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Differentially Expressed MicroRNAs Act As Inhibitors of BDNF in Prefrontal Cortex - Implications for Schizophrenia: A Dissertation

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DIFFERENTIALLY EXPRESSED MICRORNAS ACT AS INHIBITORS OF BDNF IN PREFRONTAL CORTEX – IMPLICATIONS FOR SCHIZOPHRENIA

A Dissertation Presented

By

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Dedicated to my parents Georgios Mellios and Heleni Melliou, and my grandparents

Nikolaos Mellios, Kalliopi Melliou and Vasiliki Mastorakou

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Abstract

During my thesis work I studied the expression and potential function of brain expressed microRNAs (miRNAs) in human prefrontal cortex (PFC). Initially, I used combinatorial computational analysis and microarray data to identify miRNAs that are predicted with high probability to target the human Brain Derived Neurotrophic Factor (BDNF) 3' Untranslated Region (3'UTR) and are expressed in moderate to high levels in adult human prefrontal cortex. A subset of 10 miRNAs segregating into 5 different miRNA families (miR-30a-d, miR-103/107, miR-16/195, miR-191 and miR-495) met the above criteria. I then designed a protocol to detect these miRNAs with Locked Nucleic Acid (LNA) in situ hybridization in human prefrontal cortex and determine their layer and cellular expression patterns. LNA in situ revealed differential lamina and cellular enrichment of BDNF-related miRNAs. As an example, miR-30a-5p was found to be enriched in large pyramidal neurons of layer 3, which was verified using laser capture microdissection of layer 3 pyramidal neurons and quantitative Real Time Polymerase Chain Reaction (qRT-PCR) following dissection of upper and deeper layers of human PFC. Parallel to this, I used

miRNA qRT-PCR to determine the developmental expression of miRNAs using postmortem PFC tissues ranging from embryonic age to old adulthood and compared miRNA to BDNF protein levels. My results revealed a robust inverse correlation between BDNF-related miRNAs and BDNF protein during late maturation and aging of human prefrontal cortex. In vitro luciferase assays and/or lentivirus mediated neuronal miRNA overexpression experiments validated that at least two miRNAs, miR-30a-5p and miR-195, target human BDNF 3'UTR and mediate its translational repression.

In the second part of my thesis work I measured levels of miR-30a and miR-195 in the prefrontal cortex of patients with schizophrenia and compared them with levels of BDNF protein and BDNF-related GABAergic mRNAs. According to my results differences in miR-195 levels in a subset of subjects diagnosed with schizophrenia were found to be associated with disease related changes in BDNF protein levels and deficits in BDNF dependent GABAergic gene expression.

In the last part of my work I focused on miR-30b, another member of the miR-30 family, which I found to be reduced in the prefrontal cortex of female

but not male subjects with schizophrenia. More importantly, disease related changes in miR-30b levels were strongly associated with the age of onset of the disease. Additional experiments in mouse cortex and hippocampus revealed a gender dimorphic expression pattern of this miRNA with higher expression in female brain.

Collectively, my results suggest that miRNAs could participate in novel molecular pathways that play an important role during cortical development and maturation and are potentially linked to the pathophysiology of neuropsychiatric disease.

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ABBREVIATIONS

ANOVA	Analysis of Variance
BA	Brodmann Area
bp	Base pair
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BDNF	Brain Derived Neurotrophic Factor
C	Control
CB	Calbindin
CMV	Cytomegalovirus
CSS	Citrated Saline Solution
Ct	Cycle Threshold
DAB	Diamino Benzidine
DEPC	Diethyl Pyrocarbonate
ELISA	Enzyme-Linked ImmunoSorbent Assay
F	Female
FITC	Fluoroscein Isothiocyanate
GABA	Gamma-AminoButyric Acid
GAD67	Glutamic Acid Decarboxylase 67
H3K4	Histone 3 Lysine 4
ISH	In Situ Hybridization
kD	Kilodalton
LIMK1	Lim Domain Kinase 1
LNA	Locked Nucleic Acid
M	Male
miRNA	MicroRNA
miRISC	miRNA RNA-Induced Silencing Complex
mTOR	mammalian Target Of Rapamycin
NF-H	Neurofilament H

NPY	Neuropeptide Y
NSC30	Non Silencing Control for miRNA-30a
nts	Nucleotides
P1/P4/P9	Promoter 1/4/9 (of BDNF)
p75NTR	p75 Neurotrophin Receptor
PAGE	PolyArcylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3 - Kinase
PFC	Prefrontal Cortex
PKB	Protein Kinase beta (also known as AKT1)
PLCγ	Phospolipase gamma
PMI	Postmortem Interval
PV	Parvalbumin
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
REML	Restricted Maximum Likelihood
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
RT	Room Temperature
S	Subject with schizophrenia
S/C	Subject with schizophrenia to control (ratio)
SNP	Single Nucleotide Polymorphism
SST	Somatostatin
TE	Tris- Ethylenediaminetetraacetic acid (EDTA)
trk-B	Tropomyosin-related kinase Beta
UTR	Untranslated Region

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CHAPTER I - INTRODUCTION

MicroRNAs

The consensus of the scientific community a few decades ago was that the vast majority of non-protein coding genomic regions were either extraneous DNA sequences filling the gaps between protein coding genes or just evolutionary remnants of the integration of viral DNA, and that non-coding RNAs with the exception of the well characterized ribosomal, messenger and transcript RNAs, were of limited biological importance. The finding that small nuclear RNAs (snRNAs) participate in alternative splicing of protein coding transcripts, thus greatly increasing the number and diversity of potential protein products (Tarn and Steitz 1997), was the first indication that parts of the previously named “junk DNA” are important regulators of biological complexity. In the years that followed the discovery of a plethora of small non coding RNAs demanded the modification of the central dogma of biology and revolutionized biomedical research. Surprisingly, the completion of genome projects for different species, including human, revealed that the number of protein coding genes is not necessarily higher in species that are higher in the

evolutionary ladder (Taft et al., 2007). On the other hand, the number of non-coding RNAs was shown to reflect an evolutionary hierarchy (Taft et al., 2007).

MicroRNAs (miRNAs) are a subcategory of small non coding RNAs that are evolutionary conserved and are predicted to mediate the posttranscriptional regulation of at least 30% of protein coding genes (Bartel 2004; Filipowicz et al., 2008). They are derived from longer precursor RNA molecules and their biogenesis pathway consists of two sequential cleavage events by nuclear and cytoplasmic RNase III enzymes (Lee et al., 2002; Gregory et al., 2004; Filipowicz et al., 2008). Specifically, miRNAs are initially transcribed from intergenic or intronic genomic regions to form the primary miRNA precursor molecules known as pri-miRNAs (Lee et al., 2002; Gregory et al., 2004). Pri-miRNAs contain a 5' methyl-guanosine cap and a 3' polyadenylated tail, vary greatly in length from approximately 0.1 to 100 kb and can contain a single or multiple miRNA sequences (Lee et al., 2002; Bartel, 2004). They are initially cleaved in the nucleus by the microprocessor complex, which in mammalian cells contains the RNase III enzyme Drosha

and the RNA-binding protein DGCR8 (Gregory et al., 2004; Zeng et al., 2005). The cleaved precursor is a hairpin structured RNA molecule of approximately 60-80nts and is known as pre-miRNA. Pre-miRNAs are exported to the cytoplasm through the combined action of RAN-GTPase and Exportin 5 (Lee et al., 2002; Yi et al., 2003; Lund et al., 2004). Once in the cytoplasm another RNase III enzyme called Dicer cleaves them further and produces the mature miRNA duplex, which consists of two complementary RNA strands of 18-23 nts in length (Bartel, 2004; Filipowicz et al., 2008). The miRNA duplex is then unwound through the action of an elusive helicase and only one of the two strands is loaded to the miRNA RNA-Induced Silencing Complex (miRISC), which is the miRNA ribonucleoprotein effector complex and contains among others members of the Argonaute protein family (Bartel, 2004; Filipowicz et al., 2008). The RISC complex scans mRNAs for regions of complementarity to the mature miRNA, and depending on the degree of complementarity and the type of Argonaute proteins, results in either translational inhibition or mRNA cleavage. In mammalian cells the target

regions are predominantly in the 3' Untranslated Region (3'UTR) of the mRNA (Bartel et al., 2004; Filipowicz et al., 2008).

Interestingly, recent evidence has suggested that miRNA-mediated posttranscriptional inhibition might be a dynamic process, which involves in some cases the transport of the repressed target mRNA to cytoplasmic loci called P-bodies, where the mRNA is either degraded or stored and released following specific stimuli (Chan and Slack 2006; Bhattacharyya et al., 2006), including synaptic activation in neuronal P-bodies (Cougot et al., 2008). In a similar note, in the nervous systems of flies neuronal activity results in the degradation of a component of miRISC (Ashraf et al., 2006), which is expected to reset the process of miRNA targeting. As far as the translational repression effect, which is the predominant one in mammals, it is believed that miRNAs can inhibit translation of their targets at the initiation or elongation step (Liu 2008, Filipowicz et al., 2008). However, the details of the mechanism of action of miRNAs are still under debate, especially since other functions different from translational inhibition and mRNA cleavage have been proposed, ranging from transcriptional gene silencing through chromatin

modifications (Kim et al., 2008) to miRNA-mediated translational activation (Vasoudevan et al., 2008).

Numerous studies have suggested that miRNAs are important regulators of gene expression that participate in a variety of biological processes (Chang and Mendell, 2007). For example the first two discovered miRNAs *lin-4* (Lee et al., 1993) and later *let-7* (Reinhart et al., 2000) were shown to target genes crucial for developmental timing in C-elegans (*lin-14* and *lin-28* respectively) by interacting with their 3'UTR. Subsequent discoveries of miRNAs in flies, mice and humans revealed that these well conserved small non coding RNAs participate in multiple biological pathways having to do with the control of cellular growth and apoptosis (Cimmino et al., 2005), fat metabolism (Xu et al., 2003) and cellular and chemical differentiation (Chen et al., 2004; Chang et al., 2004).

In the vertebrate nervous system miRNAs have already been shown to regulate neuronal development, differentiation and synaptic plasticity (Giraldez et al., 2005; Schratt et al., 2006; Visvanathan et al., 2007; Makeyev et al., 2007). Maternal-zygotic disruption of Dicer in zebrafish resulted in

aberrations in brain morphogenesis, which were partly rescued upon overexpression of miR-430 (Giraldez et al., 2005). Intriguingly, recent evidence has demonstrated that in mouse neuronal progenitors polarized proteins determine the levels of Ago1 and miRNA expression in daughter cells, with let-7 miRNA being not only required, but also sufficient for neuronal differentiation (Schwamborn et al., 2009). The brain enriched miR-124 was also shown to promote neuronal differentiation by inhibiting non-neuronal genes (Visvanathan et al., 2007; Makeyev et al., 2007). Furthermore, miR-134 was found to be present in mouse hippocampal dendritic spines and to regulate synaptic plasticity by inhibiting LIM Kinase 1 (Schratt et al., 2006). More importantly, brain expressed miRNA miR-132 was proposed to be responsive to neuronal activity and to regulate axonal and dendritic length (Vo et al., 2005; Weyman et al., 2008).

The number of human diseases where miRNAs are implicated keeps rising. These range from examples of miRNAs that can act as oncogenes (Gabriely et al., 2008) or onco-suppressors (Cimmino et al., 2005) to the impressive example of a single nucleotide change in sheep myostatin 3'UTR

that creates an illegitimate target site for muscle enriched miR-1 and miR-206 and results in a muscle hypertrophy phenotype (Clop et al., 2006). The potential role of miRNAs for neuropsychiatric disease, though, has only recently become apparent in the light of the findings that disruption of miRNA processing genes such as Dicer and DGCR8 can promote neurological (Kim et al., 2007; Cuellar et al., 2008; Davis et al., 2008) or psychiatric phenotypes (Stark et al., 2008). Specifically, conditional deletion of Dicer in mouse forebrain neurons resulted in increased postnatal apoptosis, which lead to reduced brain size and enlarged lateral ventricles (Davis et al., 2008). In addition, abnormalities in both axonal pathfinding and dendritic arborization were observed in this study (Davis et al., 2008), suggesting that Dicer plays a critical role in the mammalian nervous system. Additional studies with cell-specific ablation of Dicer verified its importance for the control of neuronal survival (Kim et al., 2007; Cuellar et al., 2008). Parallel to the above findings, mice that are heterozygotes for DGCR8 exhibited alterations in dendritic morphology, and a behavioral phenotype reminiscent of schizophrenia (Stark et al., 2008).The fact that the genomic region that harbors the human DGCR8

gene is disrupted in the DiGeorge microdeletion syndrome (Velo-cardio-facial syndrome) (Gothelf et al., 2007), which conveys a close to thirty fold increase in susceptibility for schizophrenia and schizoaffective disorders (Murphy et al., 1999), implies a link between miRNAs and psychiatric disease. Indeed, during the last 3 years multiple studies have provided evidence supporting the significance of miRNAs in the pathophysiology of brain disorders such as schizophrenia (Perkins et al., 2007; Beveridge et al., 2008), Alzheimer's disease (Wang et al., 2008b), Tourette's Syndrome (Abelson et al., 2005) and Parkinson's disease (Kim et al., 2007; Wang et al., 2008a).

Schizophrenia

Schizophrenia is a debilitating psychiatric disease that is known to affect approximately 1% of the population with an age of onset during late adolescence to young adulthood (Tsuang, 2000). Due to its severity and prevalence in early age schizophrenia has a major socio-economical impact in our society (Kooyaman et al., 2007; McEvoy et al., 2007). Epidemiological research, including studies in monozygotic twins, has suggested a genetic

component for schizophrenia and a large number of genes have already been linked to the disease (Karayiorgou and Gogos, 1997; Tsuang, 2000). On the other hand, most schizophrenia susceptibility genes seem to have a minor if any contribution to the pathogenesis of the disease (Tandon et al., 2008). In addition, epigenetic mechanisms have recently been suggested to be an integral part of the pathophysiology of schizophrenia (Tremolizzo et al., 2003; Tamminga et al., 2005; Huang et al., 2007). Parallel to this, prenatal and perinatal environmental stressors, such as infections and hypoxia, have been shown to increase the possibility of developing schizophrenia (Kroll, 2007; Verdoux, 2004), suggesting a neurodevelopmental aspect for this complicated mental disorder.

The age of onset of schizophrenia is between 15 to 40 years old with very few cases diagnosed at earlier ages (childhood onset schizophrenia) (Lieberman et. al., 2001) and even fewer above the age of 40 (late onset schizophrenia) (Howard et al., 2000). The age of onset of the disease varies by gender, since in females there is usually a later age of onset (Hafner et al., 1998). The symptomatology of the disease is categorized into positive

symptoms such as delusions, hallucinations and disorganized thoughts; negative symptoms such as social withdrawal, flat affect or even catatonia and anhedonia; and cognitive symptoms, such as working memory, attention allocation and executive functioning deficits (Lecrubier et al., 2007). Based on which of the above symptoms are more predominant, schizophrenia is currently classified in 5 different categories/subtypes: paranoid, catatonic, undifferentiated, disorganized and residual according to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (Andreasen et al., 1993).

Before the discovery of the first antipsychotic medications, patients with schizophrenia were held under inhumane conditions in institutions and their uncontrolled symptoms often caused families and caregivers insurmountable psychological pressure. In the midst of this desperate situation, the first proposed treatment for the disease was, ironically, lobotomy. Despite the fact that it involved extreme risk for the patient's life and resulted in catastrophic effects in their cognitive status, frontal lobotomy was considered the practice of choice by some mental institutions (Kucharski, 1984). The discovery of the

first antipsychotic medications in 1952 brought a breakthrough in the way physicians treated patients with schizophrenia, but these drugs were not devoid of dangerous side-effects and were only effective against positive symptoms (Karpenter and Koenig, 2007). The second-generation antipsychotics, although devoid of a certain type of side effects characteristic of typical antipsychotics, were far from what one would consider as a cure, especially since negative and cognitive symptoms were still not effectively alleviated (Leucht et al., 2009). However, it provided the first clue that neurotransmitters and especially dopamine might be important for the pathophysiology of the disease, since dopamine receptors were the main targets of both typical and atypical antipsychotic drugs. This lead to multiple studies that verified the relevance of dopaminergic signaling in schizophrenia (Baumeister and Francis, 2002; Toda and Abi-Dargham, 2007). Subsequent studies however, added additional layers to the complexity of the disease, since glutaminergic, and GABAergic systems proved of comparable importance (Stone et al., 2007; Conn et al., 2008; Paz et al., 2008; Akbarian et al., 1995; Lewis, 2000). Notably, the findings that pharmacological

blockade of NMDA receptors in humans can lead to schizophrenia-like psychosis or can exacerbate an existing condition (Mechri et al., 2001), paired with animal studies outlining the interplay of NMDA receptors and dopaminergic pathways (de Bartolomeis et al., 2005) suggested that the pathways affected in schizophrenia are multiple and possibly interconnected.

Notably, multiple neuroimaging, postmortem and animal model studies have provided considerable evidence suggesting that abnormal circuits within the prefrontal cortex are related to the disease (Lewis et al., 2004; Wobrock et al., 2008; Woodward et al., 2009). Furthermore, processes such as excessive synaptic elimination (McGlashan and Hoffman, 2000) and abnormal neuronal migration (Roberts, 2007) have been proposed to be linked to its pathophysiology. However, none of the studies so far have provided enough evidence to explain the pathogenesis of schizophrenia or even elucidate the mechanisms that could lead to such diverse biochemical and physiological abnormalities.

Prefrontal Cortex

The prefrontal cortex is part of the anterior frontal lobe, which is responsible for high order cognitive functions, is particularly developed in primates and especially humans (Smith and Jonides, 1999; Miller and Earl, 2000), and has been shown to be affected in psychosis (Lewis et al., 2004; Woodward et al., 2009). Subregions of the prefrontal cortex such as the dorsolateral, ventromedial and orbitofrontal prefrontal areas have been shown to contain the neuronal circuits that are responsible among others for working memory, attention allocation, goal directed behavior, social behavior and decision making (Smith and Jonides, 1999; Funahashi, 2001; Lewis and Lieberman, 2000). Not surprisingly, lesions in prefrontal areas can result to a various symptoms that reflect deficiencies in the pre-mentioned high order cognitive functions (Bogousslavsky, 1994; Haddon and Killcross, 2006; Tsuchida and Fellows, 2008).

The gray matter of the prefrontal cortex consists of six layers/laminas which can be easily discriminated using Nissl staining. The main types of prefrontal neurons are the excitatory glutaminergic pyramidal neurons and the inhibitory GABAergic neurons. Among them, GABAergic neurons have been

shown to be very diverse and can be divided in multiple subtypes based on the expression of specific neuropeptides, such as neuropeptide Y (NPY), somatostatin (SST), parvalbumin (PV) and calbindin (CB) and neurotransmitter-related enzymes such as Glutamic Acid Decarboxylase 67 (GAD67) (Tamminga et al., 2004). The inhibitory and excitatory neurons in the prefrontal cortex form connections with neurons in the same or different cortical layer (Lewis et al., 2004; Tamminga et al., 2004). For example pyramidal neurons in layer III form synapses with SST, NPY or PV containing interneurons but also with GABAergic neurons of the upper cortical layers. The orchestrated action of layer III neuronal networks are actually of specific importance since they have already been linked to working memory and attention allocation and their balance has been proposed to be disrupted in schizophrenia (Lewis et al., 2004; Tamminga et al., 2004)). In addition, subtle alterations in the size of somata and in the density of dendritic spines of layer III pyramidal neurons have been identified in postmortem brains of patients with schizophrenia (Pierri et al., 2001; Glantz and Lewis, 2000). However, the most reliable molecular deficits in the schizophrenic PFC have been detected

in transcripts expressed in GABA-expressing neurons (GABAergic transcripts) such as GAD67, NPY and SST (Akbarian et al., 1995; Lewis et al., 2004; Hashimoto et al., 2008).

Despite the fact that most human cortical areas reach their maturation before adolescence, human PFC is known for its very prolonged maturation, which does not complete until late adolescence and young adulthood, a period that coincides with the age of onset of schizophrenia and bipolar disorder (Lewis et al., 2004). Experiments in non human primates have revealed that during adolescence there is extensive and selective pruning of synaptic connections which results in a thinning of cortical gray matter (Lewis et al., 2004). The same process has recently been demonstrated by neuroimaging studies in the human brain and the degree and speed of synaptic elimination seems to be an important predictor of human intelligence (Shaw et al., 2006). The fact the synaptic loss has been already been demonstrated in schizophrenia has prompted the hypothesis that the normal process of synaptic pruning that takes place during adolescence is either accelerated or not properly “halted” in schizophrenia (McGlashan and

Hoffman, 2000). However, the molecular determinants of human PFC maturation and their importance in schizophrenia are only beginning to be understood.

BDNF

Brain Derived Neurotrophic Factor (BDNF) is a prominent member of the neurotrophin family and the second one to be discovered following Nerve Growth Factor (NGF). In the mammalian nervous system BDNF is known to influence a plethora of important processes ranging from neuronal growth, survival and maturation, to chemical differentiation and synaptic plasticity (Marty et al., 1997; Murer et al., 2001; Gorski et al., 2003; Binder et al., 2004; Chan et al., 2008). BDNF is transcribed through multiple promoter regions, which are utilized differentially depending on neuronal activity and brain region (Murer et al., 2001; Pruunsild et al., 2007). Due to the different transcription initiation sites, the subsequent alternative splicing and the utilization of two different alternative polyadenylation sites there are more than twenty divergent BDNF mRNA isoforms (Pruunsild et al., 2007).

BDNF protein is synthesized initially as a pro-neurotrophin (pro-BDNF) that varies in size between 24 and 30 kD, which is then cleaved by proteases to generate the mature 13.5 kD BDNF protein (Mowla et al., 2001; Murer et al., 2001; Fayard et al., 2005). The biological role of the precursor form is not yet completely elucidated but recent studies indicate that it is expressed in high levels in perinatal brain (Jang et al., 2009) and might play an important role in regulation of neuronal survival (Lee et al., 2001). BDNF protein is packaged in secretory vehicles and is released to the synaptic cleft in response to neuronal activity (Murer et al., 2001). There are two types of receptors that BDNF can bind: The high affinity tropomyosin related kinase beta (trk-B) and the low affinity p75 neurotrophin receptor (p75NTR) (Fayard et al., 2005). Binding to trk-B can activate multiple intracellular pathways such as the Ras/ERK, MAPK, phospholipase C gamma (PLCγ) and phosphatidylinositol 3- kinase (PI3K)/Protein Kinase B (PKB or AKT1) signaling, which can culminate in the activation of transcription factors that can affect neuronal gene expression (Murer et al., 2001). In cerebral cortex BDNF has been known to affect the maturation of both GABAergic and

glutaminergic synapses (Kohara et al, 2007; Itami et al., 2000). The importance of BDNF for cortical neurons is further supported by its role in activity dependent synaptic modulation (Lu, 2003). Furthermore, BDNF has been shown to be developmentally regulated in human brain regions such as prefrontal cortex with a substantial increase in mRNA levels happening early postnatally (Webster et al., 2002), which results in BDNF being the highest expressed neurotrophin at adulthood.

Importantly, a SNP in human BDNF gene resulting in a substitution of the amino acid valine to methionine (Val/Met) has been linked to altered cognitive performance (Egan et al., 2003), which suggests that the multiple roles of BDNF on neuronal development and maturation exert a considerable effect on brain function. On a similar note, BDNF has been implicated in the pathophysiology of multiple neuropsychiatric diseases such as depression, schizophrenia, Alzheimer's, Huntington's and Parkinson's diseases (Angelucci, 2005; Hu and Russek, 2008). In the case of schizophrenia, prefrontal BDNF expression has been shown to be reduced at the level of BDNF mRNA, yet BDNF protein levels have been shown to be either

increased, or decreased, or even unaltered (Durany et al., 2001; Weickert et al., 2003; Takahashi et al., 2000; Hashimoto et al., 2005). Despite the fact that cohort demographic factors such as the age of subjects or the experimental techniques used for BDNF protein detection (western blotting vs ELISA) might have contributed to this variability in schizophrenia-related changes in BDNF protein, no study has ever explained the above discrepancy. In addition, polymorphisms in BDNF have been shown to influence frontal lobe thickness in subjects with schizophrenia (Varnäs et al., 2008) and to be associated with age of onset of schizophrenia and response to antipsychotic medication (Krebs et al., 2000), suggesting a link between BDNF and disease progression. Furthermore, BDNF receptor trk-B was found to be reduced in the PFC of subjects with schizophrenia (Hashimoto et al., 2005). Notably, disease-related changes in trK-B but not BDNF mRNA, were associated with PV and GAD67 mRNA expression deficits (Hashimoto et al., 2005), suggesting that different components of the BDNF pathway might be participating in the pathophysiology of schizophrenia.

Goals and rationale

Despite the emerging important role of miRNAs in mammalian brain development and cortical plasticity very little is known about the expression and potential function of microRNAs in human cerebral cortex. The goal of my thesis work was originally to elucidate the spatiotemporal expression of miRNAs in human prefrontal cortex, a brain region which is responsible for high order cognitive functions and is linked to psychiatric disease. I later hypothesized that changes in cerebral cortex expressed miRNAs might play a role during PFC development and maturation by controlling important molecular regulators such as BDNF, which might have implications for psychiatric disease. Our results provide the first evidence suggesting that differentially expressed miRNAs regulate BDNF expression in human PFC, influence the variability of schizophrenia-related changes in BDNF and BDNF-dependent GABAergic gene expression and might be regulated in a gender specific way.

CHAPTER II – A SET OF DIFFERENTIALLY EXPRESSED MICRORNAS, INCLUDING MIR-30A-5P, ACT AS POST-TRANSCRIPTIONAL INHIBITORS OF BDNF IN PREFRONTAL CORTEX

The work presented in this chapter is reproduced from a study by Nikolaos Mellios, Hsien-Sung Huang, Anastasia Grigorenko, Evgeny Rogaev, and Schahram Akbarian published in Hum Mol Genet (Mellios et al., 2008a). This work was conducted under the direction of Dr. Schahram Akbarian, and it is with gratitude to him and the other authors that I reproduce these data for the purposes of this dissertation. My contribution in this work was to design and execute the majority of the experiments and write together with Dr. Schahram Akbarian the manuscript. Specifically, I carried out in silico analysis of miRNA target sites, microRNA in situ hybridization in human and mouse brain, small RNA isolation, qRT-PCR for miRNA analysis, laser capture microdissection, immunostaining in human postmortem brain, construction of multiple miRNA or reporter expressing vectors including self-inactivating lentiviral vectors, luciferase reporter assays, BDNF ELISA, solution hybridization for miRNA detection in human brain and analysis of data. Drs. Evgeny Rogev and Anastasia Grigorenko conducted the microarray experiment, Hsien-Sung Huang conducted the chromatin immunoprecipitation experiment, with the help of Anouch Matevossian, and Dr. Stephen Baker assisted in statistical analysis. Catheryne Whittle contributed to this work by generating and

maintaining rat neuronal cultures. Yin Guo contributed by conducting postmortem brain dissection and David Burns by assisting in live imaging. Mathieu Guillaume, Katerina Ikonomu, Regina Bergmeier and Simone Jäger contributed by assisting in running qRT-PCR, extracting RNA and construction of plasmids. Gulnaz Faskhutdinova contributed by assisting in reagent preparation for in situ hybridization.

Abstract

Expression of brain-derived neurotrophic factor (BDNF) is developmentally regulated in prefrontal cortex (PFC). The underlying molecular mechanisms, however, remain unclear. Here, we explore the role of microRNAs (miRNAs) as post-transcriptional inhibitors of BDNF. A sequential approach involving in silico, miRNA microarray, in situ hybridization and qRT–PCR studies identified a group of 10 candidate miRNAs, segregating into five miRNA families (miR-30a-5p/b/c/d, miR-103/107, miR-191, miR-16/195, miR-495), which exhibited distinct developmental and lamina-specific expression in human PFC. Luciferase assays confirmed that at least two of these miRNAs, miR-30a-5p and miR-195, target specific sequences surrounding the proximal

polyadenylation site within BDNF 3'-untranslated region. Furthermore, neuronal overexpression of miR-30a-5p, a miRNA enriched in layer III pyramidal neurons, resulted in down-regulation of BDNF protein. Notably, a subset of seven miRNAs, including miR-30a-5p, exhibited an inverse correlation with BDNF protein levels in PFC of subjects age 15–84 years. In contrast, the role of transcriptional mechanisms was more apparent during the transition from fetal to childhood and/or young adult stages, when BDNF mRNA up-regulation was accompanied by similar changes in (open chromatin-associated) histone H3-lysine 4 methylation at BDNF gene promoters I and IV. Collectively, our data highlight the multiple layers of regulation governing the developmental expression of BDNF in human PFC and suggest that miRNAs are involved in the fine-tuning of this neurotrophin particularly in adulthood.

Introduction

MicroRNAs (miRNAs) are evolutionary conserved small noncoding RNAs that are known to post-transcriptionally inhibit protein coding genes, by affecting

their translation and/or mRNA stability (Fillipowicz et al., 2008). They are derived from longer precursor molecules, are incorporated to the RNA-induced silencing complex (RISC) and interact with complementary regions mainly within the 3' untranslated region (3'-UTR) of their target mRNAs (Bartel, 2004). Evidence from the early days of miRNA research, and up to the present day, has suggested that the expression of some miRNAs is highly regulated in a temporal and region-specific manner and that they participate in divergent biological processes (Chang and Mendell, 2007).

In the vertebrate nervous system, miRNAs have been shown to play an important role during development (Giraldez et al., 2005) and in regulation of synaptic plasticity (Schratt et al., 2006). A subset of miRNAs are abundantly expressed in the mammalian brain (Miska et al., 2004; Bak et al., 2008) and have been implicated in numerous brain diseases (Abelson et al., 2005; Perkins et al., 2007; Kim et al., 2007; Beveridge et al., 2008; Wang et al., 2008a; Wang et al., 2008b). However, very little is known about their expression and function in the human prefrontal cortex (PFC), a brain area responsible for high order cognitive functions, which displays delayed

maturation and is disrupted in patients with psychiatric disease (Bertolino et al., 1998; Hashimoto et al., 2005).

Brain-derived neurotrophic factor (BDNF) plays a prominent role during cortical development and maturation (Gorski et al., 2003), and alterations in BDNF expression have been reported in a plethora of neuropsychiatric diseases (Angelucci et al., 2005). Interestingly, pyramidal neurons - the primary source of BDNF in cerebral cortex - express high levels of DICER, an RNAse III endoribonuclease and key molecule for miRNA biogenesis, as well as components of RISC, such as eIF2c (Lugli et al., 2005). Furthermore, the 3'-UTR of human BDNF is predicted according to computational analysis to include numerous miRNA target sites that show a high degree of conservation between different mammalian species (Lewis et al., 2003). These findings taken together, point to a potential role for miRNAs in the control of cortical BDNF expression. However, to date, this hypothesis has not yet been tested.

Here, we present evidence that multiple miRNAs, including a subset of the miR-30 family, are involved in fine-tuning of BDNF expression specifically

during late maturation and aging of human PFC. Our findings suggest that BDNF expression in human cerebral cortex is regulated by a complex system of small RNAs, which in turn display lamina-specific enrichment and are differentially regulated during development. These findings provide the first evidence for the miRNA pathway acting as a key regulator of BDNF expression during maturation and aging of human PFC.

Results

In silico analysis of putative miRNA target sites within BDNF 3'-UTR

Potential miRNA target sites within the 3 kb of BDNF 3'-UTR were identified by combining three in silico tools: TargetScan 3.1 (Lewis et al., 2003), Pictar (Krek et al., 2005; Lall et al., 2006) and RNAhybrid (Rehmsmeier et al., 2004). Altogether, 17 distinct target sites - all of which appear to be highly conserved in various mammalian species (human, chimp, dog, mouse, rat) - were found, which potentially could interact with 26 different miRNAs (Fig. 1-1 and Supplemental Table S1-3).

Next, we used an array-based approach to measure the expression of these 26 candidate miRNAs and identified 10 miRNA species that were present at moderate or high levels in the adult human parietal cortex; for the remaining miRNAs, levels were very low or indistinguishable from background (Supplemental Fig. S1-1A). We then compared our array results with published microarray data on adult human PFC (Perkins et al., 2007). Remarkably, all but one (miR-495) showed a similar order of expression in prefrontal and parietal cortex (Supplemental Fig. S1-1B).

Notably, the target site(s) for each of the 10 expressed miRNAs - which segregate into five different miRNA families (miR-103/107, miR-191, miR-16/195, miR-30a-5p/b/c/d, miR-495) - were in close vicinity to the two proximal (out of four total) BDNF 3'-UTR polyadenylation sites (Fig. 1-1); in adult cerebral cortex, the bulk of BDNF transcript extends beyond these two proximal polyA sites (Weickert et al., 2003; Liu et al., 2005). This would suggest that a large fraction of BDNF transcript could be targeted by the miRNAs listed above.

Laminar and cellular specificity of miRNAs expressed in PFC

We wanted to examine the laminar and cellular expression pattern of the predicted miRNAs in adult human PFC; BDNF transcript is found in putative pyramidal neurons positioned within layers II– VI (Huntley et al., 1992; Webster et al., 2002). Cellular labeling was weak or not discernible from background in sections processed by in situ hybridization with locked nucleic acid (LNA-ISH) for miR-1 and miR-10a (Fig. 1-2A and H and Supplemental Fig. S1-2), two miRNAs expressed at very low or non-detectable levels, respectively, according to our microarray data (Supplemental Fig. S1-1A). In contrast, miR-128a, a reportedly pan-neuronal miRNA marker (Smirnova et al., 2005), also detected at high levels in our microarray analysis (data not shown), was robustly expressed throughout the full thickness of PFC (Fig. 1-2A). Therefore, we conclude that LNA-ISH is applicable to human postmortem brain tissue, which is in accordance with previous reports (Nelson et al., 2005). Next, we studied prefrontal expression patterns of the 10 predicted miRNAs with 8 probes (due to one-base-differences, a single probe was applied for miR-103/107 and for miR-30a/d; see also

Supplemental Table S1-2). Each of the 8 probes revealed a distinct laminar expression (Fig. 1-2A and H and Supplemental Fig. S1-2). For example, miR-30a showed robust labeling in the upper cortical layers, including a subset of large, putative pyramidal neurons primarily residing in layer III (Fig. 1-2A and B). In contrast, labeling in PFC layers V and VI was very weak or non-detectable (Fig. 1-2A and E). To further confirm these lamina-specific differences, we assayed miR-30a levels by qRT–PCR separately for the upper (including layers II and III) and lower (mainly V and VI) layers of the cortex. Indeed, levels of miR-30a were ~ 2.5-fold higher in the upper when compared with the deeper layers (Fig. 1-2J). In contrast, neither BDNF mRNA (Fig. 1-2J) nor protein levels (data not shown) showed significant differences between the upper and lower layers; the latter finding may not be too surprising, however, given that BDNF protein produced in pyramidal neuron somata could potentially be distributed via their processes to other cortical layers and neuronal populations further removed from the site of synthesis (Angelucci et al., 2005).

In addition, expression of miR-30a in pyramidal neurons was further confirmed in sections processed by LNA-ISH followed by immunolabeling for a neurofilament epitope selectively expressed by a subset of pyramidal neurons (Cambell et al., 1989) (Fig. 1-2I) and by RT–PCR from laser-capture dissected pyramidal neurons (Supplemental Fig. S1-2). Expression of miR-495 was highly restricted and limited to a subpopulation of cells positioned in and around layer II (Fig. 1-2H and Supplemental Fig. S1-2). Cellular labeling for miR-16 in tissue sections was weak and mostly confined to deeper portions of cortical gray matter (Supplemental Fig. S1-2). It is possible that the high levels of miR-16 in mature erythrocytes (Rathjen et al., 2006) could have contributed to the comparatively high but variable levels of expression in whole tissue homogenates assayed by array (Supplemental Fig. S1-1).

Among all miRNAs tested, only miR-103/107 was enriched in upper cortical layers and was also expressed in white matter. This particular finding is in good agreement with a recent report showing the same laminar enrichment in superior and middle temporal gyrus (Wang et al., 2008b). Intriguingly, however, none of the miRNAs included in our study showed

discernable layer specificity in mouse cerebral cortex (Supplemental Fig. 1-2 and data not shown), suggesting that miRNA expression in cerebral cortex shows important differences between human and rodent. In conclusion, miR-30a and other members of the miR-30 family, and several additional miRNAs predicted to interact with the 3'-UTR of BDNF are abundantly expressed in adult human PFC, with distinct laminar specificity.

Validation of human BDNF 3'-UTR miRNA target sites

To determine whether the candidate miRNAs described above target the 3'-UTR of human BDNF mRNA, we constructed a luciferase reporter plasmid with a 551 bp fragment of human BDNF 3'-UTR containing all the highly predicted miRNA target sites fused to the 3' end of the luciferase gene (Fig. 1-3A). We utilized CMV-driven vectors that encode for each of the following miRNA precursors—miR-30a, miR-30b, miR-30c, miR-107, miR-191 and miR-195 (Supplemental Fig. S1-3). Since the endogenous levels of these miRNAs varied, transfection of these plasmids in HEK293 cells resulted in various degrees of overexpression of the mature miRNAs (Fig. 1-3D). It

should be noted that, in these assays, miR-16 and miR-495 were not included because of uncertain (miR-16), or highly restricted (miR-495) expression in the cortex. Furthermore, because of high sequence similarity (one single nucleotide difference), miR-30d and miR-103 are predicted to have very similar effects on BDNF 3'-UTR with miR-30a and miR-107 respectively; hence these miRNAs were not tested separately.

Due to the fact that miR-30a was enriched in layer III pyramidal neurons (Fig. 1-2), which are a critical component of prefrontal cortical neuronal networks (Lewis et al., 2004), and one of the major sites of BDNF synthesis (Huntley et al., 1992), we anticipated that this miRNA might be particularly important for the post-transcriptional inhibition of BDNF. Additionally, in silico analysis of the interaction between miR-30 miRNAs and BDNF 3'-UTR indicated a more favorable thermodynamic interaction of miR-30a compared with miR-30b and miR-30c, resulting from a higher 3' end complementarity of miR-30a with the 3'-UTR (Supplemental Fig. S1-3). Indeed, results from the luciferase assay showed that overexpression of miR-30a, but not miR-30b and miR-30c, lead to a significant decrease in activity of

the reporter (Fig. 1-3B). Among the remaining miRNAs tested, only miR-195 induced a significant reduction in luciferase activity (Fig. 1-3B). Importantly, no changes in reporter activity were observed after transfection of a miR-30a-based non-silencing control ('NSC30', Fig. 1-3B), in which the seed sequence (nt 2–7, see Fig. 1-4D for details) was mutated in order to disrupt complementarity with BDNF 3'-UTR target sites. Furthermore, the NSC30 mature sequence was not predicted to target the 551 bp of BDNF 3'-UTR, according to RNA hybrid software (data not shown).

Notably, processing of the miR-30a precursor can lead to two miRNAs, miR-30a-5p and miR-30a-3p (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008). Interestingly, both our microarray (Supplemental Fig. S1-1A) and ISH experiments (Fig. 1-2A and data not shown) indicate that expression of miR-30a-5p is much higher than miR-30a-3p in the human cerebral cortex. Therefore, we modified the miR-30a precursor sequence by replacing one or two Watson–Crick base pairings with G-U wobbles at the 5' end and vice versa for the 3' end (marked by arrows in Fig. 1-3C). The rationale was to destabilize the 5' end relative to the 3' end of the precursor, which is expected

to shift relative levels of mature miRNA towards the 5' end product (Schwarz et al., 2003). Indeed, transfection with this modified miR-30a precursor (m30a-5p, see Fig. 1-3B–D) resulted in a 3-fold increase in levels of mature miR-30a-5p, compared with transfection with the wild-type form of the precursor (Fig. 1-3D). There was a ~ 36% reduction in reporter activity in cells transfected with m30a-5p, compared with ~ 24% in cells transfected with wild-type miR-30a precursor (Fig. 1-3B). Finally, it is worth mentioning that there are two potential miR-30 interaction sites within the BDNF 3'-UTR, although the second site (Figs 1-1 and 1-3A) is likely to be less functional due to the presence of a G-U wobble in the seed sequence (first site, nts 397–402; second site, nts 680-685 from the beginning of BDNF 3'-UTR, see Supplemental Table S1-3) (Doench and Sharp, 2004). Therefore, we wanted to demonstrate that the first canonical site is sufficient to mediate the inhibitory effect of miR-30a. To this end, we transfected HeLa cells with a luciferase vector containing a 70 bp BDNF 3'-UTR sequence, which included the first target site (nucleotides 1653–1722, Genbank ID NM_170735). There was a consistent, ~35% decrease in reporter activity in cells transfected with

miR-30a precursor relative to a scrambled precursor control (n = 3 independent experiments, data not shown).

Furthermore, we co-transfected both miR-30a and miR-195, which independently reduced luciferase activity, with half the amount needed to exert the observed significant inhibitory effect (375 ng of each per well), a concentration that for miR-30a was unable to induce significant repression (data not shown). The combination of these two miRNAs even in such lower concentrations resulted in a significant (~31%) reduction in luciferase activity (Fig. 1-3B). This level of inhibition was not, however, significantly higher than the independent inhibitory effects of miR-30a (~24%) and miR-195 (~28%), so that the possibility of a synergistic effect remains to be clarified. These results indicate that a subset of miRNAs expressed in PFC, including miR-30a, can exert an inhibitory interaction with BDNF 3'-UTR sequences.

MiR-30a negatively regulates BDNF protein in neurons

The validation of miR-30a:BDNF 3'-UTR interaction with luciferase reporter assays in two different cell lines, and its higher relative expression in neurons

that are able to synthesize BDNF compared with some of the other candidate miRNAs, including miR-195, suggest that miR-30a is more likely to be a potential regulator of neuronal BDNF expression. To pursue this further, we used a lentivirus-based system to overexpress miR-30a precursor in cultured neurons (derived from E14.5 rat forebrain progenitor cells, see Materials and Methods) (Fig. 1-4A) and then assayed BDNF protein by ELISA. A lentivirus expressing miR-NSC30 (Fig. 1-4D) was also included in these experiments. Transduction efficiencies were verified with GFP expression and included a majority of cells (on average 60%) (Fig. 1-4A). Cultures transduced with miR-30a showed a 2–4-fold increase in mature miR-30a levels, as measured by qRT–PCR (Supplemental Fig. S1-4). There was a significant, ~30% decrease in BDNF protein levels in neuronal cultures overexpressing miR-30a (Fig. 1-4B). In contrast, the control - which was identical to miR-30a except for three mutations in the seed sequence (Fig. 1-4C and D) - did not alter neuronal BDNF levels. Notably, miRNAs exhibiting partial complementarity to their mRNA target (such as miR-30a: BDNF 3'-UTR, Fig. 1-4C), predominantly block translation, but additional mechanisms involving mRNA decay have

also been reported (Filipowicz et al., 2008). Therefore, we assayed BDNF mRNA in our cultures, and no changes were observed (Supplemental Fig. S1-4). We conclude that miR-30a exerts an inhibitory effect on BDNF translation in neurons.

Expression of selected miRNAs in PFC shows inverse correlation with BDNF protein during late adolescence and adulthood

Our experiments ex vivo described above strongly suggest that miR-30a-5p regulates BDNF protein levels, via interaction with a conserved sequence located in the proximal portion of BDNF 3'-UTR. Based on this observation, it could be possible that there is an inverse relationship between BDNF and miR-30a levels in (human) PFC tissue. Furthermore, it has been shown that when multiple miRNAs target a specific transcript, synergistic effects could lead to more robust target regulation when compared with each miRNA separately (Greco and Rameshwar, 2007). Given this potential cooperativity in miRNA targeting and the limitations of our luciferase assay to address this

issue, we wanted to investigate if their combined effects on BDNF levels during various stages of PFC development could be physiologically relevant.

Towards this end, we first used qRT–PCR to assay expression levels of the following miRNAs using seven sets of primers (with confirmed sequence specificity, see Materials and Methods) for qRT–PCR (miR-30a,b,c,d, miR-103/107, miR-191 and miR-195) in 37 PFC specimens (BA 10) across a wide age range, from the second trimester of pregnancy to 84 years. Tissue levels for these miRNAs were variable across the lifespan, although 5/7 miRNAs were defined by a significant increase in specimens from individuals older than 41 years, in comparison to either specimens younger than 15 years (miR-30c, miR-30d, miR-191, miR-195) (Fig. 1-5A and Supplemental Fig. S1-5), or - in the case of miR-30a - in late adolescent to young adult specimens (ages 15-41) (Fig. 1-5A and B). Importantly, PMI, brain pH, gender and other postmortem confounds (See also Materials and Methods and Supplemental Table S1-1) had no significant effect on miRNA expression (data not shown). Furthermore, miR-128a, a pan-neuronal miRNA not predicted to interact with BDNF, showed a progressive decrease during the course of PFC

development (Fig. 1-5A and Supplemental Fig. S1-5), which contrasts the observed increases in BDNF-related miRNAs (Fig. 1-5A and B and Supplemental Fig. S1-5). Given these highly dynamic differences in miRNA levels during the course of PFC development, and especially in the mature PFC, we asked whether these changes relate to BDNF protein content.

To address this question, we measured BDNF protein by ELISA in the same postmortem specimens. Our data showed that both BDNF protein and mRNA are up-regulated during the early stages of postnatal PFC development, yet appear to be discordant during late adolescence and adulthood (Fig. 1-5A, C and D). In addition, there was, as expected, a positive correlation between BDNF mRNA and protein, in the entire developmental cohort ($r = +0.379$, $p = 0.025$) and, independently, in samples less than 15 years of age ($r = +0.525$, $p = 0.021$). However, there was no significant correlation in samples more than 15 years of age (data not shown).

Strikingly, in mature (15–84 years old) PFC a highly robust, inverse correlation between the expression of the 7 miRNAs as a group and BDNF protein levels (Fig. 1-6A and B) was observed. Furthermore, there was an

inverse correlation between PFC BDNF levels for the same age group (15-84 years old), and for three miRNAs independently (miR-30a, miR-30d and miR-191) (Fig. 1-6C). These statistical associations were highly specific, because the (neuronal enriched) miR-128a - which is not predicted to target BDNF - had no correlation to BDNF protein levels in any age group (Fig. 6C and data not shown). In addition, no correlation was detected between BDNF mRNA and miRNA expression levels (data not shown). Taken together, these findings suggest that the orchestrated developmental expression of a group of miRNAs including miR-30a might exert an inhibitory effect on BDNF translation especially in the mature PFC.

Transcriptional mechanisms regulating BDNF expression in immature PFC

In contrast to the significant findings in adults, there were no significant correlations between BDNF protein and the 7 miRNAs as a group, or individually, in subjects less than 15 years old (Fig. 1-6D and data not shown). However, we noticed that levels of BDNF mRNA were increased up

to 3-fold after birth, consistent with an earlier report (Webster et al., 2002), but did not change significantly thereafter (Fig. 1-5D). These findings raise the possibility that BDNF levels at these earlier stages of PFC development are less dependent on miRNA-mediated post-transcriptional regulation and instead are regulated on the level of gene expression.

In order to address this issue, we measured levels of H3-trimethyl-lysine 4, an open chromatin mark related to transcriptional activity (Eissenberg and Shilatifard, 2006) that can be measured in postmortem brain (Huang et al, 2007) - at defined BDNF promoter sequences in PFC of fetal, child and adult samples. We assessed BDNF gene promoters I and IV (P1 and P4, Fig. 1-7A), as these are known to be epigenetically regulated in rodent cerebral cortex (Chen et al., 2003; Martinowicz et al., 2003; Tsankova et al., 2006; Nelson et al., 2008). As a control we also checked for changes of the same chromatin marker within the newly recognized promoter IX (P9, Fig. 1-7A), which reportedly shows only very low levels of activity in brain (Pruunsild et al., 2007). Indeed, our results showed a significant increase in histone methylation at BDNF P1 occurring after birth (Fig. 1-7B), and at BDNF P4

after childhood (Fig. 1-7C). In contrast, histone methylation at BDNF P9 was very low and indistinguishable from background in all samples (data not shown). These results suggest that while miRNAs exert a robust effect on BDNF levels in mature and aging PFC, chromatin remodeling and transcriptional mechanisms might play a more prominent role at the earlier developmental stages.

Discussion

Using multiple approaches, including microarray, LNA-ISH and qRT–PCR we identified a group of miRNAs that were abundantly expressed in different layers of human PFC and predicted to target a specific region within human BDNF 3'-UTR. Notably, albeit the sequence of these miRNAs is completely preserved in multiple mammalian species, there was lamina-specific expression in human but not in mouse neocortex. A subset of these miRNAs (miR-30a,b,c,d, miR-103/107, miR-191, miR-195) showed an inverse correlation with BDNF protein levels in the adult, but not in the immature human PFC. Among these miRNAs, miR-30a exerted a significant inhibitory

interaction with BDNF 3'-UTR in functional assays and decreased BDNF protein levels in neuronal culture.

The significant inverse correlation between the group of the selected miRNAs and BDNF protein levels from late adolescence to old age suggests that these miRNAs could participate in post-transcriptional fine-tuning of BDNF expression in adult PFC, including the periods of late maturation and aging. Interestingly, BDNF mRNA levels in human PFC have been shown to increase from infancy to young adult age but subsequently are maintained at roughly the same levels during adulthood and old age (Webster et al., 2002). In contrast, BDNF protein levels are reportedly reduced during the aging of human PFC (Durany et al., 2001). Therefore, our studies could potentially explain these discrepancies in age-dependent changes of BDNF mRNA and protein, by showing that BDNF protein levels in mature and aging PFC could be driven in part by the post-transcriptional regulation mediated by BDNF-related miRNAs (Fig. 1-5 and Supplemental Fig. S1-5).

In contrast, the absence of significant correlations between BDNF protein and the selected miRNAs in the younger PFC samples could be partly

attributed to a more prominent transcriptional control of the BDNF gene in the immature PFC (defined here as the fetal and 0-15 years old samples). This hypothesis is further supported by dynamic increases in open chromatin-associated histone methylation at a subset of BDNF gene promoters during postnatal PFC development (Fig. 1-7). It has to be noted, though, that due to technical limitations we did not explore the expression levels of all BDNF alternative mRNA transcripts and the chromatin status of all BDNF gene promoters. In addition to the two layers of regulation outlined in the present study - miRNA-mediated inhibition and chromatin remodeling - additional mechanisms that could affect expression and function of BDNF regulation may involve antisense non-coding transcripts originating from the BDNF locus (Pruunsild et al., 2007).

The presence of miR-30a in large layer III pyramidal neurons of human PFC, as observed in the present study by qRT–PCR, LNA-ISH and laser capture (Fig. 1-2 and Supplemental Fig. S1-2), is of particular interest given the fact that this neuronal population displays alterations in dendritic spine density (Glantz and Lewis, 2000) and soma size (Pierri et al., 2001) in

schizophrenia, a disease where deficits in BDNF levels have been reported in some postmortem cohorts (Weickert et al., 2003; Hashimoto et al., 2005). In this context it is intriguing that the developmental dynamics of miR-30a expression in human PFC include a pronounced decline in miR-30a levels during the late phase of PFC maturation (ages 15–41 years old), which coincides with the age of onset of the clinical symptomatology of psychiatric disease (Lewis et al., 2004).

According to our present study, at least seven different miRNAs could contribute to the regulation of BDNF expression in human PFC (miR-30a-5p, miR-30b,c,d, miR-103/107, miR-191, miR-195). Interestingly, miR-107 was very recently shown to be significantly down-regulated in Alzheimer's disease (Wang et al., 2008b). Furthermore, in a previous study, miR-30b and miR-195 were shown to be reduced in schizophrenia PFC (Perkins et al., 2007). Of note, miR-30a-3p was shown, by qRT-PCR, to be increased in cases of the same study; this miRNA is derived from the same precursor as miR-30a-5p, although its interactions with BDNF remain unclear. The potential role of

these miRNAs for BDNF regulation and signaling in diseased brain remains to be clarified.

It is noteworthy that the BDNF-related miRNAs that were the focus of our study are also predicted to target numerous genes related to synaptogenesis, neuronal migration, neuronal growth and differentiation, according to multiple computational analysis tools (Lewis et al., 2005; Krek et al., 2005; Lall et al., 2008). In this context, the miR-30 family of miRNAs is predicted to target multiples genes (Lewis et al., 2003; Grimson et al., 2007) implicated in the genetics or pathophysiology of schizophrenia other than BDNF; these include MAP6 (Shimizu et al., 2006), NR4A2 (Rojas et al., 2007), GRM3 (Egan et al., 2004), GRM5 (Devon et al., 2001), CNR1 (Ujike et al., 2002), NCAM1 (Barbeau et al., 1995; Sullivan et al., 2007) and NEUROG1 (Fanous et al., 2007). Given their potential interaction with multiple schizophrenia risk genes and the reported interaction between BDNF and other miRNAs important for neuronal plasticity, additional studies are needed to elucidate the potential significance of this family of miRNAs in the context of psychiatric disease.

According to the present study, there is laminar specificity for several miRNAs expressed in human PFC, including miR-30a, miR-103/107, miR-495 (Fig. 1-2 and Supplemental Fig. S1-2); for miR-103/107, this was also observed in (human) temporal neocortex (Wang et al., 2008b). In striking contrast, the same miRNAs appeared to be either expressed evenly throughout layers II–VI of mouse neocortex (including frontal areas), or in the case of miR-495, below the detection limit (Supplemental Fig. S1-2 and data not shown). It is possible that these species-related differences in cortical miRNA patterns could result in a more sophisticated lamina-specific regulation of BDNF expression in the human cortex.

Interestingly, a previous study has demonstrated that miR-134, a brain enriched miRNA that can inhibit the translation of the neurotrophin-related gene Lim-domain-containing protein kinase 1 (LIMK1) and regulate dendritic spine density, increases postnatally in mouse hippocampus, reaching its maximum levels at the age when synaptic maturation occurs (Schratt et al., 2006). Therefore, one could hypothesize that maturation processes in the mammalian brain related to neurotrophin signaling could be influenced by the

miRNA pathway. The findings presented here further support this scenario by showing that developmentally regulated miRNAs including members of the miR-30 family could modulate BDNF expression in human PFC.

Lastly, in addition to the miRNA-mediated inhibitory effects on BDNF levels as reported here, this neurotrophin might itself regulate the expression of neuronal miRNAs (Vo et al., 2005; Klein et al., 2007). Furthermore, BDNF could antagonize miRNA-mediated translational inhibition (Schratt et al., 2006), possibly by activating the tropomyosin-related protein kinase B (TrK-B)/mammalian target of rapamycin (mTOR) signaling pathway, which in turn interacts with subunits of the translation initiation complex (Filipowicz et al., 2008; Gingras et al., 1998). Interestingly, deficiency of protein kinase B (PKB or Akt1), a kinase that can activate the mTOR pathway alters neuronal morphology and leads to impaired PFC functions (Lai et al., 2006). It is therefore intriguing to speculate that miRNAs, including the ones discussed here, might participate in a molecular network involving multiple reciprocal nodes, which together orchestrate and fine-tune prefrontal BDNF expression and signaling in a developmental stage- and lamina-specific manner.

Materials and Methods

Postmortem studies

Postmortem samples from 37 subjects, obtained from the dorso-rostral pole of the frontal lobe (Brodmann's area 10), were included in this study. All procedures were approved by the review boards of the participating institutions. All brains were fresh-frozen and stored at -80ºC. The fetal, child and adolescent samples were obtained through the Brain and Tissue Banks for Development Disorders, University of Maryland (NICHD contract no. NO1-HD-8-3284). Adult samples were obtained from a brain bank located at the University of California at Davis, as described (Huang et al., 2007; Akbarian et al., 1995). Demographics, medication status and postmortem confounds, including tissue pH and RNA integrity number (RIN) are provided in Supplemental Table S1. For the cases where the age of onset was recorded as approximately twenties (20S) or thirties (30S) the age used for calculation was 20 and 30 years, respectively. For all experimental procedures, each assay included samples from all age groups. Adult brains were subjected to neuropathological examination to rule out neurodegenerative disease.

RNA isolation

Small RNAs (<200 nt) were isolated by using the mirVANA PARIS kit (Ambion), according to the manufacturer's instructions and treated with DNase I for 30 min at 37ºC. Then, samples were incubated at RT (room temperature) for 2 min in DNase I inactivating buffer (Ambion—RNAqueous kit), followed by centrifugation (13,000g) for 1.5 min and supernatant was stored at -80ºC. The mirVANA PARIS kit was also used for the extraction of large (>200nt) RNA that was used for measurement of BDNF mRNA. The mirVANA PARIS kit (Ambion) was used for total or small RNA isolation from rat neuronal cultures and the RNAqueous Micro kit (Ambion) was used for total RNA extraction in HEK-293 cells. All samples were treated with DNase I to avoid DNA contamination. For determination of RNA quality, RNA RIN were calculated using the Agilent 2100 bioanalyzer and according to manufacturer's instructions. RNA quantification amplicons were generated for 5S rRNA and the following miRNAs (see also Supplemental Table S2): (i) miR-30a-5p, (ii) miR-128, (iii) miR-103, (iv) miR-30b (v) miR-30c, (vi) miR-

30d, (vii) miR-191, (viii) miR-195, using mirVana qRT–PCR miRNA detection kit (Ambion). Applications were performed with an 7500 Applied Biosystems Real-Time PCR System and SDS software: Step 1, 95ºC x 3 min; Step 2, 95ºC x 15 s; Step 3, 60ºC x 34 s, 40 cycles (Step 2, Step 3), followed by dissociation step to obtain SYBR Green I-based melting curves. Specificity of the reactions was confirmed by melting curve analysis in conjunction with gel electrophoresis and, if necessary, subcloning and sequencing. For example, amplicons derived from miR-30a and miR-30d-specific PCR reactions (two miRNAs that differ in a single nucleotide in the middle portion of their mature sequence) yielded the correct sequence in >95% of clones (n= 37). For each sample and amplicon, cycle thresholds were averaged from triplicate reactions and normalized to 5S rRNA according to the following formula, $E^{-\text{^Ctmi}RNA}/E^{-\text{^Ct5sr}RNA}$, where E = (1+ln2/primer slope). TaqMan One-Step RT–PCR (Applied Biosystems) was used according to manufacturer's instructions for the human BDNF, and 18S rRNA and rat BDNF and 18S rRNA with primers shown in Supplemental Table S1-2.

miRNAs microarray

MiRNA expression profile was analyzed in pooled RNA samples isolated from parietal cortex of right hemisphere from 7 normal individuals (4 males with ages 41, 42, 52, 59 years and 3 females with ages 35, 44 and 57 years). Mixed RNA probes were labeled with Cy5 fluorescent dye and applied onto a mParaFloTM Human miRNA chip (LcSciences). The chip contains seven redundant regions with miRNA probes corresponding to miRNA transcripts from miRNA Registry list (Human_V4E_050630 - Based on Sanger miRNA Registry Release 7.0), non-verified miRNA probes and multiple control probes. 5S rRNA was used as a housekeeping gene for normalization control.

The data were processed with background subtraction (regression-based background mapping method), Cy5 channel normalization (Locally-weighted regression method on the background-subtracted data) and detection determination (LcSciences data analysis). Transcripts were determined as detectable if their signal intensity was higher than 3x background standard deviation, spot coefficient of variation (standard deviation/signal intensity) was

<0.5 and transcripts had at least 50% of replicate probe signals registering above the detection level.

Solution hybridization

^{32}P-UTP-labeled probes (mirVana miRNA Probe Construction Kit, Ambion) reverse antisense to the mature miRNAs and 5S rRNA were used in conjunction with the solution hybridization assay according to manufacturer's instructions (mirVana miRNA detection kit). Briefly, the small RNA sample was mixed with the probe and after hybridization in solution, samples were subjected to RNase digestion. The radiolabeled protected fragments of the probe after RNase inactivation and precipitation were separated in a denaturing polyacrylamide gel. DNA oligonucleotides used to generate the probes were as follows (without linker sequence): 5'-CTTCCAGTCGAGGATGTTTACA-3' (probe generated is the reverse complement of mature mir-30a-5p); 5'-ACTAGAGCCTTCGATT-3' (probe generated is the reverse complement of a conserved region within the 5S rRNA).

ISH with LNA-modified oligonucleotides

For LNA-ISH, 20 µm thick sections of immersion-fixed (human) or perfusion-fixed (mouse) cerebral cortex were mounted on SuperFrost-Plus slides (VWR), air-dried, then subjected to the following procedure with sterile solutions (DEPC-treated water): Washed with 1x PBS 3 x 5 min each, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at RT, then washed again in 1x PBS 3 x 5 min each, then protein was denatured using 0.2 M HCL/ 1xPBS for 10 min at RT, then washed with 1xPBS 3 x 5 min, then treated with 0.25% acetic anhydride /0.1 M triethanolamine/ 1xPBS for 10 min at RT, washed again at 1x PBS 3 x 5 min, then prehybridized in 50–100 µl hybridization buffer per section for 2 h at a specific temperature depending on probe based on the formula T_m probe -21ºC, with T_m provided by probe vendor (Exiqon) and shown in Supplemental Table S2. Each probe is a 5'-digoxigenin-labeled, 2'-O, 4'-C methylene bicyclonucleoside monomer-containing oligonucleotide (LNA, phosphoramidite). Sequences are reverse complement to the mature miRNAs (Supplemental Table S2). The 20 ml of

hybridization buffer was made of 50% deionized formamide/ 2x SSC/10% dextran sulfate/ 500 mg/ml sperm DNA/ 0.25 mg/ml yeast t-RNA/ 0.2 mg/ml BSA/ 50 mg/ml heparin/ 2.5 mM EDTA/ 0.1% Tween-20 in 2.3 ml DEPC-H20. After absorbing the prehybridization buffer with a kimwipe, 50–100 µl of hybridization buffer containing 0.17–0.25 mM of probe were added to each section and slides were covered with RNAse-free coverslips (HybriSlip, Molecular Probes) and incubated overnight at the specific temperatures (see above) in a humidified chamber. The following day, sections were washed twice in 2xSSC at RT for 15 min on a shaker, then washed in 1xSSC at 37ºC for 15 min, then washed twice with 2xSSC/formamide, then with 0.1xSSC for 30 min at the probe-specific temperature (see above), then washed with 0.1xSSC for 15 min at RT, then incubated with buffer I (0.1% Tween-20/0.1 M Tris–HCL, pH 7.5/150 mM NaCl) for 10 min at RT, then with blocking solution [10% normal goat serum/1% blocking reagent (Roche) in buffer I] for 30 min at RT, then incubated with anti-digoxigenin-alkaline phosphate-conjugated antibody (goat, Roche) diluted 1:1000 in blocking solution (150 µl/slide) and parafilm-covered slides were incubated in a humidified chamber on shaker for

3 hours at RT. Sections were then washed in buffer I 3 x 15 min each, then incubated in buffer III (0.1 M Tris–HCL, pH 9.5/0.1 M NaCl) at RT for 10 min, then 500 ml of color substrate solution (CSS) were added to each slide (CSS = nitroblue tetrazolium/BCIP stock solution (Roche) diluted 1:50 in buffer III) at RT under light-protected conditions overnight. Slides were then washed in TE buffer at RT for 10 min, then washed with 1xPBS at RT for 10 min, then with ddH20 at RT for 10 min. Finally the slides were coverslipped with 100 µl of VectaMount mounting medium (Vector Labs) for each slide, and were stored under light-protected conditions at RT for microscopic studies. Additional sections were first processed by LNA-ISH as described above, and then subjected to immunohistochemical labeling with the mouse monoclonal anti-neurofilament H (anti-SMI-32 antibody, Covance) and FITC-conjugated goat-anti mouse antibody, followed by diaminobenzidine (DAB)-based peroxidase detection with Vectastain ABC (Vector Labs).

BDNF immunoassay

Protein was extracted with the mirVANA PARIS kit according to manufacturer's instructions and after centrifugation the supernatants were used for estimation of total protein with BCA micro-kit (Pierce). BDNF levels were essayed with enzyme-linked immunosorbent assay (ELISA) and with the use of BDNF ELISA kit (Chemicon) according to manufacturer's instructions.

Immunohistochemistry, tissue dissection and laser capture microdissection procedures

Sections, 8–10 μm thick, were cut from frozen unfixed postmortem human tissue blocks (adult PFC—BA10) on a cryostat (Leica) on plain non-coated glass slides, stored at -80°C, than before staining they were dried for 2 min, fixed in 100% acetone for 2 min, air dried for 30 s, then washed in PBS and processed for immunohistochemistry with the mouse monoclonal anti neurofilament H (NF-H; SMI-32 antibody, Covance) and FITC-conjugated goat-anti mouse antibody, with intermittent washing steps. This staining procedure was limited to altogether less than 100 min, and then sections were transferred to a Arcturus Veritas microdissection instrument (Molecular

Devices) in order to collect somata of layer III NF-H immunoreactive pyramidal neurons, as defined by triangular shape and prominent, vertically oriented apical dendrite. As a control, tissue from deeper white matter was collected. Cells were collected in pools of 500–1000, using the CapSure MacroLCM Caps (Arcturus) collection caps and then transferred to RNase-free Eppendorf tubes and stored at -80ºC until further processed. RNA was extracted with the mirVana miRNA isolation kit (Ambion). In particular, the plastic membrane containing harvested cells was removed from the CapSure cap and immersed into 300–400 µl of the kit's lysis-binding buffer, then incubated in the same solution at 42ºC for 30 min with intermittent vortexing, in order to remove the laser-captured tissue from the membrane. The yield was ~5 ng/µl small RNA/pool. For dissection of upper and deeper cortical layers, superficial cortical gray matter (approximately upper one-fifth of gray matter) and white matter from frozen unfixed postmortem tissue (n = 5, ages 30, 38, 56, 61, 68 years of age) was removed and the upper (roughly corresponding to parts of layers II and III) and lower one-third (roughly

corresponding to parts of layers V and VI) of the remaining gray matter tissue was used for protein and RNA extraction.

Chromatin immunoprecipitation in postmortem tissue

Chromatin immunoprecipitation in postmortem tissue from human PFC of different age was done as described previously (Huang et al., 2007) by using 70–100 mg of tissue and with the primers shown in Supplemental Table S1-2.

Luciferase assays

Ambion's pMIR-REPORT luciferase reporter plasmid was engineered to include a 551 bp fragment of human BDNF 3'-UTR (1500–2051 nt, Genbank ID NM_170735) at the 3' end of the luciferase gene. Lipofectamine 2000 (Invitrogen) was used for transfection of HEK293 cells in 24-well plates. CMV-driven vectors containing chicken beta-actin promoter (named CAG-R-miR plasmids, see also Supplemental Fig. S1-3) and expressing miRNA precursors (750 ng per well) were cotransfected with the luciferase reporter plasmid (150 ng per well) containing the 551 nt fragment of BDNF 3'-UTR and

with Ambion's pMIR-REPORT β-galactosidase plasmid (100 ng per well) to control for transfection efficiency. Luciferase and β-galactosidase assays (Promega) were used to calculate the normalized luciferase expression. As controls 750 ng of vector expressing miR-NSC30 precursor with 3 bases difference in the seed sequence (see also below) or 750 ng of an EGFP expressing vector (control reference) were used. The overexpression of the mature miRNAs was measured with qRT–PCR and from at least two replicates.

Neuronal transduction

The pGIPZ self-inactivating lentiviral empty vector was purchased by Open Biosystems. Two sets of 111 bp oligos that encode the human miR-30a precursor or the miR-30a precursor with 3 bases difference in the seed sequence of the 5p mature miRNA (NSC30) and that contain XhoI and EcoRI restriction enzyme overhangs (purchased by Integrated DNA Technologies) were annealed and initially ligated into a double digested (XhoI, EcoRI) self-inactivating retroviral vector pSM2c by Open Biosystems (Purchased by the

shRNA Library Core Facility of UMass Medical School). After PCR and subsequent digestion this product was then ligated to the pGIPZ self-inactivating lentiviral empty vector. The final products (called pmiR30 and NSC30) are designed to drive expression of tGFP (turbo Green Fluorescent Protein) and the miRNA precursor molecule, through the same CMV RNA polymerase II promoter. The expected mature miRNA of the miR-NSC30 precursor molecule is not predicted to target BDNF mRNA at any region (RNA hybrid software). Standard methodologies were used for preparation of rat forebrain neuronal cultures from precursor cells (Huang et al., 2007), for viral production and infection (Wang et al., 2005). The production of the mature miR-30a was assayed with qRT–PCR. In addition, transduction efficiency was estimated by measuring GFP expression 4 days post-infection with epifluorescence microscopy (Nikon Eclipse E600). An average of 60% transfection efficiency was observed.

Statistical analysis

For the analysis of the Luciferase data, and after proper normalization of Luciferase activity to β-galactosidase activity and logarithmic transformation, data were evaluated using analysis of variance (ANOVA) for a mixed model by REML (restricted estimation by maximal likelihood). In the presence of significant main or interaction effects, pairwise comparisons were evaluated using Tukey Kramer adjustment for multiple comparisons. In the cases where no pairing was required, then ANOVA with post hoc Tukey was applied after ensuring normalized distribution of data. For presentation of data from age groups with different age representation and sample size and for allowing the comparison or combination of ‘relative’ changes in the values measured, weighted means were selected for data shown on Figs 1-5 and 1-6.

Figure 1-1. BDNF 3'-UTR contains numerous predicted target sites for miRNAs expressed in human cerebral cortex. Map of BDNF 3'-UTR (human) showing potential miRNA target sites conserved across mammalian species (see also Supplemental Table S1-3); target sites for miRNAs expressed in moderate to high levels in human cerebral cortex (Supplemental Fig. S1-1) are shown in black whereas the remaining predicted sites are shown in gray. Notice that miRNAs expressed in moderate to high levels are located in the vicinity of the two proximal poly-A sites in the BDNF 3'-UTR.

A

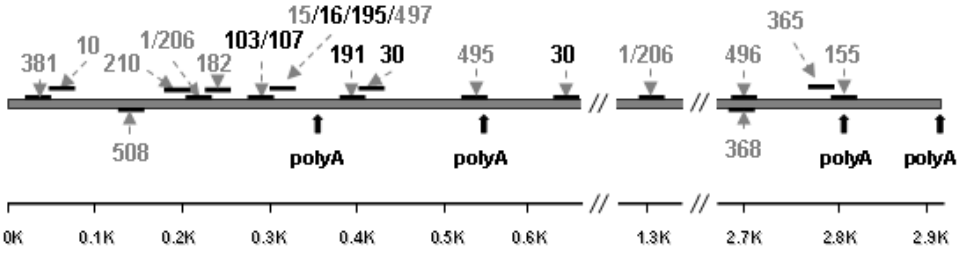
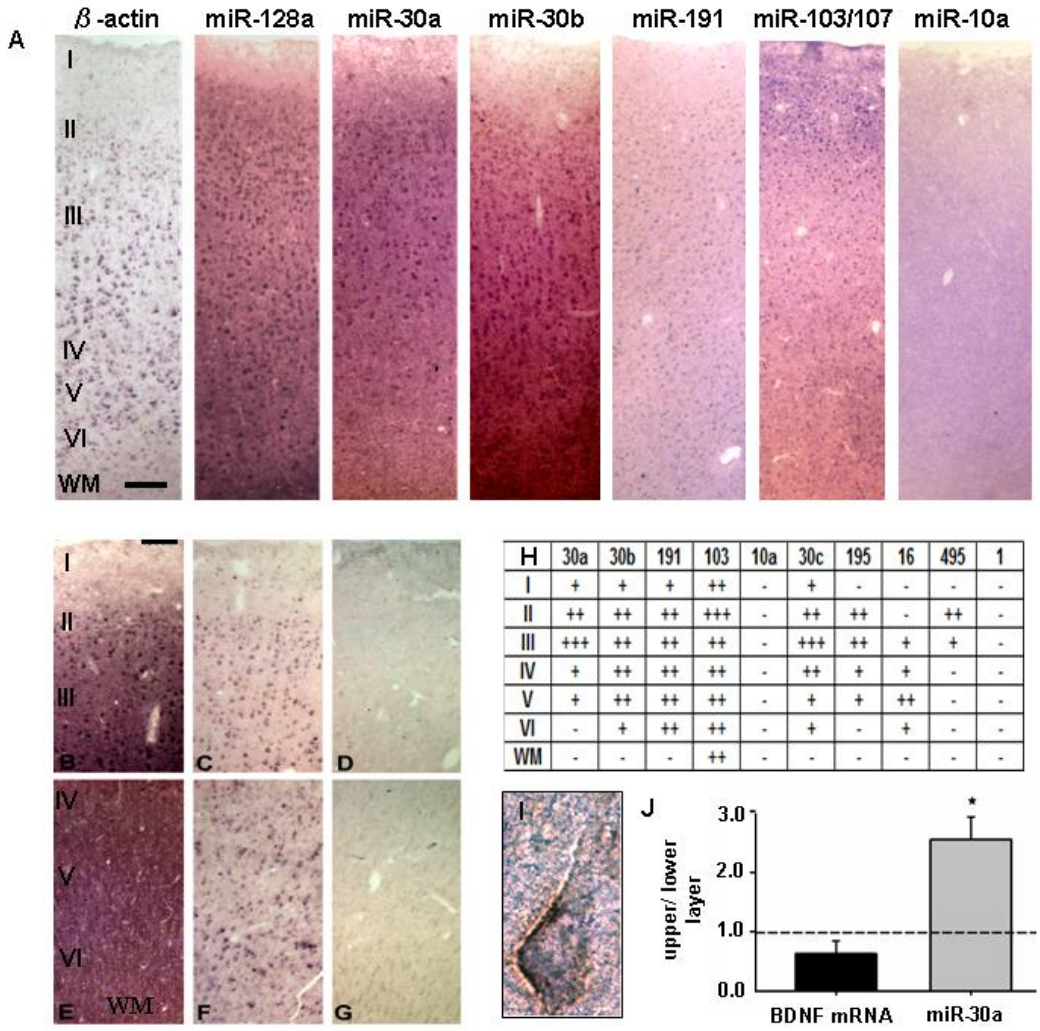

15/16/195/497
365
381
10
210
1/206
182
103/107
191
30
495
30
1/206
496
155
508
368
polyA
polyA
polyA
polyA
0K
0.1K
0.2K
0.3K
0.4K
0.5K
0.6K
1.3K
2.7K
2.8K
2.9K

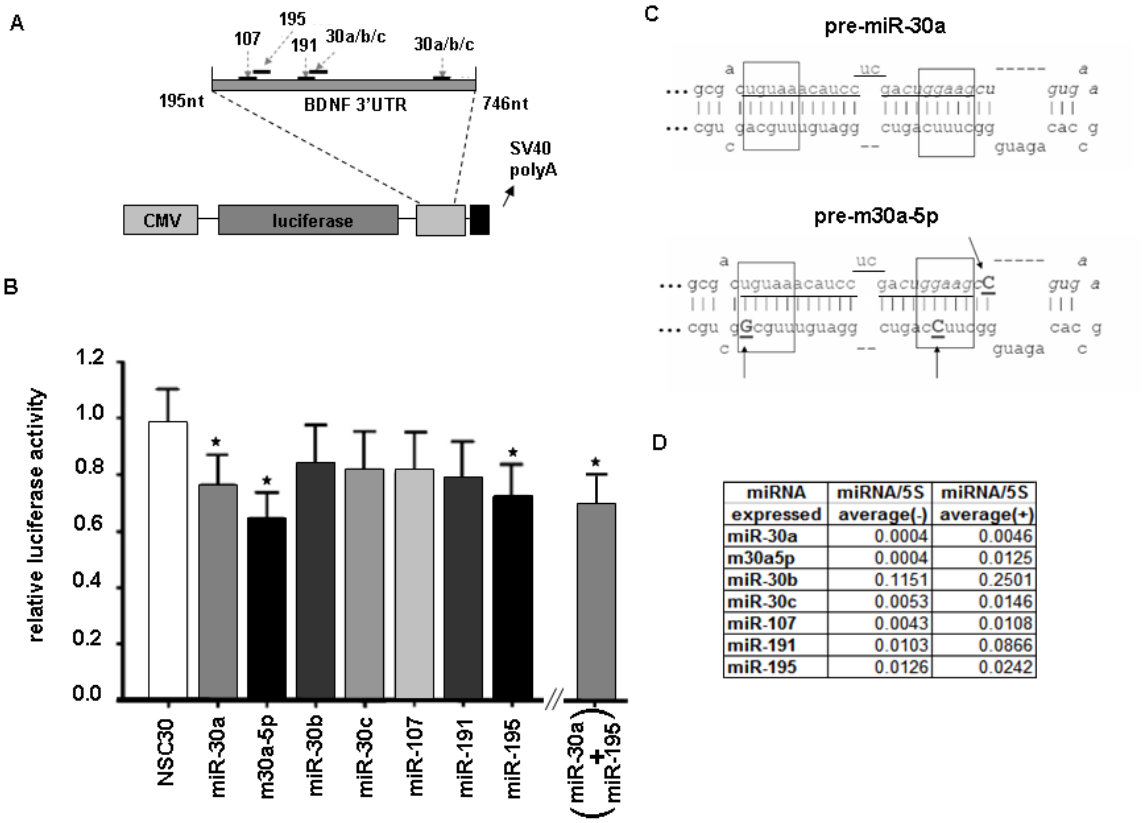
Figure 1-2. Lamina and cellular expression pattern of selected miRNAs in human PFC. (A) Representative images (from 2–6 replicates) of six-layered PFC (adult) sections processed by LNA-ISH with miRNA-specific probes, and β-actin as control. Notice lamina-specific expression patterns, including enrichment of miR-30a in layers II and III. (B–G) Additional images of upper (B–D) and deeper (E–G) cortical layers for miR-30a LNA-ISH (B and E) together with β-actin (C and F) and no probe negative control (D and G). (H) Table summarizing relative expression of 10 miRNAs across PFC layers I–VI, and underlying white matter (WM) (+++ = high, ++ = moderate, + = weak, or indistinguishable from background). Note that miR-103/107 probe detects both miRNAs due to a single nucleotide difference at their 3' end. (I) Double labeled layer III pyramidal neuron from section processed for Neurofilament-H immunohistochemistry (brown) after miR-30a LNA in situ (purple). (J) Bar graphs show qRT–PCR data for miR-30a and BDNF mRNA from dissected tissue corresponding to upper (layer II and III) and lower (layer V and VI) cortical layers from five adult samples (see Materials and Methods) shown as relative ratios (upper to lower). Notice the significant approximately 2.5-fold enrichment of miR-30a in upper layers. Bar in (A) = 200 μm, in (B–G) = 100μm. Image (I) taken at 63 x 10 magnification.



H	30a	30b	191	103	10a	30c	195	16	495	1
I	+	+	+	++	-	+	-	-	-	-
II	++	++	++	+++	-	++	++	-	++	-
III	+++	++	++	++	-	+++	++	+	+	-
IV	+	++	++	++	-	++	+	+	-	-
V	+	++	++	++	-	+	+	++	-	-
VI	-	+	++	++	-	+	-	+	-	-
WM	-	-	-	++	-	-	-	-	-	-

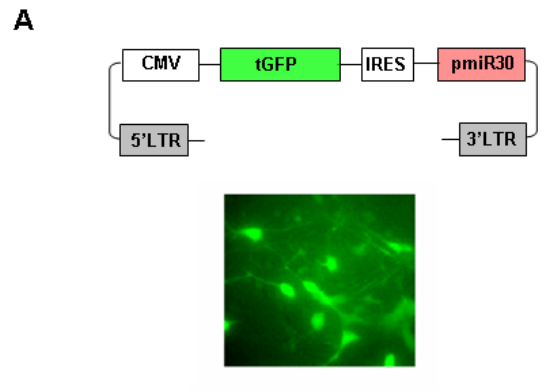
Figure 1-3. A region of human BDNF 3'-UTR is targeted by a subset of miRNAs. (A) Map of the luciferase reporter vector containing the 551 bp portion of human BDNF 3'-UTR (nts 195 to 746 from beginning of 3'-UTR, nucleotides 1500–2051, Genbank ID NM_170735), which includes target sites for the miRNAs assayed in (B). Bar graph in (B) shows results from reporter assay, expressed as luciferase units normalized to β-galactosidase units and relative to a reference control (EGFP expressing vector not containing any miRNA sequence). Last column shows relative luciferase activity following transfection of both miR-30a and miR-195 vectors – see methods for more). Bars represent the calculated means by ANOVA REML model plus standard error. Asterisks depict statistically significant differences (after post-hoc Tukey correction) compared with the (miR-30a based) non-silencing seed sequence mutant (NSC30); p = 0.010 (miR-30a); 0.0005 (m30a-5p); 0.0136 (miR-195); 0.0207 (miR-30a + miR-195). n = 2–6 independent replicates. Notice the significant reduction in reporter activity after transfection with miR-30a, m30a-5p and miR-195 expressing vectors. (C) Predicted secondary structure of the (top) wild-type pre-miRNA 30a and (bottom) modified precursor designed to preferentially express miR-30a-5p (m30a-5p in (B)). Sequence underlined represents mature miR-30a-5p miRNA, which remains unaltered in the modified precursor. Arrows demarcate nucleotides modified from wild-type. See text for further details. (D) Table summarizing expression changes of mature miRNAs after transfection of

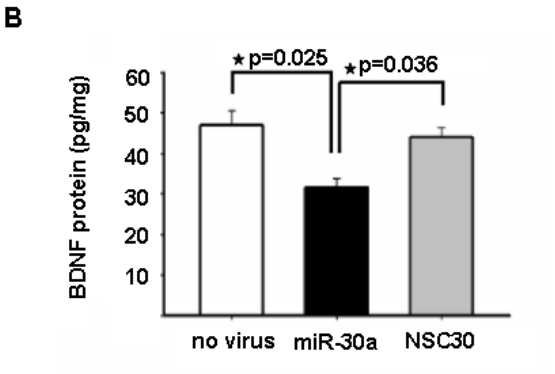
HEK293 cells with appropriate vectors (averaged from two independent experiments); (+) labels transfected cells, (-) non-transfected controls.

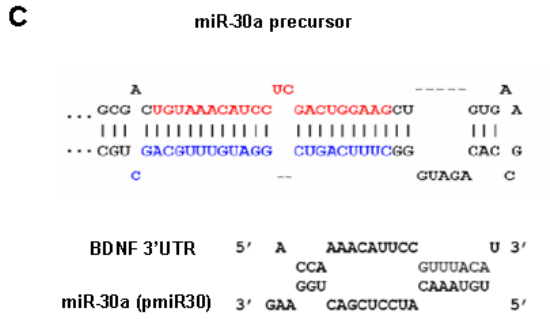


miRNA expressed	miRNA/5S average(-)	miRNA/5S average(+)
miR-30a	0.0004	0.0046
m30a5p	0.0004	0.0125
miR-30b	0.1151	0.2501
miR-30c	0.0053	0.0146
miR-107	0.0043	0.0108
miR-191	0.0103	0.0866
miR-195	0.0126	0.0242

Figure 1-4. MiR-30a mediates translational inhibition of BDNF in neurons. Neuronal cultures from rat forebrain were infected with lentiviruses that contained constructs co-expressing GFP and precursor miRNAs. (A) Top: Map of the lentiviral vector used; Bottom: Representative image of neuronal culture infected with the GFP-expressing lentiviral vector shown above. (B) Bar graphs showing BDNF protein levels (mean ± SEM) for cultures infected with miR-30a (n = 3), or miR-NSC30 (n = 3) (see text for details), and non-infected ('no virus', n = 2) cultures. Notice the significant decrease in BDNF protein in miR-30a overexpressing cultures. P-values after post-hoc Tukey/ANOVA. (C and D) Top: Secondary structures of (C) miR-30a precursor and (D) miR-NSC30 which contains a 3 base substitution in the seed sequence of miR-30a-5p. Mature miR-30a-5p is depicted in red, miR-30a-3p in blue and the nucleotide changes in the non-silencing control, NSC30, are shown in black and underlined. Bottom: Predicted interactions between the first target site in BDNF 3'-UTR (see text for details) and either (C) wild-type miR-30a-5p or (D) non-silencing precursor ('NSC30').


A
CMV
tGFP
IRES
pmiR30
5'LTR
3'LTR


B
BDNF protein (pg/mg)
60
50
40
30
20
10
★p=0.025
★p=0.036
no virus
miR-30a
NSC30


C
miR-30a precursor
BDNF 3'UTR
miR-30a (pmiR30)


D
Non-silencing control (NSC30)
BDNF 3'UTR
NSC30

Figure 1-5. Expression patterns of selected miRNAs and BDNF in PFC across the lifespan. (A) Heatmap showing relative expression levels presented as quartiles (from higher to lower: red, yellow, green, blue) of (i) control miR-128a not predicted to target BDNF, (ii) 7 microRNAs each predicted to target BDNF and expressed in human PFC (data from qRT–PCR and normalized to 5S rRNA), (iii) average of weighted means of all 7 miRNAs shown in (ii), (iv) BDNF protein as measured by ELISA and (v) BDNF mRNA as measured by qRT–PCR and normalized to 18S rRNA. All samples from human PFC (gray matter, BA10) ranging in age from 21 estimated weeks of gestation (ewg) to 84 years (y). Brain Hemisphere (H), gender (G) and tissue pH (pH) are also shown for each sample. Notice the age-related expression changes of the BDNF-related miRNAs, including the decline in miR-30a levels in late adolescent—young adult group (15–41 years of age), which are distinct from the control miR-128a. (B–D) Bar graph summarizing the developmental expression data shown in (A) for miR-30a (B), BDNF protein (C), and BDNF mRNA levels (D). Graphs represent weighted means ± SEM. (n = 7–15 per age group) normalized to 5S rRNA for miRNAs and to 18S rRNA for BDNF mRNA, for (x-axis) four different age groups ranging from fetal to adult ages (n = 7–15/age group). Notice again the robust decline in miR-30a levels in the group of samples 15–41 years old and the significant increases in BDNF mRNA after birth (0–15 year) and in BDNF protein both after birth and between 15 and 41 years of age (B). *$p < 0.05$, (ANOVA post hoc Tukey).

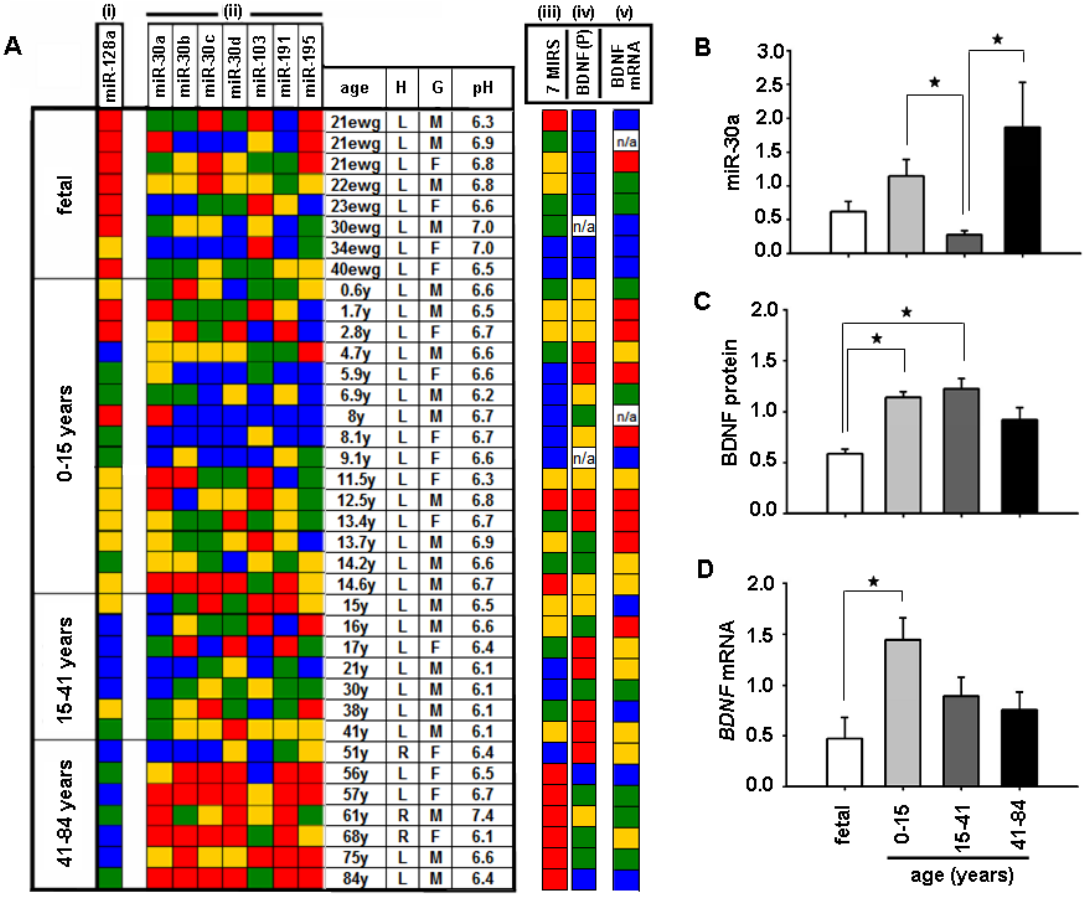
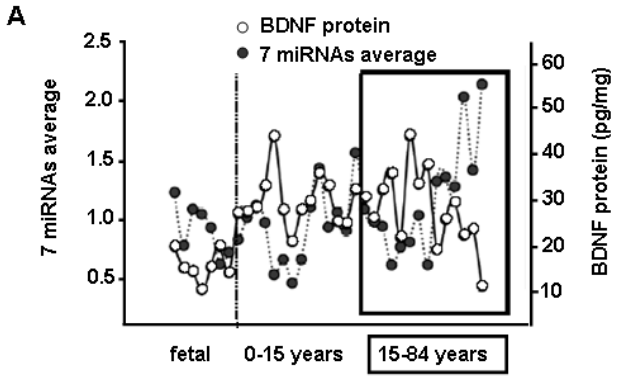
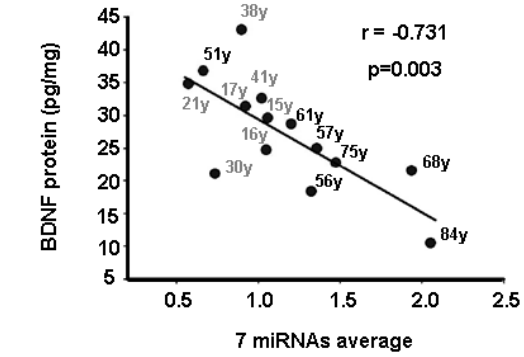

A
(i)
(ii)
(iii)
(iv)
(v)
miR-128a
miR-30a
miR-30b
miR-30c
miR-30d
miR-103
miR-191
miR-195
7 MIRS
BDNF (P)
BDNF mRNA
age
H
G
pH
fetal
0-15 years
15-41 years
41-84 years
21ewg L M 6.3
21ewg L M 6.9
21ewg L F 6.8
22ewg L M 6.8
23ewg L F 6.6
30ewg L M 7.0
34ewg L F 7.0
40ewg L F 6.5
0.6y L M 6.6
1.7y L M 6.5
2.8y L F 6.7
4.7y L M 6.6
5.9y L F 6.6
6.9y L M 6.2
8y L M 6.7
8.1y L F 6.7
9.1y L F 6.6
11.5y L F 6.3
12.5y L M 6.8
13.4y L F 6.7
13.7y L M 6.9
14.2y L M 6.6
14.6y L M 6.7
15y L M 6.5
16y L M 6.6
17y L F 6.4
21y L M 6.1
30y L M 6.1
38y L M 6.1
41y L M 6.1
51y R F 6.4
56y L F 6.5
57y L F 6.7
61y R M 7.4
68y R F 6.1
75y L M 6.6
84y L M 6.4
n/a
B
miR-30a
C
BDNF protein
D
BDNF mRNA
fetal
0-15
15-41
41-84
age (years)

Figure 1-6. Inverse correlation between BDNF protein and BDNF-related miRNAs in late adolescent and adult PFC. (A) Plot showing case-by-case the relative changes in BDNF protein (white circles) when compared with weighted average of all seven miRNA probes (black) across the life span (n = 35). Notice the consistent inverse relationship between BDNF and miRNA average from late adolescence until old age (15–84 years, box in graph). (B) Inverse correlation between BDNF protein and average from weighted means of the seven miRNAs for 15–84-year-old samples (y, year, 15–41 shown in gray, rest shown in black); r, Pearson coefficient. Notice, also, declining BDNF, and increased miRNA levels in six out of the seven oldest samples. (C) Table showing correlations between miRNAs and BDNF protein in late adolescent and adult (15–84 years old) PFC. Notice independent significant inverse correlations between miR-30a, miR-30d and miR-191 with BDNF protein (all shown in bold), and lack of inverse relationship between BDNF and a control miRNA (miR-128a, shown in italics) not predicted to target BDNF. (D) No correlation between BDNF protein and average from weighted means of the seven miRNA probes for fetal to 15-year-old samples.



C

miRNA	r	pvalue
miR-30a	**-0.55**	**0.041**
miR-30b	-0.53	0.053
miR-30c	-0.45	0.107
miR-30d	**-0.56**	**0.036**
miR-103	-0.23	0.430
miR-191	**-0.64**	**0.013**
miR-195	-0.31	0.274
miR-128a	*0.21*	*0.479*



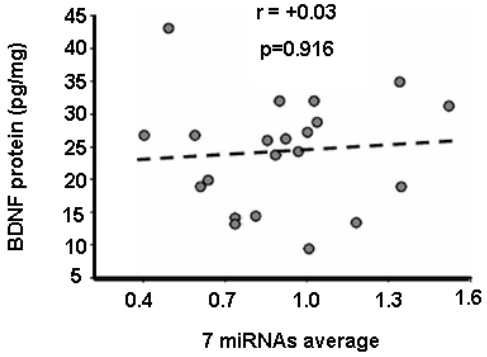
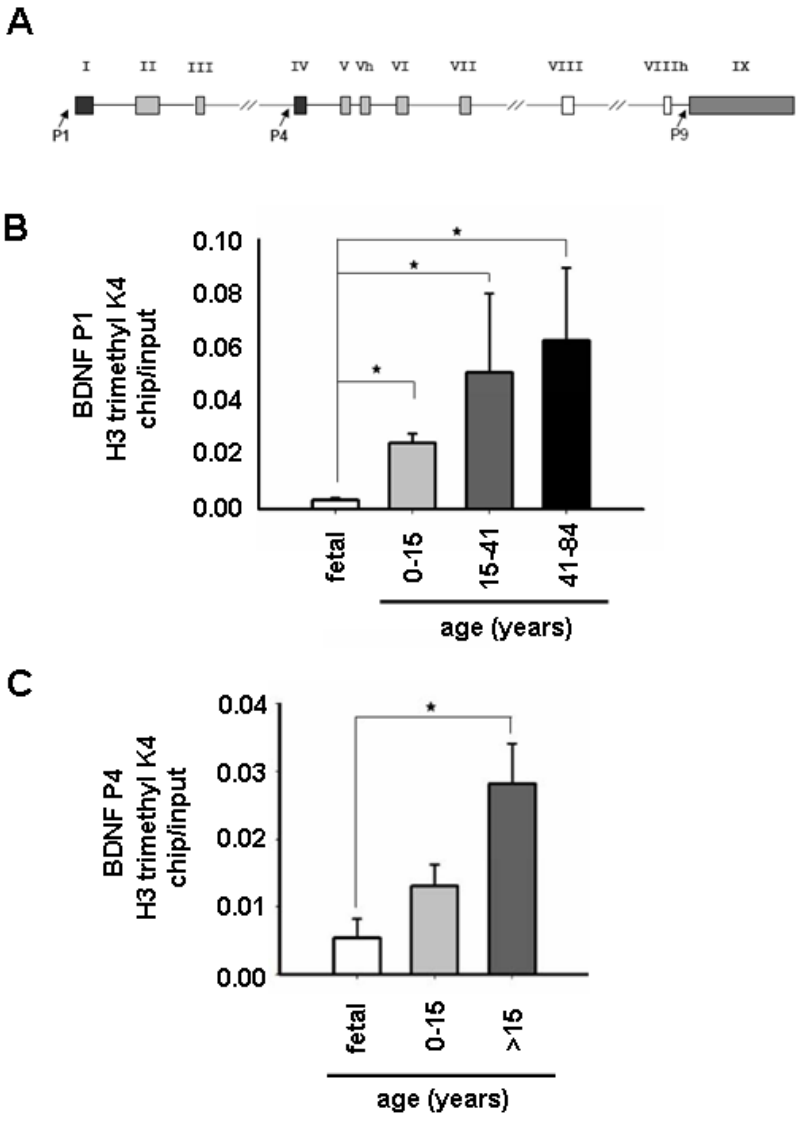
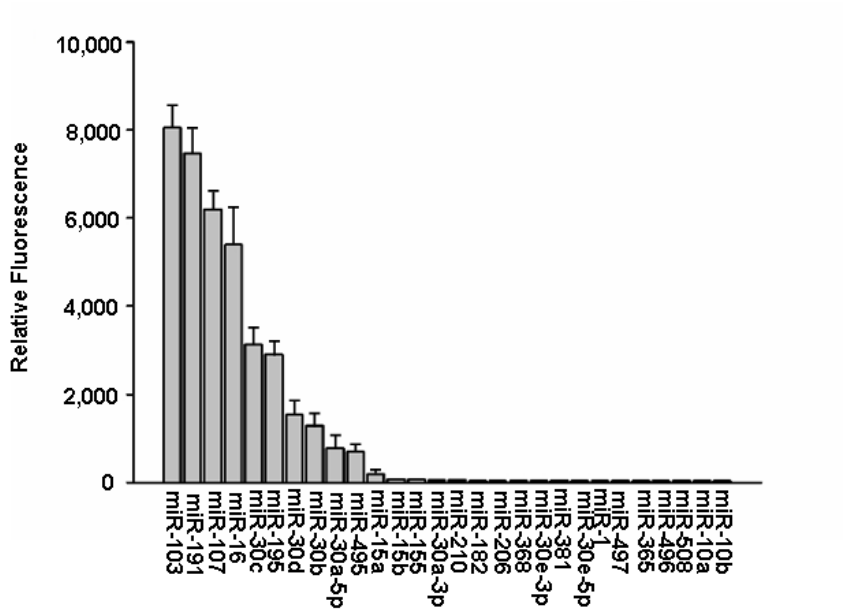


Figure 1-7. Chromatin remodeling at BDNF promoters during PFC development. (A) Illustration showing human BDNF gene, including its multiple exons and the three promoters (P1, P4 and P9, arrows) selected for chromatin immunoprecipitation studies (see text). (B and C) Developmental changes in tri-methylated histone H3-lysine 4 at BDNF P1 (B) and P4 (C). Bar graphs represent mean ± SEM of chip-to-input ratios (n = 3–6/age group). Notice significant increases in P1- and P4-associated histone methylation after birth or childhood [last two age groups merged in (C) due to limited number of samples], respectively. *p< 0.05, ANOVA post hoc Tukey.


A
I
II
III
IV
V
Vh
VI
VII
VIII
VIIIh
IX
P1
P4
P9
B
BDNF P1
H3 trimethyl K4
chip/input
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
fetal
0-15
15-41
41-84
age (years)
C
BDNF P4
H3 trimethyl K4
chip/input
0.04
0.03
0.02
0.01
0.00
*
fetal
0-15
>15
age (years)

Supplemental Figure 1-1: Expression of BDNF-related miRNAs in adult cerebral cortex. (A) Relative expression of the predicted miRNAs in adult human parietal cortex (pooled RNA from 7 samples) by microarray. Data shown as mean ± SD (spot to spot SD), with the variance referring to the array's replicate measures all after normalization to *5S* rRNA (4-5 replicates). (B) Comparison between relative abundance of the BDNF-related miRNAs in adult parietal cortex and in adult prefrontal cortex from previous microarray study. The 10 most highly expressed predicted miRNAs are shown from higher (up) to lower expression (down). Notice that 9/10 of the highly expressed in parietal cortex miRNAs are also within the top ten expressed in human prefrontal cortex (shown in bold).

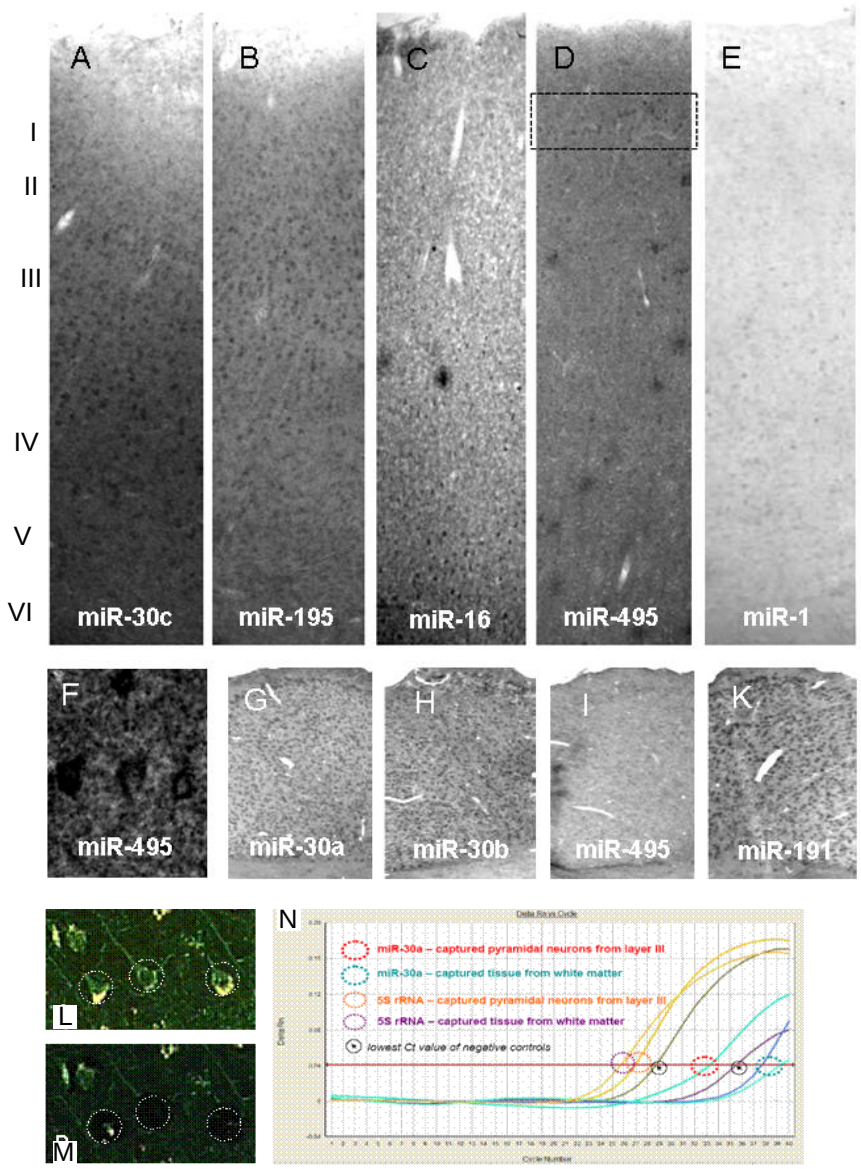
A



B

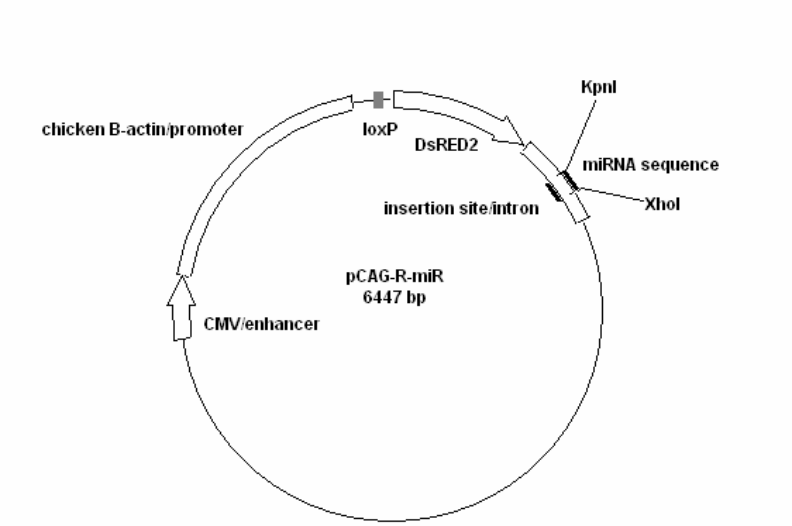
Adult Parietal Cortex (our study)	Adult Prefrontal Cortex (Perkins et al., 2007)
miR-103	miR-103
miR-191	miR-107
miR-107	miR-30c
miR-16	miR-30b
miR-30c	miR-191
miR-195	miR-30d
miR-30d	miR-16
miR-30b	miR-195
miR-30a	miR-15b
miR-495	miR-30a

Supplemental Figure 1-2: Lamina and cell specificity in miRNA expression in human PFC and mouse cerebral cortex. (A-E) Representative images from LNA in situ hybridization in postmortem fixed tissue from adult human prefrontal cortex for miRNAs 30c (A), 195 (B) 16 (C), 495 (D) and 1 (E).Layers of gray matter (I-VI) are shown. Notice the layer 2 specific enrichment for miR-495 (box) and the very low to negative signal for heart and muscle enriched miR-1. (F) Layer 2 miR-495 expressing neurons in higher magnification. (G-K) LNA in situ hybridization in mouse cerebral cortex for miR-30a, miR-30b, miR-495 and miR-191. Notice the lack of lamina specificity of mouse miR-30a. (L-N) Immunofluorescence for NF-H in acetone fixed sections from human postmortem PFC as visualized in the laser capture microdissection apparatus. (L) Before laser capturing of SMI-32 positive pyramidal neurons in layer III and (M) after laser capture of selected pyramidal neurons. (N) qRT-PCR from RNA<200nts isolated from 500-1000 laser captured pyramidal neurons of layer III and tissue fragments from white matter shows that miR-30a can be detected in the laser captured SMI-32 positive pyramidal neurons of layer III of human PFC (red dotted circle) but is undetectable in laser captured tissue fragments from the white matter (light blue dotted circle) of the same section. Note also the detection of 5S rRNA with qRT-PCR in both small RNA samples indicating that the RNA isolation procedure was successful (orange and purple dotted circle). Arrows represent the lowest Cycle Threshold (Ct) values from negative controls (no sample) for 5S rRNA (left) and miR-30a (right). Images taken at magnification: (A-E, G-K at 10X5, F at 10X63 and L,M at 10X10).


A
B
C
D
E
I
II
III
IV
V
VI
miR-30c
miR-195
miR-16
miR-495
miR-1
F
G
H
I
K
miR-495
miR-30a
miR-30b
miR-495
miR-191
L
M
N
miR-30a – captured pyramidal neurons from layer III
miR-30a – captured tissue from white matter
5S rRNA – captured pyramidal neurons from layer III
5S rRNA – captured tissue from white matter
lowest Ct value of negative controls

Supplemental Figure 1-3: MiRNA expressing vector map and computational analysis of miR-30a,b and c targeting of BDNF 3'UTR. (A) Map of the pCAG-R-miR miRNA expression vector used to drive the expression of the studied miRNAs in HEK-293 cells. The position of the CMV enhancer and chicken β-actin promoter are shown. The miRNA precursors were expressed from an intronic region following DsRED2 gene. The latter was also used to verify expression of vector (data not shown). (B) Table showing in silico predictions based on TargetScan 4.0 of targeting of human BDNF 3'UTR and 3 members of the miR-30 family. Notice the stronger complementarity between miR-30a and BDNF 3'UTR in the 3' region of the miRNA.

A

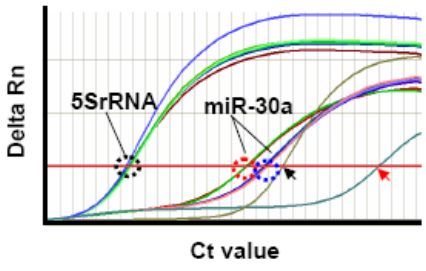


B

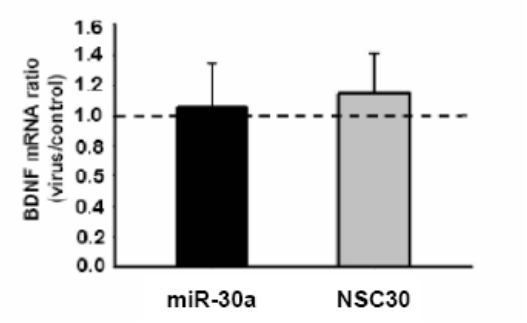
BDNF 3'UTR	5' . . . GAACCAAAACAUUCCGUUUACAU . . . 3'
miR-30a	3' GAAGGUCAGCUCCUACAAAUGU 5'
BDNF3'UTR	5' . . . GAACCAAAACAUUCCGUUUACAU . . . 3'
miR-30b	3' UCGACUCACAUCCUA---CAAAUGU 5'
BDNF 3'UTR	5' . . . GAACCAAAACAUUCCGUUUACAU . . . 3'
miR-30c	3' CGACUCUCACAUCCUA--CAAAUGU 5'

Supplemental Figure 1-4: MiR-30a and BDNF mRNA levels in rat neuronal cultures. (A) Representative results from qRT-PCR showing the lentivirus - mediated overexpression of miR-30a (dotted red circle) in neuronal cultures. Notice that the cultures infected with non silencing control (NSC30) do not exhibit higher miR-30a levels than the controls (no virus) cultures (both marked with dotted blue circle). Arrows represent qRT-PCR negative controls for (red) miR-30a and (black) 5S rRNA. (B) Bar graphs (Mean ± S.E.M.) showing *BDNF* mRNA levels (normalized to 18S rRNA) in neuronal cultures expressing miR-30a and NSC30 (N=3 per group), relative to no virus control.

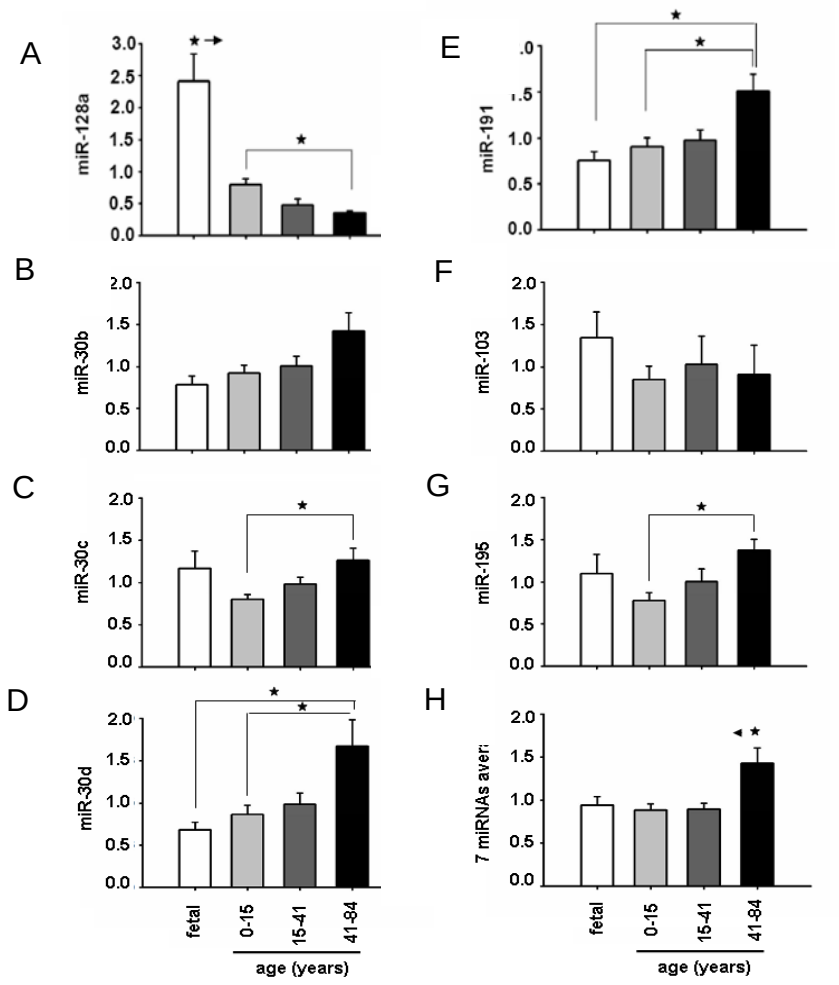
A

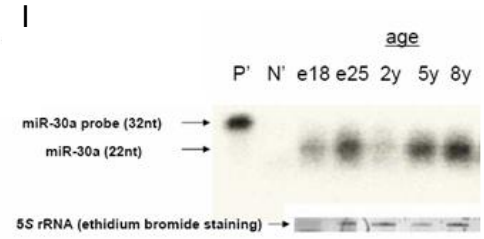

Delta Rn
5SrRNA
miR-30a
Ct value

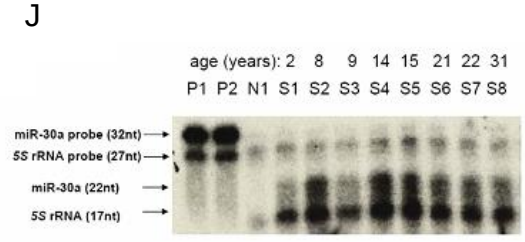
B


BDNF mRNA ratio
(virus/control)
1.6
1.4
1.2
1.0
0.8
0.5
0.4
0.2
0.0
miR-30a
NSC30

Supplemental Figure 1-5: Levels of selected miRNAs in PFC across the lifespan. (A-H) Developmental expression of selected BDNF related miRNAs (B-G), not predicted to target BDNF miR-128a (A) and the average of all 7 BDNF-related miRNAs (H) in human PFC. Graphs showing weighted means ± S.E.M of miRNA expression normalized to *5S* rRNA, for (x-axis) 4 different age groups ranging from fetal to adult ages (N = 7 -15/age group). Notice that miR-30c, miR-30d, miR-191 and miR-195 and the 7 miRNAs average show a significant increase in the older group ages 41-84. (* $p < 0.05$, ANOVA post-hoc Tukey). (I) Solution hybridization results from younger brains for miR-30a. Notice expression at 18th and 25th week of gestation (e18 and e25 respectively) and until 8 years of age (y=years). Undigested full length probe positive control (P') and no sample negative control (N') are also shown together with ethidium bromide staining for 5*S* rRNA (below). (J) Solution hybridization results with miR-30a and 5*S* rRNA probes with positive (P1,P2) and negative controls (N1) for samples from 2 to 31 years old (S1-S8).


A
miR-128a
B
miR-30b
C
miR-30c
D
miR-30d
E
miR-191
F
miR-103
G
miR-195
H
7 miRNAs aver
fetal
0-15
15-41
41-84
age (years)


I
age
P' N' e18 e25 2y 5y 8y
miR-30a probe (32nt)
miR-30a (22nt)
5S rRNA (ethidium bromide staining)


J
age (years): 2 8 9 14 15 21 22 31
P1 P2 N1 S1 S2 S3 S4 S5 S6 S7 S8
miR-30a probe (32nt)
5S rRNA probe (27nt)
miR-30a (22nt)
5S rRNA (17nt)

Supplemental Table 1-1. Postmortem Collections

Group	N	Age	PMI Mean ± S.E.M	Brain pH Mean ± S.E.M	Gender	H
		Range	hrs		M/F	R/L
Fetal	8	21-40 ewg	9.1 ± 1.3	6.7 ± 0.1	4/4	0/8
Childhood - Early Adol.	15	0-15y/o	15.8 ± 1.8	6.6 ± 0.1	9/6	0/15
Late Adol.-Adult	7	15-41 y/o	4.2 ± 2.2	6.3 ± 0.2	6/1	0/7
Old Adult	7	41-84 y/o	13.9 ± 3.4	6.6 ± 0.2	3/4	3/4

EWG, estimated weeks of gestation
PMI , postmortem interval
H, brain hemisphere

Tissues obtained from Brain and Tissue Bank for Developmental Disorders, University of Maryland (fetal, child and adolescent) and Center for Neuroscience, University of California Davis brain bank (adult).

Supplemental Table 1-2 – LNA probes and qRT-PCR primers

i) LNA probes (Exiqon)

miRNA	probe sequence (5'-3')	Tm
hsa-miR-30a-5p	cttccagtcgaggatgtttaca	73
hsa-miR-1	tacatacttctttacattcca	64
hsa-miR-128a	aaaagagaccggttcactgtga	77
hsa-miR-16	cgccaatatttacgtgctgcta	74
hsa-miR-10a	cacaaattcggatctacagggta	74
hsa-miR-103	tcatagccctgtacaatgctgct	80
hsa-miR-191	agctgcttttgggattccgttg	74
hsa-miR-495	aaagaagtgcaccatgtttgttt	71
hsa-miR-30b	agctgagtgtaggatgtttaca	71
hsa-miR-30c	gctgagagtgtaggatgtttaca	73
hsa-miR-195	gccaatatttctgtgctgcta	73

ii) mirVANA qRT-PCR probes

Catalog No (Ambion)	miRNA
AM30142	miR-30a-5p
AM30143	miR-30b
AM30144	miR-30c
AM30145	miR-30d
AM30011	miR-103
AM30026	miR-128
AM30079	miR-191
AM30083	miR-195

iii) other primers

Human

Genomic sequences for chromatin immunoprecipitation studies

Gene (HUGO)	Chromosome	Product location	Length [bp]	Primer sequence		Source	Gene transcritption start site
				Forward	Reverse		
BDNF	11p13	(-573:-675)	103	AGCCCAACAACTTTCCCTTT	GAGAGCTCGGCTTACACAGG	NT_009237.17	26530564
BDNF	11p13	(-399:-469)	71	AGCCTTTCGGGGTTCTCATTT	TCCTCTGGACCCTAGCCATA	NT_009237.17	26510394
BDNF	11p13	(-1902:-2001)	100	CGTCCATGGGGGTTTCTATT	GCCCCTTGGGTTGTTTTTAT	NT_009237.17	26467373

mRNA

Gene	Exon	Product location	Length of [bp]	Primer sequence		Source	Transcript length [bp]
				Forward	Reverse		
BDNF	E1-E2	N/A	116	N/A	N/A	Applied Biosystems	N/A
18S rRNA	N/A	(1345:1464)	150	GTTGGTGGAGCGATTTGTCT	GAACGCCACTTGTCCCTCTA	X03205.1	1869

Rat

mRNA

Gene	Exon	Product location	Length [bp]	Primer sequence		Source	Transcript length [bp]
				Forward	Reverse		
BDNF	E4	(715:832)	118	GCGCCCATGAAAGAAGCAAA	TCGTCAGACCTCTCGAACCT	NM_012513.3	4252
18S rRNA	N/A	(1335:1469)	135	CATGGCCGTTCTTAGTTGGT	GAACGCCACTTGTCCCTCTA	X01117.1	1874

Supplemental Table 1-3. In silico analysis of BDNF 3'UTR miRNA target sites using a combination of 4 different tools.

This table describes first the position of predicted miRNA target sites in the 3'UTR (5' to 3') of human BDNF mRNA (NM_170735)and the evolutionary conservation of the target sites in human (Hs), mouse (Mm), rat (Rn) and dog (Cf). Information for BDNF 3'UTR sequences across the 4 mammalian species, obtained from TargetScan 3.1 website (http:www.targetscan.org/mamm_31/)are shown, followed by the miRNA registry (miRBAse) accession id (http://microrna.sanger.ac.uk/). In red the part of the target site that interacts with 2^{nd}-7^{th} nt 5' of the miRNA. Secondary structure of the predicted 3'UTR – miRNA duplexes were determined with RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). In blue the seed sequence of each miRNA. MiRNA target sites within human BDNF 3'UTR were also determined by PicTar software [(http://pictar.bio.nyu.edu/), based on conservation in mammals and chicken (Lall et al., 2006). Those that were predicted by Pictar software are specified.

1. Position in BDNF 3'UTR = 50-55
hsa-miR-381 conserved miRNA

TargetScan 3.1 | **miRBase Accession No**

BDNF 3' UTR	miR-381
Hs: …UAU**UUGUAU**AUA…	(MI0000789)
Mm:…UAU**UUGUAU**AUA…	(MI0000798)
Rn: …UAU**UUGUAU**AUA…	(MI0003546)
Cf: …UAU**UUGUAU**AUA…	(N/A)

RNAhybrid

```
  BDNF   5'   U    CAAAAA  A   AU       U 3'
               GAGA      UU UCU  UUGUAUA
               CUCU      AA GGG  AACAUAU
miR-381  3' UGU    CG      C             5'
```

Pictar: Not predicted

2. Position in BDNF 3'UTR = 65-70
hsa-miR-10a,b conserved miRNAs

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-10a,10b
Hs: …UAA**CAGGGU**AAA…	(MI0000266),(MI0000267)
Mm:…UAA**CAGGGU**AAA…	(MI0000685),(MI0000221)
Rn: …UAA**CAGGGU**AAA…	(MI0000841),(MI0000842)
Cf: …UAA**CAGGGU**AAA…	(N/A)

RNAhybrid

```
  BDNF   5′ G    AAAUUAUCU     U  AUAUACAUA         A 3′
             ACAA          AUUUG AU          ACAGGGUA
             UGUU          UAAGC UA          UGUCCCAU
miR-10a  3′ G                    C  GA                 5′
```

Pictar: Predicted site

3. Position in BDNF 3'UTR = 145-150
hsa-miR-508 *non conserved miRNA

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-508
Hs: …UUC**UACAAU**CUA…	(MI00003195)
Mm…UUC**UACAAU**CUA…	(N/A)
Rn:…UUC**UACAAU**CUA…	(N/A)
Cf: …UUC**UACAAU**CUA…	(N/A)

RNAhybrid

```
  BDNF  5′  A   AGUA   U    UC       U 3′
            UAC    CAG  GGU  UACAAUC
            AUG    GUU  CCG  AUGUUAG
miR-508 3′ AG   AG     UU            U 5′
```

Pictar: Not predicted

4. Position in BDNF 3'UTR = 196-201
hsa-miR-210 conserved miRNA

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-210
Hs: …U-G**CGCACA**ACU…	(MI0000286)
Mm:…U-G**CGCACA**ACU…	(MI0000695)
Rn: …U-G**CGCACA**ACU…	(MI0000950)
Cf: …UUG**CGCACA**ACU…	(N/A)

RNAhybrid

```
BDNF 3'UTR 5' C   AA GGAAACA     UU       A 3'
               CAG  G        GUCAU  GCGCACA
               GUC  C        CAGUG  UGCGUGU
   miRNA   3' A   GG GA                   C 5'
```

Pictar: Not predicted

5. Position in BDNF 3'UTR = 220-225
hsa-miR-1(1-2), hsa-miR-206 conserved miRNAs

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR1-1,1-2,206
Hs: …AUU**ACAUUC**CUU…	(MI0000651),(MI0000437),(MI0000490)
Mm …AUU**ACAUUC**CUC…	(MI0000139),(MI0000652),(MI0000249)
Rn: …AUU**ACAUUC**CUC…	(MI0003489), (N/A) , (MI0000948)
Cf: …AUU**ACAUUC**CUC…	(N/A)

RNAhybrid

```
BDNF   5' C       UAAAAAG   GCA         U 3'

           ACA ACU       UCU   UUACAUUCC
           UGU UGA       AGA   AAUGUAAGG
miR-1  3' A   A                         U 5'
```

Pictar: Predicted site

6. Position in BDNF 3'UTR = 252-257
hsa-miR-182 conserved miRNA

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-182
Hs: …CCG**UUGCCA**AGA…	(MI0000272)
Mm …CCG**UUGCCA**AGA…	(MI0000224)
Rn: …CCG**UUGCCA**AGA…	(N/A)
Cf: …CCG**UUGCCA**AGA…	(N/A)

RNAhybrid

```
5' BDNF 5' U          GU              A 3'
           UGUG GUUU  UGCCGUUGCCAAG
           ACAC CAAG  AUGGUAACGGUUU
miR-182 3'      U                     5'
```

Pictar: Predicted site

7. Position in BDNF 3'UTR = 299-304
hsa-miR-103 (1-2), hsa-miR-107 conserved miRNAs

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-103-1,103-2,107
Hs: …GCA**UGCUGC**UUU…	(MI0000109),(MI0000108),(MI0000114)
Mm …GCA**UGCUGC**UUU…	(MI0000587),(MI0000588),(MI0000684)
Rn: …GCA**UGCUGC**UUU…	(MI0000888),(MI0000887),(MI0000890)
Cf: …GCA**UGCUG**CUUU…	(N/A)

RNAhybrid

```
 BDNF   5'  AAAUAAUAAAU            U 3'
                        UGC AUGCUGCU
                        AUG UACGACGA
miR-103 3'  AGUAUCGGGAC   U          5'
```

Pictar: Predicted site

8. Position in BDNF 3'UTR = 300-305
hsa-miR-15a,b, hsa-miR-16(1-2), hsa-miR-195, hsa-miR-497 conserved miRNAs

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-15a,b,16-1, 16-2,195,497
Hs: …CAU**GCUGCU**UUA…	(MI0000069),(MI0000438),(MI0000070) (MI0000115),(MI0000489),(MI0003138)
Mm: …CAU**GCUGCU**UUA…	(MI0000564),(MI0000140),(MI0000565) (MI0000566),(MI0000237),(MI0004636)
Rn: **…**CAU**GCUGCU**UUA…	(N/A) (MI0000843),(MI0000844)
	(N/A) (MI0000939),(MI0003724)
Cf: **…**CAU**GCUGCU**UUA…	(N/A)

RNAhybrid

```
  BDNF    5′  A      AA     CA        U 3′
               UAAUA    UUG  UGCUGCU
               GUUAU    GAC  ACGACGA
miR-195   3′ CG     AAA             U 5′
```

Pictar: Predicted site

9. Position in BDNF 3'UTR = 393-398
hsa-miR-191 conserved miRNA

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-191
Hs: …CAU**UCCGUU**UAC…	(MI0000465)
Mm:…CAU**UCCGUU**UAC…	(MI0000233)
Rn: …CAU**UCCGUU**UAC…	(MI0000934)
Cf: …CAU**UCCGUU**UAC…	(N/A)

RNAhybrid

```
  BDNF    5′  U   AACCAAAAC         U 3′
              UUG          AUUCCGUU
              GAC          UAAGGCAA
miR-191   3′UC   GAAAACCC          C 5′
```

Pictar: Not predicted

10. Position in BDNF 3'UTR = 397-402
hsa-miR-30a(5p),b,c(1-2),d,e(5p) conserved miRNAs

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-30a,b,c1, c2,d,e
Hs: …UCC**GUUUAC**AUU…	(MI0000088),(MI0000441),(MI0000736) (MI0000254),(MI0000255),(MI0000749)
Mm: …UCC**GUUUAC**AUU…	(MI0000144),(MI0000145),(MI0000547) (MI0000548),(MI0000549),(MI0000259)
Rn: **…**UCC**GUUUAC**AUU…	(MI0000870),(MI0000868),(MI0000866) (MI0000871),(MI0000869),(MI0000867)
Cf: **…**UCC**GUUUAC**AUU…	(N/A)

RNAhybrid

```
  BDNF      5′  A    AAACAUUCC       U 3′
                CCA          GUUUACA
                GGU          CAAAUGU
miR-30a5p   3′ GAA   CAGCUCCUA         5′
```

Pictar: Predicted site

11. Position in BDNF 3'UTR = 552-557
hsa-miR-495 conserved miRNA

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-495
Hs: …U-G**UUUGUU**UUG…	(MI0003135)
Mm:…U-G**UUUGUU**UUG…	(MI0004639)
Rn: …U-G**UUUGUU**UUG…	(N/A)
Cf: …UUG**UUUGUU**UUG…	(N/A)

RNAhybrid

```
 BDNF     5′  U  GG      AUUUU         U 3′
              GG  G GUAU     UGUUUGUUU
              UC  C CGUG     ACAAACAAA
miR-495   3′ UU  UU A    GU              5′
```

Pictar: Not Predicted

12. Position in BDNF 3'UTR = 680-685
hsa-miR-30a(5p),b,c(1-2),d,e(5p) conserved miRNAs

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-30a,b,c1, c2,d,e
Hs: …A- U**GUUUGC**AAU…	(MI0000088),(MI0000441),(MI0000736) (MI0000254),(MI0000255),(MI0000749)
Mm: …A- U**GUUUGC**AAA…	(MI0000144),(MI0000145),(MI0000547) (MI0000548),(MI0000549),(MI0000259)
Rn: …AAU**GUUUGC**AAA…	(MI0000870),(MI0000868),(MI0000866) (MI0000871),(MI0000869),(MI0000867)
Cf: …A- U**GUUUGC**AGU…	(N/A)

RNAhybrid

```
  BDNF      5′ U  G  U    UGAA          A 3′
                UU UA GUUG    GAUGUUUGCA
                AA GU CAGC    CUACAAAUGU
miR-30a5p   3′ G  G       UC              5′
```

Pictar: Predicted site

13. Position in 3'UTR = 1321-1326
hsa-miR-1(1-2), hsa-miR-206 conserved miRNA

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR1-1,1-2,206
Hs: …AGA**CAUUCC**AAA…	(MI0000651),(MI0000437),(MI0000490)
Mm: …AGA**CAUUCC**UAA…	(MI0000139),(MI0000652),(MI0000249)
Rn: **…**AGA**CAUUCC**UAA…	(MI0003489), (N/A) ,(MI0000948)
Cf: **…**---**CAUUCC**GGA…	(N/A)

RNAhybrid

```
BDNF   5′ G     G A   GAG        A 3'
           GCAUG U UUU   ACAUUCCA
           UGUAU A GAA   UGUAAGGU
miR1   3' A     G A   A            5'
```

Pictar: Predicted site

14. Position in BDNF 3'UTR = 2704-2709
hsa-miR-368 not conserved miRNA

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-368
Hs: …UCU**CUCUAU**GGU…	(MI0000776)
Mm: …UCU**CUCUAU**GGU…	(N/A)
Rn: …UCU**CUCUAU**GGU…	(N/A)
Cf: …UCU**CUCUAU**GGU…	(N/A)

RNAhybrid

N/A

Pictar: Predicted site

15. Position in BDNF 3'UTR = 2728-2733
hsa-miR-496 conserved miRNA

TargetScan 3.1	**miRBAse Accession No**
BDNF 3' UTR	miR-496
Hs: …ACC**AUGUAA**AA-…	(MI0003136)
Mm: …ACC**AUGUAA**AA-…	(MI0004589)
Rn: …ACC**AUGUAA**AA-…	(N/A)
Cf: …ACC**AUGUAA**AAA…	(N/A)

RNAhybrid

```
 BDNF   5' A    AUUAUUCA        A 3'
            AGAU         CCAUGUAA
            UCUA         GGUACAUU
miR496  3' C    ACC             A 5'
```

Pictar: Not Predicted

16. Position in BDNF 3'UTR = 2796-2801
hsa-miR-365 (1-2) conserved miRNA

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-365-1,365-2
Hs: …-UU**GGCAUU**AAA…	(MI0000767),(MI0000769)
Mm: …-UU**GGCAUU**AAA…	(MI0000768),(MI0001645)
Rn: …-UU**GGCAUU**AAA**…**	(MI0001656), (N/A)
Cf: …AUU**GGCAUU**AAA…	(MI0001657),(MI0001647)

RNAhybrid

```
 BDNF   5'                U       A 3'
                           GGCAUUA
                           CCGUAAU
miR-365 3' UAUUCCUAAAAAUCC          5'
```

Pictar : Not Predicted

17. Position in BDNF 3'UTR = 2797-2802
hsa-miR-155 conserved miRNA

TargetScan 3.1 **miRBAse Accession No**

BDNF 3' UTR	miR-155
Hs: …UUG**GCAUUA**AAA…	(MI0000681)
Mm: …UUG**GCAUUA**AAA…	(MI0000177)
Rn: …UUG**GCAUUA**AAA**…**	(N/A)
Cf: …UUG**GCAUUA**AAA…	(N/A)

RNAhybrid

```
 BDNF   5'      A       UUAUUGAAAAAA            A 3'
                 AUUAUG              AUUGGCAUUAA
                 UAGUGC              UAAUCGUAAUU
miR-155 3' GGGGA                                5'
```

Pictar: Predicted site

CHAPTER III – MOLECULAR DETERMINANTS OF DYSREGULATED GABAERGIC GENE EXPRESSION IN THE PREFRONTAL CORTEX OF SUBJECTS WITH SCHIZOPHRENIA

The work presented in this chapter is reproduced from a study by Nikolaos Mellios, Hsien-Sung Huang, Anastasia Grigorenko, Stephen Baker, Marzena Galdzicka, Edward Ginns, and Schahram Akbarian published in Biol Psychiatry (Mellios et al., 2008b). This work was conducted under the direction of Dr. Schahram Akbarian, and it is with gratitude to him and the other authors that I reproduce these data for the purposes of this dissertation. My contribution in this work was to design and execute the miRNA-related experiments and write together with Dr. Schahram Akbarian the manuscript. Specifically, I carried out small RNA isolation, including PAGE purification of small RNAs, qRT-PCR for miRNA analysis, BDNF ELISA, part of the mouse chronic antipsychotic treatment and analysis of data. Hsien-Sung Huang, who is a first co-author conducted the GABAergic gene qRT-PCRs in mouse and human brain, chromatin immunoprecipitation experiments, and part of the

mouse chronic antipsychotic treatment. Drs. Edward Ginns and Marzena Galdzicka conducted the NPY genotyping experiment and Dr. Stephen Baker assisted in statistical analysis. Anouch Matevossian assisted in chromatin immunoprecipitation experiments. Yin Guo contributed by conducting postmortem brain dissection. Simone Jäger contributed by assisting in running qRT-PCR.

Abstract

Background: Prefrontal deficits in gamma-aminobutyric acid (GABA)ergic gene expression, including neuropeptide Y (NPY), somatostatin (SST), and parvalbumin (PV) messenger RNAs (mRNAs), have been reported for multiple schizophrenia cohorts. Preclinical models suggest that a subset of these GABAergic markers (NPY/SST) is regulated by brain-derived neurotrophic factor (BDNF), which in turn is under the inhibitory influence of small noncoding RNAs. However, it remains unclear if these mechanisms are

important determinants for dysregulated NPY and SST expression in prefrontal cortex (PFC) of subjects with schizophrenia.

Methods: Using a postmortem case-control design, the association between BDNF protein, NPY/SST/PV mRNAs, and two BDNF-regulating microRNAs (miR-195 and miR-30a-5p) was determined in samples from the PFC of 20 schizophrenia and 20 control subjects. Complementary studies were conducted in cerebral cortex of mice subjected to antipsychotic treatment or a brain-specific ablation of the *Bdnf* gene.

Results: Subjects with schizophrenia showed deficits in NPY and PV mRNAs. Within-pair differences in BDNF protein levels showed strong positive correlations with NPY and SST and a robust inverse association with miR-195 levels, which in turn were not affected by antipsychotic treatment or genetic ablation of *Bdnf*.

Conclusions: Taken together, these results suggest that prefrontal deficits in a subset of GABAergic mRNAs, including NPY, are dependent on the regional supply of BDNF, which in turn is fine-tuned through a microRNA (miRNA)-mediated mechanism.

Introduction

Schizophrenia is a complex psychiatric disorder with genetic (Porteous et al., 2008; Allen et al., 2008) and epigenetic (Huang et al., 2007; Tsankova et al., 2007) factors potentially contributing to its pathophysiology, which has been linked among others to aberrant inhibitory synaptic function in the prefrontal cortex (PFC) (Lewis et al., 2004; Gonzalez-Burgos et al., 2008). Interestingly, multiple studies have revealed deficits in the expression of gamma-aminobutyric acid (GABA)ergic transcripts such as neuropeptide Y (NPY), somatostatin (SST), parvalbumin (PV), and glutamic acid decarboxylase 67 (GAD67) in the prefrontal cortex of patients with schizophrenia (Akbarian et al., 1995; Guidotti et al., 2000; Fatemi et al., 2005; Hashimoto et al., 2008). Two interneuron subtypes, including PV-positive fast-spiking neurons forming synapses with perisomatic domains of pyramidal neurons and non fast-spiking NPY-positive and SST-positive neurons targeting pyramidal neuron distal dendrites (Lewis et al., 2004; Gonzalez-Burgos et al., 2008), are pivotal for the synchronization of prefrontal neuronal networks, which are disrupted in schizophrenia (Spencer et al., 2003; Cho et al., 2006; Uhlhaas et al., 2006).

Furthermore, brain-derived neurotrophic factor (BDNF), a potential schizophrenia susceptibility gene (Durany et al., 2001; Egan et al., 2003; Angelucci et al., 2005), and its receptor tropomyosin related kinase B (TRK-B) could be important regulators of the GABAergic transcriptome in mammalian cerebral cortex (Gorski et al., 2003; Hashimoto et al., 2005; Glorioso et al., 2006). Based on studies in *Bdnf* mutant mice (Glorioso et al., 2006), NPY and SST messenger RNA (mRNA) expression is dependent on BDNF, but this link has not yet been explored in schizophrenia postmortem studies.

MicroRNAs (miRNAs) are small noncoding RNAs that are evolutionarily conserved and are predicted to target at least one third of protein coding genes (Bartel et al., 2004; Filipowicz et al., 2008). They are derived from longer precursor molecules through a combined action of the nuclear microprocessor complex and the cytoplasmic RNAase III enzyme Dicer (Bartel et al., 2004; Filipowicz et al., 2008). The mature product of approximately 20 nucleotides (nts) in length is loaded to the RNA-induced silencing complex (RISC) and targets areas predominantly in the 3' untranslated region (UTR), mediating translational repression or mRNA

degradation, depending on the degree of complementarity (Bartel et al., 2004; Filipowicz et al., 2008). The emerging important role of miRNAs in various cellular processes and their implication in a plethora of human diseases (Chang et al., 2007) has made them a new promising field of molecular epigenomics (Chuang and Jones, 2007). Furthermore, miRNAs have been proposed to account for part of the variability in gene expression in human cerebral cortex (Zhang and Su, 2008); to display remarkable resistance to the effects of temperature, pH, and prolonged storage (Chen et al., 2008); and to be stable and consistent biomarkers in postmortem studies (Szafranska et al., 2008). We have previously shown that the expression of BDNF in adult human PFC is inversely correlated to a subset of miRNAs predicted to target conserved regions within human BDNF 3' UTR, with two species in particular, miR-30a-5p and miR-195, having the most pronounced inhibitory effect on BDNF translation (Mellios et al., 2008a).

In this case-control study, we determined the expression and potential interactions of NPY, SST, and PV mRNAs with BDNF protein levels in 20 subjects with schizophrenia, including the putative influence of miR-30a-5p

and miR-195 microRNAs. Our results show for the first time that alterations in NPY and SST, but not PV, mRNA in PFC of schizophrenia subjects are modulated by BDNF protein, which in turn is negatively regulated by miR-195. Furthermore, we show that the cerebral cortex of mice with a central nervous system (CNS)-specific conditional ablation of BDNF exhibits deficits in NPY and SST mRNAs without concomitant changes in miR-195 levels. Therefore, a small noncoding RNA, miR-195, could be an important modifier of BDNF-related GABAergic deficits in schizophrenia.

Results

Altered expression of GABAergic transcripts in PFC of subjects with schizophrenia

To determine if the reported deficits in a subset of GABAergic transcripts in PFC of patients with schizophrenia (Gabriel et al., 1996; Hashimoto et al., 2008) could be recapitulated in our cohort of 20 matched pairs (Table 2-1),

we measured with qRT-PCR mRNA levels for NPY, SST, and PV. Our results revealed significant deficits (NPY, PV) or a trend for decrease (SST) in mRNA levels of the schizophrenia subjects (Fig. 2-1A–C). To rule out that these alterations were due to differences in the level of the normalization gene, B2M, we reanalyzed NPY transcript changes for eight randomly selected matched pairs using two additional reference genes (18S rRNA and GUSB) (Peltier et al., 2008). The within-pair differences in NPY levels based on each of these two additional reference genes were highly correlated with the B2M-based values (18S rRNA: r = +0.87, p= 0.005; GUSB: r= +0.85, p = 0.008, with r = Pearson correlation coefficient).

Disease-specific changes in prefrontal NPY and SST mRNAs are related to within-pair differences in BDNF protein

Next, we wanted to explore the molecular mechanisms that could underlie the observed deficits in NPY, SST, and PV expression in the schizophrenia cohort of this study. Based on studies in genetically engineered mice, expression of NPY and SST in cerebral cortex is dependent on BDNF

(Glorioso et al., 2006), but it is not known whether a similar mechanism plays a role in schizophrenia. To address this issue, we measured BDNF protein by ELISA in our cohort and determined BDNF protein and NPY, SST, and PV mRNA levels in schizophrenia subjects relative to their matched control subjects (S/C). Although no significant changes in BDNF protein in this cohort were observed (data not shown), there were significant positive correlations between within-pair differences in BDNF and NPY or SST mRNA levels (Fig. 2-1D and 2-1E). However, there was no correlation between BDNF and PV S/C ratios (Fig. 2-1F). Therefore, changes in BDNF protein levels in schizophrenia preferentially affect prefrontal NPY and SST but not PV gene expression.

MiR-195, a BDNF-targeting microRNA, is an upstream effector of BDNF and BDNF-Regulated GABAergic gene transcripts in schizophrenia

From the above findings, one could draw two conclusions. First, prefrontal BDNF levels had a significant effect on a subset of GABAergic gene transcripts. Second, within-pair differences in BDNF proteins levels showed

considerable variability (Fig. 2-1D–F). However, based on our previous study, BDNF levels in adult human PFC are regulated by a distinct set of microRNAs targeting multiple conserved miRNA sites in the BDNF 3' UTR (Mellios et al., 2008a). Each BDNF-related miRNA displays developmental and/or layer-specific expression in the PFC (Mellios et al., 2008a). Notably, among these differentially expressed miRNAs, two in particular, miR-30a-5p and miR-195, exert a more pronounced posttranscriptional inhibition on BDNF (Mellios et al., 2008a). Therefore, we hypothesized that aberrant expression of one or both of these two miRNAs in schizophrenia subjects might contribute to the disease-related changes in BDNF protein and BDNF-regulated NPY and SST gene expression.

To examine this, we first extracted small RNAs (<200 nts) from the 42 brains included in this study and measured miR-195, miR-30a-5p, and for normalization 5S rRNA by qRT-PCR. Overall, miR-195 levels in the PFC of subjects with schizophrenia did not differ significantly to those of their matched controls (Fig. 2-2A). However, the <200 nts RNA pool used for the above analyses includes, in addition to the mature microRNAs (which range

between 18 nts and 23 nts in size), the precursor microRNAs molecules such as the pre-miRNAs (60–70 nts) and a subset of primary microRNAs transcripts (>100 nts) (Bartel et al., 2004; Filipowicz et al., 2008), which might in some cases be detected by qRT-PCR. Importantly, only the mature microRNAs are capable of posttranscriptional inhibition of target genes (Bartel et al., 2004; Filipowicz et al., 2008). Therefore, we wanted to measure specifically mature miR-195 levels in our schizophrenia cohort. To address this issue, additional tissue samples were obtained from a subset of 18 brains or 9 matched pairs (selected beforehand from the same cohort and based on availability of tissue), and <40 nts RNA was purified by polyacrylamide gel electrophoresis (see Methods and Materials). Of note, 5S rRNA, used for normalization in qRT-PCRs utilizing the <200 nts RNA (see above), was not present in the <40 nts RNA fraction. Therefore, miR-195 levels in the <40 nts RNA pool were assayed by qRT-PCR and normalized to miR-191, previously determined as an ideal normalizer for miRNA qRT-PCR analysis in human tissues (Peltier et al., 2008). In addition, miR-191 is highly expressed across neuronal layers II to VI human PFC (Mellios et al., 2008a) and not affected in

schizophrenia (Perkins et al., 2007). Our results showed a significant decrease in mature miR-195 in the <40 nts RNA pool of these nine schizophrenia subjects (Fig. 2-2B), which suggests that the disease-related decrease in miR-195 expression, at least in a subset of schizophrenia subjects, occurs at the level of mature miRNA. We next compared the case-control ratios for miR-195 with that of BDNF protein to examine if any disease-related variability in BDNF protein could be attributed to this BDNF targeting microRNA. Notably, there was a robust inverse correlation between miR-195 and BDNF protein ratios (schizophrenia/control subjects) after controlling for the presence of significant outliers (r= -0.710, p < 0.001) (Fig. 2-2C – see also methods for statistical analysis). Interestingly, within-pair changes in miR-195 also showed a modest inverse correlation with NPY (r = -0.230) and SST mRNA changes (r = -0.253).

Due to the fact that RNA integrity number (RIN) measurements were done after match pairing, we wanted to ensure that RNA quality parameters were not a major confounding factor of our results. Toward this end, we excluded all subjects with RIN < 6 and reanalyzed data for the remaining 13 matched

pairs (26 samples total). There was a significant positive correlation between within-pair differences in BDNF protein and both NPY (r = +0.489, p= 0.045) and SST (r = +0.560, p = 0.023) mRNA and a significant inverse correlation between BDNF protein and miR-195 (r = -0.523, p = 0.033) (Fig. 2-3A–C). In addition, there was again no significant correlation between within-pair differences of BDNF protein and PV mRNA and miR-30a (data not shown). Notably, in the subset of 13 matched pairs, the inverse correlation between miR-195 and NPY/SST mRNAs was more robust than in the 20 pairs (NPY: r = -0.542, p = 0.028; SST: r = -0.3824, p = 0.099). Therefore, the variability in NPY and SST mRNA changes in schizophrenia could be partly attributed to a negative regulatory effect of miR-195 on prefrontal BDNF protein levels (Fig. 2-3D). In addition, paired t test revealed a significant reduction in NPY (t = -2.492, df = 12, p = 0.028) and trends for reduction for SST (t = -1.897, df = 12, p = 0.082) and PV (t = -1.885, df = 12, p = 0.084) in schizophrenia; groupwise analysis using Wilcoxon signed rank test showed significant decrease only for NPY (p = 0.024). In summary, these results are comparable with the initial analysis on the 20 matched pairs.

Furthermore, the within-pair changes in BDNF protein levels were not correlated to those of miR-30a-5p (Fig. 2-2D). Of note, our qRT-PCR, utilizing the <200 nts RNA pools, was suggestive for an approximately 20% increase in miR-30a-5p in schizophrenia, but subsequent analyses of the mature miR-30a-5p in the <40 nts RNA pools, as described above for miR-195, revealed no consistent changes (data not shown). Therefore, the disease-related changes in prefrontal miRNA expression in a subset of schizophrenia subjects do not appear to be accompanied by a consistent change in mature miR-30a-5p levels.

To address the potential effect of demographics in our data, we performed statistical analysis (Spearman correlation) to determine whether within-pair differences in BDNF protein, NPY, SST, PV mRNA, and miR-195 and miR-30a-5p were correlated to tissue pH, autolysis time, or age. No significant correlations were observed. However, additional analysis was applied (Mann-Whitney test) to determine potential effects of gender and brain hemisphere. Surprisingly, a significant effect of brain hemisphere was observed for BDNF (S/C lower in left, $p = 0.028$) and opposite trends for an effect of gender on

SST (lower in male subjects, p = 0.078) and PV S/C ratios (lower in female subjects, p = 0.078) were identified. Of note, a left hemisphere specific increase in miR-30a-5p was found in <250 nts RNA samples, but subsequent analysis of mature miR-30a-5p levels did not verify this effect (see above). However, due to the relatively small number of left hemisphere (n=9) and female pairs (n =7) and the lack of tissue from both hemispheres of each sample, additional studies are needed to address the issue of potential hemisphere or gender effects in BDNF, miRNA, or GABAergic gene expression changes in the PFC of subjects with schizophrenia.

Lastly and despite the fact that there was no correlation between age of onset in the 20 schizophrenia subjects and any of the genes examined (data not shown), there was a strong positive correlation between miR-195 levels and age of onset specifically for subjects diagnosed with the chronic paranoid subtype of the disease (n = 7, r = +0 .927, p = 0.003, Pearson's correlation).

Alterations in GABAergic mRNAs and BDNF-Related microRNAs in Cerebral Cortex of Adult BDNF-Deficient Mice

Previous studies had suggested that BDNF could regulate NPY and SST transcription, but its effects on other GABAergic transcripts might not be as direct (Hashimoto et al., 2005; Glorioso et al., 2006). To validate these effects, we measured levels of NPY, SST, PV, and GAD67 mRNAs in embryonic and adult cerebral cortex of mice with a CNS specific ablation of BDNF (see Methods and Materials). There was a decrease in NPY and SST, but not PV and GAD67, mRNAs, specifically in adult mutant mice (Fig. 2-4A). Furthermore, to examine if miR-195 could itself be regulated by BDNF as part of a feedback loop, which has been previously reported for other brain-expressed miRNAs and their target genes (Klein et al., 2007), we measured levels of miR-195 and miR-30a-5p in adult *Bdnf*-deficient mice. No changes in miR-195 were observed, but there was a significant increase in miR-30a-5p (Fig. 2-4B). These data suggest that BDNF could be involved in feedback regulatory loops with members of the miR-30 family but not with miR-195.

Analysis of Potential Genetic and Epigenetic Determinants of Disease-Related Changes in GABAergic Transcriptome

Furthermore, we examined the potential influence of genetic polymorphism in NPY mRNA expression in our cohort by genotyping three SNPs in the proximity of the NPY promoter, two of which were previously shown to regulate mRNA expression and response of normal subjects to stress (Zhou et al., 2008). There was no significant effect of any of these SNPs on NPY mRNA expression (Supplemental Fig. S2-1) and they were equally represented in cases and control subjects (data not shown). Lastly, we used chromatin immunoprecipitation to determine case-control differences in (open chromatin-associated) trimethylated histone H3-lysine 4 (trimethyl-H3K4) in the promoter regions of NPY, SST, and PV and used previously published data (Huang et al., 2007) on GAD67 for the same samples. We found a significant positive correlation between disease-specific changes in GAD67 transcript and trimethyl-H3K4 levels at its promoter but no significant correlations for NPY, SST, and PV (Supplemental Fig. S2-2). We conclude that the deficits in NPY, SST, and PV expression in PFC of schizophrenia

subjects are not related to histone methylation changes at the corresponding promoters, while this type of epigenetic modification could contribute to the decrease in GAD67 expression that has been reported in multiple studies (Akbarian et al., 1995; Guidotti et al., 2000; Fatemi et al., 2005; Hashimoto et al., 2008).

Chronic Antipsychotic Treatment Does Not Affect Expression of BDNF-Related miRNAs in Mouse Cerebral Cortex

Previous studies on the effect of chronic haloperidol treatment in rat cortex had not shown any significant effects on the BDNF-related miRNAs miR-195 and miR-30a-5p (Perkins et al., 2007). We repeated the experiment in adult mice and included a treatment group for the atypical antipsychotic clozapine. Chronic treatment of adult mice with typical and atypical antipsychotics had no significant effect on miR-195 and miR-30a-5p expression in mouse cerebral cortex (Supplemental Fig. S2-3 and data not shown). However, due to the lack of detailed information on pharmacological treatment and the fact that our cohort consisted mainly from subjects treated with typical

antipsychotics, these preclinical studies do not exclude the possibility that in human PFC miR-195 levels are indeed influenced by antipsychotic treatment.

Discussion

Using multiple approaches, we uncovered a regulatory cascade that is involved in dysregulated GABAergic gene expression in the prefrontal cortex of subjects with schizophrenia. Importantly, we showed that miR-195, a small RNA interacting with the 3' UTR of BDNF transcript (Mellios et al., 2008a), contributes to the regulation and variability of BDNF protein levels, which in turn may influence the disease-related deficits in NPY and SST mRNAs. Interestingly, open chromatin-associated histone (H3-lysine 4) methylation was maintained at the NPY and SST loci in the affected subjects, and in the case of NPY, no association with genetic polymorphisms within or around its promoter were observed. These findings imply that the miR-195/BDNF pathway affects NPY and SST mRNA levels through mechanisms independent of chromatin remodeling. This contrasts with the coordinated

regulation of mRNA expression and H3-lysine 4 methylation at the GAD67 GABA synthesis gene promoter in the same subjects. Interestingly, histone methylation at the NPY, SST, and GAD67 loci is highly regulated during an extended period of prefrontal development (Huang et al., 2007). Therefore, at least two independent mechanisms emerge as regulators of the GABAergic transcriptome in schizophrenia: one that involves microRNAs targeting BDNF that operate primarily in the mature PFC and impact levels of NPY and SST and a second linked to alterations in promoter-associated histone methylation, which are indicative of compromised PFC development and specifically affect GAD67 levels.

Notably, miR-195 changes in diseased PFC were inversely associated with those of BDNF protein, while miR-195 levels remained unaltered in *Bdnf*-deficient mice, which suggests that miR-195 operates upstream and fine-tunes BDNF protein levels in the cortex. On the other hand, NPY and SST mRNAs are reduced in mice lacking *Bdnf* and also in our clinical samples, which further supports the notion that BDNF signaling is essential for orderly expression of these two GABAergic peptides. In contrast to the robust deficits

in NPY and SST in adult *Bdnf* mutant cortex (Glorioso et al., 2006), according to the present study, *Bdnf* deficiency did not impact expression of these RNAs in perinatal brain. This finding adds to the notion that dysregulation of at least a subset of GABAergic markers is primarily related to BDNF levels in the adult cortex and less affected by developmental mechanisms.

The positive correlation between miR-195 levels in the PFC of subjects diagnosed with chronic paranoid schizophrenia and the age of disease onset is of interest in the light that miR-195 expression is increased during normal aging of human PFC (Mellios et al., 2008a). However, additional studies in larger cohorts are needed to explore the possibility that mechanisms involved in altered developmental regulation of miR-195 might be linked to the emergence of psychosis. In addition, it is noteworthy that according to computational analysis (Lewis et al., 2003), miR-195 interacts with multiple gene products that are reportedly dysregulated in schizophrenia or affective disorder, such as glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1 (GRIN1) (Begni et al., 2003; Beneyto et al., 2008), gamma-aminobutyric acid receptor, alpha 1 (GABRA1) (Petryshen et al., 2005), serotonin receptor 2C

(HTR2C) (Castensson et al., 2003), and fibroblast growth factor-2 (FGF-2) (Gaughran et al., 2006) or are important components of glutaminergic, dopaminergic, and serotoninergic signaling such as glutamate receptor, metabotropic 7 (GRM7) (Ohtsuki et al., 2008), dopamine receptor D1 (DRD1) (Allen et al., 2008), and serotonin receptor 4 (HTR4) (Suzuki et al., 2003). Therefore, miR-195- mediated posttranscriptional fine-tuning could be of relevance for the disease-related alterations in multiple nodes of prefrontal molecular networks and either directly linked to the underlying pathophysiology of schizophrenia or as part of compensatory regulatory mechanisms. Furthermore, the changes in miR-195 in a subset of schizophrenia subjects of the present study affected the mature form. This finding is in good agreement with a previous report describing a trend for decreased levels for several miRNAs in schizophrenic PFC, including miR-195, which also affected the mature but not precursor forms (Perkins et al., 2007). These findings may hint to a potential defect in miRNA processing in schizophrenia. In support of this scenario, a recent study showed that conditional deletion of the microprocessor complex component, DiGeorge

syndrome critical region gene 8 (*Dgcr8*), which is part of the microRNA biogenesis pathway, results in behavioral abnormalities and perturbed dendritic spine morphology and complexity reminiscent of schizophrenia (Stark et al., 2008). Notably, the human DGCR8 gene is located within the region of microdeletion responsible for DiGeorge syndrome (also known as 22qDS or velocardiofacial syndrome) (Murphy et al., 1999; Gothelf et al., 2007; Stark et al., 2008), which confers a thirtyfold increase in the risk of developing schizophrenia and schizoaffective disorders (Murphy et al., 1999; Gothelf et al., 2007).

One limitation of our study is that we did not measure the expression of the multiple BDNF RNA transcripts. It would be of interest to determine which BDNF mRNA transcripts contribute the most to the variability in BDNF protein expression in human PFC and whether mechanisms, such as alternative polyadenylation, could influence the effect that miRNAs have on BDNF translation. In addition, we had previously shown using locked nucleic acid (LNA) in situ hybridization that miR-195 is differentially expressed in different cortical layers in adult human PFC (Mellios et al., 2008a). While miR-195

appears to be enriched in a subset of pyramidal neurons of human PFC (data not shown), additional studies will be necessary to explore the interrelation between BDNF protein and miR-195 on the cellular level. Furthermore, it has to be noted that BDNF protein and SST mRNA, whose disease-related changes were strongly associated, were not significantly altered in our cohort. Of note, in previous studies measuring BDNF protein and/or mRNA in the cerebral cortex of subjects with schizophrenia, both increases and decreases relative to control subjects have been reported (Durany et al., 2001; Weickert et al., 2003; Takahashi et al., 2000). Taken together these findings suggest that alterations in BDNF levels per se might not be a consistent marker of the disease, or that differences in the demographics of separate cohorts, such as the percentage of subjects diagnosed with each schizophrenia subtype, might be important confounding factors. However, based on our findings we propose that changes in BDNF protein levels in subjects diagnosed with schizophrenia are at least in some degree influenced by miR-195 expression.

Importantly, our findings suggest that up to half of the disease-related variability in BDNF levels and a part of the variability in disease-related

changes in NPY and SST levels are attributable to miR-195 (Fig. 2-3D). However, the identification of additional miRNA-mediated effects, including potential synergistic interactions between individual microRNAs (Mellios et al., 2008a) and other regulatory mechanisms controlling BDNF and NPY and SST expression, warrants further investigation.

In conclusion, our findings support the hypothesis that disease-related changes in NPY and SST, but not PV, mRNA expression are influenced by BDNF protein levels. More importantly, the current study introduces miR-195 as a novel regulator of prefrontal BDNF expression in schizophrenia. More broadly, the findings presented here indicate that the molecular pathology of psychosis could be related to a complex interplay of protein coding and noncoding transcripts that eventually culminate in a finite set of final common pathways, including gene expression in inhibitory interneurons.

Materials and Methods

Postmortem Brains

A total of 40 postmortem brain samples from 20 subjects diagnosed with schizophrenia and 20 controls were used in this study. All procedures were approved by the Institutional Review Board of the University of Massachusetts Medical School. Each sample from a disease case was matched to a control according to gender, age, postmortem interval (PMI), and hemisphere. Demographics, medication status and postmortem confounds, including RNA Integrity Number (RIN) are provided in Table 2-1. All samples included in this study had a RIN ≥ 4.0, which has been proposed as a minimum standard for postmortem RNA quality (Lipska et al., 2006). Each sample was from the pole of the frontal cortex (rostral portion of BA10 cut through the full vertical thickness of the cortex) and collected from a brain bank at the University of California at Davis (Dr. Edward G. Jones, Center for Neuroscience, University of California at Davis). The matching process had been completed prior to the experiments. Diagnosis of schizophrenia was based on DSM-IVR, and control brains had no history of psychiatric or neurological disease, as previously described (Akbarian et al., 1995). For the cases where the age of onset was recorded as twenties (20S) or thirties (30S)

the age used for calculation was 20 and 30 years, respectively. For all experimental procedures tissue from a case was processed in parallel together with its matched control, using aliquots of the same solutions, buffers, probes, etc.

BDNF Immunoassay

Protein was extracted with the mirVANA PARIS Kit (Ambion) according to manufacturer's instructions, and after centrifugation, the supernatants were used for estimation of total protein with BCA Micro-kit (Pierce). Brain-derived neurotrophic factor levels were essayed with enzyme-linked immunosorbent assay (ELISA) and with the use of BDNF ELISA Kit (Chemicon) according to manufacturer's instructions.

RNA Isolation

Total RNA was isolated by using RNeasy Lipid Tissue Mini Kit (Qiagen) and then treated with DNase I (Ambion). Small RNAs (<200 nts) were isolated by using the mirVANA PARIS Kit (Ambion), according to the manufacturer's

instructions and as described before (Mellios et al., 2008a). For isolation of <40 nts RNA, the flashPAGE Fractionator System (Ambion) was used according to manufacturer's instructions. Briefly, 5 µg of total RNA was run for 12 minutes at 75 mV and RNA from the lower running buffer was purified using flashPAGE Reaction Clean-Up Kit (Ambion).

RNA Quantification

The mirVana qRT-PCR miRNA Detection Kit (Ambion) was used for measuring human miR-195 in samples of <200 nts and <40 nts RNA. For each sample and amplicon, cycle thresholds were averaged from triplicate reactions and normalized to either 5S ribosomal RNA (rRNA) (<200 nts RNA) or miR-191 (<40 nts RNA). The miRCURY LNA microRNA PCR System (Exiqon) was used for quantification of miRNA expression in mouse RNA samples. In this case, duplicate reactions were used and data were normalized again to 5S rRNA. TaqMan One-Step RT-PCR (Applied Biosystems) was used according to manufacturer's instructions for human and mouse NPY, SST, PV, β-2 microglobulin (B2M), BDNF, and 18S rRNA

with primers shown in Supplemental Table 2-1. Custom primers (Applied Biosystems) were used for human β-glucuronidase (*GUSB)* RT-PCR (primer set hs99999908_m1 of Applied Biosystems Taqman gene assays).

Genotyping

Neuropeptide Y single nucleotide polymorphism (SNP) genotyping was performed using direct sequencing and also matrixassisted laser desorption/ionization mass spectrometry (Sequenom), in conjunction with SpecroDesign software (Sequenom) for polymerase chain reaction (PCR) and MassEXTEND primers (Sequenom).

Chromatin Immunoprecipitation in Postmortem Tissue

Postmortem tissue (70 mg to 100 mg) was subjected to chromatin immunoprecipitation as described before (Huang et al., 2007) and histone methylation levels at specific promoter sequences measured by qRT-PCR, using the primers shown in Supplemental Table 2-1. Anti-H3-trimethyl-lysine 4 (anti-H3K4me3) antibodies (Upstate) were used.

Animal Studies

For antipsychotic drug studies, adult male C57BL/6 mice, 10 to 15 weeks of age, were treated for 21 days with once daily intraperitoneal injections of saline or haloperidol (0.5 mg/kg) or clozapine (5 mg/kg) (Sigma) and then killed 1 hour after the last treatment. The Nestin-Cre transgenic line was used for a CNS-specific conditional ablation of *Bdnf* before E14.5 (Rios et al., 2001). The mutant genotype was Nestin-Cre+, BDNF2lox/2lox, and the control animals from the same outbred colony had the genotype Nestin-Cre+, BDNF+/+. Brains were harvested at E19.5 and postnatal weeks 14 to 15.

Statistical Analyses

The Shapiro Wilks goodness of fit test for normality was used to assess compliance with the bivariate normality assumption. In the case of S/C ratio correlations for the 20 matched pairs, where normality parameters were met, Pearson's correlation was used. Differences in gene expression between the 20 subjects diagnosed with schizophrenia and their matched controls were

analyzed with Wilcoxon Signed Rank test, due to lack of normality that was not corrected following logarithmic transformation. Likewise, Spearman's correlation was applied for the analysis of association between within-pair differences (S/C ratios) of BDNF, NPY, SST and miR-195 in the selected 13 matched pairs with RIN>6. In addition, the Hadi outlier identification and estimation procedure of the statistical software package SYSTAT11, which uses an algorithm that identifies outliers and corrects correlations after eliminating outlier values, was applied, in order to achieve better estimates of the correlational parameters between BDNF protein and miRNA S/C ratios. It has to be noted, also, that Grubb's test also identified two outliers, whose exclusion from our data resulted in comparable correlations between BDNF protein and miRNA S/C ratios, however the Hadi outlier elimination output does not allows us to recognize the exact outliers that it recognized and used for correcting correlations.

Table 2-1 – Demographics of human postmortem brains

	Schizophrenics												Controls						
Pair	No.	Gender	Age at Death, y	PMI, h	Brain pH	RIN	Lateral	Age at Onset ,y	Duration of illness, y	Diagnosis	Medication	Cause of Death	No.	Gender	Age at Death, y	PMI, h	Brain pH	RIN	Lateral	Cause of Death
1	**1605**	M	70	24.3	7.9	8.2	R	19	51	P	N	Cardiac	**1919**	M	61	25.5	7.4	7.1	R	Cardiac
2	**1986**	M	32	26.5	6.5	8.3	R	16	16	U*	N	Peritonitis	**2066**	M	39	27.8	6.5	4.3	R	Traumatic arrest
3	**2042**	F	69	7.3	6.1	6.7	R	35	34	U	N	Cardiac	**1796**	F	69	7.3	6.0	7.9	R	Cardiac
4	**2043**	M	55	21.2	6.3	6.3	L	20	35	U	N	Cancer	**1901**	M	51	21.5	6.2	6.8	L	Cardiac
5	**1620**	F	70	11.2	7.3	5.4	R	25	45	U	U**	Cardiac	**1713**	F	68	7.0	6.1	4.6	R	Respiratory failure
6	**1541**	M	72	9.0	6.0	7.4	L	24	48	U	N	Cardiac	**1604**	M	75	7.5	6.6	7.2	L	Cardiac
7	**1679**	M	79	5.5	6.3	5.4	L	30	49	P	N	Cardiac	**1644**	M	81	8.0	6.6	7.6	L	Cardiac
8	**2291**	M	60	26.0	6.5	7.8	R	22	38	U	N	Cardiac	**1591**	M	63	23.5	6.3	8.3	R	Cardiac
9	**2045**	M	23	11.0	6.2	5.0	L	17	6	P	N	Suicide	**2168**	M	21	17.0	6.1	4.3	L	Auto accident
10	**2033**	M	87	8.5	6.2	7.6	L	38	49	U	N	Cardiac	**1786**	M	90	8.5	6.0	8.1	L	Cardiac
11	**2232**	F	61	15.0	6.3	5.0	L	32	29	P	N	Unknown	**1756**	F	57	16.5	6.7	6.5	L	Cardiac
12	**2191**	F	58	7.5	6.2	7.6	L	25	33	U	N	Cardiac	**1609**	F	56	8.0	6.5	6.3	L	Cardiac
13	**2326**	M	40	13.5	6.4	6.9	L	33	7	U	N	Cardiac	**2338**	M	41	18.0	6.1	6.7	L	Cardiac
14	**2506**	F	47	32.0	7.2	4.0	R	20S	27	U	U**	ST	**2694**	F	48	27.0	7.1	6.9	R	LT
15	**1964**	M	48	19.5	6.2	7.3	R	18	30	D	N	ST	**2619**	M	48	20.2	6.8	7.3	R	ST
16	**2941**	M	48	27.0	6.9	8.4	R	18	30	S	A	Unknown	**2664**	M	43	26.0	7.1	8.4	R	ST
17	**2384**	M	50	6.3	6.8	8.2	R	36	14	P	N	ST	**1856**	M	54	11.0	7.0	8.3	R	LT
18	**2789**	F	59	14.8	6.6	7.4	R	30S	29	P	N	LT	**2248**	F	64	19.3	6.8	7.9	R	ST
19	**3274**	F	59	13.3	6.4	6.4	R	30S	29	P	N	Suicide	**3253**	F	57	16.0	6.6	7.2	R	LT
20	**2545**	M	64	8.5	6.2	6.6	R	18	46	S	A	ST	**1858**	M	59	13.5	6.4	5.4	R	ST

Abbreviations

S: Schizophrenia

P: Chronic paranoid schizophrenia

U: Chronic undifferentiated schizophrenia

D: Chronic disorganized schizophrenia

U*: Chronic undifferentiated schizophrenia with childhood onset

N: Neuroleptics

U**: Unmedicated

A: atypical antipychotics

LT: Long-term medical condition

ST: Sudden medical condition

Figure 2-1. Alterations in GABAergic transcripts in schizophrenia correlate with changes in BDNF protein. (A–C) Graphs showing case and control values based on qRT-PCR after logarithmic (natural log) transformation (each case connected to its matched control by dotted line); NPY (A), SST (B), and PV (C) mRNAs normalized to B2M in 20 PFC samples (BA 10) of patients with schizophrenia and 20 matched control subjects (S and C respectively). Notice the significant decrease in NPY and PV in patients with schizophrenia. *$p < 0.05$ as indicated, Wilcoxon signed-rank test (D–F) Correlations between within-pair changes (schizophrenia/control subject, S/C) of (y axis, natural log scale) NPY, SST, and PV mRNAs normalized to B2M and (x axis, natural log scale) prefrontal BDNF protein levels as determined by ELISA. Notice strong positive correlations for the within-pair changes in BDNF protein and (D) NPY and (E) SST but not (F) PV mRNA. r = Pearson correlation coefficient.

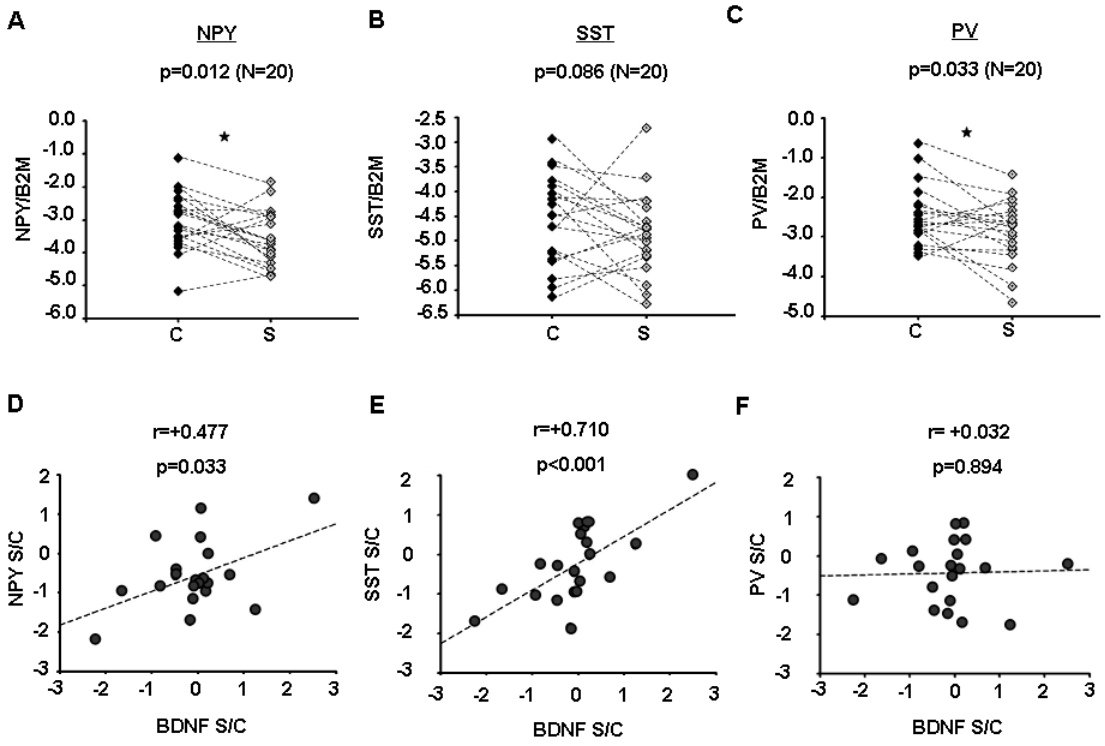

A
NPY
p=0.012 (N=20)
NPY/B2M
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
C
S
B
SST
p=0.086 (N=20)
SST/B2M
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
-6.5
C
S
C
PV
p=0.033 (N=20)
PV/B2M
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
C
S
D
r=+0.477
p=0.033
NPY S/C
BDNF S/C
E
r=+0.710
p<0.001
SST S/C
BDNF S/C
F
r= +0.032
p=0.894
PV S/C
BDNF S/C

Figure 2-2. Inverse correlation between BDNF protein and miR-195. (A) Graph showing case and control data (see also Fig. 1) of miR-195 levels as measured by qRT-PCR and after normalization to 5S rRNA in PFC of 20 patients with schizophrenia (S) and their matched control subjects (C). (B) Graph showing case and control levels of mature miR-195 levels as measured by qRT-PCR from PAGE purified RNA <40 nts in length and after normalization to miR-191 in PFC of nine patients with schizophrenia (S) and their matched control subjects (C). *p* values shown in (A) and (B) are according to Wilcoxon signed-rank test. (C–D) Correlation between within-pair changes (S/C) of miR-195 (C) and miR-30a in (D) with BDNF protein ratios in the same postmortem cohort. Notice the significant inverse correlation between miR-195 and BDNF S/C (outlier values shown with arrow). r = Pearson correlation coefficient.

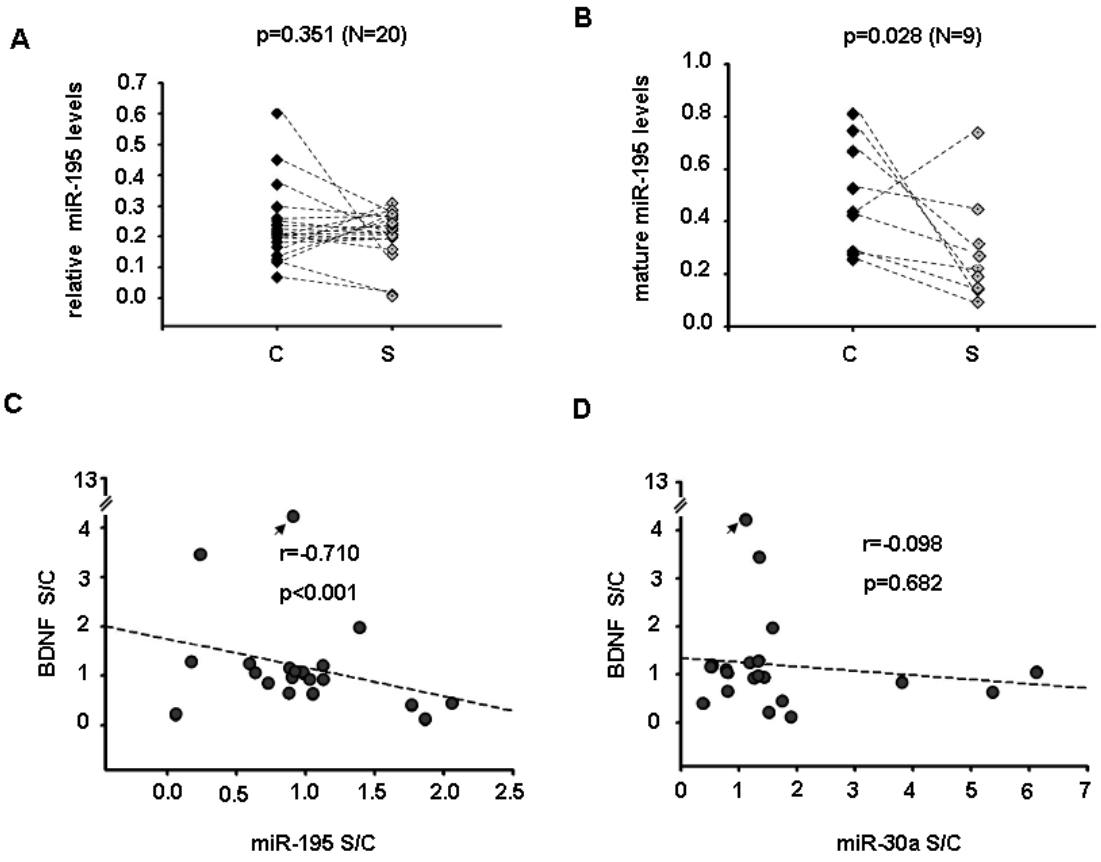

A
p=0.351 (N=20)
relative miR-195 levels
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C
S
B
p=0.028 (N=9)
mature miR-195 levels
1.0
0.8
0.6
0.4
0.2
0.0
C
S
C
BDNF S/C
13
4
3
2
1
0
r=-0.710
p<0.001
0.0
0.5
1.0
1.5
2.0
2.5
miR-195 S/C
D
BDNF S/C
13
4
3
2
1
0
r=-0.098
p=0.682
0
1
2
3
4
5
6
7
miR-30a S/C

Figure 2-3. Molecular determinants of dysregulated NPY and SST expression in schizophrenia. (A–C) Correlations between within-pair changes (schizophrenia/control subject, S/C) of BDNF protein and NPY (A) or SST (B) mRNA (both in natural log scale) and of miR-195 and BDNF protein (C) in 13 selected matched pairs with RIN > 6 (see text). (D) Schematic illustration of disease-related interrelations between miR-195, BDNF, NPY, and SST in human PFC. The percentages of variability attributed to each gene expression change (r^2x100) for both the 13 and 20 matched pairs are shown (r^2x100 for 13 pairs - r^2x100 for 20 pairs). Notice also the absence of outliers seen in Figure 2-2C,D in the selected matched pairs of optimum RNA quality. See statistical analysis for method of choice for transforming data.

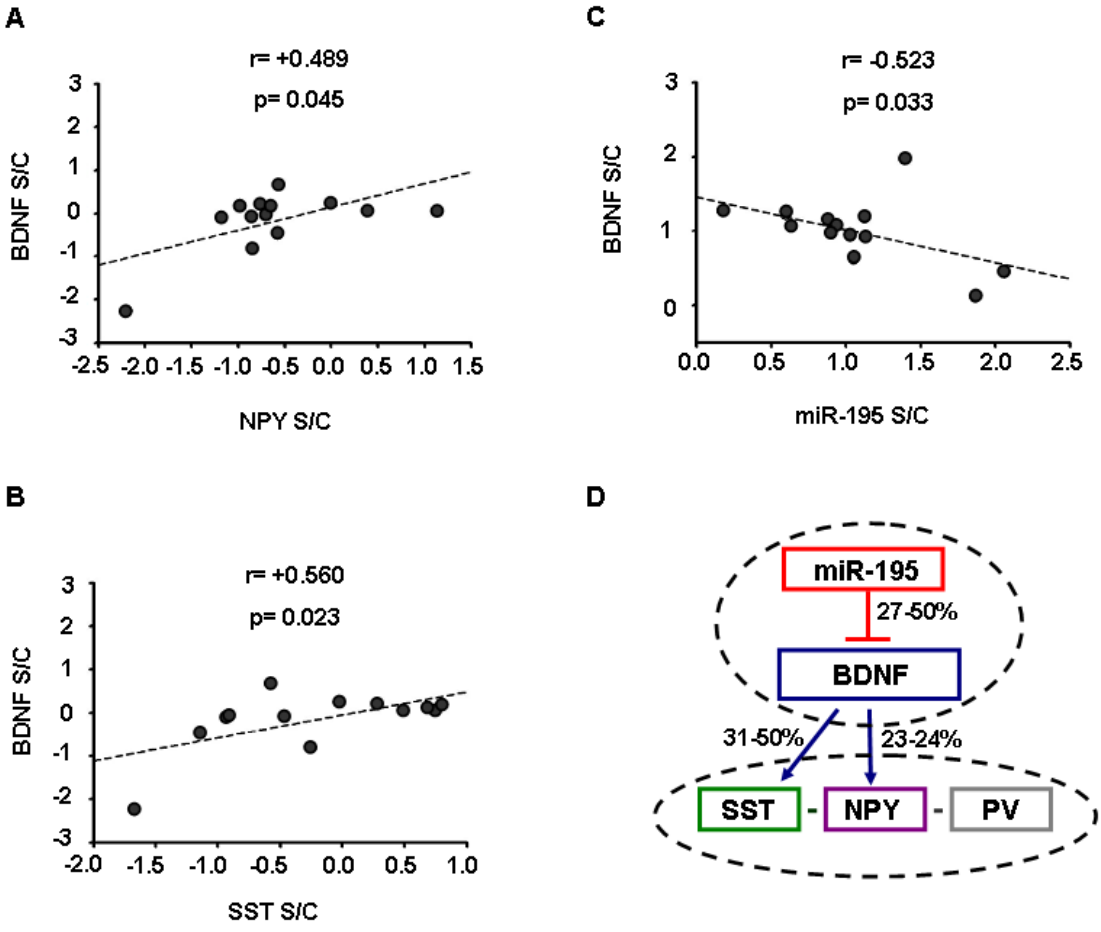
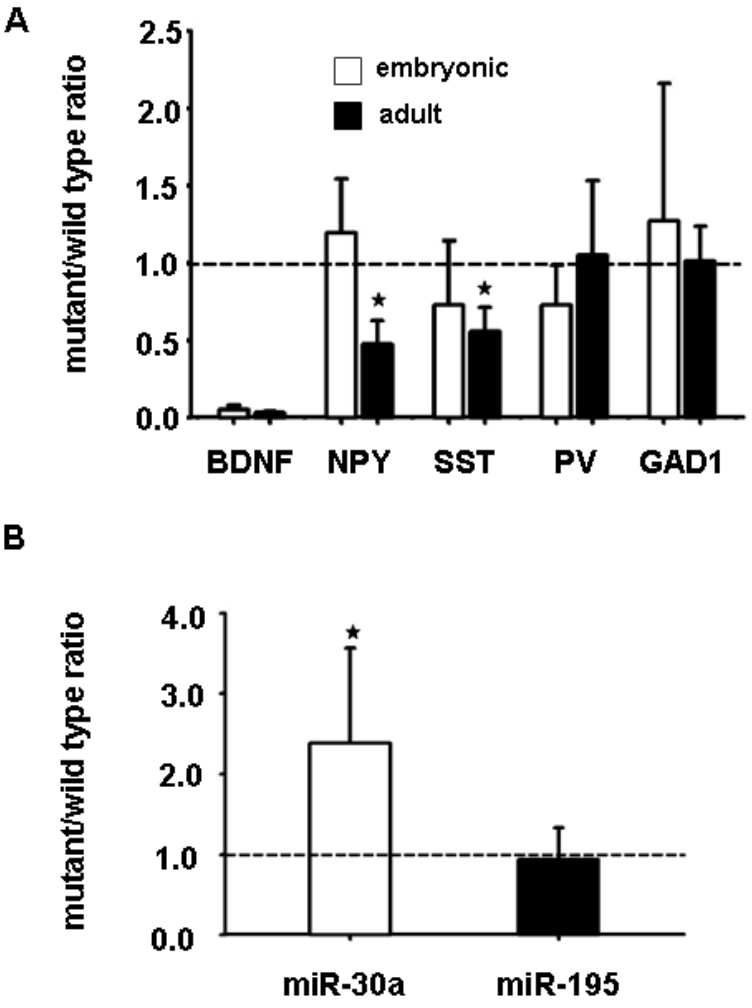
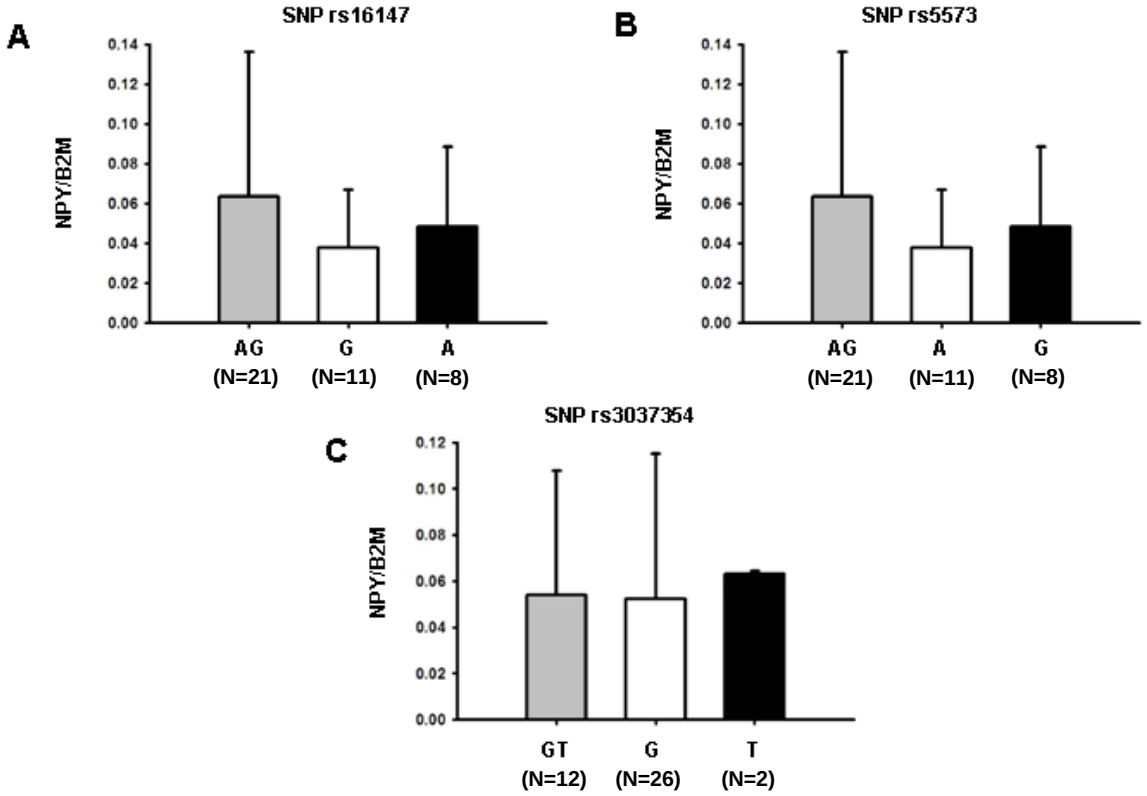

A
r= +0.489
p= 0.045
BDNF S/C
NPY S/C
B
r= +0.560
p= 0.023
BDNF S/C
SST S/C
C
r= -0.523
p= 0.033
BDNF S/C
miR-195 S/C
D
miR-195
27-50%
BDNF
31-50%
23-24%
SST
NPY
PV

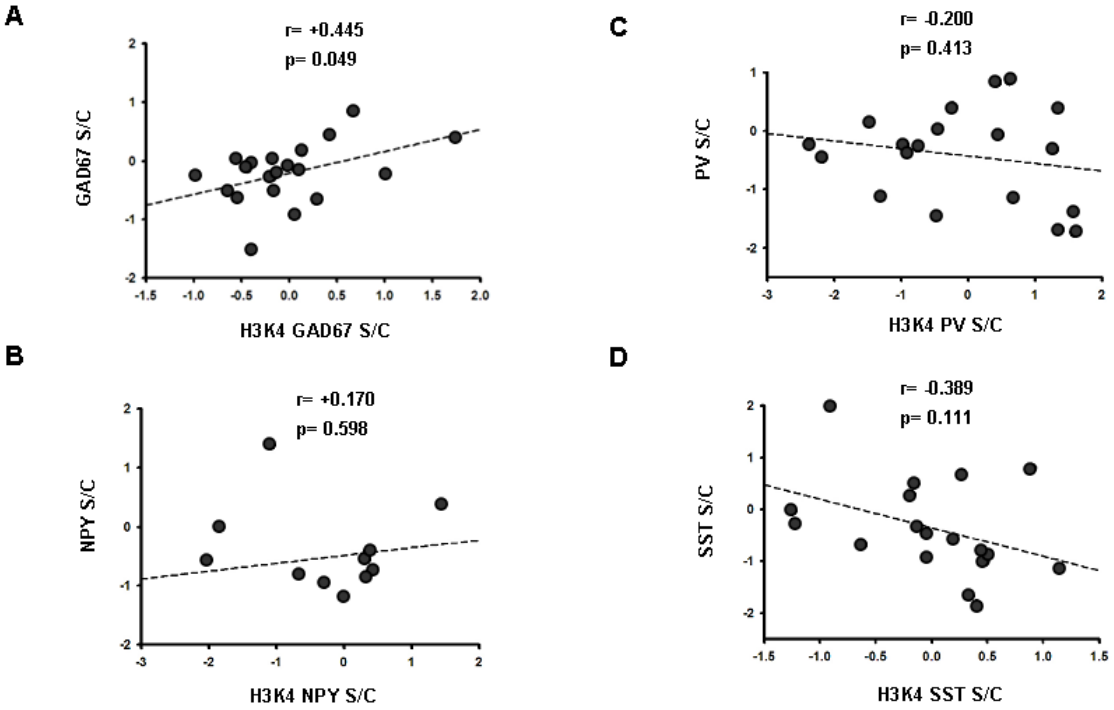
Figure 2-4. Expression of GABAergic transcripts and BDNF-related miRNAs in the cortex of BDNF deficient mice. (A) Graph showing the ratio of *Bdnf* knockout mice (mutant) to wildtype control mice (wildtype) shown as mean SD for BDNF, NPY, SST, PV, and GAD1 mRNA based on qRT-PCR data and after normalization to 18S rRNA (n = 5–7). Notice the expected robust reduction in BDNF mRNA in mutant mouse cortex and the decrease in NPY and SST mRNA in adult mutant mice only. Asterisk depicts statistically significant difference (one way t test, $p < 0.05$). (B) Expression of BDNF-related miRNAs in the cortex of *Bdnf* deficient mice. Graph showing the ratio of adult BDNF knockout mice (mutant) to control mice (wildtype) shown as mean ± SD for miR-30a (n=7) and miR-195 (n=9) based on qRT-PCR data and after normalization to 5S rRNA. Notice the increase in miR-30a levels in BDNF knockout mice but no change in miR-195. Asterisk depicts statistically significant difference (one way t test, $p < 0.05$).


A
mutant/wild type ratio
2.5
2.0
1.5
1.0
0.5
0.0
embryonic
adult
BDNF
NPY
SST
PV
GAD1
B
mutant/wild type ratio
4.0
3.0
2.0
1.0
0.0
miR-30a
miR-195

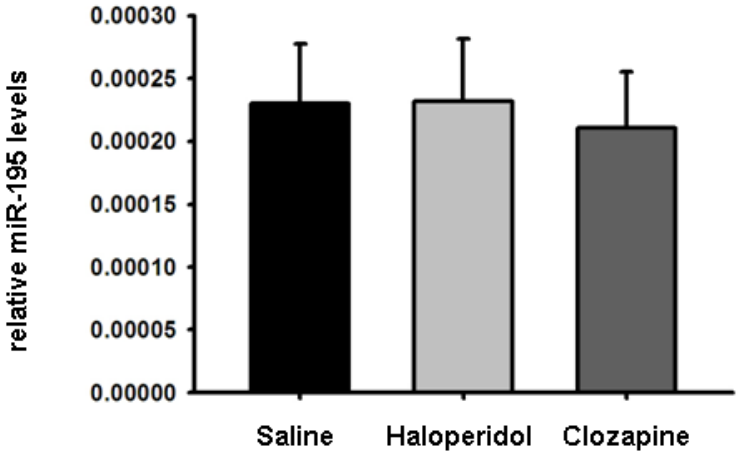
Supplemental Figure 2-1 No significant effect of NPY SNPs in PFC NPY mRNA expression. (A-C) Genotyping results shown as mean ± SD for each of three SNPs in the proximity of human NPY promoter region shown on x-axis and NPY mRNA levels (normalized to B2M) in human PFC in 40 samples of our cohort (21 control and 19 cases) are shown. Number of samples per genotype are also shown.


A
SNP rs16147
NPY/B2M
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AG (N=21)
G (N=11)
A (N=8)
B
SNP rs5573
NPY/B2M
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AG (N=21)
A (N=11)
G (N=8)
C
SNP rs3037354
NPY/B2M
0.12
0.10
0.08
0.06
0.04
0.02
0.00
GT (N=12)
G (N=26)
T (N=2)

Supplemental Figure 2-2. Association between open chromatin marker H3K4 and mRNA changes of GABAergic genes in PFC of subjects with schizophrenia. (A-D) Correlation between S/C ratios of mRNA and H3K4 levels for (A) GAD67, (B) NPY, (C) PV and (D) SST. Notice the significant positive correlation between GAD67 mRNA and H3K4 S/C ratios. r = Pearson correlation coefficient. GAD67 mRNA and H3K4 data are based on (Huang et al., 2007).


A
r= +0.445
p= 0.049
GAD67 S/C
H3K4 GAD67 S/C
B
r= +0.170
p= 0.598
NPY S/C
H3K4 NPY S/C
C
r= -0.200
p= 0.413
PV S/C
H3K4 PV S/C
D
r= -0.389
p= 0.111
SST S/C
H3K4 SST S/C

Supplemental Figure 2-3. Levels of miR-195 in mouse cortex are not affected by antipsychotic treatment. Graph represents mean ± SD based on qRT-PCR results from total RNA samples for miR-195 (normalized to 5S rRNA) after chronic treatment (see methods) with Haloperidol and Clozapine.


relative miR-195 levels
0.00030
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
Saline
Haloperidol
Clozapine

Supplemental Table 2-1: Primer sequences

Species **Genomic sequences for chromatin immunoprecipitation studies**

Species	Gene (HUGO)	Chromosome	Product location	Length [bp]	Primer sequence		Source	Gene transcritption start site
					Forward	Reverse		
Human	NPY	7P15.1	(567:633)	67	GCAATTCTCTTTCCCCTTCC	GATCAACGCTGACAGCAGAG	NT_007819.15; GI:51475902; CON 20-AUG-2004	23619482
	SST	3q28	(226:307)	82	AAGAGCTTCGGGAGCTGAG	CCATTGGTTTGGACGTAAGG	NT_005612.14; GI:37550867; CON 23-AUG-2004	93883264
	PV	22q13.1	(-117:-4)	114	CTGGTCCTCTCCAATCCAAA	GAAGATGGACCCCCTGAAAT	NC_000022.8; GI:51511751; CON 24-AUG-2004	35540023
	B2M	15q21-q22.2	(-318:-220)	99	GGGCACCATTAGCAAGTCAC	GGCGCTCATTCTAGGACTTC	NT_010194.16; GI:37540936; CON 20-AUG-2004	1579241

mRNA

	Gene	Exon	Product location	Length of [bp]	Primer sequence		Source	Transcript length [bp]
					Forward	Reverse		
Human	NPY	E4	(373:474)	102	TGTGGTGATGGGAAATGAGA	CTGCATGCATTGGTAGGATG	NM_000905.2; GI: 31542152; PRI: 06-NOV-2005	551
	SST	E2	(286:411)	126	AGCTGCTGTCTGAACCCAAC	CCATAGCCGGGTTTGAGTTA	NM_001048.3; GI: 71979669; PRI: 06-NOV-2005	632
	PV	E3-E4	(152:270)	119	CGGCCTGAAGAAAAAGAGTG	CTGGGGAGAAGCCTTTTAGG	NM_002854.2; Gi: 55925656; PRI 22-Nov-2004	572
	B2M	E1-E2	(123:272)	150	CCAGCGTACTCCAAAGATTCA	TGCTCCACTTTTTCAATTCTCTG	NM_004048.2; GI: 37704380; PRI 27-OCT-2004	987

mRNA

	Gene	Exon	Product location	Length [bp]	Primer sequence		Source	Transcript length [bp]
					Forward	Reverse		
Mouse	Npy	E3-E4	(341:436)	96	GATGAGGGTGGAAACTTGGA	GATGAGGGTGGAAACTTGGA	NM_023456.2; GI: 27754168; ROD 06-NOV-2005	561
	Sst	E1-E2	(185:302)	118	CCCAGACTCCGTCAGTTTCT	GGGCATCATTCTCTGTCTGG	NM_009215.1; GI: 6678034; ROD 16-OCT-2005	599
	Pv	E3-E4	(134:242)	109	AAAAAGAACCCGGATGAGGT	CTGAGGAGAAGCCCTTCAGA	NM_013645.3; GI: 118130845; ROD 09-DEC-2007	904
	Bdnf	E2	(716:833)	118	GCGCCCATGAAAGAAGTAAA	TCGTCAGACCTCTCGAACCT	NM_007540.3; GI: 34328441; ROD: 23-MAY-2005	4261
	18S rRNA		(1331:1464)	134	CATGGCCGTTCTTAGTTGGT	GAACGCCACTTGTCCCTCTA	X00686.1; GI: 53990; ROD 10-APR-1991	1869

CHAPTER IV: MICRORNA-30B IS REDUCED IN THE PREFRONTAL CORTEX OF FEMALE SUBJECTS WITH SCHIZOPHRENIA AND DISPLAYS GENDER DIMORPHIC EXPRESSION IN MOUSE BRAIN

This work is still in preparation, with provisional authors being Nikolaos Mellios, Jun Xu and Schahram Akbarian. My contribution to this work was to conceive the hypothesis, design the project together with Dr. Schahram Akbarian and execute all miRNA-related experiments. Specifically I isolated RNA from human and mouse samples, including PAGE purified small RNA, performed miRNA qRT-PCR, analyzed data and prepared figures and manuscript with the help of Dr. Schahram Akbarian. Dr. Jun Xu provided brain samples from mouse frontal cortex and dorsal hippocampus and determined estrous stage of female mice. Yin Guo contributed by conducting human postmortem brain dissection.

Abstract

Epidemiological, clinical and biological studies have provided evidence of an important role of gender and female hormones in schizophrenia. However, very little is known at the molecular level about pathways that might be disrupted in the disease in a gender-specific manner. In this study we show by using a case-control approach, that miR-30b, a miRNA abundantly expressed in human prefrontal cortex (PFC), is reduced in female but not male subjects diagnosed with schizophrenia. Notably, we demonstrate that disease-related deficits in miR-30b expression in human PFC are strongly associated to the age of onset of the disease. Intriguingly, levels of miR-30b in mouse cortex display a gender-dimorphic expression, which is even more pronounced in mouse dorsal hippocampus. Collectively our data provide the first evidence of a miRNA been differentially expressed in female verses male mouse brain, and suggest that a deficit in miR-30b expression in human PFC is limited to female subjects with schizophrenia.

Introduction

It is known that the age of onset of schizophrenia, its progression and patient's response to antipsychotic medication are different between males and females (Angermeyer and Kuhn, 1988; Hafner et al., 1998; Seeman, 1997). Specifically women develop schizophrenia approximately 5 years later than men with better premorbid functionality and slower course of illness (Angermeyer and Kuhn, 1988; Hafner et al., 1998). In addition, numerous studies have provided a link between estrogen and psychiatric disease. For example during phases of reduced estrogen activity such as in menopause and postpartum periods women are more likely to suffer a relapse of psychosis or present with the first psychotic episode respectively (Kendell et al., 1987; Seeman, 1997). Notably, the stages of menstrual cycle that are characterized by low estrogen levels are also linked to higher incidence of relapses in women diagnosed with psychiatric diseases including schizophrenia (Bergemann et al., 2007). On the other hand, an improvement on chronic psychiatric symptomatology and a reduction in relapse rates has been suggested to be more likely during high estrogen menstrual cycle

stages (Riecher-Rössler et al., 1994) and during pregnancy (Chang et al., 1986), where estrogen levels also rise. More importantly, estrogen supplementation has been proved to be an affective adjunctive therapeutic option for schizophrenia (Kulkarni et al., 2008).

MicroRNAs are evolutionary conserved small non-coding RNAs that have been shown to mediate the posttranscriptional regulation of a plethora of protein coding genes (Bartel, 2004; Filipowicz et al., 2008). They are derived from longer precursor molecules (pri and subsequently pre miRNAs) which are cleaved to generate the mature miRNA forms of approximately 20nt in length (Bartel, 2004; Filipowicz et al., 2008). They are abundantly expressed in the mammalian nervous system (Miska et al., 2004; Bak et al., 2008) and have been shown to be important for neuronal development and synaptic plasticity (Giraldez et al., 2005; Schratt et al., 2006). Although gender dimorphic expressions of miRNAs has been recently reported in mouse spleen (Koturbash et al., 2008), rat liver (Cheung et al., 2009) and in human serum (Chen et al., 2008) the effect of gender on brain miRNA levels has not

been examined and its relevance to psychiatric disease has not been addressed.

We have previously shown that differentially expressed miRNAs, including miR-30 family member miR-30a-5p, act as inhibitors of BDNF in human prefrontal cortex (Mellios et al., 2008a) and that one BDNF-targeting miRNA, miR-195, is an important molecular determinant of schizophrenia-related changes in BDNF and BDNF-regulated GABAergic genes (Mellios et al., 2008b). However, in the later study we failed to identify any consistent disease-related changes in miR-30a-5p in the PFC of subjects with schizophrenia, despite the fact that miR-30a-5p was shown in our first study to inhibit BDNF translation in vitro and to be inversely correlated with BDNF protein levels during the maturation and aging of human PFC (Mellios et al., 2008a). We conducted analyses to determine if any other members of the miR-30 family analyzed in our first study displayed disease-related changes in the PFC of subjects with schizophrenia. Surprisingly, we found an unexpected pattern of results for the randomly selected as control miR-30b, a miRNA that was shown to be expressed in higher levels in human prefrontal cortex and

with a different laminar enrichment compared to miR-30a-5p (Mellios et al., 2008a).

Intriguingly, using a case-control design we report that miR-30b is reduced in the prefrontal cortex of female but not male subjects with schizophrenia. Spurred by this serendipitous finding, we demonstrate that miR-30b displays a gender dimorphic expression in mouse frontal cortex and dorsal hippocampus. Interestingly, we show that the disease-related gender-specific deficit in prefrontal miR-30b expression is at the level of mature miRNA and that it is associated with the age of onset of the schizophrenia. Our results show that a brain expressed miRNA is differentially regulated in male and female brains and that it is altered in a gender-specific manner in schizophrenia.

Results

Gender-specific deficits in miR-30b expression in the prefrontal cortex of subjects with schizophrenia

We had previously shown that miR-30b is abundantly expressed in human prefrontal cortex (Mellios et al., 2008a). In addition a previous study had reported a trend for decreased miR-30b levels in the prefrontal cortex of subjects with schizophrenia based on microarray data in a non-matched pair cohort and qRT-PCR data in 4 cases and 4 controls (Perkins et al., 2007). We examined the expression of miR-30b in the prefrontal cortex of 30 subjects diagnosed with schizophrenia and 30 matched controls derived from two independent cohorts (20 matched pairs from first and 10 matched pairs from second cohort) (Table 3-1) using qRT-PCR from small (<200nts) RNA enriched samples. Intriguingly, our results demonstrated that the reduction in miR-30b levels in the PFC of subjects with schizophrenia was gender specific, with a significant reduction in miR-30b levels in female but not male subjects with schizophrenia (Fig. 3-1A, B). Specifically, in 9 out of 12 female matched pairs there was a reduction of more than 20% in miR-30b levels (expressed as schizophrenic case to control miR-30b ratios - S/C miR-30b ratio) (Fig. 3-1A). Demographic or RNA quality factors (see also Table 3.1) such as age, postmortem interval (PMI), RNA Integrity Number (RIN) and

brain pH were not correlated to miR-30b levels (data not shown). Our results suggest that the disease-related deficit in miR-30b expression is limited to female subjects.

Mature but not precursor prefrontal miR-30b levels are reduced in female subjects with schizophrenia

In order to confirm that the changes in miR-30b levels in the PFC of female subjects of schizophrenia are at the level of mature miRNA, we re-dissected tissue from 12 cases and controls (7 female and 5 male matched pairs) selected based on their availability, and used polyacrylamide gel electrophoresis (PAGE) to enrich for RNAs smaller than 40nts. Due to the fact that 5S rRNA, which was used as a normalizer in our initial qRT-PCR analysis is excluded because of its size from the <40nts RNA pool, we used miR-191, which has been shown to be an ideal normalizer for miRNA quantification in human postmortem tissues (Peltier et al., 2008). Our results from these independent RNA extractions and with a different normalization gene were comparable to our initial data (Fig. 3-2A) and there was a significant

correlation between S/C miR-30b ratios as determined by the two methods (Figure 3-2B). Due to the fact that precursor miRNA molecules are much larger than 40nts, the miR-30b levels measured with this method represent the mature miRNA. Interestingly, 6 out of 7 female subjects with schizophrenia, were once again shown to have reduced levels of miR-30b relative to their matched controls, but no differences were seen in male (Fig. 3-2A).

Previous studies have suggested that alterations of mature miRNA levels in schizophrenia are not accompanied by analogous changes in the levels of their precursors (Perkins et al., 2007; Beveridge et al., 2008). We used qRT-PCR to determine the levels of pri-miR-30b and pre-miR-30b in a subset of cases and controls (N=17 and N=15 matched pairs respectively). Due to the fact that pri-miR-30b includes the sequence of pre-miR-30b our primers for pre-miR-30b measure both molecules so are referred to as pre/pri-miR-30b. Our results showed that with the exception of a trend for an increase in pri-miR-30b levels in male cases, there was no difference in miR-30b precursor expression (Fig. 3-2C). Of note mature miR-30b levels were still significantly

reduced in this subset of samples (Figure 3-2D). We conclude that the deficit in miR-30b levels in the PFC of female subjects with schizophrenia is limited to mature miRNA.

Associations between age of onset of schizophrenia and changes in prefrontal miR-30b levels

The age of onset of schizophrenia in our two cohorts ranged between 16 and 38 years old, with the majority of data being from the first cohort due to unavailability of age of onset information for a subset of cases of the second cohort (Table 3.1). We plotted the S/C miR-30b ratios from both the initial (<200nts RNA samples) and secondary measurement (<40nts RNA samples) to the age of onset of schizophrenia. Our results revealed an inverted U shape association between miR-30b levels and age of onset in the 23 out of 30 matched pairs, for which age of onset information was available (Fig. 3-3A). We arbitrarily separated our data into two groups: one group of less and one of more than 25 years of age, since this was the approximate age of onset after which the shape of the curve changes direction (Figure 3-3A).

Separate analysis of the two groups showed that the first 10 earlier onset cases exhibited a weak inverse correlation to disease-related changes in miR-30b ($r = -0.477$, $p = 0.163$ – Fig. 3-3B) and the last 13 later onset cases exhibited a significant positive correlation to S/C miR-30b ratios ($r = +0.606$, $p = 0.028$ – Fig. 3-3C). Notably, a robust positive correlation was found between age of onset and S/C mature miR-30b levels in female samples as measured in the <40nts samples ($r = 0.863$, $p = 0.012$, 7 matched pairs – Fig. 3-3D), although a weak trend ($r = +0.437$, $p = 0.279$, 8 matched pairs) was found between age of onset and S/C miR-30b ratios as determined in the <200nt RNA samples. No correlation between age of onset and miR-30b was found for male samples (data not shown). In addition, duration of illness was not associated with any of the S/C miR-30b ratios (data not shown). Our data suggest that in adult onset subjects with schizophrenia, reduced levels of miR-30b are linked to an earlier age of onset, whereas in adolescent and young adult onset cases increased levels of miR-30b could be associated with earlier age of onset. The presence, though, of a positive correlation between the changes in miR-30b levels and age of onset only in female

cases, which display a later average age of onset in our cohorts (see Table 3-1) might have contributed to the associations observed in our study.

Gender-dimorphic expression of miR-30b in mouse prefrontal cortex and dorsal hippocampus.

Although in our human PFC control samples the differences between miR-30b levels in male and female were not consistent (approximately 20% less miR-30b in male control PFC for the first cohort, but no difference for second cohort – data not shown), we wanted to determine if there is any gender effect on miR-30b levels in mouse frontal cortex and if this effect was pronounced in other brain areas, such as hippocampus. Towards this end, we first measured with qRT-PCR the levels of miR-30b, together with the neuronal enriched miR-100 and X-chromosome encoded miR-222 in samples from mouse frontal cortex. Our results revealed a modest yet significant higher expression of miR-30b in mouse female frontal cortex. On the other hand, there were no changes in miR-100 levels, whereas a trend for higher miR-222 levels was observed in female frontal cortex. We then measured

miR-30b levels with qRT-PCR in samples from dorsal hippocampus. Intriguingly, there was an approximately 2 fold significantly higher expression of hippocampal miR-30b in female mice. We then determined through vaginal smears the estrous cycle of the mice used and compared miR-30b levels in female cortex and dorsal hippocampus during diestrus, proestrus, estrus and metestrus (Supplemental Fig. 3-1). There was no significant effect of estrous cycle stage on female miR-30b levels (ANOVA $p>0.05$), although the highest mean of miR-30b levels was observed during estrus in both cortex and dorsal hippocampus (Supplemental Fig. 3.1). Our results suggest the presence of gender dimorphism in miR-30b expression in mouse brain.

Potential effects of chronic antipsychotic treatment on miR-30b expression

In order to determine if chronic antipsychotic treatment might influence cortical miR-30b levels we measured miR-30b expression in the cerebral cortex of mice treated with typical antipsychotic haloperidol or atypical antipsychotic clozapine. Our results showed no significant reduction in

cortical miR-30b levels (Supplemental Fig. 3.2) following chronic antipsychotic treatment. It has to be noted, also, that in our human cohorts the majority of cases (N=23 out of 30) were treated with typical antipsychotics, with a few cases being unmedicated (N=5 out of 30) and only 2 having received atypical antipsychotics (Table 3.1), so that the differences on mean miR-30b levels between these 3 groups cannot be properly evaluated. However, due to the fact that in both our mouse pharmacological study and in our human cohorts the mean levels of miR-30b were actual lower in antipsychotic naïve cortical samples (Supplemental Fig. 3.2), our data suggest that it is unlikely that the observed deficits in miR-30b expression are a result of antipsychotic treatment.

Conclusion

Using a case control design our study provides the first evidence of a miRNA displaying a gender specific deficit in the prefrontal cortex of subjects with schizophrenia. We show that miR-30b is reduced in PFC of female subjects with schizophrenia and that his deficit is at the level of mature miRNA.

Notably, we provide evidence of an association between age of onset of schizophrenia and disease-related changes in miR-30b. Furthermore, we show that miR-30b displays a pronounced gender dimorphic expression pattern in dorsal hippocampus as well as a modest gender dimorphic expression in mouse frontal cortex.

The consistency of the deficit in miR-30b expression in female subjects with schizophrenia as measured with two independent RNA extraction and qRT-PCR normalization protocols, and the intriguing association of miR-30b disease-related levels to the age of onset of the disease warrants in our opinion further studies to determine if this miRNA is a reliable molecular marker of schizophrenia. A previous study that had reported alterations in a subset of miRNAs in the PFC of schizophrenia cases included miR-30b in the list of miRNAs that were found to be reduced by microarray (Perkins et al., 2007). Intriguingly, despite the fact that no effect of gender was reported, our re-analysis of supplemental material from this study reveals a more robust reduction in female subjects with schizophrenia (Perkins et al., 2007 and data not shown).

Furthermore, our finding that the levels of precursor miR-30b molecules were not significantly altered is in accordance with two previous studies, where changes in miRNA expression in the parietal and prefrontal cortex of subjects with schizophrenia were at the level of mature miRNA (Perkins et al., 2007; Beveridge et al., 2008). It has been suggested that a defect in the processing of pri-miRNAs can greatly increase the possibility of developing schizophrenia as evident but the approximately 30-fold greater risk for the disease in patients with a microdeletion which includes the miRNA processing gene DGCR8 (Murphy et al., 1999; Gothelf et al., 2007) and by the behavioral and neuroanatomical changes observed in forebrain specific DGCR8 deficient mice, which are reminiscent of psychosis (Stark et al., 2008). However, an increase in pri-miR-30b levels predominantly in male cases with schizophrenia, which would be expected if pri-miR-30b was not effectively cleaved yet normally transcribed, implies that for female cases there are either compensatory mechanisms that alter miR-30b transcription masking any pri-miRNA processing defect, or that the potential cause of miR-30b changes has to do with subsequent steps in miRNA biogenesis that affect the

stability of mature miRNA molecules. Further studies are needed to pinpoint the exact mechanisms behind miR-30b alterations in the PFC of female subjects with schizophrenia.

Interestingly, a recent study estimated that miR-30b is transcribed as an initial approximately 40kb precursor sequence which also includes miR-30d miRNA (Saini et al., 2008). The expression of these two miR-30 family miRNAs seems to be positively correlated (Mellios et al., 2008a and data not shown), although in schizophrenia no changes in miR-30d expression have been reported (Perkins et al., 2007). It has to be noted also that the genomic region which encodes miR-30b/miR-30d precursor is at the subtelomeric region of the long arm of chromosome 8 (8q24.22), which is known to be characterized by instability and frequent changes in copy number variation (Blenkiron et al., 2007).

Interestingly, in our previous study we found that miR-30a expression is developmentally regulated and it can act as an inhibitor of BDNF expression in human prefrontal cortex (Mellios et al., 2008a). However, our analysis (Mellios et al., 2008b) and a previous study (Perkins et al., 2007) found

inconsistent changes in miR-30a expression in schizophrenia both for miR-30a-5p (increased in our <250nts RNA sample analysis but not changed in <40nt mature miRNA analysis and no significant changes in microarray measurements by Perkins et al., 2007). Moreover, miR-30a-3p, which is derived from the same precursor as miR-30a-5p but is expressed in lower levels in human PFC (Mellios et al., 2008a), was reported to be reduced by qRT-PCR but not microarray (Perkins et al., 2007). In addition, a recent study identified miR-30c, another member of miR-30 family, as one of the strongest responsive miRNAs to lithium treatment in mice (Zhou et al., 2008). Given the fact that miRNAs of the same family share high sequence similarities it is of particular interest that several different members of the miR-30 family are been linked to human brain function and psychiatric disease.

Despite the fact that miR-30b levels exhibit a gender dimorphic expression in mouse brain with a more pronounced difference between female and male levels in dorsal hippocampus compared to frontal cortex, there was no significant effect of estrous cycle stage on miR-30b expression. On the other hand, in silico analysis predicts multiple estrogen responsive

elements in human, mouse and rat predicted miR-30b/miR-30d precursor (data not shown). In addition, a recent study has shown that miR-30b is the highest expressed miRNA in maturing oocytes (Murchison et al., 2007), which are under clear influence of female hormones. Future experiments are under preparation to determine if these estrogen responsive sequences are functional and if in mammalian brain miR-30b expression is directly influenced by estrogen levels or by hormonal-independent mechanisms that can lead to gender dimorphism (Xu and Disteche, 2006; Arnold, 2004).

To sum up, our data so far provide evidence that miR-30b is a gender dimorphic miRNA in mouse brain and that it is selectively reduced in the PFC of female subjects diagnosed with schizophrenia. More importantly we show that the deficit in miR-30b expression in female cases is strongly associated to the age of onset of the disease and that it is at the level of mature miR-30b. Collectively our results suggest that miRNAs might play an important role in gender-related variability in gene expression which could potentially be partly contributing to differences between female and male subjects diagnosed with schizophrenia.

Materials and Methods

Postmortem Brains

A total of 60 postmortem brain samples from 30 subjects diagnosed with schizophrenia and 30 controls were used in this study. All procedures were approved by the Institutional Review Board of the University of Massachusetts Medical School. Each sample from a disease case was matched to a control according to gender, age, postmortem interval (PMI), and hemisphere. Demographics, medication status and postmortem confounds, including RNA Integrity Number (RIN) are provided in Table 3-1. All samples included in this study had a RIN ≥ 4.0, which has been proposed as a minimum standard for postmortem RNA quality (Lipska et al., 2006). Each sample was from the pole of the frontal cortex (rostral portion of BA10 cut through the full vertical thickness of the cortex) and collected from a brain bank at the University of California at Davis for the first cohort (Dr. Edward G. Jones, Center for Neuroscience, University of California at Davis) and from the brain bank at Maryland Psychiatric Research Center (Baltimore,

Maryland) for the second cohort (Matched pairs 1-7 and 13-25 from first cohort and 8-12 and 26-30 for second –Table 3-1). The matching process had been completed prior to the experiments. Diagnosis of schizophrenia was based on DSM-IVR, and control brains had no history of psychiatric or neurological disease, as previously described (Akbarian et al., 1995). For all experimental procedures, tissue from a case was processed in parallel together with its matched control, using aliquots of the same solutions, buffers, probes, etc.

Animal Studies

C57BL/6 mice were used for all animal experiments. For antipsychotic drug studies, adult male mice, 10 to 15 weeks of age, were treated for 21 days with once daily intraperitoneal injections of saline or haloperidol (0.5 mg/kg) or clozapine (5 mg/kg) (Sigma, St. Louis, Missouri) and then killed 1 hour after the last treatment. To dissect mouse frontal cortex, the brain was positioned in a coronal brain matrix and a block between 4mm and 2mm (anterior/posterior, all coordinates relative to Bregma; Paxinos and Franklin,

2001) was removed. The block was then placed on a flat surface on ice, posterior side up. The dorsal portion (~1mm wide), containing both frontal association cortex and prelimbic cortex, was collected. The rest of the brain was transferred from the matrix into a beaker containing dry ice in isobutane. Once frozen, cortical samples were stored at -80ºC until processing. Tissue from dorsal hippocampus was isolated on a cryostat with a 0.5mm micro-puncher based on the following coordinates: (-1.2 anterior/posterior, ±1.0 medial/lateral, 1.2 dorsal/ventral. Estrus cycle was determined after microscopic examination of vaginal smear.

RNA Isolation

Total and small RNAs (<200 nts) were isolated by using the mirVANA PARIS Kit (Ambion), according to the manufacturer's instructions and as described before (Mellios et al., 2008a). For isolation of <40 nts RNA, the flashPAGE Fractionator System (Ambion) was used according to manufacturer's instructions. Briefly, 5 μg of total RNA was run for 12 minutes at 75 mV and RNA from the lower running buffer was purified using flashPAGE Reaction

Clean-Up Kit (Ambion). In this case total RNA was isolated by using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, California) and then treated with DNase I (Ambion).

RNA Quantification

The mirVana qRT-PCR miRNA Detection Kit (Ambion) was used for measuring human miR-30b in samples of <200 nts and <40 nts RNA. For each sample and amplicon, cycle thresholds were averaged from triplicate reactions and normalized to either 5S ribosomal RNA (rRNA) (<200 nts RNA) or miR-191 (<40 nts RNA). TaqMan miRNA assays (Applied Biosystems) were used according to manufacturer's instructions for determining miR-30b, miR-100 and miR-222 levels in mouse frontal cortex and dorsal hippocampus (normalized to snoRNA202). TaqMan One-Step RT-PCR (Applied Biosystems) was used according to manufacturer's instructions for quantification of human pri-miR-30b and pre/pri-miR-30b and 18S rRNA with the following primers: GTGAATGCTGTGCCTGTTC and GCCTCTGTATACTATTCTTGCCA for pri-miR-30b and

CATGTAAACATCCTACACTCAGCT, ATCCACCTCCCAGCCAAT for pre/pri-miR-30b and primers shown on Supplemental Table 2.1 for 18S rRNA.

Table 3.1. Demographics of human postmortem brains and miR-30b case to control ratios.

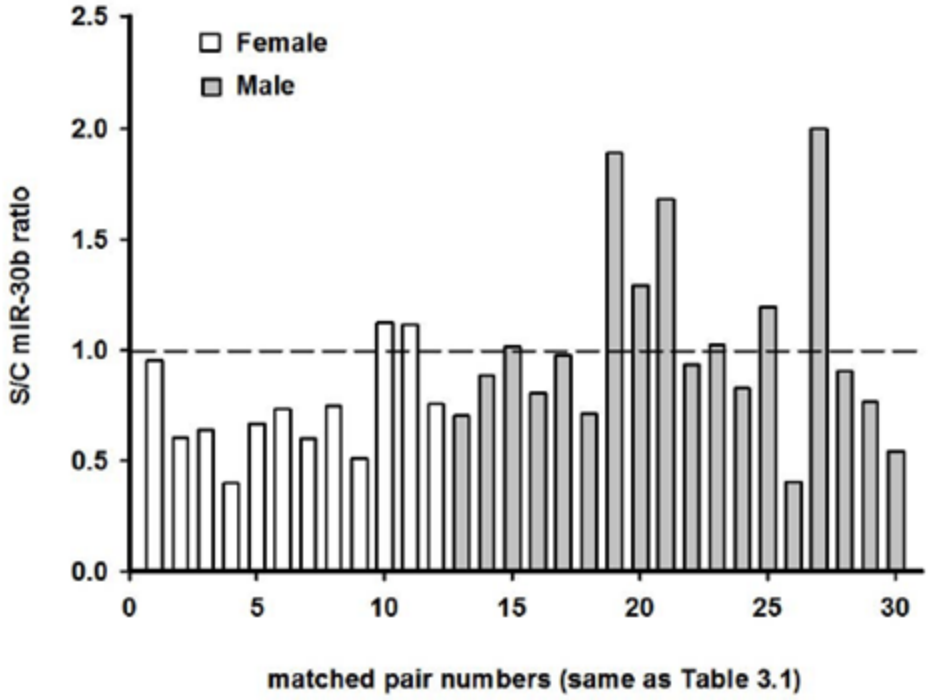
	Schizophrenics													Controls							
Pair	No.	Gender	Age at Death, y	PMI, h	Brain pH	RIN	Lateral	Age at Onset ,y	Duration of illness, y	Diagnosis	Medication	Cause of Death	miR-30b S/C ratio	No.	Gender	Age at Death, y	PMI, h	Brain pH	RIN	Lateral	Cause of Death
1	**2042**	F	69	7.3	6.1	6.7	R	35	34	U	N	Cardiac	**0.953**	**1796**	F	69	7.3	6.0	7.9	R	Cardiac
2	**1620**	F	70	11.2	7.3	5.4	R	25	45	U	U**	Cardiac	**0.603**	**1713**	F	68	7.0	6.1	4.6	R	Resp. failure
3	**2232**	F	61	15.0	6.3	5.0	L	32	29	P	N	Unknown	**0.639**	**1756**	F	57	16.5	6.7	6.5	L	Cardiac
4	**2191**	F	58	7.5	6.2	7.6	L	25	33	U	N	Cardiac	**0.399**	**1609**	F	56	8.0	6.5	6.3	L	Cardiac
5	**2506**	F	47	32.0	7.2	4.0	R	20S	27	U	U**	ST	**0.665**	**2694**	F	48	27.0	7.1	6.9	R	LT
6	**2789**	F	59	14.8	6.6	7.4	R	30S	29	P	N	LT	**0.735**	**2248**	F	64	19.3	6.8	7.9	R	ST
7	**3274**	F	59	13.3	6.4	6.4	R	30S	29	P	N	Suicide	**0.598**	**3253**	F	57	16.0	6.6	7.2	R	LT
8	**13**	F	40	21	6.4	8	R	N/A	N/A	N	U**	Cardiac	**0.749**	**27**	F	45	18	6.65	7.9	R	Accident
9	**21**	F	45	14	6.05	4	L	N/A	N/A	U	N	Cardiac	**0.507**	**39**	F	43	19	6.55	5.8	L	Cardiac
10	**32**	F	58	3	6.8	6.9	L	29	28	U	U**	Cardiac	**1.122**	**26**	F	59	14	6.45	7.4	L	Pulm.Emb.
11	**24**	F	39	18	6.85	5.6	R	N/A	N/A	N	N	Pulmon.Emb.	**1.115**	**25**	F	33	16	6.75	6.9	R	Cardiac
12	**37**	F	33	3	6.35	7.9	L	N/A	N/A	N	U**	Drug intox.	**0.755**	**36**	F	30	10	6.85	6.5	R	Accident
13	**1605**	M	70	24.3	7.9	8.2	R	19	51	P	N	Cardiac	**0.705**	**1919**	M	61	25.5	7.4	7.1	R	Cardiac
14	**1986**	M	32	26.5	6.5	8.3	R	16	16	U*	N	Peritonitis	**0.885**	**2066**	M	39	27.8	6.5	4.3	R	Traum.Arrest
15	**2043**	M	55	21.2	6.3	6.3	L	20	35	U	N	Cancer	**1.016**	**1901**	M	51	21.5	6.2	6.8	L	Cardiac
16	**1541**	M	72	9.0	6.0	7.4	L	24	48	U	N	Cardiac	**0.806**	**1604**	M	75	7.5	6.6	7.2	L	Cardiac
17	**1679**	M	79	5.5	6.3	5.4	L	30	49	P	N	Cardiac	**0.976**	**1644**	M	81	8.0	6.6	7.6	L	Cardiac
18	**2291**	M	60	26.0	6.5	7.8	R	22	38	U	N	Cardiac	**0.715**	**1591**	M	63	23.5	6.3	8.3	R	Cardiac
19	**2045**	M	23	11.0	6.2	5.0	L	17	6	P	N	Suicide	**1.890**	**2168**	M	21	17.0	6.1	4.3	L	Accident
20	**2033**	M	87	8.5	6.2	7.6	L	38	49	U	N	Cardiac	**1.291**	**1786**	M	90	8.5	6.0	8.1	L	Cardiac
21	**2326**	M	40	13.5	6.4	6.9	L	33	7	U	N	Cardiac	**1.681**	**2338**	M	41	18.0	6.1	6.7	L	Cardiac
22	**1964**	M	48	19.5	6.2	7.3	R	18	30	D	N	ST	**0.932**	**2619**	M	48	20.2	6.8	7.3	R	ST
23	**2941**	M	48	27.0	6.9	8.4	R	18	30	S	A	Unknown	**1.023**	**2664**	M	43	26.0	7.1	8.4	R	ST
24	**2384**	M	50	6.3	6.8	8.2	R	36	14	P	N	ST	**0.827**	**1856**	M	54	11.0	7.0	8.3	R	LT
25	**2545**	M	64	8.5	6.2	6.6	R	18	46	S	A	ST	**1.196**	**1858**	M	59	13.5	6.4	5.4	R	ST
26	**40**	M	44	14	6.6	7.9	L	N/A	N/A	U	U**	Fatty liver	**0.407**	**11**	M	46	19	6.7	8	L	Cardiac
27	**14**	M	59	13	7.05	8.4	R	N/A	N/A	N	N	Pulmon.Emb.	**2.000**	**35**	M	52	12	6.7	7.8	R	Pulm. Emb.
28	**1**	M	53	11	6.75	8.2	R	N/A	N/A	U	N	Cardiac	**0.905**	**7**	M	55	17	6.9	7.9	R	Cardiac
29	**29**	M	32	21	6.7	6.7	R	28	4	P	N	Narcotic intox.	**0.768**	**38**	M	30	22	6.75	7.5	R	Asthma
30	**16**	M	34	16	6.9	7.5	R	26	6	P	N	Suicide	**0.543**	**28**	M	31	15	6.6	6.3	R	Cardiac

Abbreviations

S: Schizophrenia
P: Chronic paranoid schizophrenia
U: Chronic undifferentiated schizophrenia
D: Chronic disorganized schizophrenia
U*: Chronic undifferentiated schizophrenia with childhood onset
N: Neuroleptics
U**: Unmedicated
A: atypical antipychotics
LT: Long-term medical condition
ST: Sudden medical condition

Figure 3-1. Deficits in miR-30b expression in the prefrontal cortex of female but not male subjects with schizophrenia. (A) Graph showing the ratio of miR-30b levels of subjects diagnosed with schizophrenia to their matched controls (referred to as S/C ratio) for 30 matched pairs taken from 2 independent cohorts (see table 3-1 for each matched pair number demographics, including brain hemisphere) and presented according to their gender (white bars for female and gray bars for male matched pairs). Notice the disease-related reduction in miR-30b levels (evident by <1 S/C ratios) in the majority of female but only in a subset of male matched pairs. (B) Graph showing mean ± SEM for S/C miR-30b ratios of female and male matched pairs. Notice the significant reduction in miR-30b levels in female subjects with schizophrenia (S/C ratio significantly less than 1). Asterisk depicts p value based on one tailed t-test.

A


Female
Male
S/C mIR-30b ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
matched pair numbers (same as Table 3.1)

B

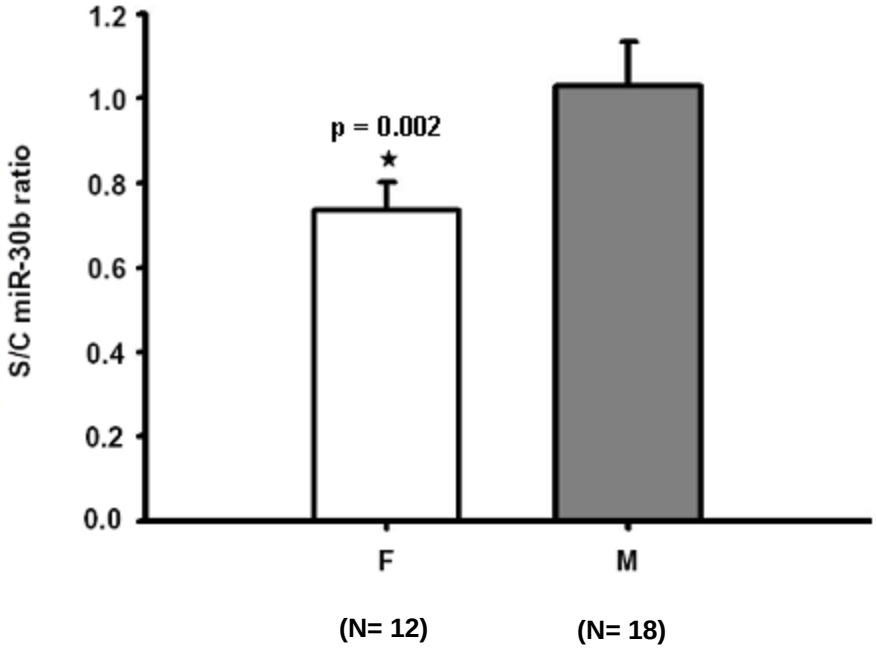

S/C miR-30b ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.002
★
F
M
(N= 12)
(N= 18)

Figure 3-2. Disease related alterations in miR-30b expression are consistent and at the level of mature miRNA. (A) Comparison between case to control S/C miR-30b ratios determined using <200nts enriched RNA samples normalized to 5S rRNA (shown as black bars) and PAGE purified <40nts enriched RNA samples derived from independent dissection of the same tissues (all from cohort 1) and normalized to miR-191 (shown as grey bars - seven female and five male matched pairs were selected based on availability of tissue). Notice the consistency in miR-30b deficits in female subjects with schizophrenia. (B) Association between S/C miR-30b ratios between <200nts and <40nts RNA samples (black circles show female, and gray show male pairs). Notice significant positive correlation. (C) Disease-related changes in precursor miR-30b levels (based on primers specific for pri-miR-30b and for both pre and pri-miR-30b and normalized to 18S rRNA) in female and male matched pairs (N=7 matched pairs for female, and N=8-10 matched pairs for male). (D) Disease-related changes in miR-30b levels in <200nts samples normalized to 5S rRNA (N=7 matched pairs for female and 13 matched pairs for male) and <40nts PAGE purified samples normalized to miR-191 (N=7 matched pairs for female and 5 matched pairs for male). All samples in (D) were from the same cohort as in (C). Notice significant or close to significant reduction in miR-30b only in female subjects in <250nts and <40nts samples, respectively, in this subset of samples. Asterisk denotes statistically significant difference based on t-test ($p<0.05$ – significant and close to significant p values also indicated).

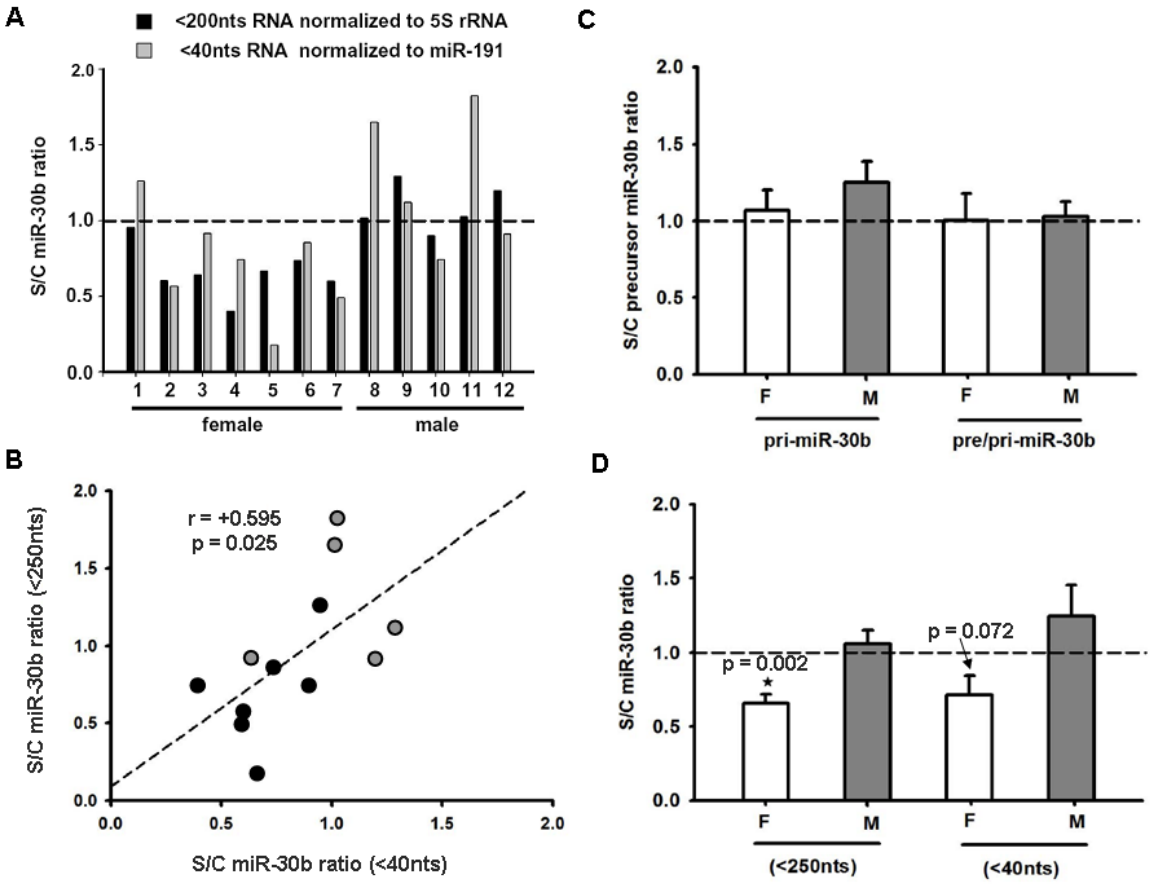

A
<200nts RNA normalized to 5S rRNA
<40nts RNA normalized to miR-191
S/C miR-30b ratio
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
female
male
C
S/C precursor miR-30b ratio
F M F M
pri-miR-30b
pre/pri-miR-30b
B
r = +0.595
p = 0.025
S/C miR-30b ratio (<250nts)
S/C miR-30b ratio (<40nts)
D
S/C miR-30b ratio
p = 0.002
p = 0.072
(<250nts)
(<40nts)

Figure 3-3. Association between age of onset of schizophrenia and PFC deficits in miR-30b levels. Graphs showing associations between age of onset of disease (x-axis) and S/C miR-30b ratio (y-axis), based on qRT-PCR using either <200nts enriched RNA samples (A-C) or <40nts enriched RNA samples (D). Female samples are represented with black circles and male samples with gray circles. (A) Association between age of onset and S/C miR-30b ratio for the 30 matched pairs. Notice inverted U shape. (B) Association between age of onset and S/C miR-30b ratio for subjects with age of onset less than 25 years. (C) Association between age of onset and S/C miR-30b ratio for subjects with age of onset more than 25 years. Notice significant positive correlation. (D) Association between age of onset and S/C miR-30b ratio measured in PAGE purified <40nts RNA samples for female matched pairs only. Notice the strong positive correlation. r = Pearson's correlation coefficient.

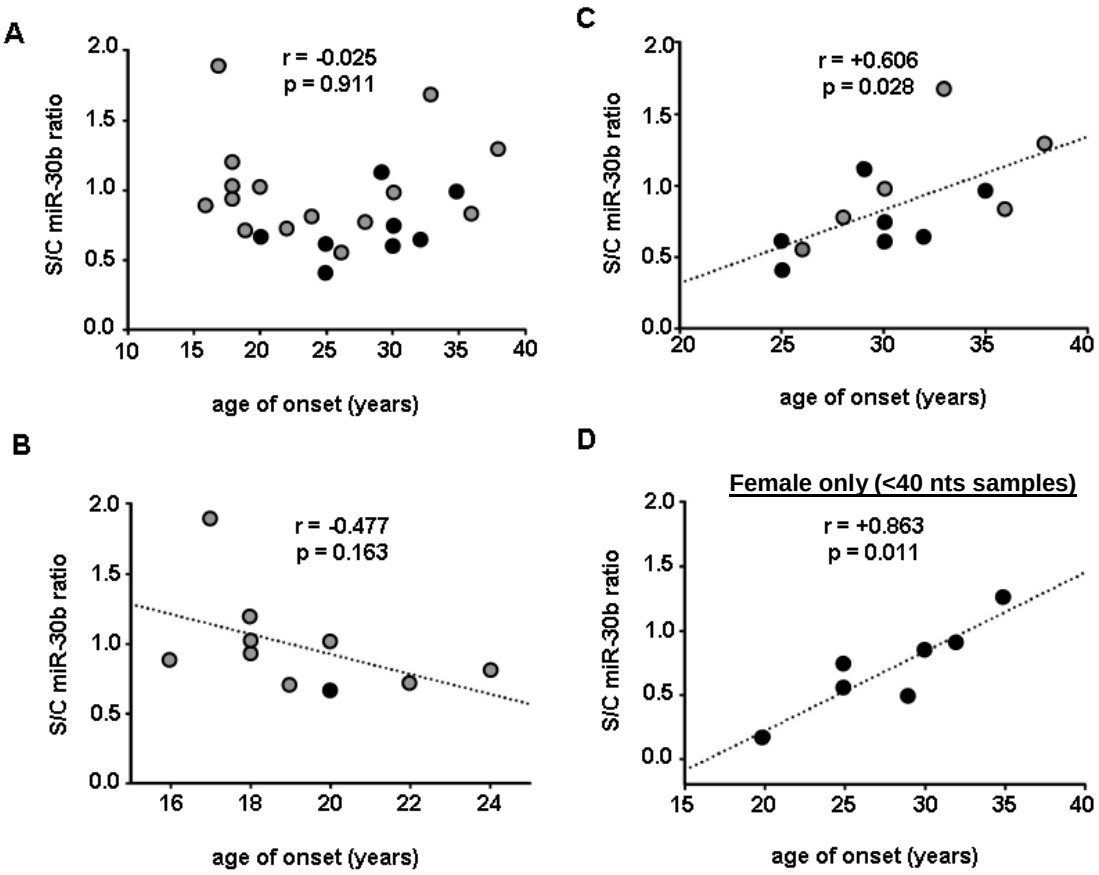

A
r = -0.025
p = 0.911
S/C miR-30b ratio
age of onset (years)
C
r = +0.606
p = 0.028
S/C miR-30b ratio
age of onset (years)
B
r = -0.477
p = 0.163
S/C miR-30b ratio
age of onset (years)
D
Female only (<40 nts samples)
r = +0.863
p = 0.011
S/C miR-30b ratio
age of onset (years)

Figure 3-4. **Gender - dimorphic expression of miR-30b in mouse frontal cortex.** Graph showing mean ± SEM of miR-30b levels (based on qRT-PCR and normalized to mouse snoRNA202). Notice the higher levels of miR-30b in female (F, white bars – N=16) compared to male (M, gray bars – N=6) frontal cortex. Another brain enriched miRNA (miR-100) is shown as a control, whereas a trend for higher expression in female mouse frontal cortex for X-chromosome encoded miR-222 is also seen. P values shown are calculated based on one tailed t-test; asterisk denotes statistical significance.

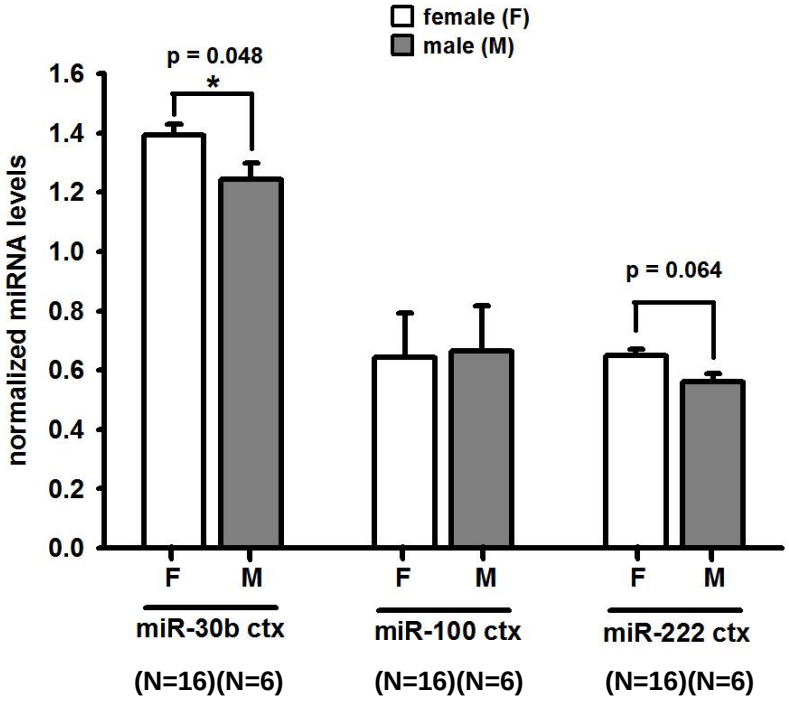
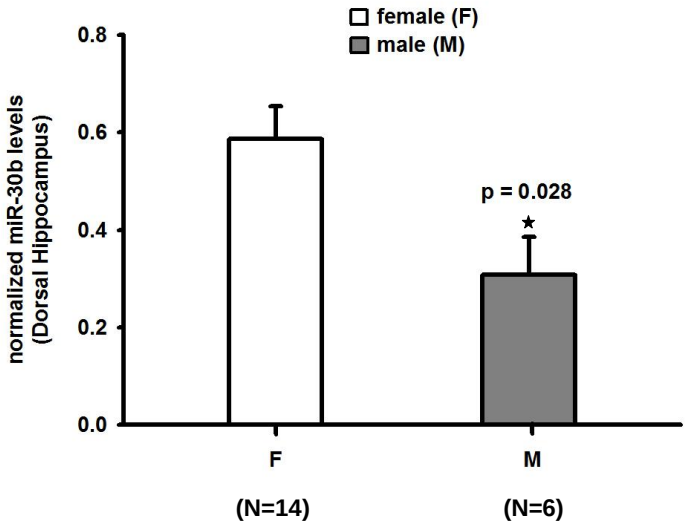
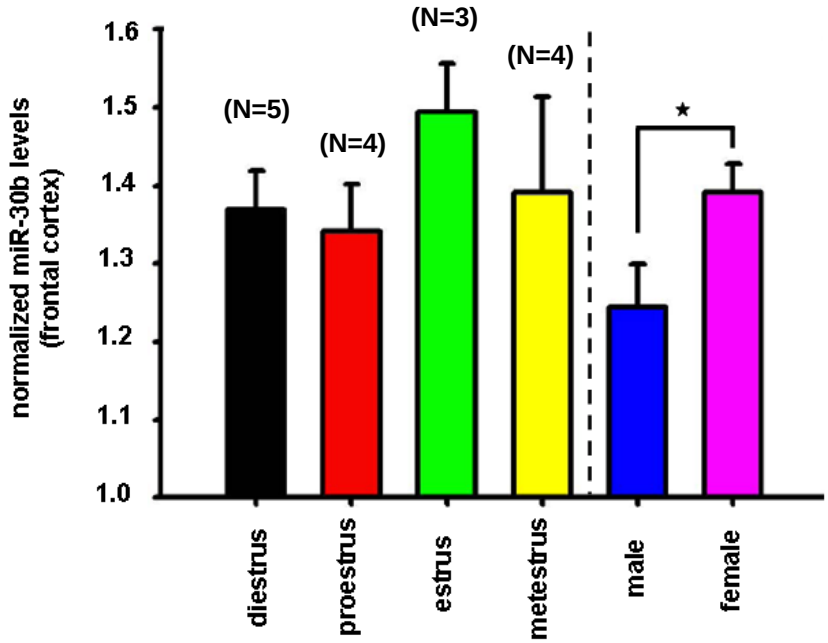

female (F)
male (M)
p = 0.048
*
p = 0.064
normalized miRNA levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
F
M
F
M
F
M
miR-30b ctx
(N=16)(N=6)
miR-100 ctx
(N=16)(N=6)
miR-222 ctx
(N=16)(N=6)

Figure 3-5. **Pronounced gender dimorphism in miR-30b expression in mouse dorsal hippocampus.** Graph showing mean ± SEM of miR-30b levels (based on qRT-PCR and normalized to mouse snoRNA202). Notice an approximately 2 fold higher expression of miR-30b in female (F, white bars – N=14) compared to male (M, gray bars – N=6) dorsal hippocampus. P values shown are calculated based on Wilcoxon Signed Rank Test; asterisk denotes statistical significance.

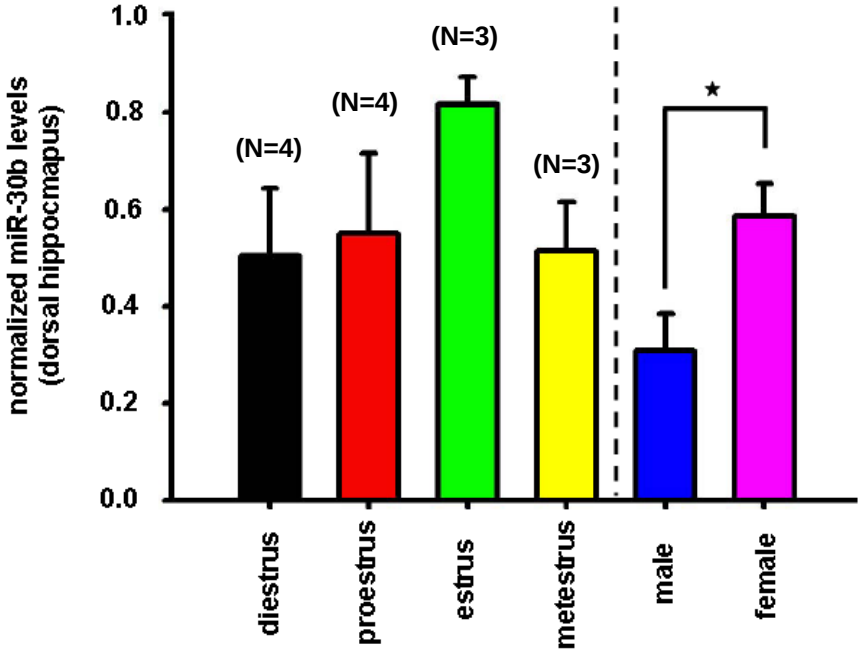

female (F)
male (M)
normalized miR-30b levels
(Dorsal Hippocampus)
0.8
0.6
0.4
0.2
0.0
p = 0.028
★
F
M
(N=14)
(N=6)

Supplemental Figure 3-1. Changes in miR-30b expression in frontal cortex and dorsal hippocampus during different stages of estrus cycle. Graph showing mean ± SEM of miR-30b levels (based on qRT-PCR and normalized to mouse snoRNA202) during the four different estrus cycle stages (diestrus, proestrus, estrus, metestrus as determined by vaginal smear and shown on graph as black, red, green and yellow bars respectively) in frontal cortex and dorsal hippocampus. Numbers of samples per group (N) are indicated. Expression of miR-30b in males and females are also shown for comparison (blue bar and pink bar respectively). Asterisk denotes statistical significant difference between male and female miR-30b expression in frontal cortex and dorsal hippocampus based on one-tailed t-test and Wilcoxon Signed Rank test, respectively (data reproduced from Figures 3.4 and 3.5).

A

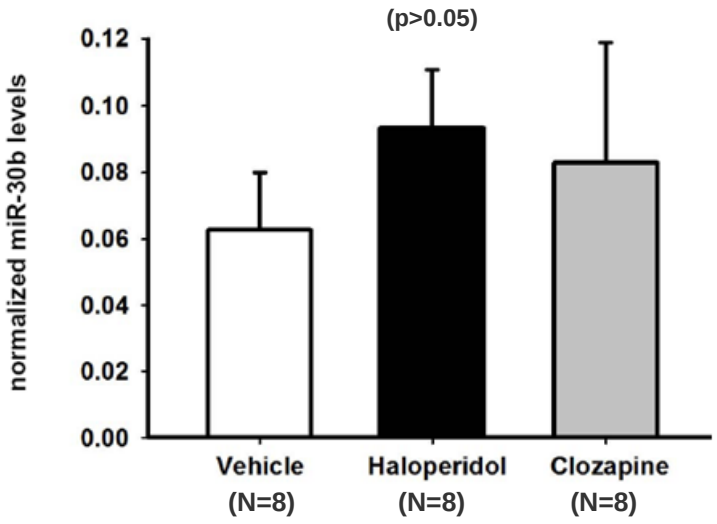

normalized miR-30b levels
(frontal cortex)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
(N=5)
(N=4)
(N=3)
(N=4)
★
diestrus
proestrus
estrus
metestrus
male
female

B


normalized miR-30b levels
(dorsal hippocmapus)
1.0
0.8
0.6
0.4
0.2
0.0
(N=4)
(N=4)
(N=3)
(N=3)
★
diestrus
proestrus
estrus
metestrus
male
female

Supplemental Figure 3-2. **Potential effects of chronic antipsychotic treatment on miR-30b expression in mouse cortex and human prefrontal cortex.** (A) Graph represents mean ± SE based on qRT-PCR results for miR-30b (normalized to 5S rRNA) after chronic treatment (N=8 for each group - see also methods) with Haloperidol and Clozapine. ANOVA revealed no significant differences ($p>0.05$). (B) Table showing mean ± SE and number (N) of S/C miR-30b ratios for unmedicated cases (N=5), cases treated with typical antipsychotics (N=23) and cases treated with atypical antipsychotics (N=2).

A



B

Groups	Mean +/- SEM (N)
Unmedicated cases	0.678 +/- 0.125 (N=5)
Typical Antipsychotics	0.948 +/- 0.086 (N=21)
Atypical Antipsychotics	1.109 +/- 0.087 (N=2)

CHAPTER V – GENERAL DISCUSSION

The findings discussed in this dissertation provide the first evidence that miRNAs act as inhibitors of BDNF during the maturation and aging of human prefrontal cortex and can contribute to the variability of the changes in BDNF protein levels and BDNF-dependent GABAergic gene expression in schizophrenia. Furthermore, we show that a subset of BDNF-related miRNAs display a distinct laminar and cellular specificity in their expression in human prefrontal cortex, which is not observed in mouse cortex. In addition, we show for the first time that one of these miRNAs, miR-30b, is expressed in higher levels in female mouse brain and is reduced in female subjects with schizophrenia. Furthermore, our results suggest that BDNF-related miRNAs inhibit BDNF at a posttranscriptional level. Our functional assays revealed that most of the BDNF-related miRNAs have a modest contribution to the inhibition of BDNF translation, with miR-30a-5p and miR-195 having a significant effect. However, the robust inverse correlation of the group of BDNF-related miRNAs to BDNF protein levels after late

adolescence raises the possibility of a synergistic effect of multiple miRNAs for the control of this important neurotrophin.

The intriguing cellular, laminar and developmental specific pattern for miR-30a that was described in our first project, suggests that miRNAs involved in the regulation of important neuronal genes, such as BDNF, might participate in a more complex spatio-temporal control of gene expression in human brain. In the case of BDNF for example, this might ensure that different cellular populations or cortical layers express different levels of BDNF protein, despite having comparable BDNF mRNA expression. This provides an additional posttranscriptional control in the spatial resolution of BDNF, which might have important implications due to BDNF's multiple effects on neuronal growth, survival, differentiation and neuronal plasticity (Marty et al., 1997; Murer et al., 2001; Gorski et al., 2003; Binder et al., 2004; Chan et al., 2008). In a similar note, a differential miRNA-mediated posttranscriptional regulation of BDNF during development, maturation and aging of human PFC, adds a new layer of temporal control of BDNF expression, which might be a critical parameter for ensuring the

smoothness and accuracy of the plethora of neurotrophin-related molecular changes that take place at specific time-points in human PFC.

The findings described in chapter 3 (Mellios et al., 2008b) suggest that prefrontal changes in miR-195 levels are inversely correlated to disease-related changes in BDNF protein levels, which in turn are associated with NPY and SST mRNA changes. This appears initially to be counter-intuitive since previous postmortem studies suggest a reduction in BDNF, NPY and SST expression and a trend for decrease in miR-195 levels (Weickert et al., 2003; Hashimoto et al., 2008; Perkins et al., 2007). In addition, our study also found a reduction in miR-195 but only in a subset of subjects with schizophrenia and no significant changes in BDNF protein levels. However, the only consistent findings of a reduction in BDNF expression are at the level of mRNA (Weickert et al., 2003; Hashimoto et al., 2005), and protein levels have been shown to be either increased, or decreased or not changed (Durany et al., 2001; Weickert et al., 2003; Takahashi et al., 2000). Furthermore, the study that showed a reduction in BDNF protein levels in schizophrenia (Weickert et al., 2003) used western blotting and analyzed only mature BDNF protein levels, whereas the study that

showed an increase in BDNF protein levels used ELISA (Durany et al., 2001), which is expected to detect both precursor and mature BDNF protein. It is known that BDNF precursor (pro-BDNF) is cleaved to generate the mature protein (Mowla et al., 2001; Murer et al., 2001; Fayard et al., 2005). Because the immediate product of BDNF translation is BDNF precursor protein, we chose to detect BDNF protein in our study through ELISA, so as to have a better estimate of total translation, and by comparison miRNA-mediated translational inhibition. Therefore, apart from any potential demographic and technical confounds that are common to different postmortem cohorts, it is possible that although BDNF mRNA is reduced in the PFC of subjects with schizophrenia, miR-195 deficits in a subset of subjects could alleviate miR-195 mediated translational inhibition of BDNF mRNA, so that precursor BDNF protein levels are not overall significantly altered. Subsequently, miRNA-independent posttranscriptional mechanisms related to the cleavage of BDNF precursor protein (Mowla et al., 2001; Murer et al., 2001; Fayard et al., 2005) might potentially further influence the disease-related mature BDNF levels in a subset of cases. The latter in conjunction with BDNF-independent mechanisms could then allow for the bigger percentage of

subjects with schizophrenia that display reduced NPY and SST mRNA levels. Furthermore, due to the moderate effect of miR-195 mediated inhibition of BDNF translation the cases that do have reduced miR-195 levels (a subset only), might not display a significant increase in BDNF protein levels, but just a shift in BDNF protein expression from a reduced to a similar to normal expression. It is, therefore, possible that miR-195 changes are a compensatory regulatory mechanism aiming at normalizing BDNF protein levels, which potentially takes place in some subjects diagnosed with schizophrenia, creating thus an increased variability in the disease-related changes in BDNF protein levels. In partial agreement with this hypothesis that changes in miR-195 levels in human PFC of subjects with schizophrenia might explain part of the discrepancy and variability in disease-related changes in BDNF protein levels reported in previous studies is the finding that miRNAs in general are important determinants of the variability in human cortical gene expression (Zhang and Su, 2008).

Combining the results presented in Chapters 2-4, we could conclude that the biological roles of the three miRNAs that were the main focus of the three studies (miR-30a, miR-195 and miR-30b) are quite distinct. Based on our data,

the most important function for miR-30a seems to be the fine-tuning of BDNF protein levels during maturation and aging of normal human PFC, with no significant impact on BDNF protein changes that occur in subjects with schizophrenia. On the other hand, miR-195 appears to have a pronounced effect on regulating the disease-related variability in BDNF protein levels and to have a limited impact on BDNF protein levels during development. Last but not least, miR-30b, which did not show any significant inhibitory effect on BDNF translation, is of unique importance since it is the first miRNA found to display a gender dimorphic expression in the mammalian brain and to be specifically reduced in female subjects with schizophrenia. Interestingly, according to computational analysis (Lewis et al., 2003; Grimson et al., 2007), miR-30b is predicted to target multiples genes linked to the pathophysiology of schizophrenia other than BDNF, such as NCAM1 (Barbeau et al., 1995; Sullivan et al., 2007), NR4A2 (Rojas et al., 2007), GRM3 (Egan et al., 2004) and GRM5 (Devon et al., 2001).

One limitation of our study presented at chapter 2 (Mellios et al., 2008a) was that measurements for all BDNF-related miRNAs were not repeated in PAGE-purified <40nts RNA samples due to limited availability of samples from

embryonic and young brains. However, in our last two studies we found that for some miRNAs, such as miR-30b, there is a very good correlation between the levels measured using <200nts and <40nts samples (Mellios et al., 2008b), whereas for others such as miR-195 this correlation is weaker (data not shown). Furthermore, because the inverse correlation between BDNF protein and miR-195 during late maturation and aging of human PFC was not significant as was for miR-30a-5p, and because miR-30a was expressed in pyramidal neurons that synthesize BDNF (Mellios et al., 2008a), we decided to test only miR-30a in neuronal cultures. However, given the inverse correlation between the disease-related changes in BDNF protein and miR-195 in subjects diagnosed with schizophrenia (Mellios et al., 2008b), future studies might be needed to address the effect of miR-195 - mediated translational inhibition on BDNF in neuronal cultures. In that case, it would be of interest to measure potential changes in cellular morphology or synaptic plasticity as a result of neuronal overexpression of miR-195 and mir-30a, so as to address the physiological importance of these BDNF-related miRNAs.

Furthermore, it has to be noted that schizophrenia is a very heterogeneous disorder and the different disease subtypes that are put in one cohort and examined together might not necessarily be a result of the same pathogenic mechanism. In addition, the progressive character of the disease and the different age of onset in males and females create an additional confounding factor, which even when controlled partially with match pairing, might lead to significantly different results in the concentrations of molecules studied. It is, therefore, of great importance that future studies using postmortem cohorts take into account gender, disease subtype, brain hemisphere, anatomical subregions within PFC examined and tissue quality parameters when attempting to uncover molecular alterations in schizophrenia.

Despite the fact that our studies presented in chapters 2 and 3 focused only on miRNAs that could target BDNF, it is possible that other neurotrophins or neurotrophin receptors are under the control of miRNAs. Indeed one study has already shown that miRNAs regulate the levels of the neurotrophin receptor tropomyosin-related kinase C in human neuroblastoma cells (Laneve, et al., 2007). However, computational analysis of the 3'UTR of neurotrophins 3 and 4

reveals only a limited number of conserved miRNA target sites (Lewis et al., 2003), which reduces the possibility of these two neurotrophins being targeted by the miRNA machinery.

At this point I would like to discuss the future perspectives of miRNA research on elucidating important molecular mechanisms of mammalian and especially human brain, which might prove to be instrumental for understanding the pathophysiology of neuropsychiatric disease. As mentioned in the introduction of this dissertation a plethora of studies including those described here have provided links between miRNAs and schizophrenia (Perkins et al., 2007; Beveridge et al., 2008, Mellios et al, 2008b), Alzheimer's disease (Wang et al., 2008b), Tourette's Syndrome (Abelson et al., 2005) and Parkinson's disease (Kim et al., 2007; Wang et al., 2008a). The fact that many neuropsychiatric disorders such as schizophrenia have tens or even hundreds of protein coding genes being indirectly linked to their pathophysiology, but with each one having a very small contribution overall, opens a window for potential novel regulatory elements that might regulate multiple genes and potentially multiple pathways. One candidate for such a "molecular multitasking" function is the family of miRNA

molecules, each of which is estimated to target multiple protein coding genes and biological pathways. It is therefore not accidental that disruption of miRNA processing in mouse models results to phenotypes that are reminiscent of neurological and psychiatric diseases (Kim et al., 2007; Cuellar et al., 2008; Stark et al., 2008, Davis et al., 2008). However, it has to be noted that disruption of pri-miRNA processing in mice heterozygotic for DGCR8 influences the levels of a subset only of mature miRNAs (Stark et al., 2008), which contradicts the initially hypothesized role of DGCR8 as a RNA binding protein that participates in the processing of all miRNAs. This is of importance in the light of the link between DGCR8 locus and schizophrenia in subjects with DiGeorge microdeletion syndrome (Murphy et al., 1999; Gothelf et al., 2007). Future studies are needed aiming at elucidating this link by determining which miRNAs are responsive to DGCR8 deficiency and by examining the potential effect of antipsychotics on miRNA processing.

On the other hand, it has to be noted that any alteration in miRNA expression in any given disorder, even if verified in multiple disease cohorts, should not be translated as a proof of this miRNA participating in the

pathogenesis of the disease. Given the refined spatio-temporal regulation of miRNA expression in human brain as described in our study (Mellios et al., 2008a), it is possible that aberrant brain developmental or maturational deficits that occur as part of the pathogenesis of the disease, could disrupt normal miRNA expression. In addition, it is possible that disease-related changes in gene expression including miRNAs could be a result of compensatory mechanisms aiming at ameliorating the disrupted biological pathways. However, even in the case that a miRNA “signature” characteristic of a disease is just an epiphenomenon, there is potential for such a molecular marker to be used for diagnosis and even prognosis of the disease, an application which has already been reported for other diseases and especially cancer (Mitchell et al., 2008; Wang et al., 2009; Jackson, 2009). In the case of miR-30 family miRNA, there is evidence that they are among the miRNAs that are found in detectable levels in the blood (Fan et al., 2008; Mitchell et al., 2008), which warrants further studies aiming at determining if blood or serum levels of miR-30 family miRNAs reflect their expression in the brain. Furthermore, endogenous miRNAs seem to be very resistant to blood RNases, and they are not significantly influenced by storage,

temperature and pH changes of tissue material (Chen et al., 2008; Szafranska et al., 2008), which would make them ideal as biomarkers.

Another intriguing potential that needs to be further investigated is the possibility that the cellular, laminar and developmental specific expression of certain miRNAs in the human brain might be a key aspect of human brain evolution. It has been suggested that non-coding RNA sequences differ more that coding ones between humans and other primates (Prabhakar et al., 2006; Beniaminov et al., 2008). It is therefore, very tempting to speculate that the regional and temporal specific fine-tuning of multiple genes needed to start, maintain or terminate specific events during brain development and maturation, could be one of the parameters contributing to the more sophisticated functions of the human brain.

Concluding, in our attempts to shed light to new molecular regulators of brain function and disease, we uncovered that miRNAs are not only expressed in an orchestrated manner in the human brain, but also regulate important molecular components for brain development and maturation that have implications for psychiatric disease. Although the studies presented in this

dissertation have added only a very small piece to the puzzle of small RNA function and brain disease, we are optimistic that subsequent work using in vivo animal experimental approaches and new technologies such us sequencing of small RNA, will uncover exciting aspects of miRNA function pertaining to brain function and disease.

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