Identification of microRNA-132/212 function *in vivo*

By

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CERTIFICATE OF APPROVAL

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This thesis is dedicated to my grandfathers, George Krahn and Stanley R. Magill, whose lives exemplified the hard work, passion, and character it takes to triumph through the challenges that life presents and whose memories motivated me when graduate school was difficult

And written while my mind was filled with thoughts of my son

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LIST OF ABBREVIATIONS

AcGFP	*Aequorea coerulscens* GFP
Ach	acetylcholine
AchE	acetylcholinesterase
Ago	argonaute
AIDA	(*RS*)-1-Aminoindan-1,5-dicarboxylic acid
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
ANOVA	analysis of variance
APT	acyl-protein thioesterase
APV	(2*R*)-amino-5-phosphonopentanoate
ATP	adenosine triphosphate
BAF	barrier to autointegration factor
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
BS	brainstem
CAF	chromatin assembly factor
CaMK	Ca^{2+}/calmodulin-dependent kinase
cAMP	5’:3’-cyclic adenosine monophosphate
CB	cerebellum
CBP	CREB binding protein
CCR4	C-C chemokine receptor type 4
cDNA	complementary DNA
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPEB	cytoplasmic polyadenylation element binding protein

CPP	(6)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (drug)
CPP	conditioned place preference (method)
Cre	Cre recombinase
CRE	cAMP response element
CREB	cAMP response element binding protein
CS	conditioned stimulus
CtBP	c-terminal binding protein
d	days
Dcx	doublecortin
DIV	days *in vitro*
DNA	deoxyribonucleic acid
DPI	days post-injection
DGCR8	DiGeorge syndrome critical region gene 8
dsRNA	double-stranded RNA
E	embryonic day
eGFP	enhanced GFP
eIF	eukaryotic initiation factor
ERK	extracellular regulated MAPK
F Ctx	frontal cortex
F(-neo)	floxed miR-212/132 mouse without the PGK-neo cassette
Flox	floxed miR-212/132 mouse with the PGK-neo cassette
Flox-Ctrl	flox mouse infected with a control virus
Flox-Cre	flox mouse infected with a Cre-expressing virus
FLPe	flippase

FMRP	fragile-X mental retardation protein
FRT	flippase recognition target
G/R	green/red
GABA	gamma aminobutyric
GAP	GTPase activating protein
GEF	GTPase exchange factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumor
GKO	miR-212/132 germline knockout mouse
GnRH	gonadotropin releasing hormone
GW182	trinucleotide repeat containing 6A
Hb-EGF	heparin-binding epidermal growth factor
hCG	human chorionic gonadotropin
HCMV	human cytomegalovirus
HDAC	histone deacetylase
Hip	hippocampus
HUVEC	human umbilical vein endothelial cell
Hox	homeobox
HSD	honestly significant difference
HSV	herpes simplex virus
IFN	interferon
KO	knockout
KPBS	potassium PBS

KPBS-T	KPBS-tween20
KSHV	Kaposi's sarcoma-associated herpes virus
KSRP	KH-type splicing regulatory protein
LEC	lymphatic endothelial cell
LH	luteinizing hormone
LNA	locked nucleic acid
LPS	lipopolysaccharide
LTP	long term potentiation
M/P Ctx	medial/parietal cortex
MAM	methylazoxymethanol
MAPK	mitogen activated protein kinase
MB	midbrain
MeCP	methyl CPG-binding protein
MEF	myocyte enhancer factor
mEPSC	minature excitatory post-synaptic current
miRNA	microRNA
miR	microRNA
MMP	matrix metalloproteinase
MOV10	moloney leukemia virus 10
MRE	microRNA response element
mRNA	messenger RNA
MSK	ribosomal protein S6 kinase
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
Neo	neomycin

NeuN	neuronal nuclei (specific marker)
NMDA	N-Methyl-D-aspartic acid
OB	olfactory bulb
Occ Ctx	occipital cortex
PABP	polyA binding protein
PACT	PRKRA protein kinase, interferon-inducible dsRNA-dependent activator
Pax	paired box
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PFA	paraformaldehyde
PGK-neo	phosphoglycerate kinase promoter driving neomycin resistance gene
PKA	protein kinase a, also known as cAMP-dependent protein kinase
PolII	RNA polymerase II
polyA	polyadenylation
POMC	proopiomelanocortin
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTBP	Polypyrimidine tract binding protein
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
REST	RE1-silencing transcription factor
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

RNAi	RNA interference
rtTA	reverse tetracycline-controlled transactivator
SACO	serial analysis of chromatin occupancy
SCC	squamous cell carcinoma
SCN	suprachiasmatic nucleus
SD	standard deviation
SEM	standard error of the mean
shRNA	short-hairpin RNA
Str	striatum
TGF	transforming growth factor
Thal	thalamus
TLX	nuclear receptor subfamily 2, group E, member 1
TORC	transducer of regulated CREB-binding protein
TRBP	TAR RNA-binding protein
TRE	tetracycline response element
TU	thiouracil
um	microns
UPRT	*T. Gondi* uracil phosphoribosyltransferase
UTR	untranslated region
WT	wild type
WT-Ctrl	WT mouse injected with mCherry control virus
WT-Cre	WT mouse injected with mCherry and GFP-Cre viruses

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Finally, and most importantly, I am incredibly grateful for the love, kindness, and sounding board given to me by my best friend, my wife, and the love of my life, Pamela. You make life worth living. Thanks to my family and friends for their unwavering support. I especially want to thank my mother, who provided my educational foundation, and Mr. Baumer, who believed in me when I really needed it. Let's celebrate!

Preface

I have prepared this dissertation in accordance with the guidelines set forth by the Neuroscience Graduate Program in the School of Medicine, Oregon Health and Science University. My thesis is comprised of a general introduction, two chapters of original data, and a discussion of my findings. Chapter 2 is the manuscript of a paper that was originally published in *PNAS*. Chapter 3 contains follow up experiments that arose from our findings in Chapter 2, as well as experiments to behaviorally characterize the germline miR-212/132 knockout mouse. Chapter 4 is a summary and discussion of the results. The references are formatted in the *Cell Journal* style, so one can easily identify the papers to which I am referring without having to search through pages of references.

All the experiments and data analysis presented in this thesis was performed by the author except for the following: *Daniel Lioy*, *Richard Goodman*, and *Gail Mandel* designed the floxed allele. *Lulu Cambronne*, with assistance from *Barbara Leighton,* designed, validated and performed all the experiments with the sensors, except for the *in vivo* injections and imaging in Chapter 2. *Barbara Leighton* assisted with mouse husbandry in the late stages of this project and some of the BrdU quantification. *Rukayat Taiwo* assisted with some of the stereotactic injections, immunohistochemistry, and image analysis in Chapter 2. *Bryan Luikart* designed and cloned the mCherry retrovirus construct and assisted with the seizure experiment. *Eric Washburn*, of the Vollum Viral Core, packaged all the retroviruses used in this dissertation. *James Stafford* in Matt Lattal's lab performed the fear conditioning and extinction experiments in the germline miR-212/132 knockout mice. *David Dietz* in Eric Nestler's lab performed the cocaine-induced conditioned place preference experiments.

Abstract

For any species to survive, it must be able to respond to its environment, responses that are mediated by changes in the output of neural networks. Plasticity within neural networks, which is thought to underlie memory formation, arises from changes in the way individual neurons respond to the input they receive. The cyclic AMP response element binding protein (CREB) is a transcription factor that activates genes in response to neuronal activity and is necessary for long term potentiation (LTP). CREB activation increases the expression of several microRNAs. microRNAs are small RNA molecules that post-transcriptionally regulate gene expression. This dissertation is focused on identifying the *in vivo* function of one CREB-regulated microRNA locus, the miR-212/132 locus, which has been implicated in neural plasticity *in vitro*.

Newborn neurons in the dentate gyrus of the adult hippocampus rely upon CREB signaling for their differentiation into mature granule cells and their integration into the dentate network. CREB activation increases the expression of miR-132 and miR-212. In cultured cortical and hippocampal neurons, miR-132 functions downstream from CREB to mediate activity-dependent dendrite growth and spine formation in response to a variety of signaling pathways. To investigate whether miR-132 and/or miR-212 contribute to the maturation of dendrites in newborn neurons in the adult hippocampus, we inserted LoxP sites surrounding the miR-212/132 locus and specifically targeted its deletion by stereotactically injecting a retrovirus expressing Cre recombinase. Deletion of the miR-212/132 locus caused a dramatic decrease in dendrite length, arborization, and spine density. The miR-212/132 locus may express up to four distinct microRNAs, miR-132 and -212, and their reverse strands, miR-132* and -212*. Using ratiometric microRNA sensors, we determined that miR-132 is the predominantly active product in hippocampal neurons. We conclude that miR-132 is required for normal dendrite maturation

in newborn neurons in the adult hippocampus and suggest that this microRNA also may participate in other examples of CREB-mediated signaling.

Selective loss of miR-212/132 decreased dendrite outgrowth and spine formation in newborn neurons. However, the expression of miR-212/132 in other brain regions and the impact of miR-212/132 germline knockout (GKO) on adult neurogenesis and behavior remain unknown. We report that miR-132 is expressed in a rostral to caudal pattern in the brain of wild type mice, and that the absolute level of miR-132 is higher than miR-212. Additionally, the loxP sites and neomycin cassette in floxed miR-212/132 mice attenuated miR-212/132 expression, but the neomycin cassette, not the reduced miRNA expression, reduced dendrite outgrowth in floxed mice. GKO mice have no deficit in dendrite outgrowth of newborn hippocampal neurons, including following seizures. Although GKO mice have normal proliferation within the dentate gyrus, the survival of newborn neurons is decreased compared to wild-type mice. Behaviorally, the GKO mice perform equivalent to wild-type mice in the open field test, the novel object test, a spatial learning task, context and cue-dependent fear conditioning, fear memory retrieval, and fear memory extinction paradigms. However, injection of HSV-GFP-Cre into the nucleus accumbens of floxed miR-212/132 mice increased cocaine-induced conditioned place preference, suggesting the miR-212/132 contributes in the rewarding properties of cocaine. Finally, I present *in vitro* evidence that Sprouty1 is a direct miR-132 target.

Together, these studies demonstrate that miR-132 promotes dendrite outgrowth, spine formation, and survival of newborn neurons *in vivo* and that products of the miR-212/132 locus contribute to the rewarding properties of cocaine.

CHAPTER 1

Introduction

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Chapter 1.1: A history of neuronal plasticity

For any species to survive, it must be able to respond and adapt to its environment. This adaptation occurs constantly and has effects that can be observed on different timescales, from immediate responses, like reflexively withdrawing your hand from a hot plate, to long-term responses, like remembering how to obtain food or avoid harmful situations. Behavioral responses that are necessary for survival are mediated by changes in the output of neural networks. In order to respond in a meaningful way to changing environments, these networks tune their output in response to sensory stimuli, a concept known as neural plasticity. Experiments on the macaque visual cortex by Hubel and Wiesel provide one of the most striking examples of neural plasticity. Using radiolabeled amino acids to trace the input from each eye into the visual cortex, they observed equal input from each eye, generating the classic black and white striped images of ocular dominance columns (Hubel et al. 1977). However, upon sensory deprivation of one eye, the inputs became unequal, and the majority of the visual cortex receives input from the non-deprived eye. Indeed, even simple organisms, like the sea slug *Aplysia californica*, have the ability to increase or decrease how they respond to sensory stimuli (Kandel 2001). The work described in this dissertation is focused on increasing our knowledge of the cellular and molecular mechanisms that mediate neuronal plasticity.

Plasticity within neural networks, which is hypothesized to underlie memory formation, ultimately arises from changes in the way individual neurons respond to the input they receive. The first observation of such a change was in granule cells of the dentate gyrus in rabbit hippocampus, a brain region that is required for memory formation (Bliss et al. 1973). Bliss and Lomo stimulated the perforant path, which contains input fibers from the entorhinal cortex to the dentate gyrus, at high frequency and recorded the amplitude and response latency in populations of granule neurons using extracellular electrodes. They found that the amplitude

increased, and the latency to respond decreased, compared to baseline responses following high frequency stimulation. Amazingly, this potentiation of the granule neuron response was maintained for up to ten hours following stimulation.

The implications of Bliss and Lomo's findings are profound. They suggest that the functional properties of a neuron can be altered for an extended period of time in response to input received over the course of only three to four seconds. These findings, along with subsequent similar studies, drove scientists to begin questioning what were the cellular and molecular mechanisms that mediate this phenomenon. There is a strong interest in identifying these mechanisms because neuronal potentiation, and its reverse, neuronal depression, are thought to be the cellular correlate of learning memory.

In the decade prior to the discovery of neuronal potentiation/plasticity, studies of memory formation suggested that both protein synthesis and transcription were required for long-term memory formation. It had been postulated that learning and memory were dependent on proteins [for an in depth review of the literature, see: (Davis et al. 1984)], and the first experimental evidence suggesting a role for protein synthesis was published in 1963 (Flexner et al. 1963). In this, and subsequent studies, pharmacological inhibition of protein synthesis was accomplished using antibiotics and their derivatives. In the original study, puromycin, an aminoglycoside antibiotic that functions by inhibiting translation, was injected bilaterally into the hippocampus of mice one day after they were trained on a Y-maze, where the mouse learned to avoid the arm in which they received a small electric shock. The mice were tested three days later, and it was found that mice that received puromycin did not retain the memory of the shock, in contrast to those that received saline injections. Further studies using other classes of protein synthesis inhibitors such as cycloheximide and anisomycin

confirmed the essential role of protein synthesis in long-term memory (Agranoff et al. 1966; Squire et al. 1974).

Around the same time that protein synthesis was discovered to be necessary for long-term memory, pharmacological approaches were also being used to investigate whether transcription was necessary for long-term memory. The idea that RNA synthesis, or gene transcription, could underlie learning and memory was formally put forward by Samuel Barondes in a lecture to the American Psychological Association in 1964, which was subsequently published in Nature (Barondes 1965). In 1967 Bernard Agranoff was the first person to show that RNA synthesis was necessary for memory formation. In his experiments, he trained goldfish to avoid a shock by swimming to a different location. Immediately after training, he intracranially injected 2 ug of Actinomycin-D, an inhibitor of RNA synthesis. The goldfish that received the inhibitor had impaired performance in the shock avoidance test four days later (Agranoff et al. 1967). Further experiments in goldfish using another, less toxic inhibitor of RNA synthesis confirmed the role of transcription in memory (Neale et al. 1973). While translating these results to mammals was prohibited by the toxicity of Actionmycin-D (Squire et al. 1970), use of a different inhibitor of RNA Polymerase II, α-amanitin, allowed confirmation that transcription was necessary for learning and memory of a passive avoidance task in mice (Thut et al. 1974).

Bliss and Lomo's observation that neurons undergo sustained physiological changes in response to activity occurred at the same time as the aforementioned studies demonstrating that protein synthesis and transcription were necessary for learning and memory. In the discussion of their work, Bliss and Lomo postulated that the neurophysiological changes they observed in response to activity could be the cellular correlates of learning and memory (Bliss et al. 1973). Combining this idea with the studies showing that protein synthesis and transcription

were necessary for learning and memory raised the question of how neuronal activity could drive changes in gene transcription. There must be many signals that a stimulated by neuronal activity and could subsequently drive transcription, but what was it?

One potential candidate was 5':3'-cyclic adenosine monophosphate (cAMP), discovered nearly a decade earlier by Earl Sutherland (Sutherland et al. 1958; Rall et al. 1958). cAMP was known to be stimulated by epinephrine, which could initiate glycogen breakdown (Posner et al. 1962; Rall et al. 1958). Work in Edward Kreb's lab studying glycogen metabolism demonstrated that cAMP was necessary to activate phosphorylase, the enzyme that mediates glycogen breakdown (Krebs et al. 1959). This cAMP-dependent activation was mediated by activation of phosphorylase kinase (Posner et al. 1965), however, efforts to purify phosphorylase kinase caused the kinase to become inactive. By adding back individual fractions from the purification, the activity of phosphorylase kinase could be restored, suggesting that the fraction contained a cAMP-dependent kinase (Soderling et al. 1970). Purification of the factor identified a cAMP-dependent protein kinase that was the same kinase that had been discovered to be responsible for casein and protamine phosphorylation and subsequently came to be known as protein kinase A (PKA) (Walsh et al. 1968). Thus, cAMP was a factor that could integrate external signals and drive changes in cellular function via activation of PKA, however, the relationship between PKA and gene transcription remained unknown for some time.

The association of the cAMP pathway with gene transcription was first suggested by studies investigating the effects of estrogen on the rat uterus. Oscar Hechter made the observation that cAMP, like estrogen, could stimulate RNA synthesis and protein production in cultured rat uterine horns; furthermore, he demonstrated that this effect could be blocked by treatment with Actinomycin D, suggesting that cAMP was stimulating transcription (Hechter et al. 1967). Studies investigating the regulation of phosphoenolpyruvate carboxykinase (PEPCK),

the rate-limiting enzyme in gluconeogenesis, demonstrated that cAMP activated the enzyme in a manner that was also dependent on Actinomycin D, which identified the first individual gene whose transcription was stimulated by cAMP (Yeung et al. 1968). However, it would be more than a decade before clues revealing how stimulation of the cAMP/PKA pathway activated gene expression would emerge.

Richard Hanson's lab, studying PEPCK, and Richard Goodman's lab, studying the hormone somatostatin, were both utilizing improvements in molecular techniques to study cAMP-mediated gene regulation. They found that PEPCK and somatostatin mRNA levels increased following injection of cAMP or treatment with drugs that activate cAMP signaling (Iynedjian et al. 1977; Montminy et al. 1986a; Lamers et al. 1982). By cloning promoter fragments of the PEPCK and somatostatin promoter, they, along with Howard Goodman's lab studying the enkephalin promoter, isolated regions of the promoter that mediated cAMP-dependent transcription (Short et al. 1986; Montminy et al. 1986b; Comb et al. 1986). By comparing promoter sequences of other known cAMP-regulated genes, Marc Montminy, working in the Goodman lab, discovered the cAMP response element (CRE), an eight nucleotide palindrome, 5'-TGACGTCA-3'. Once the CRE was identified, the race to identify the proteins that linked cAMP to gene activation via the CRE was on. Using DNase footprinting assays and sequence specific DNA affinity chromatography, the Montminy lab purified CREB protein and demonstrated that it was phosphorylated by PKA (Montminy et al. 1987). By screening placental cDNA libraries with radiolabeled CRE oligonucleotides, Jim Hoeffler cloned the cDNA for CREB and characterized its primary features, including the DNA-binding leucine zipper and the N-terminal transactivational domain (Hoeffler et al. 1988). Further studies demonstrated that CREB was activated by phosphorylation from multiple kinases and that its activation by PKA was mediated via phosphorylation at Ser-133 (Gonzalez et al. 1989; Gonzalez and Montminy

1989). These studies defined a signaling cascade linking increases in cAMP to gene activation via PKA-mediated CREB phosphorylation.

As it became clear that the cAMP signaling through CREB could alter gene expression, the question arose as to whether this same pathway could mediate cellular changes in response to neuronal activity, as had been functionally observed by Bliss and Lomo. At the time, it was known that neurotransmitters could drive changes in mRNA levels, as exemplified by dopamine's repression of pro-opiomelanocortin expression (Chen et al. 1983). In addition, treatment of PC12 cells with nicotine, which stimulates acetylcholine receptors, could induce expression of c-*fos* in a manner dependent on Ca^{2+} influx (Greenberg et al. 1986). Further studies in the Greenberg lab demonstrated that membrane depolarization and calcium influx could activate CREB-dependent gene transcription through Ca^{2+}-calmodulin dependent kinases (Sheng et al. 1990; 1991). In addition to neurotransmitter administration and membrane depolarization, neurotrophins, such as BDNF, can activate CREB (Finkbeiner et al. 1997). CREB activation can occur through CaMK pathways as well as Ras-dependent pathways. These findings are important because they suggest that activation of CREB-dependent gene transcription is a point of functional convergence between multiple signaling pathways mediating responses to neuronal activity and neuromodulators [for review see: (Shaywitz et al. 1999)].

Studies of neuronal potentiation in *Aplysia* demonstrated that the CREB-mediated detection of cellular signals through multiple pathways was necessary for long-term memory formation (Kandel 2001). In addition, CREB is activated in response to physiological neuronal activity *in vivo* (Ginty et al. 1993) and is essential for long-term memory in multiple animal models (Josselyn et al. 2005). While the mechanisms of these pathways, and their relevance in mammalian systems, have been more thoroughly elucidated through the years, the idea that

CREB-dependent transcription is crucial for learning and memory has held true. Indeed, in humans, mutations in CREB Binding Protein (CBP), a co-activator necessary for CREB-dependent gene transcription (Chrivia et al. 1993), cause Rubinstein-Taybi Syndrome, which is characterized by mental retardation and facial and limb abnormalities (Petrij et al. 1995). The story, however, doesn't end with CREB. In order to understand the cellular mechanisms that underlie neuronal adaptation, one must identify and determine the function of the genes that are activated by CREB, the targets that actually facilitate long-lasting neuronal plasticity (Carlezon et al. 2005).

To address this question, the Goodman lab developed a technique, termed Serial Analysis of Chromatin Occupancy (SACO), to identify all the genes regulated by CREB (Impey et al. 2004). SACO was a powerful technique because it was the first sequencing-based approach to identify targets of a transcription factor in mammalian cells and did not rely on hybridization, like previous experiments using microarrays. Furthermore, a sequencing-based approach allows genome-wide assessment of CREB binding, and is not limited by the number of probes on an array. The results of the screen demonstrated that CREB regulated thousands of transcripts, among which were a group of microRNAs, small RNA molecules that were recently discovered, one of which, is the focus of this thesis. Our knowledge of the cellular and molecular mechanisms underlying neuronal plasticity has grown exponentially since the initial extracellular recordings of neuronal potentiation in the dentate by Bliss and Lomo, however, we still have a long way to go to understand how neurons change in response to activity and the functional implications of those changes. The work presented in this dissertation is focused on understanding how the gene products of one CREB-regulated microRNA locus, encoding miR-132 and miR-212, function *in vivo*, and adds another chapter to our understanding of how neurons change in response to activity.

Chapter 1.2: microRNAs

The central dogma of molecular biology stated that information flows from DNA to RNA to protein (Crick 1970). In the classical model, a gene is encoded by DNA in the nucleus and is transcribed by RNA polymerase into mRNA. Ribosomes bind to the mRNA and translate it into protein. The protein then performs its function within the cell, such as sensing external signals, catalyzing chemical reactions, or providing structural support. However, the molecular revolution of the last fifty years has dramatically enhanced our understanding of, and appreciation for, the complex regulation that occurs during this information transfer from the DNA blueprint to the functional cellular machinery, which includes both RNA and proteins. Regulation occurs at seemingly every level of this process. Proteins undergo numerous modifications where enzymes attach or remove molecules that can control their function or stability. These modifications include things such as phosphorylation, ubiquitination, acetylation, methylation, glycosylation, sumolyation and palmitolation, among others. DNA can also be modified directly by methylation and indirectly by epigenetic changes that alter the accessibility of DNA to the transcriptional and regulatory machinery. The focus of this thesis, however, is on miRNAs, which regulate mRNA.

mRNA transcripts can be regulated by multiple processes. The first chance for regulation occurs during transcription by RNA Polymerase II when the primary mRNA transcript undergoes 5' capping. Following capping, the RNA is spliced together, removing the introns. This is a highly regulated process that is often used to provide tissue specificity, with different splice isoforms being expressed in different tissues. Once PolII arrives at the polyA signal, the transcript is cleaved and released from the transcription complex. The polyA tail is then constructed by polyA polymerase and the transcript is actively transported out of the nucleus.

Following the regulation that takes place during capping, splicing, polyadenylation and export of the transcript from the nucleus, the mRNA is then ready to be transported to the ribosome where it can be translated into protein. At this point one of three primary outcomes occurs: 1) the transcript is translated into protein; 2) the transcript is degraded; or 3) the transcript remains stable, but translation is inhibited. Regulatory factors are known to play a key role in all of these processes. For translation to occur, the initiation complex must be activated, beginning with phosphorylation of eIF-2 and recruitment of the initiator tRNA to the 5' cap of the transcript (Traugh et al. 1976). Other proteins, such as CPEB, can bind to the mRNA transcripts in the cytosol and promote their translation by inducing polyadenylation (Lin Wu et al. 1998). Alternatively, deadenylating proteins, such as CCR4, can remove the polyA tail and promote transcript degradation (Tucker et al. 2001). Finally, proteins can inhibit translation, as occurs when cytosolic aconitase binds to the ferritin mRNA (Klausner et al. 1993; Rouault et al. 1990). However, towards the end of the last century, a previously unrecognized class of molecules that function to regulate mRNA transcripts was discovered, and turned out to be an essential component of many cellular processes. These small RNA molecules are miRNAs.

Chapter 1.2.1: microRNA biogenesis and mechanism of action

microRNAs are short 20-25 nucleotide (nt) RNA molecules that do not code for proteins, but rather modulate the expression and stability of mRNA transcripts. The first miRNA was identified as a gene responsible for developmental mutations in the nematode, *C. elegans* (Wightman et al. 1993; Lee et al. 1993). The Ambros and Ruvkun labs discovered that lin-4 produced two short transcripts, one 22 and the other 61 nucleotides, that were complementary to sequences in the 3'UTR of lin-14, a gene that must be down-regulated in *C. elegans* development. They argued that the lin-4 gene product, the first miRNA, functioned by silencing

the lin-14 mRNA through an RNA-RNA interaction. This initial observation, however, was thought to be specific to nematodes and not a phenomenon that occurred in higher organisms. That remained the status quo for seven years until the characterization of the second miRNA, let-7 (Reinhart et al. 2000). Again using developmental studies in *C. elegans*, loss of let-7 was shown to inhibit developmental progression, while overexpression of let-7 caused the nematode larva to progress more rapidly towards adulthood. Additionally, by analyzing interactions between let-7 mutants and five other mutants, it was hypothesized that miRNAs may function by regulating multiple target genes. Phylogenetic comparison of let-7 expression across multiple species revealed that let-7 was conserved from *C. elegans* all the way to *Homo sapiens*, firmly establishing the presence of miRNAs in higher vertebrates (Pasquinelli et al. 2000).

The intriguing studies of lin-4 and let-7 in *C. elegans*, and their extension to vertebrates, raised several important questions for the field, including how many miRNAs are present in the genome, how are miRNAs generated, and how do they silence their targets? Several labs began large-scale efforts to clone small RNAs by first purifying low-molecular weight RNA, then ligating adapters, amplifying and then sequencing the library (Lagos-Quintana et al. 2002; 2001; Lau et al. 2001; Lee et al. 2001). The development of high-throughput sequencing technologies has facilitated deep sequencing of small RNAs and led to a dramatic increase in the number of cloned miRNAs, now numbering more than 17,000 (Kozomara et al. 2010).

In addition to identifying miRNAs, determining how miRNAs are formed has been a foundational question within the field, one that benefited greatly from work already underway to establish the mechanisms that mediate RNA interference (RNAi). RNAi is the process by which delivery of double-stranded RNA (dsRNA) into a cell causes silencing of the transcripts that contain a directly complementary sequence (Fire et al. 1998). Whereas dsRNA used for RNAi experiments is typically transfected, injected into the cell, or delivered as a short-hairpin

RNA (shRNA) in a virus, miRNAs are encoded in the genome. Primary miRNAs (pri-miRNA) are found on PolII-generated, capped and polyadenylated transcripts that contain stem-loop structures (Cai et al. 2004; Lee et al. 2004). These stem-loop structures are typically 60-80 nt long and can be found in multiple locations within the transcript. They can be located by themselves or in clusters on non-protein coding transcripts and can also be found within introns, exons and the 3'UTR of protein coding transcripts (Rodriguez et al. 2004). While still in the nucleus, the stem-loop structure of the pri-miRNA is cleaved to form the precursor miRNA (pre-miRNA) by Drosha in a process that requires DGCR8 (Denli et al. 2004; Landthaler et al. 2004; Gregory et al. 2004; Han et al. 2004). The pre-miRNA is exported from the nucleus by Exportin 5 (Yi et al. 2003; Lund et al. 2004; Bohnsack et al. 2004). Once in the cytosol the pre-miRNA is processed in parallel with siRNAs (exogenous dsRNA) introduced in RNAi experiments. The pre-miRNA is cleaved into a 22 nt, double-strand mature RNA by the RNase III domain containing protein, Dicer (Knight et al. 2001; Ketting et al. 2001; Grishok et al. 2001; Hutvágner et al. 2001; Bernstein et al. 2001). The double-stranded mature miRNA associates in an ATP-dependent manner with a miRNA-ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) (Hammond et al. 2000; Hutvágner et al. 2002; Khvorova et al. 2003; Schwarz et al. 2003; Yoda et al. 2010). Argonaute (Ago) proteins, which in humans are expressed as four isoforms, form the core components of the RISC (Peters et al. 2007; Kawamata et al. 2010; Hammond et al. 2001). Ago proteins directly bind to the miRNA and its target mRNA to mediate silencing. In addition, it has been shown that Dicer, TRBP and PACT can facilitate loading of the miRNA into the RISC (Lee et al. 2006; Kok et al. 2007). Whether the guide strand of the double-stranded mature miRNA or its complementary strand, the star strand, are incorporated into RISC, is thought to be determined by the thermodynamic stability of the base pairing at the 5' end

(Khvorova et al. 2003; Schwarz et al. 2003), although both can be loaded into the RISC and mediate biological functions (Packer et al. 2008a).

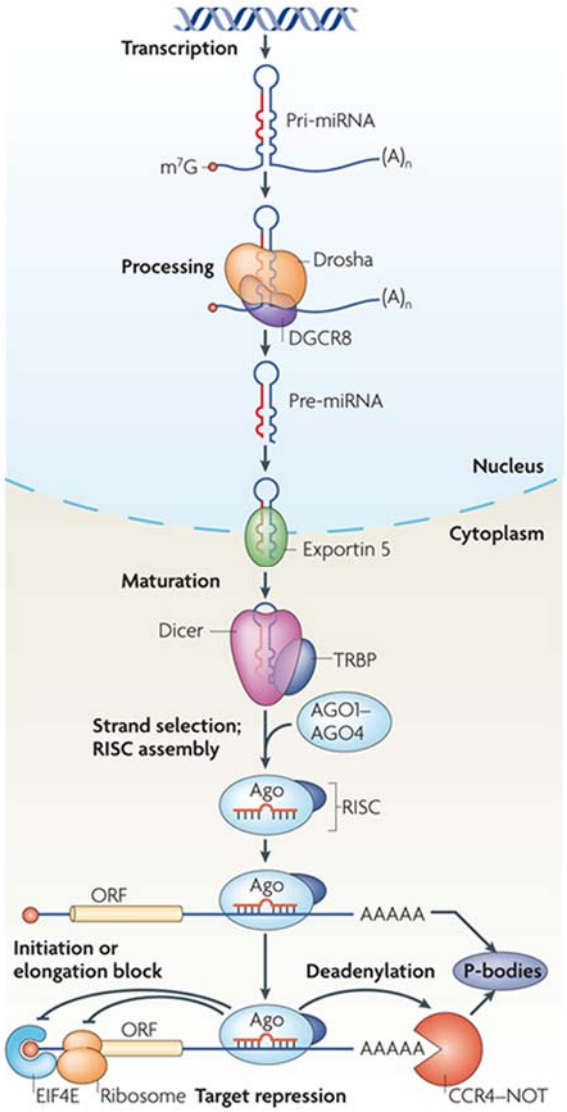

Figure 1.1: Schematic of miRNA biogenesis and function. miRNAs transcribed in the nucleus as primary miRNAs (Pri-miRNA). Pri-miRNAs are processed by Drosha into precursor miRNAs (Pre-miRNA). The Pre-miRNA is exported from the nucleus by Exportin5 and cleaved by Dicer into the mature 20-22 nt double-stranded miRNA. The mature miRNA is unwound, and the functional strand, which can be either the sense or star strand, is incorporated into the RISC with its target mRNA. The miRNA-RISC silences target mRNA by translational inhibition or mRNA degradation. Figure from (Inui et al. 2010).

Once the mature miRNA has been incorporated into the RISC, the Ago-miRNA complex binds to the target mRNA. In plants, miRNAs bind to exactly complementary sequences on their target mRNAs (Hamilton et al. 1999; Rhoades et al. 2002), however, in mammals, the situation is considerably more complex due to the non-perfect complementarity of most target sites (Lewis et al. 2003). Target sites are classically found in the 3'UTR, however, functional sites in both the 5'UTR and coding sequence have been identified (Lytle et al. 2007; Duursma et al. 2008; Forman et al. 2008; Tay et al. 2008). Mammalian miRNA binding sites are typically found in the 5' region of the 3' UTR and have a high degree of Watson-Crick base pairing at nucleotides 2-8 from the 5' end, termed the 'seed' sequence of the miRNA (Lewis et al. 2005). In addition, there is often base pairing found between nucleotides 13-17, however, mismatches can occur throughout the binding site (Brennecke et al. 2005). Given the importance of base pairing in miRNA target recognition, a number of bioinformatic approaches to identifying targets have been developed (Lewis et al. 2005; Krek et al. 2005) [for review, see: (Watanabe et al. 2007)]. Unfortunately, these sequence- and thermodynamically-based predictions are often quite variable between algorithms and have little overlap in the targets they predict for a particular miRNA (Vo et al. 2010b). These observations have highlighted the importance of using biochemical methods to identify miRNA targets, which should yield new insights into what additional factors determine the functionality of a putative miRNA binding site.

Once the miRNA:RISC complex binds its target site, it silences translation from the mRNA transcript through two primary mechanisms, translational inhibition or mRNA degradation. Translational inhibition was the first mechanism postulated for miRNA function due to the observation that following lin-4 expression, lin-14 protein levels decreased without a concurrent decrease in mRNA levels (Wightman et al. 1993). However, more recent studies have suggested that, in addition to translational inhibition, lin-14 mRNA is degraded as well

(Bagga et al. 2005). Regardless, numerous studies have shown that protein levels can be decreased by miRNAs without changing mRNA levels (Seggerson et al. 2002) [for review: (Huntzinger et al. 2011)]. Early investigations into the mechanism of translational silencing suggested that the inhibition happened after translation initiation, in part because the target mRNAs were found in polysome fractions (Olsen et al. 1999). However, additional studies have shown that translation can be inhibited prior to mRNA association with polysomes, suggesting that inhibition can occur prior to initiation (Humphreys et al. 2005; Pillai et al. 2005). Mechanistically, inhibition of translational initiation is thought to be caused by interactions between the Ago protein directly, or its associated protein, GW182 (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005), and the m^7G 5' cap (Kiriakidou et al. 2007); this association is thought to compete with eIF4E binding, thus preventing initiation.

In addition to translational inhibition, degradation of the mRNA transcripts was hypothesized to be an additional mechanism miRNAs could use to silence translation. This idea took hold following microarray studies that showed transcripts containing putative miRNA binding sites were decreased following overexpression of the miRNA (Lim et al. 2005). It has now been shown that GW182 can recruit poly-A binding protein (PABP) and CAF1, the decapping proteins DCP1 and DCP2 as well as the deadenylase protein complex consisting of CCR4 and Not1 (Fabian et al. 2009; Behm-Ansmant et al. 2006). Decapping and removal of the poly-A tail cause destabilization and degradation of the mRNA transcript (Wu et al. 2006; Giraldez et al. 2006). It remains unknown what determines whether a miRNA causes translation inhibition or mRNA decay, although it has recently been hypothesized that there is a continuum between these mechanisms. The continuum begins with a miRNA initially inhibiting translation by inhibiting ribosome association with the mRNA and competing off eIF4E, thus blocking new initiation (Djuranovic et al. 2011). This initial inhibition could then progress to mRNA

degradation following recruitment of decapping and deadenylating proteins, however, future experiments will be needed to determine whether miRNAs function through such a continuum.

miRNAs have been shown to be essential for a diverse array of biological functions, which often involve defining particular cell states or mediating transitions from one state to another. Since their discovery in *C. elegans,* where they were shown to be necessary for larval development, miRNAs have been shown to play important roles in other aspects of biology including differentiation, proliferation, cancer formation, immune function, stem cell maintenance, and neurodegenerative processes, among others (Eacker et al. 2009; Tiscornia et al. 2010; Metzler et al. 2004; Esquela-Kerscher et al. 2006; Chen et al. 2004a; Davidson-Moncada et al. 2010). Because of their ability to target many transcripts, a single miRNA can facilitate a biological function by acting at multiple levels in a signaling cascade, as exemplified by miR-26a, which targets Cyclin D2 and Cyclin E2 (Kota et al. 2009). Cyclin D2 activates E2F, which activates Cyclin E2 to drive the cell cycle forward from G1 to S phase (Vermeulen et al. 2003). Thus, by targeting both Cyclins, miR-26a can inhibit the signaling cascade at multiple levels, and prevent cell cycle progression. In addition to targeting a signaling pathway at multiple levels, an individual miRNA can target genes in multiple pathways that must be down-regulated to facilitate a developmental transition, as exemplified by miR-1, which is up-regulated during the differentiation of a muscle progenitor cell to fully differentiated skeletal muscle. miR-1 mediates this transition by silencing both HDAC4 and Pax7 (Chen et al. 2010; 2006). Thus, miRNAs can use multiple strategies to accomplish their biological functions.

Chapter 1.2.2: microRNAs in the developing nervous system

Within the nervous system, miRNAs have been shown to be essential for development and have been associated with numerous disease states. There are hundreds of miRNAs expressed in the

brain (Kim et al. 2004; Sempere et al. 2004; Miska et al. 2004; Krichevsky et al. 2003; Lagos-Quintana et al. 2002), and knockout of zebrafish Dicer, a protein essential for cleavage of the pre-miRNA into the mature miRNA, causes defects in the nervous system. These defects include disorganization during neural tube formation, abnormalities at the mid-brain/hind-brain border, and impaired retinal development (Giraldez et al. 2005). In mice, Dicer knockout is embryonic lethal, prior to neurulation (Bernstein et al. 2003). However, embryonic lethality can be circumvented by conditionally knocking out Dicer in the mouse brain at later timepoints [for review see: (Fineberg et al. 2009)]. In the developing mouse telencephalon, miRNAs are necessary to promote differentiation of neuronal progenitors, but not for their initial proliferation (De Pietri Tonelli et al. 2008). In the cerebellum, loss of Dicer causes Purkinje cell death and cerebellar degeneration leading to ataxia (Schaefer et al. 2007). In the striatum, Dicer knockout in neurons expressing dopamine receptors (DR-1) causes a decrease in brain and neuron size as well as ataxia and fore/hind-limb clasping (Cuellar et al. 2008). In CamKII-expressing neurons throughout the cortex and hippocampus, Dicer knockout decreases dendritic branch and spine length; in addition, it causes a reduction in brain size due to increased apoptosis (Davis et al. 2008). Knockout of Dicer in astrocytes using an mGFAP-Cre driver that primarily targets the cerebellum caused non-cell autonomous neuronal dysfunction leading to ataxia, epilepsy and early death (Tao et al. 2011). While all these studies are limited due to Dicer's ability to perform other functions, such as chromatin remodeling (Volpe et al. 2002), taken together, they suggest that miRNAs are necessary for brain development, neuronal survival, and normal brain functioning.

The first role identified for a specific miRNA in neurons suggested that miRNAs play a role in defining neuronal identity. Again using *C. Elegans* as a model, the miRNA lsy-6 was identified and shown to be essential for establishing left-right asymmetry of chemosensory

neurons (Johnston et al. 2003). Consistent with a role for miRNAs in determining neuronal fate, members of the miR-200 family are necessary for terminal differentiation of olfactory neurons (Choi et al. 2008). While promoting differentiation, miRNAs can also influence neuronal patterning, as is the case with miR-196, which is encoded within a cluster of Hox genes (Mansfield et al. 2004). Hox genes are essential in establishing patterning within the developing nervous system and are well characterized with respect to their role in motor neuron organization in the spinal cord [for review see: (Dasen et al. 2009)]. Interestingly, miR-196 is encoded from three loci, each within a Hox gene cluster, Hox-A, -B and –C. The miRNA regulates the corresponding Hox8 gene from each cluster (Yekta et al. 2004), which provides robustness to the Hox8 expression pattern (Hornstein et al. 2005). Together these examples demonstrate that individual miRNAs contribute to neuronal identity.

Two other well-characterized miRNAs in neuronal development are miR-9/9* and miR-124, which promote the differentiation of neural progenitors to mature neurons [for review see: (F.-B. Gao 2010; Vo et al. 2010a)]. In *in vitro* differentiation assays, miR-9 inhibits neural stem cell proliferation and promotes differentiation by inhibiting the transcription factor TLX (Zhao et al. 2009a). *In vivo* studies using miR-9-2 and miR-9-3 double knockout mice have demonstrated that miR-9 controls the balance between proliferation and differentiation in the developing telencephalon by targeting several different transcription factors and RNA binding proteins at different stages of development (Shibata et al. 2011). Interestingly, both miR-9/9* and miR-124 are targets of the RE-1 silencing transcription factor (REST), which silences neuronal genes in non-neural cells (Conaco et al. 2006). As a neuron differentiates, REST levels decrease, allowing expression of neuronal genes, including miR-9* and miR-124. These miRNAs work together to facilitate the transition to a mature neuron by silencing non-neuronal target genes, and in particular, the SWI/SNF chromatin remodeling complex protein BAF53a (Yoo et al. 2009). The

BAF53a chromatin-remodeling complex maintains neuronal progenitor identity (Lessard et al. 2007). Silencing of BAF53a by miR-9* and miR-124 allows BAF53b to incorporate into the chromatin remodeling complex, which then promotes a mature neuronal state. In addition to silencing BAF53a and promoting a neuronal chromatin state, miR-124 also targets the pre-mRNA splicing repressor PTBP1, which suppress neuronal-specific RNA splicing (Makeyev et al. 2007). By silencing PTBP1, miR-124 facilitates the neuronal splicing of PTBP2 and the GABA(B) receptor. These studies provide examples of how specific miRNAs can determine neuronal identity. Interestingly, they do this by controlling transcription factors, chromatin remodeling factors and RNA splicing factors—indirectly regulating gene expression at multiple levels through post-transcriptional repression of their direct targets.

Chapter 1.2.3: microRNAs in neurons

In addition to their essential role in neuronal development, miRNAs are also important gene regulators in neurons [for reviews see: (Kosik 2006; Schratt 2009; Saba et al. 2010; Vo et al. 2010a; Siegel et al. 2011)]. miRNAs are uniquely positioned to contribute to the structural and physiological changes that mature neurons undergo in response to changes in activity for several reasons. First, miRNAs are expressed in mature neurons. Second, they are transported into the dendritic compartment and specific miRNAs are enriched at the synapse. Third, they undergo rapid turnover in the nervous system, allowing functional levels of an individual miRNA to change quickly as neurons adapt to sensory input. These traits allow miRNAs to provide local control of translation, which is essential for plasticity in neurons and is especially important given their large cell size (Sutton et al. 2006). Examples demonstrating these characteristics and expounding on the role of miRNAs in the mature neurons are given below.

Following the initial discovery of miRNAs, there was a concerted effort to characterize tissue specific miRNA expression, including in the brain. Early studies used a cloning approach to identify miRNAs in the brain, as well as other tissues (Lagos-Quintana et al. 2002). Subsequently, array technology was developed to identify miRNA expression and used to characterize expression throughout development and in the adult brain. These studies identified several neuronal miRNAs that were expressed more during development, such as miR-9, miR-19b, and miR-178, as well as miRNAs that were enriched in the adult brain, such as miR-128 (Krichevsky et al. 2003; Miska et al. 2004). Furthermore, components of the miRNA processing and silencing machinery, such as Dicer and RISC, are localized and functional in dendrites and synapses, suggesting that in addition to neuronal expression, miRNA localization is also important to their function within the nervous system (Lugli et al. 2005; Ashraf et al. 2006). Additional studies have characterized specific subsets of miRNAs that are found in synapses, dendrites and axons (Natera-Naranjo et al. 2010; Lugli et al. 2008; Kye et al. 2007; Siegel et al. 2009). Interestingly, the turnover of miRNAs in neurons is much faster than in non-neuronal cells, with an average half-life of thirty minutes to one hour in neurons compared to greater than six hours, and possibly as long as twenty-four hours in non-neuronal cells, such as ES cells, NIH-3T3, hepatocytes and glia (Gatfield et al. 2009; Krol et al. 2010). The combination of expression, localization and rapid turnover put miRNAs in the perfect position to help facilitate the cell state transitions that mature neurons experience in response to activity.

A number of specific miRNAs have been found to influence the physiology and morphology of dendrites and synapses on mature neurons. miR-132, the focus of this thesis and described extensively below, was one of the earliest identified neuronal miRNAs to influence dendritic morphology (Vo et al. 2005). It is unique from other well-characterized neuronal miRNAs, in that it promotes dendrite and spine outgrowth (Impey et al. 2010; Wayman et al.

2008). In contrast, miR-124, -125b, -134, and -138, all reduce spine volume and impair neuronal facilitation. In *Aplysia* sensory neurons, application of serotonin, which mediates sensitization of the gill-withdrawal reflex in the sea slug, causes a rapid reduction in miR-124 levels (Rajasethupathy et al. 2009). Consistent with this, overexpression of miR-124 in the pre-synaptic sensory neuron impairs facilitation of the post-synaptic motor neuron, while inhibition of miR-124 promotes facilitation. miR-124's negative effect on facilitation is mediated by direct targeting of the CREB 3'UTR (Rajasethupathy et al. 2009). miR-134 also negatively regulates synaptic plasticity by targeting CREB within the context of a functionally-null SIRT1 mutant mouse (Gao et al. 2010b). The SIRT1 mutant, which has impairments in LTP and contextual fear conditioning, also has elevated levels of miR-134 and decreased CREB levels. Knockdown of miR-134 in the SIRT1 mutant mouse rescues the LTP impairment and the defect in fear conditioning (Gao et al. 2010b). In addition to targeting CREB, miR-134 also targets LimK1, an enzyme that promotes dendritic spine formation through its regulation of actin dynamics (Schratt et al. 2006). Overexpression of miR-134 in hippocampal neurons decreased dendritic spine width, but had no effect on spine density or dendritic arborization. In contrast to the negative effect on spine width, the Schratt group reported that miR-134 expression was induced by activity through a MEF2-dependent mechanism and promoted dendrite outgrowth of cultured neurons by silencing of Pumilio2 (Fiore et al. 2009). A study of miRNA expression following high frequency stimulation that induced LTP in dentate gyrus granule cells saw no change in miR-134 levels (Wibrand et al. 2010), however, members of the miR-134 family were induced in the dentate gyrus following seizures, although miR-134 itself, was not (Luikart et al. 2011b).

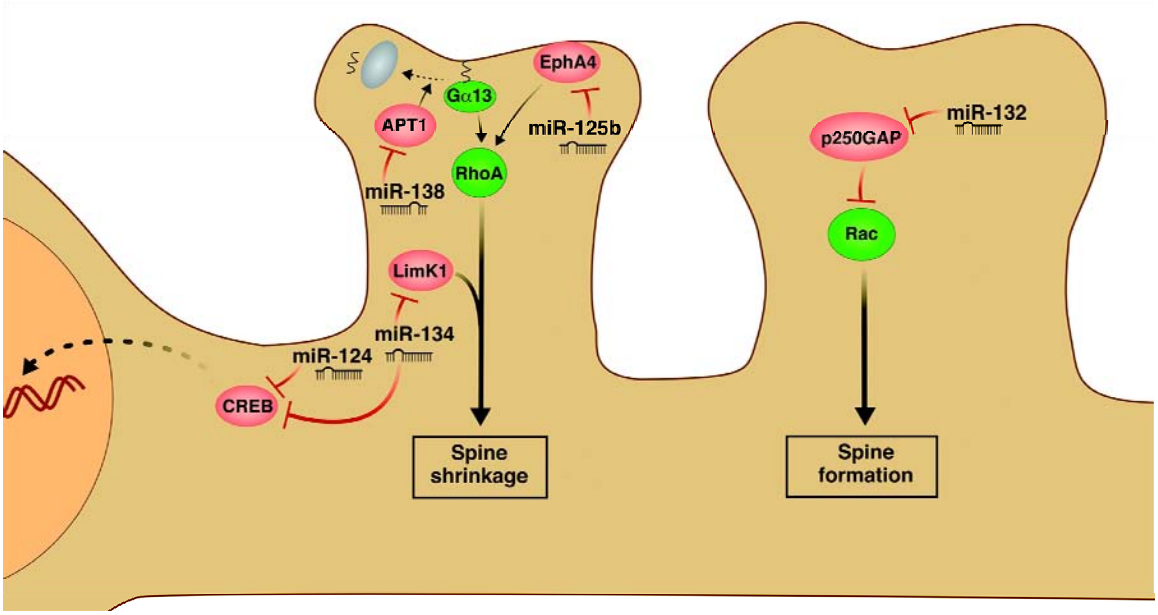

Figure 1.2: miRNA regulation of spine morphology. miR-124, miR-125b, miR-134, and miR-138 all negatively regulate spine size, while miR-132 promotes spine growth. Figure modified from (Siegel et al, 2011).

Along with miR-124 and -134, miR-138 and -125b also negatively regulate spine size. When overexpressed in cultured hippocampal neurons, miR-125b caused the formation of long and thin spines, a sign of immaturity (Edbauer et al. 2010). Consistent with this, there was a decrease in the miniature excitatory post-synaptic current (mEPSC) frequency and amplitude, confirming that there is a decrease in synaptic transmission. miR-125b may mediate this effect by targeting EphA4, although the evidence supporting this was limited to reporter experiments. Interestingly, the Fragile X Mental Retardation Protein (FMRP) binds both miR-125b and its target, NR2A. FRMP mediated suppression of NR2A expression is partially dependent on miR-125b (Edbauer et al. 2010). Like miR-125b, miR-138 decreases spine volume and mEPSC amplitude in cultured hippocampal neurons (Siegel et al. 2009). miR-138 targets acyl-protein thioesterase 1 (APT1), a protein that removes palmitate from its targets, allowing them to be trafficked from the plasma membrane to endosomal compartments (Linder et al. 2007). APT1 removes palmitate from $G_{\alpha 13}$, which leads to the activation of Rho-dependent signaling and cytoskelatol remodeling (Bhattacharyya et al. 2000). Blocking miR-138 allows APT1 to be expressed, which cleaves the palmitate from $G_{\alpha 13}$ and removes it from the membrane, leading to spine enlargement. In the presence of miR-138, APT1 translation is inhibited, and $G_{\alpha 13}$ remains in the membrane, decreasing spine volume (Siegel et al. 2009). Together, these four miRNAs inhibit spine growth and neuronal facilitation, which raises the question of what happens to them during spine outgrowth in response to neuronal activity.

Local protein synthesis and degradation are both necessary within dendrites for activity-dependent neuronal changes as well as learning and memory (Sutton et al. 2006). Because numerous miRNAs, as well as their functional machinery, are localized in dendrites, they are well positioned to regulate this local protein synthesis. Indeed, one of the RISC components, MOV10, is degraded in response to NMDA receptor activation (Banerjee et al. 2009). This loss

of miRNA-mediated inhibition allows local synthesis of three enzymes that are important mediators of spine outgrowth, CamKII, Limk1, and lysophospholipase1 (Lypla1). Limk1 and Lypla1 are regulated by miR-134 and miR-138, respectively. Interestingly, neuronal activity accelerates the decay of miR-124, miR-134, and miR-138 (Krol et al. 2010). Thus, by inducing proteasomal degradation of RISC and mature miRNAs, neuronal activity removes local inhibition in the dendrites and facilitates local protein synthesis of proteins that promote spine formation.

Chapter 1.3: microRNA-212/132

microRNA-132 was first identified as one of the early brain-specific miRNAs using tissue specific cloning (Lagos-Quintana et al. 2002). Subsequently, a genome wide-screen to characterize all CREB target genes identified three CREB response elements in the miR-212/132 locus (Impey et al. 2004; Vo et al. 2005). Treatment with forskolin, an activator of adenylyl cyclase, increased CREB binding at response elements in the miR-212/132 promoter and in between miR-212 and -132, inducing expression of the miRNAs (Rongkun Shen, unpublished data). The miR-212/132 locus is in an intergenic region on the long arm of mouse chromosome 11 (qB5) and the short arm of human chromosome 17 (p13.3). This highly conserved locus lies on the plus strand between Ovca2 and Dph1, which are overlapping genes located on the minus strand ~2kb downstream, and Hic1, which is ~3kb upstream on the minus strand. Ovca2 is a putative serine-hydrolase and a potential tumor suppressor gene that is expressed in normal ovarian epithelial cells and lost in human ovarian tumors and tumor cell lines (Schultz et al. 1996; Wiper et al. 1998; Azizi et al. 2006). In addition to disruption of the locus in ovarian cancer, allelic imbalance in this region is observed in some esophageal squamous cell carcinomas (Huang et al. 2000). Interestingly, the genomic regions reported to be lost in these human cancers would also result in the loss of miR-212 and miR-132. Dph1, also known as Ovca1 and DPH2L1, overlaps the 5'

end of Ovca2, and is also downstream from miR-212 and miR-132. Dph1 is a protein that participates in the first step of diphthamide biosynthesis (Nobukuni et al. 2005). Diphthamide modifies a histidine on elongation factor 2 (eEF2), which is the target of ADP-ribsolyating toxins such as diptheria toxin (Nobukuni et al. 2005). Homozygous knockout mice lacking Dph1 die *in utero* and exhibit multiple defects in growth and organogenesis [for review see: (Chen et al. 2005)]. Hic1, a transcription factor located ~3kb upstream of miR-212/132, is a tumor suppressor gene that is hypermethylated in numerous cancers and regulates cell growth and proliferation in conjunction with p53 (Chen et al. 2004b). It regulates different sets of target genes, including SIRT1, through its co-repressors, CtBP and NuRD (Zhang et al. 2007; Deltour et al. 2002; Van Rechem et al. 2010) [for reviews see: (Dehennaut et al. 2009; Fleuriel et al. 2009)].

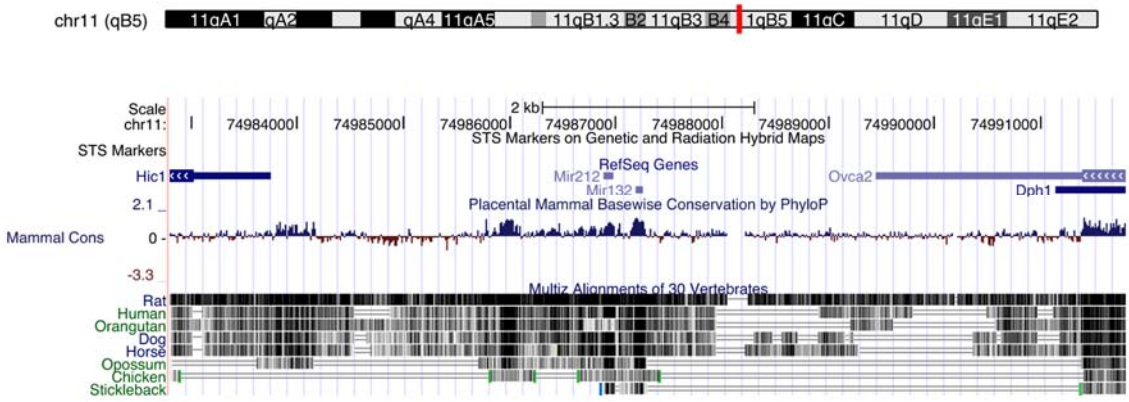

Figure 1.3: Mouse miR-212/132 genomic locus and conservation. The miR-212/132 locus is in a highly conserved intergenic region on the long arm of chromosome 11 (qB5). Pre-miR-212 is roughly 300 bp upstream of pre-miR-132. The locus is bordered by Ovca2 and Dph1, which are ~2kb downstream on the minus strand. Hic1 is ~3kb upstream, also on the minus strand. Image and data from the USCS Genome Browser.

The primary miR-212/132 transcript is a non-coding RNA that is independent from the transcripts that code for the genes that flank the miR-212/132 locus. Canonical CREB- and REST-binding sites lie between the two miRNA sequences, and two additional CREB binding sites are located approximately 100 and 150 bases upstream of miR-212. Two transcripts encode primary miR-212/132. The first, Isoform 1, is 5.1kb and is expressed in brain and testis. The second, Isoform 2, is only 2.3kb and is expressed in brain, testis, heart, and mammary stroma, but not in kidney or mammary epithelial cells (Ucar et al. 2010). Both isoforms of the primary transcript contain pre-miR-212/132 and are processed into separate pre-miR-212 and pre-miR-132 transcripts in the nucleus, presumably by Drosha (Ensembl gene ID for pre-miR-212: ENSMUST00000083656, and pre-miR-132: ENSMUSG00000065537).

miR-132 is expressed at high levels in brain and testis, and at lower levels in a number of other tissues and cell types, including macrophages, tumor endothelial cells, ovarian granulose cells, gonadotrope cells, and mammary stromal cells (Ucar et al. 2010; Anand et al. 2010; Shaked et al. 2009; Fiedler et al. 2008; Yuen et al. 2009; Luers et al. 2010). Within the brain, its expression has a rostral to caudal pattern, being highest in the forebrain and lowest in the cerebellum (Olsen et al. 2009). The expression of miR-132 increases post-natally from day one to day twenty-eight, where it reaches its maximum, which is the same as the expression level in the adult brain (Nudelman et al. 2010; Impey et al. 2010).

Chapter 1.3.1: Activity-dependent induction of miR-212/132

miR-132 was the first activity-dependent miRNA identified. Since its discovery, a wide variety of stimuli and activity forms have been shown to increase miR-132 and miR-212 expression. BDNF was the first of a number of trophic factors and extracellular signals found to induce miR-132 expression (Vo et al. 2005). In addition to being activated by BDNF, miR-132 is activated by

depolarization of cultured hippocampal neurons with KCl or treatment with the $GABA_A$ antagonist, bicuculline, which increases spontaneous activity levels in the culture. The increase in miR-132 occurs in both the soma as well as the dendrites of cultured hippocampal neurons (Wayman et al. 2008). In addition to being induced in cell culture, high-frequency stimulation of the perforant path *in vivo* in rats increased miR-132 and miR-212 expression in the dentate gyrus (Wibrand et al. 2010). In mice, seizures, as well as several behavioral paradigms associated with neuronal activation, induced miR-132 in specific brain regions. For example, contextual fear conditioning, which causes neuronal activation in the hippocampus, odor-exposure, which causes neuronal activation in the olfactory bulb, and cocaine injection, which causes neuronal activation in the striatum, all drive increases in miR-132 in the respective brain regions (Nudelman et al. 2010). Interestingly, induction of the pre-miR-132 transcript is much more robust than the mature transcript, which suggests that the processing of miR-132 from the pre-miRNA to the mature miRNA is regulated, however, the factors that mediate this regulation remain unknown (Nudelman et al. 2010; Wibrand et al. 2010). Finally, exposure of mice to light induces miR-132 in the suprachiasmatic nucleus (SCN), a brain region important for regulation of the circadian clock (Cheng et al. 2007).

Outside the nervous system, miR-132 is also regulated by external stimuli, including trophic factors and viruses. For instance, treatment of human umbilical cord endothelial cells with bFGF or VEGF can induce miR-132 expression (Anand et al. 2010). In addition, expression of both miR-132 and miR-212 is induced by treating LβT2 cells, a mouse gonadotropin cell line, with gonadotropin releasing hormone (GnRH), or by treating primary mouse ovary granulosa cells with luteinizing hormone (LH) or human chorionic gonadotropin (hCG) (Fiedler et al. 2008; Yuen et al. 2009). miR-132 is expressed at low levels in primary human macrophages, yet its expression can be robustly induced by treatment with lipopolysaccharide (LPS). LPS treatment

also induces miR-132 expression in mouse bone marrow and splenocytes (Shaked et al. 2009). In addition to trophic factors and toxins, viruses can induce expression of miR-132. For instance, infection of primary lymphatic endothelial cells with Kaposi's sarcoma-associated herpesvirus (KSHV) stimulates miR-132 expression. In a similar manner, infection of monocytes with herpes simplex virus-1 (HSV1) or human cytomegalovirus (HCMV) also induces miR-132 (Lagos et al. 2010).

All these stimuli that induce the expression of miR-132 and miR-212 use signaling pathways that ultimately converge on CREB. Indeed, transfection with a dominant negative CREB construct blunts the induction of miR-132 (Wayman et al. 2008; Cheng et al. 2007). The pathways linking the external stimuli to CREB have been most thoroughly elucidated within neurons. The induction of miR-132 by bicuculline in hippocampal cultures can be blocked by APV, an NMDA receptor blocker (Wayman et al. 2008). However, induction of miR-132 by BDNF was not blocked despite concurrent application of APV and CNQX, an AMPA receptor blocker (Kawashima et al. 2010). Similarly, miR-132 expression was induced *in vivo* by high frequency stimulation of the perforant path in rats, despite administration of CPP, a different NMDA antagonist (Wibrand et al. 2010). While treatment with CPP had no effect on pre-miR-132 induction, it slightly, but significantly, increased the levels of mature miR-132 compared to high frequency stimulation alone. Interestingly, treatment with AIDA, a Group I mGluR antagonist, blocked the induction of both the pre- and mature miR-132 in response to high-frequency stimulation *in vivo*. These findings suggest that different stimuli can induce miR-132 expression through diverse receptors and pathways, including mGluRs, and possibly NMDARs.

Several different signaling pathways have been identified that transmit the initial stimulus to the final step of CREB activation. miR-132 induction in cultured neurons by treatment with BDNF or bicuculline can be blocked by UO126 or PD184352, drugs that block the

MEK-ERK signaling pathway (Wayman et al. 2008; Remenyi et al. 2010). Activation of the MEK-ERK pathway in response to BDNF treatment can be inhibited by pre-treatment with the glucocorticoid, dexamethasone, which blocks the induction of miR-132 by BDNF (Kawashima et al. 2010). Furthermore, blockade of CaM kinase signaling with KN-62 also inhibited miR-132 induction (Wayman et al. 2008). The induction of miR-132 and miR-212 by BDNF was dramatically impaired in cultured cortical neurons from mice lacking MSK1 and MSK2, downstream kinases in the ERK pathway that can directly phosphorylate CREB (Remenyi et al. 2010). Interestingly, treatment with the PI3K inhibitor, LY294002, did not affect the induction of miR-132 by BDNF (Kawashima et al. 2010). Taken together, these studies demonstrate that varying stimuli signaling through the MEK-ERK-MSK and CaMK pathways converge on CREB activation to induce the expression of miR-132 and miR-212.

Neuronal activity can also affect the stability of a miRNA. Stability of a mature miRNA can be measured by assaying for the mature miRNA with Taqman qPCR after treatment with a transcription blocker such as α-Amantin or Actinomycin D. For several of the well-characterized neuronal miRNAs such as miR-124, miR-134 and miR-138, glutamate treatment promotes their degradation in cultured hippocampal neurons or ES cells differentiated to mature neurons (Krol et al. 2010). This effect of glutamate is mediated by NMDA and AMPA receptors, as it can be blocked by addition of CPP and NBQX. In stark contrast, 10 uM bath application of glutamate stabilizes mature miR-132. Furthermore, application of the glutamate receptor antagonists NBQX and CPP promote the degradation of miR-132. This occurs with our without glutamate treatment, suggesting that the basal level of glutamate in the cultures is enough to promote miR-132 stability. This observation is particularly fascinating when considered within the context of local protein synthesis and degradation discussed above. For instance, when a synapse is activated, the RISC component MOV10 is degraded by the proteasome removing

miRNA inhibition from CamKII, Limk1, and Lypla1, allowing them to be synthesized locally, which promotes synaptic strengthening and spine growth (Banerjee et al. 2009). Thus, differential stability of miRNAs allows them to provide rapid and local regulation to fine-tune synaptic strength in response to extracellular inputs.

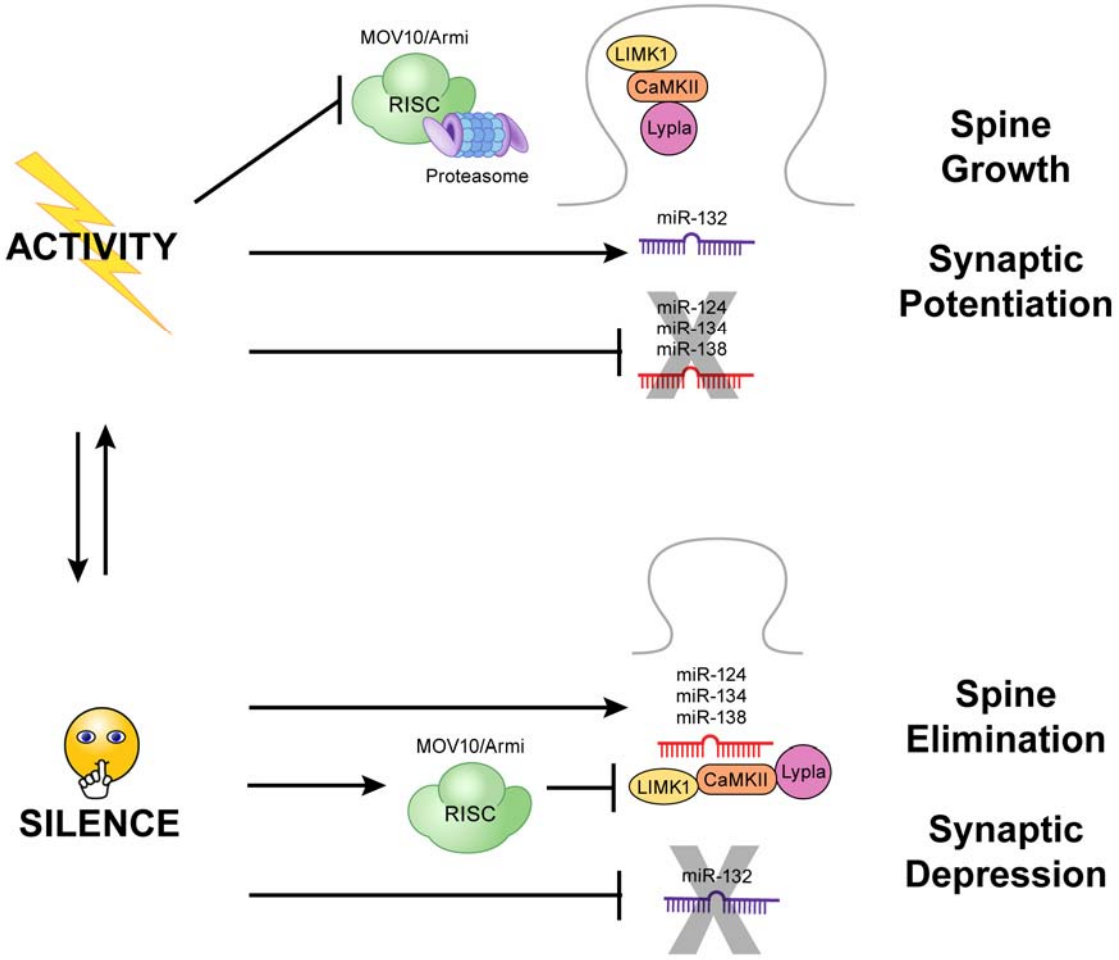

Figure 1.4: Differential consequences for miRNAs in response to activity. Activity promotes degradation of MOV10 by the proteasome, degradation of miR-124, -134, and -138, and stabilizes miR-132. Lack of activity promotes miR-132 degradation and stabilizes the RISC as well as miR-124, -134, and -138. These changes may mediate the balance between synaptic potentiation and depression in response to activity. Figure modified from from (Vo et al, 2010a).

Chapter 1.3.2: miR-212/132 functions within the nervous system

miR-132's function was first investigated in cultured cortical neurons. Overexpression of miR-132 caused a significant increase in neurite length and number, indicating that it was sufficient to stimulate neurite outgrowth (Vo et al. 2005). Consistent with this observation, transfection of a 2'O-methyl anti-sense miR-132 inhibitor reduced the number and length of neurites in cultured cortical neurons. Prediction algorithms identified p250GAP, a Rac/Rho GTPase activating protein (GAP), as a potential miR-132 target due to sequence complementarity in its 3'UTR. Overexpression of miR-132 decreased expression of p250GAP in cortical neuron cultures, while transfection with a miR-132 inhibitor increased p250GAP expression. Furthermore, knockdown of p250GAP caused an increase in neurite length and outgrowth identical to the phenotype caused by overexpression of miR-132, suggesting that miR-132 targeting of p250GAP mediated neurite outgrowth.

To address whether miR-132 was necessary for activity-induced dendrite outgrowth, dissociated hippocampal cultures and organotypic hippocampal cultures were treated with bicuculline and transfected with a miR-132 inhibitor. Inhibition of miR-132 blocked the increase in dendritic length and branching stimulated by bicuculline (Wayman et al. 2008). Additionally, transfection of p250GAP with a 3'UTR containing a mutation in the miR-132 binding site blocked the bicuculline-induced increase in dendritic length and branching, suggesting that inhibition of p250GAP by miR-132 was necessary for dendritic outgrowth in response to activity. Further experiments demonstrated that p250GAP inhibits the small GTPase Rac. Following stimulation with bicuculline and the subsequent down-regulation of p250GAP by miR-132, Rac becomes active and mediates dendritic outgrowth. Interestingly, this same pathway is also functional in a pituitary gonadotrope cell line, LβT2, where GnRH stimulates miR-132 and miR-212, which downregulates p250GAP and inducing neurite outgrowth and increasing cell migration (Godoy

et al. 2011). Thus, miR-132 is a key regulator of dendritic plasticity *in vitro* through its regulation of p250GAP.

In addition to influencing dendritic outgrowth, miR-132 can also influence plasticity within dendritic spines. In contrast to the effects of miR-125b, miR-134, and miR-138, overexpression of miR-132 in dissociated hippocampal cultures increases dendritic spine density (Impey et al. 2010). Furthermore, transfection with a 2'O-methyl miR-132 inhibitor decreased the spine density and the frequency of mEPSCs in dissociated hippocampal cultures. Within organotypic slice cultures, the 2'O-methyl miR-132 inhibitor blocked the increase in bicuculline-stimulated spine density, but had no effect on spine density in the absence of stimulation. Experiments utilizing dominant-negative small GTPases demonstrated that the effect of miR-132 on spine density, like its effect on dendritic outgrowth, is mediated by Rac activation due to p250GAP silencing. Rac responds to a balance between inhibition via p250GAP and activation by its GTPase exchange factor (GEF), Kalirin-7. At baseline activity, the balance likely favors the GAP, keeping Rac activity at a low level and stabilizing neuronal structure. However, neuronal activation increases miR-132 levels, decreasing p250GAP, and allowing the Rac-GEF, Kalirin-7, to increase Rac activity. Rac stimulates another small GTPase, Pak, which leads to spine formation (Impey et al. 2010). Consistent with these results, transfection of cultured cortical neurons with a double-stranded miR-132 inhibitor attenuated the BDNF-induced increase in NR2A, NR2B and GluR1, although no experiments were done to determine if these glutamate receptors were directly regulated by miR-132 (Kawashima et al. 2010). These are likely indirect effects, and would be expected if miR-132 were necessary for spine strengthening—fewer or weaker spines following BDNF treatment in the presence of the miR-132 inhibitor would likely correlate with a decrease in the number of glutamate receptors. Thus, by modulating the balance in actin

remodeling through its regulation of p250GAP, miR-132 may fine-tune dendritic spine density to the level of activity a neuron receives.

miR-132 clearly impacts neuronal morphology in culture, and several groups have used different approaches to test the neurophysiological consequences of these changes. Following overexpression of miR-132 in hippocampal neurons, Edbauer et al observed a slight decrease in spine density, but also an increase in spine width (Edbauer et al. 2010). Recording from these neurons, they observed an increase in both frequency and amplitude of mEPSCs, suggesting that, despite the decrease in spine density, overexpression of miR-132 caused an increase in synaptic strength. Recording from autaptic hippocampal neurons that had been infected with a miR-132 overexpressing lentivirus, Lambert et al found a slight, but significant, increase in the paired-pulse ratio (0.8 to 1.0) (Lambert et al. 2010). Following a synaptic depression protocol, they found that mEPSC amplitude in autaptic neurons overexpressing miR-132 did not decrease as much as control. Interestingly, while they report that there was no change in mEPSC amplitude at baseline, their data show a significant increase in the baseline mEPSC during the synaptic depression protocol. This would be consistent with reports from other groups overexpressing miR-132, which found an increase in mEPSC amplitude and frequency, although the effect could be specific to the autaptic culture. Lambert et al also report no difference in basal release probability, in the refilling or size of the readily releasable pool, in the frequency of mEPSCs, or in Ca^{2+}-dependent release. While the results from the autaptic cultures could be questioned given their internal inconsistency and the high level of miR-132 overexpression, coupling the physiological findings from Edbauer et al and Impey et al with all the morphological studies suggest that miR-132 participates in mediating synaptic strengthening and spine formation.

In a study that was conducted concurrent to the work reported in this thesis, the Obrietan group generated a transgenic mouse overexpressing miR-132 under control of a tetracycline responsive element (Hansen et al. 2010). Crossing the miR-132 transgenic with a CamKII-tTA mouse led to a 7-fold increase in miR-132 expression. Consistent with the previously mentioned morphological studies, overexpression of miR-132 led to an increase in spine density on the dendrites of CA1 pyramidal neurons. They also saw a decrease in MeCP2 expression, which has been shown to be a target of miR-132 and will be discussed in Chapter 1.3.4 (Klein et al. 2007). Interestingly, the miR-132 overexpressing mice have impaired discrimination in the novel object recognition test, a hippocampal-dependent task. These results suggest that synaptic changes mediated by miR-132 contribute to the molecular processes underlying learning and memory.

Regulation of the circadian rhythm is an important physiological process that is exquisitely sensitive to light-induced neuronal activity. As mentioned above, exposure to light can stimulate miR-132 expression in the SCN (Cheng et al. 2007). Interestingly, miR-132 levels within the SCN, but not within the cortex, fluctuate in a circadian fashion, and this fluctuation is abrogated in mice that have mutations that preventing entrainment to light. Mice that are housed continuously in the dark shift their activity from a 24 hr rhythm to 23.7 hr; however, exposure to light rapidly resets their circadian clock. Injection of a miR-132 antagomir into the SCN prevented clock resetting in response to light, suggesting that light-induced miR-132 expression was necessary for clock resetting. Furthermore, Cheng et al demonstrated that miR-132 targeted Rfx4 and Per1 to mediate this effect. To determine miR-132's actions in regulating the circadian clock, transgenic mice overexpressing miR-132 in the SCN were tested for clock resetting in response to light and found to be impaired as well (Alvarez-Saavedra et al. 2010). MeCP2, Ep300, and Jarid1a, which are involved in chromatin remodeling and regulation, as well

as Btg2 and Paip2a, which are involved in translational control, were all validated to be direct miR-132 targets and were down regulated in the miR-132 overexpressing mice. Interestingly, light induced the activity of both miR-132 and these target genes in the SCN of wild type mice. These data support a model where light activation of the SCN induces the expression of miR-132 target genes, which epigenetically remodel the SCN neurons into an "light-on state". However, miR-132 expression is also induced by light. Once miR-132 is processed into its mature form, it begins to silence the translation of its target genes, causing their expression to be transient, and ultimately allowing the neurons to return to their "light-off state" until reactivated by light. Thus, miR-132, by regulating the duration of its target's expression, facilitates the transition of the SCN neurons between a "light-on" and "light-off" state.

Taken together, these studies suggest a dynamic role for miR-132 in modulating activity-dependent neuronal plasticity. However, many of these studies are limited in that they are based primarily on experiments using cultured neurons, leaving the *in vivo* relevance of the findings unknown. The *in vivo* studies that have been conducted only examine expression of miR-132 or rely on overexpression of miR-132. The notable exception to this would be the studies by the Kenny (discussed below in Chapter 1.3.4) and Obrietan groups (Cheng et al. 2007) that inject lentiviral-expressed miRNA sponges or antagomirs *in vivo*, respectively. Our data using miRNA sensors suggest that typical antagomir overexpression levels are high enough to silence miRNAs other than the intended miRNA. Thus, there is the potential for non-specific or off-target effects. A cleaner approach to understanding miR-212/132 function would be to use a conditional knockout, which is the approach we took in the experiments described in this dissertation.

Chapter 1.3.3: miR-212/132 functions in non-neural cells

Outside the nervous system, miR-132 has been reported to play important roles in immune responses, angiogenesis and mammary gland development. There is extensive interaction between the nervous system and the immune system [for review see: (Sternberg 2006)]. In the periphery, acetylcholine (Ach) released from the vagus, and other parasympathetic nerves, binds to its receptor on activated macrophages and inhibits the translocation of NF-κB into the nucleus. Because NF-κB promotes the expression of pro-inflammatory genes in macrophages, inhibition of its translocation to the nucleus decreases the expression of pro-inflammatory genes, resulting in a net anti-inflammatory effect. Interestingly, 24 hrs after treatment of splenocytes with the bacterial endotoxin, LPS, there is a decrease in the expression and functional activity of acetylcholinesterase (AchE), the enzyme that inactivates Ach (Shaked et al. 2009). AchE expression is increased in the first three hours following LPS treatment, but then drops dramatically. This suggests the possible presence of a feedback loop where LPS initially induces pro-inflammatory genes through NF-κB and removes the anti-inflammatory signal from Ach by increasing AchE. However, 12 hrs following LPS treatment, AchE levels decrease, leading to an increase in Ach levels, which promotes an anti-inflammatory response, thus limiting the extent and duration of inflammation. The delayed timecourse of AchE degradation raised the possibility that miRNAs induced by LPS could target AchE and mediate the feedback loop. Using predication algorithms, Shaked et al identified miR-132 as a putative regulator of the AchE 3'UTR. Indeed, treatment with LPS increased miR-132 expression in macrophages and *in vivo* inhibition of miR-132 by injection of an anti-miR-132 LNA increased in serum AchE. Following *in vitro* validation of AchE as a direct target of miR-132, Shaked et al generated a transgenic mouse with a mutation in the miR-132 binding site of the AchE 3'UTR. The transgenic mouse had higher levels of AchE and inflammatory cytokines, which could be decreased to WT levels by

administration of nicotine, an AchR agonist. Furthermore, the transgenic mouse had increased body temperature, a surrogate marker for inflammation, compared to control mice after treatment with LPS. These data suggest that miR-132 can inhibit AchE in macrophages, increasing Ach levels, which promotes an anti-inflammatory response. Interestingly, Ach enhances learning and memory and plays a role in synaptic potentiation in the hippocampus [for review see: (Hasselmo 2006)], raising the possibility that miR-132 down-regulation of AchE in the brain may be one of the mechanisms underlying the dendritic and spine outgrowth phenotypes described above.

The anti-inflammatory effects of miR-132 expression also occur in response to viral infection, although through a different mechanism. Infection of lymphatic endothelial cells (LEC) with KSHV or monocytes with HSV-1 or HCMV increases the expression of both miR-132 and miR-212 (Lagos et al. 2010). The increase in miR-132 expression occurs, like in the nervous system, through a MEK-ERK-CREB pathway. Interestingly, they identified the transcriptional co-activator, p300, as a direct target of miR-132. p300 has widespread effects throughout the genome because it functions as a co-activator for multiple transcription factors, including the pro-inflammatory NF-κB as well as the anti-viral interferon response factors. Reductions in p300 decrease expression of inflammatory and anti-viral genes such as IL-1β, IL-6, and ISG15. This dampens the immune response, promoting viral infection. Consistent with this observation, LECs transfected with a miR-132 inhibitor had decreased viral load and increased IFN-β production following KSHV infection. Additionally, treatment with a miR-132 inhibitor prevented the decrease in p300 that occurs following KSHV infection. Thus, by inducing miR-132 expression, viruses usurp the host cellular machinery to promote viral infection by stimulating a widespread dampening of the immune response through p300 down-regulation.

In addition to functioning in immune responses, miR-132 has also been implicated as an important factor in blood vessel development. Treatment of human umbilical vein endothelial cells (HUVEC) or embryonic stem cells with VEGF or bFGF leads to the formation of endothelial networks in culture and is used as a model of angiogenesis. A screen for miRNAs that were up regulated during this process identified miR-132 as the most highly up regulated miRNA (Anand et al. 2010). Overexpression of miR-132 in the HUVEC model of angiogenesis increased tube formation, while inhibition of miR-132 inhibited tube formation *in vitro*. In addition, mice given a systemic dose of a miR-132 anatagomir had decreased blood vessel growth into Matrigel plugs seeded with bFGF, an *in vivo* model of angiogenesis. Anand et al went on to show that miR-132 directly targeted another GAP protein, p120RasGAP, to mediate this effect. Furthermore, injection of a miR-132 antagomir into the developing retina inhibited normal blood vessel formation. These findings suggest that miR-132 mediates pro-angiogenic signals by silencing p120RasGAP, thus facilitating Ras activation and subsequent neovascularization.

Finally, miR-132 and miR-212 are necessary for normal mouse mammary gland development. In studies conducted independently, but concurrent with those described in this dissertation, Ucar et al generated a miR-212/132 knockout mouse to investigate the role of miR-132 in development (Ucar et al. 2010). The knockout mice were generated using homologous recombination. The final targeted allele replaced the miR-212/132 transcript with an internal ribosomal entry sequence (IRES) followed by a LacZ-pA reporter construct. The endogenous miR-212/132 promoter, which was upstream of the IRES-LacZ-pA, was left intact. Despite normal maternal mouse behavior, pups of homozygous miR-212/132 KO mothers failed to grow as fast as pups with heterozygous or control mothers, and most of the pups from the KO mothers died within 5 days. Interestingly, when KO pups were placed with a WT mother, they grew at a normal rate and lived a normal life span. Based on these findings, the mammary

glands were examined, which revealed that the miR-212/132 KO female mice had impaired mammary ductal outgrowth during puberty. The few ducts that did form during puberty were able to proliferate and differentiate during lactation, however, the developmental impairment of ductal formation resulted in inadequate milk production and subsequent impairment in pup growth and survival. miR-132 is expressed only in the mammary stroma and not the epithelia. Its expression increases during puberty and decreases following the completion of ductal development at 10 weeks. Using mammary epithelial transplant experiments from wild-type to knockout and vice versa, Ucar et al demonstrated that loss of miR-212/132 in the stroma inhibited ductal outgrowth during mammary development. In addition to the impaired ductal outgrowth, miR-212/132 KO mice had less collagen deposition in the mammary stroma than wild-type mice. Collagen in the extracellular matrix is important for ductal outgrowth and is regulated by matrix metalloproteinases (MMP). Using prediction algorithms, MMP-9 was identified and validated as a direct target of miR-212 and miR-132. Both miRNAs silence MMP-9 through the same site in the MMP-9 3'UTR. Consistent with this, miR-212/132 KO mice had increased MMP-9 expression in the mammary stroma. MMP-9, in addition to breaking down collagen, can also activate TGF-β signaling. Hyperactive TGF-β signaling, which was also observed in the miR-212/132 KO mice, can inhibit ductal outgrowth (S. D. Robinson et al. 1991). Taken together, these data suggest that miR-212 and miR-132 function to promote mammary ductal outgrowth potentially through regulation of MMP-9 in the mammary stroma.

Chapter 1.3.4: miR-212/132 in disease states

Changes in miR-132 and miR-212 expression have been observed in many different diseases ranging from vascular/immune/neoplastic to metabolic and neurological disorders. For instance, miR-132 is up regulated in hemangiomas and tumor endothelium (Anand et al. 2010).

Furthermore, administration of anti-miR-132 nanoparticles decreases tumor volume and neovascularization *in vivo* in a mouse orthotopic model of breast carcinoma via its inhibition of p120RasGAP. miR-132 is also up regulated in tissue from human abdominal aortic aneurysms (Liu et al. 2010). Given miR-132's role in macrophages and anti-inflammatory responses, it is not surprising that it is up regulated in circulating blood mononuclear cells from rheumatoid arthritis (RA) patients (Pauley et al. 2008). Interestingly, though, miR-132 is not expressed highly in synovial fluid samples containing synoviocytes and mononuclear cells from RA patients (Murata et al. 2010).

miR-132 and miR-212 expression change in a number of neoplastic conditions. For instance, miR-212 is down regulated in non-small cell lung cancer, which allows increased expression of its direct target, PED, an anti-apoptotic protein that is overexpressed in many human tumors (Incoronato et al. 2010). The miR-212 locus is hypermyethylated and its expression is decreased in human gastric carcinoma, where it normally targets MeCP2 and Myc (Wada et al. 2010; Xu et al. 2010). In gastrointestinal stromal tumors (GIST) with PDGFRA mutations, miR-132 is up regulated, while it is down regulated in GIST with KIT mutations (Haller et al. 2010). Both miR-132 and miR-212 are up regulated in oral squamous cell carcinomas (SCC), and miR-212 regulation of its target, Hb-EGF, may play a role in mediating resistance to chemotherapy in some head and neck SCC (Hatakeyama et al. 2010; Scapoli et al.; Wong et al. 2008). Low levels of miR-132 are part of a miRNA signature that predicts which osteosarcomas will respond to treatment with the DNA-alkylating agent, Ifosfamide (Gougelet et al. 2010). Finally, both miR-132 and miR-212 are overexpressed in pancreatic adenocarcinoma, where they both directly target the retinoblastoma tumor suppressor (Park et al. 2011).

In addition to neoplastic conditions, miR-132 and miR-212 are altered in several metabolic disorders. For instance, in BTBR-ob/ob mice, a validated model of obesity associated

type two diabetes, miR-132 and -212 are both significantly up regulated in pancreatic islets compared to lean controls (Zhao et al. 2009b). In the Goto-Kakizaki rat, a non-obese type two diabetes model, miR-132 and -212 are also up regulated in the pancreatic islets, further suggesting they may play a role in the pathogenesis of type two diabetes (Esguerra et al. 2011). Omental fat accumulates during the development of the metabolic syndrome. In a study comparing 25 obese patients to 25 non-obese controls, miR-132 levels were decreased in the blood and omental fat of obese patients relative to non-obese controls (Heneghan et al. 2011). Consistent with this, a smaller study comparing 9 control patients to 6 patients with newly diagnosed type two diabetes found decreased levels of miR-132 in the omental fat of the type two diabetics (Klöting et al. 2009). Interestingly, they observed fewer macrophages in the omental fat of the control patients with higher miR-132 expression, correlating miR-132 expression with decreased inflammatory infiltration into the adipose tissue. In contrast to the obese patients and type two diabetes models where low miR-132 levels in omental fat correlates with increasing disease severity, miR-132 levels in visceral adipose tissue from patients with non-alcoholic fatty liver disease (NAFLD) are higher in the more severe non-alcoholic steatohepatitis (NASH) compared to the less severe, non-NASH subjects (Estep et al. 2010). Lastly, miR-212, which is expressed in intestinal epithelial cells and is induced in response to high alcohol intake, is thought to play a role in maintaining gut epithelial integrity by regulating the tight junction protein Zona Occluding 1 (Tang et al. 2008).

Given miR-132's important roles in neuronal development and function, it is reasonable to think that alterations in its levels may be associated with certain neurological diseases. Rett Syndrome, an autism spectrum disorder afflicting young girls, is caused by mutations in MeCP2, which is a target of miR-132 (Klein et al. 2007). Expression of miR-132 decreases MeCP2 levels, which reduces BDNF expression, leading to a subsequent decrease in miR-132, allowing MeCP2

levels to increase. Thus, miR-132 is thought to function as an important homeostatic regulator of MeCP2 levels. In addition to Rett Syndrome, miR-132 also has been implicated in pre-conditioning for stroke, possibly through its regulation of MeCP2 (Lusardi et al. 2010). Pre-conditioning consists of administering sub-lethal ischemia, which protects the brain from subsequent ischemia. Pre-conditioning decreases the level of miR-132 leading to an increase in MeCP2 protein levels, suggesting that miR-132 levels may influence response to cerebral ischemia (Lusardi et al. 2010). miR-132 and miR-212 were also down regulated in autopsy samples from the pre-frontal cortex of patients with schizophrenia, and miR-212 levels were reduced in hippocampus and pre-frontal cortex in a mouse model of schizophrenia (Stark et al. 2008; Kim et al. 2010). These studies suggest that miR-132 and miR-212 may be involved in the pathogenesis of neuropsychiatric disorders and recovery from stroke.

If miR-132 is important in maintaining synaptic homeostasis, it is also possible that it could be an important factor in neurodegenerative disease. Hereditary autosomal-dominant Parkinson's disease can be caused by point mutations in α-synuclein, including the A30P mutation. Mice overexpressing the A30P mutation develop motor symptoms and neuronal lesions similar to those observed in Parkinson's disease. Interestingly, both miR-132 and miR-212 were decreased in brainstem samples taken from the A30P transgenic mouse compared to wild-type, suggesting that the loss of miR-132 may correlate with the neurodegenerative phenotype in these mice (Gillardon et al. 2008). In Huntington's disease, which is characterized by striatal degeneration, the data regarding miR-132 expression is contradictory. While miR-132 is up regulated in Huntington's autopsy samples, it is decreased in two different mouse models of the disease (Packer et al. 2008a; Lee et al. 2010b). Like Huntington's, the data in human patients with Alzheimer's disease has also been contradictory. For instance, Lukiw saw no change miR-132 levels in five human autopsy samples from Alzheimer's patients, however, in a

larger study with fifteen autopsy samples from Alzheimer's patients, Cogswell et al found a significant decrease in both miR-132 and miR-212 levels in the hippocampus (Cogswell et al. 2008; Lukiw 2007). Another study found that miR-212 levels were negatively correlated with the severity of Alzheimer's, suggesting that it may be involved in Alzheimer's pathogenesis (Wang et al. 2010).

miR-212 has also been implicated in animal models of cocaine addition. Cocaine is an addictive drug that blocks the dopamine transporter and inhibits dopamine reuptake. Knockout of Ago2 in dopamine receptor 2 expressing neurons inhibits self-administration of cocaine in mice, suggesting that miRNAs are important in the rewarding properties of cocaine (Schaefer et al. 2010). Rats given extended access to cocaine exhibit behaviors similar to human cocaine addicts, including increasing motivation to obtain cocaine and increasing consumption over time, making it one of the best animal models of cocaine addiction. Extended access rats have increased levels of miR-212 in their striatum (Hollander et al. 2010). Incredibly, striatal injection of a lentivirus overexpressing miR-212 reduces cocaine self-administration, while injection of an LNA-miR-212 antagomir increases self-administration, suggesting that miR-212 functions within the striatum to limit cocaine self-administration. Overexpression of miR-212 increased the levels of activated Raf1, likely by inhibiting its direct target, SPRED1, which normally inhibits Raf1. Activated Raf1 increases phospho-CREB levels, possibly through an up regulation of TORC signaling. Consistent with this, overexpression of TORC1 in the striatum, like miR-212, decreased cocaine self-administration. These data suggest that miR-212 attenuates cocaine self-administration by potentiating CREB-TORC signaling.

In addition to its actions on CREB-TORC signaling, miR-212 also inhibits cocaine self-administration in the extended access model through repression of MeCP2 (Im et al. 2010). Like miR-212, MeCP2 levels are increased in the striatum of rats given extended access to cocaine.

Knockdown of MeCP2 decreases cocaine self-administration and increases expression of both miR-132 and miR-212. In addition, knockdown of MeCP2 and overexpression of miR-212 both decrease BDNF expression. Because BDNF overexpression can increase cocaine self-administration, and treatment with anti-BDNF antibodies decreases cocaine self-administration, it is possible that overexpression of miR-212 decreases cocaine self-administration through an MeCP2-mediated decrease in BDNF. However, it remains unclear as to why both miR-212 and MeCP2 are up regulated in the extended access model. Given the proposed mechanism that miR-212 inhibits MeCP2 expression, one would expect that the increased level of miR-212 in the extended access model would cause a decrease of MeCP2. However, the opposite is true, and MeCP2 expression is elevated. While more work needs to be done to sort out the molecular mechanisms underlying cocaine self-administration, the findings raise the possibility that increasing miR-212 levels could be a way to attenuate cocaine addiction, and provides support to the idea that changes in miR-212 expression, and possibly miR-132, are involved in cocaine addiction. In conclusion, changes in miR-132 and miR-212 expression have been observed in human samples as well as animal models from numerous diseases, which suggests that increasing our understanding of their functions may be relevant to designing new therapies in the future, or at the very least, increase our understanding of the pathophysiology of these diseases.

Chapter 1.4: Using mouse genetics to study microRNAs

microRNAs have been difficult to study *in vivo* for several reasons [for review see: (Park et al. 2010)]. First, many miRNAs, like miR-9 or miR-124, are transcribed from multiple locations in the genome. Knocking out one locus may have no phenotype, or only a partial phenotype, because expression from other locus could compensate for the loss. For instance, knockout of

two copies of miR-9 (miR-9-2 and miR-9-3) reduces miR-9 levels by 75% and only produces a mild phenotype, thus, a triple knockout may be needed to fully understand the function of miR-9 (Shibata et al. 2011). Second, miRNAs can be expressed within large clusters, as exemplified by miR-134, which is part of the miR-379-410 cluster. This cluster contains 37 miRNAs within ~35kb region on mouse chromosome 12qF1. Knockout of an individual miRNA within this region could easily alter the expression of adjacent miRNAs. However, knockout of the whole region would make it difficult to determine which miRNA was responsible for any observed phenotype. Finally, paralogous miRNAs can cooperate genetically. For instance, while knockout of the miR-106b-25 cluster produces no obvious phenotype, double knockout of the miR-106b-25 and miR-17-92 cluster produces a different, and more severe, phenotype than knockout of the miR-17-92 cluster alone (Ventura et al. 2008). Thus, miRNAs have proven difficult to study using genetic approaches because they are encoded in multiple locations within the genome, in clusters, and can engage in genetic cooperation.

Chapter 1.5: Adult hippocampal neurogenesis

The adult mammalian brain was thought to be structurally static, a dogma that persisted until the late twentieth century [for review see: (Gross 2000)]. Cracks in the dogma began to emerge with the development of new technologies. Following the development of tritiated thymidine, a radiolabeled nucleotide that is incorporated into DNA of dividing cells, Joseph Altman began a series of experiments where he was able to show, using autoradiographs of adult mammalian brain injected with tritiated thymidine, that neurons and neuroblasts were labeled with the radioactive thymidine, raising the possibility that new neurons were born in the adult brain (Altman 1962; Altman et al. 1966). Altman's results were questioned because he could not prove that the tritiated cells were actually neurons, and not some other type of cell. However,

studies by Michael Kaplan using electron microscopy demonstrated that the radiolabeled cells had dendrites and contained synapses, confirming that they were neurons (Kaplan et al. 1977). Still, the dogma that the brain was structurally static remained prevalent until studies led by Fernando Nottebohm demonstrated the importance of adult neurogenesis in bird song [for review see: (Nottebohm F, 1985)], and finally, the development of bromodeoxyuridine (BrdU) staining. BrdU is a thymidine analog that is incorporated into the DNA of dividing cells and can be used in parallel with immunohistochemistry, allowing co-labeling with cell-specific markers (Nowakowski et al. 1989). Using BrdU labeling, the Gage lab was able to demonstrate unequivocally that new neurons were generated within the dentate gyrus of the rodent hippocampus (Kuhn et al. 1996). They extended their findings into humans by administering BrdU to terminal cancer patients and examining post-mortem samples, where they found BrdU labeling of neuronal cells in the hippocampus, suggesting that adult neurogenesis occurs in the human hippocampus (Eriksson et al. 1998). Studies using atmospheric carbon-14 levels to retrospectively birthdate cells suggest that most neurogenesis in the human brain occurs very early in life (Spalding et al. 2005; Bhardwaj et al. 2006), however, these same methods demonstrate that neurogenesis continues within the human dentate gyrus until roughly twenty years old (Jonas Frisén, unpublished data). Finally, the use of stereotactically injected retroviruses, which only infect dividing progenitor cells, has allowed detailed characterization of adult neurogenesis in the dentate and demonstrated that newborn neurons survive for long periods of time and functionally integrate into the dentate gyrus (Zhao et al. 2006; van Praag et al. 2002). Thus, the development of novel methods has firmly established adult hippocampal neurogenesis as a real phenomenon and contributed to the demise of the dogma that the brain is a static structure.

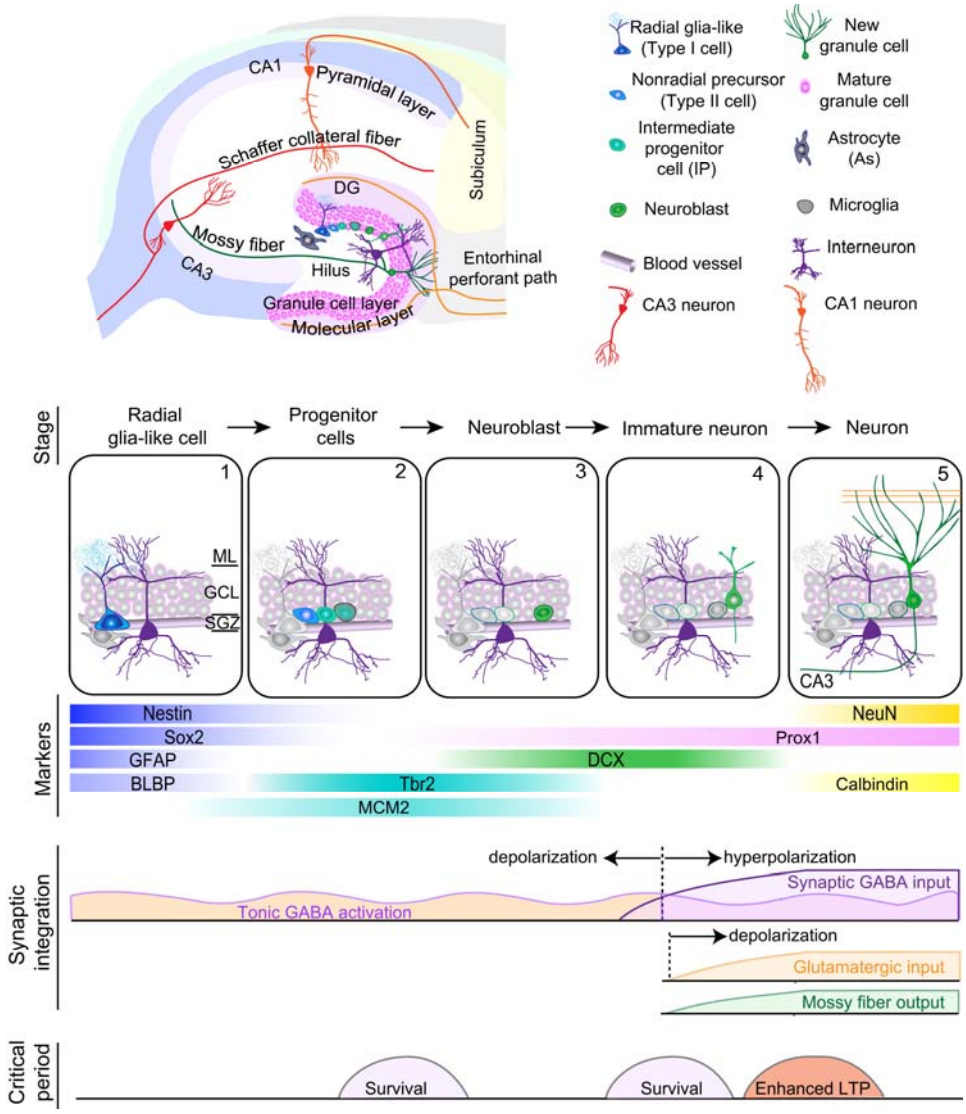

Figure 1.5: Schematic of neurogenesis in the adult dentate gyrus. Newborn neurons are generated from radial glia-like cells, also known as Type 1 cells, in the subgranular zone of the adult dentate gyrus (1). These cells undergo asymmetric cell division and give rise to progenitor cells, also known as Type 2 cells (2). The progenitors give rise to neuroblasts, which have proliferative potential (3). A neuroblasts undergoes differentiation to become an immature neuron (4) and after 4-6 weeks, a mature neuron (5). The interneurons provide GABAergic input and promote differentiation of the newborn neuron. Cell specific markers for each stage are shown, as well as the physiological properties and critical periods. Neurons that survive to 4 weeks integrate into the dentate circuitry and can last throughout the life of the rodent. Adult hippocampal neurogenesis occurs in many species, including humans. From (Ming and Song, 2011).

The development and maturation of newborn neurons in the adult hippocampus undergoes a stereotyped process that passes through many of the same developmental milestones as the developing nervous system, however, the process is much slower than in embryonic development [for review see: (Ming et al. 2005; 2011; Overstreet-Wadiche and Westbrook 2006)]. Radial glia-like cells, also called Type 1 cells, that express GFAP are self-renewing stem cells within the subgranular zone that divide and give rise to neural progenitor cells (Seri et al. 2001). These neural progenitor cells, also known as transiently amplifying cells or Type 2 cells, express Tbr2 and proliferate in response to exercise (Hodge et al. 2008). Once terminal differentiation of the progenitor occurs, the immature neuron expresses doublecortin (Dcx), a microtubule binding protein (Brown et al. 2003). Over the course of three weeks the immature neuron undergoes maturation, migrating into the inner granular layer, sending out dendrites into the molecular layer, forming synaptic connections, and projecting an axon to CA3 (Hastings et al. 1999; Zhao et al. 2006).

Adult neurogenesis is a highly regulated process, with numerous stimuli influencing the process at each stage of neuronal maturation [for review see: (Zhao et al. 2008)]. Maintenance of the of radial-glia stem cell pool is dependent on Notch signaling driving the expression of Sox2 and activation of the canonical Wnt pathway by TLX (Qu et al. 2010; Ehm et al. 2010). GABA, which comes primarily from interneurons, depolarizes the progenitor cells due to the high internal chloride concentration and promotes their differentiation (Tozuka et al. 2005; Markwardt et al. 2009). The GABA-mediated depolarization activates CREB, which promotes dendritic outgrowth and newborn neuron survival (Jagasia et al. 2009). The survival of newborn neurons is also dependent on the expression of NMDA receptors 2-3 three weeks after they are born, thus providing further evidence that activity during differentiation is required for newborn neuron survival (Tashiro et al. 2006). Finally, newborn neurons are hyper-excitable, with a

resting membrane potential around -45 mV, which lowers the threshold for LTP induction and produces more robust potentiation (Overstreet-Wadiche and Westbrook 2006; Schmidt-Hieber et al. 2004). The resting membrane potential decreases to around -70 mV by 4 weeks after birth. Interestingly, exposure to an enriched environment during 1-3 weeks post-injection increased the survival of newborn neurons and increased their activation in response to re-exposure to the enriched environment (Tashiro et al. 2007). However, exposure to an enriched environment during the fourth week post-injection or at four months post-injection had no effect on survival or activation upon re-exposure, suggesting that the period of hyperexcitability during weeks 1-3 post-birth is a critical period for the newborn neurons to encode sensory information.

In addition to activity, a number of behaviors and pathological conditions also affect the proliferation and the survival of newborn neurons. For instance, exercise increases proliferation of newborn neurons, and housing in an enriched environment increases survival (van Praag et al. 2002; Kempermann et al. 1997). In contrast, aging, stress, and inflammation decrease newborn neuron proliferation (Kuhn et al. 1996; Monje et al. 2003; Tanapat et al. 1998). Treatment with anti-depressants or lithium increases neurogenesis (Santarelli et al. 2003; Chen et al. 2000), while opiates and methamphetamine decrease neurogenesis (Kahn et al. 2005; Teuchert-Noodt et al. 2000).

Although adult neurogenesis is clearly a highly plastic process influenced by a wide variety of stimuli, the function of the newborn neurons has been controversial. However, recent studies may be leading to a more unified understanding of their function within the dentate gyrus [for review see: (Aimone et al. 2011; Sahay et al. 2011b)]. Early studies used x-ray irradiation of the brain or administration of the antimitotic drug, methylazoxymethanol (MAM) to inhibit adult neurogenesis. Because the first study demonstrating that ablation of

neurogenesis with MAM impaired hippocampal-dependent learning in the eye-blink conditioning paradigm (Shors et al. 2001), more than 50 different behavioral tests have been done using these inhibitors of neurogenesis in an attempt to determine the function of newborn neurons in learning and memory [See Table S1 in: (Deng et al. 2010)]. These tasks include varying conditions of the Morris water maze, the Barnes maze, contextual, cue and trace fear conditioning, eye blink conditioning, working memory tasks, place recognition tasks, passive avoidance tasks, and spatial discrimination. These studies found that ablation of hippocampal neurogenesis impaired performance on some tasks, but not others, including some hippocampal-dependent tasks. These inconsistent results created controversy regarding the function of newborn neurons. More recent studies have utilized genetic approaches to ablate newborn neurons. Treating adult transgenic mice expressing thymidine kinase under control of GFAP-promoter with ganciclovir causes cell death of radial glial cells and ablates neurogenesis (Saxe et al. 2006). These mice have impaired contextual fear condition, a hippocampal-dependent task, but show no impairment in cued conditioning, which is not hippocampal-dependent. Two other genetic approaches use inducible promoters to drive expression of a toxic gene selectively in the radial glial stem cells in the adult mouse. Imayoshi et al crossed the Nestin-CreER^{T2} mouse to a mouse expressing a flox-stop diphtheria toxin under control of the neuron specific enolase promoter (Imayoshi et al. 2008). Administration of tamoxifen to the adult mouse activated Cre expression in the Nestin-positive radial glial cells and effectively ablated neurogenesis. Tamoxifen treated mice had impaired performance on the Barnes maze, a test of spatial learning. Finally, Dupret et al crossed a Nestin-rtTA mouse with a TRE-Bax mouse (Dupret et al. 2008). Administration of doxycycline to adult mice activates the expression of Bax expression in the Nestin-positive radial glial cells, ablating neurogenesis. Doxycycline treated mice had impaired performance in the variable start water maze. These

findings suggest that neurogenesis plays an important role in performance on certain hippocampal-dependent tasks.

For some time, it has been thought that the dentate gyrus functions to spatially separate patterns (Gilbert et al. 2001). How newborn neurons in the dentate circuitry contribute to this function has been a nagging question. Several computational models led to describing how the hyperexcitability of newborn neurons during their critical period could contribute to dentate function [for review see: (Aimone and Gage 2011)]. Recent behavioral data from the Hen lab demonstrates that genetically increasing the survival of newborn neurons by knocking out Bax in adult radial glial cells using the Nestin-CreER^{T2} mouse improves the performance of mice on a task were they have to distinguish between two similar, but slightly different, environments (Sahay et al. 2011a). Interestingly, these mice had normal performance in common hippocampal learning tasks including object recognition, spatial learning, contextual fear conditioning and extinction learning, which suggests that some of the classic tasks used to test the function of newborn neurons may not be the best experiments for identifying newborn neuron function, and likely contributed to the inconsistent results seen in previous studies. Combining these data with findings from computational studies, Aimone et al propose that newborn neurons allow greater resolution of new memories, which improves discrimination between objects (Aimone et al. 2011). Sahay et al take the idea further, suggesting that increases in neurogenesis improve pattern separation, which facilitates discrimination and cognitive flexibility, while decreases in neurogenesis impair pattern separation, which allows generalization (Sahay et al. 2011b). Boldly extrapolating to human diseases, they hypothesize that too much neurogenesis could contribute to the pathophysiology of disorders characterized by excessive attention to detail, like autism or obsessive-compulsive disorder, while too little neurogenesis could contribute to disorders with excessive generalization like anxiety, post-

traumatic stress disorder, and mild cognitive impairment. While much remains to be done to fully understand the function of newborn neurons in the dentate, it is becoming clear that they contribute to pattern separation.

Chapter 1.5.1: microRNAs in adult neurogenesis

Adult neurogenesis is a highly regulated process, and as such, it is likely that miRNAs are involved in regulating the proliferation and maturation of newborn neurons in the dentate gyrus. However, to date, only a handful of studies have addressed this issue. In adult neural stem cells, miR-184 targets Numblike and suppresses its expression, which promotes differentiation of neural progenitor cells (Liu et al. 2010a). Consistent with this, knockdown of miR-184 *in vivo* increases the number of proliferating, BrdU-positive, cells and decreases the number of differentiating Dcx-positive cells. Thus, miR-184 contributes to the balance between proliferation and differentiation of neural stem cells in the dentate gyrus. Functioning in a similar manner, miR-137 targets Ezh2, a histone methyl-transferase, and promotes the differentiation of neural progenitor cells (Szulwach et al. 2010). Like miR-184, knockdown of miR-137, *in vivo,* increases the number of proliferating, BrdU-positive, cells and decreases the number of differentiating Dcx-positive cells in the dentate. In addition to miR-137 and miR-184, miR-124 also promotes the differentiation of adult neural stem cells, although its function has been described in the subventricular zone (Cheng et al. 2009). miR-124 directly targets Dlx2, Jag1, and Sox9, genes that have been implicated in neural progenitor self-renewal (Nyfeler et al. 2005). Lastly, Luikart et al performed an array on dentate gyrus to identify miRNAs whose expression changed following seizures (Luikart et al. 2011b), which increase the number of newborn neurons as well as the size of their dendritic arbors (Overstreet-Wadiche et al. 2006). They identified several up regulated miRNAs, including miR-132, -137, -212, -335, -337-3p, -338-

3p, -543-3p and -762. These studies suggest that miRNAs are important in regulating the balance between proliferation and differentiation of neural stem cells in the dentate, as well as in the response to seizures.

Finally, in a study conducted in parallel with the work presented in this dissertation, *Luikart et al* investigated the function of miR-132 in the maturation of newborn neurons in the dentate *in vivo* (Luikart et al. 2011a). Using a viral mediated reporter of miRNA activity as well as cell sorting from cell-type specific GFP-expressing transgenic mice, they found that miR-132 expression increases from very low levels in radial glial cells to intermediate levels with in Dcx-positive immature neurons and finally increases to high levels in mature neurons within the dentate gyrus. Furthermore, knockdown of miR-132 using a retroviral sponge decreased spine density on distal dendrites of 21 day old neurons and decreased the frequency, but not amplitude, of spontaneous EPSCs. The amplitude of evoked AMPA currents was decreased in newborn neurons expressing the miR-132 sponge as determined from paired recordings of age-matched granule neurons. Finally, microarray experiments comparing gene expression in PC12 cells expressing the miR-132 sponge with control cells identified up regulation of a large number of genes implicated in immune function, including IL-6, as potential miR-132 targets.

In conclusion, there has been an explosion in the knowledge of the cellular and molecular mechanisms underlying neuronal plasticity since the initial observations by Bliss and Lomo. Numerous discoveries have paved the way to the present, including the identification of PKA, CREB, and miRNAs as well as the characterization of newborn neurons in the adult brain. However, we still have a long way to go to fully appreciate how neurons change in response to activity and the functional implications of those changes. The work presented in this dissertation is focused on understanding how the gene products of one CREB-regulated

microRNA locus, encoding miR-132 and miR-212, function *in vivo*, and adds another chapter to our understanding of how neurons change in response to activity.

CHAPTER 2

microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus

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Chapter 2.1: Abstract

Newborn neurons in the dentate gyrus of the adult hippocampus rely upon cAMP response element binding protein (CREB) signaling for their differentiation into mature granule cells and their integration into the dentate network. Among its many targets, the transcription factor CREB activates expression of a gene locus that produces two microRNAs, miR-132 and miR-212. In cultured cortical and hippocampal neurons, miR-132 functions downstream from CREB to mediate activity-dependent dendritic growth and spine formation in response to a variety of signaling pathways. To investigate whether miR-132 and/or miR-212 contribute to the maturation of dendrites in newborn neurons in the adult hippocampus, we inserted LoxP sites surrounding the miR-212/132 locus and specifically targeted its deletion by stereotactically injecting a retrovirus expressing Cre recombinase. Deletion of the miR-212/132 locus caused a dramatic decrease in dendrite length, arborization, and spine density. The miR-212/132 locus may express up to four distinct microRNAs, miR-132 and -212 and their reverse strands miR-132* and -212*. Using ratiometric microRNA sensors, we determined that miR-132 is the predominantly active product in hippocampal neurons. We conclude that miR-132 is required for normal dendrite maturation in newborn neurons in the adult hippocampus and suggest that this microRNA also may participate in other examples of CREB-mediated signaling.

Chapter 2.2: Introduction

The dentate gyrus of the hippocampus supports neurogenesis throughout life, and the newborn neurons generated at this site form functional synapses, integrate into the dentate network, and contribute to complex spatial learning (van Praag et al. 2002; Dupret et al. 2008). Adult hippocampal neurogenesis is stimulated by spatial learning, physical exercise, and treatment with antidepressant drugs (Tronel et al. 2010; van Praag et al. 1999; Santarelli et al. 2003). Conversely, aging, psychosocial stress, and excess adrenal glucocorticoids are linked to decreases in this type of neurogenesis (Gould et al. 1997; Kuhn et al. 1996; Cameron et al. 1994). Interestingly, these decreases may be reversible, and many of the stimuli that promote neurogenesis also promote dendritic growth and increased arbor complexity (Jin et al. 2003). This response suggests that these newborn neurons have a high degree of plasticity that is strongly influenced by external stimuli.

One potential mechanism underlying the dendritic plasticity of newborn hippocampal neurons involves the transcription factor cAMP response element binding protein (CREB), which mediates signals from a variety of signal-transduction cascades. Several studies have shown that blocking CREB leads to a dramatic decrease in hippocampal neurogenesis and dendritic arborization in newborn neurons (Nakagawa et al. 2002; Jagasia et al. 2009), although disparate results also have been reported (Gur et al. 2007). Because CREB can activate thousands of genes, it is difficult to associate specific targets with particular CREB functions. In an effort to characterize these targets, we developed a method termed “Serial Analysis of Chromatin Occupancy” to identify CREB-binding sites on a genome-wide level (Impey et al. 2004). Many noncoding RNAs, including one that encoded two microRNAs, miR-212 and miR-132, were among the CREB targets. Introduction of miR-132 into primary cortical neurons stimulated robust neurite outgrowth, whereas inhibition of miR-132 blunted neurite outgrowth under basal

conditions and blocked the response to BDNF (Vo et al. 2005). We demonstrated that these effects occurred, at least in part, through the actions of miR-132 on the GTPase-activating protein, p250GAP, which inhibits the activities of Rac and Cdc42. Subsequent studies using more mature cultures showed that miR-132 and p250GAP mediated the dendritic growth and spine formation induced by depolarization and synaptic activity (Wayman et al. 2008; Impey et al. 2010). Thus, we proposed that miR-132 was both necessary and sufficient for activity-stimulated changes in dendritic growth and spine formation in neuronal cultures. Other microRNAs mediate similar effects in culture (Schratt et al. 2006; Siegel et al. 2009; Edbauer et al. 2010); however, it has not been determined whether miR-132 or any of these other microRNAs have these actions *in vivo*.

Knockout mice have proven to be an excellent tool for analyzing gene function *in vivo*. However, few brain microRNAs have been studied using this method, largely for technical reasons. Many brain microRNAs, such as miR-124, are transcribed from multiple locations in the genome, complicating the generation of effective knockouts. Some brain microRNAs, such as miR-134, are located in clusters, making it difficult to delete one microRNA without affecting the expression of others. In other situations, as exemplified by miR-9 and -9*, biological functions have been ascribed to both strands of the microRNA, complicating interpretation of which microRNA is responsible for a particular outcome (Packer et al. 2008). miR-132, however, is transcribed from only a single locus and is generated from a precursor that encodes only a limited number of other microRNAs. To determine which of the four potential microRNAs were generated from the miR-212/132 locus, we developed a set of bidirectional ratiometric sensors that were capable of distinguishing their activities at single-cell resolution. Ablation of the miR-212/132 locus dramatically reduced dendritic length, branching, and spine density in newborn hippocampal neurons in young adult mice. Furthermore, we show that miR-132 is the primary

functional product of the miR-212/132 locus in these cells, suggesting that it is a key mediator of the dendritic phenotype.

Chapter 2.2: Material and Methods

Mice: Floxed miR-132/212 mice were generated by Ozgene. A targeting vector was generated from three fragments, a 0.8-kb LoxP arm, a 6.3-kb 3' homology arm, and a 7.6-kb 5' homology arm. As part of the LoxP arm, a PGK-Neo-pA selection cassette was included for clonal selection. This cassette was immediately flanked by flippase recognition target (FRT) sites, allowing its later removal using flippase recombinase. The linearized targeting vector was transfected into Bruce4 embryonic stem cells, which are derived from a C57BL6 mouse (Köntgen et al. 1993). To screen for correct integration of the targeting construct, we used 5' and 3' probes generated from C57BL6 genomic DNA. A neo2 probe was used to test for additional random integration of the targeting vector, which was evaluated by Southern blotting. Following homologous recombination and selection with neomycin, the clonal floxed ES cells were injected into Balb/C blastocysts to generate chimeric mice. These mice were crossed into C57Bl6 mice to attain germline transmission of the floxed allele. Mice used in this study had a mixed background of C57Bl6 and Balb/C but had been backcrossed into C57Bl6/J mice for at least two generations. Rosa-YFP reporter mice (Jackson Laboratory, Strain 006148) and Deleter-Cre (Jackson Laboratory, Strain 006054) were obtained from the Jackson Laboratory. Mice were housed in accordance with the Oregon Health and Science University Institutional Animal Care and Use Committee (OHSU IACUC) regulations.

Viruses: The CAG-GFP-Cre retrovirus (Tashiro et al. 2006) was a generous gift from F. Gage (Salk Institute, La Jolla, CA). The mCherry virus was generated by introducing a BstBI site into pSie (Ge

et al. 2006) by ligating a linker into the BamHI/XbaI digested vector. pSie and FUGW then were digested with XhoI and BstBI to isolate the retroviral backbone from pSie and the ubiquitin promoter, GFP, and the woodchuck posttranscriptional regulatory element fragment from FUGW. These fragments were ligated to generate pRubi. GFP then was replaced with mCherry using the EcoRI/BamHI sites in pRubi to generate redRubi, which was used to produce the mCherry virus. Retroviral and lentiviral preparations were produced using standard techniques (Lois et al. 2002; Yee et al. 1994). Lentiviruses used for the sensor injections were titered by flow cytometry.

Stereotactic Injections: Stereotactic injections were performed as previously described (Cetin et al. 2006). Briefly, 8-wk-old homozygous floxed male mice were placed in a cage with a running wheel the day before injection. Mice were anesthetized with isoflurane, and 2 µL of concentrated virus was injected at y = −1.9 mm, x = ± 1.1 mm, z = −2.3 and −2.5 mm relative to bregma at a rate of 0.2 µL/min. Mice were housed in a cage with a running wheel until analysis. All experiments were approved by the OHSU IACUC.

Immunohistochemistry: Mice were perfused with 4% paraformaldehyde in PBS with 4% sucrose and were postfixed overnight. The brain was cut into 100-µm sections with a vibratome. Free-floating sections were permeabilized with 0.4% Triton X-100 in PBS and blocked with 5% horse serum (Jackson ImmunoResearch). Sections were stained overnight at 4°C with the following primary antibodies: rabbit anti-GFP conjugated to Alexa Fluor 488 (1:500; Invitrogen), mouse anti-mCherry (1:5,000; Clontech), mouse anti-GFAP (1:500; Millipore), mouse anti-NeuN (1:1,000; Chemicon), and guinea pig anti-Doublecortin (1:500; Chemicon). The sections were incubated for 6 h at room temperature with secondary antibodies: anti-mouse Cy3 (1:200;

Jackson ImmunoResearch) and anti-guinea pig Alexa Fluor 647 (1:200; Invitrogen). Sections were imaged on a Zeiss LSM710 confocal microscope using a Plan-Apo 20× objective (dendritic arborization and sensors) and a Plan-Apo 63× objective with a 3× digital zoom (spines). Dendritic length, branching, and spine density were quantified blind by manually tracing through Z-stacks using National Institutes of Health ImageJ (National Institutes of Health); DCX/GFP-positive neurons were counted with MetaMorph (Molecular Devices).

Flow cytometry: Flowcytometry analysis was performed on the Aria II (BDBiosciences). Asingle 488-nm laser excited both green fluorescent protein from Aequorea coerulscens (AcGFP) and Discosoma sp. Express-1 (DsRedEx1). Cells were gated to exclude debris, and a standard doublet-exclusion was performed. Compensation was calculated automatically for each experiment using no color, AcGFP-only, and DsRedEx1-only controls. AcGFP levels were detected with FL1 and a 530/30 filter, and DsRedEx1 levels were detected with FL2 and a 585/42 filter. For SH-SY5Y cells, 1×10^4 red-positive cells were evaluated per condition, and $1–5 \times 10^3$ red-positive neurons were evaluated per condition. Before flow analysis, cells were trypsinized and passed through a 35-μm mesh strainer. Data were analyzed and plotted with FlowJo. Sensor ratios were normalized to Scrambled microRNA recognition elements (MRE) controls from each experiment, and the SEM from multiple experiments is represented.

Cell Culture: SH-SY5Y cells were grown in 1:1 DMEM:F12 medium supplemented with 10% FBS. Lipofectamine 2000 was used according to the manufacturer's instructions to transfect the sensors alone or with RNA oligos. For retinoic acid treatment, the medium on transfected cells was replaced the day after transfection with fresh medium containing 10 μM retinoic acid. The next day, the medium was replaced again with fresh medium containing 10 μM retinoic acid.

Cells were collected 48 h after the application of the first treatment. Dissociated hippocampal cultures were obtained from 21d embryonic rat pups and were cultured in Neurobasal medium supplemented with B-27 and 1 mM Glutamax (Invitrogen). Neurons were grown either on plates or on acid-washed glass coverslips that had been coated with 0.5 mg/mL poly-L-lysine. Sensors were either nucleofected for flow experiments (2.5 × 106 neurons per nucleofection) before seeding or were transfected with Lipofectamine 2000 for older microscopy experiments.

Chapter 2.4: Results

Chapter 2.4.1: Generation of a miR-212/132 floxed mouse

miR-132 is located 200 bases downstream from miR-212 in Ensembl gene ID ENSMUSG00000065537. Canonical CREB- and RE1-silencing transcription factor (REST)-binding sites lie between the two microRNA sequences, and two additional CREB-binding sites are located ~100 and 150 bases upstream (Figure 2.1A). We generated a targeting vector with parallel LoxP sites 0.4 kb upstream and downstream from the miR-132 gene, creating a LoxP arm spanning 0.8 kb of the first intron. A PGK-Neo-pA cassette flanked by flippase recognition target (FRT) sites was included for clonal selection. Following selection with neomycin and confirmation of the correct integration site by Southern blot, clonal ES cells were injected into Balb/C blastocysts. Chimeric mice were crossed into a C57Bl6 mouse, and germline transmission of the floxed locus was confirmed by PCR. Expression of mature miR-132 and miR-212 in the cortex of the floxed mice was reduced by 46% and 69%, respectively (Figure 2.1C), and a similar reduction occurred in the hippocampus (Figure 2.2). Presumably, this hypomorphic phenotype is caused by the retention of the neomycin cassette. The homozygous floxed miR-212/132 mice were found to be viable, fertile, and grossly normal. The expression of

mature miR-132 was considerably higher than the expression of miR-212 in these mice as well as in the control C57Bl6 strain (Figure 2.1C). Crossing the floxed mouse with a ubiquitous Deleter-Cre mouse caused genomic deletion of the miR-212/132 locus, as assessed by PCR, and completely eliminated expression of mature miR-132 and miR-212 (Figure 2.1B, C).

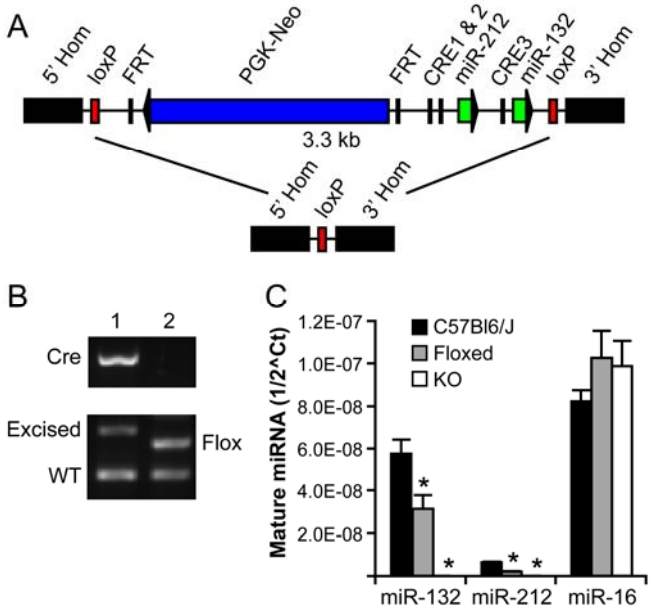

Figure 2.1: Generation and validation of floxed miR-212/132 mice. (*A*) Schematic of miR-212/132 targeting vector before and after Cre-mediated excision. The floxed mice have a PGK-Neo cassette upstream of the miR-212/132 locus. A REST binding site is located between CRE1 and CRE2 and between miR-212 and CRE3. (*B*) PCR analysis showing that the floxed allele is excised only when Cre is present (lane 1) in heterozygous floxed mice. (*C*) Mature miR-132 and miR-212 are decreased in the cortex of floxed mice compared with C57Bl6/J mice and are absent in the knockout mice generated by crossing the floxed mouse with a Deleter-Cre mouse (miR-132: $f_{2,15}$ = 20.3, P < 0.001; Tukey's post hoc test, P < 0.01 for all comparisons; miR-212: $f_{2,15}$ = 98.8, P < 0.001; Tukey's post hoc test, P < 0.01 for all comparisons; error bars show SEM). There was no change in the level of miR-16, an unrelated microRNA.

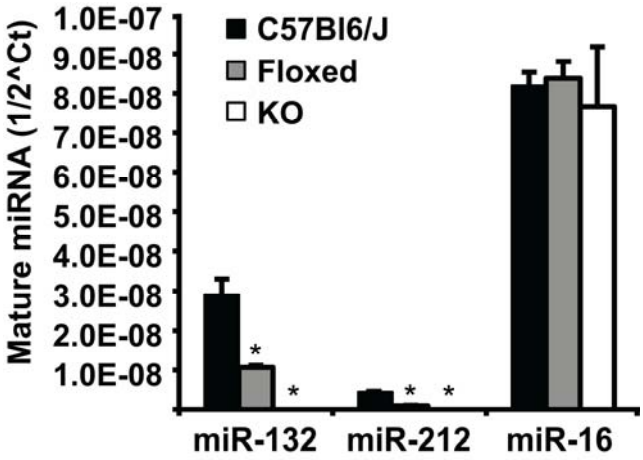

Figure 2.2: Mature miR-132 and -212 expression in the hippocampus of adult mice. miR-132 and -212 are decreased in the hippocampus of floxed mice compared with C57Bl6/J mice and are absent in the knockout mice generated by crossing the floxed mouse with a Deleter-Cre mouse as determined using Taqman assays (miR-132: $f_{2,12} = 24.6$, $P < 0.001$; Tukey's post hoc test, *$P < 0.01$ for all comparisons; miR-212: $f_{2,12} = 40.2$, $P < 0.001$; Tukey's post hoc test, *$P < 0.01$ for all comparisons; error bars indicate SEM). There was no change in the level of miR-16, an unrelated microRNA. n = 6 C57Bl6/J mice, 6 floxed mice, 3 knockout mice.

Chapter 2.4.2: Knockout of miR-212/132 in Newborn Hippocampal Neurons Decreases Dendritic Length and Arborization

To determine whether miR-132 influences dendritic length and arborization *in vivo*, we stereotactically injected the dentate gyrus of homozygous floxed miR-212/132 mice with a retrovirus expressing a GFP-Cre fusion protein (Tashiro et al. 2006). The GFP-Cre retrovirus was active when injected into the dentate gyrus, as monitored by its effect on mice containing a floxed stop allele upstream of YFP in the ROSA26 locus (Figure 2.3) (Srinivas et al. 2001). Along with the GFP-Cre retrovirus, we coinjected a retrovirus expressing mCherry, which allowed visualization and quantification of dendritic length, branching, and spines. C57Bl6/J mice also were injected with the mCherry virus alone as a control. To promote hippocampal neurogenesis (van Praag et al. 1999), mice were housed with a running wheel from 1 d before injection until they were killed 21 d postinjection. Dendritic length and arborization were quantified in newborn neurons from floxed mice coexpressing GFP-Cre and mCherry (Flox-Cre) and were compared with neighboring newborn neurons in the same image expressing mCherry alone (Flox-Ctrl) as well as with newborn neurons in C57Bl6/J mice that had been injected with the mCherry virus alone (WT) (Figure 2.4). The GFP-Cre virus had no effect on WT newborn neurons (Figure 2.5). The Flox-Cre newborn neurons had dramatically decreased dendritic arbors compared with neighboring Flox-Ctrl neurons. Furthermore, the Flox-Ctrl neurons (which express about half the levels of miR-132 and miR-212 as WT neurons) had decreased length and arbors compared with WT mice, suggesting that the degree of dendritic growth depends upon the level of miR-212/132 products. Total dendritic length was decreased by 33% in the Flox-Ctrl and 76% in the Flox-Cre newborn neurons relative to WT neurons (Figure 2.4G; n = 27 or 28 cells from three or four mice; one-way ANOVA, $f_{2,79} = 83.9$, $P < 0.001$; Tukey's post hoc test, $P < 0.001$ for all comparisons). Total dendritic branching decreased by 31% in the Flox-Ctrl and by

66% in the Flox-Cre newborn neurons relative to WT neurons (Figure 2.4A–F and H, one-way ANOVA, $f_{2,79} = 68.0$, $P < 0.001$, Tukey's post hoc test, $P < 0.001$ for all comparisons). To ensure that Flox-Cre newborn neurons, which often had only one dendrite, were not radial glial cells, we immunostained for glial fibrillary acidic protein (GFAP), Doublecortin (DCX), and the neuron-specific protein NeuN. We found that 96% of Flox-Cre newborn neurons costained with NeuN, and 90% costained with DCX, whereas no newborn neurons costained with GFAP, suggesting that the Flox-Cre neurons are indeed immature granule neurons and not radial glial cells (Figure 2.6). Overall, these results indicate that microRNAs encoded by the miR-212/132 locus regulate dendritic length and arborization in newborn hippocampal neurons *in vivo*.

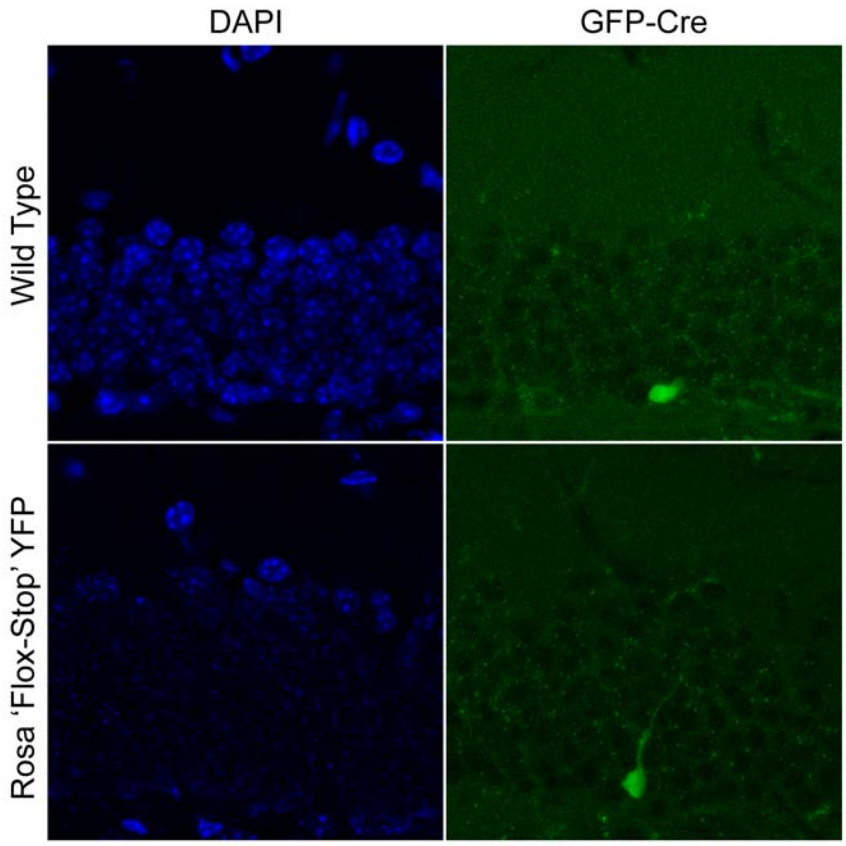

Figure 2.3: Injection of GFP-Cre retrovirus into the dentate gyrus causes cleavage of a floxed allele *in vivo*. At 7 d postinjection, GFP staining was restricted to the nucleus of a WT newborn neuron. However, in a Rosa-'Flox-Stop'-YFP mouse, GFP was seen in the primary dendrite emerging from the newborn neuron as well as in the cell body, indicating that Cre is active and the floxed STOP codon was excised, allowing soluble YFP expression throughout the neuron.

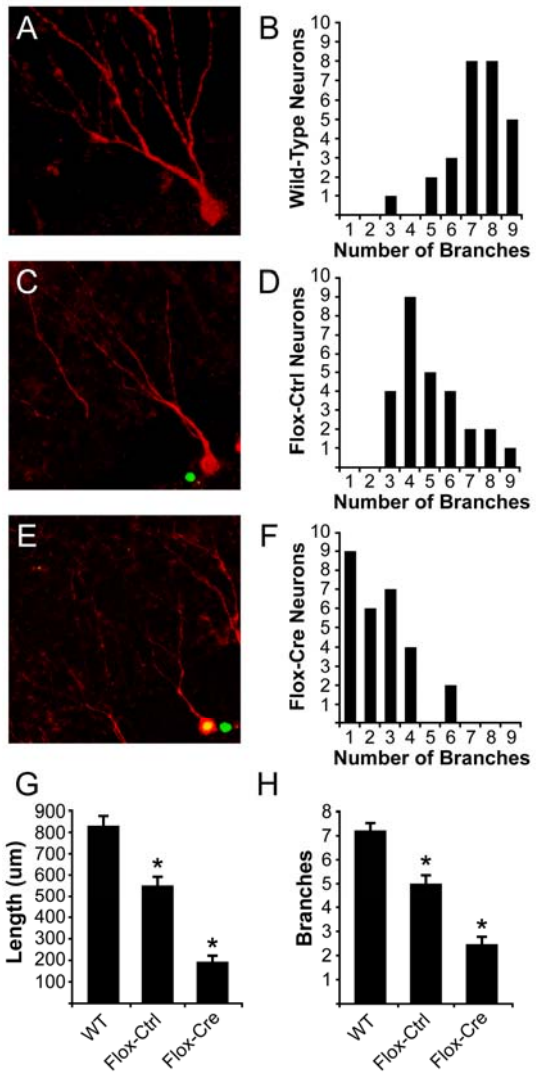

Figure 2.4: Loss of miR-212/132 decreases dendritic length and branching in newborn neurons in the adult hippocampus. Representative neurons are shown 21 d postinjection. (*A*) C57Bl6/J mouse injected with mCherry virus. (*C* and *E*) Floxed miR-212/132 mouse injected with mCherry and GFP-Cre (*C* shows a neuron from a floxed mouse that did not coexpress GFP-Cre.). (*B*, *D*, *F*, and *H*) Dendritic branching is decreased in Flox-Ctrl and Flox-Cre mice ($f_{2,79}$ = 68.0, P < 0.001; Tukey's post hoc test, P < 0.001 for all comparisons). (*G*) Total dendritic length is decreased in Flox-Ctrl and Flox-Cre neurons ($f_{2,79}$ = 83.9, P < 0.001; Tukey's post hoc test, P < 0.001 for all comparisons; error bars show SEM).

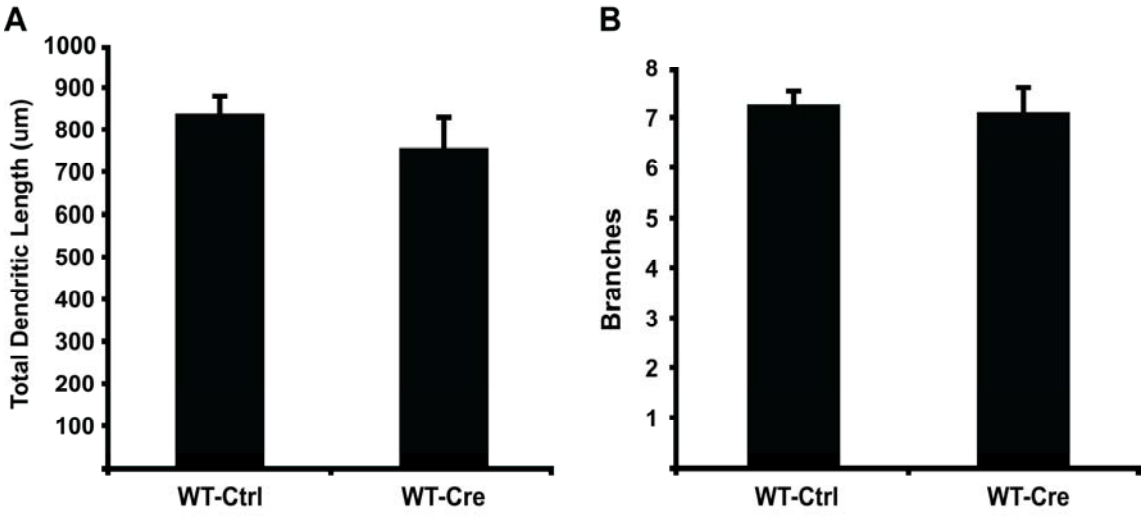

Figure 2.5: The GFP-Cre retrovirus does not influence dendritic length or branching in WT mice. C57Bl6/J mice were injected with mCherry retrovirus alone (WTCtrl) or with mCherry and GFP-Cre viruses (WT-Cre). The mice were sacrificed 21 d postinjection. Dendritic length (A) and branching (B) did not differ between mCherry-positive newborn neurons (WT-Ctrl, n = 27) and mCherry, GFP-Cre double-positive newborn neurons (WT-Cre, n = 10). Error bars show SEM.

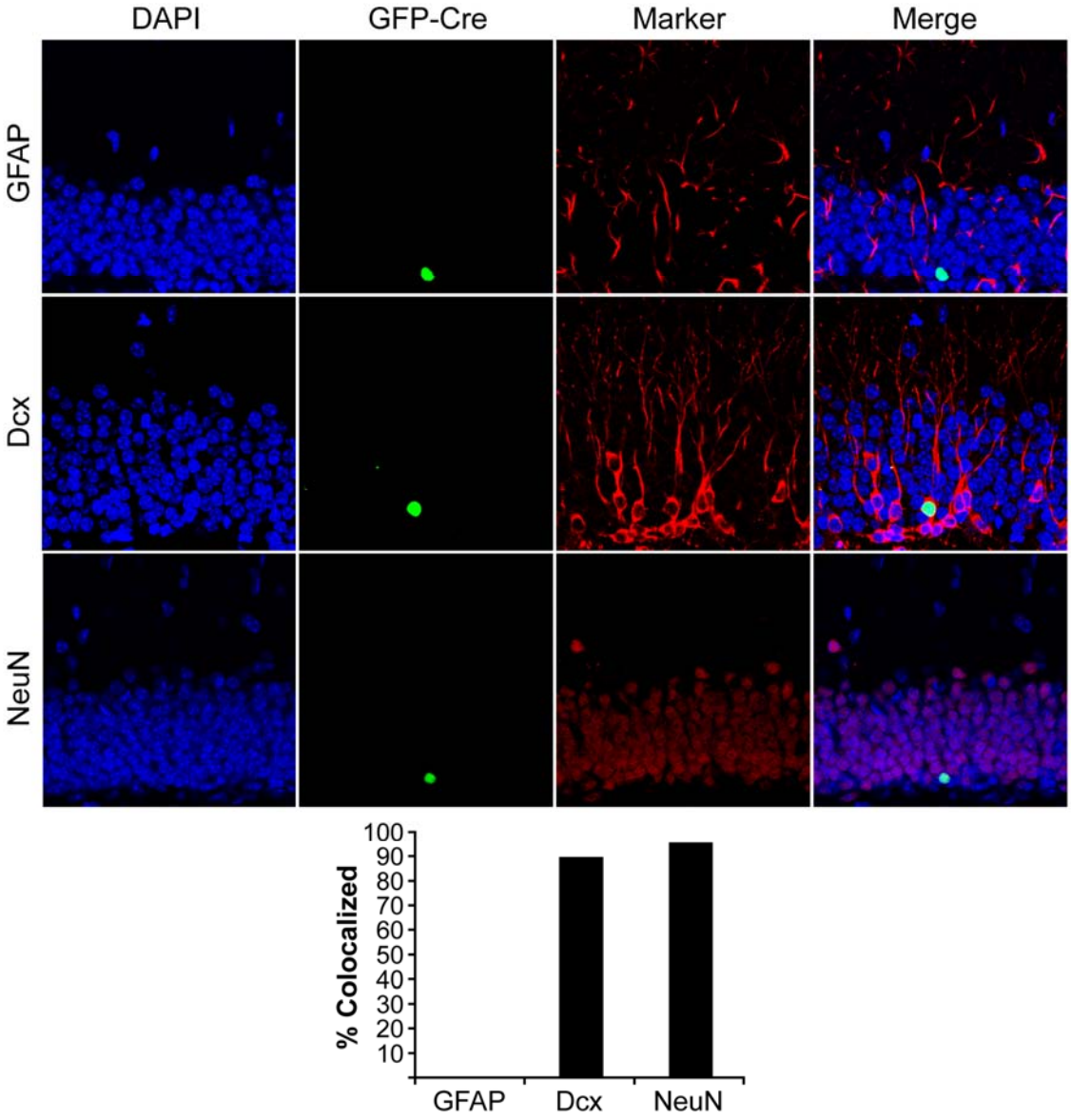

Figure 2.6: GFP-Cre retrovirus infects newborn granule neurons but not radial glial cells. Representative confocal sections showing that GFP-Cre colocalizes with doublecortin (DCX) and the neuron-specific protein NeuN, but not with glial fibrillary acidic protein (GFAP). GFP-Cre was never found to colocalize with GFAP, whereas 90% of GFP-Cre cells colocalized with doublecortin and 95% colocalized with NeuN (n = 49 neurons from four mice for each antibody).

Chapter 2.4.3: Knockout of miR-212/132 reduces spine density on newborn hippocampal neurons

We next examined whether products from the miR-212/132 locus affected dendritic spine formation. The severity of the dendritic phenotype prevented us from assessing spine density in many Flox-Cre newborn neurons because they lacked secondary dendrites and their dendritic arbor barely extended beyond the granule cell layer (Figure 2.4F). Consequently, we quantified spine density on second- and third-order dendrites in the inner half of the molecular layer. Flox-Cre neurons that had secondary dendrites had a 24% decrease in spine density compared with WT neurons and a 20% decrease compared with Flox-Ctrl newborn neurons (Figure 2.7; n = 30 dendrites from three or four mice; one-way ANOVA, $f_{2,87} = 11.1$, $P < 0.001$; Tukey's post hoc test, $P < 0.01$ WT or Flox-Ctrl compared with Flox-Cre).

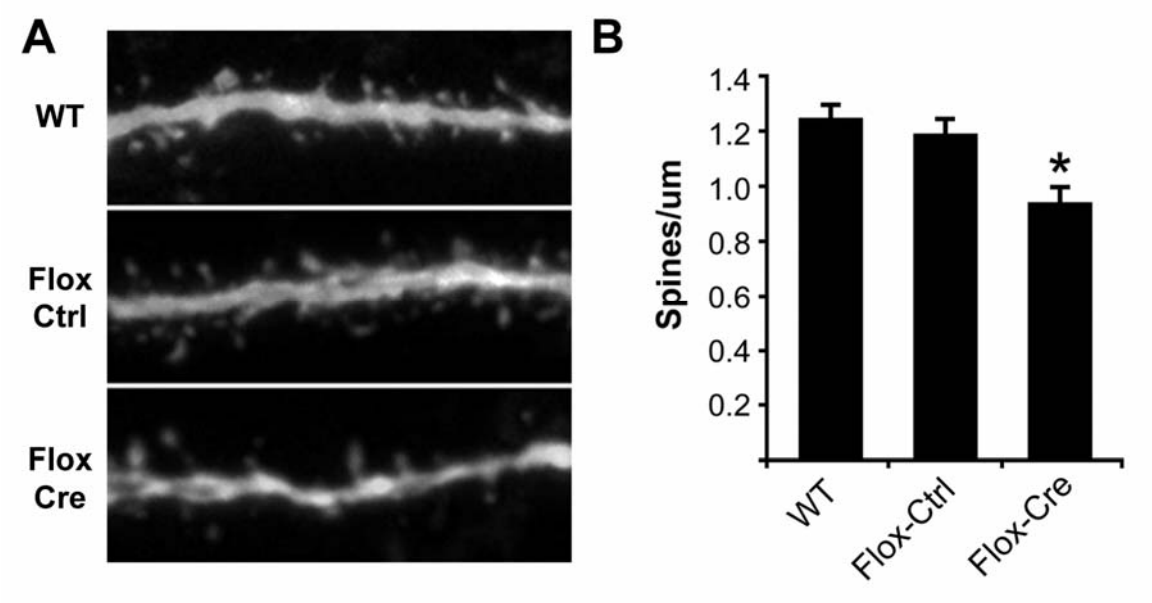

Figure 2.7: Ablation of miR-212/132 decreases spine density. Dendritic spine density on second- and third-order dendrites in the inner molecular layer of newborn granule neurons is decreased by 24% in Flox-Cre neurons ($f_{2,87} = 11.1$, $P < 0.001$; Tukey's post hoc test, *$P < 0.01$ WT or Flox-Ctrl compared with Flox-Cre). Error bars show SEM.

Chapter 2.4.4: miR-132 is the predominant microRNA generated from the miR-212/132 cluster

Deletion of the miR-212/132 locus ablated four potential microRNAs, miR-132, -212, -132* and -212*. To determine which of these microRNAs are functionally important in the newborn hippocampal neurons, we developed a set of bidirectional ratiometric sensors capable of distinguishing each potential microRNA. These sensors feature a bidirectional promoter to express simultaneously distinct transcripts encoding a green fluorescent protein from *Aequorea coerulscens* (AcGFP) and a red fluorescent protein, *Discosoma sp.* Express-1 (DsRedEx1) (Figure 2.8A). In the 3' UTR of the AcGFP transcript, we incorporated three perfectly complementary microRNA recognition elements (MRE) that corresponded to the mature microRNA of interest (Figure 2.8A). Thus, the level of AcGFP expression reflects the activity of the microRNA in a particular cell. We minimized the effects of variable expression levels and nonspecific transcriptional regulation by normalizing the AcGFP level to the internally expressed DsRedEx1 that lacked MREs. This approach provided a ratiometric measure that represents the activity of the specific microRNA with single-cell resolution.

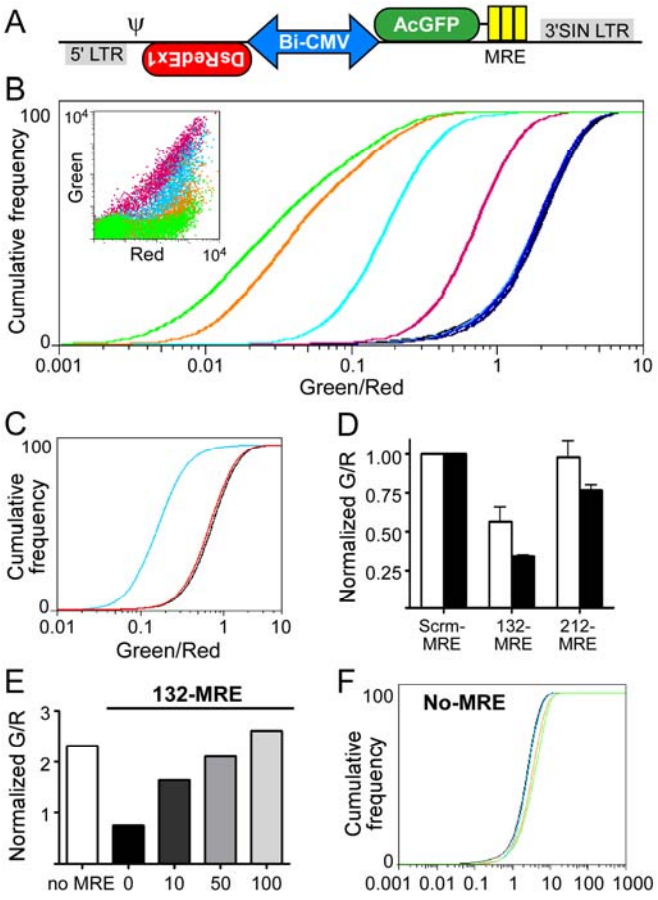

Figure 2.8: Ratiometric sensors for microRNA activity (legend next page).

Figure 2.8: Ratiometric sensors for microRNA activity. (*A*) A bidirectional promoter (Bi-CMV) simultaneously drives expression of AcGFP whose 3′ UTR contains three MREs. DsRedEx1 is expressed in the antisense direction as an internal control. Lentiviral packaging elements include *psi* and self-inactivating (SIN) LTRs. (*B*) SH-SY5Y neuroblastoma cells expressing the 132-MRE sensor were transfected with increasing amounts (up to 10 nM total) of miR-132 mimic and a miR-Scrm mimic (pink = 0 nM; cyan = 0.1 nM; orange = 1.0 nM; green = 10 nM). The G/R ratio in 1×10^4 individual cells per condition was assessed using flow cytometry and graphed as a cumulative frequency distribution. Black and blue lines represent the response of the negative control No-MRE sensor to the same concentrations of miR-132 mimic. (*C*) The 132-MRE sensor responded to the exogenous (0.1 nM) miR-132 mimic (blue) but not to the highly related miR-212 mimic (red). (*D*) 132-MRE and 212-MRE sensors were expressed in SH-SY5Y cells under growth (white bars) and differentiation (black bar) conditions (1% serum + 10 ng/mL retinoic acid for 2 d). The G/R ratios were normalized to that of the Scrm-MRE negative control. (*E*) Addition of 2′O-methyl inhibitors specific to miR-132 confirmed that the 132-MRE sensor detected endogenous miR-132 in SH-SY5Y cells. (*F*) The same concentrations of 2′O-methyl inhibitors did not affect expression of the No-MRE control vector. Experiment performed by Lulu Cambrone.

To evaluate its dynamic range, we expressed the sensor for miR-132 (132-MRE) with increasing amounts of miR-132 mimic in SH-SY5Y neuroblastoma cells and used flow cytometry to obtain ratiometric green/red (G/R) measurements in 1×10^4 individual red-positive cells per condition (Figure 2.8B). A decreased G/R ratio indicated microRNA activity. Notably, we detected endogenous miR-132 in these cells (Figure 2.8B, pink lines compared with black lines) and observed a dose-dependent decrease in the G/R measurement of 132-MRE that corresponded to increasing amounts of expressed miR-132 mimic (Figure 2.8B, cyan, orange, and green lines, respectively). Importantly, the miR-132 mimic had no effect on a control sensor lacking MREs (designated "No-MRE"; Figure 2.8B, dark blue lines compared with black lines). Sensors for miR-212 and miR-212* had similar responses, indicating that both could be used to detect their cognate microRNAs (Figure 2.9A). MREs containing mismatches at positions 9–11 responded in the same manner but with a narrower dynamic range.

To assess their specificity, we tested various combinations of sensors and microRNA mimics. Importantly, expression of scrambled microRNA mimics (miR-Scrm) did not change the ratios, and the mimics were highly specific for particular sensors (Figure 2.9B). Notably, 132-MRE could even distinguish between ectopically expressed miR-132 and miR-212 mimics (Figure 2.8C), which have identical seed sequences and whose mature sequences differ by only four nucleotides (Figure 2.9C). Using Taqman assays, we confirmed that endogenous expression of miR-132 and miR-212 fell well within the range of the ability of the sensors to distinguish the ectopically expressed mimics (Figure 2.10A).

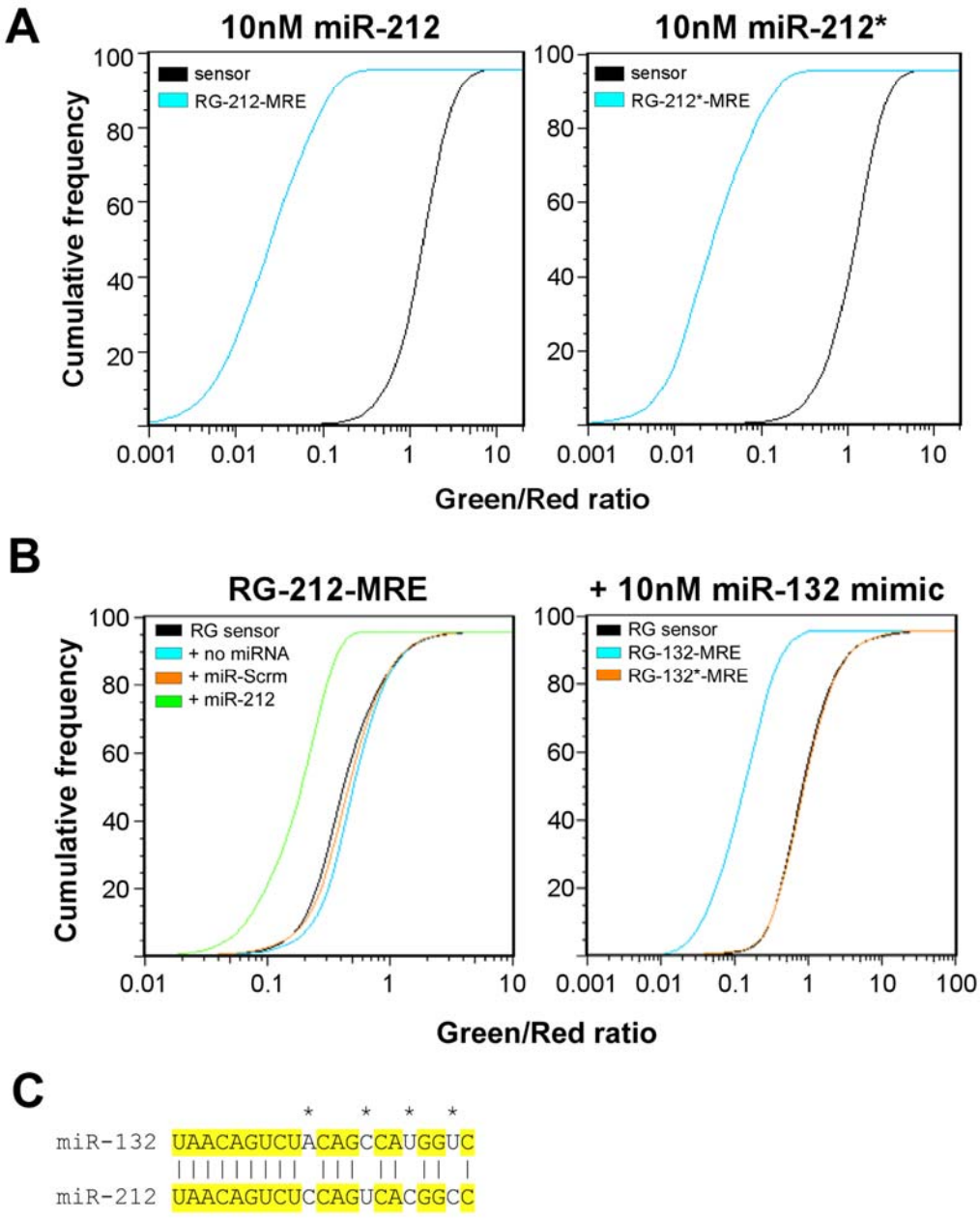

Figure 2.9: Range and specificity of microRNA sensors (legend next page).

Figure 2.9: Range and specificity of microRNA sensors. (A) Range and responsiveness of miR-212 and miR-212* sensors. The 212-MRE and 212*-MRE sensors responded to ectopic expression of miR-212 and miR-212*, respectively, with similar dynamic ranges similar to those in 132-MRE. (B) Specificity of sensors. (Left) 212-MRE sensor responded when miR-212 was ectopically expressed in SH-SY5Y cells but not a scrambled microRNA duplex (miR-Scrm). (Right) Expression of ectopic miR-132 affected the 132-MRE sensor but not the 132*-MRE sensor. (C) miR-132 and miR-212 homology. miR-132 and miR-212 have identical seed sequences and differ by only four nucleotides. Experiment performed by Lulu Cambrone.

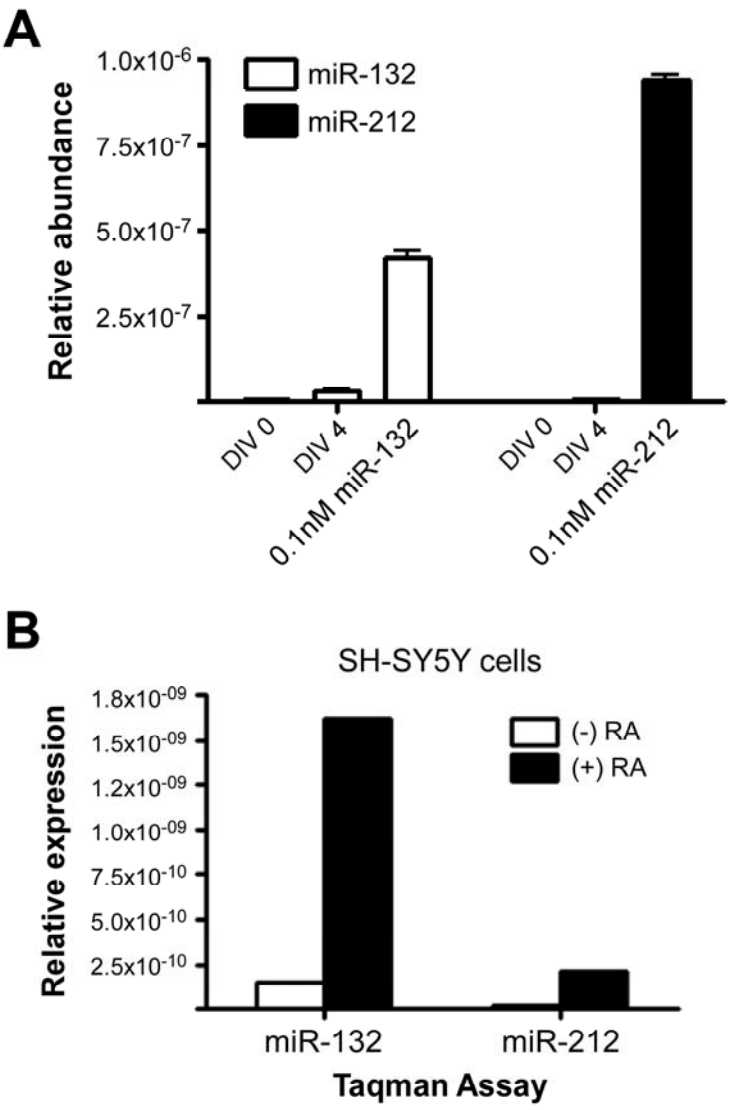

Figure 2.10: miR-132 expression in hippocampal cultures and SH-SY5Y cells. (A) Taqman assays comparing the endogenous levels of mature miR-132 and miR-212 in E21 rat hippocampal cultures (labeled DIV 0 and DIV 4) and SH-SY5Y cells transfected with 0.1 nM microRNA mimic. DIV, days in vitro. (B) Induction of endogenous miR-132 and miR-212 in SH-SY5Y cells. Cells were cultured for 48 h in growth medium (white bar) or in differentiation medium (black bar) consisting of 1% serum plus 10 µM retinoic acid. Total RNA was isolated, and Taqman assays were performed to measure the abundance of endogenous miR-132 and miR-212. Experiment performed by Lulu Cambrone.

We next tested whether the sensors could detect induced microRNA activity. Using Taqman assays, we had detected miR-132 and a much lower level of miR-212 in SH-SY5Y cells under basal conditions (Figure 2.10B). Treatment with retinoic acid produced equivalent fold-increases in both miR-132 and miR-212; however, the absolute level of miR-132 was considerably higher (Figure 2.10B). We applied our sensors to the same paradigm and, under basal conditions, saw a decreased G/R ratio between 132-MRE and a sensor with a scrambled MRE (Scrm-MRE) and no significant difference between Scrm-MRE and 212-MRE (Figure 2.8D). Upon treatment with retinoic acid, the G/R ratio of 132-MRE decreased further, and we observed a small but significant decrease of the G/R ratio in 212-MRE (Figure 2.8D). This observation suggests that the sensors were capable of detecting endogenous and inducible microRNA activity. To confirm that the decrease in the observed G/R ratio was the result of miR-132 activity, we used increasing concentrations of a 2'O-methyl miR-132 inhibitor and observed a corresponding return of the ratio back to the No-MRE sensor control (Figure 2.8E). Importantly, the inhibitor had no significant effects on the No-MRE sensor control (Figure 2.8F).

To establish the specificity and sensitivity of the sensors in primary neurons, we performed Taqman assays for miR-132 and miR-212 on a population of dissociated hippocampal neurons that had been freshly dissected or cultured for 4d. In both cases, we observed a greater abundance of miR-132 than miR-212 (Figure 2.11A). To monitor the activity of each microRNA, we introduced the sensors 132-MRE, 132*-MRE, 212-MRE, and 212*-MRE into dissociated hippocampal cultures and used flow cytometry to assay the G/R ratio either the next day or after culturing for 3–5 d in vitro (DIV 3–5) (Figure 2.11B–E). Approximately 1–5 × 10^3 individual red-positive live cells were evaluated per condition for each experiment, and experiments were repeated with seven different cultures. At DIV 1, we did not observe significant differences in the G/R ratio for any of the microRNAs. In contrast, at DIV 3–5 the

sensors reported a significant level of miR-132 activity but did not detect significant miR-132*, -212, or -212* activity (Figure 2.11B–E). This result was confirmed using quantitative confocal microscopy of individual neurons at DIV 1 (Figure 2.12) and DIV 4 (Figure 2.11F) in which imaging conditions were calibrated based on green-only and red-only controls (Figure 2.13). Moreover, the predominance of miR-132 activity in these neurons extended to DIV 17 (Figure 2.12).

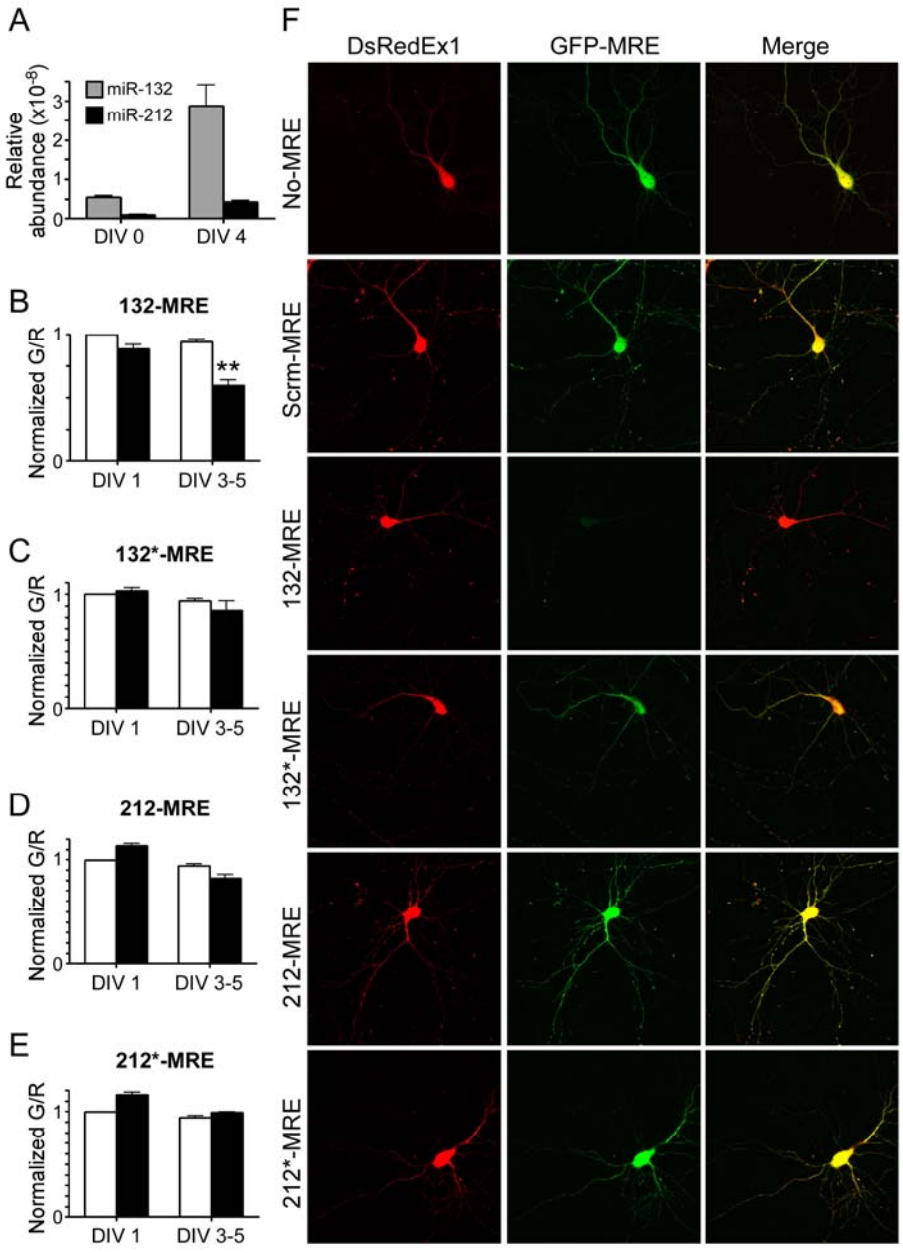

Figure 2.11: miR-132 is the predominant functional product of the miR-212/132 locus in primary hippocampal neurons (legend next page).

Figure 2.11: miR-132 is the predominant functional product of the miR-212/132 locus in primary hippocampal neurons. (*A*) Taqman assays from DIV 0 and 4 dissociated hippocampal cultures measuring the relative abundance of mature miR-132 and miR-212. (*B*–*E*) Sensors (black bars) for each of the putative microRNAs from the miR-212/132 locus were expressed in dissociated hippocampal cultures and assessed with flow cytometry after DIV 1 or DIV 3–5. The median G/R ratios of 5×10^3 neurons were normalized to that of control sensors (white bars) in the same experiment, and the SEM from seven cultures is shown. $^{**}P < 0.01$; ANOVA $P < 0.001$. (*F*) Representative confocal images of the sensors in DIV 4 neurons. Scrm-MRE control corresponds to *C. elegans* miR-239b. Experiment performed by Lulu Cambrone.

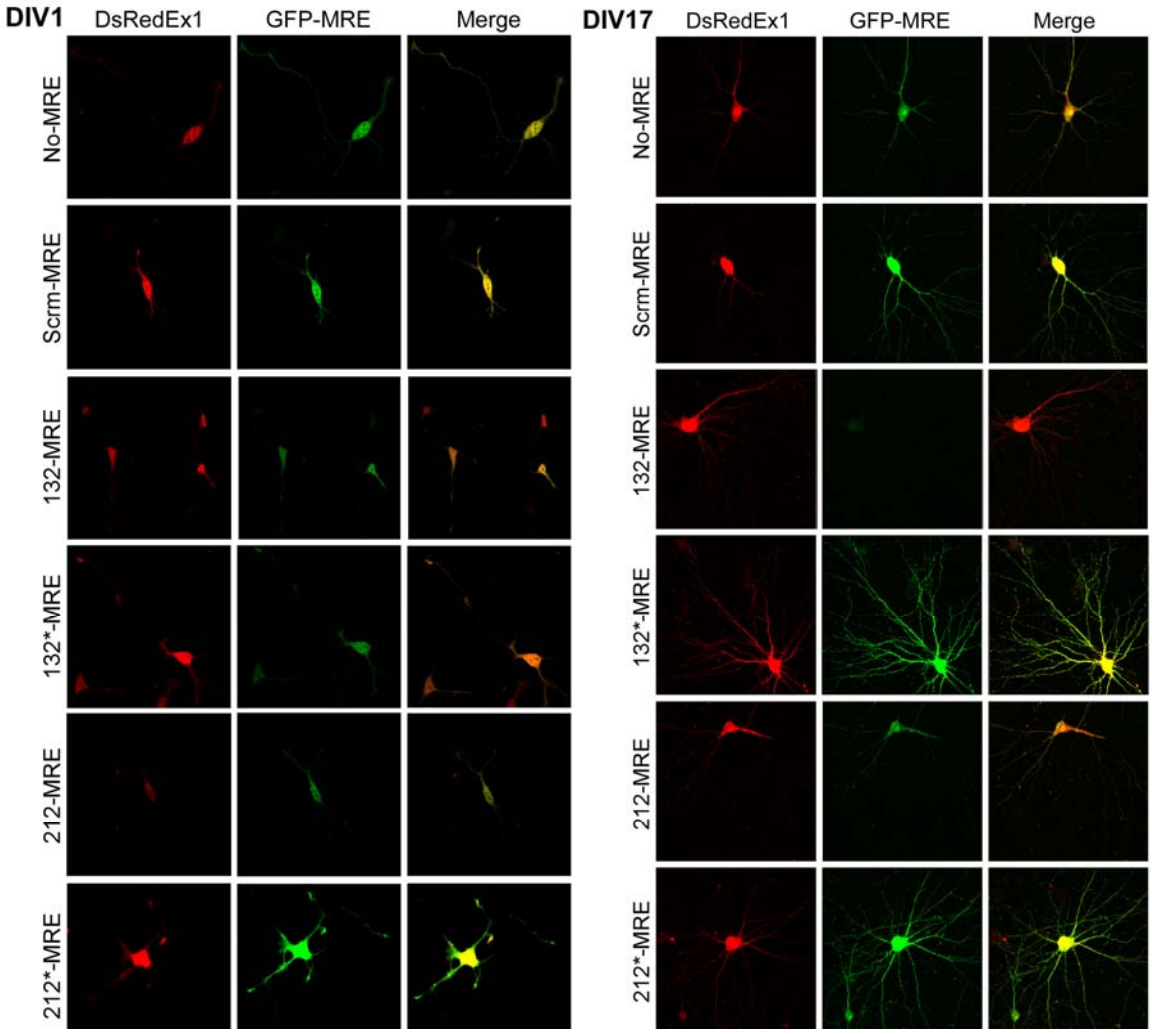

Figure 2.12: DIV 1 and DIV 17 dissociated hippocampal cultures transfected with sensors. Experiment performed by Lulu Cambrone.

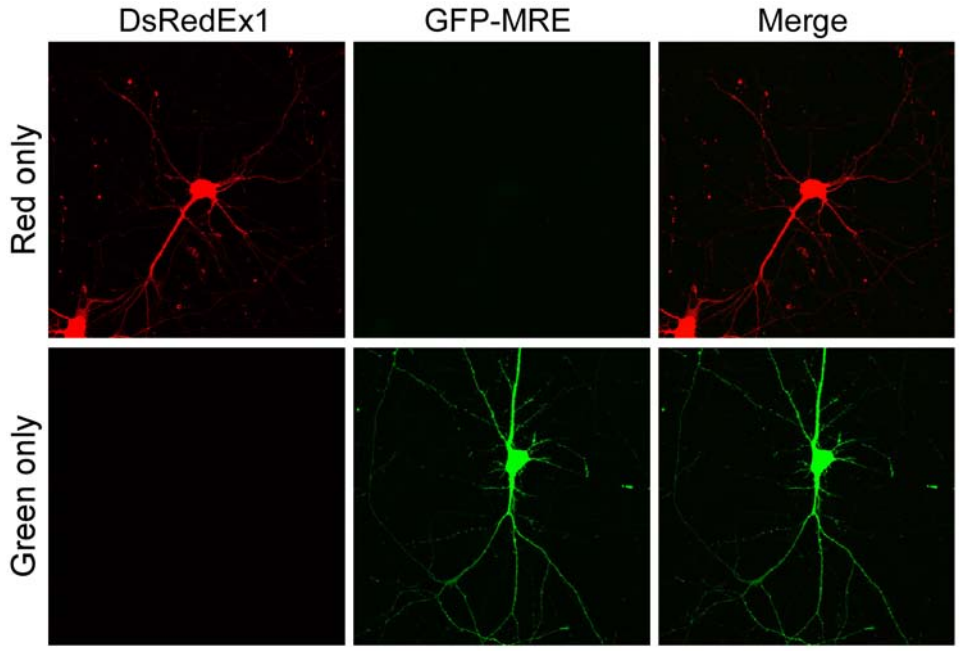

Figure 2.13: Confocal Images of red-only and green-only controls. Settings for both flow cytometry and microscopy were adjusted for every experiment using controls that expressed red (Upper Row) and green (Lower Row) as single colors. Experiment performed by Lulu Cambrone.

To determine whether miR-132 was responsible for the observed dendritic growth, arborization, and spine phenotypes in newborn neurons, we asked which microRNAs were active in DCX-positive neurons in the dentate gyrus of young adult C57Bl6/J mice. The expression of the red fluorescent protein from the (−) strand was insufficient to monitor transduced cells after lentiviral infection, so we compared the percentage of DCX-positive neurons that expressed GFP (indicating low microRNA activity) after infection with equal titers of lentiviral sensors. With the control Scrm-MRE, 36% of the DCX-positive cells showed GFP expression (Figure 2.14), and a similar percentage was observed for the 212-MRE, 132*-MRE, and 212*-MRE sensors. However, only 4% of the DCX-positive cells in mice injected with the 132-MRE sensor expressed GFP, indicating that miR-132, but not miR-132*, -212, or -212*, is active in immature granule neurons. Thus, we propose that the lack of miR-132 is responsible for the phenotypic changes in the adult newborn neurons.

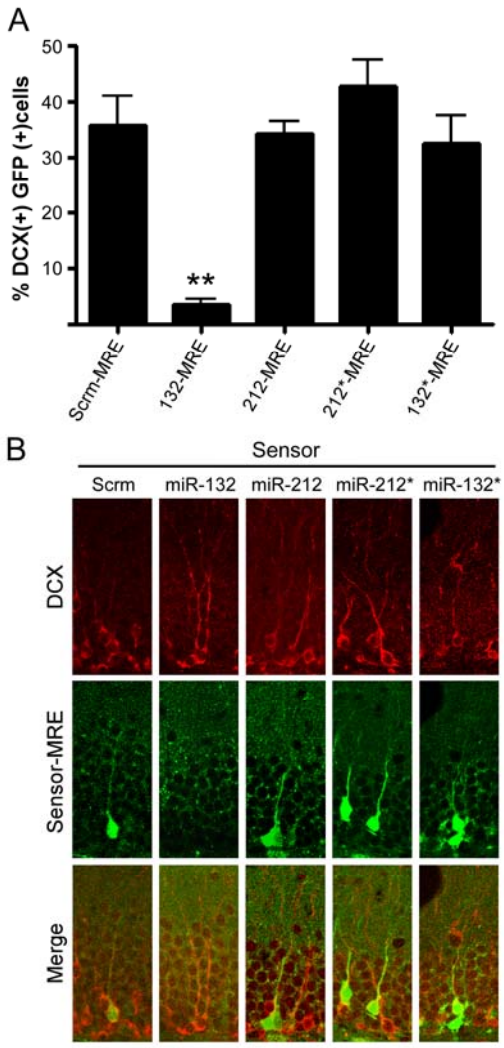

Figure 2.14: miR-132 is the primary functional product of the miR-212/132 locus in immature granule neurons *in vivo*. The dentate gyrus of WT mice was stereotactically injected with lentiviral microRNA sensors, and immature granule neurons were detected with DCX staining (Alexa Fluor 647). (*A*) The mean percentage of DCX-positive neurons that also express GFP ($f_{4,106}$ = 23.4; Dunnett's multiple comparison post hoc test **P < 0.01; Error bars show SEM). (*B*) Representative confocal images (*n* = 15 sections from two or three mice per condition). Data analyzed blind by Lulu Cambrone

Chapter 2.5: Discussion

MicroRNAs are essential for normal brain development and for establishing the functional connectivity of the brain, as evidenced by the decreased dendritic complexity, altered spine morphology, and cognitive defects caused by disruption of the microRNA processing machinery (Davis et al. 2008; Stark et al. 2008). Linking individual microRNAs to specific brain functions has been difficult, especially in the context of intact animals. As a result, most studies have relied on cell-culture models and have used microRNA mimics, inhibitors, or sponges (Ebert et al. 2010), all of which have the potential for off-target effects and can produce partial phenotypes. Furthermore, these approaches have a mixed record in recapitulating the effects of genetic knockouts or even can have opposite effects (Carè et al. 2007). Generating knockout models has been challenging because brain microRNAs often are transcribed from multiple genetic loci or are located within clusters.

Ablation of miR-212/132 is comparatively simple because a single deletion affects, at most, four potential microRNAs. Still, understanding such a deletion requires establishing which of the four microRNAs from the miR-212/132 locus are functional. To address this question, we developed a series of ratiometric fluorescent sensors specific for each possible microRNA. Surprisingly, the sensors revealed that only one of the four potential microRNAs miR-132, is functional in hippocampal neurons. Thus, we could use the conditional knockout mouse strain that we developed to determine the function of miR-132 alone.

To determine the consequences of deleting the miR-212/132 locus in vivo, we examined newborn neurons within the young adult hippocampus. Hippocampal neurogenesis is exquisitely sensitive to spatial learning tasks, exposure to an enriched environment, and physical exercise. Studies have shown extensive changes in dendritic arborization and spine formation in newborn neurons that persist for several months after training for just 6 d in a Morris water

maze (Tronel et al. 2010). This morphological plasticity is much more evident in newborn neurons than in mature neurons, providing us with a sensitized system for examining the contribution of miR-132.

To test whether miR-132 regulates dendritic maturation in vivo, we injected Cre-expressing retroviruses into the dentate gyrus of floxed miR-132 mice and examined the morphology of the newborn neurons. By using retroviral Cre infection, we could prevent microRNA expression acutely, eliminating the possibility of compensating effects during brain development that can occur with a germline knockout. Newborn neurons expressing Cre had a profound dendritic phenotype characterized by a reduction in dendritic length, branching, and spine density. Notably, our sensors confirmed that miR-132 activity predominated in these specific neurons. Together with our previous findings, these results suggest that miR-132 plays an important role in neuronal development and maturation in vivo.

Loss of CREB signaling has been shown to impair dendritic growth and arborization of newborn hippocampal neurons (Jagasia et al. 2009). Our findings demonstrate that ablating a single CREB target, miR-212/132, causes a similar dendritic phenotype in newborn hippocampal neurons, suggesting that microRNAs from this locus mediate some of these CREB effects. This notion is consistent with our previous experiments using neuronal cultures in which we found that miR-132 inhibitors largely blocked the effects of CREB on dendrite maturation. Previously, we had identified p250GAP as a target mediating miR-132 effects on dendritic outgrowth in cultured neurons; whether p250GAP is sufficient for these effects *in vivo* is under investigation. Other miR-132 targets have been identified, including methyl CpG-binding protein 2 (MeCP2), p120RasGAP, and p300 (Anand et al. 2010; Klein et al. 2007; Lagos et al. 2010), and it will be interesting to determine whether any of these contribute to the dendritic phenotype.

In addition to its role in dendrite maturation, CREB is necessary for the survival of newborn granule neurons (Nakagawa et al. 2002). Thus, it will be important to determine whether newborn neuron survival depends on miR-132. The effects of miR-132 on dendritic growth and neuronal survival could be interdependent; a large proportion of the newborn hippocampal neurons undergo apoptosis (Biebl et al. 2000), and survival may depend on whether immature neurons become functionally integrated into circuits. A severe dendritic phenotype caused by loss of CREB signaling or miR-132 expression could prevent integration, rendering these cells more susceptible to elimination.

Two recent papers implicated striatal miR-212 as an essential component of the mechanism underlying cocaine addiction (Hollander et al. 2010; Im et al. 2010). Our data indicated that mature miR-132 is expressed at a significantly higher level than mature miR-212 in hippocampus and cortex. Although low levels of mature miR-212 expression could be detected, functional activity, as measured using the sensors, is minimal under basal conditions, suggesting a potential threshold (Brown et al. 2007). Without the information provided by the microRNA sensors, it would have been difficult to rule out the possibility that the low level of miR-212 expression results from different PCR primer efficiencies. However, the expression and sensor data combined indicate that miR-212 levels are considerably lower than those of miR-132. Whether the levels of miR-212 are sufficient to mediate a functional response is uncertain. An advantage of the sensors is that they allow monitoring of specific microRNA activity in individual cells, as we demonstrated using the immature neuron marker, DCX. It will be interesting to determine whether miR-212 is functionally active in dorsal striatal neurons.

In conclusion, we have demonstrated that miR-132 mediates the dendritic outgrowth of newborn neurons in the adult hippocampus in vivo. Furthermore, we have shown that miR-132 is the primary functional product of the miR-212/132 locus in both hippocampal cultures and

newborn granule neurons. Thus, we have identified a mechanism whereby CREB signaling through microRNAs can promote the growth and maturation of newborn neurons in the hippocampus. Future studies will elucidate the generality of this pathway and how it may underlie activity-dependent changes in other parts of the brain.

CHAPTER 3

Further studies of miR-212/132 expression and function

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Chapter 3.1: Abstract

Selective loss of miR-212/132 decreased dendrite outgrowth and spine formation in newborn neurons. However, the expression of miR-212/132 in other brain regions and the impact of miR-212/132 germline knockout (GKO) on adult neurogenesis and behavior remain unknown. Here, I report that miR-132 is expressed in a rostral to caudal pattern in the brain of wild type mice, and that the absolute level of miR-132 is higher than miR-212 in the striatum. Additionally, the loxP sites and neomycin cassette in floxed miR-212/132 mice attenuate miR-212/132 expression, and the neomycin cassette by itself reduces dendrite outgrowth in floxed mice. GKO mice have no deficit in dendrite outgrowth of newborn hippocampal neurons, including following seizures. While GKO mice have normal proliferation within the dentate gyrus, the survival of newborn neurons is decreased compared to wild-type mice. Behaviorally, the GKO mice perform equivalent to wild-type mice in the open field test, the novel object test, a spatial learning task, context and cue-dependent fear conditioning, fear memory retrieval, and fear memory extinction paradigms. However, injection of HSV-GFP-Cre into the nucleus accumbens of floxed miR-212/132 mice increased cocaine-induced conditioned place preference, suggesting the miR-212/132 participates in the rewarding properties of cocaine. Finally, I present *in vitro* evidence that Sprouty1 may be a direct miR-132 target. Together, these studies enhance our knowledge of miR-132 expression and function, and raise questions regarding the mechanism of miR-132 action in adult neurogenesis.

Chapter 3.2: Introduction

The miR-212/132 locus is necessary for dendrite outgrowth and spine formation in newborn hippocampal neurons. Furthermore, experiments with microRNA sensors suggest that miR-132 is the primary functional product of the miR-212/132 locus *in vitro* and *in vivo* (Figure 2.11, 2.12, 2.14). Mature miR-132 is expressed 8-10 fold higher than miR-212 in multiple brain regions and cell types (Figure 2.1C, 2.2, 2.10B), strengthening the idea that miR-132 is the primary functional product of the miR-212/132 locus. However, expression of miR-132 and miR-212 could vary depending on brain region or cell type. Experiments in the Kenny lab demonstrated that miR-212 expression in the striatum regulates self-administration of cocaine (Hollander et al. 2010; Im et al. 2010), raising the possibility that the striatum may be a brain region with differential expression of miR-132 and miR-212. The sequence similarity between miR-132 and miR-212 (Figure 2.9C) might allow them to regulate the same targets, or more concerning, might cause overexpression of one to silence targets of the other. Similarly, inhibitors of one could inhibit the other. Thus, region-specific characterization of miR-132 and miR-212 expression could determine if they are differentially expressed throughout the brain.

In newborn hippocampal neurons, miR-132 and -212 expression correlates with dendrite outgrowth (Figure 2.1C, 2.4). Whether this correlation reflects causation remains unknown. Other factors could explain the phenotype, such as the presence of the PGK-neomycin cassette in the miR-212/132 locus, or background differences between the floxed miR-212/132 mice and wild type mice. Thus, it is essential to determine whether the expression level of miR-132 controls the extent of dendrite outgrowth. Alternatively, there may be a threshold level of miR-132 expression that is necessary to allow dendrite outgrowth to occur; once that threshold is reached, the extent of dendrite outgrowth is determined by other stimuli, such as hormones, neuropeptides, or neuronal activity.

As dendrites of newborn neurons extend into the molecular layer, they establish synaptic connections with incoming axons from the entorhinal cortex (EC) and local interneurons. Synapse formation requires anterograde, retrograde and bidirectional signaling between two neurons, a process that often involves contact-dependent signaling [for review see: (Shen et al. 2010)]. While miR-132 expression within newborn neurons promotes dendrite outgrowth and spine formation (Luikart et al. 2011a; Magill et al. 2010), it is unknown whether miR-212/132 in the surrounding cells also influences newborn neuron integration. Given the likelihood that contact-dependent signaling is involved, it is possible that the difference between miR-212/132 expression level in the newborn neuron and the surrounding cells, or the input cells in the EC, influences synapse formation and dendrite outgrowth in the newborn neuron. Germline knockout of miR-212/132 could address this question by eliminating the difference in expression level, which would provide insights into whether miR-212/132 functions in a cell-autonomous, or non-cell autonomous, manner.

Neurogenesis within the adult brain is a highly regulated process that responds to stimuli, such as exercise or environmental enrichment that increase the proliferation and survival of newborn neurons (Kempermann et al. 1998; van Praag et al. 1999). Because miR-132 promotes dendrite outgrowth, it would be interesting to determine if it also impacts survival of newborn neurons. In addition to exercise, seizures increase the number of newborn neurons in the adult dentate gyrus, which may contribute to hippocampal dysfunction due to aberrant synaptic connections (Parent et al. 1997). Seizures also promote dendrite outgrowth and accelerate the maturation of newborn neurons (Overstreet-Wadiche et al. 2006). Finally, miR-132 and -212 are increased in the dentate following seizures (Luikart et al. 2011b; Nudelman et al. 2010), which raises the possibility that miR-212/132 mediates seizure-induced changes in dendrite morphology.

Neuronal activity increases miR-132 expression, which raises the question of whether it is a component of the cellular and molecular machinery that facilitate learning and memory. Interestingly, identified miR-132 targets are important for cognitive performance. For example, overexpression or deletion of MeCP2 in humans can produce cognitive impairment [for review see: (Moretti et al. 2006)]. Because miR-132 participates in a feedback loop with BDNF to fine-tune MeCP2 levels (Klein et al. 2007), pathological increases or decreases in miR-132 could lead to cognitive impairment due to insufficient, or excessive, MeCP2. In addition, mutations in the miR-132 target, p300, as well as the functionally similar protein, CBP, have been identified in patients with Rubinstein-Taybi Syndrome (Petrij et al. 1995; Roelfsema et al. 2005; Zimmermann et al. 2007). Indeed, transgenic overexpression of miR-132 impaired performance in a novel object recognition task (Hansen et al. 2010). Whether ablation of miR-132 affects learning and memory, or other cognitive tasks, remains unknown.

In the present study, we report that miR-132 is expressed in a rostral to caudal pattern in the brain, and that the absolute level of miR-132 is considerably higher than miR-212 in striatum. Within floxed miR-212/132 mice, the loxP sites and neomycin cassette attenuate miR-212/132 expression, and retention of the neomycin cassette by itself impairs dendrite outgrowth. GKO mice have no deficit in dendrite outgrowth of newborn hippocampal neurons, including following seizures, which suggests that miR-212/132 regulates dendrite outgrowth in a non-cell autonomous manner or there is genetic compensation in the GKO mice. While GKO mice have normal proliferation within the dentate gyrus, the survival of newborn neurons is decreased compared to wild-type mice. Behaviorally, the GKO mice perform equivalent to wild-type mice in the open field test, the novel object test, a spatial learning task, context and cue-dependent fear conditioning, fear memory retrieval, and fear memory extinction paradigms. However, injection of HSV-GFP-Cre into the nucleus accumbens of floxed miR-212/132 mice

increased cocaine-induced CPP. Finally, I present *in vitro* evidence that Sprouty1 is a direct miR-132 target.

Chapter 3.3: Materials and Methods

qPCR: RNA was isolated using Trizol and treated for 30 min with DNAse I (Ambion). All quantitative Real-Time PCR for mature miRNAs was performed with Taqman Assay Kits (Applied Biosystems) starting with 10 ng of RNA and following the manufacturer's protocol.

Mice: The germline knockout of miR-212/132 (GKO) was generated by crossing a CMV-Cre 'Deleter' mouse (Jackson Laboratory, Strain 006054) with the floxed mice described in Chapter 2. Following germline transmission, the Cre allele was bred out, and homozygous GKO mice were generated.

The neomycin cassette, which is flanked by FRT sites, was removed from the floxed miR-212/132 mouse by crossing to a β-Actin-FLPe 'Deleter' mouse (Jackson Laboratory, Strain 005703). Following excision of the neomycin cassette and germline transmission of the floxed miR-212/132 locus lacking the neomycin cassette, the FLPe was bred out of the mice, and homozygous floxed miR-212/132 mice were obtained [abbreviated F(-neo) below].

The GKO mice were crossed with POMC-eGFP transgenic mice to generate POMC-GFP/GKO mice (Cowley et al. 2001).

BrdU administration: One day prior to BrdU injection, mice were placed in a larger cage (standard rat house) with a running wheel and house. On the day of injection, 8 week old C57Bl6/J mice (n=5) or miR-212/132 KO/KO mice (n=6) were injected twice, separated by 4.5 hrs, intraperitoneally, with 200 mg/kg dose of BrdU suspended at 10 mg/mL in normal saline.

Following injection, the mice were housed in the cage with a running wheel and house until analysis.

BrdU Immunohistochemistry: Mice were perfused with PBS and fixed with 4% PFA, 4% sucrose in PBS. They were post-fixed overnight in 4%PFA/4% Sucrose. 100 um free floating sections were cut on a vibratome. Sections were washed 2 times for 10 min in KPBS-T at room temperature on rocker. Antigen retrival was conducted using 2N HCl in KPBS-T for 45 min at 37C on a slow rocker/shaker. Sections were washed for 10 min in KPBS pH 8.5, then washed for 10 min in KBS-T, followed by 30 min of blocking in KPBS-T with 5% Normal Horse Serum rocking. They were subsequently stained with rat monoclonal anti-BrdU (Abcam, ab6326) 1:400 in KPBS with 2.5% Horse Serum overnight 4°C, given two quick rinses with KPBS and washed 3x 10 min in KPBS, followed by staining with secondary Dylight 488 anti-rat 1:200 for 6 hr RT rocking. Again, the sections were given two quick rinses with KPBS and washed 3x 10 min in KPBS. They were mounted with Prolong Gold Antifade (Invitrogen)

Imaging: Sections were imaged using a Zeiss LSM710 confocal microscope with a 20x Plan Apo 0.8 NA objective. Images were collected using a Z-stack to get three optical sections at 15 um intervals and a 3 x 2 tile scan to visualize the whole dentate gyrus. Image resolution was 512 x 512/tile, 2x averaging, 1% of the 488 laser and 706 digital gain.

Analysis: Images were analyzed blind using ImageJ. BrdU positive cells located in the subgranular layer of the dentate gyrus from anatomically similar sections were counted. The number of BrdU cells was divided by the length of the dentate to determine the density of BrdU positive cells.

Chapter 3.3.1: Behavioral experimental methods

All behavioral analyses were done between 7AM and 1PM.

Open Field Test: Naïve 6-8 week old mice were placed in a 14 inch by 14 inch by 14 inch plexiglass box (test arena) for 30 minutes and monitored with video recording equipment in the morning. Mouse location, distance and velocity were quantified using Stereoscan 3D Software (Clever Sys. Inc, Reston, VA). The open field apparatus was cleaned with 70% ethanol followed by a water rinse between each mouse.

Spatial Memory and Novel Object Tests: Naïve 6-8 week old mice were recorded in a 30 minute open field test arena on Day 1. On Days 2 and 3, the mice were placed in the open field again for 30 minutes. On Day 4, the mice were placed into the open field test arena for 5 minutes, which had been supplemented with three plastic toys, a man, a horse, and a cow (Figure 3.7), in three corners of the arena, while one corner was left empty. After 5 minutes, the mice were then placed in a holding cage inside a brown paper bag for 5 minutes. This was repeated for two more rounds. Prior to placing the mouse in the cage for the fourth round, the cow was moved to the empty corner. Time spent exploring each object was quantified (Spatial Memory Test). The mouse was placed back in the holding cage for 5 more minutes. Prior to returning the mouse to the test arena for the fifth round, the horse was replaced with a gorilla (Novel Object). The mouse was then placed in the test arena for a fifth five minute segment. Time spent exploring each object was quantified (Novel Object Test).

Conditioned Place Preference (CPP): CPP was performed as previously described (Russo et al. 2009; Maze et al. 2010). Floxed miR-212/132 mice containing the neomycin cassette were

placed into a test chamber consisting of two environmentally distinct chambers for 20 minutes and allowed unrestricted access to both chambers. The time spent in each chamber was tracked using a photo-beam to assess inherent bias to the chambers. The mice were then stereotactically injected with HSV-GFP-Cre or HSV-GFP into the nucleus accumbens using the method described in Chapter 2. Forty eight hours following surgery , the mice were given four 30 minute training sessions during which 7.5 mg/kg cocaine or saline was paired with one side of the chamber. The mice were tested one day later with a 20 minute session where they could roam through both chambers in a drug free state. The CPP score is the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber.

Chapter 3.3.2: Fear conditioning

Conditioning: The mice were placed in the context chamber for 2 minutes, and the CS was presented for 30 seconds. The last 2 seconds were paired with a .35mA shock. Following the shock, the mice remained in the context for an additional 30 seconds.

Testing and Extinction: To test for contextual fear, the mice were exposed to the context for six minutes, during which they were not shocked. They were then removed from the context and returned to their home cage for two hours. The mice were subsequently placed in modified context for two minutes, at which point the CS was presented for three minutes. The modified context had floors and lighting that were different from the conditioning context. The mice remained in the modified context for one minute after the cue was presented and were tested 1, 14, 19, 20 and 21 days after conditioning. This allowed testing for long term memory (Day 14) and extinction (Day 19-21).

Data Analysis: Fear memory was determined by measuring freezing behavior, which was defined as no movement for greater than three seconds. Freezing was quantified using the infrared activity monitor and binned into two minute blocks.

Chapter 3.3.3: Seizures

Seizures: Twelve week old mice were pretreated with a 1 mg/kg dose of 0.125 mg/mL scopalamine methyl nitrate (Sigma). Twenty minutes later, they were given 300 mg/kg dose of 10 ug/mL pilocarpine hydrochloride (Bausch & Lomb). Following pretreatment and the initial dose of pilocarpine, 150 mg/kg doses of pilocarpine were given every twenty minutes until the mice had multiple stage 3-5 seizures, as measured using the Racine scale (Racine 1972). This was considered status epilepticus. After three seizures that were stage 3 or greater, 1 mg/mL diazepam was administered to stop the seizure activity. Mice were sacrificed 14 days after the seizures were induced.

Chapter 3.4: Results

Chapter 3.4.1: miR-132 is expressed in a rostral to caudal pattern in the adult brain

To determine if there was spatial variation in the expression of miR-132, the brain of an eight week old C57Bl6/J mouse was dissected. RNA was purified and Taqman PCR was used to determine the level of miR-132 expression. miR-132 was expressed highly in all brain regions (27 cycles in spinal cord). However, it exhibited a rostral to caudal pattern of expression (Figure 3.1), being highest in the olfactory bulb and frontal cortex (24 cycles). Expression decreased progressing rostrally through medial/parietal cortex, occipital cortex, striatum, hippocampus,

midbrain, brainstem, cerebellum, and spinal cord. This pattern is consistent with reports from other labs (Olsen et al. 2009).

To determine whether miR-132 and miR-212 were differentially expressed in the striatum, I dissected striatum and performed Taqman PCR for miR-132 and miR-212 from a wild type mouse (C57Bl6/J). The expression of mature miR-132 was 12-fold greater than mature miR-212 (Figure 3.2), consistent with other brain regions and cell types (Figure 2.1C, 2.2, 2.10B). This suggests that miR-132 may mediate some of the phenotypes previously attributed to miR-212 in the striatum.

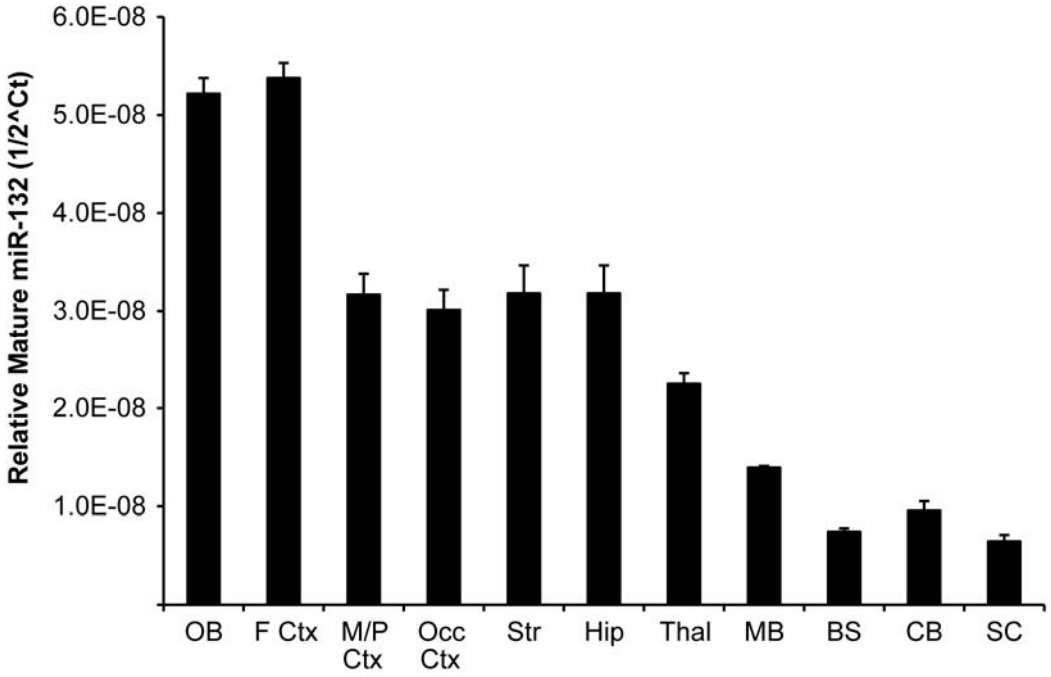

Figure 3.1: miR-132 is expressed in a rostral to caudal pattern in C57Bl6 mice. OB, olfactory bulb; F Ctx, frontal cortex; M/P Ctx, medial/parietal cortex; Occ Ctx, occipital cortex; Str, striatum; Hip, hippocampus; MB, mid-brain; BS, brainstem; CB, cerebellum; SC, spinal cord. Error bars are standard deviation of three technical replicates (n = 1 mouse).

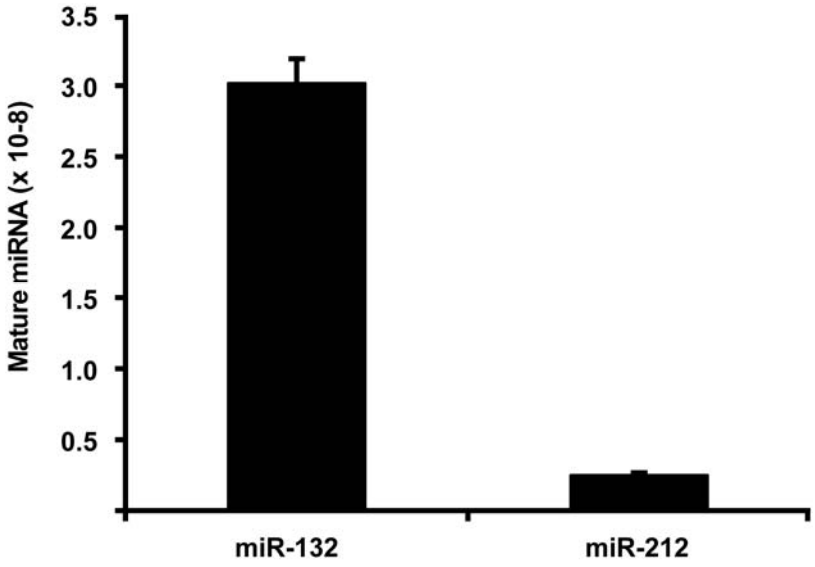

Figure 3.2: Mature miR-132 and miR-212 expression in striatum. Mature miR-132 was expressed 12-fold higher than mature miR-212 as determined using Taqman Assays. Error bars are standard deviation of three technical replicates (n = 1 mouse).

Our initial studies of the floxed miR-212/132 mouse found that miR-132 and miR-212 were both reduced by 50-60% in cortex and hippocampus (Figure 2.1, 2.2), indicating that the floxed mouse was a hypomorph. To determine whether the hypomorphic phenotype was caused by the neomycin cassette, we crossed the floxed miR-212/132 mice to a FLPe-Deleter, which excised the neomycin cassette. miR-132 and miR-212 levels in cortex (Figure 3.3) and hippocampus (data not shown) were still less than wild type following removal of the neomycin cassette. The miR-212 hypomorphic phenotype is partially, but significantly, rescued following removal of the neomycin cassette. Nevertheless, miR-212 is still significantly reduced compared to wild type. There was no significant difference in the expression of an unrelated miRNA, miR-16. Expression levels were obtained using Taqman Assays, and the difference for each miRNA was analyzed across genotypes using ANOVA followed by Tukey's HSD for post hoc testing (WT: n = 7 mice; Floxed: n = 7 mice; F(-neo): n = 7 mice; KO: n = 4 mice; **miR-132**: $f_{2,24}$ = 14.5, P < 0.001; Tukey's post hoc tests: WT vs Flox P = 0.011, WT vs F(-neo) P = 0.101, Flox vs F(-neo) P = 0.712; **miR-212**: $f_{2,24}$ = 48.4, P < 0.001; Tukey's post hoc tests: WT vs Flox P < 0.001, WT vs F(-neo) P = 0.021, Flox vs F(-neo) P < 0.001; For all comparisons with the KO, P < 0.001).

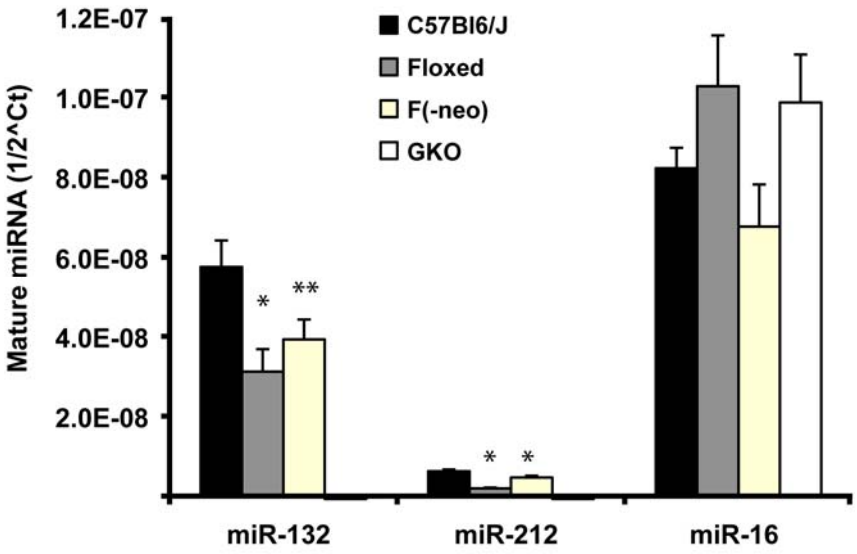

Figure 3.3: Mature mir-132 and miR-212 expression in cortex by genotype. Mature miR-132 and miR-212 levels in cortex are reduced in both the floxed miR-212/132 mouse containing the neomycin cassette as well as the F(-neo) mouse, which lacks the neomycin cassette, but retains the loxP sites. There was no change in the unrelated miRNA, miR-16. WT: n = 7 mice; Floxed: n = 7 mice; F(-neo): n = 7 mice; KO: n = 4 mice. Statistics: **miR-132**: $f_{2,24}$ = 14.5, P < 0.001; Tukey's post hoc tests: WT vs Flox P = 0.011, WT vs F(-neo) P = 0.101, Flox vs F(-neo) P = 0.712; **miR-212**: $f_{2,24}$ = 48.4, P < 0.001; Tukey's post hoc tests: WT vs Flox P < 0.001, WT vs F(-neo) P = 0.021, Flox vs F(-neo) P < 0.001; Error bars are SEM. *P < 0.05, **p = 0.102; For all comparisons with the GKO, P < 0.001.

Chapter 3.4.2: Newborn neuron dendrite length and branching are not impaired in the germline miR-212/132 knockout mouse

Selective miR-212/132 knockout in newborn hippocampal neurons attenuates dendrite length and branching (Magill et al. 2010). To determine whether this phenotype was a cell-autonomous function within the newborn neuron, we stereotactically injected the dentate gyrus of GKO mice with an mCherry expressing retrovirus. The mice were housed in a large cage with a running wheel one day prior to injection and remained in that cage until sacrificed. The mice were sacrificed 21d post-injection, perfused, and fixed overnight. Immunostaining and analysis was conducted as in Chapter 2. Surprisingly, dendrite length and branching in the newborn neurons from GKO mice were equivalent to wild type (Figure 3.4). Newborn neurons in the GKO mice had an average dendrite length of 965 um (SEM = 90 um; n = 9 cells) and an average of 8.2 branches (SEM = 0.8, n = 9 cells). Thus, miR-212/132 regulate dendrite outgrowth of newborn neurons in a non-cell autonomous manner or genetic compensation occurs in the GKO mice.

Chapter 3.4.3: Newborn neuron dendrite length and branching are not impaired in the Floxed(-neo) miR-212/132 mouse

miR-132 and miR-212 expression levels are decreased by approximately 40% in the F(-neo) mice as well as the floxed mice (Figure 2.1, 2.2, 3.3), implying that both strains are hypomorphs with respect to miR-212/132 expression. Furthermore, even in the absence of Cre expression, dendrite outgrowth was decreased in the floxed mouse that contained the PGK-neomycin cassette (Figure 2.4C, D). To determine whether the extent of dendrite outgrowth was influenced by the presence of the neomycin cassette independently of miR-132 and miR-212 expression, I stereotactically injected an mCherry expressing retrovirus into the dentate gyrus of a F(-neo) mouse. The mice were housed as above and were sacrificed 21d post-injection,

perfused, and fixed overnight. Immunostaining and analysis was conducted as in Chapter 2. Interestingly, dendrite length and branching in newborn neurons from the F(-neo) mice were equivalent to wild type (Figure 3.4), suggesting that the neomycin cassette by itself caused the decreased dendrite outgrowth. Newborn neurons in the F(-neo) mouse had an average dendrite length of was 947 um (SEM = 78 um; n = 13 cells) and an average of 8.1 branches (SEM = 0.6, n = 13 cells). Thus, we cannot conclude that the 40% reduction in the level of miR-212/132 expression is sufficient to cause the intermediate dendrite phenotype. Rather, there may be a threshold level of miR-132 necessary for dendrite growth.

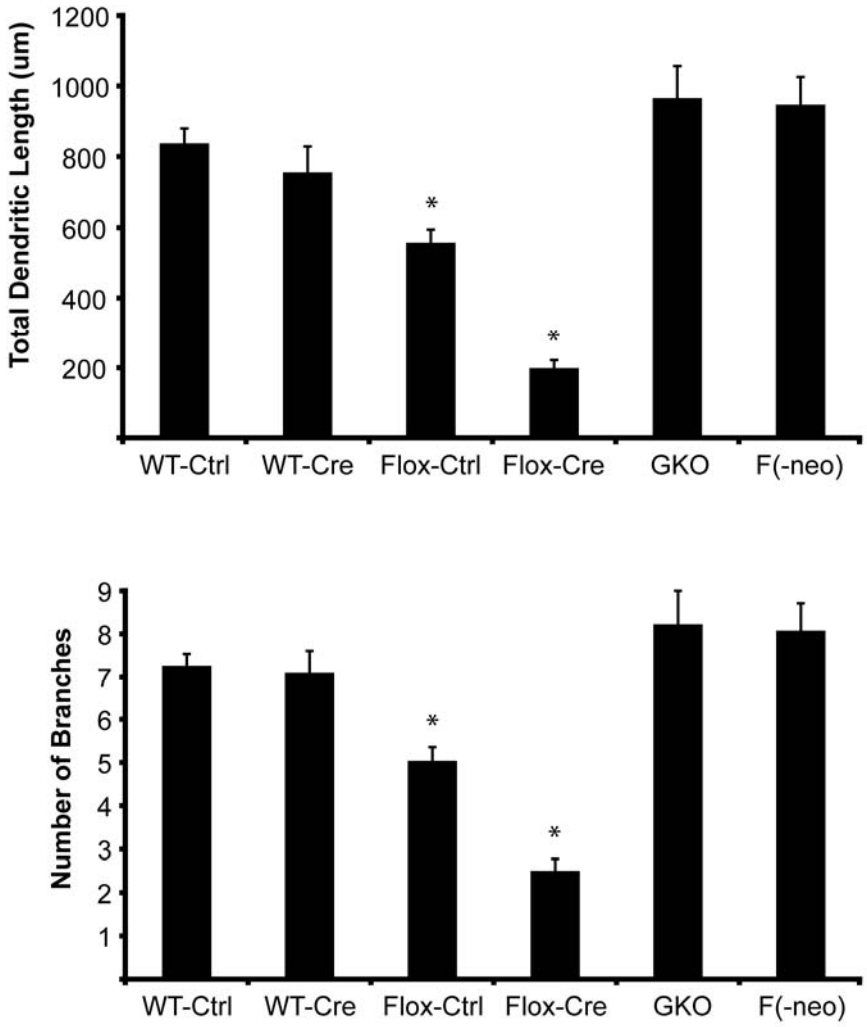

Figure 3.4: Summary of dendrite length and branching by genotype. WT-Ctrl, neurons from wild-type mouse infected with mCherry virus (n = 27 cells, 3 mice); WT-Cre, neurons from wild-type mice infected with mCherry and GFP-Cre viruses (n = 10 cells, 3 mice); Flox-Ctrl, neurons from floxed miR-212/132 mice with neomycin cassette infected with mCherry virus (n = 27 cells, 4 mice); Flox-Cre, neurons from floxed miR-212/132 mice with neomycin cassette infected with mCherry and GFP-Cre viruses (n = 28 cells, 4 mice); GKO, neurons from miR-212/132 germline knockout infected with mCherry virus (n = 9 cells, 3 mice); F(-neo), neurons from floxed miR-212/132 mouse lacking the neomycin cassette infected with mCherry virus (n = 13 cells, 1 mouse). Error bars are SEM. ANOVA *$p < 0.05$ (for details statistics, see Chapter 2.4.2).

Chapter 3.4.4: Survival, but not proliferation, of newborn neurons is reduced in the germline miR-212/132 knockout mouse

To determine whether miR-212/132 affected proliferation or survival of newborn neurons, C57Bl6/J (n=5) and GKO (n = 6) eight week old mice were given IP injections of 200 mg/kg BrdU. One day prior to BrdU injection, mice were placed in a large cage with a running wheel. Injections were given twice on one day, separated by 4.5 hrs. Following injection, the mice were housed in the cage with a running wheel and house until analysis. Two wild type and three GKO mice were sacrificed at 48 hrs; the other three wild type and three miR-212/132 KO mice were sacrificed four weeks post-injection. The brains were perfused, fixed, and immunostained for BrdU. Sections were quantified by counting cells in the subgranular layer and dividing the number of cells by the length (mm) of the subgranular layer to obtain BrdU cell density. At 48 hrs post-injections, there was no statistically significant difference between WT and GKO mice (Figure 3.5), suggesting that ablation of miR-212/132 has no effect on newborn neuron proliferation (WT, n = 2; GKO, n = 3; 20 sections/mouse were quantified; Student's T-Test, 2-tailed, P = 0.62). However, at 28 days post-injection, the BrdU cell density was significantly decreased in the GKO mice (Figure 3.5), suggesting that ablation of miR-212/132 decreased the survival of newborn neurons in the adult hippocampus (WT, n = 3; GKO, n = 3; 20 sections/mouse were quantified; 2-tailed Student's T-Test, P = 0.02).

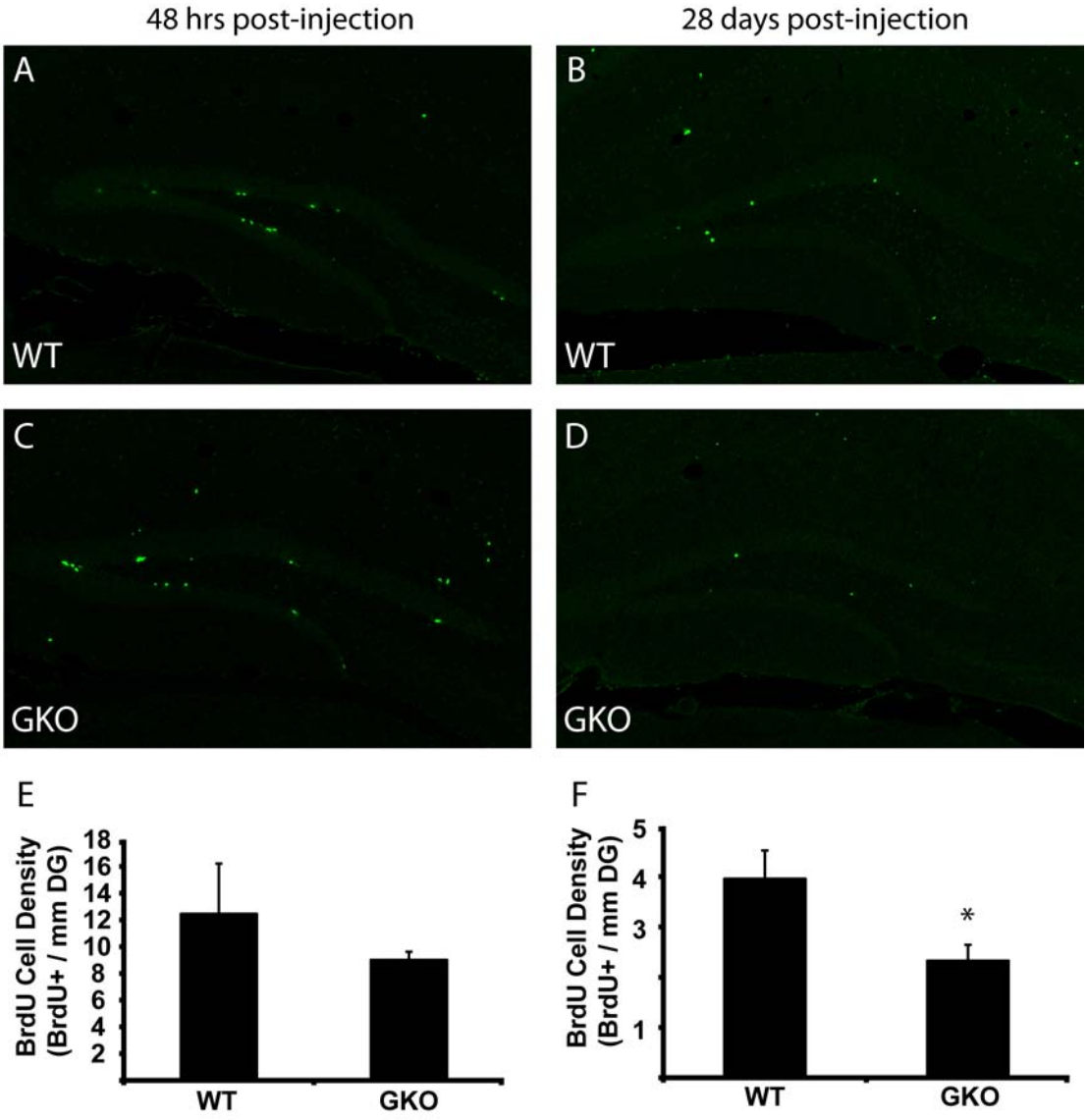

Figure 3.5: Ablation of miR-212/132 decreases survival of newborn neurons. GKO and WT mice were injected with BrdU and analyzed at 48 hrs and 28 days post-injection. (A-D) Representative images of WT and GKO dentate gyrus. (E) There was no difference in BrdU cell density at 48 hrs post-injection, suggesting that proliferation was normal in the GKO mice (WT, n = 2; GKO, n = 3; 20 sections/mouse were quantified; 2-tailed Student's T-Test, P = 0.62). (F) BrdU cell density was significantly decreased in GKO mice compared to WT controls at 28 days post-injection (WT, n = 3; GKO, n = 3; 20 sections/mouse were quantified; 2-tailed Student's T-Test, P = 0.02).

Chapter 3.4.5: miR-212/132 knockout in a kindling seizure model has no effect on dendrite outgrowth of newborn neurons

POMC-GFP mice express GFP transiently during maturation of adult born hippocampal neurons (Overstreet et al. 2004). The GFP expression peaks around day 14, allowing it to serve as a marker of immature granule neurons. Seizures increase the number of newborn cells as well as dendrite length (Overstreet-Wadiche et al. 2006). To determine whether miR-212/132 mediated the increase in dendrite length following seizures, I crossed the POMC-GFP mice to GKO mice and generated POMC-GFP/GKO mice. POMC-GFP/GKO and littermate controls were tested using a kindling model of seizures. The POMC-GFP/GKO mice, like the controls, had increased neurogenesis and dendrite outgrowth (Figure 3.6). There were no gross differences in the neurogenic response to seizures, suggesting, that miR-212/132 does not mediate dendrite outgrowth in newborn neurons in the GKO mice, which is consistent with my results using retroviruses (Figure 3.4). Alternatively, the ability of miR-212/132 to promote dendrite outgrowth may occur via a non-cell autonomous mechanism.

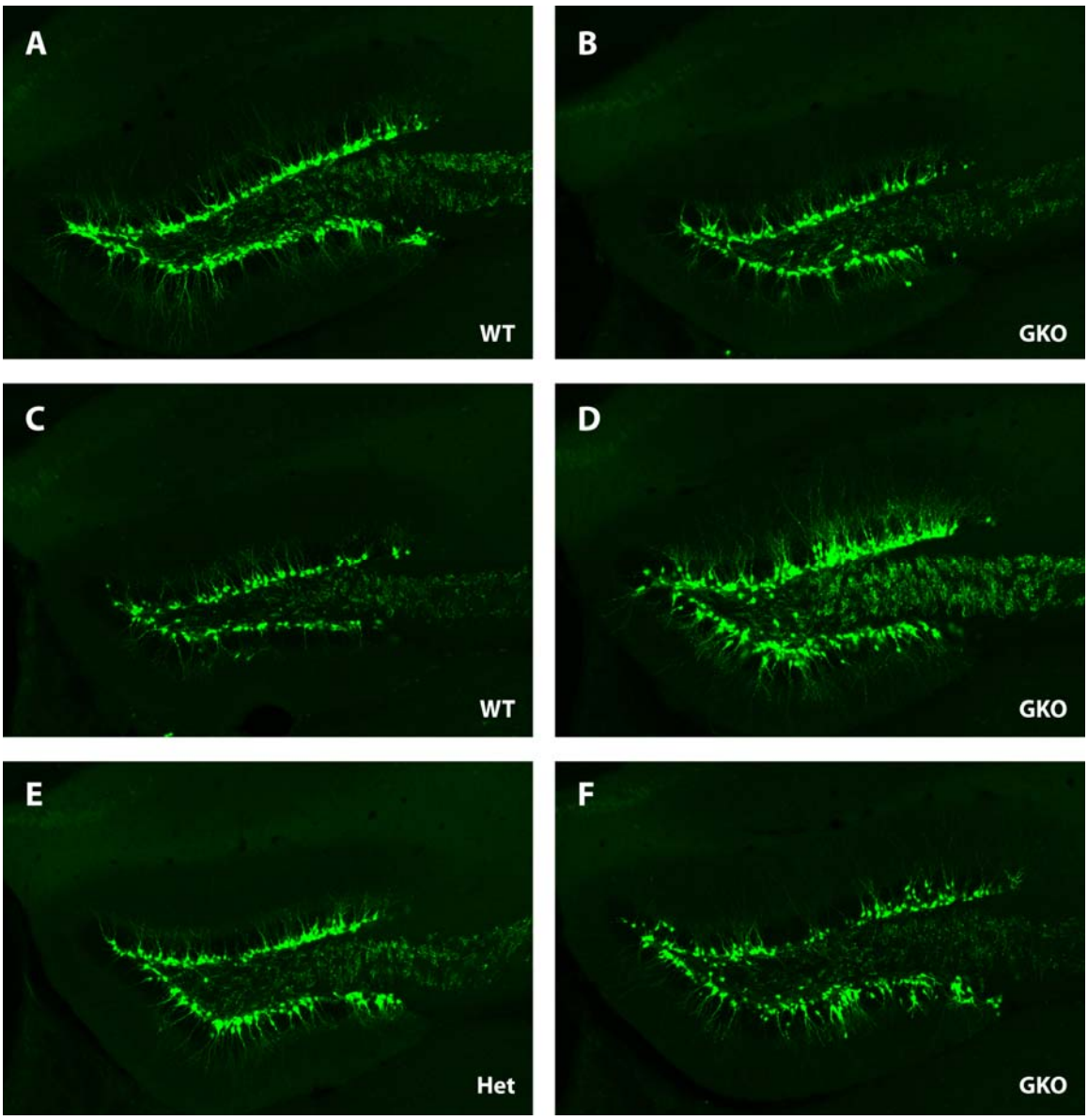

Figure 3.6: Seizures increase dendrite outgrowth of newborn neurons in GKO mice. Sample images of the dentate gyrus from POMC-GFP/GKO mice (GKO), POMC-GFP/WT (WT) and heterozygous mice (Het). There were no gross differences between the genotypes. All mice had increased proliferation and dendrites that extended through the entire the molecular layer. (GKO, n = 3 mice; Het, n = 1 mouse; WT, n = 2 mice).

Chapter 3.4.6: miR-212/132 knockout mice are not impaired in simple learning tasks

To determine whether there were changes in the baseline activity of GKO mice, we performed an open field test on wild-type (n = 3) and GKO mice (n = 3). There was no difference in the total distance traveled during a 30 min open field test between the genotypes (Figure 3.8B). Thus, any differences on subsequent tests should not be influenced by changes in baseline activity.

To determine whether the GKO mice had learning and memory defects, I tested them in novel object recognition and spatial memory tasks. Both the novel object and spatial memory tasks are thought to hippocampus-dependent (Kesner et al. 1993; Ennaceur et al. 1997). The mice are habituated to the chamber without objects for three days for 30 minutes. On the fourth day, the mice are placed in the chamber with three objects for 5 minutes and then returned to a holding cage for 5 minutes. This is repeated three times (Rounds 1-3, Figure 3.7). On the fourth round, one of the objects was moved, and the amount of time spent with the moved object was measured (Round 4, Figure 3.7). In this spatial memory task, a wild type mouse will spend more time with the moved object than with the objects that are in the same place. The mouse is then removed from the test arena for five minutes. One object is replaced with a novel object, and the mouse is returned to the test arena (Round 5, Figure 3.7). Wild type mice will spend more time with the novel object. There was no difference between the wild type and GKO mice in either the novel object or spatial memory tests (Figure 3.8C, D).

	Test Arena	Holding Cage	Arena Layout
1st Round *Habituation 1*	5 min		Empty Horse Man Cow
		5 min	
2nd Round *Habituation 2*	5 min		Empty Horse Man Cow
		5 min	
3rd Round *Habituation 3*	5 min		Empty Horse Man Cow
		5 min	
4th Round *Spatial Memory Test*	5 min		***Cow*** Horse Man Empty
		5 min	
5th Round *Novel Object Test*	5 min		Cow ***Gorilla*** Man Empty
		5 min	

Figure 3.7: Schematic of novel and spatial memory tests. The mouse is habituated to the chamber for 30 minutes for three days. On the fourth day, the mouse undergoes the five rounds of testing outlined here.

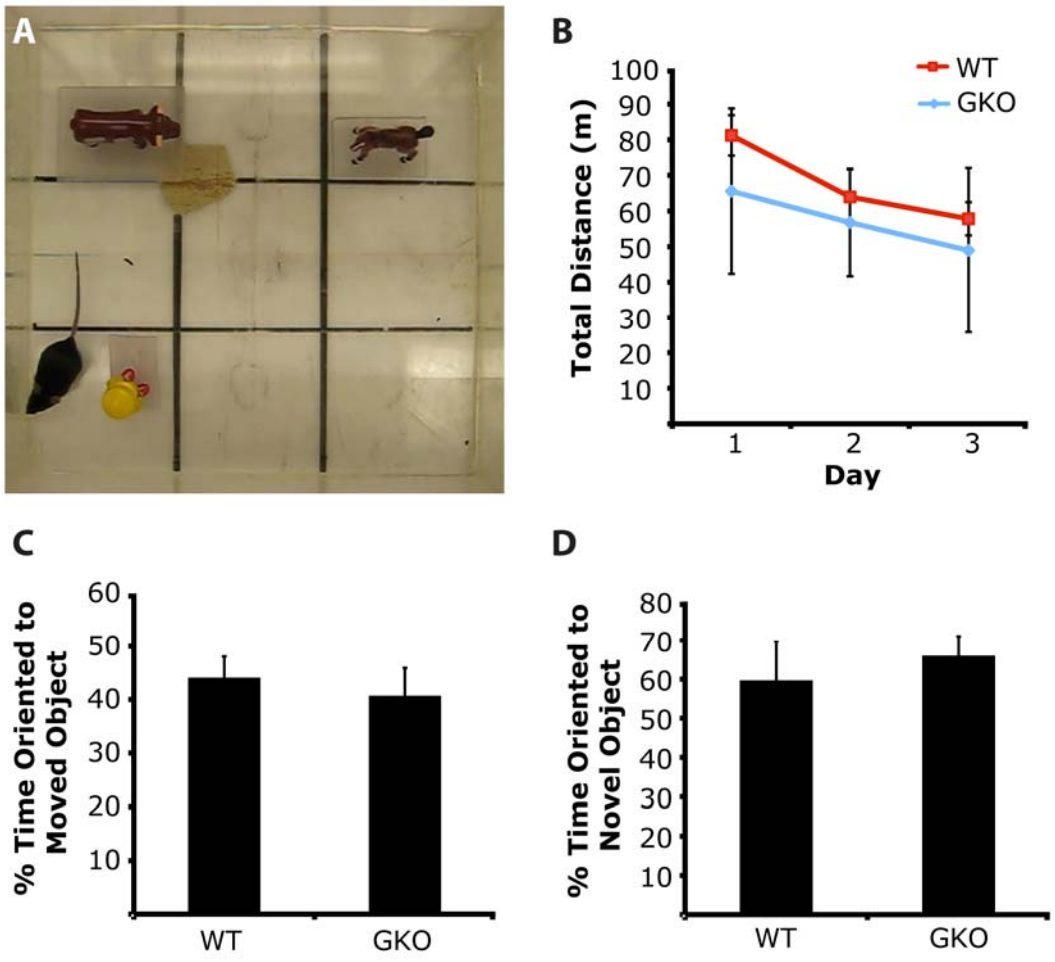

Figure 3.8: Ablation of miR-212/132 has no effect on learning tasks. (A) Image of the testing arena. There is no difference in (B) baseline activity, (C) performance in the spatial learning task, or (D) performance in the novel object recognition task between wild type (n = 3) and GKO mice (n = 3). Error bars are SD.

Chapter 3.4.7: miR-212/132 knockout has no effect on fear conditioning, retrieval or extinction

To determine whether miR-212 and miR-132 were involved in the formation of fear memory, we tested GKO, heterozygous GKO, and wild-type littermate controls in context and cue-dependent fear conditioning. There was no significant difference in baseline activity within the context (data not shown) or in fear conditioning between any of the genotypes (Figure 3.9). To assess long-term memory formation, we retested the mice at 14 days post-training. Again, there was no difference between genotypes. Finally, to determine if there was impairment in extinction of fear memory, the mice were tested at 19, 20, and 21 days post-conditioning. Again, there was no difference across genotypes. Together, these data suggest that mice lacking miR-212 and miR-132 from birth are able to form long-lasting fear memories and to extinguish them equivalently to wild type mice.

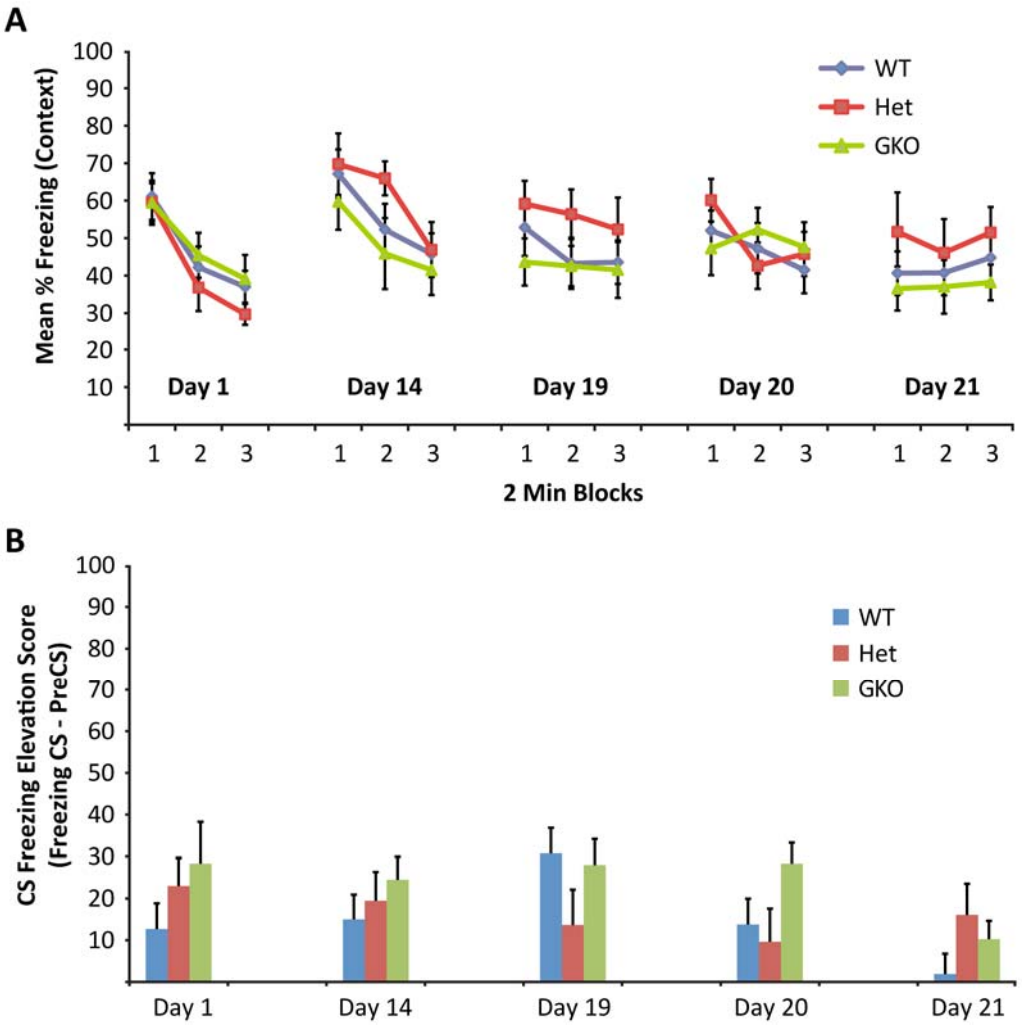

Figure 3.9: Mice lacking miR-212/132 are able to form and extinguish long-term fear memories. (A) Percent of time freezing when mice were placed into the context on the indicated days. Percent freezing was binned into 2 minute blocks throughout the six minute test session. (B) CS Freezing Elevation score is the percent increase in freezing after the CS presentation compared to freezing in the time prior to CS presentation. There was no difference between genotypes (WT, n = 16, Hets, n = 9, GKO, n = 11 mice). Experiments performed in the Lattal lab by James Stafford.

Chapter 3.4.8: miR-212/132 ablation in the nucleus accumbens enhances cocaine-dependent conditioned place preference

As discussed earlier, experiments in the Kenny lab overexpressing or inhibitng miR-212 in rat striatum suggested that miR-212 regulated cocaine self-administration (Im et al. 2010; Hollander et al. 2010). Cocaine-self-administration varied inversely with the level of miR-212 in the striatum. To determine whether miR-212/132 expression altered cocaine-induced CPP, floxed miR-212/132 mice containing the neomycin cassette were stereotactically injected with HSV-GFP or HSV-GFP-Cre in the nucleus accumbens shell. Mice injected with HSV-GFP-Cre had increased CPP compared to mice injected with HSV-GFP (n = 8-9 mice per group, Student's Test, 2-tailed, $p < 0.05$). This suggests that loss of miR-212/132 increases the rewarding properties of cocaine, consistent with the findings from the Kenny lab.

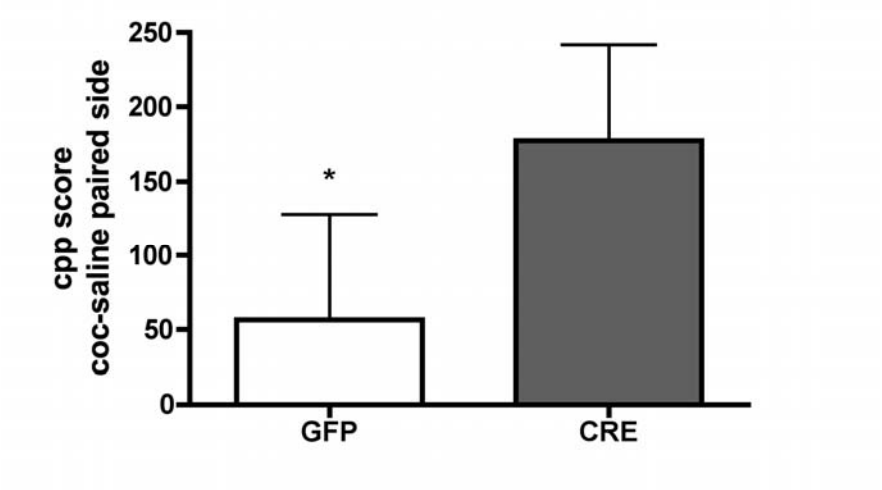

Figure 3.10: miR-212/132 ablation in the nucleus accumbens enhances cocaine-dependent conditioned place preference. Floxed miR-212/132 mice injected with HSV-Cre (n = 9) show an enhanced sensitivity to cocaine (7.5mg/kg) when compared to HSV-GFP control mice (n = 8) as assessed using the conditioned place preference test (CPP). Data is represented as a CPP score (cocaine paired chamber- saline paired chamber) on test day. Student's Test, 2-tailed, *$p < 0.05$. Experiments performed in the Nestler lab by David Dietz.

Chapter 3.4.9: miR-132 targets Sprouty1 *in vitro*

Sprouty1 was identified in our lab as a potential miR-132 target by an Ago2-immunopreciptiation from SH-SY5Y cells that had been transfected with miR-132 mimics (Kunal Chaudray, unpublished data). Sprouty proteins negatively regulate Ras-MAPK signaling (Reich et al. 1999; Casci et al. 1999; Hanafusa et al. 2002). Sprouty1 is expressed in the brain and limb buds during development (Minowada et al. 1999; Zhang et al. 2001). Recent reports demonstrate that Sprouty 1 negatively regulates differentiation of mouse embryonic stem cells to a neural lineage and also negatively regulates angiogenesis, both of which are associated with increases in miR-132 expression (Sabatel et al. 2010; Jung et al. 2011; Lee et al. 2010). Furthermore, I identified a putative miR-132 binding site (MRE) in the Sprouty1 3'UTR by sequence analysis (Figure 3.11A). To determine whether Sprouty1 was regulated by miR-132, I cloned the Sprouty1 3'UTR downstream of luciferase in the pISO vector. HEK293 cells were transfected with the reporter construct and TK-renilla to normalize transfection efficiency. Addition of 5 nM miR-132 mimic, but not a negative control mimic, decreased the luciferase signal of the WT-Spry1 3'UTR reporter by 52% (Figure 3.11B), indicating that Sprouty1 is a miR-132 target (n = 5; 4 wells were averaged per experiment). To determine whether miR-132 regulated Sprouty1 directly, I mutated five bases in the seed sequence of the putative MRE (Figure 3.10A) and repeated the luciferase assays. Transfection of 5 nM miR-132 only inhibited the Sprouty1 Mut-MRE 3'UTR by 10% (Figure 3.11C), suggesting that miR-132 regulates the Sprouty1 3'UTR through the identified MRE. Interestingly, miR-132 levels increase in rat hippocampal neuron cultures between E21 and DIV7 (Figure 3.11D), while at the same time Sprouty1 levels decrease (Figure 3.11E).

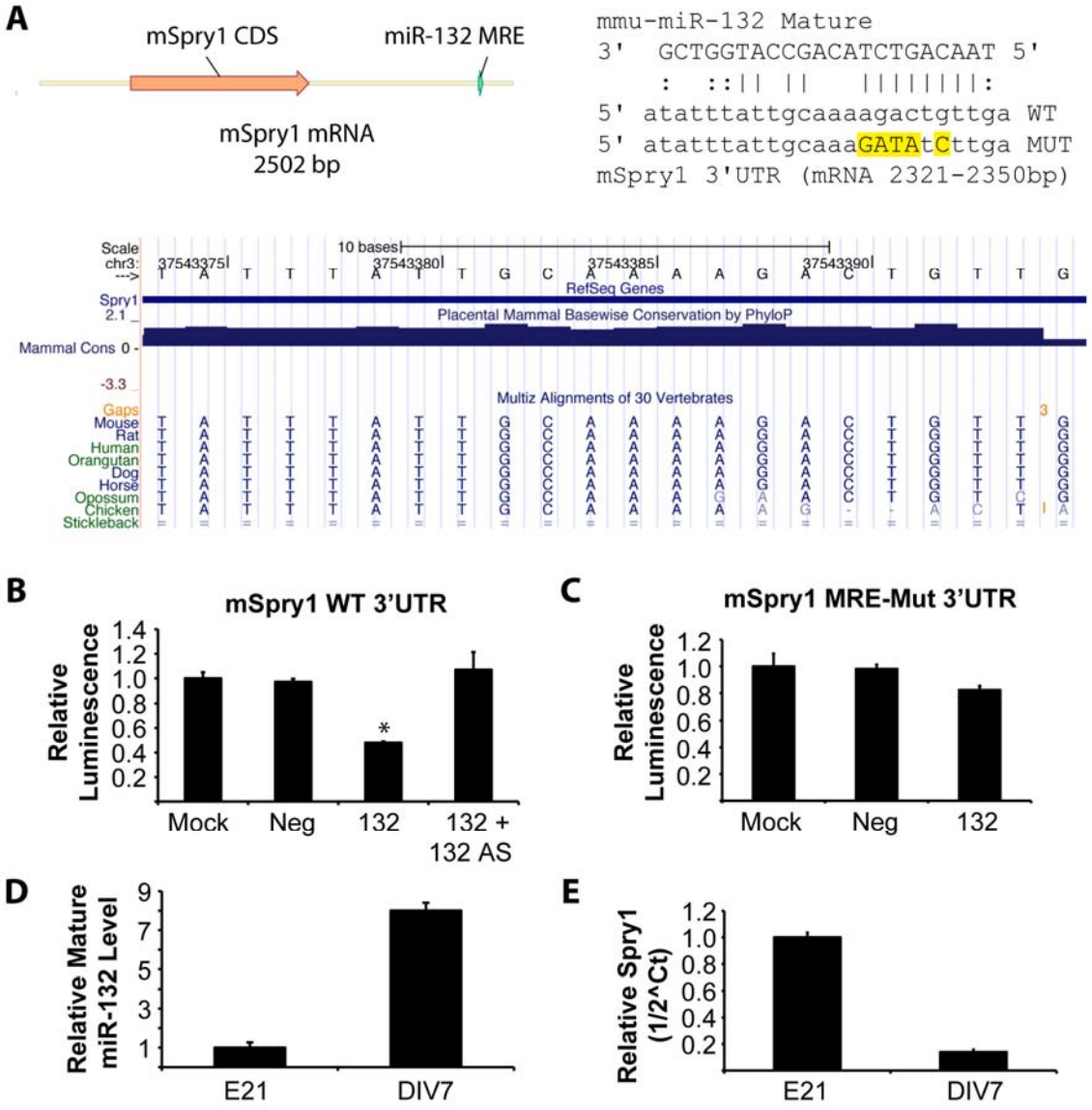

Figure 3.11: Sprouty1 3'UTR is regulated by miR-132 (legend on next page)

Figure 3.11: Sprouty1 3'UTR is regulated by miR-132. (A) A schematic of mouse Sprouty1 mRNA shows the location of the miR-132 MRE (Left). There is strong complementarity in the seed region as well as four complementary bases in nucleotides 13-17 (Right). Mutated bases in the mutant MRE are in caps and highlighted. The miR-132 binding site is highly conserved. (B) 5nM miR-132 mimic in HEK293 cells inhibits the Sprouty1 3'UTR reporter, but a negative mimic or addition of an antisense miR-132 2' O-Methyl inhibitor (132 AS) has no effect (n = 5). (C) Mutation of the miR-132 MRE in the Sprouty1 3'UTR attenuates the inhibition of Sprouty1 3'UTR by miR-132 (n = 3). (D) miR-132 expression increases in rat hippocampal neurons in culture between E21 (DIV0) and DIV7. (E) Sprouty1 expression decreases during the same time period. Error bars are SD. ANOVA *$p < 0.05$.

Chapter 3.5: Discussion

Determining the timing and location of microRNA expression is foundational to understanding its function. In this chapter, I demonstrated that miR-132 is expressed at high levels throughout the brain, with the highest expression in the olfactory bulb and frontal cortex. It decreases in a rostral to caudal manner, but is still highly expressed, even in the spinal cord. Additionally, mature miR-132 is expressed 8-10 fold higher than miR-212 in cortex, hippocampus, and striatum. Furthermore, experiments with the miRNA sensors (Chapter 2) suggest that miR-132 is the primary functional product of the locus. This differential expression of two mature miRNAs that share a primary transcript suggests that the processing of miR-132 and miR-212 is regulated post-transcriptionally. Indeed, there are two known proteins, KH-type splicing regulatory protein (KSRP) and hnRNP A1, that regulate miRNA processing by binding to stem-loop sequences and promoting processing of the stem-loop by Drosha. hnRNP A1 binds to the stem-loop of miR-18a, a component of the miR-17-92 cluster, and selectively promotes its processing from the primary transcript, without influencing the expression of the other miRNAs in the cluster which are on the same transcript (Guil et al. 2007). KSRP is a component of the Drosha/DGCR8 complex and binds to the stem-loop to promote the processing of several miRNAs from their primary transcript, including let-7a-1 and let-7g (Trabucchi et al. 2009). Interestingly, KSRP binds to guanine-rich sequences, especially those that have three consecutive guanines, however, its binding to RNA is negatively regulated by cytosine nucleotides (García-Mayoral et al. 2008). While both miR-132 and miR-212 have a GGG on the 5' end of their stem-loop (Figure 3.12), the GGG in the miR-212 stem-loop is flanked by multiple cytosine residues. The miR-212 stem-loop has eight cytosine residues, nearly 50% of its total length, while miR-132 only has two cytosine residues, and they are two nucleotides away from the GGG sequence. Thus, given the different nucleotide compositions of the stem-loops of miR-

212 and miR-132, it is possible that KSRP, or some other regulatory protein, binds to the stem-loop and selectively enhances the expression of miR-132.

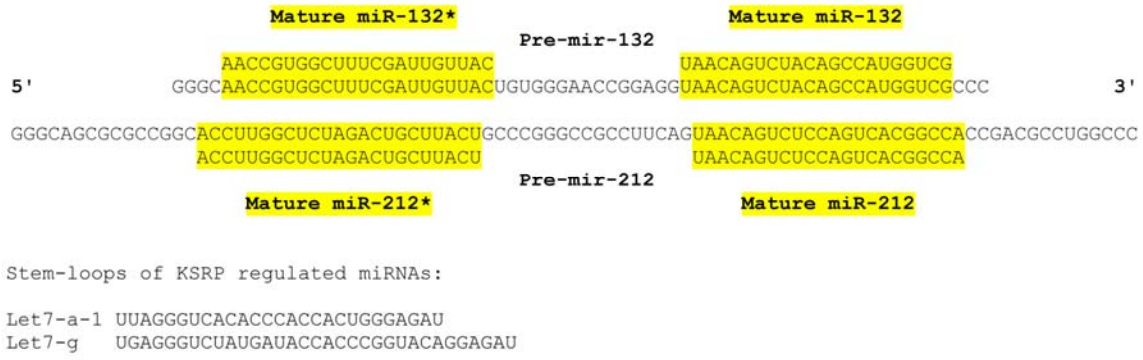

Figure 3.12: Sequence of pre-miR-132 and pre-miR-212. Note the difference in the number of cytosine residues in the stem-loop. Like let-7a-1 and let-7g, both miR-132 and miR-212 contain a putative KSRP-binding GGG motif in their stem-loops, however, it is possible that all the cytosine residues in the miR-212 stem-loop inhibit KSRP binding, which could inhibit the processing of miR-212 by Drosha and explain the differential expression of mature miR-132 and miR-212.

The floxed mice used in the experiments demonstrating that miR-132 mediated the dendrite outgrowth of newborn neurons in the adult hippocampus were hypomorphic with regards to miR-132 and miR-212 expression compared to wild type mice. Because dendrite outgrowth was also decreased compared to wild type mice, it was reasonable to hypothesize that the level of miR-132 expression directly correlated with extent of dendrite outgrowth. However, dendrite outgrowth of newborn neurons in the F(-neo) was equivalent to wild type mice, despite the somewhat lower levels of miR-132 and miR-212. Importantly, while the final levels of miR-132 and miR-212 were still hypomorphic in the F(-neo) mouse, they were higher than in the original floxed mouse that contained neomycin. This suggests that the presence of the PGK-neomycin cassette interferes with RNA transcription or processing to decrease miRNA levels which in turn cause the decreased dendrite branching in the floxed mouse. Another possibility that cannot be ruled out is that there was a selection bias in the neurons that were imaged from the wild type and F(-neo) mice. The size of hippocampal granule neurons varies throughout the hippocampus, with neurons in the dorsal arm of the dentate typically being larger than those in the ventral arm. Neurons for the comparison between Flox-Ctrl and Flox-Cre were always traced from the same image; thus, there was no opportunity for selection bias. However, neurons imaged and traced for the WT or the F(-neo) mice were selected because of their brightness and ease of tracing, which could lead to a selection bias. Nonetheless, the data suggest that the neomycin cassette was likely the cause of the difference in dendrite outgrowth between the wild type mice the floxed mice. The implication then, due to the reduced level of miR-132 and miR-212 in the F(-neo) mice, is that they may be threshold of miRNA needed to silence the targets that mediate the dendrite outgrowth phenotype. The F(-neo) mouse has enough of the miRNA to function equivalent to wild type, however, when the miRNA is completely removed, the dendrite outgrowth phenotype emerges.

Given that miR-212/132 ablation in the floxed mouse impairs dendrite outgrowth, we hypothesized that there would also be impaired dendrite outgrowth in newborn neurons of GKO mice. To our surprise, the dendrites in the GKO mouse had dendrite arbors equivalent to wild type or F(-neo) mice (Figure 3.4). Furthermore, the GKO mice had increased proliferation and dendrite outgrowth following seizures that were grossly equivalent to WT mice. The simplest explanation for this is that some form of compensation occurs, whether adaptive neuronal changes due to the loss of miR-212/132 from conception, or compensation for miR-212/132 function by other genes. However, it is also possible that the dendrite outgrowth mediated by miR-132 in newborn neurons occurs in a non-cell autonomous manner that is dependent on a difference between miR-132 expression in the newborn neuron and the surrounding cells, a concept explored in depth in Chapter 4.

While the GKO mice showed no difference from wild type with respect to proliferation and dendrite outgrowth of newborn neurons, there was a significant reduction in survival (Figure 3.5). This is particularly interesting when considering whether the lack of a dendrite outgrowth phenotype in GKO mouse is due to genetic compensation or a non-cell autonomous function of miR-132. Most microRNAs are thought to regulate many targets, so it would be plausible that miR-132 regulates one set of targets that cell-autonomously mediate survival and another set of targets that non-cell autonomously mediates dendrite outgrowth.

The idea that miR-132 mediates different responses to activity through multiple targets highlights the key challenge facing the miRNA field—target identification. To date, most studies have relied on prediction algorithms that are known to produce many false positives. Once a target is identified, *in vitro* studies are done with reporter assays to identify the binding site and validate the target. Finally, a biological function is shown to be dependent on that target and the miRNA, and the story is published. However, in reality, rather than regulating one target to

mediate one function, miRNAs are thought to regulate hundreds of target genes. Without a robust and accurate method for target identification, it is difficult to fully appreciate the function of an individual miRNA. In the case of miR-132, I was able to validate that Sprouty1 is a novel target (Figure 3.11). Interestingly, a recent report found that Sprouty1 inhibited neural differentiation of embryonic stem cells (Jung et al. 2011). Thus, it is possible that miR-132 regulates dendrite outgrowth through one set of targets, including p250GAP, and promotes survival through a different set of targets, potentially including Sprouty1. Definitive proof of this will require future experiments where the selective miR-132 knockout in the floxed mouse is rescued by knockdown of putative target genes, which will allow us to determine whether miR-132's regulation of dendrite outgrowth and survival occurs through distinct targets that function independently.

CHAPTER 4

Discussion and Conclusions

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Chapter 5: Discussion and Conclusions

Identification of miR-212/132 function *in vivo*

MicroRNAs are necessary for normal brain development and have been implicated in neural plasticity in culture [for review see: (Park et al. 2010; Siegel et al. 2011)]. However, genetic approaches to studying individual miRNAs *in vivo* have been complicated due to expression from multiple loci or within clusters. Interestingly, this is true for many of the well-characterized neuronal miRNAs, such as miR-9, -124, and -134. In contrast, miR-212/132 is only found in a single locus, simplifying the use of genetic approaches. In these studies, we demonstrated that the CREB target, miR-212/132, functions to promote dendrite outgrowth, spine formation, and survival of newborn neurons in the adult hippocampus *in vivo.*

During the maturation of newborn neurons in the adult hippocampus, GABA depolarizes the neuron and activates CREB-dependent gene expression, which promotes dendrite outgrowth and survival of newborn neurons (Jagasia et al. 2009). Our studies suggest that miR-132 is the primary functional product of the miR-212/132 locus, and is downstream of, and necessary for, CREB-mediated neuronal maturation. Our findings that miR-132 mediates newborn neuron maturation is the first demonstration of an *in vivo* function for miR-132 using a genetic approach and firmly establishes miR-132 as an important CREB effector. Future experiments overexpressing miR-132 in parallel with a dominant-negative CREB will allow us to determine whether miR-132 is sufficient to mediate CREB-dependent dendrite outgrowth and survival of newborn neurons.

Germline ablation of miR-212/132 did not alter dendrite morphology in contrast to selective knockout in newborn neurons with a Cre-expressing retrovirus. Several possible mechanisms could explain why this is the case. The simplest explanation is that genetic

compensation occurs when miR-132 is never expressed. An alternative explanation is that miR-132 mediates the dendrite outgrowth phenotype through a non-cell autonomous mechanism. Within the floxed mouse, a Cre-expressing, miR-132 null, newborn neuron, integrates into a dentate parenchyma that is filled with dendrites and axons from post-mitotic neurons that express miR-132. In contrast, newborn neurons in the GKO mouse integrate into a dentate where none of the surrounding cells express miR-132. In the floxed mouse, there is a difference in expression of miR-132 between the newborn neuron and the surrounding cells, however, in the GKO mouse there is no difference in miR-132 levels between the newborn neuron and surrounding cells. Thus, it may be the difference in miR-132 expression level between the two cells that produces the impaired dendrite outgrowth in the Cre-expressing, miR-132 null, newborn neurons. The GKO mice lack this difference in expression, which may explain why they have normal dendrite outgrowth. The concept that this type of difference could be important in dendritic spine outgrowth is exemplified by neuroligin1 (Bernardo Sabatini, unpublished data, presented at 2011 Vollum Seminar). Neuroligins are transmembrane proteins that bind to neurexins and mediate synapse formation and plasticity through contact-mediated signaling (Choi et al. 2011) [for review see: (Craig et al. 2007)]. Neuroligins and neurexins are highly regulated by alternative splicing and different splice variants form different types of synapses, either glutamatergic or GABAergic (Rowen et al. 2002). Neuroligin1 knockout mice had no gross phenotype (Varoqueaux et al. 2006) and had normal spine formation in the hippocampus (Sabatini, unpublished). However, neuroligin1 expression within an individual neuron in the context of the neuroligin1 knockout produced a significant increase in spine formation. Thus, it was the difference in neuroligin1 expression level between the two neurons that facilitated increased spine formation. miR-132 could regulate neuroligins, or other functionally similar proteins, in a comparable manner, thus leading to the lack of phenotype within the GKO

newborn neurons. Future experiments overexpressing miR-132 in newborn neurons within the context of the GKO mice could determine if this is the case. If miR-132 overexpression produced an increase in dendrite outgrowth and spine formation in the GKO mice, it would support a model where miR-132 provides an activity-dependent competitive advantage for dendrite outgrowth and synapse formation.

Regulation of miR-212/132 expression and implications for addiction

Knockout of the miR-212/132 locus eliminated four putative miRNAs, miR-132, -132*, -212 and -212*. Using miRNA sensors, we were able to demonstrate that miR-132 is the primary active product from the locus, both *in vitro* and *in vivo*, and that the other three miRNAs had very little, if any, activity. This directly correlated with experiments examining the absolute level of miRNA expression. Future experiments are needed to directly prove that miR-132 is the only functional product of the locus. To determine if this is the case, floxed mice could be infected with a retrovirus expressing Cre along with each miRNA. Rescue of the dendrite phenotype by re-expression of miR-132, but not miR-212, would definitively demonstrate that miR-132 was responsible. Alternatively, if miR-132 and miR-212 both rescued the phenotype, it would suggest that they have overlapping functions, which is plausible, particularly in light of their sequence similarity.

Mature miR-132 is expressed higher than miR-212 in every brain region and cell type that we examined, however, the mechanism that mediates the differential expression is unknown. miR-132 and miR-212 are processed from the same primary transcript, which implies that the differential expression of the two miRNAs is determined post-transcriptionally and may vary in a temporal or cell-type specific manner. Processing can be regulated during the transition from pri- to pre-miRNA or pre-miRNA to mature miRNA [for review see: (Newman et

al. 2010)]. As discussed in Chapter 3.5, both pre-miR-132 and -212 stem loops contain the GGG motify that KSRP recognizes to promote the processing of pri-miRNAs to pre-miRNAs, however, the pre-miR-212 stem loop is nearly 50% cytosine residues, which negatively regulate binding and processing by KSRP (Figure 3.11) (García-Mayoral et al. 2008). Future experiments are needed to determine whether there is a difference in the induction of pre-miR-132 and pre-miR-212 in response to activity. These experiments will demonstrate if the differential expression of mature miR-132 and -212 occurs at the transition from pri- to pre-miRNA.

In addition to regulation at the pri- to pre-miRNA transition, miRNAs can also be regulated at the pre- to mature transition. For example, lin-28 binds to pre-let-7 and recruits TUT4, a poly(U) polymerase, to poly-uridylate pre-let-7, thus, promoting its degradation prior to processing into the mature miRNA by Dicer (Heo et al. 2009; Lehrbach et al. 2009; Hagan et al. 2009). Interestingly, bicuculine treatment increases the expression of pre-miR-132 five-fold more than mature miR-132, suggesting that miR-132 is regulated during the transition from pre- to mature miR-132 [see Figure 1A in: (Wayman et al. 2008)]. It is plausible that this could occur through a mechanism similar to lin28 regulation of pre-let-7. Our data demonstrate, however, that mature miR-132 and miR-212 have the same fold-increase following stimulation, which suggests that the differential processing likely occurs at the pri- to pre-miRNA transition.

We demonstrated that miR-132 is the primary functional miRNA from the miR-212/132 locus, and that the other miRNAs expressed from this locus have little activity in neurons *in vitro* and *in vivo*. Interestingly, lentiviral overexpression of miR-212 in the striatum reduced cocaine self-administration, while injection of a miR-212 LNA inhibitor increased cocaine self-administration (Hollander et al. 2010; Im et al. 2010). Consistent with these results, knockout of miR-212/132 in the nucleus accumbens of floxed mice increased the rewarding properties of cocaine in a CPP paradigm. However, expression of Cre knocks out both miR-212 and miR-132,

preventing us from knowing which miRNA is responsible for the effect. The sensor and expression data suggest that miR-132 is the functional miRNA from the locus. Given the sequence similarity between miR-132 and miR-212, it is possible that overexpression or inhibition of miR-212 in the Kenny experiments non-specifically modulated miR-132 levels. Future experiments using the floxed miR-212/132 mice or the GKO mice should allow us to determine which miRNA actually mediates the effect on cocaine self-administration. We would predict that the GKO mice would have increased cocaine self-administration. Thus, experiments expressing miR-132 or miR-212 within the striatum of the GKO mouse may help determine which miRNA, or if both miRNAs, regulate cocaine self-administration. If it turns out that genetic compensation occurs in the GKO mice, the experiments could be conducted with Cre-expressing viruses in the floxed mice. Cocaine shares common reward pathways with other drugs of abuse (Nestler 2008). Thus, future experiments are needed to determine whether miR-212, or miR-132, provide a common mechanism underlying addiction to other drugs of abuse, and whether modulation of miR-212 or -132 levels could have therapeutic potential.

miR-132 in synaptic homeostasis

Evidence from multiple labs indicates that microRNAs play an important role in synaptic homeostasis [for review see: (Siegel et al. 2011; Schratt 2009)]. Synaptic homeostasis is the activity-dependent balance between neuronal potentiation, leading to synaptic strengthening and spine formation, and neuronal depression, leading to synaptic weakening and elimination—neuronal changes that are thought to be foundational to learning and memory. Interestingly, as discussed in the background (Figure 1.4), activity stimulates miR-132 expression while promoting the degradation of miRNAs that inhibit spine growth or synaptic transmission, such as miR-124, -134, and -138 (Krol et al. 2010). In contrast, NMDA blockers stabilize the

expression of miR-124, -134, and -138 and promote the degradation of miR-132. Thus, miRNAs function on both sides of the balance between synaptic potentiation and synaptic depression. However, activity also caused the RISC subunit MOV10, an RNA helicase, to be degraded by the proteasome, relieving miRNA-mediated inhibition of pro-synaptic genes (Banerjee et al. 2009). This raised the question as to how miR-132 could facilitate synaptic strengthening and growth if the RISC was non-functional following activity? One possibility is that miR-132 primarily silences its targets within the nucleus or cytoplasm, and thus would not be affected by MOV10 degradation locally within dendrites. However, miR-132 is present in dendrites, increasing the likelihood that it is functional within the dendrites, and thus, would also be affected by the degradation of MOV10 (Wayman et al. 2008). A second possibility is that timing differences account for the change (Vo et al. 2010a). Immediately following a burst of activity, miRNA inhibition is relieved due to MOV10 degradation, allowing short-term plasticity. For long-term changes to occur, activity leads to CREB activation and increases miR-132 transcription. By the time miR-132 is processed and trafficked to the dendrites, the RISC is reassembled and functional, allowing miR-132 to silence p250GAP, and its other targets, to mediate long-term synaptic changes. A third possibility is that there are unique RISCs located within dendrites. Mammals express four Ago isoforms (Kim et al. 2009). Interestingly, Ago1 and Ago2 are localized in dendrites, and Ago3 and Ago4 in axons (Hengst et al. 2006; Cougot et al. 2008). It is possible that dendrites contain both RISCs that promote synaptic strengthening, possibly in a MOV10-independent manner, as well as RISCs that inhibit synaptic strengthening, which might be MOV10 dependent. A final possibility is that RNA binding proteins mediate miRNA function, exemplified by FMRP, which is necessary for miR-125b silencing of NR2A (Edbauer et al. 2010). Future experiments will be necessary to determine which idea, or combination of ideas, allows miR-132 to promote synaptic strengthening.

miR-132 targets

microRNAs function by post-transcriptionally silencing target mRNAs. Interestingly, miR-132 regulates several targets that are modifiers of G-protein mediated signaling cascades. p250GAP, the first identified miR-132 target, mediates dendrite outgrowth *in vitro* (Vo et al. 2005). p250GAP is a Rac GAP, which inhibits the action of Rac. When miR-132 silences p250GAP, Rac is activated and signals through Pak to increase the formation of dendritic spines (Impey et al. 2010). miR-132 also regulates another GAP, p120RasGAP, in endothelial cells to mediate angiogenesis (Anand et al. 2010). p120RasGAP inhibits Ras activity. Thus, increases in miR-132 silence p120RasGAP, increase Ras activity, and promote angiogenesis. Interestingly, miR-132 is upregulated in hemangiomas and treatment with miR-132 inhibitors reduced angiogenesis in a cancer model, suggesting that miR-132 inhibition may have potential as an anti-angiogenic therapy. Finally, in Chapter 3, I demonstrated that the 3'UTR of Sprouty1, another negative modifier of Ras signaling, is regulated by miR-132. Thus, by targeting negative regulators of Ras signaling, miR-132 could amplify its own expression, generating a feedforward loop. This model is supported by findings from the Kenny lab suggesting that miR-212 inhibits SPRED1, leading to the activation of Raf1, which is also in the Ras pathway. Thus, miR-132 and miR-212 may function to amplify their own expression by silencing negative regulators of the CamKK-Ras-MEK-ERK pathway. They do this at multiple levels in the pathway and through different target genes. This may be functionally significant for synaptic plasticity because diffuse Ras-ERK signaling is necessary for AMPA receptor exocytosis in spines, an essential step in LTP induction (Patterson et al. 2010).

Given miR-132's role in synaptic homeostasis, we hypothesized that it might also be important for cognitive functioning. Indeed, miR-132 targets MeCP2 and p300, both of which

are mutated in diseases characterized by mental retardation (Klein et al. 2007; Lagos et al. 2010). MeCP2 mutations cause Rett syndrome (Moretti et al. 2006), and p300 mutations are associated with Rubinstein-Taybi syndrome (Roelfsema et al. 2005; Zimmermann et al. 2007). Surprisingly, we observed no change in cognitive function in the GKO mice, despite testing in a series of learning and memory experiments. This could be caused by genetic compensation or miR-132 could function in a non-cell autonomous manner, similar to what was presented as a potential explanation for the lack of a dendrite phenotype in the GKO mice (discussed above). Collaborative experiments with Marcello Wood's lab are under way to determine whether specific ablation of miR-212/132 in the hippocampus of floxed mice influences learning and memory. The results of these experiments should help determine whether genetic compensation occurs in the GKO mice, and clarify the role of miR-132 in learning and memory.

In addition to promoting dendrite outgrowth and spine formation, possibly by amplifying Ras signaling, miR-132 regulates survival of newborn neurons. Although the GKO mice had normal dendrite outgrowth and proliferation, survival of newborn neurons was decreased. This raises the possibility that different sets of target genes could mediate survival and dendrite outgrowth. The miR-132 targets that mediate dendrite outgrowth could function in a non-cell autonomous manner, while other targets that promote survival could function cell autonomously. Future experiments will need to be conducted to identify the miR-132 targets that regulate cell survival. Currently, prediction algorithms are the primary method for identifying miRNA targets, but they are notorious for producing false positives and often do not include real targets (Vo et al. 2010b). Improvements in biochemical and empirical methods should allow identification of the complete set of miR-132 targets in the future.

Generation of the floxed miR-212/132 and GKO mice provided a new tool to identify and test putative targets *in vivo*. Experiments are underway to determine whether p250GAP

mediates the miR-132 dendrite phenotype *in vivo*. For these experiments, we injected the dentate of F(-neo) mice with a GFP-Cre retrovirus along with an mCherry retrovirus containing an shRNA against p250GAP or a control shRNA. If p250GAP is the target that mediates dendrite outgrowth, we expect the phenotype to be rescued by knockdown with shp250GAP. These mice also provide a means to look at transcriptome-wide changes in gene expression in the absence of miR-212/132. This could be particularly informative by coupling with TU-tagging, a method that allows isolation of RNA from specific cell types (Miller et al. 2009). In a TU-tagging experiment, 4-thiouracil is administered to the animal and taken up specifically by cells that express *T. gondii* uracil phosphoribosyltransferase (UPRT). The 4-thiouracil is incorporated into RNA, which can be labeled with biotin and efficiently purified from total RNA using streptavidin beads. RNA-sequencing from a small number of cells, as few as 250, can be used to identify changes in RNA expression. In the case of newborn neurons, we could inject either a UPRT alone or a UPRT-IRES-Cre retrovirus into the floxed mice. Dissection of the dentate, and purification of RNA from the UPRT-infected newborn neurons would allow tracking of the transcriptional changes that occur following deletion of miR-132. Changes in gene expression could be analyzed at different stages during the maturation of newborn neurons or following different stimuli, such as exercise, stress or anti-depressants. Network analysis of changes in gene expression could identify modules of gene expression that are changed in the presence or absence of miR-212/132 and dramatically enhance our understanding of the gene networks that are regulated by miR-212/132 in newborn neurons. While this approach will not identify miR-132 targets that are regulated solely by translational arrest, it could provide information on targets regulated via mRNA degradation.

Discrepancy between different miR-212/132 knockout mice

While the work presented in this dissertation was being performed, another lab generated a miR-212/132 knockout independently (Ucar et al. 2010). Ucar et al demonstrated that knockout of miR-212/132 caused a defect in mammary gland development, which impaired the mother's ability to nurse her pups, leading to the death of pups with knockout mothers, regardless of genotype. We did not observe any difference in the mortality of pups born and raised by GKO mothers compared to all other genotypes in our colony, despite careful recording of any pups that died (data not shown). There are several differences between the GKO mice and the Ucar knockout mice. First, the Ucar mice have an IRES-lacZ-pA cassette inserted into the miR-212/132 locus. In contrast, our GKO mice only have one loxP site remaining in the miR-212/132 locus. It is possible that the cassette inserted by Ucar et al disrupted the genes that are located close to the miR-212/132 locus, which include the tumor suppressors Ovca2 and Hic1, as well as Dph1. Second, the Ucar mice were fully backcrossed to both 129/Sv as well as a C57Bl6/N backgrounds. They observed the mammary phenotype in both lines. In contrast, our GKO mice have a mixed background of C57Bl6/J and Balb/C. The mixed background could protect against the mammary phenotype, which may not emerge until the mice are fully backcrossed. Alternatively, the mixed background GKO mice may have a partial phenotype that we have not seen because we have not examined the mammary glands. Nevertheless, pups of GKO mothers survive as well as those cared for by wild-type or heterozygous mothers. In conclusion, the difference between our GKO mice and the Ucar mice could be caused by genetic changes introduced at the locus, background differences between the strains, or some factor that has yet to be appreciated.

Other possible functions of miR-132

Viruses usurp host cellular machinery to promote viral infection by stimulating a widespread dampening of the immune response (Lagos et al. 2010). miR-132 is up-regulated following viral infection in splenocytes, bone marrow, and macrophages, and mediates the anti-inflammatory response, in part, by directly targeting p300. Interestingly, single allele mutations in p300, or its functional homolog CBP, are found in 39% of diffuse large B-cell lymphomas and 41% of follicular lymphomas (Pasqualucci et al. 2011). Thus, the level of p300/CBP expression is important because haploinsufficiency can cause disease. Given that p300 and CBP have many overlapping functions, it is surprising that loss of only one allele can lead to disease. It is possible that viral infection may play a role in the lymphoma pathogenesis by inducing miR-132 and decreasing the level of p300, which may exacerbate mutations in CBP and promote proliferation or transformation in lymphoma.

miR-132 has also been noted to be induced in macrophages following exposure to lipopolysacharride (LPS). Thus, the idea that miR-132 is a neural specific miRNA is likely to be incorrect and may reflect the fact that neurons are perpetually in an at least partially activated state.

Conclusion

The studies presented in this dissertation demonstrate that miR-132 regulates dendritic plasticity in newborn neurons in the adult hippocampus and participates in the molecular processes underlying the rewarding aspects of cocaine. They also describe the generation of the first conditional knockout of a brain enriched microRNA. Together, these studies add another chapter to our understanding of how microRNAs contribute to neuronal plasticity.

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