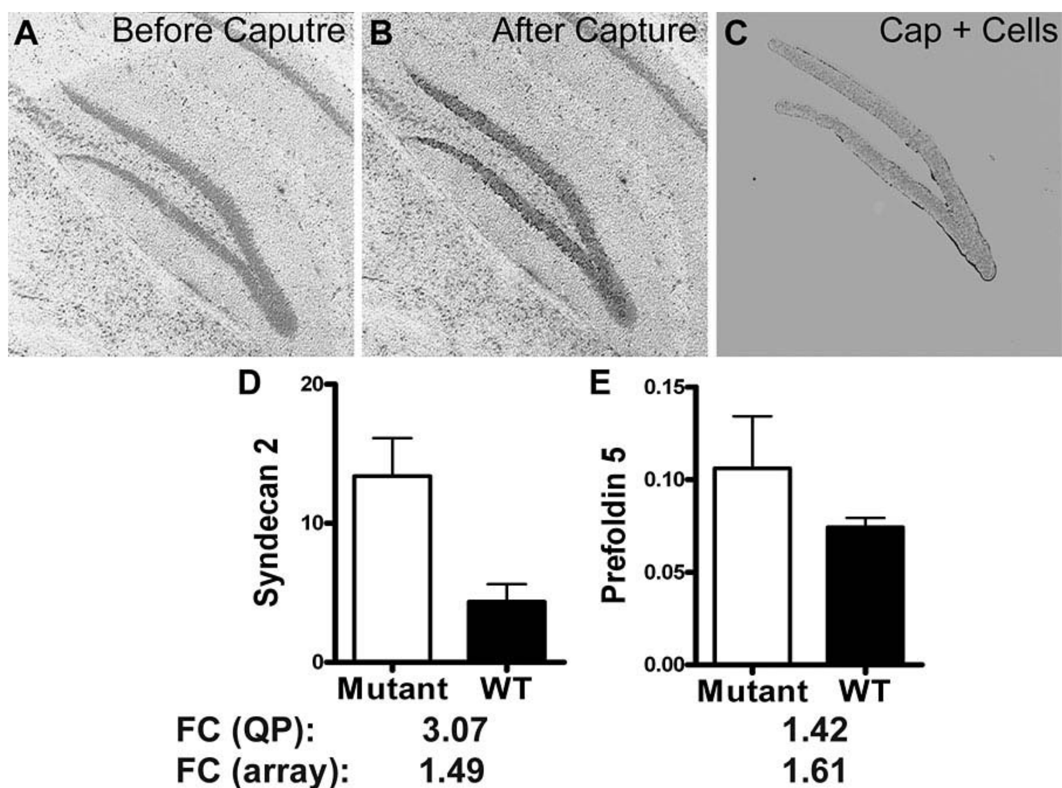


Figure 5.5. Gene expression analyses of LCM-isolated DG neurons indicate altered expression of genes related to synaptogenesis. (A-C) Bright field images demonstrate the process of isolating DG granule neurons from cresyl violet-stained brain sections using LCM. (C) Isolated neurons (dark) were melted into the cap during the LCM procedure and used for RNA isolation. (D-E) Real time PCR analyses confirmed the differential expression of two of the candidate genes, Syndecan 2 and Prefoldin 5. The fold changes determined by real time PCR are consistent with those determined by microarray analyses ($p<0.05$; n=3 experiments with 4 mice/genotype).

5.5 Discussion

With the identification of *MECP2* as the gene responsible for RTT, it becomes critically important to understand the role of MeCP2 in postnatal neural development. In this study, we provide strong evidence that while the lack of MeCP2 does not affect the production of NSCs, it does significantly impair subsequent steps in the maturation of neurons. First, we determined that the expression of specific markers defining the transition from immature to mature neurons was delayed in MeCP2-deficient NSCs. Second, we showed that the age-dependent shift in the expression pattern of synaptophysin in the molecular layer of the hippocampus did not occur in *Mecp2* KO brains. Third, using single neuronal labeling, we found that there were striking defects in the development of dendritic spines and synaptogenesis in DG of KO mice. Finally, the expression levels of several genes that are likely to be important for synaptogenesis, such as Prefoldin 5 and Syndecan 2, were found to be significantly altered in the DG of the hippocampus in *Mecp2* KO mice. This finding points toward a potential mechanism for the epigenetic effects mediated by ablating MeCP2 control of gene expression.

The important role of MeCP2 as an epigenetic regulator at later stages of neural differentiation, in contrast to that reported for Xenopus (Kishi and Macklis, 2004; Stancheva, 2003), is consistent with the normal development of the brain until birth both for RTT patients

and *Mecp2* KO mice. While our results demonstrate clearly that the initial generation of new neurons in the DG was not altered, later steps in synapse formation were significantly perturbed. Although further efforts to examine the effects on early stages of neurogenesis in the SVZ could be useful, our studies, together with those of Kishi and Macklis (Kishi and Macklis, 2004), strongly support the view that MeCP2 is not critical for the production of new, immature neurons in either the embryonic or postnatal mammalian brains.

The progressive acquisition of protein markers followed by the loss of their expression provides a well-defined system to measure the development and differentiation of newborn neural progenitor cells. During normal development, DCX is expressed transiently in immature neurons, and co-expression of DCX and NeuN (DCX^+NeuN^+) marks the end of the early immature neuronal stage. Subsequently, the expression of NeuN, but not DCX, is the hallmark of matured DG neurons (Brown et al., 2003). It seems likely that the age-dependent decrease in the DCX^+NeuN^- immature population and increase in DCX^-NeuN^+ mature population that we observed in both control WT and *Mecp2* KO mice are due to both the decrease in cell proliferation and the increase in total number of granule neurons that occurs from 4 to 8 weeks of age. DCX^+NeuN^+ “transitioning neurons” can be detected as early as 12 days post-differentiation in the DG, and reach their highest level about three days later (Brown et al., 2003). The lower percentage of “transitioning neurons” observed in 4-week-old *Mecp2* KO mice compared to WT mice could result from delayed maturation of DCX^+NeuN^- immature neurons into the “transitioning neuron” stage. The WT adult DG had a lower percentage of “transitioning neurons” than young mice, indicating that the DG is more mature; however, this reduction is much attenuated in adult KO mice, suggesting that more immature neurons either become “stuck” or stay longer in the “transitioning neuron” stage and thus their transition into a mature

stage is delayed. This hypothesis is consistent with previous reports indicating a greater percentage of immature neurons in the olfactory epithelium of *Mecp2* KO mice (Matarazzo et al., 2004), and with histone H3 lysine-9 acetylation and methylation patterns seen in MeCP2-deficient RTT human brains that correspond to an arrest at the transitioning stage of neuronal maturation (Kawasaki et al., 2005). Future studies will be needed to define how MeCP2 regulates the expression of genes critical for this transitioning stage.

Synaptophysin staining has been widely used to indicate the numbers and distribution of neuronal synapses in mammalian brains (Li et al., 2002). However, while most studies have focused on determining the level of expression, we observed two distinct staining patterns (small positive spots and large clusters) in the developing mouse brain. These synaptophysin patterns change in a regulated manner in WT mice, with young brains exhibiting more large clusters than adult brain. The presence of large clusters could, therefore, correlate with immature stages of neuronal plasticity, when synaptophysin may not be well distributed to synaptic sites. If this is the case, the persistence of clustered synaptophysin staining in adult *Mecp2* KO mice would be consistent with the notion that newborn neurons are "stalled" from further maturation in the absence of MeCP2. This stalling might be due to deficits in the neuronal transport mechanism or abnormalities in cytoskeleton structure in the absence of functional MeCP2 protein. In fact, we have found that the expression levels of several cytoskeleton-related factors are altered in *Mecp2* mutant DG neurons (Table 5.1). Interestingly, we also observed that clustered synaptophysin staining was retained in the stratum lucidum of the hippocampus, which contains the terminals of CA3 neurons (data not shown). Further analyses of other brain regions should shed light on whether the lack of MeCP2 as an epigenetic factor leads to a general impairment of synaptic

development, as reflected by synaptophysin staining, and results in global deficits in neuronal maturation throughout the brain.

Dendritic spine density is a morphological indicator of neuronal maturation in hippocampal granule cells, and reduced spine density is correlated with impaired maturation of DG neurons (Zhao et al., 2006). Abnormalities in the morphology and density of dendritic spines have been found both in RTT patients and other neural developmental disorders, such as Down's syndrome, Fragile X syndrome, and Autism (Fiala et al., 2002), however whether *Mecp2* mutations affect dendritic development in animal models is currently not clear. To clarify the current controversy between human and mouse studies (Belichenko et al., 1997b; Kishi and Macklis, 2004; Moretti et al., 2006), we used a retrovirus to mark single newborn neurons on the DG. Our findings of the reduced dendritic spine density in newly generated neurons in the DG are thus consistent with similar findings in human pathologies, suggesting that this abnormal dendritic development may be a common point of vulnerability that leads to the neurological deficits caused by these genetic disorders. Further experiments analyzing the dendritic morphology of eGFP^{+} neurons at earlier time points will help to define how MeCP2 regulates the development of dendritic spines. The apposition of postsynaptic and presynaptic terminal components is a prerequisite for a functional synapse. Although we did not observe a significant difference in the percentage eGFP^{+} spines apposed to presynaptic terminals distinguished by synaptophysin, the evaluation of impairments in the number of functional synapses and potential abnormalities in synaptic transmission will require future electrophysiological analyses. Remarkably, the altered morphology seen in DCX^{+} immature neurons, including abnormal orientation of the processes (Fig. 5.1), was not found in GFP^{+} new neurons at 4-week post-labeling. This phenomenon might be due to the preferential death of morphologically altered

immature neurons or, alternatively, the abnormal orientation might correspond to a characteristic of an immature stage of neuronal maturation within the first week of differentiation. New neurons display horizontal processes, or short processes similar to those seen in *Mecp2* KO brains and develop vertical processes during later stages of development (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). Further analyses of viral-labeled cells at early stages of neuronal differentiation will confirm this hypothesis.

Despite extensive effort, only a few genes, including Brain-Derived Neurotrophic Factor (Chen et al., 2003b; Martinowich et al., 2003), DLX5 and 6 (Horike et al., 2005), and inhibitor of differentiation (ID1, 2, 3, and 4) genes (Peddada et al., 2006) have been shown to be regulated by MeCP2. Therefore, the identification of additional genes that are either directly or indirectly regulated by MeCP2 in neurons is a critical step forward in delineating what role this epigenetic regulator of gene expression plays in the development of the brain, as well as other tissues. Several of the candidate genes we identified are involved in cytoskeleton structure formation, such as Prefoldin 5, Arpc3, Syndecan 2, etc (Table 5.1). Prefoldin 5 is involved in actin and tubulin folding. Mutations of prefoldins result in an abnormal cytoskeleton (Hartl and Hayer-Hartl, 2002). Syndecan 2 is a transmembrane heparin sulfate proteoglycoprotein that binds extracellular matrix components and growth factors and is expressed at the mature dendritic spines of hippocampal neurons. Like that of MeCP2, the expression of Syndecan 2 coincides with dendritic spine maturation (Ethell and Yamaguchi, 1999). Exogenous Syndecan 2 expression induces increased dendritic spine formation, whereas blocking Syndecan 2 phosphorylation by the EphB2 receptor results in reduced spine density (Ethell et al., 2001). Further mechanistic analyses will determine whether these genes are targets of MeCP2 and functional consequences their altered expression in *Mecp2* KO neurons

In summary, our data indicate that MeCP2 is not critical for the early stages of neurogenesis, but is important for neuronal maturation in the postnatal brain. The fact that adult *Mecp2* KO brains are more similar to immature WT brains than to mature WT brains suggests that MeCP2 is critical for regulating the transition of neurons from immature to mature stages.

5.6 Acknowledgements

Images in this paper were generated in the University of New Mexico Cancer Center Fluorescence Microscopy Facility, supported as detailed on the webpage (http://hsc.unm.edu/crtc/microscopy/facility.html). We thank R. Lee and G. Phillips for technical assistance. We thank Dr L.A. Cunningham for advice in stereotaxic grafting. We thank L.C. Tafoya, and Drs. M.C. Wilson, C.F. Valenzuela, P. Jin, X. Li, and H. van Praag for critical reading of the manuscript and helpful comments. We thank M.L. Gage and S.M. Wimberly for editorial assistance. This work was funded by research grants from RSRF, IRSA, Oxnard Foundation, Autism Speaks, NIH/NCRR/COBRE, and funds from UNM School of Medicine.

5.7 Tables

Table 5.1. Genes that are expressed at different levels in hippocampal granule neurons of *Mecp2* KO mice.

Gene Symbol	Gene Description	dChip Fold Change* (Range)	Drop Confidence# (Range)	Gene Bank IDs
Genes expressed at higher levels				
Pfdn5	Prefoldin 5	1.61	94.5 - 99.0%	NM_020031.1
1110013I11Rik	RIKEN cDNA 1110013I11 gene	2.58	99.80%	BF608615
Uchl1	Ubiquitin carboxy-terminal hydrolase L1	1.53	83.6 – 90.9%	NM_011670.1
Sfxn3	Sideroflexin 3	1.34	85.7 - 87.6%	NM_053197.1
AL024345	expressed sequence AL024345	1.42	76.2 - 85.7%	AV295157
5031401C21Rik	RIKEN cDNA 5031401C21 gene	1.42	98.0 - 98.4%	AW537061
Osbpl9	Oxysterol binding protein-like 9	1.36	87.6 - 89.3%	AI875733
Zipro1	Zinc finger proliferation 1	1.58	97.5 - 99.2%	AI326272
Arpc3	Actin related protein 2/3 complex, subunit 3	1.49	2.5 - 83.6%	BC013618.1
Sdc2	Syndecan 2	1.49	60.5 - 85.7%	NM_008304.1
1500011H22Rik	RIKEN cDNA 1500011H22 gene	1.37	26.7 - 83.6%	BC019498.1
1110020P15Rik	RIKEN cDNA 1110020P15 gene	1.44	63.9 - 83.6%	BF681728
Genes expressed at lower levels				
Hmgb1	High mobility group box 1	-1.46	83.6 - 87.6%	C78183

Note: * Reference fold changes were obtained by dChip analysis; #, Confidence values were obtained by Drop analysis; the ranges are the maximum and minimum if multiple probe sets were returned.

CHAPTER 6:
MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase Mind Bomb-1

Richard D. Smrt[1], Keith E. Szulwach[2], Rebecca L. Pfeiffer[1], Xuekun Li[1], Weixiang Guo[1], Manavendra Pathania[3], Zhao-Qian Teng[1], Yuping Luo[1], Junmin Peng[2], Angelique Bordey[3], Peng Jin[2*] and Xinyu Zhao[1*]

[1]Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131;

[2]Department of Human Genetics and Graduate Program in Genetics and Molecular Biology, Emory University School of Medicine, Atlanta, GA;

[3]Department of Neurosurgery, Yale School of Medicine, New Haven, CT 06520-8082.

(Published in Stem Cells 2010;28:1060–1070)

6.1 ABSTRACT

A final step of neurogenesis is the maturation of young neurons, which is regulated by complex mechanisms and dysregulation of this process is frequently found in neurodevelopmental disorders. MicroRNAs have been implicated in several steps of neuronal maturation including dendritic and axonal growth, spine development, and synaptogenesis. We demonstrate that one brain-enriched microRNA, miR-137, has a significant role in regulating neuronal maturation. Overexpression of miR-137 inhibits dendritic morphogenesis, phenotypic maturation, and spine development both in brain and cultured primary neurons. On the other hand, a reduction in miR-137 had opposite effects. We further show that miR-137 targets the MIB1 protein through the conserved target site located in the 3' untranslated region of *Mib1* mRNA. MIB1 is an ubiquitin ligase known to be important for neurodevelopment. We show that exogenously expressed MIB1 could partially rescue the phenotypes associated with miR-137 overexpression. These results demonstrate a novel miRNA-mediated mechanism involving miR-137 and MIB1 that function to regulate neuronal maturation and dendritic morphogenesis during development.

6.2 INTRODUCTION

In both embryonic and adult brains, neurogenesis is initiated from the neuronal fate specification of neural stem cells. To function properly, new neurons have to integrate into appropriate neural networks and establish correct communication with other neurons. A critical step in development is neuronal maturation, which is characterized by dendritic and axonal growth, synaptogenesis, neuronal and synaptic pruning, and modulations of neurotransmitter sensitivities (Waites et al., 2005; Webb et al., 2001). The process of neuronal maturation is regulated by complex mechanisms that are still unclear, and deficits in this step are evident in many neurodevelopmental disorders such Rett Syndrome, Fragile X syndrome, and autism, etc (Fiala et al., 2002).

MicroRNAs (miRNAs) are small non-coding RNAs that can modulate gene expression at the post-translational level by targeting messenger RNA (mRNA), which leads to either reduced translation efficiency or cleavage of the target mRNAs. miRNAs are known to be involved in many cellular processes, such as proliferation, differentiation, apoptosis, and metabolism (Carninci et al., 2005; Chang and Mendell, 2007; Gangaraju and Lin, 2009; Liu and Zhao, 2009b). Despite the fact that 70% of detectable miRNAs are expressed in the brain, where half that number are either brain specific or enriched (Cao et al., 2006). There have been few functional studies of miRNA in the nervous system. Recent evidence has shown that many miRNAs act locally at the neuronal dendritic spines (Lugli et al., 2008; Smalheiser and Lugli, 2009). Both miR-134 and miR-138 are known to regulate dendritic patterning and spine morphogenesis by regulating protein translation at the synapse (Fiore et al., 2009; Schratt et al., 2006; Siegel et al., 2009). In addition, brain-specific miR-124 is localized at presynaptic terminal

of Aplysia and regulates synaptic plasticity by regulating transcription factor CREB (Rajasethupathy et al., 2009). Recently, a neuronal activity-dependent miRNA, miR-132, is found to regulate dendritic development by targeting a Rho family GTPase-activating protein, p250GAP (Wayman et al., 2008). Therefore, small noncoding miRNA pathways could be an important and novel mechanism regulating mammalian neurodevelopment.

In this study we show that a neuron-enriched miRNA, miR-137, has a significant role in the phenotypic maturation and dendritic morphogenesis of young neurons. We establish that miR-137 regulates the translation of the mouse homolog of Drosophila Mind bomb 1 (MIB1), an ubiquitin ligase known to be important for neurogenesis and neurodevelopment (Choe et al., 2007; Itoh et al., 2003; Ossipova et al., 2009). Finally, we show that exogenously expressed MIB1 can partially rescue the phenotypic deficits associated with miR-137 overexpression. These data suggest that functional interaction between miRNA and MIB1 plays an important modulatory role in neuronal development.

6.3 MATERIALS AND METHODS

Animals

All animal procedures were performed according to protocols approved by the University of New Mexico Animal Care and Use Committee. Wildtype C57/B6 mice were used for in vivo and in vitro studies. For histological analyses, mice were euthanized by intraperitoneal injection of sodium pentobarbital. Mice were then perfused with saline followed by 4% PFA. Brains were

dissected out, post-fixed overnight in 4% PFA, and then equilibrated in 30% sucrose. Forty-micrometer brain sections were generated.

Isolation and differentiation of adult hippocampal neuroprogenitors

Adult hippocampal neuroprogenitors (A-94-NSCs) was characterized previously and NSC proliferation and differentiation were carried out as described (Palmer et al., 1997). (Zhao et al., 2003).

Relative quantification of mature miRNAs by Taqman miRNA real-time PCR

Mature miRNA expression was assayed using Applied Biosystems' TaqMan microRNA assays or individual TaqMan miRNA assays) were performed according to protocols provided by the vendor (Lao et al., 2006). Detailed methods are provided in the Supplemental Data. Undifferentiated A94 NSCs were run in parallel with lineage specific differentiated A94 NSCs as paired samples. Data from the replicate experiments on undifferentiated A94 NSCs was then pooled and a single analysis of miRNA expression in each NSC lineage relative to undifferentiated NSCs was determined within the SDS v1.2 RQ manager to obtain the reported values.

miRNA in situ hybridization

In situ hybridization on 10 µm thick serial cryosections was carried out as outlined previously with a few modifications (Obernosterer et al., 2007). For hybridization 0.1 µl of 25 □M DIG- or FITC-labeled LNA probe (Exiqon) was added to 100□L hybridization buffer and applied to the tissue at 50-60 °C overnight (~20°C below the predicted melting temperature (T_m)

of probe:miRNA). Slides were mounted in Aquamount and visualized using confocal microscopy. Detailed methods are provided in the Supplemental Data

Nucleic acid and expression constructs

Control miRNA (miR-Con), miR-137, anti-miR-137, and anti-miRNA control (anti-miR-Con) were purchased from either GenePharma (Shanghai, China) or Ambion (AM17100, AM17110, AM17000, and AM17010, Austin, TX). MIB1 expression plasmid was described previously (Choe et al., 2007). *Mib1* shRNA and non-silencing control was purchased from Qiagen/SABiosciences (KM26177G). Lentivirus-sh-Control was created by cloning the SureSilencing non-silencing control shRNA cassette into the HpaI and ClaI sites of lentiviral vector. The generation and validation of this Lentivirus-sh-Control was described previously (Barkho et al., 2008; Li et al., 2008). PCR based generation of the miR-137 shRNA driven by a U6 Pol III promoter was done as described in our publications (Barkho et al., 2008; Li et al., 2008). Detailed methods and sequences of primers are provided in the Supplemental Data. Retroviral vector expressing both miR-137 and eGFP was engineered by deleting the original HpaI and ClaI sites in the CAG-EGFP vector (Smrt et al., 2007; Zhao et al., 2006) and inserting new HpaI and ClaI sites 5'-upstream from the CAG promoter. The sh-miR-137 or the sh-miR-Control cassette were digested from Lentiviral vectors (see above) and inserted between the HapI and ClaI sites of the retroviral vectors. The lentiviral and retroviral vectors expressing sh-miR-137 or sh-miR-Control were then verified by sequencing.

Production of retrovirus expressing miR-137 and in vivo retroviral grafting

Retrovirus production was performed as described previously (Smrt et al., 2007; Zhao et al., 2006). Detailed methods are provided in the Supplemental Data.

Maturation analysis of retroviral labeled new neurons

Immunohistochemistry and confocal imaging analysis on floating brain sections were carried out as described (Smrt et al., 2007). Floating brain sections containing eGFP+ cells were selected for staining and matched by DG region. The primary antibodies used were chicken anti-GFP (Invitrogen, #A10262), mouse NeuN (Chemicon, MAB377), and rabbit anti-doublecortin (DCX, Cell Signaling, #4604). The secondary antibodies used were anti-chicken Alexa Fluor 488 (Invitrogen, #A11039), goat anti-mouse Alexa Fluor 647 (Invitrogen, #A21236), and goat anti-rabbit Alexa Fluor 568 (Invitrogen, #A11036).

For dendritic branching analysis on 300 µm thick floating brain sections, GFP+ neurons were imaged on a LSM 510 confocal with a 20x/oil objective. Z-stacks of GFP+ dendrites were captured at 8µm intervals and the dendrites and the cell body of single GFP+ neurons were analyzed by Neurolucida software (MicroBrightField, Inc.). Roughly 30-50 neurons per DG were traced. Data were extracted for Sholl analysis, total dendritic length, branch number, and dendritic end number for each GFP+ neuron. Neurons were selected for analysis based on expression of GFP throughout the cell body and its processes. Cells were excluded if they exhibit excessive overlapping with adjacent GFP expressing neurons, their morphology is not intact, they have membrane varicosities, or they show signs of cell death such as compacted chromatin structure revealed by DAPI staining.

For dendritic spine density analyses and quantification of stage-specific neuronal markers, 1-in-3 of 40 µm floating brain sections containing eGFP+ cells (120 µm apart,

approximately 8 brain sections) were used for immunohistological staining using established protocols (Smrt et al., 2007). For the dendritic spine analysis above, the spine width was also manually measured as the distance of a straight line drawn across the widest part of the spine head using Image-J software (NIH Image).

Isolation of primary neurons from mouse embryos and transfection of cultured neurons

Hippocampal neurons were isolated from E17.5 fetal mice, and grown as described previously (Tafoya et al., 2006; Washbourne et al., 2002). Hippocampal neurons from wildtype E17.5 fetal mice were grown as dispersed mixed cell cultures, as established by the Wilson lab (Washbourne et al., 2002). Hippocampal neurons were transfected on day 4 (DIV 4) as they are undergoing dendritic and axonal morphogenesis during this time. Transfection was performed as described (Tafoya et al., 2006). 48hours after transfection, the neurons were fixed and stained as described below. Transfection efficiencies were 1-2%.

Morphological analysis of transfected neurons

Immunostaining of transfected neurons was performed as previously described (Barkho et al., 2008; Li et al., 2008; Smrt et al., 2007; Zhao et al., 2003). The primary antibody used was MAP2ab (mouse, 1:500, Sigma). The secondary antibody used was Cy3 (donkey anti-mouse, 1:500, Sigma). Low transfection efficiencies (1-2% neurons) permit imaging and quantification of single GFP expressing neurons. GFP expressing neurons were imaged with an Olympus BX51 upright microscope with 20x/oil immersion lens, a motorized stage, and digital camera. Dendritic traces were performed in real time using Neurolucida (MicroBrightField, Inc.) image analysis

software. The axon is identified using Map2 staining where the only process not positive for Map2 is the axonal process (Jugloff et al., 2005).

3'-UTR dual luciferase assays of miR-137 target mRNA

3'-UTR sequences of candidate mRNAs were PCR amplified directly from proliferating aNSC first strand cDNA generated from 5 μg TRIZOL-isolated total RNA using oligo-dT SuperScript III reverse transcription according to the manufacturer's protocol (Invitrogen, Cat. #1808-093). These primer sequences are available upon request. All primers were designed incorporating XhoI and NotI restriction sites and 4 bp of extra random sequence to aid in restricting digestion. XhoI- and NotI-digested PCR products were cloned into XhoI- and NotI-digested psiCHECK-2 dual luciferase vector (Promega, Cat# C8021) and were later transferred by XhoI/NotI double digestion and T4 DNA ligation into a pIS2 renilla luciferase vector modified with the addition of an XhoI restriction site and deletion of the SpeI restriction site. miR-137 target site deletion was done using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cat. #2000518) to delete UUCGUUAU. Briefly, E17 hippocampal neurons were cultured (as described above), and co-transfected by Lipofectamine 2000 with pIS2 Renilla luciferase vector containing the *Mib1* 3'UTR, pIS0 firefly luciferase as a transfection control, and miR-Con, miR-137, anti-miR-137, or anti-miR-Con. All co-transfections used a total of 1 μg of plasmid DNA and 50ng of shRNA. At 48 h after transfection, the cell culture medium was removed and cells were lysed with 20 μl of 1X passive lysis buffer at room temperature for 15 min and luciferase expression was detected using the Dual-Luciferase Reporter 1000 System (Promega, Cat# E1980) per the manufacturer's protocol. R-luc activity was normalized to F-luc activity to account for variation in transfection efficiencies, and miR-137–mediated knockdown

of R-luc activity was calculated as the ratio of normalized R-luc activity in the U6-miR-137-shRNA treatments to normalized R-luc activity in the U6-neg-shRNA treatments. Luciferase experiments were repeated at least three times.

Production of lentivirus and infection of E17 hippocampal neurons

Lentiviruses were produced as described previously (Barkho et al., 2008; Barkho et al., 2006; Li et al., 2008). To study the effects of miR-137 on development of dendrites in cultured neurons, 1:1 solution of virus containing supernatant and Neurobasal A medium (Invitrogen) supplemented with 25 nM glutamate, 0.5 mM L- glutamine, and 1% antibiotics was added to the neurons 1 day after plating. After 24hours, the medium was replaced with fresh virus containing solution described above, and was incubated for an additional 24 hours. Infected neurons were collected in cell lysis buffer for western blot analysis.

Western blot analysis

Protein samples were separated on SDS-PAGE gels and then transferred to PVDF membranes (Millipore). Membranes were processed following the ECL Western Blotting protocol (GE Healthcare, Cat #RPN2106). Anti-MIB1 antibody (M20a) (Choe et al., 2007) were used at a 1:1000 dilution. HRP-labeled secondary antibodies were obtained from Sigma (A0545). For loading controls, membranes were stripped and reprobed with the antibody against GAPDH (Ambion AM4300).

Statistical analysis

All statistical analyses were performed using unpaired, two-tailed, Student's t-test. The data bars and error bars indicate mean ± standard error mean. (s.e.m). Sholl analysis was analyzed using a multivariate analysis of variance (MANOVA) using SPSS statistical software (SPSS version 17, SPSS Inc., Chicago, Ill, USA).

6.4 RESULTS

6.4.1 miR-137 is enriched in neurons

To identify lineage specific miRNAs that may regulate development and function of neurons in the postnatal hippocampus, we profiled mature miRNA expression in adult hippocampal neuroprogenitors (A94-NSCs) differentiated into either neuronal or astrocytic lineages and compared the miRNA profile in undifferentiated A94-NSCs (Supplemental Figure S6.1A,B). We then quantitatively identified miRNAs that were enriched specifically in the neuronal lineage relative to the astrocytic lineage (Figure 6.1A). Several miRNAs, particularly miR-185, 27b, 182, 137, 29b, 132, and 146, showed enrichment in neurons, but not astrocytes or undifferentiated NSCs; among these miRNAs, miR-137 was previously found to be enriched in synaptosomes isolated from rat forebrains (Siegel et al., 2009; Silber et al., 2008). We further confirmed that the expression levels of miR-137 increased during neuronal differentiation of A94-NSCs (Figure 6.1B), and miR-137 expression levels were significantly higher in isolated primary neurons compared with aNSCs (Figure 6.1C). The highly enriched expression of miR-137 in the neuronal lineage suggests that it may have important functions in neuronal development.

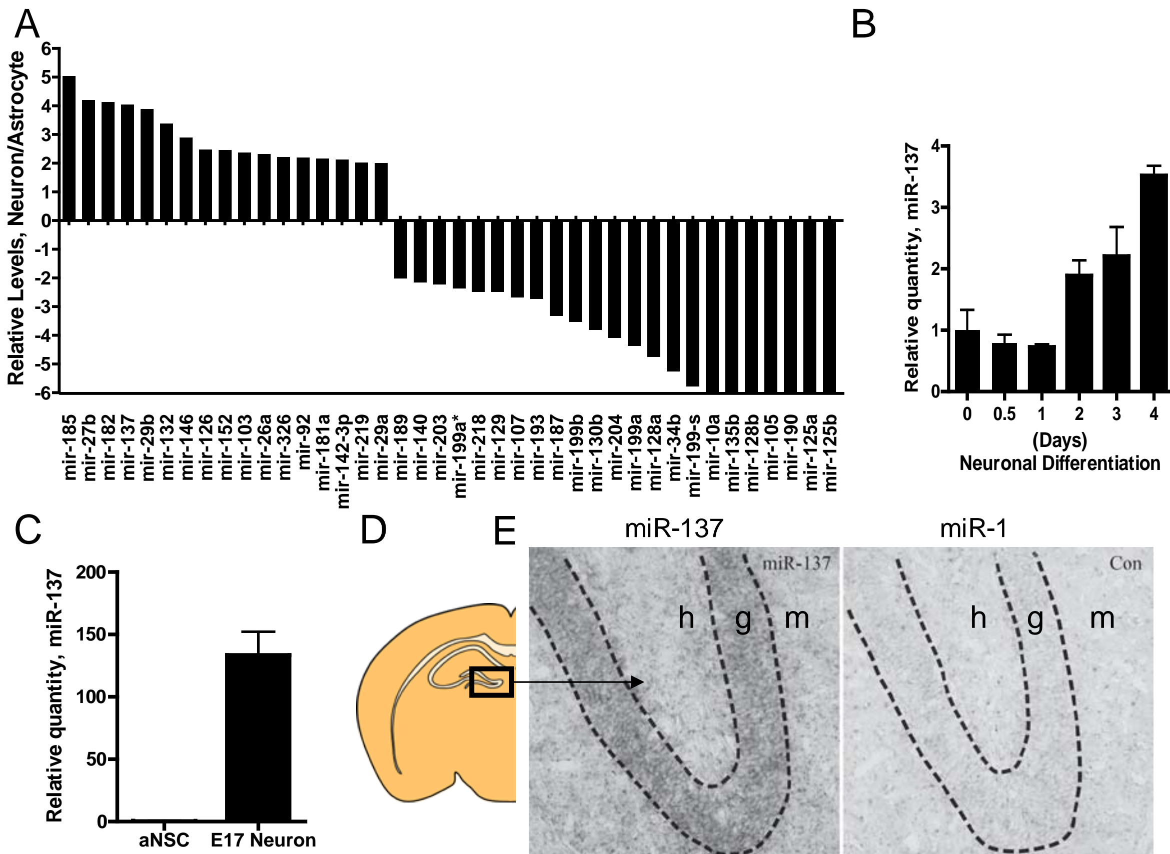


Figure 6.1. miR-137 is enriched in neurons and is expressed in the dentate gyrus and the molecular layer of the hippocampus. (**A**) Identification of lineage specific miRNAs in A94-NSCs. Plotted are the ratios of RQ values determined by comparing A94-NSCs differentiated toward the neuron lineage to undifferentiated A94-NSCs over RQ values determined by comparing A94-NSCs differentiated toward the astrocyte lineage to undifferentiated A94-NSCs (Figure S1A and S1B). Ratios≥8 were set to a value of 8. (**B**) miR-137 expression during neuronal differentiation of A94-NSCs for 0.5, 1, 2, 3, and 4 days (miR-137 expression calibrated to undifferentiated A94-NSCs, n=3, mean ± 95% CI). (**C**) Enrichment of miR-137 in E17 neurons as compared to mouse primary aNSCs (miR-137 expression calibrated to mouse primary aNSCs, n=3, mean ± SEM). (**D-E**) Hybridization with a miR-137-specific probe showed an enrichment of miR-137 within the DG and molecular layer of the hippocampus compared with miR-1, which is expressed at low levels in the CNS (h, hilus; g, dentate gyrus; m, molecular layer).

miR-137 is known to be expressed in the brain and enriched at the synaptic compartment (Siegel et al., 2009; Silber et al., 2008). We reasoned that if miR-137 is indeed a mediator of neurodevelopment and function, it should be expressed in neurons of the adult hippocampus, which is a region of the brain exhibiting significant plasticity and continuous production of new neurons. Thus we chose to examine the cellular localization of miR-137 in the adult

hippocampus. Hybridization with an miR-137-specific probe showed an enrichment of miR-137 within the dentate gyrus (DG) and molecular layer of the hippocampus compared with miR-1, a miRNA that is expressed at low levels in the central nervous system (CNS) (Figure 6.1D and E). Together, these data and the published literature (Siegel et al., 2009; Silber et al., 2008; Smalheiser and Lugli, 2009) suggest that miR-137 may play functional roles in neurons, perhaps during the formation of connectivity between neurons in the hippocampus.

6.4.2 miR-137 regulates dendritic development and phenotypic maturation of new neurons in vivo

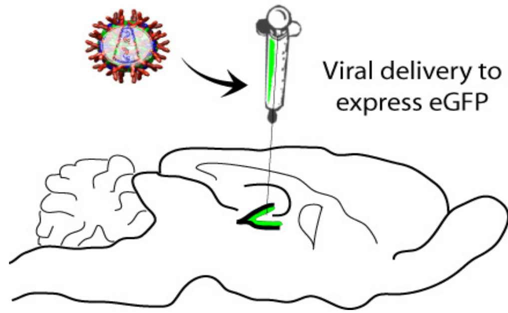
Recently, miRNAs were found to be expressed at the synapse and play an important role in dendritic patterning and spine morphogenesis (Lugli et al., 2008; Schratt et al., 2006; Siegel et al., 2009). To determine whether elevated miR-137 levels in neurons can affect neuronal maturation and dendritic morphogenesis, we overexpressed miR-137 in newborn cells of the adult DG using retrovirus-mediated gene delivery (Smrt et al., 2007). This method, referred to as the single-cell genetic approach (Song et al., 2005; van Praag et al., 2002), makes use of recombinant retroviruses capable of specifically infecting dividing cells (Figure 6.2A). Because postnatal neurogenesis persists in the adult hippocampus, this method allows us to deliver a transgene specifically to newborn cells in the DG and perform a detailed morphological and phenotypic analysis on these newly generated neurons (Smrt et al., 2007; Song et al., 2005; Zhao et al., 2006). Using stereotaxic microinjection surgery, we grafted retrovirus expressing both eGFP and small hairpin miR-137 (sh-miR-137) into one hemisphere of the adult mouse DG, and injected a retrovirus carrying nonsilencing small hairpin control (sh-Con) into the contralateral hemisphere of the same animal (Figure 6.2A and B). At 4 weeks post-injection (4wpi), one

cohort of injected animals was used to generate thick (300-μm) sections, which preserves the dendritic arborization of eGFP-positive neurons and enables extensive morphological analysis. Individual eGFP-expressing neurons in these sections were imaged using confocal microscopy. To precisely evaluate the dendritic complexity of eGFP+ neurons, we reconstructed the eGFP+ dendritic arbor in 3 dimensions, rather than the traditionally flattened 2 dimensions, for image analysis using the Image Stack Module of Neurolucida analysis software (MicroBrightField, Inc.) (Figure 6.2C and D, Supplemental Figure S6.2A and B). Since previous reports have indicated that miR-137 has no affect on dendritic spine volume (Siegel et al., 2009), we performed quantitative analysis using established parameters for assessing neuronal dendritic development (Duan et al., 2007; Zhao et al., 2006). Sholl analysis indicated that miR-137-overexpressing neurons exhibited significantly reduced dendritic complexity compared to sh-Control-expressing neurons ($F(1,65) = 8.78$, $p = 0.004$, multivariate analysis of variance) (Figure 6.2E). In addition, miR-137-overexpressing neurons exhibited significantly reduced average dendritic length ($n = 3$ animals, $p < 0.05$), number of nodes (branch points) ($n = 3$, $p < 0.05$), and number of dendritic endpoints ($n = 3$, $p < 0.05$) compared with young neurons expressing sh-Control (Figure 6.2F-H).

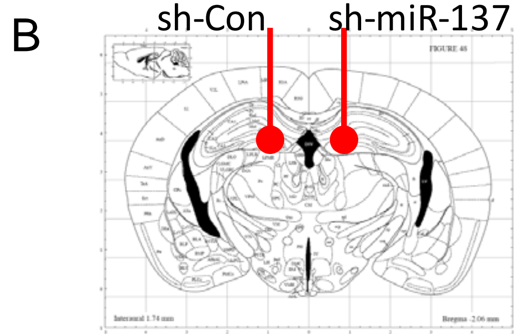
Dendritic spine density increases as neurons mature, making the spine density a good indicator of neuronal maturation (Ge et al., 2006; Zhao et al., 2006). On the other hand, altered spine density is a common characteristic of abnormal synaptic development in a variety of neurological disorders such as Fragile-X and Rett syndrome (Fiala et al., 2002; Smrt et al., 2007). We therefore used another cohort of virus-injected animals to generate 40-μm thin sections for dendritic spine analysis. To determine whether overexpression of miR-137 leads to deficits in spine morphogenesis, we analyzed the dendritic spine density of newborn GFP-

expressing DG granule neurons at 4 weeks post-injection, a time at which labeled new neurons are believed to exhibit the dendritic morphology of mature neurons (Ge et al., 2006; Smrt et al., 2007; van Praag et al., 2002; Zhao et al., 2006). To maximize consistency of our analyses, we focused on dendritic fragments 25-100 um from the cell body of each eGFP+ neuron. To quantify spine density, we counted the number of spines protruding from the dendrite within each 10-μm segment of dendrites. Quantitative analysis showed that miR-137-overexpressing neurons exhibited a 17% reduction in dendritic spine density compared with sh-Control-expression neurons ($n = 3$, $p < 0.01$) (Figure 6.2I-K). The widths of dendritic spines in miR-137-overexpressing neurons were no different from sh-Control-overexpressing neurons (Supplemental Figure S6.3).

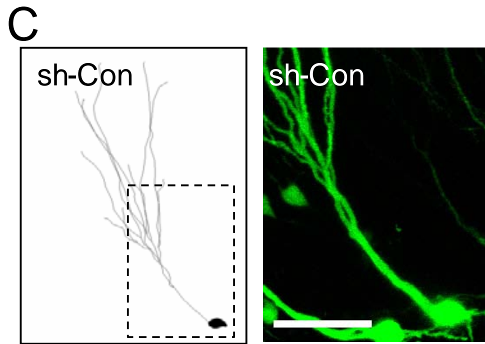
A



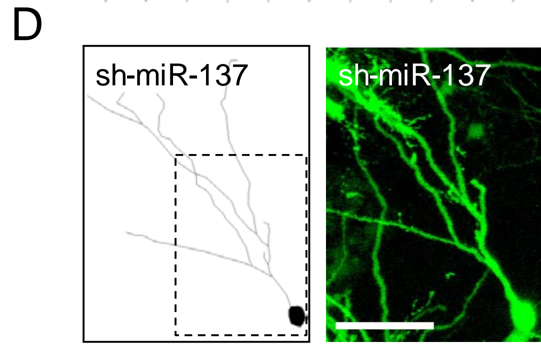
B



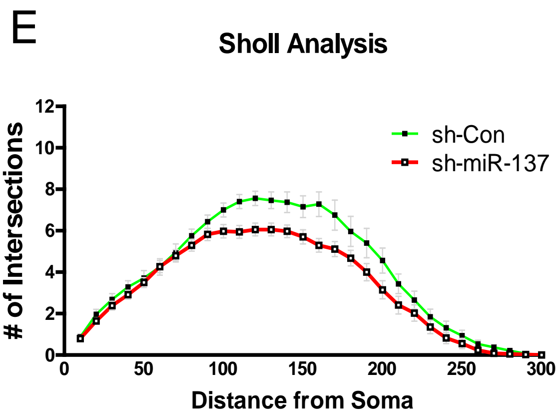
C



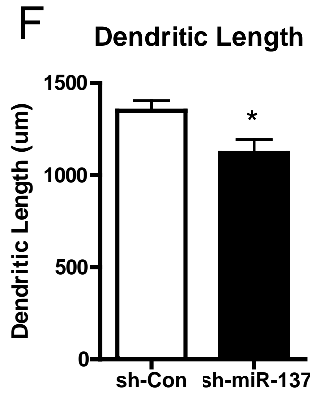
D



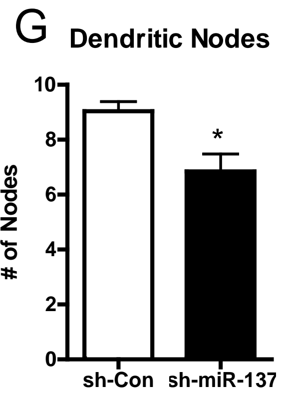
E



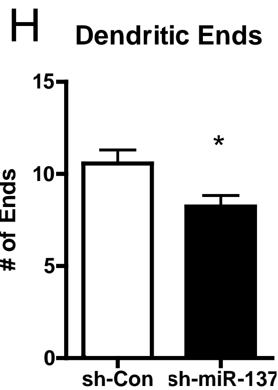
F



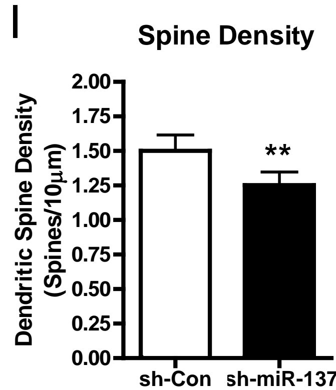
G



H



I



J K

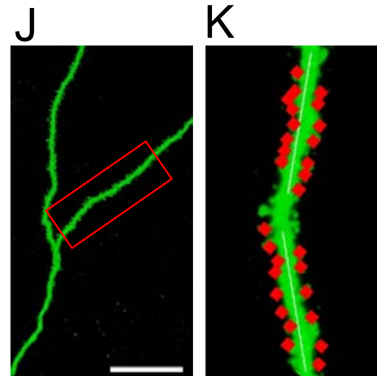


Figure 6.2. miR-137 regulates dendritic development and phenotypic maturation of new neurons in vivo. (**A**) A schematic diagram showing the retroviral vector used for in vivo miR-137 expression. miR-137 (sh-miR-137) or control miR (sh-Con) was expressed as a short hairpin under U6 RNA Polymerase III promoter while eGFP was expressed under a chicken β-actin (CAG) promoter. (**B**) Schematic diagram showing that control virus (sh-Con) was injected into the left hemisphere, and retrovirus expressing miR-137 (sh-miR-137) was injected into the right hemisphere. (**C, D**) Confocal z-stacks showing eGFP-expressing neurons at 4 weeks post-injection (4 wpi) with representative traces from both the sh-Con (C) and sh-miR-137 condition (D) (scale bar = 50 μm). (**E**) Neurons overexpressing sh-miR-137 show reduced dendritic complexity compared with controls, as determined by Sholl analysis. (**F-H**) Neurons overexpressing sh-miR-137 show reduced dendritic length (F), number of nodes (branch points, G), and dendritic ends (H). (**I**) Neurons overexpressing sh-miR-137 show reduced dendritic spine density. (**J**) Confocal z-stacks showing eGFP-expressing dendrites (scale bar = 20 μm). (**K**) A representative dendritic segment used for spine density analysis (* = $p < 0.05$)

New neurons in the DG express development stage-specific markers that define their maturation (Figure 6.3A) (Ming and Song, 2005; Zhao et al., 2008). Using immunocytochemistry for doublecortin (DCX, an immature neuronal marker) and neuronal nuclear antigen (NeuN, a mature neuronal marker) immunostaining, new neurons in the DG were categorized into 3 subpopulations: immature neurons (DCX+ only), transitioning neurons (DCX+ and NeuN+), and mature neurons (NeuN+) (Brown et al., 2003; Smrt et al., 2007). We then determined whether new neurons overexpressing miR-137 had a developmental phenotype that could be measured by the expression of stage-specific markers. Thus, we analyzed retrovirus-labeled newborn neurons at 4 weeks post-injection, a time when many virus-labeled cells have differentiated into mature neurons (Figure 6.3A-C). We found that miR-137-overexpressing cells differentiated into fewer eGFP+ neurons (either DCX+eGFP+ and/or NeuN+eGFP+ cells) in general compared with sh-Control-overexpressing cells (Supplemental Figure S6.4). We then quantified the proportion of each type of neuron among total eGFP+ neurons. The results summarized in Figure 6.3D show that neurons overexpressing miR-137 displayed a significant difference in the proportion of immature vs. mature neurons compared with neurons overexpressing control (sh-Con). Specifically, miR-137-overexpressing neurons had an 80% decrease in the proportion of mature neurons (NeuN+, $n = 3$, $p < 0.05$) compared

with control. Additionally, there was a 19% decrease in the proportion of transitioning neurons (NeuN+/DCX+, n = 3, p > 0.05) and a 62% increase in the proportion of immature neurons (DCX+, n = 3, p = 0.05) compared with control. Therefore, this indicates that elevated levels of miR-137 alter the sequential events leading to the development of a mature DG granule neuron. Taken together, these in vivo data suggest that increased expression of miR-137 in newborn neurons results in decreased dendritic development.

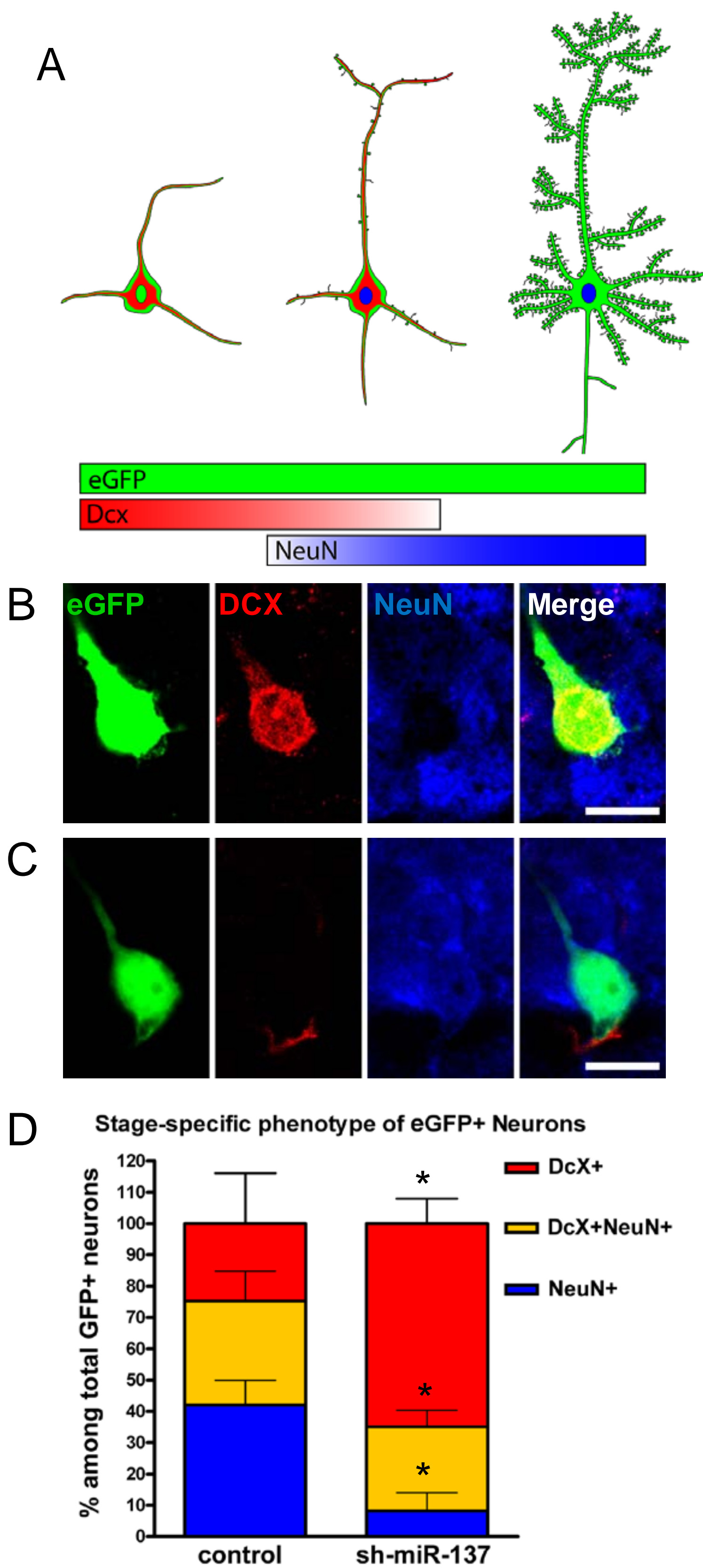


Figure 6.3. Overexpression of miR-137 leads to altered neuronal maturation of new neurons in vivo. (**A**) Illustration showing the stage-specific neuronal markers that can be used to identify the maturation state of developing DG granule neurons. (**B, C**) Confocal images showing two representative eGFP-expressing neurons in the DG: a relatively immature eGFP neuron (B) expressed DCX (immature marker) but not NeuN (mature neuron) and a relatively mature eGFP+ neuron (C) expressed NeuN but not DCX. (**D**) The miR-137-overexpressing neuron population had decreased proportions of NeuN+ only (blue) mature neurons and of DCX+/NeuN+ (yellow) transitioning neurons, but increased proportion of DCX+ only (red) immature neurons compared with control (* = p < 0.05).

6.4.3 miR-137 regulates neuronal dendritic development in vitro

Since retrovirus infects only dividing cells, overexpression of miR-137 in dividing neuroprogenitors may affect initial neuronal differentiation, which might indirectly inhibit neuronal maturation. To investigate whether miR-137 overexpression affects neuronal maturation independent of its effect on the neuronal differentiation of neuroprogenitors, we turned to a well-established in vitro cultured primary neuron system and used both gain-of-function and loss-of-function methods. Hippocampal neurons serve as a good model for studying molecular mechanisms controlling dendritic and spine development, because they form elaborate dendritic trees, functional synapses, and they can respond to both chemical and electrical stimulations (Fletcher et al., 1994; Goslin and Banker, 1989; Okabe et al., 1998). We isolated neurons from the hippocampi of E17.5 mouse embryos and plated them into serum-free medium to limit astrocyte proliferation. To modulate miR-137 expression, we overexpressed miR-137 using two different gain-of-function assays. First, we expressed miR-137 as a small hairpin RNA plasmid using a lentiviral vector that also expresses eGFP (Figure 6.4A) (Li et al., 2008). Second, we cotransfected neurons with miR-137 synthetic double-stranded RNA and an eGFP expression plasmid. Concurrently, we performed a loss-of-function assay to knock down endogenous miR-137 in cultured hippocampal neurons using a 2'-O-methylated antisense oligonucleotide.

At 48 hours post-transfection, eGFP-expressing neurons were imaged, and the morphology of the soma, dendrites, and axons were manually traced and measured using Neurolucida (MicroBrightField, Inc.) image analysis software (Figure 6.4B and C). Transfected eGFP+ neurons had clearly identifiable dendrites and axons, and axons were distinguished from

dendrites by two characteristics: axons are the longest among all processes and have negative staining for MAP2, a somatodendritic marker (Jugloff et al., 2005). The morphology of cultured hippocampal neurons is not as uniform as developing neurons in the DG of the hippocampus, which may account for the variability we saw in neuronal morphometry. However, the morphological differences between neurons expressing miR-137 and controls were apparent (Supplemental Figure S6.5A, B). Neurons transfected with plasmid expressing sh-miR-137 had a significantly reduced dendritic complexity ($F(1,52) = 5.15$, $p < 0.05$ multivariate analysis of variance) (Figure 6.4D) and 23% reduction in total dendritic length ($n = 3$, $p < 0.05$) (Figure 6.4E) compared with sh-Control-transfected neurons. Consistent with this result, neurons transfected with synthetic miR-137 also showed a 23% reduction in dendritic length and reduced dendritic complexity compared with miR-Control-transfected neurons ($n = 3$, $p < 0.01$) (Figure 6.4F and G). On the other hand, neurons transfected with a specific inhibitor of miR-137 (anti-miR-137) had increased dendritic complexity ($F(2,51) = 3.58$, $p = 0.036$ multivariate analysis of variance) (Figure 6.4F) and a significant 25% increase in total dendritic length ($n = 3$, $p < 0.01$) (Figure 6.4G) compared with control anti-miR (anti-miR-Con)-transfected neurons. The total number of dendritic ends and nodes had a similar trend of a reduction in neurons transfected with miR-137 and an increase in anti-miR-137-transfected neurons; although these differences did not reach statistical significance (Supplemental Figure S6.4C and D). These loss-of-function and gain-of-function data in primary neurons further support our in vivo observation that high levels of miR-137 inhibit neuronal dendritic development.

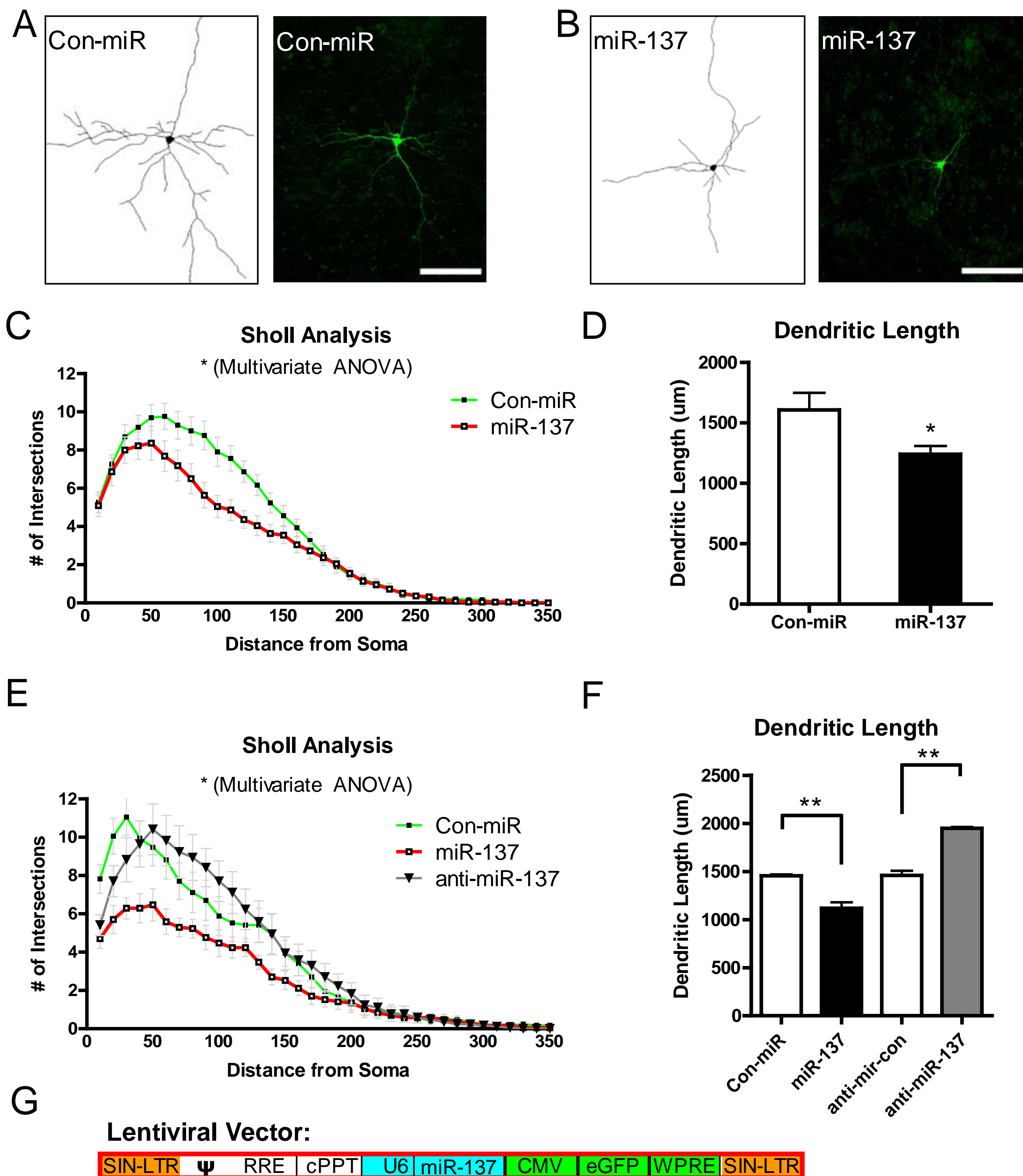


Figure 6.4. miR-137 is important for dendritic development in vitro. **(A, B)**, E17 primary hippocampal neurons were transfected with lentiviral vectors expressing either control Con (A) or miR-137 (B), as well as eGFP. Single eGFP-expressing neurons were shown next to their representative traces (scale bar = 50 µm; 20x/oil). **(C)**, Sholl analysis showing neurons overexpressing sh-miR-137 had reduced dendritic complexity compared with neurons overexpressing sh-Control. **(D)**, Neurons overexpressing sh-miR-137 had reduced total dendritic length compared with controls. **(E)**, Sholl analysis showing that neurons overexpressing miR-137 had reduced dendritic complexity compared with controls, whereas neurons transfected with an anti-miR-137 had opposite effect. **(F)**, Neurons overexpressing miR-137 had reduced total dendritic length compared with neurons overexpressing miR-Control. On the other hand, neurons transfected with anti-miR-137 showed increased dendritic length compared to neurons transfected with anti-miR-Control. **(G)** A schematic diagram showing the lentiviral vector used for miR-137 expression. miR-137 (sh-miR-137) or control miR (sh-Con) was expressed as a short hairpin under U6 RNA Polymerase III promoter while eGFP was expressed under a CMV promoter. (* , $p < 0.05$, ** , $p < 0.01$)

6.4.4 Mind Bomb-1 is a Translational Target of miR-137

To determine how miR-137 affects dendritic morphology during neuronal development, we first took a bioinformatic approach to identify the potential mRNA targets of miR-137. We referenced TargetScan 4.1, PicTar, and miRanda to compile a set of potential candidate targets (John et al., 2004; Krek et al., 2005; Lewis et al., 2003a). Next, we selected a subset of targets for further analyses based on 3 criteria: conservation, context score of target "seed sequences," and known relevance to dendritic morphogenesis and neuronal development. Among the top candidate miR-137 targets are a mouse homolog of Drosophila mind bomb 1 (MIB1), histone H3K27 methyltransferase Ezh2, EphA7, and chromatin modulator NcoA3 (Table S1). We first cloned the 3'-untranslated region (3'-UTR) of these four candidates containing the predicted miR-137 target site from mouse cDNA into a Renilla luciferase (R-luc) reporter construct (see Figure 6.5B for example). This allowed us to assess protein translation of these targets regulated through their 3'-UTR. This 3'-UTR R-luc constructs along with a firefly luciferase (f-luc) control plasmid were cotransfected into HEK 293 cells. We found that miR-137 could repress the translation of luciferase through these 3'-UTR. Then we selected the expression plasmids of the top two candidates, Mib-1 and Ezh2, to transfect into primary neurons. We found that MIB1, but not EZH2, could promote dendritic morphogenesis, similar to the effect of anti-miR-137. MIB1 was a particularly interesting candidate because it was previously shown to be enriched in the postsynaptic compartment by mass spectrometry (Choe et al., 2007; Sheng and Hoogenraad, 2007). In addition, in our initial functional screening by overexpressing these candidate targets in cultured primary neurons, MIB1 demonstrated the most dramatic effect on promoting neuronal dendritic length (data not shown). We therefore decided to further investigate whether MIB1 is a functional target of miR-137. To further test whether miR-137 could target *Mib1*, we cloned the

3'-untranslated region (3'-UTR) of *Mib1* containing the predicted miR-137 target site, from mouse cDNA into a Renilla luciferase (R-luc) reporter construct (Figure 6.5B). This allows us to assess MIB1 protein translation regulated through its *Mib1* 3'-UTR. This 3'-UTR R-luc construct along with a firefly luciferase (f-luc) control plasmid were cotransfected into cultured primary neurons. We found that overexpression of miR-137 suppressed over 50% of the R-luc activity in primary neurons at 48 hours post-transfection ($n = 7$, $p < 0.001$) (Figure 6.5C). On the other hand, transfected anti-miR-137 led to a 28% increase in R-luc activity compared with the anti-miR control (anti-miR-Con, $n = 3$, $p < 0.05$) (Figure 6.5D). To further validate the interaction between miR-137 and its target *Mib1* 3'-UTR, we mutated the seed sequence of miR-137 located within the *Mib1*-3'-UTR reporter (Figure 6.5,B lower panel). This mutation substantially alleviated the miR-137-mediated suppression of luciferase activity, suggesting that the action of miR-137 is specific to the miR-137 seed region within the *Mib1*-3'-UTR ($n = 5$, $p < 0.001$) (Figure 6.5E).

To investigate the effect of miR-137 on endogenous MIB1 expression in neurons, we used the lentivirus expressing sh-miR-137 (Figure 6.4A) to infect cultured primary neurons. Lentivirus transduction allows us to achieve relatively high expression efficiency (~50%) in mouse primary neurons (Figure 6.5F). Neurons infected by sh-miR-137 expressing virus had a 13% decrease in endogenous MIB1 expression compared with neurons infected by control virus (sh-Con, $n = 3$, $p < 0.05$) (Figure 6.5G). Taken together, these data suggest that miR-137 regulates the protein expression of MIB1 through the 3'-UTR of *Mib1*.

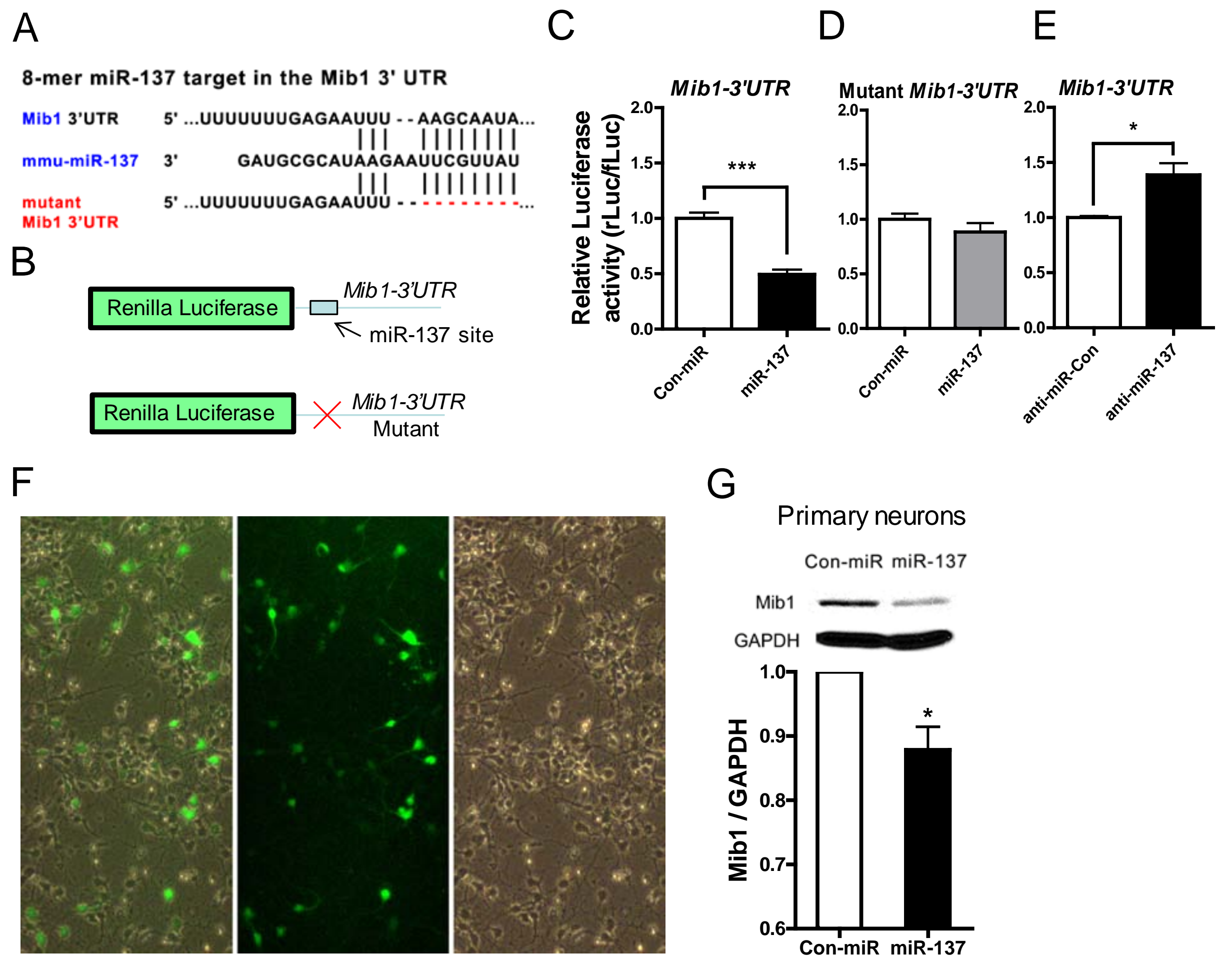


Figure 6.5. Mib1 is a functional target of miR-137. (**A**) A miR-137 target site was found in the *Mib1* 3' untranslated region (3'UTR) as predicted by TargetScan software. The Mutant *Mib1* 3'UTR used in B-E with miR-137 site deleted is shown. (**B**) Schematic diagram showing the predicted seed region where miR-137 is expected to bind the rLuc-*Mib1* 3'-UTR (upper), and the mutated version lacking the binding site for miR-137. (**C**) *Mib1*-3'-UTR–dependent expression of a Renilla luciferase reporter gene (R-luc) was suppressed by miR-137 over 50% in DIV6 primary neurons at 48 hours post-transfection ($n = 7$, $p < 0.001$). The 3'-UTR-dependent Renilla luciferase (R-Luc) activities were normalized to control firefly luciferase (f-Luc) activities in the result of miR-137 coexpression was calculated relative to the miR-Con in C-E. (**D**) The mutant *Mib1*-3'-UTR alleviated the miR-137-mediated suppression of luciferase activity, suggesting that the action of miR-137 is specific to the miR-137 seed region within the *Mib1*-3'-UTR ($n = 5$, $p < 0.001$). (**E**) *Mib1*-3'-UTR–dependent expression of R-Luc was enhanced 28% by anti-miR-137 ($n = 3$, $p < 0.05$). (**F**), 10x fluorescence and bright field images showing high infection efficiency of lentivirus expressing sh-miR-137 (also eGFP) in E17 primary cortical neurons. (**G**) Primary neurons infected with lentivirus expressing sh-miR-137 had reduced Mib1 protein expression compared with neurons infected with lentivirus expressing sh-Con at 48 hours post-infection. (*, $p < 0.05$; ***, $p < 0.001$)

6.4.5 Expression of MIB1 rescues the miR-137-mediated reduction in dendritic complexity

Since we have confirmed that miR-137 targets MIB1, we performed additional experiments to determine whether MIB1 could rescue the miR-137 overexpression-induced reduction in dendritic complexity. We showed that overexpression of MIB1 led to significant increases in both dendritic length (n = 5, 41% +/-0.12 SEM, $p < 0.01$) and dendritic complexity ($F(1,36) = 23.00$, $p < 0.001$) compared with control vector-transfected neurons (Figure 6.6A and B). On the other hand, using a specific small hairpin RNA (shRNA) against *Mib1* (Supplemental Figure S6.6), we also showed that acute knockdown of MIB1 led to a significant decrease in both dendritic length (37.5% +/-0.09 SEM, n = 3, $p < 0.05$) and dendritic complexity ($F(1,47) = 49.00$, $p < 0.001$) compared with control shRNA-transfected neurons (Figure 6.6A and B). Then, we cotransfected neurons with MIB1 expression plasmid and a synthetic miR-137 and show that MIB1 overexpression partially rescued the miR-137-mediated reduction both in dendritic length (n = 4, 6% difference between "Mib1+miR-137" and "Control GFP", 33% difference between "Mib1+miR-137" and "miR-137") (Figure 6.6A) and in dendritic complexity ($F(1,35) = 18.51$, $p < 0.001$) (Figure 6.6C). These data suggest that miR-137 regulates dendritic morphogenesis in developing neurons, at least in part, by translational regulation of MIB1 (Figure 6.6D).

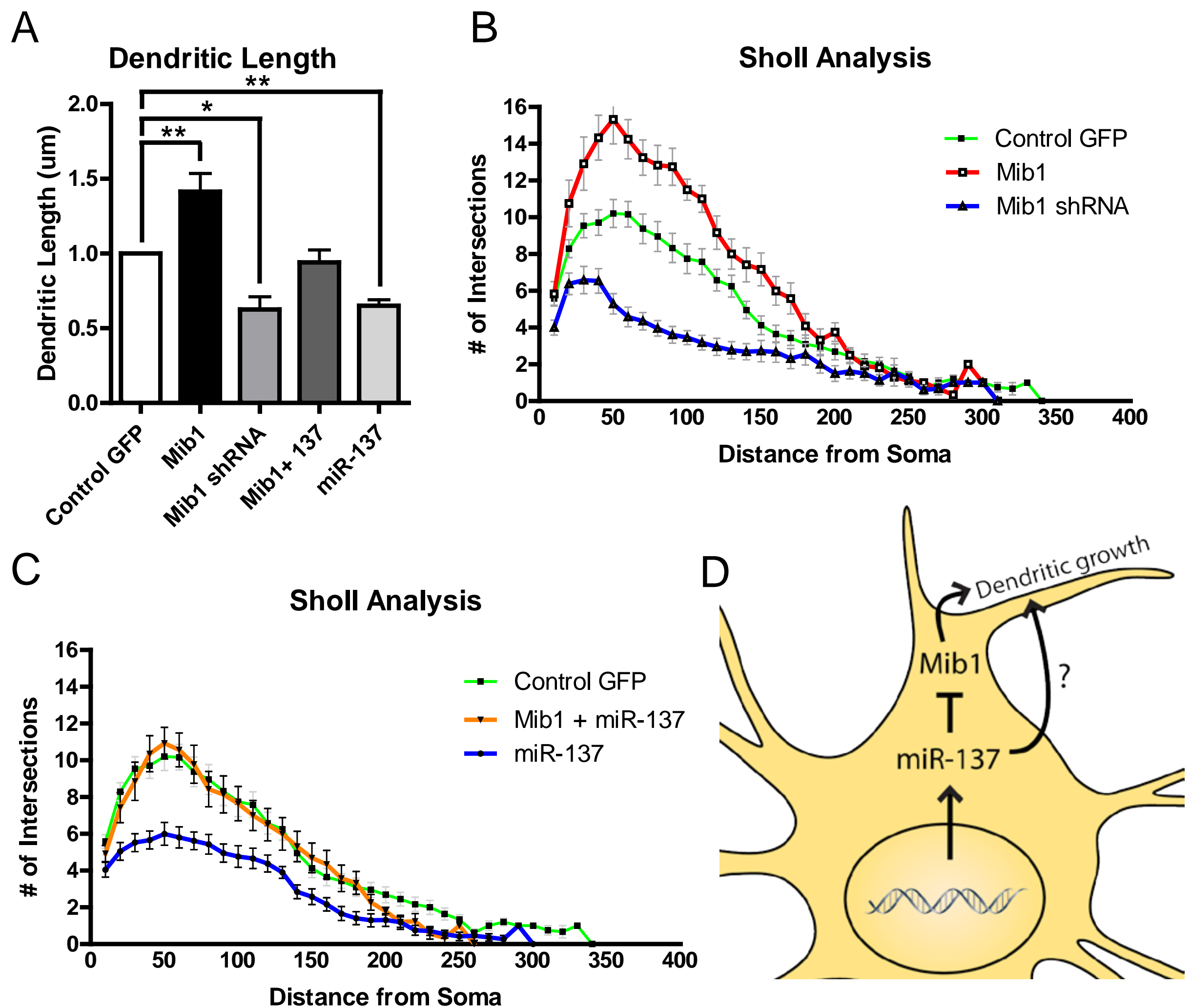


Figure 6.6. MIB1 could rescue the neuronal maturation deficits associated with miR-137 overexpression in vitro. (**A**) Overexpression of MIB1 enhanced dendritic length, whereas acute knockdown of MIB1 (*Mib1* shRNA) reduced dendritic length. MIB1 expression partially rescued the miR-137-mediated reduction in dendritic length in cultured primary neurons. (**B**) Overexpression of MIB1 enhanced the dendritic complexity of cultured neurons, whereas acute knockdown of MIB1 reduced dendritic complexity**.** (**C**) MIB1 could rescue the miR-137-mediated reduction in dendritic complexity ($F(1,35) = 18.51$, $p < 0.001$). (**D**) A hypothetic model illustrating that miR-137 may regulate dendritic morphogenesis in developing neurons, at least in part, by translational regulation of MIB1. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

6.5 DISCUSSION

The potential functions of miRNAs acting locally at the neuronal dendritic spines are just beginning to be explored (Smalheiser and Lugli, 2009). Several miRNAs are found to be localized and functioning in dendrites and synapses. Among them, miR-132 and miR-137 are both enriched in dendritic spines, but whereas miR-132 represses spine volume, miR-137 shows no effect on spine volume (Siegel et al., 2009). Therefore it is likely that individual miRNAs at the synapse play specialized roles in dendritic morphogenesis and synaptic development. Although the specific mechanisms underlying miRNA regulation of neuronal development are not fully clear, current experimental evidence suggest that miRNAs can have functions during all stages of neuronal development, including neural stem cell proliferation, neuronal fate specification, neurite outgrowth, and spine development (Liu and Zhao, 2009a). In the case of miR-137, we believe that it may play a dual role in neurogenesis. miR-137 is found to induce differentiation of adult mouse neural stem cells as well as mouse oligodendroglioma-derived stem cells and human glioblastoma multiform-derived stem cells (Silber et al., 2008). Studies from our lab have shown that miR-137 also plays a role in the proliferation and differentiation of adult neural stem cells by targeting histone H3K27 methyltransferase Ezh2 (Szulwach et al., 2010). Interestingly, we found that miR-137 regulates dendritic morphogenesis by targeting MIB1, not EZH2 (Supplemental Table S6.1). These studies suggest that miR-137 plays different roles during the early and late stages of adult neurogenesis in the hippocampus. Additionally, our ISH data (Figure 6.1) shows miR-137 expression is widespread in the DG, suggesting that miR-137 may function in both developing and mature neurons. In fact, Siegel et al have found miR-137 is one of the miRNAs enriched in the synpatosome postnatal mature neurons (Siegel et al.,

2009). Interestingly, double ISH/immunohistochemistry shows miR-137 overlaps with presynaptic synapsin, indicating that miR-137 may also function in the pre-synaptic compartment, in addition to the dendritic and postsynaptic compartment. It is well known that local protein synthesis is important for synaptic transmission and plasticity. In mature neurons miR-137 may regulate the translation of a subset of proteins that are important for neuronal activity-dependent protein expression and synaptic plasticity, similar to what has been found for miR-132 (Vo et al., 2005). Thus, an interesting question to pursue would be whether miR-137 can mediate neuronal activity-dependent dendritic development. It is also possible that miR-137 has effects on the maintenance and survival of neurons. One could speculate that miRNAs in the dendrite may participate in the regulation of local protein translation and modify synaptic plasticity at the synaptic compartment; however, a complete story of how miRNAs and their dendritic target mRNAs regulate dendritic morphogenesis and synaptic development has yet to be told and remains a critical area of future neurodevelopment studies.

The most challenging step in determining the function of miRNA is to identify their downstream mRNA targets. Based on bioinformatic databases, we know that each miRNA can have many potential mRNA targets, yet whether these predicted mRNA targets are functional in the context of miRNA-mediated gene regulation remains to be determined. However, only a small number of these predicted targets are true targets. In addition to MIB1, several additional miR-137 targets are strongly associated with neuronal development and synaptic function including Ezh2, EphA7, EphB2, NcoA3, Shank2, and Snap23. Even though some of these predicted targets did not show obvious functional rescue in our initial screening, we cannot rule out the possibility that miR-137 could function by modulating the translation of these genes to a

lesser extent. Further experiments to identify additional miR-137 targets will give us a more complete picture of miR-137 function during neurodevelopment

An interesting question to pursue would be whether miR-137 can mediate neuronal activity-dependent dendritic development. Although neurons contain many potential targets of miR-137, we chose to follow ubiquitin ligase MIB1, because mass spectrometry has shown it to be enriched in the postsynaptic compartment (Choe et al., 2007; Sheng and Hoogenraad, 2007). *Mib1* was first cloned and studied in zebrafish (Itoh et al., 2003), and the loss of MIB1 led to reduced lateral inhibition of Notch signaling, which in turn triggered changes in the number of progenitors and neuronal differentiation during zebrafish CNS development (Itoh et al., 2003). MIB1 is an E3 ubiquitin ligase and promotes ubiquitination and internalization of the Notch ligand Delta, leading to Notch pathway activation. In mammals, the function of MIB1 is not fully clear. In one study, MIB1 was found to activate the Notch pathway in embryonic mice, and *Mib1* mutant mice exhibit deficits in neurogenesis and resemble mice lacking Notch signaling components (Koo et al., 2005). MIB1 was also shown to be phosphorylated by PAR-1, resulting in MIB1 degradation and stimulation of neuronal differentiation in mammalian neuronal progenitors (Ossipova et al., 2009). Similarly, Notch activation by MIB1-positive newborn neurons and intermediate progenitors in mice functions to ensure the maintenance of stem cell properties of radial glia during neurodevelopment (Yoon et al., 2008). A recent study shows that MIB1 inhibits dendritic development in cultured rat cortical neurons (Choe et al., 2007). The precise role of MIB1 in mammalian dendritic development is unknown; however, the importance of MIB1 in neuronal maturation is clearly demonstrated by both the published literature and our data.

The possible role of the E3 ubiquitin ligase MIB1 in neuronal maturation is intriguing, albeit vastly speculative. The ubiquitin pathway is best known for its role in marking target proteins for specific proteolysis by proteasomes; however, the ubiquitin pathway may also be involved in regulating the abundance of postsynaptic receptors (Burbea et al., 2002). A neuronal deficiency of UBE3A, an ubiquitin protein ligase involved in protein degradation, causes Angelman syndrome, which is characterized by severe mental retardation. In recent studies, UBE3A was found to localize to the synapse, and its deficiency resulted in abnormal dendritic and spine morphology (Dindot et al., 2008; Lu et al., 2009). MIB1, on the other hand, seems to be involved in protein trafficking rather than protein degradation (Itoh et al., 2003). It may modify postsynaptic receptors or other regulatory molecules at the synapse and alter their intracellular localization, hence its involvement in dendritic patterning in developing and mature neurons. The fact that MIB1 can rescue the dendritic deficits associated with miR-137 overexpression further supports the positive effects of MIB1 on mammalian dendritic morphogenesis and its role as one of the downstream effectors of miR-137. Understanding this pathway may also shed light on the molecular mechanism underlying neurodevelopmental disorders associated with neuronal dendritic deficits.

6.6 SUMMARY

Our goal is to understand how noncoding miRNAs regulate development and functions of neurons. We have discovered a number of miRNAs that are enriched in neuronal lineage, relative to either astrocytic lineage or undifferentiated NSCs. Here we show that one of these miRNAs, miR-137, has an important modulatory role in dendritic morphogenesis during neuronal

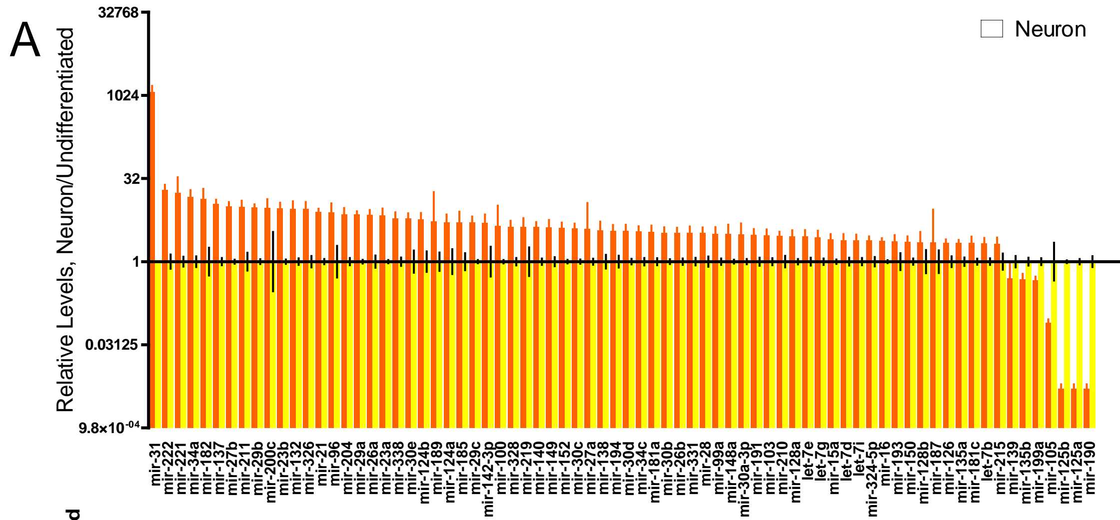
development both in vivo and in vitro. We find that overexpression of miR-137 using the "single-cell genetic approach" in newborn neurons of the adult hippocampus results in reduced dendritic complexity and spine density; however, since the single-cell genetic approach specifically targets proliferating cells prior to neuronal differentiation, we also confirmed that overexpression of miR-137 has the same effect on postmitotic cultured hippocampal neurons. Both overexpression and inhibition of miR-137 have significant but opposite effects on dendritic complexity. Therefore, our data indicate that proper expression of miR-137 is required for the normal dendritic development of hippocampal neurons.

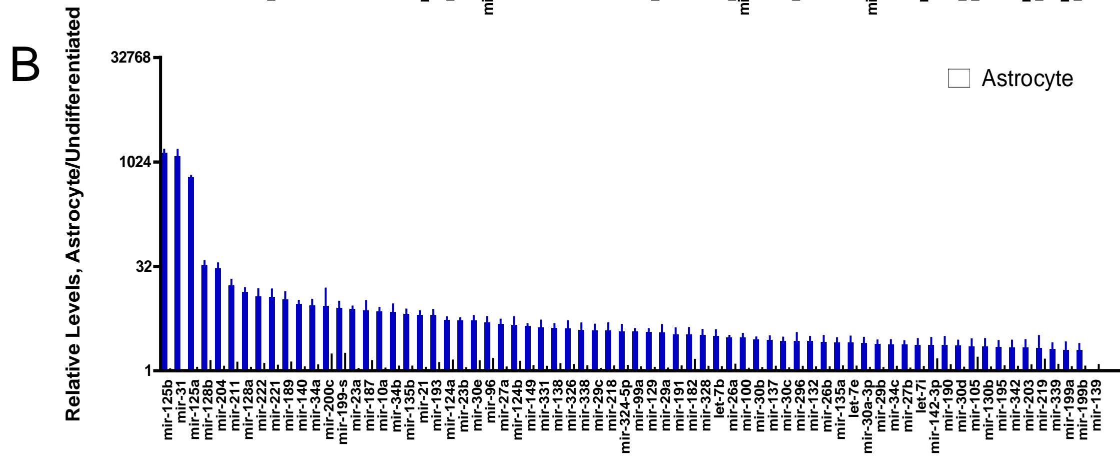
6.7 ACKNOWLEDGEMENTS

We would like to thank the members of the Zhao and Jin Laboratories for their helpful discussions. We thank F. H. Gage for providing us the lentiviral vector and retroviral vector used to engineer the expression vectors for miR-137. Thanks to Michael C. Wilson and Lawrence Tafoya for providing training in neuronal culture methods. P.J. is supported by grants from the International Rett Syndrome Foundation and the NIH (NS051630 and MH076090). P.J. is the recipient of a Beckman Young Investigator Award, Basil O'Connor Scholar Research Award, and Alfred P. Sloan Research Fellow in Neuroscience. X.Z. is supported by grants from the International Rett Syndrome Foundation and the NIH (MH080434 and MH078972). X.L. was a recipient of the Autism Speaks Postdoctoral Fellowship. R.S. is supported by a Minority Supplement to NIH grant (MH080434). R.L.P. is supported by the NIH/GM060201-funded Initiatives to Maximize Student Diversity (IMSD) program.

6.8 SUPPLIMENTAL FIGURES

Supplemental Figure S6.1





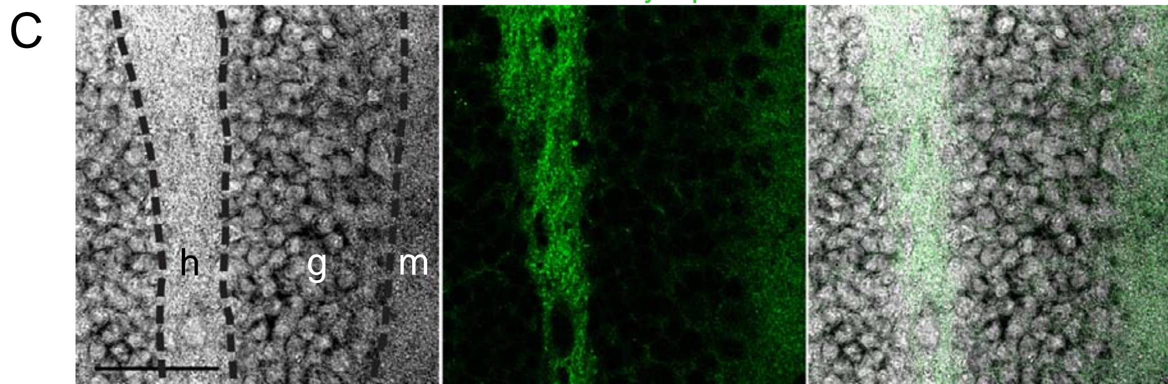


Figure S6.1. A-B, Profile of mature miRNA expression in adult hippocampal neuroprogenitors (A94-NSCs) differentiated into either neuronal or astrocytic lineages compared to undifferentiated progenitors. C, Double ISH/immunohistochemistry to show miR-137 overlaps with pre-synaptic synapsin (h, hilus; g, dentate gyrus; m, molecular layer).

Supplemental Figure S6.2

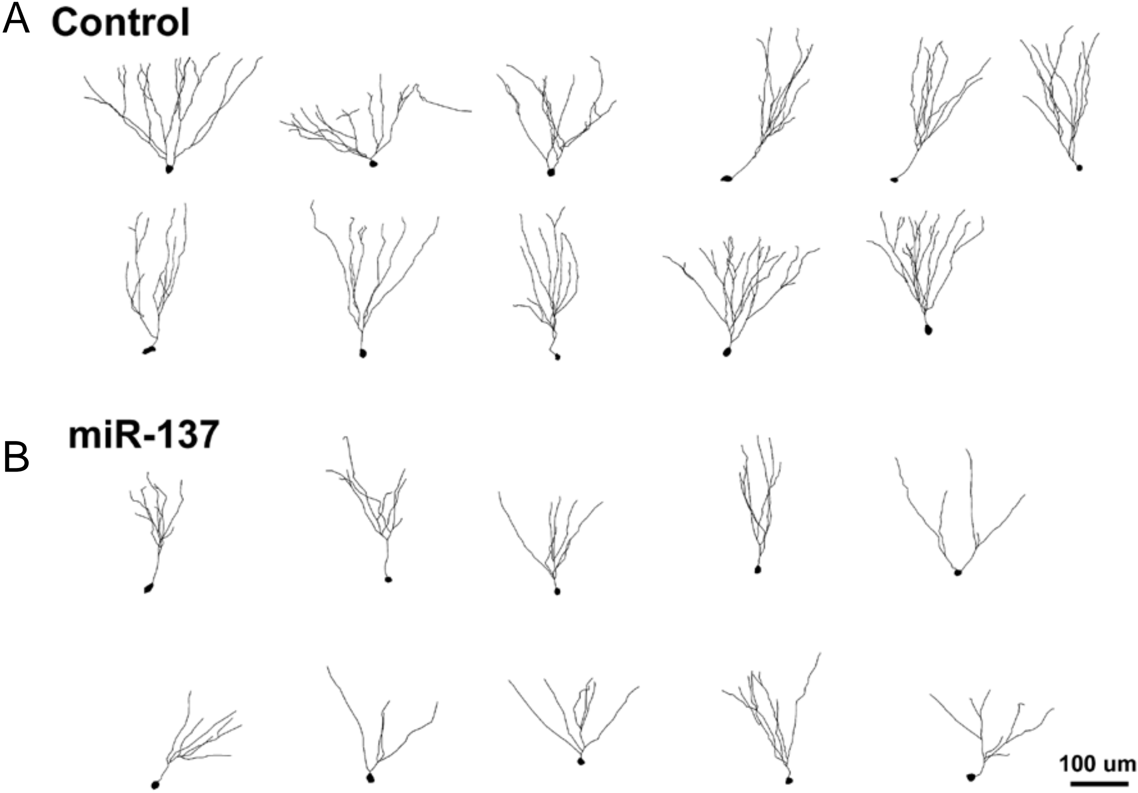


Figure S6.2. Sample traces of in vivo virus-infected neurons used for analysis in Figure 3.

Supplemental Figure S6.3

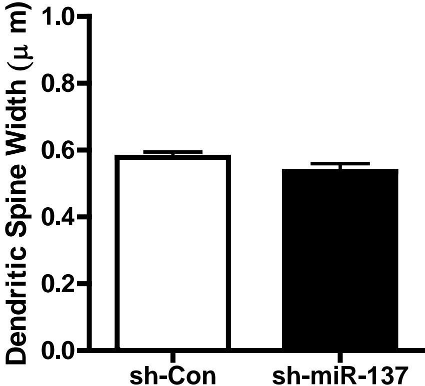


Figure S6.3. The widths of dendritic spines in miR-137-overexpressing neurons were no different from sh-Control overexpressing neurons at 4 weeks post-labeling.

Supplemental Figure S6.4

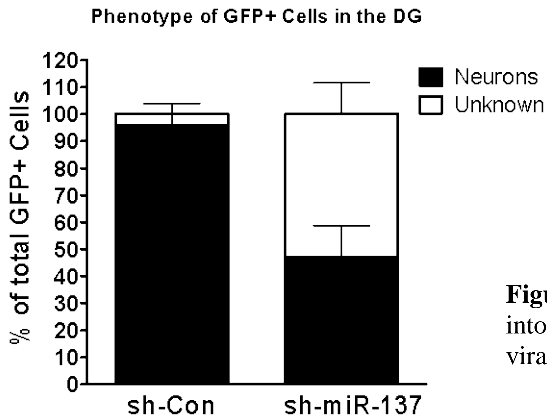
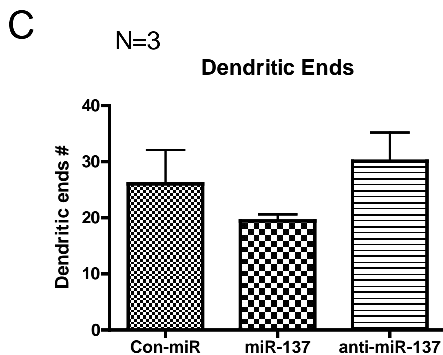


Figure S6.4. Fewer miR-137-overexpressing cells differentiated into neurons (both NeuN+ and DCX+) analyzed at 4 weeks post viral labeling.

Supplemental Figure S6.5



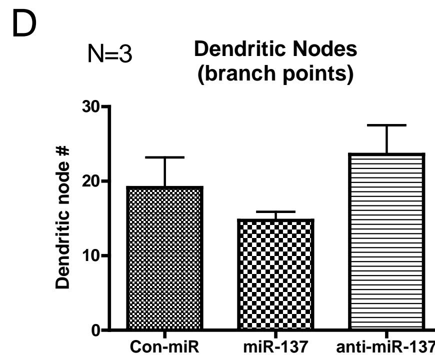


Figure S6.5. miR-137 overexpression led to reduced dendritic complexity in primary hippocampal neurons. A-B, Sample traces of E17 hippocampal neurons transfected with miR-137 used in Figure 5. C, Additional parameters used to assess dendritic morphology (non-significant).

Supplemental Figure S6.6

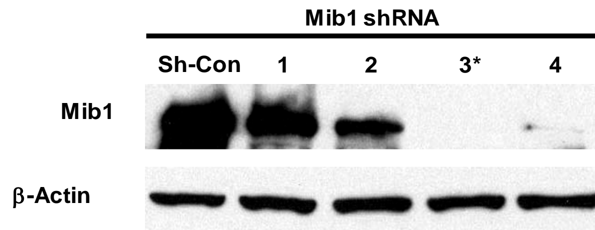


Figure S6.6. Validation of Mib1 shRNA using Western blotting. Mib1 expression vectors and 4 different Mib1 shRNA plasmids were co-transfected into HEK293 cells. The cell lysate was analyzed for mouse Mib1 expression using Western blotting. Among the 4 shRNAs tested, number 3* showed the best knockdown efficiency and was used for Figure 6 experiments.

Supplemental Table S6.1

Table S6.1. Top candidate mRNA targets of miR-137.

mRNA Targets	Luciferase Activity		Western Blot	Dendritic Complexity	Relevance
	293 cells	Hippo Neurons			
MIB1	↓	↓	↓	↓	Notch signaling. Embryonic Development. Dendritic development in hippocampal neurons.
Ezh2	↓	N/A	N/A	—	Nervous system development, stem-cell proliferation and differentiation
EphA7	↓	N/A	N/A	N/A	Nervous system development, apoptosis, proliferation. Synaptogenesis.
NCOA3	↓	N/A	N/A	N/A	nuclear receptor coactivator. The protein has histone acetyltransferase activity, recruits p300/CBP-associated factor and CREB binding protein as a multisubunit coactivation complex.

↓ Decreased

— No Change

N/A Not Analyzed

CHAPTER 7:
Summary and Critique

7.1 Summary

Dendrites and the dendritic spines of neurons are the key sites of synaptic input and connectivity in the brain and have been recognized as the locus of long-term synaptic plasticity. The development of dendrites and spines in mammals is a complex process that requires specific molecular events that function to signal neuronal differentiation, dendritic morphology, and synaptogenesis. These events are tightly regulated by genetic and epigenetic mechanisms, including DNA methylation, chromatin remodeling, and the noncoding RNA-mediated process. It has been shown that the altered spine morphology associated with pathological conditions can affect the properties of an individual neuron, the neural networks, and mental function as a whole. In fact, dendritic spine distribution and structure is abnormal in many diseases and injuries, as well as many forms of mental retardation; however, spine characterizes can also be potentiated by neuronal activities and an enriched environment. Understanding the pathways that lead to altered spines in pathological conditions will provide researchers with a better understanding of the conditions that contribute to normal dendritic spine and synaptic formation during development and learning in the adult brain. Despite the fact that several protein pathways have been identified as critical players in spine development and pathology, the molecular pathogenesis of aberrant spine morphology in these diseases has yet to be clearly and comprehensively elucidated. The goal of this thesis is to determine how epigenetic factors influence the key characteristics of neuronal maturation in developing neurons of the adult hippocampus to further elucidate the pathophysiology of abnormal spine morphogenesis in MR.

In the previous chapters, I described how a *Mecp2* mutant mouse model can be used to investigate the role of MeCP2-mediated epigenetic regulation of hippocampal development (Smrt et al., 2007). The data collected in this study showed that MeCP2 is not critical for the early stages of neurogenesis, but is important for neuronal maturation and spine distribution in the postnatal hippocampus. In a separate study, I showed that a brain-enriched microRNA, miR-137, has a significant role in regulating neuronal maturation in vivo and in vitro by modulating the expression of a MIB1 (Smrt et al., 2010). The data indicated that proper expression of miR-137 is required for normal dendritic development of hippocampal neurons.

In summary, this research shows that epigenetic control of expression pathways, which include MeCP2 and non-coding microRNAs, are required for normal dendrite development of hippocampal neurons, and demonstrate how alterations in epigenetic and noncoding RNA-mediated processes can result in morphological and phenotypic abnormalities that are a fundamental characteristic MR, such as that seen in fragile X, autism, and Rett syndrome. The first major discovery of this work is that MeCP2 epigenetically regulates a number of specific miRNAs in adult brain-derived neural stem cells (NSCs) under both proliferating and neuronal differentiating conditions. One of the miRNAs is miR-137. MeCP2 was found to bind directly to the genomic region proximal to miR-137, and absence of MeCP2 binding to this region correlated with an altered chromatin state and enriched miR-137 expression (Szulwach et al., 2010). Additionally, this finding shows that miR-137 is significantly upregulated in the absence of MeCP2 in the adult mouse brain and primary neurons (see APPENDIX A). The second major discovery of this work is that miR-137 itself has a profound effect on the development of dendritic structure and dendritic spine distribution in newborn neurons of the hippocampus and in post-mitotic cultured neurons from mice. These data support the idea that miR-137 may exert

its biological effects, at least in part, through its interaction with MIB1 (Smrt et al., 2010). The overall model of this research is that epigenetic and non-coding RNA pathways, particularly the crosstalk between these pathways (Smrt et al., 2010; Szulwach et al., 2010) influence the morphogenesis of dendrites and dendritic spines. Thus, because MeCP2 can alter expression of specific miRNAs, including miR-137, I propose that altered expression of miRNAs may contribute to the dendrite and dendritic spine pathogenesis observed in Rett Syndrome (Figure 7.1 and 7.2).

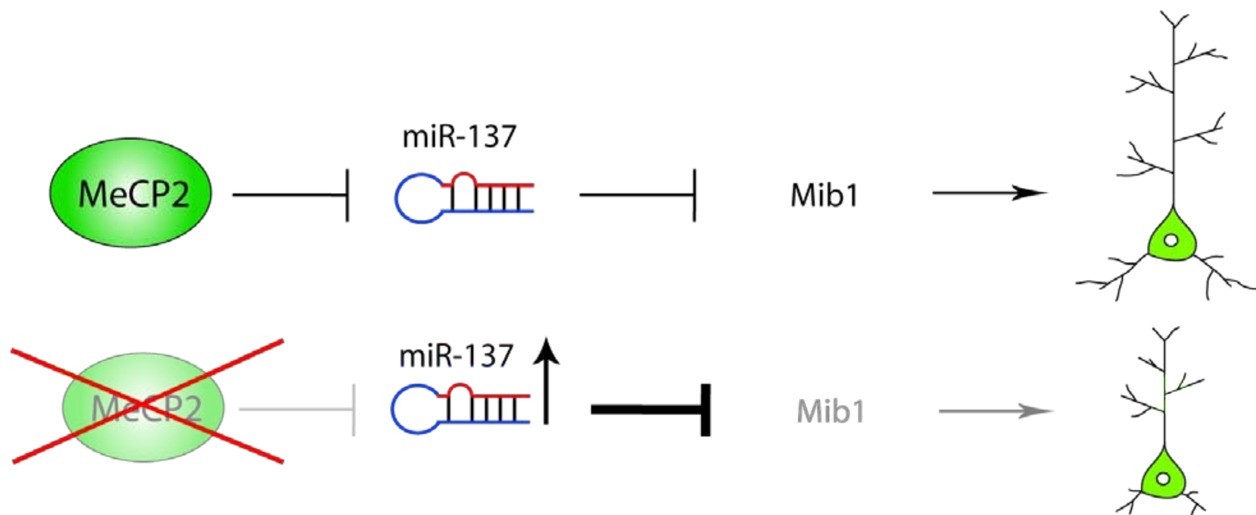


Figure 7.1. A simple model based on my dissertation research (Smrt et al., 2010) showing how crosstalk between epigenetic (MeCP2) and non-coding RNA (miR-137) pathways may converge on a protein coding gene (Mib1) to influence the morphogenesis of dendrites and dendritic spines (Smrt et al., 2010). A more complex model is shown in Figure 7.2.

Although these novel discoveries strengthen the link between epigenetic and non-coding RNA pathways, much is still unknown about the diversity of epigenetic and non-coding RNA molecules that may be interacting to shape the expression of developmentally relevant protein coding genes. Furthermore, many of these pathway interactions may only play a functional role in a specific cell type, or at a specific developmental period. It is important to realize the complexity of epigenetic and non-coding RNA pathways during development. For instance, a single epigenetic regulator such as MeCP2 can affect the expression of multiple miRNAs

(Szulwach et al., 2010) along with the expression of coding genes, and miRNAs such as miR-137 have multiple predicted targets (Figure 7.2). My dissertation research is only the “tip of the iceberg” in terms of drawing a direct link between epigenetic and non-coding RNA pathways and their role in dendritic development.

In the remainder of this chapter, I address some specific concerns and limitations surrounding my research, and expand on some strategies I have used to address many of those concerns. I also speculate on additional experimental procedures that may strengthen my dissertation research and present alternative possibilities that may explain my findings. Finally, I discuss the future directions of my research.

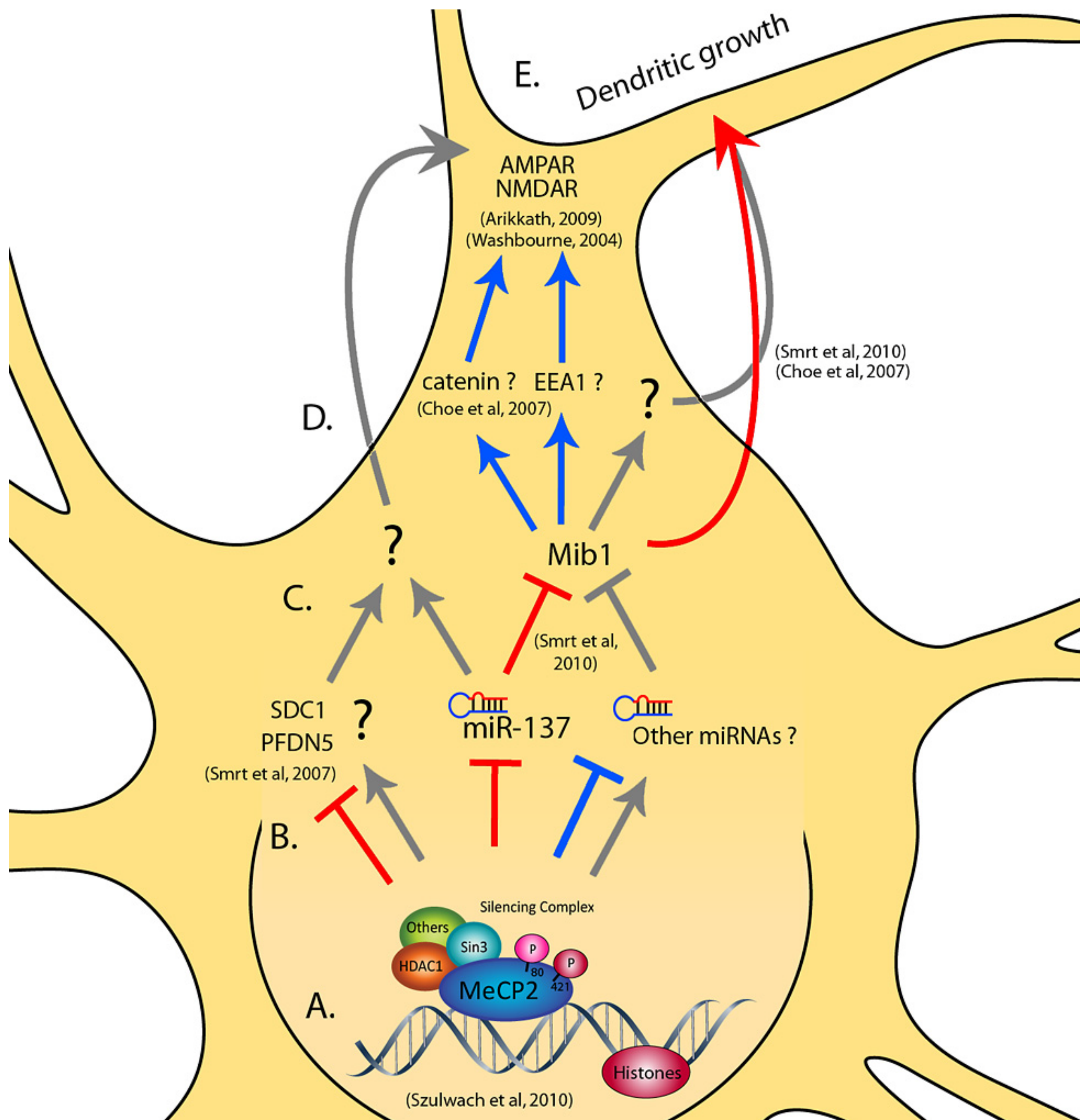


Figure 7.2. Detailed overall model summarizing this dissertation research showing how crosstalk between epigenetic (MeCP2) and non-coding RNA (miR-137) pathways may involve a complex series of pathways that ultimately influence the morphogenesis of dendrites and dendritic spines (Smrt et al., 2010; Szulwach et al., 2010). A. miR-137 expression is correlated with global changes in histone acetylation which could persist throughout neurogenesis and affect miR-137 expression during maturation. Additionally, activity dependant phosphorylation of MeCP2 can influence its association with DNA. B. MeCP2 modulates miR-137, but may also modulate the expression of other miRNAs and unknown factors. C. miR-137 has been shown to regulate MIB1 expression, but it may also regulate other factors important for neuronal maturation. D. MIB1, a ubiquitin ligase, may interact with other molecules like EEA and cateninis. E. Molecules downstream of MIB1, EEA1, and catenins may interact with synaptic molecules such as SDC1 (Smrt et al., 2007) and receptors such as NMDA and AMPA to influence dendritic morphology and dendritic spine development. **Red lines** indicate pathways confirmed in this dissertation research, **blue lines** indicate pathways confirmed in other papers, and **grey lines** are possible pathways that have yet to be confirmed.

7.2 Critical analysis of my dissertation research

7.2.1 Limitations and concerns surrounding cultured neurons

Both the reviewers of my manuscript (Smrt et al., 2010) and members of my dissertation committee have raised concerns about cultured primary neurons as a model for neurodevelopment. Although primary neurons dissociated from an embryonic brain and grown in culture dishes are accepted as a valuable model in neuroscience to study the biological properties of neurons (Goslin and Banker, 1989), cultured neurons have many inherent differences compared to those neurons which function in a living brain. The primary concern regarding these differences is how they may affect the conclusions drawn from experiments using neuronal culture. One can imagine that if the procedure for isolating primary hippocampal neurons involves stripping developing neurons of their normal chemical and physical contacts, they must somehow be different.

This concern is certainly a “no brainer” (pun intended) as the conditions between *in vitro* (dish) and *in vivo* (brain) are different in notable ways. Specifically, primary neurons are cultured in a standardized culture medium which contains factors needed for metabolism and survival of neurons in culture; however this rich medium could not fully recapitulate all of the factors found in a specific brain microenvironment, which interacts with a specific group of neurons. Formation of functional neural networks is a fundamental characteristic of brain development. In culture, post-mitotic neurons are taken out of their native networks, and asked to form new synapses and network connections. Although formation of synapses and networks are highly characterized in cultured neurons, the number and complexity of networks in the standard culture method could certainly not rival the brain. Another difference between cultured neurons and the

brain is that cultured primary neurons suffer from increased degeneration over time. After about 3-4 weeks in culture, hippocampal neurons rapidly lose their synaptic connections and begin to die. This is different from the living brain where many of our neurons must maintain synaptic connectivity and continue to function for a lifetime. Changes in any of these variables may have an impact on the outcome of in vitro experiments. For that reason, we favor in vivo experiments to complement the compelling evidence we gather from primary neuronal culture.

In my study, I used both cortical and hippocampal neuronal cultures taking advantage of the strength of both. As I mentioned in the manuscript, cortical culture yield more neurons with higher purity of neurons (>90%), which allows for biochemical and molecular pathway analysis. On the other hand, hippocampal neurons, generally containing >70% astrocytes, are more suitable for neuronal maturation analysis. However, I am aware of the limitation of both culture systems. Between cortical and hippocampal neuronal isolation, which are two common primary culture methods that both share a nearly identical isolation protocol, cortical cultures can be considered the most heterogeneous in terms of the variety of cell types cultured. For example, cortical cultures contain all the cell types and brain regions of the neocortex. If the experimenter is not careful during dissection, cortical cultures may even contain striatum, hippocampus, and olfactory bulb neurons. However if the dissection is performed correctly, with a strict criteria for removing the additional brain structured mentioned, the cortical preparation will still contain neurons of various cortical regions, birthdates, neurochemical function, and epigenetic background. Thus, it is important to determine how the variability, or heterogeneity, in cortical cultures will impact the question that is being asked. One of the major benefits of hippocampal cultures is that they are far more homogeneous than cortical cultures. Specifically, hippocampal cultures of E17.5 mouse embryos contain primarily CA1 neurons, as the dentate gyrus has not

formed at this early developmental time point. Although mouse hippocampal cultures isolated at E17.5 are more homogenous than cortical cultures isolated at the same time, they are not devoid of other cell types. Thus, I have made an effort to produce cultures that are enriched with neurons, making cellular and biochemical analysis of neurons possible (See Appendix Figure A.6). Despite the advantages of hippocampal cultures, they produce a lower yield of neuronal product in terms of total number of neurons compared to isolated cortex. This can limit the number of conditions that can be held in a single experiment. Many experiments (e.g. ChIP, WB, etc) prefer a generous amount of starting product which may favor cortical preparations.

7.2.2 Limitations and concerns surrounding in vivo retroviral targeting

One effective way for me to study gene regulation in neuronal development is to manipulate young neurons residing in the living brain. The method gaining popularity among neurodevelopmental biologists, and heavily used in my thesis work, is the single-cell genetic approach. This system makes use of recombinant retroviruses capable of specifically infecting dividing cells in vivo. Although this method has tremendous advantages due to its ability to deliver a transgene specifically to newborn cells in the DG, there is a caveat that should be considered when using this method to perform morphological and phenotypic analysis of differentiated neurons. Specifically, since retrovirus infects dividing cells, overexpression of transgenes in dividing neuroprogenitors may affect initial neuronal differentiation, which might affect subsequent neuronal maturation. One could speculate that ectopic gene expression under a retroviral promoter during proliferation and differentiation of neuroprogenators may disrupt the cell's normal biochemical balance during that stage of neurogenesis and interfere with subsequent stages in neuronal maturation. For example, when retroviruses are used to deliver a

transgene (for example GFP, or miR-137) into a cell, the experimenter doesn't have control over the genomic location the transgene will be inserted. Although researchers suggest retrovirus can select active chromatin sties for integration of their viral DNA (Coffin et al., 1990; Naldini et al., 1996), these mechanisms are poorly understood and viral DNA integration into the genome can sometimes lead to unstable mutations in the genome of the host cell(s) (Harbers et al., 1984; Soriano et al., 1987). Another potential confound is that it is unknown if the retrovirus may cause long-lasting changes in dividing or differentiating cells could impact the development of maturing neurons. To address these limitations and confirm that retroviral delivery of miR-137 affects dendritic morphology during neuronal maturation, and not early stages of neurogenesis such as proliferation or differentiation, I expressed miR-137 in post-mitotic hippocampal neurons. The limitations I described above have not eliminated the use of retroviral-mediated gene delivery to study neuronal maturation, but they are important to consider when thinking about additional experiments that can validate your findings. In Future Directions section 7.4 below, I will discuss a specific molecular modification to the retroviral system that can be used to circumvent the caveats I mentioned here.

7.3 Additional studies that could strengthen my dissertation research

7.3.1 Validation of MeCP2 target genes in neurons

In Smrt et al 2007, I showed a number of possible gene targets that were upregulated in the absence of MeCP2. It is a common belief that identification of gene targets that are directly or indirectly regulated by MeCP2 in neurons is an important effort in determining how MeCP2

exerts it effect on gene expression in the brain (Smrt et al., 2007). Although my research contained expression profiles of mRNAs that were differentially regulated in the hippocampus of mice lacking MeCP2, we did not perform ChIP on a subset of those genes. Performing ChIP would allow us to determine of MeCP2 directly regulated their expression, or if gene expression regulation was an indirect consequence of altered MeCP2 expression. To follow up on genes regulated by MeCP2, a gain or loss of function approach could be used to confirm if these genes are important for specific developmental stages of the neuron, specifically dendrite and dendritic spine development. If these MeCP2-regulated genes were found to be linked to dendritic morphology, we would express them in the *Mecp2* KO to attempt to rescue the mouse *Mecp2* KO phenotype. Additionally, genes that were confirmed to be down-regulated in the absence of MeCP2 may be pathway targets of my later miRNA studies. These studies could help identify some the factors that are involved in the morphological phenotype observed in previous studies (Smrt et al., 2007).

7.3.2 How MeCP2 regulates miR-137 in neurons

Unpublished data from my dissertation research project show that expression of miR-137 is upregulated in the absence of MeCP2 in neuronal cultures and cortical brain tissue (Appendix Figure A.1). Although these data suggest a link between epigenetic and non-coding RNA pathways, these data do not explain the epigenetic mechanism of how MeCP2 may function to modulate the expression of non-coding RNAs, specifically miR-137. The data (unpublished, Appendix Figure A.5) showed no enrichment of MeCP2 on genomic region surrounding miR-137 both in mouse hippocampal cultures and mouse adult cortex. This suggests that although miR-137 levels are elevated in the absence of MeCP2, MeCP2 may not directly regulate miR-

137 by direct genomic binding in post-mitotic neurons. In this section I will propose a number of possibilities that may explain why elevated miR-137 levels are observed in the absence of MeCP2.

In proliferating adult neural stem cells, elevated expression of miR-137 was found in the absence of MeCP2 (Szulwach et al., 2010). To determine if MeCP2 directly interacts with genomic regions proximal miR-137, MeCP2-specific ChIP followed by real-time PCR was performed in aNSCs. It was found that elements just upstream of miR-137 were enriched 3-fold suggesting MeCP2 has a direct association with regions proximal to miR-137. In aNSC, the interaction of MeCP2 with miR-137 involves SOX2, an important transcriptional regulator specifically expressed in stem cells but not in differentiated neurons. This data suggests that MeCP2 is involved in establishing the chromatin state so that transcription can be coordinated in stem cells by factors such as SOX2. It was found by Szulwach et al 2010 that Ezh2 is a functional target of miR-137. EZH2 is a H3-K27 methytransferase and part of the Polycomb group (PcG) of complexes that is related to the maintenance of the “bivalent chromatin state” of stem cells. The “bivalent chromatin state” essentially “primes” genes for cell/tissue specific expression, but holds them in check by opposing chromatin modifications (Boyer et al., 2006; Lee et al., 2006). Regulation of Ezh2 by miR-137 may be important for establishing and maintaining the bivalent chromatin state of stem cells, by keeping appropriate levels of H3-K27-Tri-Me3. A decrease in EZH2 expression resulting from elevated miR-137 is correlated with a global decrease in H3-K27-Tri-Me3 levels that can change the chromatin state of aNSCs (Szulwach et al., 2010). These changes may persist throughout neurogenesis even when aNSCs differentiate into neurons when Ezh2 is no longer expressed.

I propose that reduced H3-K27-Tri-Me3 levels in differentiating KO NSCs leads to increased expression of specific developmental genes, such as miR-137 in neurons. There are several ways to test this hypothesis. To test if the amount of H3-K27-Tri-Me3 bound to miR-137 genomic region is altered in the absence of MeCP2, I could perform a H3-K27-Tri-M3e histone-specific ChIP in proliferating NSCs, differentiating NSCs and primary neurons lacking MeCP2 compared to WT controls. I will then determine whether exogenous MeCP2 or Ezh2 can rescue the binding of H3-K27-Tri-M3e to the miR-137 genomic region using ChIP. These experiments will show whether H3K27-Me3 levels are altered in KO neurons and whether it is result of lacking MeCP2. Another possibility is that H3-K9-Ac, which is associated with transcription activation, is increased in miR-137 genomic region of *Mecp2* KO aNSCs and such increase may persist into the neuronal lineage. It was found previously that H3-K9-Ac was increased surrounding the miR-137 gene in proliferating aNSCs lacking MeCP2 (Szulwach et al., 2010). Therefore, I could also use a histone-specific ChIP to determine if H3-K9-Ac is altered in neuronal differentiating aNSCs lacking MeCP2 compared to WT controls. These data will help us to determine if decreased MeCP2 and increased miR-137 expression early in development may be related to altered establishment of the chromatin state leading to the altered miR-137 expression observed in neurons lacking MeCP2.

Alternate possibilities are that MeCP2 indirectly regulates miR-137 expression by regulating an unknown factor (Figure 7.2). For instance, MeCP2 may maintain expression of miR-137 by repressing a transcriptional activator of miR-137. Additionally, MeCP2 has been shown to be a transcriptional activator for a small subset of genes. Thus, MeCP2 could activate a repressor to maintain miR-137 expression. Another possibility based on recent literature showing the role of MeCP2 in activity dependant gene regulation, is that MeCP2 may selectively regulate

miR-137 in an activity dependant manner (See section 7.4.1 about activity-dependant regulation). Thus, detection of genomic binding of MeCP2 may only be possibe under activated conditions. In any of these testable scenarios, lack of MeCP2 would lead to increased miR-137 levels (Appendix Figure A.1).

7.3.3 Other potential targets of miR-137

Most miRNAs bind to their target 3'UTR with imperfect complementarity as they function as translational repressors (Guo et al., 2010). This is in contrast to RNA interference (RNAi), where RNAi is a sequence-specific gene silencing mechanism (Hannon, 2002) that uses small interfering RNAs (siRNAs) to interact with target mRNAs. siRNAs and miRNAs are similar with respect to their biogenesis, molecular characteristics, and functions (He and Hannon, 2004). As described in Chapter 2.4, both miRNAs and siRNAs are 21-25 nucleotides in length, share the RNase-III processing enzyme, Dicer, and interact with the effector complex, RISC. miRNAs and siRNAs seem to differ most noticeably in their origin and target recognition. First, miRNA precursors are genetically encoded whereas siRNAs are formed from long dsRNAs that can be generated from endogenous RNAs that can anneal to form dsRNA (Hannon, 2002). Second, miRNAs bind to target 3'UTRs through imperfect complementarity, often at multiple sites (Guo et al., 2010). In contrast, siRNA mostly binds only one site forming a perfect duplex with the target. It seems that the similarities and differences between miRNA and siRNA have been extensively explored; we have yet to identify all the miRNA-interacting complexes and to understand the specific actions that distinguish miRNA and siRNA and their respective in vivo targets. Thus, the small similarities and differences between these two regulatory mechanisms

will certainly emerge as the precise mechanisms of individual non-coding RNAs and associated protein complexes are revealed.

This introduction sets the stage for a major concern surrounding my research, which is focused on the effect of a single miRNA on neurodevelopment when there are so many targets of any single miRNA based on the non-complementary characteristics of miRNA targeting. Why select miR-137 when there are a number of miRNAs expressed in developing neurons and quite a few of them might be regulated by MeCP2? This question has been raised for a number of reasons. Since the study of miRNA function in the brain is a novel pursuit in neuroscience, scientific peers and critics are particularly interested in knowing more about how they can optimize selection criteria and target validation. Fortunately, despite the fact that miRNAs are only recently becoming a popular target for neurodevelopmental studies, researchers have long been using molecular tools to detect RNAs. These tools, along with advances in high-throughput microarray screening and popularization of bioinformatic databases have accelerated our ability to examine miRNA expression in the brain, and enabled researchers to propose potential mechanisms for miRNA function. I will first describe a somewhat standardized process for identifying miRNA's and their functional targets followed by how I identify miR-137 and its target MIB1.

In my studies, we first looked at the expression of miRNAs using miRNA-specific microarrays in brain tissue, post-mitotic neurons, undifferentiated neural stem cells, and neural stem cells that are differentiated into neurons or astrocytes. This kind of sampling enabled us to identify a subset of miRNAs that are enriched in cells derived from the neuronal lineage. This is precisely how miR-137 became noticed as a neuronal enriched miRNA that may be important for development. The next step is to assess the subset of miRNAs that have been revealed by the

array. This selection process can be based on factors such as the degree of expression, or relevance to development. When considering miRNAs that are relatively uncharacterized, one may chose to study a miRNA based on its expression levels in a certain tissue or cell type. However, with the increasing amount of literature on miRNA in the brain, researchers can now chose miRNAs to study based on relevance to their research and potentially discover novel mechanisms. For example, miR-137 was highly uncharacterized at the time we noticed it on arrays, and was chosen based on its neuronal specific expression. Once the miRNA(s) have been selected, their expression levels can be confirmed by qRT-PCR. The next step involves the mining of reputable bioinformatic databases. The internet is populated with many miRNA prediction databases (mentioned in Chapter 6) that use genetic sequences and mathematical algorithms to generate a list of possible interactions between a mature miRNA and specific sequences of the 3'UTR of a target gene. It has been reported that this method for target prediction is very efficient. One may consider using defined selection criteria when choosing a target. For example, a subset of potential miRNA targets can be chosen for further analysis based on conservation, context score of target ''seed sequences,'' and known relevance to dendritic morphogenesis and neuronal development. Next, it is a common practice in miRNA research to take the subset of potential targets and validate them using luciferase assays. This assay is designed to confirm that the miRNA binds to the 3'UTR of a target gene to regulate translation. The 3'UTR of the potential target genes can be fused to a luciferase reporter, and cells of interest can be co-tranfected with the reporter and miRNA(s) of interest. To further confirm that the miRNA regulates translation of the target gene by binding the predicted "seed" region, many researchers have modified the seed region on the 3'UTR of the target. Based on luciferase activity data, identified targets can be further assayed using western blots. For example, one

would overexpress or knockdown miRNA in cells and assay protein expression of the target gene. Overall, this process allows researchers to discover the expression profiles of miRNAs in a tissue of interest, and then derive a one or more protein targets that play a role in the function of the cell.

Previously, I used this process to show miR-137 regulates neuronal development by translational regulation of MIB-1 (Smrt et al., 2010). However, during this process many other miRNAs and potential miR-137 targets were identified. Other miRNAs identified during our initial screening may also be important for neurodevelopment and dendritic morphogenesis. Following up on additional miRNAs or miR-137 targets is considered a "lack of studies" because it is likely that some of the other miRNAs may be related to the pathway I have identified, and that additional miR-137 targets function to shape dendritic and spine development of the neuron. However, although there may be many potential targets of miR-137, only a few are likely to be true targets of miR-137 and have a function in neuronal maturation. For example, it was criticized that I identified SHANK2 as a potential target of miR-137, but didn't perform additional experiments to confirm that gene as a target. SHANK2 is known to specifically play a role in spine morphogenesis and function, and I found spine morphology was no different in neurons overexpressing miR-137 compared to controls suggesting Shank2 is not a functional target of miR-137. However, although SHANK2 is not a likely target for miR-137-mediated dendritic maturation, it could potentially mediate miR-137 function in other neuronal development aspects. In addition, some of the other potential miR-137 targets may also play a role in dendrite morphogenesis to some extent. This additional information not only may shed light on the role of miRNAs in neuronal maturation, but also can provide an additional

dimension to the complexity of the miRNA regulatory pathway. Thus, these unexplored data I have generated can be used to propose future experiments.

7.3.4 Cell type-specific and subcellular localization of miRNA

The combination of fluorescent *in situ* hybridization (FISH) and immunohistochemical colocalization method is used by many researchers as a tool to characterize the cell type-specific and subcellular localization of miRNAs and the proteins they potentially regulate. The major technical limitation to this approach is obtaining antibodies and RNA probes that can each label their target effectively when used together. It seems that specific combinations of antibodies and probes can be challenging to optimize. Using FISH to characterize the subcellular localization of miRNA may give clues to its function and could be used to help researchers identify a potential miRNA pathway. For example when studying dendritic or spine development, it's informative to know if a specific miRNA is expressed in the dendrites, cell body, or nucleus. ISH combined with immunohistochemistry could confirm that the miRNA and its proposed target are colocalized within a cellular compartment. In the case of my dissertation research, the co-localization of miR-137 and MIB-1 in neurons could further support my model. In addition, identification of where the interaction of miR-137 with MIB1 takes place would provide further mechanistic insight to the relationship between miR-137 with MIB1. Is it in the dendrite, in the spine, or in the cell body? Are they selectively colocalized proximal to translation machinery? For example, I hypothesize that miR-137 and MIB1 localized in the dendrite near or in the dendritic spine because it has been shown that translation of proteins important for synaptic function take place locally at the dendrite and at the dendritic spine (Sutton and Schuman, 2005), and miR-137 has been shown to be enriched in synaptic fractions (Lugli et al., 2008). I further

speculate that this interaction of miR-137 and MIB1 takes place proximal to polysomes located in the dendrites and dendritic spine. This hypothesis could be tested in neurons by using FISH along with the appropriate probes. This additional information could show where this subcellular interaction takes place, and add to the understanding of how miR-137 shapes dendritic development through its interaction with MIB1.

7.3.5 Potential function of MIB1 in neuronal maturation

MIB1 is an ubiquitin ligase known to be important for neurogenesis and neurodevelopment (Choe et al., 2007; Itoh et al., 2003; Ossipova et al., 2009). MIB1 is a E3 ubiquitin ligase and promotes ubiquitination and internalization of the Notch ligand Delta, leading to Notch pathway activation. In mice, notch activation by MIB1-positive newborn neurons and intermediate progenitors functions to ensure the maintenance of stem cell properties of radial glia during neurodevelopment (Yoon et al., 2008). Although we know MIB1 is important for neuronal maturation (Choe et al., 2007), the molecular mechanism(s) underlying the effect of MIB1 on dendritic development are unclear. One proposed hypothesis is that the effect of MIB1 on neurite outgrowth could be explained to some degree by the effect of MIB1 on Notch signaling (Choe et al., 2007). Because Notch has been shown to reduce neurite morphology, activation of Notch by MIB1 cannot explain my results as published in Smrt et al 2010. I have found that overexpression of MIB1 leads to increased dendrite complexity and acute knockdown leads to reduced dendrite complexity. Choe and colleagues also recognized that MIB1 may regulate neurite morphogenesis through pathways other than Notch. For example, MIB1 was proposed to regulate neurite morphogenesis by its functional interaction with p35/CDK5, where CDK5 activity can stimulate the degradation of MIB1, however it is not

known if this interaction is transient because the majority MIB1 and p35/CDK5 are not localized in the same compartment (Choe et al., 2007).

My research reports an increase of dendritic complexity when over expressing MIB1 while others have reported a decrease in dendritic complexity, however the importance of MIB1 in neuronal maturation is clearly demonstrated by both the other published literature and our data (Choe et al., 2007; Smrt et al., 2010). The reason for this discrepancy is likely due to the cell-type, and developmental time-point we used. For example, the data shown in Choe et al 2007 was mostly in cortical neurons, where transfection was performed and morphology was analyzed at a time when neurons have made few synaptic connections, and at an earlier time than in my studies. In my studies, hippocampal neurons were used, which are more homogeneous and can become far more complex compared to cortical neurons, and additional morphological analysis were used which included dendritic length, branch points, dendritic ends, and dendritic complexity by sholl analysis. Although MIB1 is highly expressed in both embryos and adults, one possibility is that the function of MIB1 may vary in cell/tissue specific manner and over a developmental time course. There are multiple ways that MIB1 can regulate neuronal maturation (Figure 7.2). For example, it was discovered that many other proteins that may interact with MIB1 also have a dramatic effect neuronal morphology. One such protein is FAM, a mammalian orthologue of the Drosophola protein fat facets. FAM is a deubiquitinating enzyme that can remove ubiquitin tags from substrates. FAM/faf has been proposed to control vesicle trafficking (Chen et al., 2003a), synaptic plasticity (Chen et al., 2002), and Notch signaling (Overstreet et al., 2004). Expression of FAM/faf in neurons causes an increase of synaptic boutons in neuromuscular junctions (DiAntonio et al., 2001). The interaction of ubiquitin ligases and deubiquitinating enzymes is an important mechanism for modulation of the ubiquitination of

protein targets (Nijman et al., 2005), and this interaction could play a underlying role in effect on MIB1 on neuronal maturation in my dissertation research.

The ubiquitin pathway is best known for its role in marking target proteins for specific proteolysis by proteasomes; however, the ubiquitin pathway may also be involved in regulating the abundance of postsynaptic receptors (Burbea et al., 2002). Using affinity purification and mass spectrometry, MIB1 was found to interact with membrane trafficking proteins such as early endosomal antigen 1 (EEA1), Rab11-interacting proteins, and synaptosomal associated protein of 25 kDa-like protein (SNAP25) (Choe et al., 2007). For example, knocking down EEA1 in hippocampal slice cultures using EEA1 antibodies has been shown to increase AMPA, NMDA, and kainite mediated excitatory post-synaptic currents, which may be due to impairment of internalization of specific and glutamate receptors at the synapse (Selak et al., 2006), and EEA1 is colocalized with these receptors during synapse formation (Washbourne et al., 2004). Catenins have also be suggested to interact with MIB1 by affinity purification (Choe et al., 2007), and it is known that alpha-, beta- and delta-catenin are involved in synaptic function and dendritic morphogenesis (Arikkath, 2009). Thus, the possible role of the E3 ubiquitin ligase MIB1 and its interaction with trafficking proteins like EEA1 or catenins to shape neuronal maturation is intriguing, albeit speculative. It is unknown what effect MIB1 may have on the function of its proposed interactome such as EEA1 and catenins. Hence, I propose that MIB1 is involved in dendritic patterning in neuronal development through its modification of postsynaptic or cell-adhesion molecules at the synapse. Further studies can be conducted in vitro to confirm the physical and functional interaction of MIB1 with these other proteins. For example, affinity chromatography and subsequent mass spectrometry has been used to identify MIB1 interacting proteins, followed by affinity columns made of GST-MIB1 to isolate target proteins (Choe et al.,

2007). To test if these molecules interact with MIB1 to shape dendritic morphology in neurons, the proteins predicted to interact with MIB1 can be co-transfected with MIB1 in neurons to determine if they can alter or rescue the effect of MIB1 on dendritic morphology. Understanding this pathway may shed light on the molecular mechanism that leads to the results I see when manipulating MIB1 expression, and the underlying neurodevelopmental disorders associated with neuronal dendritic deficits.

7.4 Future directions of this project

I have made significant discoveries during my dissertation research that have led to multiple publications in peer-reviewed scientific journals. The results of my work have also generated many new questions and future directions that can be pursued by other scientists in our laboratory and related scientific fields. These future directions are the focus of this section.

7.4.1 Activity-dependant epigenetic regulation (MeCP2) of miRNA

An important characteristic of MeCP2 is that its function is regulated in a neuronal activity-dependent manner. For example, addition of potassium chloride (KCl) will depolarize cultured neurons and lead to reduced association of MeCP2 with its known target promoter of *Bdnf*, and also leads to an increase in *Bdnf* transcription (Chen et al., 2003b). The activity-dependent function of MeCP2 is regulated by protein phosphorylation, which is known to be an important posttranslational modification that can modulate the function of a protein by adding a phosphate group to serine, tyrosine, or threonine residues. MeCP2 is predicted to be

phosphorylated in at least 10 different sites and the the most studied sites are Serine 421 (S421) and S80. MeCP2 can be phosphorylated at S421 and dephosphorylated at S80 under activity-dependant conditions and is associated with promoter binding to methylated DNA. MeCP2 is phosphorylated at S80 and dephosphorylated at S421 under resting conditions and such phosphorylation inhibits binding methylated DNA (Chao and Zoghbi, 2009; Zhou et al., 2006). Recently, it was found that mutations of specific phosphorylation sites on MeCP2, such as S80A, can recapitulate the loss of function phenotype whereas a S421A/S424A double mutant displays phenotypes observed in the gain of function model (Tao et al., 2009). This research demonstrates the significance of posttranscriptional changes to MeCP2 and how transcriptional regulation by MeCP2 can respond to neuronal activity. We know that MeCP2 S421 phosphorylation regulates the activity dependant induction of *Bdnf* transcription (Zhou et al., 2006); however, much is unknown about the role of activity-dependant phosphorylation of MeCP2 on the expression of specific MeCP2-mediated genes. It was also shown that KCl treatment can induce miR-132 expression, which targets the *Mecp2* 3'UTR and inhibits its translation (Klein et al., 2007), demonstrating an activity dependant link between MeCP2 and miRNA. Thus, I propose that activity-dependant phosphorylation of MeCP2 may be a mechanism by which miRNA expression is modulated in developing neurons. It is possible that direct MeCP2-dependant epigenetic regulation of non-coding RNAs during neuronal development requires depolarization of the cell, such as the depolarization of neurons induced by KCl in previous MeCP2 studies (Chen et al., 2003b; Martinowich et al., 2003; Zhou et al., 2006). That is, MeCP2 may have an activity-dependant role in regulating of non-coding RNA expression that we have not previously explored. Activity dependant regulation of miR-137 by MeCP2 could explain why we see an upregulation of miR-137 in the absence of MeCP2, yet MeCP2 does not appear to bind the

region upstream of miR-137 using ChIP. A potential future experiment to explore this mechanism is to stimulate neuronal cultures with KCL and determine if MeCP2 can bind to genomic regions proximal to miR-137 and compare those results to non-stimulated cultures.

7.4.2 Synergistic effects of multiple miRNAs

The effect of multiple miRNAs on single gene targets and also multiple miRNAs on multiple gene targets is an understudied area concerning miRNA functions in the brain. One aim is to address the problem of complexity in the network of miRNA-mRNA interactions that may play a role neurodevelopment. For example, a potential future experiment would first use bioinformatics to identify multiple miRNAs that may converge on a single gene target to regulate translation. For example, I previously showed that developing neurons express miR-137 and miR-301, and a bioinformatic approach confirmed that are both predicted to bind the *Mib1* 3'UTR (Smrt et al., 2010). Interestingly, altered miR-301 and miR-137 levels are found in the absence of MeCP2. The hypothesis for this potential future experiment is that both of these miRNAs may function together to regulate the expression of MIB1 during the process of neuronal development. However, the complexity of such an interaction should be considered carefully. For example, do these miRNAs (137 and 301) interact with *Mib1* in the same sub-cellular compartment, and at the same time during development? One possibility is that they both work together, at the same time during neuronal maturation to regulate MIB1 protein expression. An alternate possibility is that they work at different periods, or in different sub-cellular compartments. For example, miR-137 is known to by enriched in the synaptic compartment (Lugli et al., 2008), where it is speculated to play a regulatory role on local gene translation, but perhaps miR-301 is has its maximum regulatory effect in a non-synaptic fraction of the cell. I

speculate that such combinations of altered miRNA expression could contribute to the phenotype of Rett syndrome, and could be the focus of a future direction. This "convergence" of multiple miRNA's on a gene target could then be expanded to look at the effect these multiple miRNAs have on other targets related to neurodevelopment. Solving the complexity of these regulatory networks will shed light on the mechanisms of developmental pathways, and help us to understand the pathogenesis of diseases like Rett syndrome.

7.4.3 How MeCP2-regulated miRNAs may regulate synaptic function

In my previous work, I show that miR-137 overexressing neurons have morphological abnormalities, including reduced dendritic complexity and spine density (Smrt et al., 2010). The functional relevance of how a modest miR-137-mediated decreased dendritic complexity and decreased spine density may affect the function of neurons and synapses remains to be explored. Because the synapse is the locus of communication between neurons, one possibility is that reductions in dendrite length, complexity, and spine density lead to fewer total synapses on the neuron. A neuron with fewer synapses, all else being equal, could affect the capacity to which that neuron responds to the surrounding circuitry. Specifically, fewer spines and smaller dendrites could result in fewer synapses which would lead to reduced connectivity to surrounding neurons. A modest change in dendritic complexity and spine density may be detrimental to overall neuronal connectivity and function when considered in the context of an entire neural network, such as the hippocampus. However, I do not imply that a modest morphological change alone can interfere with functional circuitry development and lead to severe cognitive and motor impairment. It may be possible that changes in other molecular and functional characteristics of the neuron lead to the phenotype I observe in neurons

overexpressing miR-137. Specifically, it has been shown that there is a shift in the balance between synaptic excitation and inhibition in Layer 5 pyridimal neurons in *Mecp2*-mutant mice (Dani et al., 2005). Additionally, deficits in long-term potentiation (LTP) in cortical and hippocampal synapses have been reported in *Mecp2*-mutant mice (Asaka et al., 2006; Moretti et al., 2006). It has also been demonstrated that duplication or knock down of MeCP2 can lead to altered glutamatergic synapse numbers in hippocampal cultures (Chao et al., 2007). Recently it was shown that *Mecp2*-mutant mice have weaker excitatory synapses and fewer connections in Layer 5 neurons (Dani and Nelson, 2009). Thus, these data suggest there is a relationship between neural circuits and the RTT phenotype, and that MeCP2 expression is critical for the proper formation and balance of excitatory and inhibitory synapses. It is possible that miR-137 may therefore alter the synaptic function and connectivity in hippocampal neurons. To determine if overexpression of miR-137 leads to synaptic deficits similar to those seen in *Mecp2*-mutant mice, a retrovirus carrying GFP and miR-137 can be injected into the mouse hippocampus as described in Smrt et al 2010. Hippocampal slice cultures can be prepared from the brains of these mice, and whole-cell recordings can be performed in GFP-expressing new dentate granule neurons. Spontaneous action potential firing can be measured, which has been shown to be significantly decreased in *Mecp2*-mutant mice (Dani et al., 2005). In addition spontaneous excitatory and inhibitory synaptic currents can be measured in these slice cultures, as excitatory synaptic charge has been found to be decreased and inhibitory synaptic charge has been found to be decreased in *Mecp2*-mutant mice (Dani et al., 2005). Quantal release of individual glutamatergic vesicles can be studied by looking at spontaneous mini excitatory postsynaptic currents (mEPSCs) and individual GABA vesicles by spontaneous mini inhibitory postsynaptic currents (mIPSCs), which have also been found to be modestly altered in *Mecp2*-mutant mice

(Dani et al., 2005). These studies could help us understand how miR-137 may alter the synaptic function in hippocampal neurons. However, electrophysiology methods alone may not tell the complete story. It is possible that miR-137 may be important for the development of excitatory and inhibitory synapse distribution. The effect of miR-137 on the formation of glutamatergic synapses can be determined using immunohistochemisty in mouse cultured hippocampal neurons. To do this, the localization of developmentally-regulated synaptic proteins that are known to cluster at the synapse can be stained: 1) Excitatory post-synaptic proteins PSD95, and NMDA and AMPA receptor subunits GluR2 and NR2b, 2) inhibitory synaptic post-synaptic proteins GABA Receptor A, 3) excitatory pre-synaptic proteins scaffolding protein Bassoon, and neurotransmitter transporters VGLUT1 and VGLUT2, and 4) the inhibitory pre-synaptic protein GAD67. This study will determine if altering the expression of miR-137 leads to changes in the distribution of excitatory and inhibitory synaptic proteins. Overall, these studies will help us to understand how MeCP2 regulates miRNAs, such as miR-137, may affect synaptic function. Additionally, these studies may associate the miR-137 pathway as an underlying mechanism that leads to the mysterious altered balance between excitation and inhibition observed in Rett syndrome research.

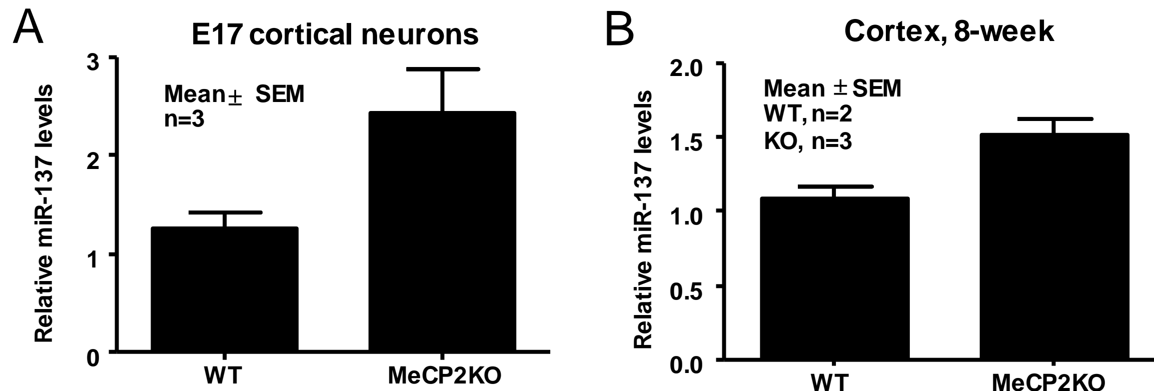
7.4.4 Studying in vivo miRNA functions using mouse genetics

Previously, I take a gain-of-function approach to study the effect of miR-137 on dendritic and dendritic spine development (Smrt et al., 2010). However, we still don’t know the effect of knocking out, or reducing miR-137 expression. Thus, a major future direction to my dissertation research is to further characterize the importance of miR-137 in dendrite and dendritic spine development. I hypothesize that knocking out miR-137 *in vivo*, will increase dendritic

complexity, which is consistent with what I observed previously (Smrt et al., 2010) when I knocked down miR-137 *in vitro*. To test this, a novel miR-137 conditional knockout must be generated. These mice can be used to extrapolate meaningful information on the function of miR-137 in neurodevelopment. We also don't know the effects of manipulating miR-137 expression in different brain regions and at specific developmental periods. Because a novel miR-137 conditional knockout or overexpressing mouse will possess the potential for conditional loss or gain of miR-137 function, researchers can probe the effect of miR-137 at specific developmental periods, or within specific brain regions. For example the conditional miR-137 mice could be crossbred with a mouse expressing CRE-ER^{T2} fusion protein under a developmentally or tissue specific promoter, such that the effects of gain or loss of miR-137 can be studied in a subset of cells when CRE-mediated recombination is induced by Tamoxifen (Lagace et al., 2007). Additionally, conditional miR-137 knockout or overexpressing mice could be crossbred with *Mecp2* mutant mice, and functional or behavioral studies could be performed without the invasive use of intracranial virus injection surgery. This proposal is one solution to the limitations of in vitro culture system and *in vivo* retroviral injection I used in my previous research.

APPENDIX A:
SUPPLEMENTAL DATA

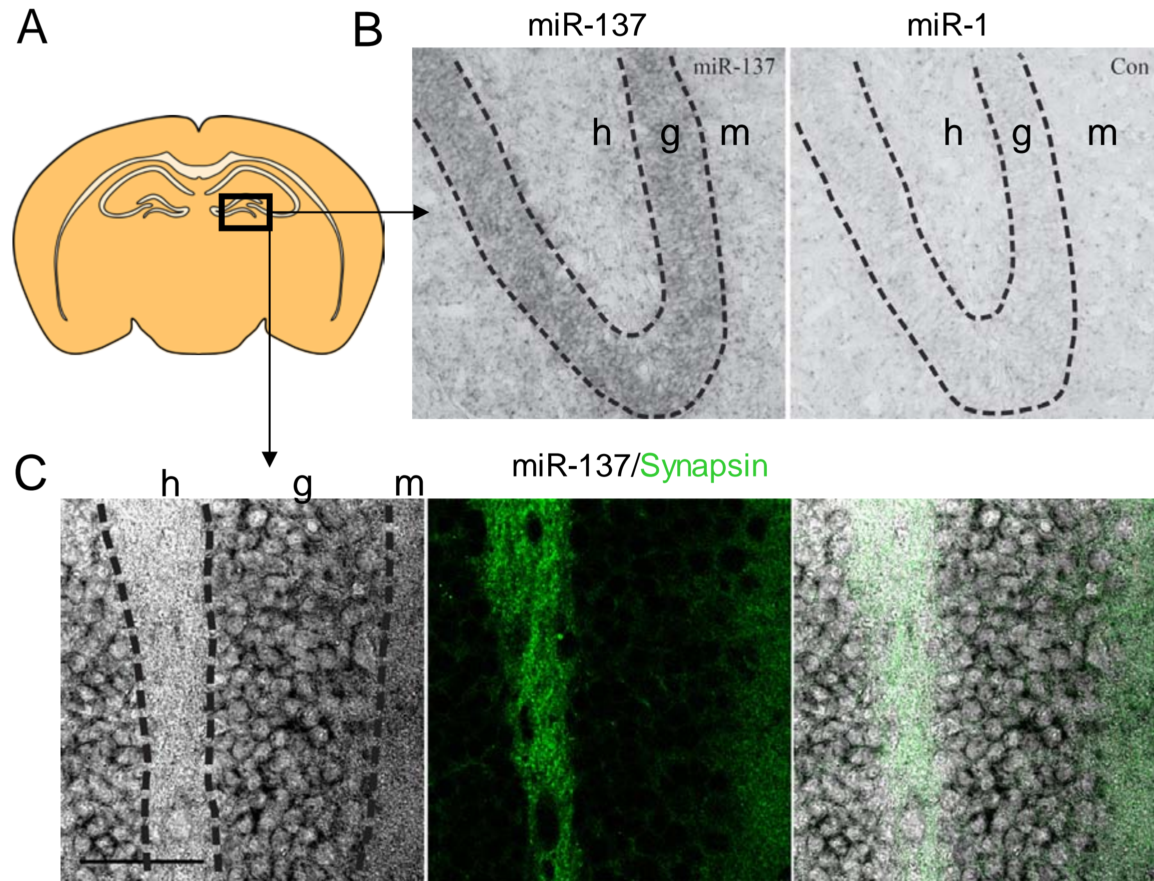
A.1. miR-137 is upregulated in MeCP2-deficient neurons



Goal of the experiment: I have previously shown that MeCP2 deficiency leads to reduced neuronal maturation in young neurons of postnatal brains; however gene expression analyses yielded limited information about the potential downstream effector(s) (Smrt et al., 2010). The goal of these experiments is to determine if miR-137 expression levels is changed in the absence of MeCP2. To determine whether MeCP2 regulates neuronal maturation through noncoding miRNAs, our collaborators obtained miRNAs expression profiles in NSC derived from young adult *Mecp2* KO and control littermate mice that were differentiated into neurons for 72 hours. A subset of miRNAs, such as miR-137, miR-301, and miR-187, etc exhibited showed altered expression in the absence of *Mecp2* compared to WT controls (Szulwach et al., 2010). In these experiments, I isolated all primary neurons and brain tissues and miR-137 levels were determined by Keith Szulwach at the Jin lab.

Results: We looked at altered expression of a subset of miRNAs in adult brain tissue from both wildtype and KO animals. Among the candidate miRNA's displaying altered expression, miR-137 exhibited increased expression in both E17 primary cultures and brain tissue of *Mecp2* WT and KO animals. (**Figure A.1 A, B**), suggesting that the expression of miR-137 is modulated by MeCP2.

A.2. miR-137 is enriched in the dentate gyrus of the hippocampus



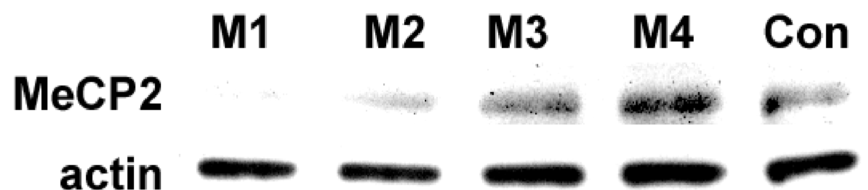
Goal of the experiment: It has been shown that miR-137 is expressed in the brain and enriched at the synaptic compartment (Siegel et al., 2009; Smalheiser and Lugli, 2009). I rationed that if miR-137 is a mediator of MeCP2, it should be expressed in neurons in the adult DG of adult hippocampus which is affected by MeCP2 deficiency (Smrt et al., 2007). The goal of this experiment is to determine if miR-137 is localized in the adult hippocampus. The brain tissues were isolated and prepared for ISH by me at UNM. The tissue was then sent to Yale and ISH was done by our collaborator and co-author Manov. Note that ISH in Figure A.2 was published in my paper described in chapter 6 (Smrt et al., 2010).

In-situ hybridization: Following transcardial perfusion, brain tissue is equilibrated in 30% sucrose, then embedded in OCT (Tissue Tek) and frozen in liquid nitrogen-cooled isopentane. 10 µm thick serial cryosections were cut in the sagittal plane on a Leica CM3050S cryomicrotome and stored at -20C until hybridization could begin. In situ hybridization was carried out as outlined previously with a few modifications (Obernosterer et al., 2007). Following tissue processing, slides are air-dried, then fixed in 4% PFA and washed in DEPC-treated PBS. Slides were then acetylated (590ml DEPC water, 8ml triethanolamine, 1050 µl 37% HCl and 1.5 ml acetic anhydride), washed in PBS, treated with Proteinase K (5 µg/ml) and washed again. Slides were then prehybridized (50% formamide, 5X SSC, 5X Denhardt's, 200µg/ml yeast RNA, 500

µg/ml salmon sperm DNA, 0.4g Roche blocking reagent and 1.75 ml DEPC water) for 4-8 hours at RT. For hybridization 0.1 µl of LNA DIG- or FITC-labeled probe was added to the hybridization buffer (same as prehybdrization buffer but with 500 µl 10% CHAPS, 100 µl 20% Tween and 1.15ml DEPC water) and applied to the tissue at 50-60C overnight (~20C below the predicted melting temperature (T_m) of probe:miRNA). Following hybridization, slides were washed in 5X and 0.2X SSC at 60C, followed by buffer B1 (0.1M Tris pH 7.5/0.15M NaCl) at RT. Sections were then blocked in 10% FCS in B1 and probed with anti-DIG/FITC-HRP antibodies as well as anti-synapsin and anti-neurofilament H. Following incubation in primary antibodies, slides were washed in B1 and then equilibrated in buffer B3 (0.1M Tris pH 9.5/0.1M NaCl/50Mm $MgCl_2$) for 10 minutes. Developer solution (100mg/ml NBT, 50 mg/ ml BCIP, 24 mg/ml levamisol and 10% Tween in B3) was then added to the tissue for ~4hours RT. The reaction was stopped with washes in PBT, and sections were then probed with dye-coupled secondary antibodies. Following final washing steps, slides were mounted in Aquamount and visualized using confocal microscopy (UNMHSC Fluorescence Microscopy Shared Resource).

Results: Hybridization with the miR-137-specific probe showed an enrichment of miR-137 within the DG and molecular layer of the hippocampus compared to the miR-1, an miRNA that is expressed at low levels in CNS (**Figure A.2 A,B**). In the molecular layer, miR-137 expression is overlap with the expression of the synaptic protein synapsin. This data, along with previous reports from other labs suggests miR-137 my play a functional role in the connectivity of neurons in the hippocampus, and may be particularly important for developing neurons in the hippocampus.

A.3. Characterization of *Mecp2*-siRNA



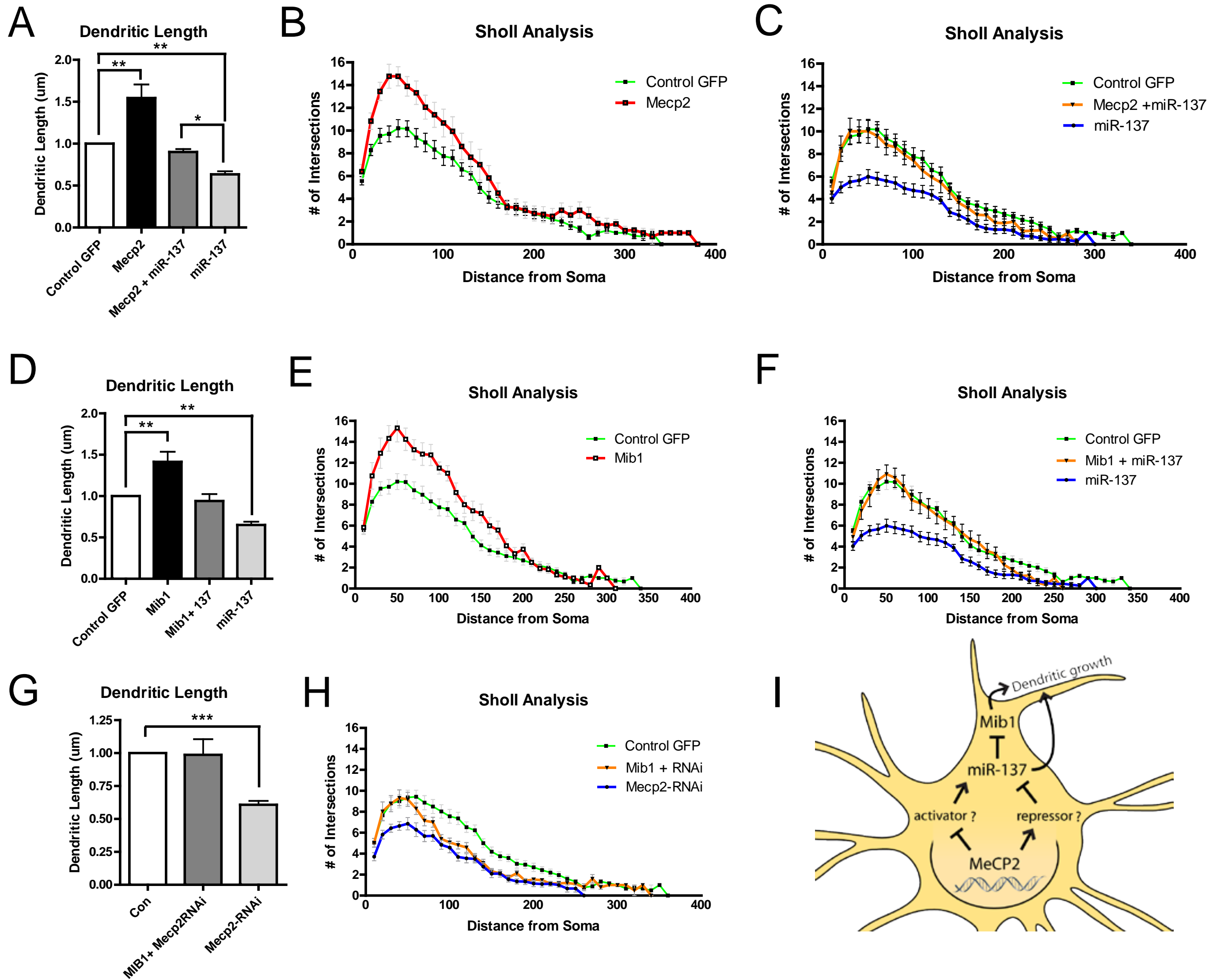
Goal of the experiment: To determine which *Mecp2* siRNA is the most effective for knocking down expression of MeCP2.

Cells: 293 cells were cultured in 6cm dishes, and transfected using a CaCl2 transfection method which results in ~100% infection efficiency. The cells were collected for protein analysis (western blot).

DNA constructs: *Mecp2* expression vector in pCDNA3 was a gift from Dr Qiang Chang (Univ Wisconsin Madison), *Mecp2* shRNA (co-express eGFP) expression vector was purchased from SABioscience and one of the four shRNAs was selected based on in vitro efficacy assays.

Results: M1-4 are four constructs purchased from SABiosciences. This data demonstrates M1 is more effective.

A.4. MeCP2 and mir137 rescue (morphology data)



Goal of the experiment: I hypothesized that MeCP2-regulated miR-137 modulates neuronal dendritic development through MIB1. If this is correct, both MeCP2 and MIB1 would be capable of rescuing the dendritic developmental deficits resulting from miR-137 overexpression. The rescue of MIB1 has been published and is described in Chapter 6. The rescue by MeCP2 has not been published and is exclusively described here.

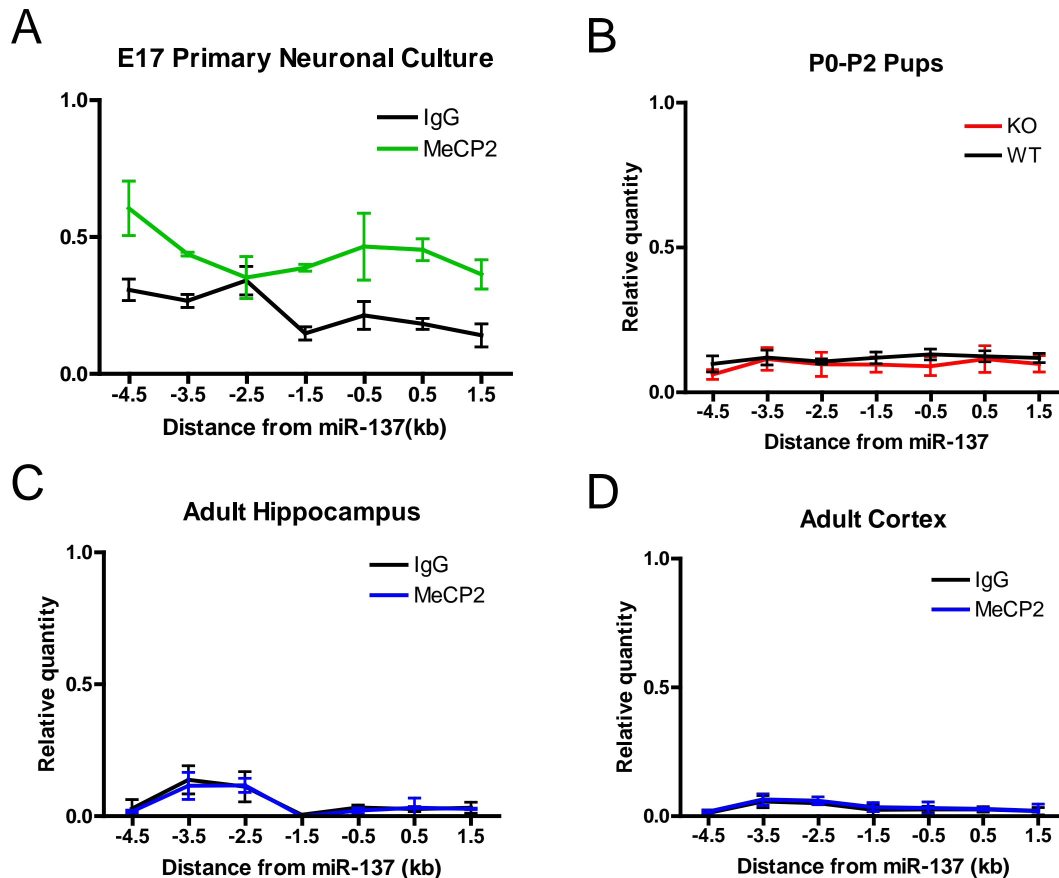
Methods: The methods for cell culture, transfection, and DNA are described previously (Chapter 6). *Mecp2* expression vector in pCDNA3 was a gift from Dr Qiang Chang (Univ Wisconsin Madison), *Mecp2* shRNA (co-express eGFP) expression vector was purchased from SABioscience and one of the four shRNAs was selected based on in vitro efficacy assays.

Results: First, I demonstrated that overexpression of MeCP2 in neurons led to an increase in dendritic length of 32% ($n = 4$, $p < 0.01$), as well as increased dendritic complexity ($F(1,42) = 7.439$, $p = 0.009$) (**Figure A.4 A,B**), consistent with previous findings (Jugloff et al., 2005). Next, I explored whether MeCP2 could rescue the miR-137-induced deficits in dendritic development. To this end, *Mecp2* expression plasmid was cotransfected with synthetic miR-137 RNA. My data show that MeCP2 could rescue the miR-137-mediated reduction both in dendritic length ($n = 4$, 1% difference between MeCP2+miR137 and control, 33% difference between MeCP2+miR137 and miR-137) (**Figure A.4 A**) and in dendritic complexity ($F(1,36) = 18.15$, $p < 0.001$) (**Figure A.4 C**). Figures D-F are already published and described in Chapter 6.

Finally, I investigated whether MIB1 could rescue MeCP2 deficiency-induced deficits in dendritic development. To confirm that MeCP2 expression levels were important for dendritic development in my model system, I knocked down endogenous MeCP2 in cultured neurons using RNAi (**Supplemental Figure S6.6**). I found that acute knockdown of MeCP2 led to a 39% reduction in dendritic length ($n = 8$, $p < 0.0001$) and a significant decrease in dendritic complexity ($F(1,37) = 9.707$, $p = 0.004$) in hippocampal neurons compared with control RNAi (**Figure A.4 G, H**). I then showed that MIB1 expression could indeed rescue the *Mecp2* RNAi-induced reduction both in dendritic length ($n = 3$, 1% difference between Mib1+RNAi and control, 38% difference between Mib1+RNAi and *Mecp2* RNAi) (**Figure A.4 G**) and in dendritic complexity ($F(1,36) = 11.83$, $p = 0.002$) (**Figure A.4 H**).

Thus, my data suggests that miR-137, which is a potential downstream effector of MeCP2, regulates dendritic morphogenesis in developing neurons, at least in part, by translational regulation of MIB1 (**Figure A.4 I**).

A.5. MeCP2/mir-137 ChIP assay in adult brain and cultured post-mitotic neurons



Goal of the experiment: To determine if MeCP2 directly interacts with genomic regions proximal to miR-137, our collaborators and I performed MeCP2-specific chromatin immunoprecipitation (ChIP) followed by real-time quantitative PCR across a 7 kb region surrounding *miR-137* that included most of the highly conserved upstream sequences. I isolated chromatin from mouse brains and primary neurons and sent these samples to collaborator Keith Szulwach and Peng Jin at Emory to finish ChIP.

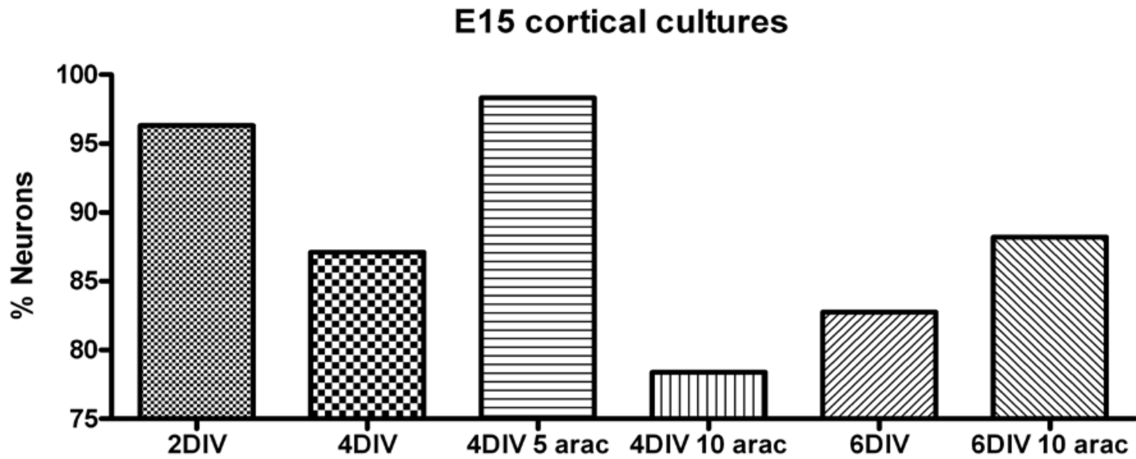
Isolation of chromatin from mouse brains or neurons: The ChIP methods are previously described in a paper I co-authored (Szulwach et al., 2010). For cultured neurons, E17 cortex was used. For P0-P2 pups, a litter was obtained from a Het *Mecp2* mom and chromatin was isolated from each brain separately. For adult mouse brain, dissected hippocampi and cortex from *Mecp2* WT or KO animals were separated and chromatin was isolated. Each animal is considered n=1, a total of n=3 or greater are presented in the data. Note, the ChIP was performed for all samples (including both published aNSCs, and unpublished neurons and brain tissue) at the same time.

Results: Immunoprecipitation of chromatin chemically crosslinked to DNA in primary cortical neurons using two different MeCP2-specific antibodies demonstrated non-significant enrichment of MeCP2, compared to IgG, in the genomic regions proximal to *miR-137* (**Figure A.5 A**). Additional ChIP assays were performed on developing brains (Postnatal day 2) and adult brain regions (hippocampus and cortex), and again found no enrichment of MeCP2 in this genomic

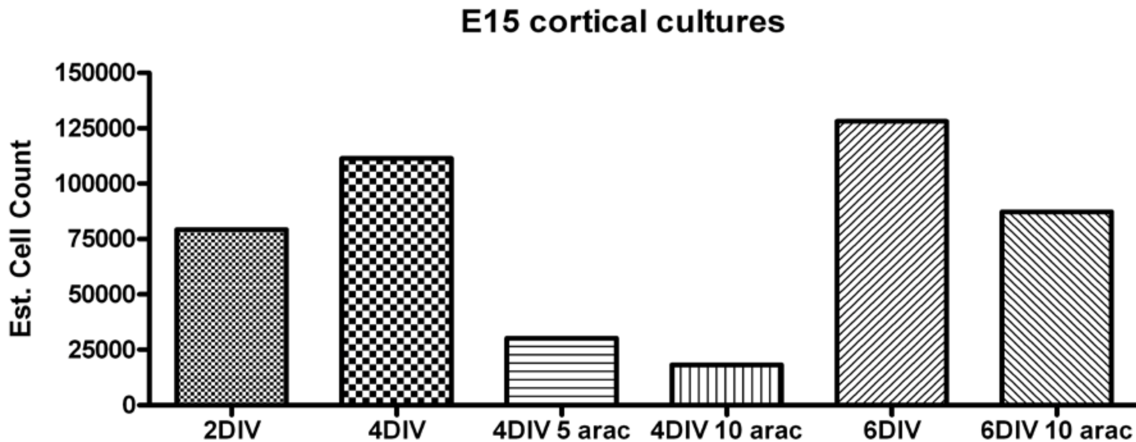
regions (**Figure A.5 B-D**). Therefore, increased expression of miR-137 in the absence of MeCP2 may not be due to direct binding of MeCP2 to miR-137.

A.6. Characterization of neuronal cultures

A.



B.



Goal of the experiment: To determine the characteristics and optimize the conditions of primary neuronal culture. For example, what is the percentage of neurons with respect to total cells that make up a standard neuronal culture? At what timepoint is the percentage of neurons the highest? Finally, does AraC enrich the percentage of neurons? Defining these parameters enables me to produce an enriched neuronal population which serves as a model system to conduct extensive biochemical analysis of neurons.

Cells: Cortical neurons from wildtype E15 fetal mice were grown as dispersed mixed cell cultures, as established by the Wilson lab (Washbourne et al., 2002). Cortical neurons were fixed

and stained for NeuN (neuronal marker) and dapi (nuclear marker) at 2, 4, 6, and 9 days in culture. Stereology was used unbiased sampling and quantification of total cell and neuronal cell counts. For araC treatment, fresh araC was added to the neuron media (10ug/mL) for 48 hours, then the media was changed to non-araC containing media.

Results: Early timpoints (DIV2) yield the highest percentage of neurons (**Figure A.6 A**). Additionally, although araC enriches the neuronal population (**Figure A.6 A**), it also kills a large amount of total cells including neurons (**Figure A.6 B**).

APPENDIX B:
UNPUBLISHED MANUSCRIPT

Age-Dependent dynamic alteration of wild type neurons in a Novel *Mecp2* Heterozygous Mosaic Mouse Model

Richard D. Smrt, Rebecca L. Pfeiffer, and Xinyu Zhao

Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque,

NM 87131;

(This manuscript is in preparation for submission.)

B.1. ABSTRACT

Mutations and altered expression of the x-linked MeCP2 protein leads to Rett's syndrome one of the Autistic Spectrum Disorders (ASDs). MeCP2 deregulation has also been found in autism. Most RTT patients are females who are mosaic in the MeCP2 deficiency due to random X Chromosome Inactivation (XCI). Given the critical function of MeCP2 in postnatal brain development and its clear link to autism, it is important to determine whether MeCP2 mutation affects XCI. Our data suggests that neurons expressing WT MeCP2 in *Mecp2* heterozygote females have a survival advantage over mutant neurons. To determine whether MeCP2 affects XCI, we developed a novel *Mecp2 mosaic* mouse model, in which one X chromosome expresses WT *Mecp2* and also expresses GFP while the mutant X chromosome does not. Due to random XCI, the female mosaic mice have both WT (GFP+) and mutant (GFP-) *Mecp2* neurons that can be distinguished by GFP fluorescence. To characterize XCI skewing in our newly generated mouse model, we evaluated the HET mosaic mice at three separate time points: 3 months, 6 months, and 9 months after birth. Using this mouse model, we have determined that MeCP2 deficiency leads to reduced neuronal survival rather than skewed XCI in *Mecp2* heterozygote mice. Given the important function of MeCP2 in postnatal brain development understanding how *Mecp2* mutation affects XCI skewing in neurons will likely provide critical insight in understanding the etiology of Rett syndrome.

B.2. INTRODUCTION

ASDs, including autism, Asperger disorder, Rett syndrome (RTT), childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified, affect one in 166 children in the US. Among ASDs, only RTT has been clearly linked to a known gene, X-linked *MECP2* gene (Amir et al., 1999). *MECP2* mutations and reduced expression of MeCP2 protein have also been found in autistic patients (Samaco et al., 2004). These data suggest that MeCP2 deficiency not only is responsible for classic RTT, but is also involved in the etiology of autism. Autism affects more boys than girls, with male to female ratio about 4 to 1 (Volkmar et al., 1993). This gender difference, together with the discovery of X Chromosome rearrangements in autistic patients (Ishikawa-Brush et al., 1997; Rao et al., 1994; Thomas et al., 1999) suggests the involvement of genes on the X Chromosome. It is our premises that by studying the function of X-linked MeCP2 in neurodevelopment, we will gain critical knowledge in not only RTT but also the more complex autism.

RTT is primarily caused by mutations in the X-linked *MECP2* gene encoding a methyl CpG binding protein that binds methylated DNA and repress gene transcription. Most RTT patients are females who are heterozygote and mosaic in the MeCP2 deficiency due to random X Chromosome inactivation. Mammalian female cells inactivate one of the two X Chromosomes, and the genes on the inactive X Chromosome, with a few exceptions, are not expressed. X Chromosome inactivation occurs during early embryonic development, and it is generally irreversible (Plath et al., 2002). While some studies found skewed X Chromosome inactivation (one X Chromosome is more active than the other X) in *Mecp2* heterozygote females (Braunschweig et al., 2004; Young and Zoghbi, 2004), another study does not fully support this notion(Metcalf et al., 2006). Skewed X Chromosome inactivation has been shown in both autism (Talebizadeh et al., 2005) and X Chromosome linked mental retardation (Plenge et al., 2002). Given the important function of MeCP2 in postnatal brain development and reduced MeCP2 expression is frequently found in autistic brains (Samaco et al., 2005), it is important to determine whether MeCP2 mutation affect X Chromosome inactivation pattern and whether neurons expressing abnormally lower levels of MeCP2 have negative effect on other neurons.

Mecp2 mutant mice have provided valuable models in understanding the function of MeCP2 in postnatal neuronal development, however most RTT patients are heterozygote females and male RTT patients generally die in early infancy. In contrast to the dosage compensation mechanism in Drosophila and C. elegans, mammalian female cells inactivate one of the two X Chromosomes, and the genes on the inactive X Chromosomes, with a few exceptions, are not expressed (Plath et al., 2002). X Chromosome inactivation occurs before the completion of gastrulation during early embryonic development, and it is generally random and irreversible (Plath et al., 2002). Several groups have shown that both human and mouse *Mecp2* heterozygote females are mosaic in the *Mecp2* mutation and exhibit a uniform distribution of mutant cells. However, there is a preferential inactivation of the mutant allele in more than 60% heterozygote females, with only 20-40% cells expressing mutant MeCP2 (Braunschweig et al., 2004; Young and Zoghbi, 2004). The severity of neurological deficits in both human and mice is partially dependant on the X Chromosome inactivation patterns (Braunschweig et al., 2004; Young and Zoghbi, 2004). How do heterozygote brains with more than half of their neurons expressing functional MeCP2 have such severe neurological deficits? Young et al has demonstrated that there is a survival advantage for neurons expressing the wild type *Mecp2* allele compared to those expressing the mutant allele, suggesting that the skewed ratio of mutant *Mecp2* expressing

neurons versus wild type *Mecp2* expressing neurons could be due to the survival disadvantage of mutant neurons (Young and Zoghbi, 2004). However, the problem is further complicated by the findings that first, no significant neuronal death has been found in RTT brains (Armstrong, 2002), and second, in RTT patients or *Mecp2* mutant mice, MeCP2 protein expression levels in wild type *Mecp2* expressing neurons are lower than that of wild type cells of normal brains (Braunschweig et al., 2004). These observations suggest that MeCP2 mutation in heterozygote females not only affects the development of neurons expressing the mutant allele but also neurons expressing the wild type allele. Given the important function of MeCP2 in postnatal brain development and reduced MeCP2 expression is frequently found in autistic brains (Samaco et al., 2005), it is important to determine whether MeCP2 mutation affects X Chromosome inactivation patterns and whether neurons expressing abnormally lower levels of MeCP2 have a negative effect on other neurons. Our MeCP2 heterozygote females containing both normal and mutated neurons in the same brains provide a unique model for us to understand how neuron-neuron interactions regulate neuronal maturation and brain function. Neuronal maturation is a complex process that is regulated by many genetic and epigenetic factors and is significantly influenced by inter-cellular signaling between neurons and between neurons and glia (Waites et al., 2005; Webb et al., 2001). It is during this major postnatal maturation period that RTT manifests itself. Recent studies suggest that RTT may result from a defect in synaptogenesis (Belichenko et al., 1997b; Cohen et al., 2003; Kishi and Macklis, 2004; Matarazzo et al., 2004). MeCP2 expression is correlated with neuronal maturation, with an increase in protein levels immediately before and during synapse formation and reaching its highest level in mature neurons (Balmer et al., 2003; Jung et al., 2003; Shahbazian et al., 2002b). Both RTT patients and *Mecp2* mutant mice have excess immature neurons in the olfactory epithelium, and reduced transition of immature neurons into mature neurons (Matarazzo et al., 2004). RTT patients have underdeveloped neuronal axons and dendrites (Armstrong, 1997; Armstrong et al., 1995; Kaufmann et al., 1997). Using retrovirus-based in vivo neuron labeling, we have demonstrated that newly matured neurons in *Mecp2* mutant mice develop fewer synaptic spines (Smrt et al., 2007). Since each neuron interacts with many other neurons, intercellular signaling, including neuron-neuron and neuron-glia interaction, is critical for both development and function (Waites et al., 2005; Webb et al., 2001). To date, most MeCP2 studies focus on the intrinsic properties of MeCP2 in cells. In order to have a complete picture of how postnatal neural development is regulated by MeCP2, it is critical and essential to know whether and how *Mecp2* mutation affects the function of surrounding wt-*Mecp2* expressing neurons that share the microenvironment and/or form interactions with mutant neurons in a mosaic *Mecp2* system.

In this study, we show no difference in the percentage of MeCP2 expressing neurons at 3 months after birth. However at 6 and 9 months, the percentage of MeCP2/GFP positive neurons is significantly altered in the experimental mosaic mouse compared to the GFP control female mouse.

B.3. MATERIALS AND METHODS

Animals

All animal procedures were performed according to protocols approved by the University of New Mexico Animal Care and Use Committee. The *Mecp2* KO mice (*Mecp2*$^{tm1.1Jae}$) used in this study were created by deleting exons 3 containing the MBD domain of *Mecp2* (Chen et al.,

2001b). These mice have been bred over 40 generations on to ICR background. They start to show neurological symptoms between 5 and 7 weeks of age and die before 10 weeks of age. The mosaic mouse line was created by cross breeding Tg-GFPx mice from the Jackson labs with *Mecp2*$^{+/-}$ female mice. By crossing the WT male (Tg-GFP^{+}/*Mecp2*$^{+}$) with a *Mecp2* heterozygous female (*Mecp2*$^{+/-}$), some of the female mosaic offspring will be heterozygous for GFP and MeCP2 (Tg-GFP$^{+/-}$/*Mecp2*$^{+/-}$). Specifically, these mosaic offspring express the WT *Mecp2* gene and *Gfp* on one x-chromosome and no *Gfp* and no *Mecp2* on the other (See Figure B.1A). For histological analyses, mice were euthanized by intraperitoneal injection of sodium pentobarbital. Mice were then perfused with saline followed by 4% PFA. Brains were dissected out, post-fixed overnight in 4% PFA, and then equilibrated in 30% sucrose. Forty-micrometer brain sections were generated.

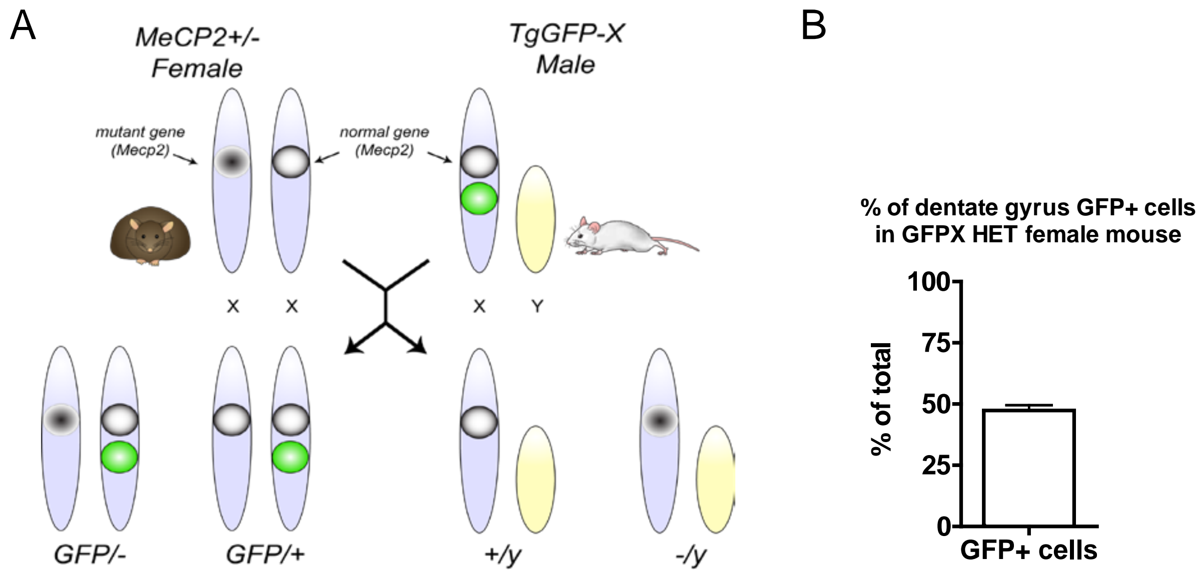


Figure B.1. Breeding strategy and quantification of GFP+ cells. A. Breeding diagram showing how the mosaic mouse model is generated. B. 50% of cells express GFP in the dentate gyrus of the hippocampus in this X-linked GFP mouse model. To quantify, roughly 100 cells from a single z-plane (n=4) were analyzed for GFP expression using ImageJ software. A One-sample t-test confirms the actual mean (47.35 +/- 2.33, P=0.32) is not different from the expected mean of 50%.

Immunohistochemistry:

Immunostaining was conducted according to previous works (Barkho et al., 2008; Silber et al., 2008; Smrt et al., 2007; Zhao et al., 2003). Mouse Neun (Chemicon 1:5000) and MeCP2 Chicken (Emory, 1:5000). The secondary antibodies used were AF488 (Molecular Probes, 1:500) to amplify the endogenous GFP, Cy3 (donkey anti-mouse, 1:500, Sigma), and Mouse AF647 (Molecular Probes, 1:500). After the primary and secondary antibodies were rinsed the sections were then stained with Dapi and mounted on glass slides using DABCO for coverslipping. The DABCO helps to prevent fading from microscope exposure.

Confocal microscopy and quantification:

Images of brain sections were taken using a 63x/1.4 Oil DIC Plan-apochronial lens (Zeiss LSM510META Confocal Microscope). The proportions of GFP/MeCP2expressing neurons

were counted using the computer program Metamorph. The images obtained from confocal microscopy were merged and 3 concurrent frames were chosen based upon image quality. These 3 frames were then stamped with a 600x600 box. The number of Neun+ cells were then counted and recorded followed by the total number of GFP expressing cells. The counts were done as previously described (Zhao et al. 2003).

Statistical analysis

All statistical analyses were performed using unpaired, two-tailed, Student's t-test. The data bars and error bars indicate mean ± standard error mean. (s.e.m).

B.4. RESULTS

GFP+/- control mice express GFP in 50% of cells in the hippocampus

To determine if female Tg-GFPx mice, which are heterozygous for the X-linked GFP gene, can faithfully demonstrate random X-Chromosome inactivation in neurons, we quantified the percentage of GFP expressing neurons. Specifically, expression of GFP in neurons was determined by counting the number of neurons that are GFP-positive (GFP+NeuN+) over the total number of neurons (NeuN+) in the dentate gyrus. We show that approximately 50% of neurons express GFP in the dentate gyrus of the hippocampus in this X-linked GFP mouse model (Figure B.1B). This data suggests that control heterozygous Tg-GFPx mice in our model undergo random X-Chromosome inactivation in the hippocampus, consistent with previous data that was conducted in other anatomical regions (Hadjantonakis Non-invasive sexing of preimplantation stage mammalian embryos 1998).

To determine if our control GFP mosaic female (Tg-GFP$^{+/-}$/*Mecp2*$^{+/+}$) mice expressed GFP in neurons and astrocytes of the cortex and hippocampus, we stained brain tissue sections with antibodies against early and late neuronal markers, and astrocytes. Using confocal microscopy, we found that brain tissue was mosaic for GFP expression, and that all three markers could be found to be colocalized with GFP (Figure B.2 A-C)

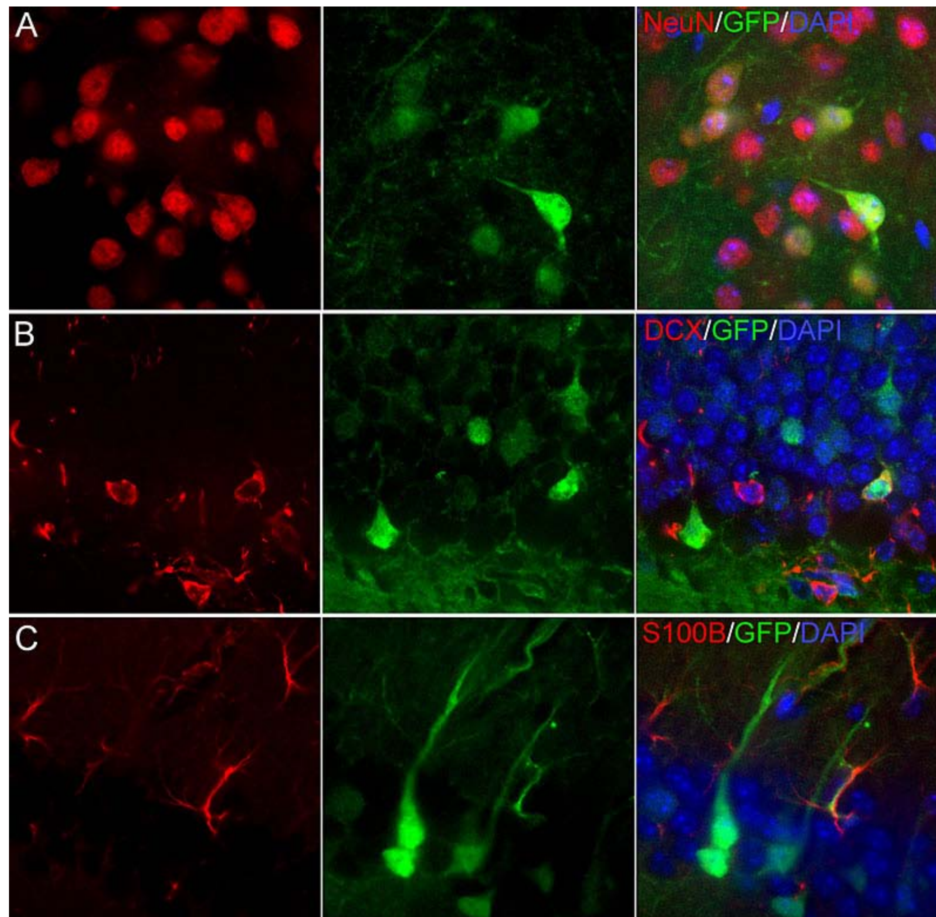


Figure B.2. Tissue staining showing the co-localization of GFP with other astrocytic/neuronal markers in the brain. A. GFP+ neuron colocalized with NeuN in the cortex. B. GFP+ early neuron colocalized with DCX in the hippocampus. C. GFP+ cell colocalized with S100B in the hippocampus.

GFP-positive neurons express MeCP2 in the *Mecp2* mosaic mouse model

To determine if neurons in the *Mecp2*$^{+/-}$ mice have a survival deficit, we generated a novel *Mecp2* mouse model by crossing a *Mecp2*$^{+/-}$ female with a Tg-GFPx male mouse (Figure B.1A). The resulting progeny include males which are *Mecp2* WT or *Mecp2* KO, females which are *Gfp*$^{+/-}$ and *Mecp2*$^{+/+}$ (Tg-GFP$^{+/-}$/*Mecp2*$^{+/+}$: used as controls), and females which are GFP+/- and *Mecp2*+/- (Tg-GFP$^{+/-}$/*Mecp2*$^{+/-}$) experimental condition, also referred to in this paper as the "mosaic mouse model"). The expression of GFP in all the female progeny is mosaic, in which cells expressing GFP indicate that the active X-chromosome contains the *Gfp* gene. Cells that are negative for GFP indicate the X-chromosome containing GFP is silenced. In the Tg-GFP$^{+/-}$ /*Mecp2*$^{+/-}$ mosaic mice, the GFP protein expression is found to be linked to wild-type MeCP2 in all neurons of the dentate gyrus (Figure B.3B-C). These results indicate that wild-type MeCP2 and GFP are located on the same X-chromosome in the mosaic model and GFP is consistently expressed in conjunction with MeCP2 in adult animals. This design allows us to genotype MeCP2 expressing cells through GFP fluorescence.

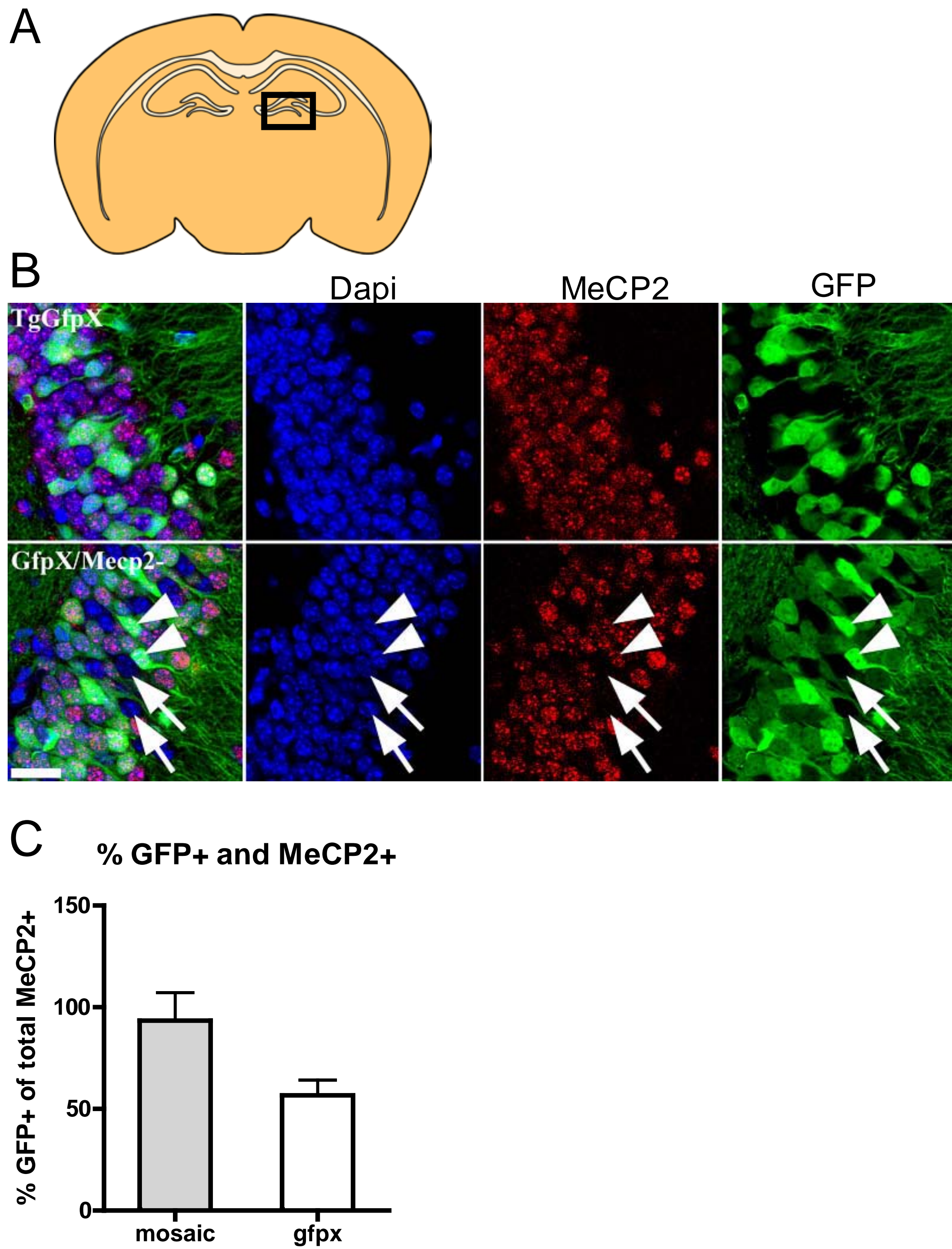


Figure B.3. Tissue staining showing the colocalization of MeCP2 and GFP in the dendate gyrus (DG) of the hippocampus. A. Illustration showing a representative coronal section of the mouse brain. The location of the DG is indicated by a square. B. Comparison of the mosaic mouse (GFPX/MeCP2) and the control (TgGFPX). Arrowhead: Cells positive for both MeCP2 and GFP. Arrow: Cells negative for MeCP2 and GFP. C. Quantification of cells colocalized with MeCP2 and GFP in the mosaic mouse and the TgGFPX control. In the mosaic mouse, it is expected that all MeCP2+ cells are also GFP+.

MeCP2 expression varies in heterozygous neurons over time

In order to characterize XCI skewing in our newly generated mouse model, we evaluated the HET mosaic mice at three separate time points: 3 months, 6 months, and 9 months after birth. At 3 months, our data show that the percentage of neurons expressing both MeCP2 and GFP in the hippocampus are the same for both the $GFP^{+/-}$/*Mecp2*$^{+/-}$ mosaic mice and the $GFP^{+/-}$/*Mecp2*$^{+/+}$ control mice (Figure B.4A). At 6 months, the percentage of neurons expressing both MeCP2 and GFP has significantly decreased in the experimental mosaic mouse compared to the GFP control female mouse (Figure B.4B). However at 9 months, the overall MeCP2/GFP positive neurons is significantly higher in the experimental mosaic mouse than in the GFP control female mouse (Figure B.4C).

To extend our characterization of XCI skewing in the brain, we next looked in the cortex of our mosaic mouse model. At 3, 6, and 9 months, there is no significant difference in the number of GFP/MeCP2 positive neurons between the experimental and control conditions (Figure B.4D-F). These data suggest that there is genetic skewing in the hippocampus of the MeCP2 mosaic mice that favor to the WT allele at late adult developmental time points; however, there is no effect of X-chromosome skewing in the cortex.

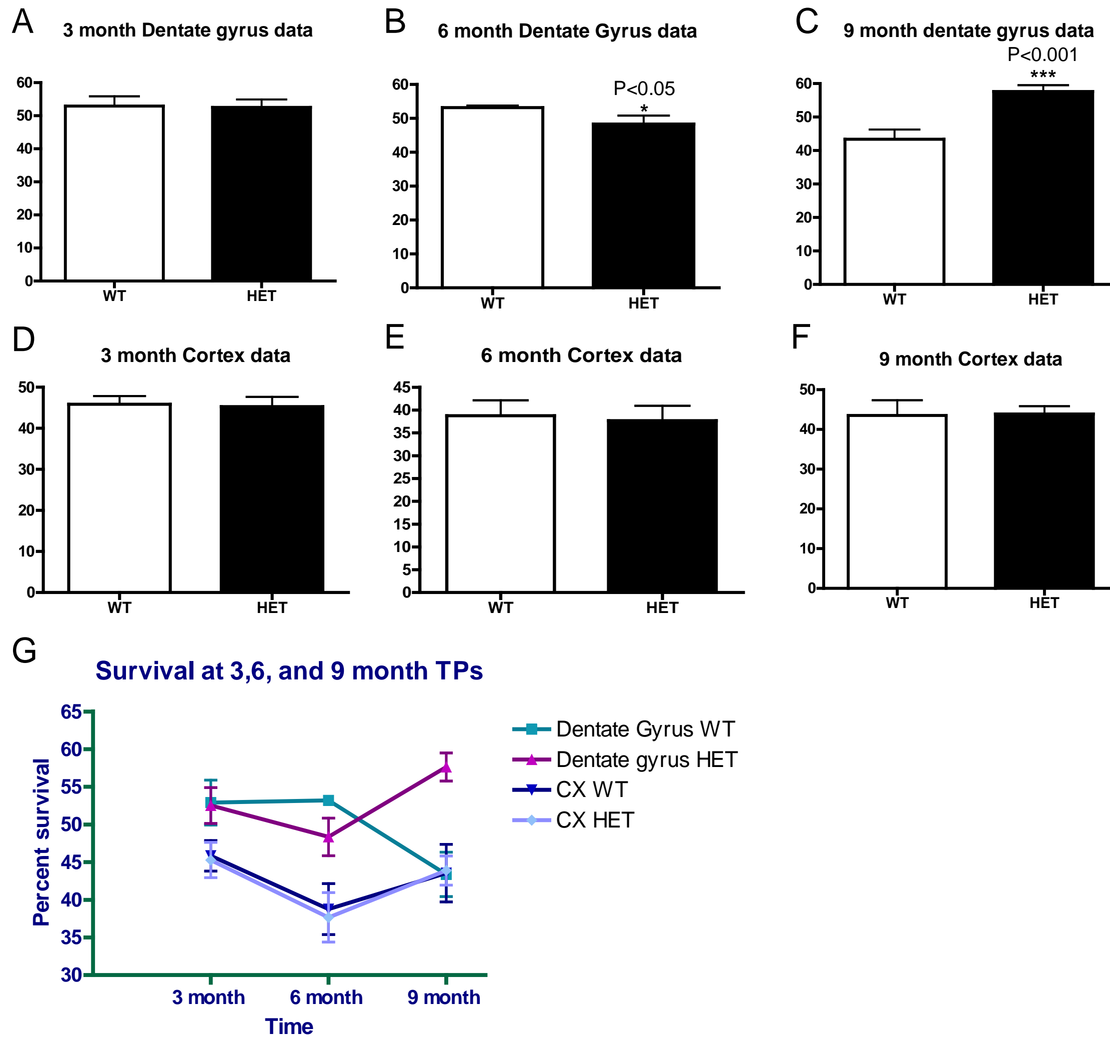


Figure B.4. Quantification of the percentage of GFP+ neurons in the hippocampus and cortex. A-C. The percentage of GFP+ neurons in the hippocampus relative to the total number of neurons analyzed. D-F. The percentage of GFP+ neurons in the cortex relative to the total number of neurons analyzed. G. A color line graph describing the same data shown in A-F.

Survival of neurons in TgGFP-X/*Mecp2* mosaic mice is not affected by GFP expression

To confirm GFP alone does not play a role in the genetic skewing, we observe the hippocampus at the later time points, we found that the number of GFP/MeCP2 positive neurons in the experimental mosaic mouse model are no different than the number of MeCP2 positive neurons in *Mecp2* heterozygous female mice that do not co-express GFP (Figures B.5A,B). This suggests that XCI in the adult *Mecp2* mosaic mouse is similar to the traditional non-GFP expressing *Mecp2* heterozygous mice.

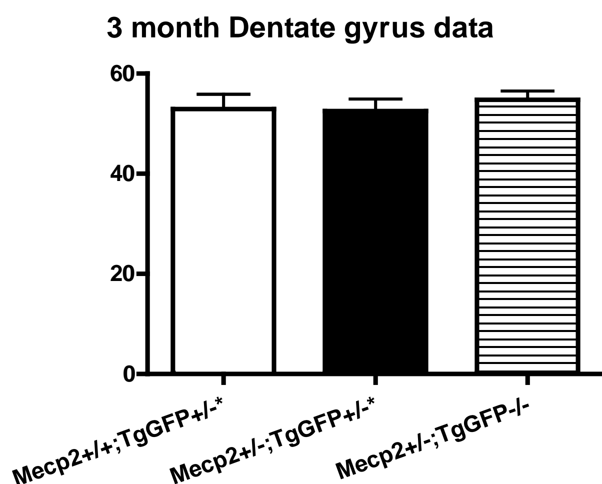


Figure B.5. Comparison of GFP+ and GFP- *Mecp2* heterozygous mice. A. The percentage of GFP+ neurons (for *Mecp2*$^{+/+}$/TgGFP$^{+/-}$ and *Mecp2*$^{+/-}$/TgGFP$^{+/-}$) and MeCP2+ neurons (for *Mecp2*$^{+/+}$/TgGFP$^{-/-}$) in the hippocampus of 3mo old mice relative to the total number of neurons analyzed. B. The percentage of GFP+ neurons (for *Mecp2*$^{+/+}$/TgGFP$^{+/-}$ and *Mecp2*$^{+/-}$/TgGFP$^{+/-}$) and MeCP2+ neurons (for *Mecp2*$^{+/+}$/TgGFP$^{-/-}$) in the hippocampus of 6mo old mice relative to the total number of neurons analyzed

B.5. DISCUSSION

By the creation and analysis of a novel mosaic mouse, we have discovered that X-chromosome inactivation is skewed in favor of the normal *Mecp2* allele over time. However, our data found from our three month time point suggests that skewing does not take place during the primary X-chromosome inactivation early in the mouse female's development. The fact that the level of skewing is actually in favor of the mutant allele at the 6 month time point indicated that the mutant MeCP2 expressing neurons may have a negative effect on their WT neighbors, though overtime this is corrected by a higher proliferation of the WT neurons. At the 9 month time point however there was a significantly higher proportion of WT neurons in the hippocampus, suggesting there is skewing occurring in the hippocampus, which is consistent with previous reports of XCI in *Mecp2* hereozygous females (Braunschweig et al., 2004).

Studies using *Mecp2* mutant mice have greatly advanced our knowledge of RTT pathogenesis, however, most RTT patients are heterozygous females and are mosaic for the MeCP2 mutation. *Mecp2* mosaic female mice, containing neurons both with and without mutation of MeCP2, can be distinguished by GFP fluorescence (Fig B.3), providing a unique model to study how neuron-neuron interactions regulate neuronal maturation and function, and how deficits of a subpopulation of neurons may contribute to autism spectrum disorders. We anticipate the results of this study will set the stage for further mechanistic investigations that will significantly sharpen our understanding of the pathogenesis of RTT. For example, if mutations in *Mecp2* affect the development and function of wild type MeCP2 expressing neurons in the same brain region, blocking the negative effects of mutant neurons on wild type MeCP2 expressing neurons or supplying wild type neurons with the factors that are limited in heterozygote brains may ultimately lead to stopping or reversing the progression of neurological

deficits in RTT females. In summary, X-chromosome skewing may play an important role in the severity presented by Rett syndrome patients. Therefore better understanding of XCI in Rett Syndrome may provide valuable insight into the development of the hippocampus and provide researchers with answers for the cognitive pathogenesis of RTT.

B.6. ACKNOWLEDGEMENTS

We would like to thank the members of the Zhao for their helpful discussions. We thank Dr Peng Jin for providing us the TgGFP-X mice. X.Z. is supported by grants from the International Rett Syndrome Foundation and the NIH (MH080434 and MH078972). X.L. was a recipient of the Autism Speaks Postdoctoral Fellowship. R.S. is supported by a Minority Supplement to NIH grant (MH080434). R.L.P. is supported by the NIH/GM060201-funded Initiatives to Maximize Student Diversity (IMSD) program.

REFERENCES

Aimone, J.B., and F.H. Gage. 2004. Unbiased characterization of high-density oligonucleotide microarrays using probe-level statistics. *J Neurosci Methods*. 135:27-33.

Aimone, J.B., J.L. Leasure, V.M. Perreau, and M. Thallmair. 2004. Spatial and temporal gene expression profiling of the contused rat spinal cord. *Exp Neurol*. 189:204-21.

Akbarian, S., R.Z. Chen, J. Gribnau, T.P. Rasmussen, H. Fong, R. Jaenisch, and E.G. Jones. 2001a. Expression pattern of the Rett syndrome gene MeCP2 in primate prefrontal cortex. *Neurobiol Dis*. 8:784-91.

Akbarian, S., R.Z. Chen, J. Gribnau, T.P. Rasmussen, H. Fong, R. Jaenisch, and E.G. Jones. 2001b. Expression pattern of the Rett syndrome gene MeCP2 in primate prefrontal cortex. *Neurobiol Dis*. 8:784-91.

Alvarez, V.A., and B.L. Sabatini. 2007. Anatomical and physiological plasticity of dendritic spines. *Annu Rev Neurosci*. 30:79-97.

Amir, R.E., I.B. Van den Veyver, M. Wan, C.Q. Tran, U. Francke, and H.Y. Zoghbi. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl- CpG-binding protein 2. *Nat Genet*. 23:185-8.

Antar, L.N., R. Afroz, J.B. Dictenberg, R.C. Carroll, and G.J. Bassell. 2004. Metabotropic glutamate receptor activation regulates fragile x mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. *J Neurosci*. 24:2648-55.

Antar, L.N., J.B. Dictenberg, M. Plociniak, R. Afroz, and G.J. Bassell. 2005. Localization of FMRP-associated mRNA granules and requirement of microtubules for activity-dependent trafficking in hippocampal neurons. *Genes Brain Behav*. 4:350-9.

Arikkath, J. 2009. Regulation of dendrite and spine morphogenesis and plasticity by catenins. *Mol Neurobiol*. 40:46-54.

Armstrong, D. 1997. Recent developments in neuropathology--electron microscopy--brain pathology. *Eur Child Adolesc Psychiatry*. 6 Suppl 1:69-70.

Armstrong, D., J.K. Dunn, B. Antalffy, and R. Trivedi. 1995. Selective dendritic alterations in the cortex of Rett syndrome. *J Neuropathol Exp Neurol*. 54:195-201.

Armstrong, D.D. 2002. Neuropathology of Rett syndrome. *Ment Retard Dev Disabil Res Rev*. 8:72-6.

Asaka, Y., D.G. Jugloff, L. Zhang, J.H. Eubanks, and R.M. Fitzsimonds. 2006. Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome. *Neurobiol Dis*. 21:217-27.

Ashley, C.T., J.S. Sutcliffe, C.B. Kunst, H.A. Leiner, E.E. Eichler, D.L. Nelson, and S.T. Warren. 1993. Human and murine FMR-1: alternative splicing and translational initiation downstream of the CGG-repeat. *Nat Genet*. 4:244-51.

Ashraf, S.I., A.L. McLoon, S.M. Sclarsic, and S. Kunes. 2006. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. *Cell*. 124:191-205.

Azuara, V., P. Perry, S. Sauer, M. Spivakov, H.F. Jorgensen, R.M. John, M. Gouti, M. Casanova, G. Warnes, M. Merkenschlager, and A.G. Fisher. 2006. Chromatin signatures of pluripotent cell lines. *Nat Cell Biol*. 8:532-8.

Bagni, C., and W.T. Greenough. 2005. From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. *Nat Rev Neurosci*. 6:376-87.

Bakker, C.E., C. Verheij, R. Willemsen, R.v.d. Helm, F. Oerlemans, M. Vermey, A. Bygrave, A. Hoogeveen, B.A. Oostr, E. Reyniers, K.D. Boule, R. D'Hooge, P. Cras, D.v. Velzen, G. Nagels, J.-J. Martin, P.P.D. Deyn, J.K. Darby, and P.J. Willem. 1994. Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. *Cell*. 78:23-33.

Balasubramaniyan, V., E. Boddeke, R. Bakels, B. Kust, S. Kooistra, A. Veneman, and S. Copray. 2006. Effects of histone deacetylation inhibition on neuronal differentiation of embryonic mouse neural stem cells. *Neuroscience*. 143:939-51.

Balmer, D., J. Goldstine, Y.M. Rao, and J.M. LaSalle. 2003. Elevated methyl-CpG-binding protein 2 expression is acquired during postnatal human brain development and is correlated with alternative polyadenylation. *J Mol Med*. 81:61-8.

Barbato, C., C. Giorgi, C. Catalanotto, and C. Cogoni. 2008. Thinking about RNA? MicroRNAs in the brain. *Mamm Genome*. 19:541-51.

Barkho, B.Z., A.E. Munoz, X. Li, L. Li, L.A. Cunningham, and X. Zhao. 2008. Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. *Stem Cells*. 26:3139-49.

Barkho, B.Z., H. Song, J.B. Aimone, R.D. Smrt, T. Kuwabara, K. Nakashima, F.H. Gage, and X. Zhao. 2006. Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. *Stem Cells Dev*. 15:407-21.

Barreto, G., A. Schafer, J. Marhold, D. Stach, S.K. Swaminathan, V. Handa, G. Doderlein, N. Maltry, W. Wu, F. Lyko, and C. Niehrs. 2007. Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature*. 445:671-5.

Barski, A., S. Cuddapah, K. Cui, T.Y. Roh, D.E. Schones, Z. Wang, G. Wei, I. Chepelev, and K. Zhao. 2007. High-resolution profiling of histone methylations in the human genome. *Cell*. 129:823-37.

Bassell, G.J., and S.T. Warren. 2008. Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. *Neuron*. 60:201-14.

Battaglia, A. 2005. The inv dup(15) or idic(15) syndrome: a clinically recognisable neurogenetic disorder. *Brain Dev*. 27:365-9.

Belichenko, P.V., B. Hagberg, and A. Dahlstrom. 1997a. Morphological study of neocortical areas in Rett syndrome. *Acta Neuropathol*. 93:50-61.

Belichenko, P.V., B. Hagberg, and A. Dahlstrom. 1997b. Morphological study of neocortical areas in Rett syndrome. *Acta Neuropathol (Berl)*. 93:50-61.

Belichenko, P.V., E. Masliah, A.M. Kleschevnikov, A.J. Villar, C.J. Epstein, A. Salehi, and W.C. Mobley. 2004. Synaptic structural abnormalities in the Ts65Dn mouse model of Down Syndrome. *J Comp Neurol*. 480:281-98.

Belichenko, P.V., A. Oldfors, B. Hagberg, and A. Dahlstrom. 1994. Rett syndrome: 3-D confocal microscopy of cortical pyramidal dendrites and afferents. *Neuroreport*. 5:1509-13.

Benzer, S. 1967. BEHAVIORAL MUTANTS OF Drosophila ISOLATED BY COUNTERCURRENT DISTRIBUTION. *Proc Natl Acad Sci U S A*. 58:1112-1119.

Berg, J.S., N. Brunetti-Pierri, S.U. Peters, S.H. Kang, C.T. Fong, J. Salamone, D. Freedenberg, V.L. Hannig, L.A. Prock, D.T. Miller, P. Raffalli, D.J. Harris, R.P. Erickson, C. Cunniff, G.D. Clark, M.A. Blazo, D.A. Peiffer, K.L. Gunderson, T. Sahoo, A. Patel, J.R. Lupski,

A.L. Beaudet, and S.W. Cheung. 2007. Speech delay and autism spectrum behaviors are frequently associated with duplication of the 7q11.23 Williams-Beuren syndrome region. *Genet Med*. 9:427-41.

Bernstein, B.E., A. Meissner, and E.S. Lander. 2007. The mammalian epigenome. *Cell*. 128:669-81.

Bernstein, B.E., T.S. Mikkelsen, X. Xie, M. Kamal, D.J. Huebert, J. Cuff, B. Fry, A. Meissner, M. Wernig, K. Plath, R. Jaenisch, A. Wagschal, R. Feil, S.L. Schreiber, and E.S. Lander. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*. 125:315-26.

Bestor, T.H. 2000. The DNA methyltransferases of mammals. *Hum Mol Genet*. 9:2395-402.

Bestor, T.H., and B. Tycko. 1996. Creation of genomic methylation patterns. *Nat Genet*. 12:363-7.

Bhattacharya, S.K., S. Ramchandani, N. Cervoni, and M. Szyf. 1999. A mammalian protein with specific demethylase activity for mCpG DNA. *Nature*. 397:579-83.

Bienvenu, T., and J. Chelly. 2006. Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. *Nat Rev Genet*. 7:415-26.

Bird, A. 2002a. DNA methylation patterns and epigenetic memory. *Genes Dev*. 16:6-21.

Bird, A. 2002b. DNA methylation patterns and epigenetic memory. *Genes Dev*. 16:6-21.

Bourne, J.N., K.E. Sorra, J. Hurlburt, and K.M. Harris. 2007. Polyribosomes are increased in spines of CA1 dendrites 2 h after the induction of LTP in mature rat hippocampal slices. *Hippocampus*. 17:1-4.

Boyer, L.A., K. Plath, J. Zeitlinger, T. Brambrink, L.A. Medeiros, T.I. Lee, S.S. Levine, M. Wernig, A. Tajonar, M.K. Ray, G.W. Bell, A.P. Otte, M. Vidal, D.K. Gifford, R.A. Young, and R. Jaenisch. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*. 441:349-53.

Braunschweig, D., T. Simcox, R.C. Samaco, and J.M. LaSalle. 2004. X-Chromosome inactivation ratios affect wild-type MeCP2 expression within mosaic Rett syndrome and Mecp2-/+ mouse brain. *Hum Mol Genet*. 13:1275-86.

Brennecke, J., D.R. Hipfner, A. Stark, R.B. Russell, and S.M. Cohen. 2003. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell*. 113:25-36.

Brown, J.P., S. Couillard-Despres, C.M. Cooper-Kuhn, J. Winkler, L. Aigner, and H.G. Kuhn. 2003. Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol*. 467:1-10.

Burbea, M., L. Dreier, J.S. Dittman, M.E. Grunwald, and J.M. Kaplan. 2002. Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in C. elegans. *Neuron*. 35:107-20.

Busard, H.L., W.O. Renier, F.J. Gabreels, H.H. Jaspar, J.L. Slooff, A.J. Janssen, and U.J. Van Haelst. 1987. Lafora disease: a quantitative morphological and biochemical study of the cerebral cortex. *Clin Neuropathol*. 6:1-6.

Bushati, N., and S.M. Cohen. 2007. microRNA functions. *Annu Rev Cell Dev Biol*. 23:175-205.

Cajal, S.R. 1891. Sur la structure de l'ecorce cérébrale de quelques mammifères. *La Cellule*. 7:125-176.

Cao, X., G. Yeo, A.R. Muotri, T. Kuwabara, and F.H. Gage. 2006. Noncoding RNAs in the mammalian central nervous system. *Annu Rev Neurosci*. 29:77-103.

Carninci, P., T. Kasukawa, S. Katayama, J. Gough, M.C. Frith, N. Maeda, R. Oyama, T. Ravasi, B. Lenhard, C. Wells, R. Kodzius, K. Shimokawa, V.B. Bajic, S.E. Brenner, S. Batalov, A.R. Forrest, M. Zavolan, M.J. Davis, L.G. Wilming, V. Aidinis, J.E. Allen, A. Ambesi-Impiombato, R. Apweiler, R.N. Aturaliya, T.L. Bailey, M. Bansal, L. Baxter, K.W. Beisel, T. Bersano, H. Bono, A.M. Chalk, K.P. Chiu, V. Choudhary, A. Christoffels, D.R. Clutterbuck, M.L. Crowe, E. Dalla, B.P. Dalrymple, B. de Bono, G. Della Gatta, D. di Bernardo, T. Down, P. Engstrom, M. Fagiolini, G. Faulkner, C.F. Fletcher, T. Fukushima, M. Furuno, S. Futaki, M. Gariboldi, P. Georgii-Hemming, T.R. Gingeras, T. Gojobori, R.E. Green, S. Gustincich, M. Harbers, Y. Hayashi, T.K. Hensch, N. Hirokawa, D. Hill, L. Huminiecki, M. Iacono, K. Ikeo, A. Iwama, T. Ishikawa, M. Jakt, A. Kanapin, M. Katoh, Y. Kawasawa, J. Kelso, H. Kitamura, H. Kitano, G. Kollias, S.P. Krishnan, A. Kruger, S.K. Kummerfeld, I.V. Kurochkin, L.F. Lareau, D. Lazarevic, L. Lipovich, J. Liu, S. Liuni, S. McWilliam, M. Madan Babu, M. Madera, L. Marchionni, H. Matsuda, S. Matsuzawa, H. Miki, F. Mignone, S. Miyake, K. Morris, S. Mottagui-Tabar, N. Mulder, N. Nakano, H. Nakauchi, P. Ng, R. Nilsson, S. Nishiguchi, S. Nishikawa, et al. 2005. The transcriptional landscape of the mammalian genome. *Science*. 309:1559-63.

Caudy, A.A., M. Myers, G.J. Hannon, and S.M. Hammond. 2002. Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev*. 16:2491-6.

Chahrour, M., and H.Y. Zoghbi. 2007. The story of Rett syndrome: from clinic to neurobiology. *Neuron*. 56:422-37.

Chang, S., S.M. Bray, Z. Li, D.C. Zarnescu, C. He, P. Jin, and S.T. Warren. 2008. Identification of small molecules rescuing fragile X syndrome phenotypes in Drosophila. *Nat Chem Biol*. 4:256-63.

Chang, S., R.J. Johnston, Jr., C. Frokjaer-Jensen, S. Lockery, and O. Hobert. 2004. MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. *Nature*. 430:785-9.

Chang, T.C., and J.T. Mendell. 2007. microRNAs in vertebrate physiology and human disease. *Annu Rev Genomics Hum Genet*. 8:215-39.

Chao, H.T., and H.Y. Zoghbi. 2009. The yin and yang of MeCP2 phosphorylation. *Proc Natl Acad Sci U S A*. 106:4577-8.

Chao, H.T., H.Y. Zoghbi, and C. Rosenmund. 2007. MeCP2 controls excitatory synaptic strength by regulating glutamatergic synapse number. *Neuron*. 56:58-65.

Chen, C.Z., L. Li, H.F. Lodish, and D.P. Bartel. 2004. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 303:83-6.

Chen, H., S. Polo, P.P. Di Fiore, and P.V. De Camilli. 2003a. Rapid Ca2+-dependent decrease of protein ubiquitination at synapses. *Proc Natl Acad Sci U S A*. 100:14908-13.

Chen, L., and M. Toth. 2001. Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience*. 103:1043-50.

Chen, R.Z., S. Akbarian, M. Tudor, and R. Jaenisch. 2001a. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet*. 27:327-31.

Chen, R.Z., S. Akbarian, M. Tudor, and R. Jaenisch. 2001b. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet*. 27:327-31.

Chen, W.G., Q. Chang, Y. Lin, A. Meissner, A.E. West, E.C. Griffith, R. Jaenisch, and M.E. Greenberg. 2003b. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science*. 302:885-9.

Chen, X., B. Zhang, and J.A. Fischer. 2002. A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. *Genes Dev*. 16:289-94.
Cheng, L.C., M. Tavazoie, and F. Doetsch. 2005. Stem cells: from epigenetics to microRNAs. *Neuron*. 46:363-7.
Choe, E.A., L. Liao, J.Y. Zhou, D. Cheng, D.M. Duong, P. Jin, L.H. Tsai, and J. Peng. 2007. Neuronal morphogenesis is regulated by the interplay between cyclin-dependent kinase 5 and the ubiquitin ligase mind bomb 1. *J Neurosci*. 27:9503-12.
Coffin, R.B., D.J. Velinsky, R. Devereux, W.A. Price, and L.A. Cifuentes. 1990. Stable carbon isotope analysis of nucleic acids to trace sources of dissolved substrates used by estuarine bacteria. *Appl Environ Microbiol*. 56:2012-20.
Cohen, D.R., V. Matarazzo, A.M. Palmer, Y. Tu, O.H. Jeon, J. Pevsner, and G.V. Ronnett. 2003. Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. *Mol Cell Neurosci*. 22:417-29.
Cohen, S., and M.E. Greenberg. 2008. Communication between the synapse and the nucleus in neuronal development, plasticity, and disease. *Annu Rev Cell Dev Biol*. 24:183-209.
Collins, A.L., J.M. Levenson, A.P. Vilaythong, R. Richman, D.L. Armstrong, J.L. Noebels, J. David Sweatt, and H.Y. Zoghbi. 2004. Mild overexpression of MeCP2 causes a progressive neurological disorder in mice. *Hum Mol Genet*. 13:2679-89.
Comery, T.A., J.B. Harris, P.J. Willems, B.A. Oostra, S.A. Irwin, I.J. Weiler, and W.T. Greenough. 1997. Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc Natl Acad Sci U S A*. 94:5401-4.
Dani, V.S., Q. Chang, A. Maffei, G.G. Turrigiano, R. Jaenisch, and S.B. Nelson. 2005. Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett syndrome. *Proc Natl Acad Sci U S A*. 102:12560-5.
Dani, V.S., and S.B. Nelson. 2009. Intact long-term potentiation but reduced connectivity between neocortical layer 5 pyramidal neurons in a mouse model of Rett syndrome. *J Neurosci*. 29:11263-70.
Day, M., Z. Wang, J. Ding, X. An, C.A. Ingham, A.F. Shering, D. Wokosin, E. Ilijic, Z. Sun, A.R. Sampson, E. Mugnaini, A.Y. Deutch, S.R. Sesack, G.W. Arbuthnott, and D.J. Surmeier. 2006. Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat Neurosci*. 9:251-9.
De Carlos, J.A., and J. Borrell. 2007. A historical reflection of the contributions of Cajal and Golgi to the foundations of neuroscience. *Brain Res Rev*. 55:8-16.
Detich, N., J. Theberge, and M. Szyf. 2002. Promoter-specific activation and demethylation by MBD2/demethylase. *J Biol Chem*. 277:35791-4.
DiAntonio, A., A.P. Haghighi, S.L. Portman, J.D. Lee, A.M. Amaranto, and C.S. Goodman. 2001. Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature*. 412:449-52.
Dillon, C., and Y. Goda. 2005. The actin cytoskeleton: integrating form and function at the synapse. *Annu Rev Neurosci*. 28:25-55.
Dindot, S.V., B.A. Antalffy, M.B. Bhattacharjee, and A.L. Beaudet. 2008. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Hum Mol Genet*. 17:111-8.
Ding, F., H.H. Li, S. Zhang, N.M. Solomon, S.A. Camper, P. Cohen, and U. Francke. 2008. SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice. *PLoS One*. 3:e1709.

Dockendorff, T.C., H.S. Su, S.M. McBride, Z. Yang, C.H. Choi, K.K. Siwicki, A. Sehgal, and T.A. Jongens. 2002. Drosophila lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. *Neuron*. 34:973-84.

Duan, X., J.H. Chang, S. Ge, R.L. Faulkner, J.Y. Kim, Y. Kitabatake, X.B. Liu, C.H. Yang, J.D. Jordan, D.K. Ma, C.Y. Liu, S. Ganesan, H.J. Cheng, G.L. Ming, B. Lu, and H. Song. 2007. Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell*. 130:1146-58.

Edbauer, D., J.R. Neilson, K.A. Foster, C.F. Wang, D.P. Seeburg, M.N. Batterton, T. Tada, B.M. Dolan, P.A. Sharp, and M. Sheng. 2010. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. *Neuron*. 65:373-84.

Egger, G., G. Liang, A. Aparicio, and P.A. Jones. 2004. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 429:457-63.

Elsea, S.H., and S. Girirajan. 2008. Smith-Magenis syndrome. *Eur J Hum Genet*. 16:412-21.

Esposito, M.S., V.C. Piatti, D.A. Laplagne, N.A. Morgenstern, C.C. Ferrari, F.J. Pitossi, and A.F. Schinder. 2005. Neuronal differentiation in the adult hippocampus recapitulates embryonic development. *J Neurosci*. 25:10074-86.

Ethell, I.M., F. Irie, M.S. Kalo, J.R. Couchman, E.B. Pasquale, and Y. Yamaguchi. 2001. EphB/syndecan-2 signaling in dendritic spine morphogenesis. *Neuron*. 31:1001-13.

Ethell, I.M., and Y. Yamaguchi. 1999. Cell surface heparan sulfate proteoglycan syndecan-2 induces the maturation of dendritic spines in rat hippocampal neurons. *J Cell Biol*. 144:575-86.

Fan, G., C. Beard, R.Z. Chen, G. Csankovszki, Y. Sun, M. Siniaia, D. Biniszkiewicz, B. Bates, P.P. Lee, R. Kuhn, A. Trumpp, C. Poon, C.B. Wilson, and R. Jaenisch. 2001. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. *J Neurosci*. 21:788-97.

Feng, J., Y. Zhou, S.L. Campbell, T. Le, E. Li, J.D. Sweatt, A.J. Silva, and G. Fan. 2010. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci*. 13:423-30.

Ferrer, I., I. Fabregues, J. Coll, T. Ribalta, and A. Rives. 1984. Tuberous sclerosis: a Golgi study of cortical tuber. *Clin Neuropathol*. 3:47-51.

Ferrer, I., and F. Gullotta. 1990. Down's syndrome and Alzheimer's disease: dendritic spine counts in the hippocampus. *Acta Neuropathol*. 79:680-5.

Fiala, J.C., M. Feinberg, V. Popov, and K.M. Harris. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci*. 18:8900-11.

Fiala, J.C., J. Spacek, and K.M. Harris. 2002. Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Res Brain Res Rev*. 39:29-54.

Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 9:102-14.

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*. 28:697-710.

Fiore, R., G. Siegel, and G. Schratt. 2008. MicroRNA function in neuronal development, plasticity and disease. *Biochim Biophys Acta*. 1779:471-8.

Fletcher, E.J., J. Church, and J.F. MacDonald. 1994. Haloperidol blocks voltage-activated Ca2+ channels in hippocampal neurones. *Eur J Pharmacol*. 267:249-52.

Gage, F.H. 2002. Neurogenesis in the adult brain. *J Neurosci*. 22:612-3.

Galvez, R., A.R. Gopal, and W.T. Greenough. 2003. Somatosensory cortical barrel dendritic abnormalities in a mouse model of the fragile X mental retardation syndrome. *Brain Res*. 971:83-9.

Gangaraju, V.K., and H. Lin. 2009. MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol*. 10:116-25.

Ge, S., E.L. Goh, K.A. Sailor, Y. Kitabatake, G.L. Ming, and H. Song. 2006. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature*. 439:589-93.

Gemelli, T., O. Berton, E.D. Nelson, L.I. Perrotti, R. Jaenisch, and L.M. Monteggia. 2006. Postnatal loss of methyl-CpG binding protein 2 in the forebrain is sufficient to mediate behavioral aspects of Rett syndrome in mice. *Biol Psychiatry*. 59:468-76.

Golshani, P., L. Hutnick, F. Schweizer, and G. Fan. 2005. Conditional Dnmt1 deletion in dorsal forebrain disrupts development of somatosensory barrel cortex and thalamocortical long-term potentiation. *Thalamus Relat Syst*. 3:227-233.

Goslin, K., and G. Banker. 1989. Experimental observations on the development of polarity by hippocampal neurons in culture. *J Cell Biol*. 108:1507-16.

Gothelf, D., C. Feinstein, T. Thompson, E. Gu, L. Penniman, E. Van Stone, H. Kwon, S. Eliez, and A.L. Reiss. 2007. Risk factors for the emergence of psychotic disorders in adolescents with 22q11.2 deletion syndrome. *Am J Psychiatry*. 164:663-9.

Graff, J., and I.M. Mansuy. 2009. Epigenetic dysregulation in cognitive disorders. *Eur J Neurosci*. 30:1-8.

Greenough, W.T., A.Y. Klintsova, S.A. Irwin, R. Galvez, K.E. Bates, and I.J. Weiler. 2001. Synaptic regulation of protein synthesis and the fragile X protein. *Proc Natl Acad Sci U S A*. 98:7101-6.

Grewal, S.I., and S.C. Elgin. 2007. Transcription and RNA interference in the formation of heterochromatin. *Nature*. 447:399-406.

Grossman, A.W., G.M. Aldridge, I.J. Weiler, and W.T. Greenough. 2006. Local protein synthesis and spine morphogenesis: Fragile X syndrome and beyond. *J Neurosci*. 26:7151-5.

Grutzendler, J., N. Kasthuri, and W.B. Gan. 2002. Long-term dendritic spine stability in the adult cortex. *Nature*. 420:812-6.

Guan, J.S., S.J. Haggarty, E. Giacometti, J.H. Dannenberg, N. Joseph, J. Gao, T.J. Nieland, Y. Zhou, X. Wang, R. Mazitschek, J.E. Bradner, R.A. DePinho, R. Jaenisch, and L.H. Tsai. 2009. HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature*. 459:55-60.

Gundersen, H.J., T.F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J.R. Nyengaard, B. Pakkenberg, F.B. Sorensen, A. Vesterby, and et al. 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *Apmis*. 96:379-94.

Guo, H., N.T. Ingolia, J.S. Weissman, and D.P. Bartel. 2010. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 466:835-40.

Guy, J., B. Hendrich, M. Holmes, J.E. Martin, and A. Bird. 2001. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet*. 27:322-6.

Hagberg, B., J. Aicardi, K. Dias, and O. Ramos. 1983a. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. *Ann Neurol*. 14:471-9.

Hagberg, B., J. Aicardi, K. Dias, and O. Ramos. 1983b. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. *Annals of neurology*. 14:471-9.

Hagberg, B., and I. Witt-Engerstrom. 1986. Rett syndrome: a suggested staging system for describing impairment profile with increasing age towards adolescence. *Am J Med Genet Suppl*. 1:47-59.

Hannon, G.J. 2002. RNA interference. *Nature*. 418:244-51.

Harbers, K., M. Kuehn, H. Delius, and R. Jaenisch. 1984. Insertion of retrovirus into the first intron of alpha 1(I) collagen gene to embryonic lethal mutation in mice. *Proc Natl Acad Sci U S A*. 81:1504-8.

Harris, K.M., and S.B. Kater. 1994. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci*. 17:341-71.

Hartl, F.U., and M. Hayer-Hartl. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*. 295:1852-8.

He, L., and G.J. Hannon. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 5:522-31.

Henze, D.A., N.N. Urban, and G. Barrionuevo. 2000. The multifarious hippocampal mossy fiber pathway: a review. *Neuroscience*. 98:407-27.

Higashi, Y., S. Murayama, P.G. Pentchev, and K. Suzuki. 1993. Cerebellar degeneration in the Niemann-Pick type C mouse. *Acta Neuropathol*. 85:175-84.

Hinton, V.J., W.T. Brown, K. Wisniewski, and R.D. Rudelli. 1991. Analysis of neocortex in three males with the fragile X syndrome. *Am J Med Genet*. 41:289-94.

Holliday, R., and J.E. Pugh. 1975. DNA modification mechanisms and gene activity during development. *Science*. 187:226-32.

Hoogenraad, C.C., B. Koekkoek, A. Akhmanova, H. Krugers, B. Dortland, M. Miedema, A. van Alphen, W.M. Kistler, M. Jaegle, M. Koutsourakis, N. Van Camp, M. Verhoye, A. van der Linden, I. Kaverina, F. Grosveld, C.I. De Zeeuw, and N. Galjart. 2002. Targeted mutation of Cyln2 in the Williams syndrome critical region links CLIP-115 haploinsufficiency to neurodevelopmental abnormalities in mice. *Nat Genet*. 32:116-27.

Horike, S., S. Cai, M. Miyano, J.F. Cheng, and T. Kohwi-Shigematsu. 2005. Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat Genet*. 37:31-40.

Hotta, Y., and S. Benzer. 1970. Genetic dissection of the Drosophila nervous system by means of mosaics. *Proc Natl Acad Sci U S A*. 67:1156-63.

Hsieh, J., K. Nakashima, T. Kuwabara, E. Mejia, and F.H. Gage. 2004. Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A*. 101:16659-64.

Hull, C., and R.J. Hagerman. 1993. A study of the physical, behavioral, and medical phenotype, including anthropometric measures, of females with fragile X syndrome. *Am J Dis Child*. 147:1236-41.

Hutnick, L.K., P. Golshani, M. Namihira, Z. Xue, A. Matynia, X.W. Yang, A.J. Silva, F.E. Schweizer, and G. Fan. 2009. DNA hypomethylation restricted to the murine forebrain

induces cortical degeneration and impairs postnatal neuronal maturation. *Hum Mol Genet*. 18:2875-88.
Huttenlocher, P.R. 1970. Dendritic development and mental defect. *Neurology*. 20:381.
Huttenlocher, P.R. 1974. Dendritic development in neocortex of children with mental defect and infantile spasms. *Neurology*. 24:203-10.
Irizarry, R.A., B.M. Bolstad, F. Collin, L.M. Cope, B. Hobbs, and T.P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 31:e15.
Irwin, S.A., B. Patel, M. Idupulapati, J.B. Harris, R.A. Crisostomo, B.P. Larsen, F. Kooy, P.J. Willems, P. Cras, P.B. Kozlowski, R.A. Swain, I.J. Weiler, and W.T. Greenough. 2001. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet*. 98:161-7.
Ishikawa-Brush, Y., J.F. Powell, P. Bolton, A.P. Miller, F. Francis, H.F. Willard, H. Lehrach, and A.P. Monaco. 1997. Autism and multiple exostoses associated with an X;8 translocation occurring within the GRPR gene and 3' to the SDC2 gene. *Hum Mol Genet*. 6:1241-50.
Ishizuka, A., M.C. Siomi, and H. Siomi. 2002. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev*. 16:2497-508.
Itoh, M., C.H. Kim, G. Palardy, T. Oda, Y.J. Jiang, D. Maust, S.Y. Yeo, K. Lorick, G.J. Wright, L. Ariza-McNaughton, A.M. Weissman, J. Lewis, S.C. Chandrasekharappa, and A.B. Chitnis. 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell*. 4:67-82.
Ivey, K.N., A. Muth, J. Arnold, F.W. King, R.F. Yeh, J.E. Fish, E.C. Hsiao, R.J. Schwartz, B.R. Conklin, H.S. Bernstein, and D. Srivastava. 2008. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell*. 2:219-29.
Jaenisch, R., and A. Bird. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 33 Suppl:245-54.
Jin, P., D.C. Zarnescu, S. Ceman, M. Nakamoto, J. Mowrey, T.A. Jongens, D.L. Nelson, K. Moses, and S.T. Warren. 2004. Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat Neurosci*. 7:113-7.
Johansson, B.B., and P.V. Belichenko. 2002. Neuronal plasticity and dendritic spines: effect of environmental enrichment on intact and postischemic rat brain. *J Cereb Blood Flow Metab*. 22:89-96.
John, B., A.J. Enright, A. Aravin, T. Tuschl, C. Sander, and D.S. Marks. 2004. Human MicroRNA targets. *PLoS Biol*. 2:e363.
Johnston, R.J., and O. Hobert. 2003. A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. *Nature*. 426:845-9.
Jones, P.A., and S.B. Baylin. 2002. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 3:415-28.
Jugloff, D.G., B.P. Jung, D. Purushotham, R. Logan, and J.H. Eubanks. 2005. Increased dendritic complexity and axonal length in cultured mouse cortical neurons overexpressing methyl-CpG-binding protein MeCP2. *Neurobiol Dis*. 19:18-27.
Jung, B.P., D.G. Jugloff, G. Zhang, R. Logan, S. Brown, and J.H. Eubanks. 2003. The expression of methyl CpG binding factor MeCP2 correlates with cellular differentiation in the developing rat brain and in cultured cells. *J Neurobiol*. 55:86-96.

Kanellopoulou, C., S.A. Muljo, A.L. Kung, S. Ganesan, R. Drapkin, T. Jenuwein, D.M. Livingston, and K. Rajewsky. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*. 19:489-501.

Kaufmann, W.E., and H.W. Moser. 2000a. Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex*. 10:981-91.

Kaufmann, W.E., and H.W. Moser. 2000b. Dendritic anomalies in disorders associated with mental retardation [In Process Citation]. *Cereb Cortex*. 10:981-91.

Kaufmann, W.E., C.V. Taylor, C.F. Hohmann, I.B. Sanwal, and S. Naidu. 1997. Abnormalities in neuronal maturation in Rett syndrome neocortex: preliminary molecular correlates. *Eur Child Adolesc Psychiatry*. 6 Suppl 1:75-7.

Kawasaki, H., K. Taira, and K.V. Morris. 2005. siRNA Induced Transcriptional Gene Silencing in Mammalian Cells. *Cell Cycle*. 4.

Kishi, N., and J.D. Macklis. 2004. MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. *Mol Cell Neurosci*. 27:306-21.

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*. 10:1513-4.

Klose, R.J., and A.P. Bird. 2006. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*. 31:89-97.

Kobrynski, L.J., and K.E. Sullivan. 2007. Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes. *Lancet*. 370:1443-52.

Koo, B.K., H.S. Lim, R. Song, M.J. Yoon, K.J. Yoon, J.S. Moon, Y.W. Kim, M.C. Kwon, K.W. Yoo, M.P. Kong, J. Lee, A.B. Chitnis, C.H. Kim, and Y.Y. Kong. 2005. Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development*. 132:3459-70.

Kooy, R.F. 2003. Of mice and the fragile X syndrome. *Trends Genet*. 19:148-54.

Kooy, R.F., R. D'Hooge, E. Reyniers, C.E. Bakker, G. Nagels, K. De Boulle, K. Storm, G. Clincke, P.P. De Deyn, B.A. Oostra, and P.J. Willems. 1996. Transgenic mouse model for the fragile X syndrome. *Am J Med Genet*. 64:241-5.

Krek, A., D. Grun, M.N. Poy, R. Wolf, L. Rosenberg, E.J. Epstein, P. MacMenamin, I. da Piedade, K.C. Gunsalus, M. Stoffel, and N. Rajewsky. 2005. Combinatorial microRNA target predictions. *Nat Genet*. 37:495-500.

Kriaucionis, S., and A. Bird. 2003. DNA methylation and Rett syndrome. *Hum Mol Genet*. 12 Suppl 2:R221-R227.

Krichevsky, A.M., K.C. Sonntag, O. Isacson, and K.S. Kosik. 2006. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells*. 24:857-64.

Krogan, N.J., M. Kim, A. Tong, A. Golshani, G. Cagney, V. Canadien, D.P. Richards, B.K. Beattie, A. Emili, C. Boone, A. Shilatifard, S. Buratowski, and J. Greenblatt. 2003. Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. *Mol Cell Biol*. 23:4207-18.

Lachner, M., R.J. O'Sullivan, and T. Jenuwein. 2003. An epigenetic road map for histone lysine methylation. *J Cell Sci*. 116:2117-24.

Lagace, D.C., M.C. Whitman, M.A. Noonan, J.L. Ables, N.A. DeCarolis, A.A. Arguello, M.H. Donovan, S.J. Fischer, L.A. Farnbauch, R.D. Beech, R.J. DiLeone, C.A. Greer, C.D.

Mandyam, and A.J. Eisch. 2007. Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. *J Neurosci*. 27:12623-9.
Lalande, M., and M.A. Calciano. 2007. Molecular epigenetics of Angelman syndrome. *Cell Mol Life Sci*. 64:947-60.
Lao, K., N.L. Xu, V. Yeung, C. Chen, K.J. Livak, and N.A. Straus. 2006. Multiplexing RT-PCR for the detection of multiple miRNA species in small samples. *Biochem Biophys Res Commun*. 343:85-9.
Lee, J.A., and J.R. Lupski. 2006. Genomic rearrangements and gene copy-number alterations as a cause of nervous system disorders. *Neuron*. 52:103-21.
Lee, T.I., R.G. Jenner, L.A. Boyer, M.G. Guenther, S.S. Levine, R.M. Kumar, B. Chevalier, S.E. Johnstone, M.F. Cole, K. Isono, H. Koseki, T. Fuchikami, K. Abe, H.L. Murray, J.P. Zucker, B. Yuan, G.W. Bell, E. Herbolsheimer, N.M. Hannett, K. Sun, D.T. Odom, A.P. Otte, T.L. Volkert, D.P. Bartel, D.A. Melton, D.K. Gifford, R. Jaenisch, and R.A. Young. 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell*. 125:301-13.
Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, and V.N. Kim. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 425:415-9.
Leucht, C., C. Stigloher, A. Wizenmann, R. Klafke, A. Folchert, and L. Bally-Cuif. 2008. MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. *Nat Neurosci*. 11:641-8.
Lewis, B.P., I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, and C.B. Burge. 2003a. Prediction of mammalian microRNA targets. *Cell*. 115:787-98.
Lewis, D.A., L.A. Glantz, J.N. Pierri, and R.A. Sweet. 2003b. Altered cortical glutamate neurotransmission in schizophrenia: evidence from morphological studies of pyramidal neurons. *Ann N Y Acad Sci*. 1003:102-12.
Li, C., and W.H. Wong. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A*. 98:31-6.
Li, S., I. Reinprecht, M. Fahnestock, and R.J. Racine. 2002. Activity-dependent changes in synaptophysin immunoreactivity in hippocampus, piriform cortex, and entorhinal cortex of the rat. *Neuroscience*. 115:1221-9.
Li, X., B.Z. Barkho, Y. Luo, R.D. Smrt, N.J. Santistevan, C. Liu, T. Kuwabara, F.H. Gage, and X. Zhao. 2008. Epigenetic regulation of the stem cell mitogen Fgf-2 by Mbd1 in adult neural stem/progenitor cells. *J Biol Chem*. 283:27644-52.
Li, X., and X. Zhao. 2008. Epigenetic regulation of mammalian stem cells. *Stem Cells Dev*. 17:1043-52.
Lie, D.C., S.A. Colamarino, H.J. Song, L. Desire, H. Mira, A. Consiglio, E.S. Lein, S. Jessberger, H. Lansford, A.R. Dearie, and F.H. Gage. 2005. Wnt signalling regulates adult hippocampal neurogenesis. *Nature*. 437:1370-5.
Lim, M.K., T. Kawamura, Y. Ohsawa, M. Ohtsubo, S. Asakawa, A. Takayanagi, and N. Shimizu. 2007. Parkin interacts with LIM Kinase 1 and reduces its cofilin-phosphorylation activity via ubiquitination. *Exp Cell Res*. 313:2858-74.
Lin, Y.J., L. Seroude, and S. Benzer. 1998. Extended life-span and stress resistance in the Drosophila mutant methuselah. *Science*. 282:943-6.

Ling, S.C., P.S. Fahrner, W.T. Greenough, and V.I. Gelfand. 2004. Transport of Drosophila fragile X mental retardation protein-containing ribonucleoprotein granules by kinesin-1 and cytoplasmic dynein. *Proc Natl Acad Sci U S A*. 101:17428-33.

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*. 6:433-44.

Liu, C., and X. Zhao. 2009a. MicroRNAs in adult and embryonic neurogenesis. *Neuromolecular Med*. 11:141-52.

Liu, C., and X. Zhao. 2009b. MicroRNAs in Adult and Embryonic Neurogenesis. *Neuromolecular Med*.

Logue, S.F., R. Paylor, and J.M. Wehner. 1997. Hippocampal lesions cause learning deficits in inbred mice in the Morris water maze and conditioned-fear task. *Behav Neurosci*. 111:104-13.

Lu, Y., F. Wang, Y. Li, J. Ferris, J.A. Lee, and F.B. Gao. 2009. The Drosophila homologue of the Angelman syndrome ubiquitin ligase regulates the formation of terminal dendritic branches. *Hum Mol Genet*. 18:454-62.

Lugli, G., V.I. Torvik, J. Larson, and N.R. Smalheiser. 2008. Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain. *J Neurochem*. 106:650-61.

Luikenhuis, S., E. Giacometti, C.F. Beard, and R. Jaenisch. 2004. Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice. *Proc Natl Acad Sci U S A*. 101:6033-8.

Luo, Y., G. Shan, W. Guo, R.D. Smrt, E.B. Johnson, X. Li, R.L. Pfeiffer, K.E. Szulwach, R. Duan, B.Z. Barkho, W. Li, C. Liu, P. Jin, and X. Zhao. 2010. Fragile x mental retardation protein regulates proliferation and differentiation of adult neural stem/progenitor cells. *PLoS Genet*. 6:e1000898.

Lush, M.J., Y. Li, D.J. Read, A.C. Willis, and P. Glynn. 1998. Neuropathy target esterase and a homologous Drosophila neurodegeneration-associated mutant protein contain a novel domain conserved from bacteria to man. *Biochem J*. 332 (Pt 1):1-4.

Ma, D.K., M.H. Jang, J.U. Guo, Y. Kitabatake, M.L. Chang, N. Pow-Anpongkul, R.A. Flavell, B. Lu, G.L. Ming, and H. Song. 2009. Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. *Science*. 323:1074-7.

Machado-Salas, J.P. 1984. Abnormal dendritic patterns and aberrant spine development in Bourneville's disease--a Golgi survey. *Clin Neuropathol*. 3:52-8.

Marin-Padilla, M. 1972. Structural abnormalities of the cerebral cortex in human chromosomal aberrations: a Golgi study. *Brain Res*. 44:625-9.

Marin-Padilla, M. 1976. Pyramidal cell abnormalities in the motor cortex of a child with Down's syndrome. A Golgi study. *J Comp Neurol*. 167:63-81.

Marsh, J.L., and L.M. Thompson. 2006. Drosophila in the study of neurodegenerative disease. *Neuron*. 52:169-78.

Martinowich, K., D. Hattori, H. Wu, S. Fouse, F. He, Y. Hu, G. Fan, and Y.E. Sun. 2003. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science*. 302:890-3.

Matarazzo, V., D. Cohen, A.M. Palmer, P.J. Simpson, B. Khokhar, S.J. Pan, and G.V. Ronnett. 2004. The transcriptional repressor Mecp2 regulates terminal neuronal differentiation. *Mol Cell Neurosci*. 27:44-58.

Meng, Y., Y. Zhang, V. Tregoubov, C. Janus, L. Cruz, M. Jackson, W.Y. Lu, J.F. MacDonald, J.Y. Wang, D.L. Falls, and Z. Jia. 2002. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron*. 35:121-33.

Metcalf, B.M., B.C. Mullaney, M.V. Johnston, and M.E. Blue. 2006. Temporal shift in methyl-CpG binding protein 2 expression in a mouse model of Rett syndrome. *Neuroscience*. 139:1449-60.

Michel, C.I., R. Kraft, and L.L. Restifo. 2004. Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. *J Neurosci*. 24:5798-809.

Min, K.T., and S. Benzer. 1999. Preventing neurodegeneration in the Drosophila mutant bubblegum. *Science*. 284:1985-8.

Mineur, Y.S., F. Sluyter, S. de Wit, B.A. Oostra, and W.E. Crusio. 2002. Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse. *Hippocampus*. 12:39-46.

Ming, G.L., and H. Song. 2005. Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci*. 28:223-50.

Miura, K., T. Kishino, E. Li, H. Webber, P. Dikkes, G.L. Holmes, and J. Wagstaff. 2002. Neurobehavioral and electroencephalographic abnormalities in Ube3a maternal-deficient mice. *Neurobiol Dis*. 9:149-59.

Morales, J., P.R. Hiesinger, A.J. Schroeder, K. Kume, P. Verstreken, F.R. Jackson, D.L. Nelson, and B.A. Hassan. 2002. Drosophila fragile X protein, DFXR, regulates neuronal morphology and function in the brain. *Neuron*. 34:961-72.

Moretti, P., J.M. Levenson, F. Battaglia, R. Atkinson, R. Teague, B. Antalffy, D. Armstrong, O. Arancio, J.D. Sweatt, and H.Y. Zoghbi. 2006. Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. *J Neurosci*. 26:319-27.

Morris, R.G., P. Garrud, J.N. Rawlins, and J. O'Keefe. 1982. Place navigation impaired in rats with hippocampal lesions. *Nature*. 297:681-3.

Mullen, R.J., C.R. Buck, and A.M. Smith. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. *Development*. 116:201-11.

Naldini, L., U. Blomer, F.H. Gage, D. Trono, and I.M. Verma. 1996. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 93:11382-8.

Namihira, M., K. Nakashima, and T. Taga. 2004. Developmental stage dependent regulation of DNA methylation and chromatin modification in a immature astrocyte specific gene promoter. *FEBS Lett*. 572:184-8.

Nelson, E.D., E.T. Kavalali, and L.M. Monteggia. 2006. MeCP2-dependent transcriptional repression regulates excitatory neurotransmission. *Curr Biol*. 16:710-6.

Nicoll, R.A., and D. Schmitz. 2005. Synaptic plasticity at hippocampal mossy fibre synapses. *Nat Rev Neurosci*. 6:863-76.

Nijman, S.M., M.P. Luna-Vargas, A. Velds, T.R. Brummelkamp, A.M. Dirac, T.K. Sixma, and R. Bernards. 2005. A genomic and functional inventory of deubiquitinating enzymes. *Cell*. 123:773-86.

Nimchinsky, E.A., A.M. Oberlander, and K. Svoboda. 2001. Abnormal development of dendritic spines in FMR1 knock-out mice. *J Neurosci*. 21:5139-46.

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*. 135:227-39.

Nolasco, S., J. Bellido, J. Goncalves, J.C. Zabala, and H. Soares. 2005. Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death. *FEBS Lett*. 579:3515-24.

Nomura, T., M. Kimura, T. Horii, S. Morita, H. Soejima, S. Kudo, and I. Hatada. 2008. MeCP2-dependent repression of an imprinted miR-184 released by depolarization. *Hum Mol Genet*. 17:1192-9.

O'Carroll, D., S. Erhardt, M. Pagani, S.C. Barton, M.A. Surani, and T. Jenuwein. 2001. The polycomb-group gene Ezh2 is required for early mouse development. *Mol Cell Biol*. 21:4330-6.

Oberle, I., F. Rousseau, D. Heitz, C. Kretz, D. Devys, A. Hanauer, J. Boue, M. Bertheas, and J. Mandel. 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science*. 252:1097-1102.

Obernosterer, G., J. Martinez, and M. Alenius. 2007. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc*. 2:1508-14.

Okabe, S., C. Collin, J.M. Auerbach, N. Meiri, J. Bengzon, M.B. Kennedy, M. Segal, and R.D. McKay. 1998. Hippocampal synaptic plasticity in mice overexpressing an embryonic subunit of the NMDA receptor. *J Neurosci*. 18:4177-88.

Ossipova, O., J. Ezan, and S.Y. Sokol. 2009. PAR-1 phosphorylates Mind bomb to promote vertebrate neurogenesis. *Dev Cell*. 17:222-33.

Ostroff, L.E., J.C. Fiala, B. Allwardt, and K.M. Harris. 2002. Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. *Neuron*. 35:535-45.

Overstreet, E., E. Fitch, and J.A. Fischer. 2004. Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development*. 131:5355-66.

Palmer, T.D., J. Takahashi, and F.H. Gage. 1997. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci*. 8:389-404.

Papazian, D.M., T.L. Schwarz, B.L. Tempel, Y.N. Jan, and L.Y. Jan. 1987. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from Drosophila. *Science*. 237:749-53.

Parrish, J.Z., K. Emoto, L.Y. Jan, and Y.N. Jan. 2007a. Polycomb genes interact with the tumor suppressor genes hippo and warts in the maintenance of Drosophila sensory neuron dendrites. *Genes Dev*. 21:956-72.

Parrish, J.Z., K. Emoto, M.D. Kim, and Y.N. Jan. 2007b. Mechanisms that regulate establishment, maintenance, and remodeling of dendritic fields. *Annu Rev Neurosci*. 30:399-423.

Peddada, S., D.H. Yasui, and J.M. LaSalle. 2006. Inhibitors of differentiation (ID1, ID2, ID3 and ID4) genes are neuronal targets of MeCP2 that are elevated in Rett syndrome. *Hum Mol Genet*. 15:2003-14.

Pelka, G.J., C.M. Watson, T. Radziewic, M. Hayward, H. Lahooti, J. Christodoulou, and P.P. Tam. 2006. Mecp2 deficiency is associated with learning and cognitive deficits and altered gene activity in the hippocampal region of mice. *Brain*. 129:887-98.

Penagarikano, O., J.G. Mulle, and S.T. Warren. 2007. The pathophysiology of fragile x syndrome. *Annu Rev Genomics Hum Genet*. 8:109-29.

Persico, A.M., and T. Bourgeron. 2006. Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci*. 29:349-58.

Phiel, C.J., F. Zhang, E.Y. Huang, M.G. Guenther, M.A. Lazar, and P.S. Klein. 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem*. 276:36734-41.

Phillips, J., and J.H. Eberwine. 1996. Antisense RNA Amplification: A Linear Amplification Method for Analyzing the mRNA Population from Single Living Cells. *Methods*. 10:283-8.

Pittenger, C., and R.S. Duman. 2008. Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*. 33:88-109.

Plath, K., S. Mlynarczyk-Evans, D.A. Nusinow, and B. Panning. 2002. Xist RNA and the mechanism of X chromosome inactivation. *Annu Rev Genet*. 36:233-78.

Plenge, R.M., R.A. Stevenson, H.A. Lubs, C.E. Schwartz, and H.F. Willard. 2002. Skewed X-chromosome inactivation is a common feature of X-linked mental retardation disorders. *Am J Hum Genet*. 71:168-73.

Potocki, L., W. Bi, D. Treadwell-Deering, C.M. Carvalho, A. Eifert, E.M. Friedman, D. Glaze, K. Krull, J.A. Lee, R.A. Lewis, R. Mendoza-Londono, P. Robbins-Furman, C. Shaw, X. Shi, G. Weissenberger, M. Withers, S.A. Yatsenko, E.H. Zackai, P. Stankiewicz, and J.R. Lupski. 2007. Characterization of Potocki-Lupski syndrome (dup(17)(p11.2p11.2)) and delineation of a dosage-sensitive critical interval that can convey an autism phenotype. *Am J Hum Genet*. 80:633-49.

Poy, M.N., L. Eliasson, J. Krutzfeldt, S. Kuwajima, X. Ma, P.E. Macdonald, S. Pfeffer, T. Tuschl, N. Rajewsky, P. Rorsman, and M. Stoffel. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*. 432:226-30.

Purpura, D.P. 1974. Dendritic spine "dysgenesis" and mental retardation. *Science*. 186:1126-8.

Purpura, D.P. 1975. Dendritic differentiation in human cerebral cortex: normal and aberrant developmental patterns. *Adv Neurol*. 12:91-134.

Rajasethupathy, P., F. Fiumara, R. Sheridan, D. Betel, S.V. Puthanveettil, 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*. 63:803-17.

Ramocki, M.B., and H.Y. Zoghbi. 2008. Failure of neuronal homeostasis results in common neuropsychiatric phenotypes. *Nature*. 455:912-8.

Rao, M.S., B. Hattiangady, A. Abdel-Rahman, D.P. Stanley, and A.K. Shetty. 2005. Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation. *Eur J Neurosci*. 21:464-76.

Rao, M.S., and A.K. Shetty. 2004. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *Eur J Neurosci*. 19:234-46.

Rao, P.N., K. Klinepeter, W. Stewart, R. Hayworth, R. Grubs, and M.J. Pettenati. 1994. Molecular cytogenetic analysis of a duplication Xp in a male: further delineation of a possible sex influencing region on the X chromosome. *Hum Genet*. 94:149-53.

Redmond, L., A.H. Kashani, and A. Ghosh. 2002. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron*. 34:999-1010.

Reiter, L.T., L. Potocki, S. Chien, M. Gribskov, and E. Bier. 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res*. 11:1114-25.

Riggs, A.D. 1975. X inactivation, differentiation, and DNA methylation. *Cytogenet Cell Genet*. 14:9-25.

Robertson, K.D., S. Ait-Si-Ali, T. Yokochi, P.A. Wade, P.L. Jones, and A.P. Wolffe. 2000. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet*. 25:338-42.

Robinson, T.E., and B. Kolb. 2004. Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology*. 47 Suppl 1:33-46.

Rosenthal, N., and S. Brown. 2007. The mouse ascending: perspectives for human-disease models. *Nat Cell Biol*. 9:993-9.

Rybak, A., H. Fuchs, L. Smirnova, C. Brandt, E.E. Pohl, R. Nitsch, and F.G. Wulczyn. 2008. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat Cell Biol*. 10:987-93.

Sahoo, T., D. del Gaudio, J.R. German, M. Shinawi, S.U. Peters, R.E. Person, A. Garnica, S.W. Cheung, and A.L. Beaudet. 2008. Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster. *Nat Genet*. 40:719-21.

Samaco, R.C., A. Hogart, and J.M. LaSalle. 2005. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human molecular genetics*. 14:483-92.

Samaco, R.C., R.P. Nagarajan, D. Braunschweig, and J.M. LaSalle. 2004. Multiple pathways regulate MeCP2 expression in normal brain development and exhibit defects in autism-spectrum disorders. *Human molecular genetics*. 13:629-39.

Santos-Rosa, H., R. Schneider, A.J. Bannister, J. Sherriff, B.E. Bernstein, N.C. Emre, S.L. Schreiber, J. Mellor, and T. Kouzarides. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature*. 419:407-11.

Sarna, J.R., M. Larouche, H. Marzban, R.V. Sillitoe, D.E. Rancourt, and R. Hawkes. 2003. Patterned Purkinje cell degeneration in mouse models of Niemann-Pick type C disease. *J Comp Neurol*. 456:279-91.

Schratt, G. 2009. microRNAs at the synapse. *Nat Rev Neurosci*. 10:842-9.

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*. 439:283-9.

Schubeler, D., D.M. MacAlpine, D. Scalzo, C. Wirbelauer, C. Kooperberg, F. van Leeuwen, D.E. Gottschling, L.P. O'Neill, B.M. Turner, J. Delrow, S.P. Bell, and M. Groudine. 2004. The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev*. 18:1263-71.

Schwamborn, J.C., E. Berezikov, and J.A. Knoblich. 2009. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell*. 136:913-25.

Segal, M. 2005. Dendritic spines and long-term plasticity. *Nat Rev Neurosci*. 6:277-84.

Selak, S., A.V. Paternain, M.J. Fritzler, and J. Lerma. 2006. Human autoantibodies against early endosome antigen-1 enhance excitatory synaptic transmission. *Neuroscience*. 143:953-64.

Seress, L., H. Abraham, T. Tornoczky, and G. Kosztolanyi. 2001. Cell formation in the human hippocampal formation from mid-gestation to the late postnatal period. *Neuroscience*. 105:831-43.

Seress, L., and L. Mrzljak. 1992. Postnatal development of mossy cells in the human dentate gyrus: a light microscopic Golgi study. *Hippocampus*. 2:127-41.

Shahbazian, M., J. Young, L. Yuva-Paylor, C. Spencer, B. Antalffy, J. Noebels, D. Armstrong, R. Paylor, and H. Zoghbi. 2002a. Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. *Neuron*. 35:243-54.

Shahbazian, M.D., B. Antalffy, D.L. Armstrong, and H.Y. Zoghbi. 2002b. Insight into Rett syndrome: MeCP2 levels display tissue- and cell- specific differences and correlate with neuronal maturation. *Hum Mol Genet*. 11:115-24.

Shahbazian, M.D., and H.Y. Zoghbi. 2002a. Rett syndrome and MeCP2: linking epigenetics and neuronal function. *Am J Hum Genet*. 71:1259-72.

Shahbazian, M.D., and H.Y. Zoghbi. 2002b. Rett syndrome and MeCP2: linking epigenetics and neuronal function. *Am J Hum Genet*. 71:1259-72.

Sheng, M., and C.C. Hoogenraad. 2007. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem*. 76:823-47.

Shi, Y., D. Chichung Lie, P. Taupin, K. Nakashima, J. Ray, R.T. Yu, F.H. Gage, and R.M. Evans. 2004. Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature*. 427:78-83.

Siegel, G., G. Obernosterer, R. Fiore, M. Oehmen, S. Bicker, M. Christensen, S. Khudayberdiev, P.F. Leuschner, C.J. Busch, C. Kane, K. Hubel, F. Dekker, C. Hedberg, B. Rengarajan, C. Drepper, H. Waldmann, S. Kauppinen, M.E. Greenberg, A. Draguhn, M. Rehmsmeier, J. Martinez, and G.M. Schratt. 2009. A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. *Nat Cell Biol*. 11:705-16.

Silber, J., D.A. Lim, C. Petritsch, A.I. Persson, A.K. Maunakea, M. Yu, S.R. Vandenberg, D.G. Ginzinger, C.D. James, J.F. Costello, G. Bergers, W.A. Weiss, A. Alvarez-Buylla, and J.G. Hodgson. 2008. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med*. 6:14.

Slager, R.E., T.L. Newton, C.N. Vlangos, B. Finucane, and S.H. Elsea. 2003. Mutations in RAI1 associated with Smith-Magenis syndrome. *Nat Genet*. 33:466-8.

Slegtenhorst-Eegdeman, K.E., D.G. de Rooij, M. Verhoef-Post, H.J. van de Kant, C.E. Bakker, B.A. Oostra, J.A. Grootegoed, and A.P. Themmen. 1998. Macroorchidism in FMR1 knockout mice is caused by increased Sertoli cell proliferation during testicular development. *Endocrinology*. 139:156-62.

Smalheiser, N.R., and G. Lugli. 2009. microRNA regulation of synaptic plasticity. *Neuromolecular Med*. 11:133-40.

Smrt, R.D., J. Eaves-Egenes, B.Z. Barkho, N.J. Santistevan, C. Zhao, J.B. Aimone, F.H. Gage, and X. Zhao. 2007. Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons. *Neurobiol Dis*. 27:77-89.

Smrt, R.D., K.E. Szulwach, R.L. Pfeiffer, X. Li, W. Guo, M. Pathania, Z.Q. Teng, Y. Luo, J. Peng, A. Bordey, P. Jin, and X. Zhao. 2010. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. *Stem Cells*. 28:1060-70.

Smrt, R.D., and X. Zhao. 2010. Epigenetic regulation of neuronal dendrite and dendritic spine development. *Frontiers in Biology*. 5:304–323.

Snow, W.M., K. Hartle, and T.L. Ivanco. 2008. Altered morphology of motor cortex neurons in the VPA rat model of autism. *Dev Psychobiol*. 50:633-9.

Song, H., G. Kempermann, L. Overstreet Wadiche, C. Zhao, A.F. Schinder, and J. Bischofberger. 2005. New neurons in the adult mammalian brain: synaptogenesis and functional integration. *J Neurosci*. 25:10366-8.

Soriano, P., T. Gridley, and R. Jaenisch. 1987. Retroviruses and insertional mutagenesis in mice: proviral integration at the Mov 34 locus leads to early embryonic death. *Genes Dev*. 1:366-75.

Spires, T.L., H.E. Grote, S. Garry, P.M. Cordery, A. Van Dellen, C. Blakemore, and A.J. Hannan. 2004. Dendritic spine pathology and deficits in experience-dependent dendritic plasticity in R6/1 Huntington's disease transgenic mice. *Eur J Neurosci*. 19:2799-807.

Stancheva, I., Collins,AL, Van den Veyver,IB, Zoghbi, H and Meehan, RR. 2003. A Mutant Form of MeCP2 Protein Associated with Human Rett Syndrome Cannot Be Displaced from Methylated DNA by Notch in Xenopus Embryos. *Molecular Cell*. 12:425-435.

Stark, K.L., B. Xu, A. Bagchi, W.S. Lai, H. Liu, R. Hsu, X. Wan, P. Pavlidis, A.A. Mills, M. Karayiorgou, and J.A. Gogos. 2008. Altered brain microRNA biogenesis contributes to phenotypic deficits in a 22q11-deletion mouse model. *Nat Genet*. 40:751-60.

Suetsugu, M., and P. Mehraein. 1980. Spine distribution along the apical dendrites of the pyramidal neurons in Down's syndrome. A quantitative Golgi study. *Acta Neuropathol*. 50:207-10.

Sutton, M.A., and E.M. Schuman. 2005. Local translational control in dendrites and its role in long-term synaptic plasticity. *J Neurobiol*. 64:116-31.

Suzuki, T., Q.B. Tian, J. Kuromitsu, T. Kawai, and S. Endo. 2007. Characterization of mRNA species that are associated with postsynaptic density fraction by gene chip microarray analysis. *Neurosci Res*. 57:61-85.

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*. 189:127-41.

Tafoya, L.C., M. Mameli, T. Miyashita, J.F. Guzowski, C.F. Valenzuela, and M.C. Wilson. 2006. Expression and function of SNAP-25 as a universal SNARE component in GABAergic neurons. *J Neurosci*. 26:7826-38.

Takai, D., and P.A. Jones. 2002. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A*. 99:3740-5.

Takashima, S., L.E. Becker, D.L. Armstrong, and F. Chan. 1981. Abnormal neuronal development in the visual cortex of the human fetus and infant with down's syndrome. A quantitative and qualitative Golgi study. *Brain Res*. 225:1-21.

Takashima, S., K. Iida, T. Mito, and M. Arima. 1994. Dendritic and histochemical development and ageing in patients with Down's syndrome. *J Intellect Disabil Res*. 38 (Pt 3):265-73.

Talebizadeh, Z., D.C. Bittel, O.J. Veatch, N. Kibiryeva, and M.G. Butler. 2005. Brief report: non-random X chromosome inactivation in females with autism. *J Autism Dev Disord*. 35:675-81.

Tao, J., K. Hu, Q. Chang, H. Wu, N.E. Sherman, K. Martinowich, R.J. Klose, C. Schanen, R. Jaenisch, W. Wang, and Y.E. Sun. 2009. Phosphorylation of MeCP2 at Serine 80 regulates its chromatin association and neurological function. *Proc Natl Acad Sci U S A*. 106:4882-7.

Thomas, N.S., A.J. Sharp, C.E. Browne, D. Skuse, C. Hardie, and N.R. Dennis. 1999. Xp deletions associated with autism in three females. *Hum Genet*. 104:43-8.

Tudor, M., S. Akbarian, R.Z. Chen, and R. Jaenisch. 2002. Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. *Proc Natl Acad Sci U S A*. 99:15536-41.

Tusher, V.G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 98:5116-21.

Van de Bor, V., and I. Davis. 2004. mRNA localisation gets more complex. *Curr Opin Cell Biol*. 16:300-7.

van Praag, H., A.F. Schinder, B.R. Christie, N. Toni, T.D. Palmer, and F.H. Gage. 2002. Functional neurogenesis in the adult hippocampus. *Nature*. 415:1030-4.

Vanderklish, P.W., and G.M. Edelman. 2005. Differential translation and fragile X syndrome. *Genes Brain Behav*. 4:360-84.

Vo, N., M.E. Klein, O. Varlamova, D.M. Keller, T. Yamamoto, R.H. Goodman, and S. Impey. 2005. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc Natl Acad Sci U S A*. 102:16426-31.

Volkmar, F.R., P. Szatmari, and S.S. Sparrow. 1993. Sex differences in pervasive developmental disorders. *J Autism Dev Disord*. 23:579-91.

Waites, C.L., A.M. Craig, and C.C. Garner. 2005. Mechanisms of vertebrate synaptogenesis. *Annu Rev Neurosci*. 28:251-74.

Walkley, S.U., and H.J. Baker. 1984. Sphingomyelin lipidosis in a cat: Golgi studies. *Acta Neuropathol*. 65:138-44.

Wan, L., T.C. Dockendorff, T.A. Jongens, and G. Dreyfuss. 2000. Characterization of dFMR1, a Drosophila melanogaster homolog of the fragile X mental retardation protein. *Mol Cell Biol*. 20:8536-47.

Wang, Y., R. Medvid, C. Melton, R. Jaenisch, and R. Blelloch. 2007. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet*. 39:380-5.

Washbourne, P., X.B. Liu, E.G. Jones, and A.K. McAllister. 2004. Cycling of NMDA receptors during trafficking in neurons before synapse formation. *J Neurosci*. 24:8253-64.

Washbourne, P., P.M. Thompson, M. Carta, E.T. Costa, J.R. Mathews, G. Lopez-Bendito, Z. Molnar, M.W. Becher, C.F. Valenzuela, L.D. Partridge, and M.C. Wilson. 2002. Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci*. 5:19-26.

Wayman, G.A., M. Davare, H. Ando, D. Fortin, O. Varlamova, H.Y. Cheng, D. Marks, K. Obrietan, T.R. Soderling, R.H. Goodman, and S. Impey. 2008. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. *Proc Natl Acad Sci U S A*. 105:9093-8.

Wayman, G.A., S. Impey, D. Marks, T. Saneyoshi, W.F. Grant, V. Derkach, and T.R. Soderling. 2006. Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron*. 50:897-909.

Webb, S.J., C.S. Monk, and C.A. Nelson. 2001. Mechanisms of postnatal neurobiological development: implications for human development. *Dev Neuropsychol*. 19:147-71.

Wisniewski, K.E., S.M. Segan, C.M. Miezejeski, E.A. Sersen, and R.D. Rudelli. 1991. The Fra(X) syndrome: neurological, electrophysiological, and neuropathological abnormalities. *Am J Med Genet*. 38:476-80.

Xu, X.L., Y. Li, F. Wang, and F.B. Gao. 2008. The steady-state level of the nervous-system-specific microRNA-124a is regulated by dFMR1 in Drosophila. *J Neurosci*. 28:11883-9.

Yamauchi, J., Y. Miyamoto, S. Kusakawa, T. Torii, R. Mizutani, A. Sanbe, H. Nakajima, N. Kiyokawa, and A. Tanoue. 2008. Neurofibromatosis 2 tumor suppressor, the gene

induced by valproic acid, mediates neurite outgrowth through interaction with paxillin. *Exp Cell Res*. 314:2279-88.

Yamauchi, J., Y. Miyamoto, T. Torii, R. Mizutani, K. Nakamura, A. Sanbe, H. Koide, S. Kusakawa, and A. Tanoue. 2009. Valproic acid-inducible Arl4D and cytohesin-2/ARNO, acting through the downstream Arf6, regulate neurite outgrowth in N1E-115 cells. *Exp Cell Res*. 315:2043-52.

Yang, L., R. Duan, D. Chen, J. Wang, and P. Jin. 2007. Fragile X mental retardation protein modulates the fate of germline stem cells in Drosophila. *Hum Mol Genet*. 16:1814-20.

Yoder, J.A., C.P. Walsh, and T.H. Bestor. 1997. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet*. 13:335-40.

Yoo, A.S., B.T. Staahl, L. Chen, and G.R. Crabtree. 2009. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. *Nature*. 460:642-6.

Yoon, K.J., B.K. Koo, S.K. Im, H.W. Jeong, J. Ghim, M.C. Kwon, J.S. Moon, T. Miyata, and Y.Y. Kong. 2008. Mind bomb 1-expressing intermediate progenitors generate notch signaling to maintain radial glial cells. *Neuron*. 58:519-31.

Young, J.I., and H.Y. Zoghbi. 2004. X-chromosome inactivation patterns are unbalanced and affect the phenotypic outcome in a mouse model of rett syndrome. *Am J Hum Genet*. 74:511-20.

Zhang, Y.Q., A.M. Bailey, H.J. Matthies, R.B. Renden, M.A. Smith, S.D. Speese, G.M. Rubin, and K. Broadie. 2001. Drosophila fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell*. 107:591-603.

Zhao, C., C. Aviles, R.A. Abel, C.R. Almli, P. McQuillen, and S.J. Pleasure. 2005. Hippocampal and visuospatial learning defects in mice with a deletion of frizzled 9, a gene in the Williams syndrome deletion interval. *Development*. 132:2917-27.

Zhao, C., W. Deng, and F.H. Gage. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell*. 132:645-60.

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*. 107:1876-81.

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*. 16:365-71.

Zhao, C., E.M. Teng, R.G. Summers, Jr., G.L. Ming, and F.H. Gage. 2006. Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J Neurosci*. 26:3-11.

Zhao, X., E.S. Lein, A. He, S.C. Smith, C. Aston, and F.H. Gage. 2001. Transcriptional profiling reveals strict boundaries between hippocampal subregions. *J Comp Neurol*. 441:187-96.

Zhao, X., T. Ueba, B.R. Christie, B. Barkho, M.J. McConnell, K. Nakashima, E.S. Lein, B.D. Eadie, A.R. Willhoite, A.R. Muotri, R.G. Summers, J. Chun, K.F. Lee, and F.H. Gage. 2003. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. *Proc Natl Acad Sci U S A*. 100:6777-82.

Zhou, Z., E.J. Hong, S. Cohen, W.N. Zhao, H.Y. Ho, L. Schmidt, W.G. Chen, Y. Lin, E. Savner, E.C. Griffith, L. Hu, J.A. Steen, C.J. Weitz, and M.E. Greenberg. 2006. Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron*. 52:255-69.

Ziv, N.E., and C.C. Garner. 2004. Cellular and molecular mechanisms of presynaptic assembly. *Nat Rev Neurosci*. 5:385-99.
Zoghbi, H.Y. 2003. Postnatal neurodevelopmental disorders: meeting at the synapse? *Science (New York, N.Y*. 302:826-30.