Published in final edited form as:

Neurobiol Dis. 2015 May ; 77: 228–237. doi:10.1016/j.nbd.2015.03.004.

The *BDNF Val66Met* Variant Affects Gene Expression through *miR-146b*

Pei-Ken Hsu^{a,#}, Bin Xu^{b,#}, Jun Mukai^a, Maria Karayiorgou, and Joseph A. Gogos^{a,c}

^aDepartment of Physiology and Cellular Biophysics, Columbia University, New York, NY, USA

^bDepartment of Psychiatry, Columbia University, New York, NY, USA

^cDepartment of Neuroscience, Columbia University, New York, NY, USA

Abstract

Variation in gene expression is an important mechanism underlying susceptibility to complex disease and traits. Single nucleotide polymorphisms (SNPs) account for a substantial portion of the total detected genetic variation in gene expression but how exactly variants acting *in trans* modulate gene expression and disease susceptibility remains largely unknown. The *BDNF Val66Met* SNP has been associated with a number of psychiatric disorders such as depression, anxiety disorders, schizophrenia and related traits. Using global microRNA expression profiling in hippocampus of humanized *BDNF Val66Met* knock-in mice we showed that this variant results in dysregulation of at least one microRNA, which in turn affects downstream target genes. Specifically, we show that reduced levels of miR-146b (mir146b), lead to increased *Per1* and *Npas4* mRNA levels and increased Irak1 protein levels *in vitro* and are associated with similar changes in the hippocampus of *hBDNF^{Met/Met}* mice. Our findings highlight *trans* effects of common variants on microRNA-mediated gene expression as an integral part of the genetic architecture of complex disorders and traits.

Keywords

BDNF; Val66Met; microRNA; knock-in mice

Corresponding authors: Dr. Maria Karayiorgou, Department of Psychiatry, Columbia University, 1051 Riverside Drive, New York, New York 10032, USA, mk2758@columbia.edu, Dr. Joseph A. Gogos, Department of Physiology and Cellular Biophysics, Columbia University, 630 West 168th Street, New York, New York 10032, USA, jag90@columbia.edu.

[#]These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

P-KH, BX, JAG designed the research; P-KH, BX performed the experiments and analyzed the data; JM contributed to the generation of the mouse strains; JAG supervised experiments and data analysis; P-KH, BX, MK, JAG wrote the paper.

Accession Number

The raw microarray data reported in this paper have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus under accession number GSE44817.

Introduction

Genetic and genomic variability is intimately linked to differential disease risk among individuals. Gene expression can be modified by various genetic variants ranging from large structural variants (such as CNVs) to single nucleotide polymorphisms (SNPs). It is estimated that SNPs and CNVs capture 84% and 18%, respectively, of the total detected genetic variation in gene expression (Stranger et al., 2007). Such genetic variants affecting gene expression are referred to as expression quantitative trait loci (eQTL). eQTL can affect transcription either *in trans* (by affecting expression at distally located genomic loci, including ones in different chromosomes) or *in cis* (by affecting expression of genomic loci at their immediate vicinity). While the mechanism by which *cis*-acting variants affect gene expression is well established, how exactly variants acting *in trans* modulate gene expression and contribute to disease susceptibility remains under investigation (Cookson et al., 2009). microRNAs (miRNAs) (Bartel, 2004) bind to their target mRNAs (Lewis et al., 2003) and play a fundamental role in regulating gene expression primarily through post-transcriptional gene silencing via mRNA degradation or translational repression. As such, miRNAs could play an important role in mediating *trans*-acting effects on gene expression. In that respect, the potential of miRNAs to regulate expression of multiple genes could be an important component of the genetic architecture of complex disorders and traits. We have previously shown that 22q11.2 microdeletions, rare but recurrent *de novo* CNVs that predispose to cognitive dysfunction and schizophrenia mediate their effects at least in part due to pervasive miRNA-dependent effects on gene expression (Ambros et al., 2003; Stark et al., 2008; Xu et al., 2013). This finding established that miRNA-mediated *trans* effects on gene expression as an integral part of the pathogenesis of psychiatric and cognitive disorders (Xu et al., 2012). However, whether the contribution of miRNAs to the genetic etiology of complex disorders extends beyond rare structural mutations to common risk variants remain unknown. Addressing this issue is a key step towards obtaining a comprehensive view of the role that miRNAs play on mediating the *trans* regulatory effects of various rare and common disease-predisposing genetic variants.

To investigate whether miRNAs contribute to the *trans* regulatory effects of common genetic variants, we took advantage of a common SNP at codon 66 in the pro-domain of the *Brain-Derived Neurotrophic Factor* (*BDNF*) gene, which results in substitution of a Valine by a Methionine (Val66Met), leading to disrupted transportation of both the mRNA and protein to neuronal terminals and reduced activity-dependent release of BDNF protein both *in vitro* and *in vivo* (Chen et al., 2004; Chiaruttini et al., 2009; Egan et al., 2003). *Val66Met* SNP is a human-specific common genetic variant (Tettamanti et al., 2010). The allelic frequency of *BDNF^{Met}* is ~20–30% in populations of European origin but ranges from 0% in some populations of African origin to more than 50% in Asian populations (Petryshen et al., 2010). *Val66Met* SNP has been associated with reduced volume of hippocampal and prefrontal cortical grey matter and altered performance in memory tasks (Egan et al., 2003; Hariri et al., 2003; Rybakowski et al., 2006). Moreover, there is suggestive evidence that *Val66Met* modulates risk of depression (Verhagen et al., 2010), anxiety disorders (Tocchetto et al., 2011) and schizophrenia (Neves-Pereira et al., 2005). Studies in *in vitro* neuronal cultures have shown a role of BDNF in regulating miRNA levels (Fiore et al., 2009; Huang

et al., 2012; Vo et al., 2005), suggesting that the effects of natural variation at the *BDNF* locus may be mediated via changes in miRNA levels. However, whether BDNF affects miRNA expression *in vivo* and most importantly, whether *BDNF Val66Met* SNP modulates risk of associated diseases and traits via miRNAs remains unknown. In this study we begin to address this question by utilizing a mouse model of humanized *BDNF Val66Met* variant.

Results

We have previously generated two mouse strains where the mouse *Bdnf* coding sequence was substituted by the corresponding human *BDNF* sequence carrying either a *Met* (*hBDNF^{Met}*) or a *Val* (*hBDNF^{Val}*) allele (Cao et al., 2007). To determine the impact of the humanized BDNF variant upon miRNA gene expression in *hBDNF^{Met/Met}* and *hBDNF^{Val/Val}* mouse model, we conducted a miRNA microarray-based expression profiling and identified 23 miRNAs with significantly altered expression (FDR-corrected *P*-value < 0.05) (Figure 1A and Table 1, **see Experimental methods**). Thirteen of these miRNAs were significantly upregulated in *hBDNF^{Met/Met}* mice, while 10 of them were significantly downregulated in *hBDNF^{Met/Met}* mice. Expression changes (fold change, FC) ranged from 1.29 to 0.79, except for miR-700 (FC = 1.66).

We followed up expression changes of the top three miRNAs (miR-146b, miR-700 and miR-337-3p) with absolute FC > 1.2 and FDR-corrected *P*-value < 0.005 (Figure 1A). We verified the downregulation of miR-146b and miR-337-3p by quantitative real-time PCR (qRT-PCR) in adult HPC. As compared with levels in *hBDNF^{Val/Val}* animals, miR-146b was decreased by 8% (*P* = 0.20) and 19% (*P* < 0.01) in *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* animals respectively, and miR-337-3p was decreased by 19% (*P* < 0.01) and 10% (*P* = 0.06) in *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* animals respectively (Figure 1B). qRT-PCR did not confirm the significant change in miR-700 levels in the microarray data (FC = 1.08, *P* = 0.48, *hBDNF^{Met/Met}* versus *hBDNF^{Val/Val}*) (Supplementary Figure 1). It is worth noting that both miR-146 and miR-337-3p are among the top 20 most enriched miRNAs in synaptic compartments of adult mouse forebrain, suggesting they control synaptic-related functions (Lugli et al., 2008). The expression pattern and level of miR-700 during brain development remains unknown. Since validation was attempted for only 3 of the microRNAs in Table 2, the full extent of microRNA dysregulation due to *BDNF Val66Met* SNP remains to be determined in future experiments.

We speculated that if reduced expression levels of miR-146b and miR-337-3p were due to reduction in regulated BDNF release in these animals, their expression would be induced by BDNF. To test this hypothesis, we applied physiological levels of BDNF to acute hippocampal slice preparations from wildtype animals and monitored expression levels of miR-146b and miR-337-3p following 2 hours of incubation. We found that acute BDNF stimulation increased the levels of miR-146b by 22% (*P* = 0.01). We also observed a non-significant increase in the levels of miR-337-3p (21%, *P* = 0.11) (Figure 1C). These experiments demonstrated that expression of at least miR-146b is likely to be under the direct control of BDNF and could be altered in response to reduction in regulated BDNF release.

We further conducted a functional enrichment analysis using the DAVID functional annotation tool upon the potential targets of these top altered miRNAs (FDR $p < 0.005$) as predicted by TargetScan. Among the six miRNAs analyzed, the number of predicted miR-146b targets is relatively modest but they show a highly significant enrichment of the GO term “neuron differentiation”, which is consistent with the well-established neurotrophic effects of BDNF (Table 2). In contrast, all other miRNA targets were either enriched in broad GO terms (such as regulation of transcription) or did not show any significant enrichment (Supplementary Data Sheet 1). Notably, miR-146b was initially isolated from the HPC (He et al., 2007) and was found to represent one of the most abundant miRNA in mouse HPC. In addition, miR-146b is also downregulated in a *Mecp2*-null mouse model of the Rett syndrome, which presents BDNF signaling abnormalities (Urduingio et al., 2010). Collectively, the evidence outlined above points to miR-146b as a good candidate BDNF-modulated miRNA that deserves further analysis.

To determine whether the observed miRNA dysregulation is sufficient to affect expression of downstream targets, we sought miR-146b targets whose expression levels are in turn altered by *Val66Met* SNP. Luciferase reporter clones of longest 3'UTRs of putative miR-146b targets (Table 3) were generated and the repressive effects of pre-mir-146b mimic on these clones were measured in luciferase reporter assays using N18 cells, a mouse neuroblastoma cell line. Luciferase expression from 3'UTR constructs of *Irak1*, *Traf6*, *Per1*, *Stx3*, *Syt1*, *Kctd15*, *Sort1*, *Dlgap1*, *Npas4* and *Lin28A* was significant downregulated ($P < 0.05$) (Figure 2A) by pre-mir-146b, as compared to a pre-scramble control. The repression of luciferase expression by pre-miR-146b was especially pronounced on the 3'UTR of *Irak1* (59%, $P < 0.01$), *Traf6* (81%, $P < 0.01$), *Per1* (54%, $P < 0.01$), *Lin28A* (55%, $P < 0.01$), *Npas4* (44%, $P < 0.01$), while constructs with 3'UTR of other candidate targets were modestly repressed (Figure 2A). The repressive effects of pre-mir-146b on *Per1* 3'UTR reporters were antagonized by anti-miR-146b LNA oligonucleotides, demonstrating the specificity of the luciferase assay screen for miR-146b targets (Figure 3). Overall, miR-146b was able to repress *in vitro* expression of all predicted targets through their 3'UTR sequences.

As mammalian miRNAs inhibit target expression predominantly through decreasing mRNA levels rather than repressing translation (Guo et al., 2010), we hypothesized that most of the physiologically relevant target genes *in vivo* would also be upregulated at the transcript levels in *hBDNF^{Met/Met}* animals, which have lower levels of mir-146b in HPC. Therefore, we measured and compared the expression levels of the predicted candidates in *hBDNF^{Val}* and *hBDNF^{Met}* knock-in animals by qRT-PCR. Expression levels of only *Per1* and *Npas4* (out of 13 predicted targets with good amplification signals in qRT-PCR assays) were significant influenced by genotype (ANOVA, $P < 0.01$ for *Per1*; $P < 0.05$ for *Npas4*) and were upregulated in *hBDNF^{Val/Met}* (24% for *Per1* and 33% for *Npas4*) and *hBDNF^{Met/Met}* mice (28% for *Per1* and 65% for *Npas4*) as compared to *hBDNF^{Val/Val}* mice (Table 4, left panel and Figure 2C). As a control, we tested an equal number of genes without a miR-146b seed sequence but none showed significantly elevated expression in *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* mice (Table 4, right panel). Therefore, *Per1* and *Npas4* represent particularly sensitive targets whose modulation by the *Val66Met* SNP can be readily detected at both mRNA levels *in vivo* and protein levels *in vitro*.

To investigate if miR-146-mediated repression on *Per1* and *Npas4* expression is specific and operates directly via the target sites as predicted by TargetScan (Table 3), we engineered *Per1* and *Npas4* 3'UTR luciferase reporters carrying mutated miR-146b binding sites. *Per1* 3'UTR contains two cognate miR-146b binding sites at position 190–197 (an 8-mer site) and position 505–511 (a 7mer-1A site). 3 different *Per1* 3'UTR mutants (Mut1: Site 1 mutant; Mut2: Site 2 mutant; Mut1&2: Site 1 and 2 mutants), and a *Npas4* 3'UTR mutant (Mut) with the 7mer-m8 site abrogated were generated. Compared with Wt reporter, Mut1, Mut2 and Mut1&2 in *Per1* 3'UTR increased luciferase activity by 49% ($P < 0.01$), 26% ($P < 0.01$), and 58% ($P < 0.01$) respectively, while Mut in *Npas4* 3'UTR increased the luciferase activity by 29% ($P < 0.01$) (Figure 2B). Thus both miR-146b binding sites in *Per1* 3'UTR control miR-146b-mediated regulation on *Per1* expression, although the 8mer site seems to have a larger impact. The miR-146b binding site in *Npas4* 3'UTR similarly controls miR-146b-mediated repression of *Npas4* expression. Overall, we have identified *Per1* and *Npas4* as targets of mir-146b *in vivo*. Reduced miR-146b levels in animals carrying one or two *hBDNF^{Met}* alleles result in the corresponding rise in *Per1* and *Npas4* transcript levels.

Intriguingly two other target genes (*Traf6*, *Irak1*) which were strongly affected by pre-miR-146b in luciferase assays (Figure 2A) but whose mRNA levels were unaffected in *hBDNF^{Met/Met}* mice (Table 4) have been previously identified as targets of miR-146 in human monocytes (Taganov et al., 2006). It is likely that these targets are repressed by miR-146b translationally without any changes in their transcript levels (Table 4). Indeed, Western blot assays of protein extracts from the HPC of *hBDNF^{Val}* and *hBDNF^{Met}* knock-in mice (6 pairs of 8 week-old male mice, group-housed under standard rearing condition) showed increases of Irak1 in *hBDNF^{Val/Met}* (25%, $P > 0.05$) and *hBDNF^{Met/Met}* animals (59%, $P < 0.05$) as compared to *hBDNF^{Val/Val}* animals (Figure 2D). In contrast, Traf6 levels did not vary significantly with the genotype of the mice (ANOVA, $P > 0.05$) (Figure 2D). Thus, although we did not detect altered mRNA levels, Irak1 protein levels were significantly elevated in *hBDNF^{Met/Met}* mice likely due to reduced miR-146b-mediated translational repression.

It was recently reported that BDNF application can rapidly elevate *in vitro* expression of several miRNA biogenesis proteins, including Dicer1 and Lin28A, resulting in a general increase in mature miRNA levels but a decrease in levels of let-7 family miRNAs whose degradation is promoted by Lin28A-induced pre-miRNA uridylation (Huang et al., 2012). Derepression of let-7 family miRNA targets leads to increased expression of proteins such as GluA1 and Homer2 in response to BDNF. Altered expression of miR-146b and downstream targets could therefore be secondary to changes in levels of Dicer1, Lin28A or let-7 miRNA in *hBDNF^{Met/Met}* mice. To test whether *Val66Met* regulates protein levels of microRNA biogenesis components, we used Western blot analysis to monitor levels of Dicer1, Dgcr8, Lin28A and Ago2 in six pairs of 8 week-old male mice (grouped-housed under standard rearing condition) but did not observe any genotypic differences (ANOVA, $P > 0.05$) (Figure 4). In addition, expression levels of previously reported BDNF-induced targets, GluA1 and Limk1 (Huang et al., 2012; Schratt et al., 2006) as well as let-7 family miRNAs, let-7b and miR-98 were not altered in *hBDNF^{Met/Met}* mice (Supplementary Figure 2 and 3, Table 5). These results argue against a generic effect of *Val66Met* on miRNA biogenesis and possibly

suggest a more direct mode of action on the expression control of a subset of miRNAs including miR-146b (see Discussion).

Discussion

Our results clearly demonstrated that *BDNF Val66Met* is associated with altered expression of a specific subset of miRNAs and most importantly of their downstream targets. *Val66Met* affects miRNAs levels not through miRNA biogenesis (Huang et al., 2012) but more likely through modulating BDNF-dependent transcription factor binding in regulatory regions. Among the affected miRNAs, *miR-146b* and its modulated downstream targets, which are particularly enriched in neuronal genes, are likely to have substantial contribution to the biological effects of the *Val66Met* variant. Indeed, it is well established that BDNF induces the expression of neuronal genes through activation of MAPK pathway and Akt1/2 downstream of TrkB receptor (Lyons and West, 2011), which in turn leads to activation of CREB, MEF2 and NF- κ B transcription factors (Huang and Reichardt, 2003; Minichiello, 2009; Shalizi and Bonni, 2005; Yoshii and Constantine-Paton, 2010). Recent *in vitro* studies in neuronal cultures have shown that exogenous BDNF transcriptionally activates the expression of miRNAs via CREB and MEF2 activation (Fiore et al., 2009; Vo et al., 2005). Interestingly, it was shown that *miR-146* transcription is in part controlled by NF- κ B (Perry et al., 2009; Taganov et al., 2006).

We have identified *Per1* and *Npas4* as genuine targets of *mir-146b* *in vitro* and our *in vivo* studies strongly suggest that both of them are *mir-146b* targets *in vivo*, as well. Interestingly, *Per1* is a transcriptional repressor controlling circadian rhythm (Takahashi et al., 2008). Circadian and sleep disturbances are often associated with psychiatric disorder (Lamont et al., 2007). *PER1* expression is altered in postmortem brains of schizophrenia patients (Aston et al., 2004), while its homolog *PER3* is considered a candidate gene for bipolar disorder and schizophrenia (Mansour et al., 2006; Nievergelt et al., 2006). *Npas4* is homologous to *Npas2*, which is another core component of circadian regulation. *Npas4* is required for inhibitory synapse formation in HPC as well as for activity-dependent expression of neuronal genes (Lin et al., 2008; Ramamoorthi et al., 2011). It should be noted that *Npas4* directly promotes activity-dependent transcription of *Bdnf* (Pruunsild et al., 2011) and therefore part of the observed increase in *Npas4* levels in animals carrying one or two *hBDNF^{Met}* alleles may be compensatory in nature. We also identified *Irak1* as an additional target, modulated only at the protein level. *Irak1* is an essential component downstream of Toll-like and IL-1 receptor signaling (Gottipati et al., 2008). Activation of *Irak1* enhances NF- κ B-mediated transcription and leads to elevation of innate and pro-inflammatory responses in neuron and glial cells (Li et al., 2011). Whether the *Val66Met* variant increases the risk of inflammatory insult in the central nervous system remains to be determined. Independent of underlying mechanisms, our results suggest that *BDNF Val66Met* variant may mediate its effects on psychiatric and cognitive phenotypes in part due to miRNA-dependent effects on gene expression (Stark et al., 2008; Xu et al., 2013). Because the *Bdnf* gene and the *Val66Met* variant are located on mouse chromosome 2, while *Per1*, *Npas4* and *Irak1* are on chromosomes 11, 19 and X, respectively, this represents a typical trans eQTL effect mediated via microRNAs. Integrated analysis of genetic and expression data indicates that common variants at a large number of eQTLs are associated with differential expression

of target genes either *in cis* or *in trans*. The impact of eQTLs on miRNA expression has been investigated recently in various cell types including fibroblasts, glioblastoma, liver cells and hippocampal tissues (Borel et al., 2011; Dong et al., 2010; Parsons et al., 2012; Su et al., 2011). Approximately 5–20% of miRNAs investigated are under the influence of eQTLs with half of them under the control of *cis*- and half under the control of *trans*-eQTLs. Because each miRNA can potentially modulate multiple downstream targets, eQTL control over miRNA expression could underlie coordinated expression of networks of genes. Moreover, because miRNAs often target functionally connected genes (Tsang et al., 2010; Xu et al., 2013; Zhang et al., 2009) the cumulative impact of miRNA alterations due to disease-associated eQTL variants could result in considerable cellular dysfunction, which in turn may contribute substantially towards disease risk and clinical phenotype. In that context, our results also reveal opportunities for interaction between common eQTL variants and rare mutations (such as CNVs and point mutations) that affect either individual miRNA genes or biosynthesis and action of miRNAs (Stark et al., 2008; Xu et al., 2013; Xu et al., 2008).

It is interesting to note that although *miR-146b* was able to repress *in vitro* expression of all predicted targets through their 3'UTR sequences (as indicated by our luciferase assays), only three predicted targets (30%) showed expected alterations *in vivo*. There are several potential explanations for the discrepancies observed among predicted targets (such as Lin28A and Traf6), and results from *in vitro* and *in vivo* assays. Our *in vitro* assay indicated that while these and other predicted targets represent genuine targets of *miR-146b* that could be regulated by overexpression of *miR-146b*, the interaction between *miR-146b* and target genes under more physiological conditions likely involves additional levels of complexity. For example, the accessibility of the *miR-146b* binding site may be under the control of sequestering mechanisms involving mRNA binding proteins (Kedde and Agami, 2008). In addition, it is possible that the interaction between a specific miRNA target and the cognate miRNA is very sensitive to miRNA levels and altered by competition among different miRNA targets over a limited miRNA pool (Salmena et al., 2011; Seitz, 2009). Finally, the predicted targets of *miR-146b* may not be spatially and temporally registered with miR-146 (i.e co-expressed in the same cell type and developmental window). Our findings strongly suggest that considerable caution should be exercised when extrapolating results from predicted targets and *in vitro* assays to more physiological settings.

Overall, taken together with previous results on rare structural variants (Stark et al., 2008; Xu et al., 2013), our findings highlight miRNA-mediated *trans* effects on gene expression as an integral part of the genetic architecture of complex disorders and traits caused by common disease-predisposing genetic variants.

Experimental methods

Human *BDNF* knock-in mice

Generation of *hBDNF^{Met}* and *hBDNF^{Val}* knock-in mice has been described in detail previously (Cao et al., 2007). In short, we replaced a segment in mouse *Bdnf* coding region that carries either *Val* allele or *Met* allele and encodes all the 11 amino acids that differ between mouse and human protein. This genetic manipulation generated knock-in alleles

that express human *BDNF* genes controlled by endogenous mouse *Bdnf* regulatory elements. *hBDNF^{Val/Val}*, *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* littermates were from *hBDNF^{Val/Met} X hBDNF^{Val/Met}* cross and were genotyped as described (Cao et al., 2007). All animal procedures were conducted according to protocols approved by the IACUC established by Columbia University under federal and state regulations.

miRNA microarray expression analysis

Hippocampi were acutely dissected from 5 pairs of 8 week-old male mice, which were grouped-housed and reared under standard rearing conditions. Total RNA was isolated using the mirVana miRNA isolation kit (Ambion). RNA quality was assessed with Bioanalyzer (Agilent Technologies, Palo Alto, CA) and all RNAs had a RIN > 7.0. Small RNAs (<300 nt) were then isolated and processed for microarray analysis (LC Sciences). Briefly, purified small RNAs were labeled with Cy5 (*hBDNF^{Val/Val}*) or Cy3 (*hBDNF^{Met/Met}*) fluorescent dyes and hybridized to dual-channel microarray μ ParaFlo microfluidics chips (LC Sciences) containing 569 miRNA probes to mouse mature miRNAs. Each of the spotted detection probes consisted of a nucleotide sequence complementary to a specific miRNA sequence and a long non-nucleotide spacer that extended the specific sequence away from the chip surface. The miRNA probe sequences used were from the miRBase Sequence database version 10.1. We collected hybridization images using a GenePix 4000B laser scanner (Molecular Devices) and digitized them using Array-Pro image analysis software (Media Cybernetics). Raw data were imported in ArrayAssist 5.0 (Stratagene). The microarray data were corrected by removing spots with intensity equal to or below median background and then normalized with the LOWESS (locally weighted regression) method implemented in ArrayAssist 5.5 software (Stratagene). Differentiation analysis was conducted to determine the FDR *P*-value of each miRNA gene as described previously (Stark et al., 2008).

Quantitative RT-PCR analysis of mature miRNA and coding gene expression

Total RNA samples were extracted from the hippocampi that acutely dissected from 8 week-old male mice, which were grouped-housed and reared under standard rearing conditions, using mirVana miRNA Isolation Kit (Ambion) according to manufacturer's protocol. We treated 3 μ g of total RNA from each sample with DNA-free kit (Applied Biosystems). For RT of each mature miRNA, 100 ng of treatment RNA each sample was reverse transcribed using TaqMan Reverse Transcription Kit (Applied Biosystems) and RT primer provided in the individual TaqMan MicroRNA Assay (see below for catalog numbers). A *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene-specific RT primer (reverse primer from ABI # 4352339) was also included RT reaction. For RT of coding genes, the remaining DNase-treated RNA each sample was reverse transcribed using Random Primers (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed using ABI TaqMan method in a 7900 Sequence Detection System (Applied Biosystems). For each gene/miRNA, a duplex qPCR was performed using TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems) and custom designed primer and probe set, as well as primer and probe set for *GAPDH* (custom designed primer and probe set used in qPCR of coding genes, see below; ABI # 4352339 used in qPCR of miRNA). Quantification procedures were described previously (Stark et al., 2008). All statistical analyses were

conducted in MS-Excel using Student t-test on data from at least 8 biological repeats and 5 technical repeats.

TaqMan MicroRNA Assays were purchased from Applied Biosystems. MicroRNA assay name and ID: hsa-miR-146b: 001097; mmu-miR-337: 002532; mmu-miR-700: 001634; hsa-miR-98: 000577; has-let-7b: 002619. All PCR primers and probes for coding genes were designed at Primer3 web site (<http://frodo.wi.mit.edu/>) and purchased from Sigma Genosys (Sigma-Aldrich) and the sequences can be found in Supplementary Data Sheet 2, except for *Nptx2* (Mm00479438_m1, Applied Biosystems) and *Clec16a* (Mm00624340_m1, Applied Biosystems). All target gene probes were 5' FAM and 3' BHQTM-1 Dual labeled. Mouse *GAPDH* mRNA was used as the endogenous control. The custom made *GAPDH* gene probe was 5' JOETM and 3' BHQTM-1 dual labeled. All statistical analyses were conducted in MS-Excel using Student t-test on data from at least 8 biological repeats and 5 technical repeats.

BDNF-treatment of hippocampal slices

Hippocampal slices of 250- μ m-thick were prepared as described previously (Drew et al., 2011). Briefly, 250- μ m-thick horizontal brain sections were prepared on a vibratome (Leica VT1200S) in dissection solution (in mM: sucrose 195, NaCl 10, KCl 2.5, NaH₂PO₄ 1, NaHCO₃ 25, glucose 10, MgCl₂ 5, MgSO₄ 1, CaCl₂ 0.5) from brain of 8 week-old Wt C57Bl/6J mice. Hippocampal regions were promptly cut out from ~10 horizontal brain sections and then immediately transferred to an interface chamber and allowed to recover for 1 h at 31–32 °C. Slices were then transferred to another chamber and incubated for 2 hours with pre-oxygenated artificial cerebrospinal fluid (aCSF) (bubbled with 5% CO₂/95% O₂) that had the following composition (in mM): NaCl 124, KCl 2.5, NaH₂PO₄ 1, NaHCO₃ 25, Glucose 10, MgSO₄ 1, CaCl₂ 2. In the BDNF treatment group, the aCSF also contained 50 ng/ml of BDNF (#B3795, Sigma-Aldrich). Slices were removed immediately after 2 hour incubation and total RNAs from the BDNF or sham treated slices were extracted using TRIzol (Invitrogen), following manufacturer's instruction.

miRNA target prediction

39 genes were initially predicted by both TargetScan Mammal v.4.2 and PicTar (updated March 2007) miRNA target site prediction programs. Among them, 12 target candidates (*Irak1*, *Traf6*, *Per1*, *Stx3*, *Syt1*, *Cask*, *Robo1*, *Kctd15*, *Dlgap1*, *Gria3*, *Npas4*, *Lin28A*) were initially selected for further analysis due to their roles in neural development and/or plasticity (Table 1). We included in this list also *Sort1*, which encodes sortilin, a protein required for intracellular BDNF trafficking and activity-regulated release and has a miR-146b seed sequence in the 3'UTR of its mRNA. 3 additional targets predicted by miRanda only (Betel et al., 2008) (*Akt3*, *Srrd*, *Bsn*) were also tested because they are involved in important functions in neurotransmitter production (*Srrd*), postsynaptic density (*Bsn*) and TrkB signaling (*Akt3*), which are highly related to BDNF function. 16 predicted targets of miR-146b in total were included in this analysis. It is worth noting that most of these targets are also predicted by a more updated version of TargetScan v6.2 and miRanda (released: August 2010, updated: Nov 2010) and by additional programs – mirDB (Wang, 2008) (updated: April 2012) and microT v5.0 (Maragkakis et al., 2011). In fact, almost all candidates originally predicted by both TargetScan Mammal v.4.2 and PicTar are also

predicted by 3 or more of these 5 programs (TargetScanMouse v6.0, microT v5.0, PicTar, miRanda 11/2010, mirDB 04/2012). Overall, we have tested the expression of a group of highly probable miR-146b targets in *hBDNF^{Val}* and *hBDNF^{Met}* knock-in mice.

Luciferase assays

Dual-Luciferase® Reporter Assay System and psiCHECK2 luciferase reporter construct which contains a Renilla gene as the reporter and a firefly genes as the internal control were purchased from Promega. The longest 3'UTRs of predicted miR-146b targets (*Irak1*, *Tarf6*, *Per1*, *Stx3*, *Syt1*, *Kctd15*, *Sort1*, *Dlgap1*, *Npas4*, *Lin28A*) were cloned into *XhoI* and *NotI* sites of psiCHECK2. miR-146b binding site mutant clones of *Per1* and *Npas4* were generated by PCR-based mutagenesis and subcloning. *Per1* 3'UTR mutant clone: Site Mut1 sequence (starting from position 186 in 3'UTR): TccAAGTTCagA (lower case letters denote altered nucleotide). Site Mut2 sequence (starting from position 495): GctCCCAGGTGTTacaA. *Npas4* 3'UTR mutant clone: Site Mut sequence (starting from position 309 in 3'UTR): TccGCCAGTTaca. All the clones were verified by Sanger sequencing. Mutations are predicted by RNAhybrid (Rehmsmeier et al., 2004) to disrupt the binding of miR-146b at the seeds and secondary binding sites. Luciferase assays were performed as described previously (Xu et al., 2013). Briefly, N18, a neuroblastoma cell line, was cultured in 24 well plates to 70% confluency and then transfected with various psiCHECK2 reporter constructs (100 ng per well) together with pre-mir-146 mimic or pre-scramble control oligo (1 nM = 0.5 pmol). Luciferase assays were performed 24 hr posttransfection using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. The luciferase signals were measured with a Turner BioSystems 20/20n Single-Tube Luminometer. The intensity of Renilla signal was normalized to the intensity of firefly signal for each assay. Each condition involved at least 3 biological repeats and 2 technical repeats. To control for the non-specific effects of introducing plasmids with different 3'UTRs and oligos, we normalized the luciferase level of each gene to the baseline (plasmid without 3'UTR) to control for the effect of introducing plasmids with different 3'UTRs and then compared the effect of pre-146b mimic for each gene to its corresponding pre-scramble control to determine the specific effect of pre-146b on each gene. All statistical analyses of Luciferase assays were conducted in MS-Excel with a student t-test.

Functional enrichment analysis of conserved targets of miRNAs

Conserved miRNA targets were predicted and extracted using TargetScan (<http://www.targetscan.org/>) with mouse homolog of human species. All targets were imported into The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7, <http://david.abcc.ncifcrf.gov/>) for a functional annotation using its default settings (Huang da et al., 2009).

Western blot assays

HPC from 8-wk old mice were isolated and homogenized in ice-cold modified RIPA buffer containing 1% Triton X-100, 0.2 mM EDTA, 100 mM KCl and 20 mM Tris pH 8.0 and Proteinase inhibitor cocktail (Roche). Homogenates were centrifuged at $12,000 \times g$ at 4°C for 30 min. The supernatant was saved and the protein concentration in each sample was determined by DC Protein Assay (Bio-Rad). An aliquot of the supernatant equivalent to 50

µg (Dgcr8 and Dicer1 blots) or 20 µg (blots of all other protein) proteins were resolved on 4–12% polyacrylamide gel (Bio-Rad) and then transferred onto an ECF plus membrane (Amersham Biosciences) or Immobilon-FL membrane (Millipore). Antibody hybridization was performed as described previously. (Stark et al., 2008) Primary antibodies used were Traf6 (#597, MBL), 1:1000 dilution; Irak1 (D51G7, Cell Signaling), 1:1000 dilution; Dicer1 (N1767/7, NeuroMab), 1:500 dilution; Dgcr8 (10996-1-AP, proteintech), 1:1000 dilution; Lin28A (#3978, Cell Signaling), 1:1000 dilution; Ago2/Eif2C2 (10686-1-AP, proteintech), 1:500 dilution; GluA1/GluR1 (AB1504, Millipore), 1:400 dilution; Limk1 (#3842, Cell Signaling), 1:500 dilution; β-actin antibody (A5441, Sigma-Aldrich), 1:10000 dilution; α-tubulin antibody (T5168, Sigma-Aldrich), 1:100000 dilution. Horseradish peroxidase conjugated secondary antibodies (1:5000 dilution) were subsequently used for probing the primary antibodies. The washed membrane was incubated with HRP substrate (Western Lightning Chemiluminescence Reagent, PerkinElmer Life Sciences) for 1 min, and chemiluminescence images were obtained using Alpha imaging system. Protein bands were subjected to densitometric analysis with ImageQuant (Molecular Dynamics) (Traf6 and Irak1) or NIH Image J (blots of all other proteins). Statistical analyses were conducted in MS-Excel with a student t-test upon the data from 3 biological repeats.

Database linking

The URL for data presented herein is as follows:

microRNA Database (miRBase), <http://www.mirbase.org/>

TargetScan Mammal v.4.2, http://www.targetscan.org/vert_42/

TargetScanMouse v6.2, http://www.targetscan.org/mmu_61/

PicTar prediction in vertebrates, http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertibrate.cgi

microT v.5, <http://diana.cslab.ece.ntua.gr/micro-CDS/?r=search>

miRanda, <http://www.microrna.org/microrna/home.do>

mirDB, <http://mirdb.org/miRDB/>

Primer3, <http://frodo.wi.mit.edu/>

TFSEARCH v.1.3, <http://www.cbrc.jp/research/db/TFSEARCH.html>

ConSite, <http://asp.ii.uib.no:8090/cgi-bin/CONSITe/consite>

UCSC Genome Browser, assembly Dec. 2011 (GRCm38/mm10), <http://genome.ucsc.edu/cgi-bin/hgGateway>

UCSC Genome Browser, assembly Feb. 2006 (NCBI36/mm8), <http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=327728483&clade=mammal&org=Mouse&db=mm8>

Mouse miRNA promoter (Marson et al., 2008) <http://www.cell.com/supplemental/S0092-8674%2808%2900938-0>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Megan Sribour and Yan Sun for support with the maintenance of the mouse colony and technical assistance. We are grateful to past and current members of Karayiorgou and Gogos laboratories for their helpful discussion, input and support. This work was supported by a grant from US National Institute of Mental Health grants MH67068 (to M.K. and J.A.G.), MH077235 and MH97879 (to J.A.G.), and by grants from the March of Dimes Foundation and the McKnight Endowment Fund for Neuroscience (to M.K.); B.X. was supported in part by a National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award.

Abbreviations

BDNF	brain derived neurotrophic factor
SNPs	single nucleotide polymorphisms
miRNA	microRNA
eQTL	expression quantitative trait loci

References

- Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T. A uniform system for microRNA annotation. *Rna*. 2003; 9:277–279. [PubMed: 12592000]
- Aston C, Jiang L, Sokolov BP. Microarray analysis of postmortem temporal cortex from patients with schizophrenia. *Journal of neuroscience research*. 2004; 77:858–866. [PubMed: 15334603]
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116:281–297. [PubMed: 14744438]
- Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic acids research*. 2008; 36:D149–153. [PubMed: 18158296]
- Borel C, Deutsch S, Letourneau A, Migliavacca E, Montgomery SB, Dimas AS, Vejnar CE, Attar H, Gagnebin M, Gehrig C, Falconnet E, Dupre Y, Dermitzakis ET, Antonarakis SE. Identification of cis- and trans-regulatory variation modulating microRNA expression levels in human fibroblasts. *Genome research*. 2011; 21:68–73. [PubMed: 21147911]
- Cao L, Dhillia A, Mukai J, Blazeski R, Lodovichi C, Mason CA, Gogos JA. Genetic modulation of BDNF signaling affects the outcome of axonal competition in vivo. *Current biology : CB*. 2007; 17:911–921. [PubMed: 17493809]
- Chen ZY, Patel PD, Sant G, Meng CX, Teng KK, Hempstead BL, Lee FS. Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2004; 24:4401–4411. [PubMed: 15128854]
- Chiaruttini C, Vicario A, Li Z, Baj G, Braiuca P, Wu Y, Lee FS, Gardossi L, Baraban JM, Tongiorgi E. Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:16481–16486. [PubMed: 19805324]
- Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M. Mapping complex disease traits with global gene expression. *Nature reviews. Genetics*. 2009; 10:184–194.
- Dong H, Luo L, Hong S, Siu H, Xiao Y, Jin L, Chen R, Xiong M. Integrated analysis of mutations, miRNA and mRNA expression in glioblastoma. *BMC systems biology*. 2010; 4:163. [PubMed: 21114830]

- Drew LJ, Stark KL, Fenelon K, Karayiorgou M, Macdermott AB, Gogos JA. Evidence for altered hippocampal function in a mouse model of the human 22q11.2 microdeletion. *Molecular and cellular neurosciences*. 2011; 47:293–305. [PubMed: 21635953]
- Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*. 2003; 112:257–269. [PubMed: 12553913]
- Fiore R, Khudayberdiev S, Christensen M, Siegel G, Flavell SW, Kim TK, Greenberg ME, Schrott G. Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. *EMBO J*. 2009; 28:697–710. [PubMed: 19197241]
- Gottipati S, Rao NL, Fung-Leung WP. IRAK1: a critical signaling mediator of innate immunity. *Cellular signalling*. 2008; 20:269–276. [PubMed: 17890055]
- Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010; 466:835–840. [PubMed: 20703300]
- Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, Weinberger DR. Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003; 23:6690–6694. [PubMed: 12890761]
- He X, Zhang Q, Liu Y, Pan X. Cloning and identification of novel microRNAs from rat hippocampus. *Acta biochimica et biophysica Sinica*. 2007; 39:708–714. [PubMed: 17805466]
- Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*. 2009; 4:44–57. [PubMed: 19131956]
- Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annual review of biochemistry*. 2003; 72:609–642.
- Huang YW, Ruiz CR, Eyler EC, Lin K, Meffert MK. Dual Regulation of miRNA Biogenesis Generates Target Specificity in Neurotrophin-Induced Protein Synthesis. *Cell*. 2012; 148:933–946. [PubMed: 22385959]
- Kedde M, Agami R. Interplay between microRNAs and RNA-binding proteins determines developmental processes. *Cell cycle*. 2008; 7:899–903. [PubMed: 18414021]
- Lamont EW, Legault-Coutu D, Cermakian N, Boivin DB. The role of circadian clock genes in mental disorders. *Dialogues in clinical neuroscience*. 2007; 9:333–342. [PubMed: 17969870]
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003; 115:787–798. [PubMed: 14697198]
- Li YY, Cui JG, Dua P, Pogue AI, Bhattacharjee S, Lukiw WJ. Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. *Neuroscience letters*. 2011; 499:109–113. [PubMed: 21640790]
- Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME. Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature*. 2008; 455:1198–1204. [PubMed: 18815592]
- Lugli G, Torvik VI, Larson J, Smalheiser NR. Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain. *J Neurochem*. 2008; 106:650–661. [PubMed: 18410515]
- Lyons MR, West AE. Mechanisms of specificity in neuronal activity-regulated gene transcription. *Progress in neurobiology*. 2011; 94:259–295. [PubMed: 21620929]
- Mansour HA, Wood J, Logue T, Chowdari KV, Dayal M, Kupfer DJ, Monk TH, Devlin B, Nimgaonkar VL. Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia. *Genes, brain, and behavior*. 2006; 5:150–157.
- Maragkakis M, Vergoulis T, Alexiou P, Reczko M, Plomaritou K, Gousis M, Kourtis K, Koziris N, Dalamagas T, Hatzigeorgiou AG. DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. *Nucleic acids research*. 2011; 39:W145–148. [PubMed: 21551220]
- Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N,

- Sharp PA, Bartel DP, Jaenisch R, Young RA. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell*. 2008; 134:521–533. [PubMed: 18692474]
- Minichiello L. TrkB signalling pathways in LTP and learning. *Nature reviews. Neuroscience*. 2009; 10:850–860. [PubMed: 19927149]
- Neves-Pereira M, Cheung JK, Pasdar A, Zhang F, Breen G, Yates P, Sinclair M, Crombie C, Walker N, St Clair DM. BDNF gene is a risk factor for schizophrenia in a Scottish population. *Molecular psychiatry*. 2005; 10:208–212. [PubMed: 15630410]
- Nievergelt CM, Kripke DF, Barrett TB, Burg E, Remick RA, Sadovnick AD, McElroy SL, Keck PE Jr, Schork NJ, Kelsoe JR. Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*. 2006; 141B: 234–241.
- Parsons MJ, Grimm C, Paya-Cano JL, Fernandes C, Liu L, Philip VM, Chesler EJ, Nietfeld W, Lehrach H, Schalkwyk LC. Genetic variation in hippocampal microRNA expression differences in C57BL/6 J X DBA/2 J (BXD) recombinant inbred mouse strains. *BMC genomics*. 2012; 13:476. [PubMed: 22974136]
- Perry MM, Williams AE, Tsitsiou E, Larner-Svensson HM, Lindsay MA. Divergent intracellular pathways regulate interleukin-1 β -induced miR-146a and miR-146b expression and chemokine release in human alveolar epithelial cells. *FEBS letters*. 2009; 583:3349–3355. [PubMed: 19786024]
- Petryshen TL, Sabeti PC, Aldinger KA, Fry B, Fan JB, Schaffner SF, Waggoner SG, Tahl AR, Sklar P. Population genetic study of the brain-derived neurotrophic factor (BDNF) gene. *Molecular psychiatry*. 2010; 15:810–815. [PubMed: 19255578]
- Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T. Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. *J Neurosci*. 2011; 31:3295–3308. [PubMed: 21368041]
- Ramamoorthi K, Fropf R, Belfort GM, Fitzmaurice HL, McKinney RM, Neve RL, Otto T, Lin Y. Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science*. 2011; 334:1669–1675. [PubMed: 22194569]
- Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. *Rna*. 2004; 10:1507–1517. [PubMed: 15383676]
- Rybakowski JK, Borkowska A, Skibinska M, Hauser J. Illness-specific association of val66met BDNF polymorphism with performance on Wisconsin Card Sorting Test in bipolar mood disorder. *Molecular psychiatry*. 2006; 11:122–124. [PubMed: 16222333]
- Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell*. 2011; 146:353–358. [PubMed: 21802130]
- Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. *Nature*. 2006; 439:283–289. [PubMed: 16421561]
- Seitz H. Redefining microRNA targets. *Current biology : CB*. 2009; 19:870–873. [PubMed: 19375315]
- Shalizi AK, Bonni A. brawn for brains: the role of MEF2 proteins in the developing nervous system. *Current topics in developmental biology*. 2005; 69:239–266. [PubMed: 16243602]
- Stark KL, Xu B, Bagchi A, Lai WS, Liu H, Hsu R, Wan X, Pavlidis P, Mills AA, Karayiorgou M, Gogos JA. Altered brain microRNA biogenesis contributes to phenotypic deficits in a 22q11-deletion mouse model. *Nature genetics*. 2008; 40:751–760. [PubMed: 18469815]
- Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, Redon R, Bird CP, de Grassi A, Lee C, Tyler-Smith C, Carter N, Scherer SW, Tavare S, Deloukas P, Hurles ME, Dermitzakis ET. Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science*. 2007; 315:848–853. [PubMed: 17289997]
- Su WL, Kleinhanz RR, Schadt EE. Characterizing the role of miRNAs within gene regulatory networks using integrative genomics techniques. *Molecular systems biology*. 2011; 7:490. [PubMed: 21613979]

- Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:12481–12486. [PubMed: 16885212]
- Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nature reviews. Genetics*. 2008; 9:764–775.
- Tettamanti G, Cattaneo AG, Gornati R, de Eguileor M, Bernardini G, Binelli G. Phylogenesis of brain-derived neurotrophic factor (BDNF) in vertebrates. *Gene*. 2010; 450:85–93. [PubMed: 19879341]
- Tocchetto A, Salum GA, Blaya C, Teche S, Isolani L, Bortoluzzi A, Rebelo ESR, Becker JA, Bianchini MM, Rohde LA, Leistner-Segal S, Manfro GG. Evidence of association between Val66Met polymorphism at BDNF gene and anxiety disorders in a community sample of children and adolescents. *Neuroscience letters*. 2011; 502:197–200. [PubMed: 21839144]
- Tsang JS, Ebert MS, van Oudenaarden A. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. *Molecular cell*. 2010; 38:140–153. [PubMed: 20385095]
- Urdinguio RG, Fernandez AF, Lopez-Nieva P, Rossi S, Huertas D, Kulis M, Liu CG, Croce CM, Calin GA, Esteller M. Disrupted microRNA expression caused by Mecp2 loss in a mouse model of Rett syndrome. *Epigenetics : official journal of the DNA Methylation Society*. 2010; 5:656–663.
- Verhagen M, van der Meij A, van Deurzen PA, Janzing JG, Arias-Vasquez A, Buitelaar JK, Franke B. Meta-analysis of the BDNF Val66Met polymorphism in major depressive disorder: effects of gender and ethnicity. *Molecular psychiatry*. 2010; 15:260–271. [PubMed: 18852698]
- Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH, Impey S. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:16426–16431. [PubMed: 16260724]
- Wang X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *Rna*. 2008; 14:1012–1017. [PubMed: 18426918]
- Xu B, Hsu PK, Karayiorgou M, Gogos JA. MicroRNA dysregulation in neuropsychiatric disorders and cognitive dysfunction. *Neurobiology of disease*. 2012
- Xu B, Hsu PK, Stark KL, Karayiorgou M, Gogos JA. Derepression of a neuronal inhibitor due to miRNA dysregulation in a schizophrenia-related microdeletion. *Cell*. 2013; 152:262–275. [PubMed: 23332760]
- Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M. Strong association of de novo copy number mutations with sporadic schizophrenia. *Nature genetics*. 2008; 40:880–885. [PubMed: 18511947]
- Yoshii A, Constantine-Paton M. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Developmental neurobiology*. 2010; 70:304–322. [PubMed: 20186705]
- Zhang L, Hammell M, Kudlow BA, Ambros V, Han M. Systematic analysis of dynamic miRNA-target interactions during *C. elegans* development. *Development*. 2009; 136:3043–3055. [PubMed: 19675127]

Highlights

- miR-146b is dysregulated in the hippocampus of humanized BDNFVal66Met knock-in mice
- Reduced miR-146b leads to increase of Per1, Npas4 and Irak1 in vitro and in vivo
- miRNA-mediated trans effect of common variants could contribute to complex disorders

Figure 1. miR-146b expression levels are decreased in the hippocampus of *hBDNF*^{Met/Met} mice
 (A) miRNA expression profile in hippocampus of *hBDNF*^{Met/Met} animals versus *hBDNF*^{Val/Val} animals (n = 5 each genotype). Volcano plot showing the FDR-corrected *P*-value and corresponding relative expression of each miRNA. Green dots, miR-146b and miR-337-3p; blue dot, miR-700. (B) Expression levels of miR-146b and miR-337-3p in hippocampus of *hBDNF*^{Val/Val}, *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice (n = 6 each genotype), as measured by qRT-PCR. Expression levels in *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice were normalized to *hBDNF*^{Val/Val} animals. (C) Expression levels of miR-146b and miR-337-3p in acute hippocampal slices (n = 9, from 3 animals, each treatment) treated with BDNF for 2 hours, as measured by qRT-PCR. Expression levels following BDNF treatments were normalized to mock treatment control. Results are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ANOVA with *post-hoc* Bonferroni's test (B); Student's *t*-test (C).

Figure 2. miR-146b targets, *Per1*, *Npas4* and *Irak1* are upregulated in *hBDNF*^{Met/Met} mice
 (A) Repressive effects of pre-miR-146b on 3'UTRs of a group of putative miR-146b targets (see Supplementary Data Sheet 1) were examined by a dual-luciferase reporter assay performed in N18 neuroblastoma cell line (n = 3 for each reporter). Expression value of each target reporter was normalized to no 3'UTR control. A pre-scramble oligo was used as control for each target tested. (B) Repressive effects of pre-miR-146b on *Per1* and *Npas4* 3'UTRs (left and right, respectively) were abrogated by mutations in miR-146b binding sites, as analyzed by luciferase reporter assay. Expression value of each mutant reporter was normalized to Wt 3'UTR reporter. (C) Expression levels of *Per1* and *Npas4* in hippocampus of *hBDNF*^{Val/Val}, *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice (n = 6 each genotype), as measured by qRT-PCR. Expression levels in *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice were normalized to *hBDNF*^{Val/Val} animals. (D) Western blots analysis of *Irak1* (left) and *Traf6* (right) protein levels in hippocampal lysates of *hBDNF*^{Val/Val}, *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice (n = 7 each genotype). *Upper*: Representative western blot assays of *Irak1* and *Traf6*. β -actin was used as loading control. *Lower*: Quantification of *Irak1* and *Traf6* protein levels. Expression levels in *hBDNF*^{Val/Met} and *hBDNF*^{Met/Met} mice were normalized to *hBDNF*^{Val/Val} littermates. Results are expressed as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, ANOVA with *post-hoc* Dunnett's test for multiple comparisons relative to control (A, B); ANOVA with *post-hoc* Bonferroni's test (C, left, D); Student's *t*-test (C, right).

Figure 3. The repressive effect of pre-mir-146b on *Per1* 3'UTR is specific

Pre-mir-146b significantly repressed *Per1* 3'UTR over a concentration range of 1 nM to 0.1 nM, as analyzed by luciferase reporter assay. The repressive effects of pre-miR-146b can be completely neutralized by anti-miR-146b (Exiqon) transfection ($n = 3$ for each condition) at a concentration ratio of ~1:10. Results are expressed as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$, ANOVA with *post-hoc* Dunnett's test for multiple comparisons relative to control.

Figure 4. Baseline expression levels of miRNA biogenesis enzymes are not altered in *hBDNF^{Met/Met}* mice

(A) Expression levels of Dicer1, Lin28A, Ago2 and Dgcr8 in hippocampal lysates of *hBDNF^{Val/Val}*, *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* mice (n = 6 for each genotype), as assayed by Western blots. Expression levels in *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* mice were normalized to *hBDNF^{Val/Val}* littermates. Results are expressed as mean ± SEM. $P > 0.05$, ANOVA with *post-hoc* Bonferroni's test. (B) Representative western blot assays of Dicer1, Lin28A, Ago2 and Dgcr8 in *hBDNF^{Val/Val}*, *hBDNF^{Val/Met}* and *hBDNF^{Met/Met}* lysates. α-tubulin was used as loading control.

Table 1
Significantly dysregulated microRNAs in the HPC of *BDNF^{Met/Met}* mice, as compared to *BDNF^{Val/Val}* mice

mmu-miR	FDR P-value	regulation	Fold Change	Stem-loop Accession	Chr	Chr_start	Chr_end	Strand
146b	2.83E-03	down	0.83	MI0004665	19	46417252	46417360	+
700	2.83E-03	up	1.66	MI0004684	4	134972470	134972548	-
337-3p	3.54E-03	down	0.83	MI0000615	12	110823999	110824095	+
130b	3.54E-03	up	1.17	MI0000408	16	17124154	17124235	-
127-5p ^a	4.38E-03	up	1.12	MI0000154	12	110831056	110831125	+
328	7.71E-03	up	1.23	MI0000603	8	107832264	107832360	-
291a-5p	9.73E-03	down	0.93	MI0000389	7	3218920	3219001	+
10a	1.03E-02	up	1.15	MI0000685	11	96178479	96178588	+
20b	1.40E-02	down	0.88	MI0003536	X	50095290	50095369	-
337-5p	2.51E-02	up	1.15	MI0000615	12	110823999	110824095	+
532-5p	2.61E-02	up	1.23	MI0003206	X	6825528	6825623	-
674	3.05E-02	up	1.26	MI0004611	2	117010863	117010962	+
27b	3.30E-02	down	0.92	MI0000142	13	63402020	63402092	+
874	3.70E-02	down	0.88	MI0005479	13	58124486	58124561	-
804	4.29E-02	up	1.15	MI0005203	11	50171287	50171381	-
7a-1-3p ^b	4.29E-02	down	0.83	MI0000728	13	58494140	58494247	-
485-3p ^c	4.29E-02	up	1.14	MI0003492	12	110973112	110973184	+
491	4.29E-02	up	1.15	MI0004680	4	87767944	87768029	+
721	4.29E-02	down	0.79	MI0004708	5	136851586	136851673	-
688	4.29E-02	down	0.87	MI0004653	15	102502223	102502297	-
342-3p	4.40E-02	down	0.87	MI0000627	12	109896830	109896928	+
378	4.45E-02	up	1.29	MI0000795	18	61557489	61557554	-
185	4.51E-02	up	1.09	MI0000227	16	18327494	18327558	-

^a previously mmu-miR-127*,

^b previously mmu-miR-7a*,

^c previously mmu-miR-485*

Table 2

Functional enrichment analysis of predicted miR-146b targets

Category	Term	Count	%	Fold Enrichment	P Value	Benjamini FDR
Annotation Cluster 1 Enrichment Score: 2.85						
GOTERM_BP_FAT	GO:0030182~neuron differentiation	14	8.97	3.97	4.62E-05	0.0226
GOTERM_BP_FAT	GO:0030030~cell projection organization	12	7.69	4.26	1.11E-04	0.0271
GOTERM_BP_FAT	GO:0031175~neuron projection development	10	6.41	5.19	1.25E-04	0.0246
GOTERM_BP_FAT	GO:0007409~axonogenesis	8	5.13	5.56	5.72E-04	0.0502
Annotation Cluster 2 Enrichment Score: 2.42						
GOTERM_MF_FAT	GO:0003723~RNA binding	16	10.26	2.93	3.07E-04	0.0391
Annotation Cluster 3 Enrichment Score: 2.37						
GOTERM_MF_FAT	GO:0003677~DNA binding	32	20.51	2.21	1.76E-05	0.0046
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	15	9.62	3.20	2.18E-04	0.0267
GOTERM_BP_FAT	GO:0031328~positive regulation of cellular biosynthetic process	15	9.62	3.08	3.30E-04	0.0357
GOTERM_BP_FAT	GO:0009891~positive regulation of biosynthetic process	15	9.62	3.05	3.62E-04	0.0352

Table 3

Predicted miR-146b targets selected for validation

TargetScanMouse version 6.2, 6/2012														microT version 5		PicTar 03/2007		miRanda 11/2010		mirDB 04/2012	
Target	total context+ score	Conserved sites					Poorly conserved sites					miTG score	PicTar score	mirSVR score	target score						
		total	8mer	7mer- m8	7mer- 1A	total	8mer	7mer- m8	7mer- 1A												
Irak1	-0.77	2	2	0	0	1	0	0	1	0.98	8.79	-1.65	94								
Traf6	-0.53	3	3	0	0	0	0	0	0	>0.99	6.28	-0.72	95								
Per1	-0.31	0	0	0	0	2	1	0	1	0.43	2.52	-0.66									
Stx3	-0.25	1	1	0	0	0	0	0	0	0.82	1.06	-0.46									
Syt1	-0.22	1	1	0	0	1	0	0	1	0.92	4.44	-1.15									
Cask	-0.21	1	0	1	0	1	0	0	1	0.80	1.02	-0.48	65								
Robo1	-0.19	1	0	0	1	0	0	0	0	0.83	1.30	-0.52									
Kctd15	-0.17	1	1	0	0	0	0	0	0	0.57	5.00	-0.20									
Sort1	-0.13	1	1	0	0	0	0	0	0			-0.22	50								
Dlgap1	-0.09	0	0	0	0	1	0	1	0	0.76	0.75	-0.18									
Srrd	-0.08	0	0	0	0	1	0	1	0	0.44	1.56	-0.03									
Bsn	-0.07	0	0	0	0	1	0	0	1	0.46		>-0.01									
Gria3	-0.03	0	0	0	0	1	1	0	0	0.78	0.83	>-0.01									
Npas4	-0.02	1	0	1	0	0	0	0	0	0.56	2.02	-0.02									
Lin28A	-0.01	0	0	0	0	1	0	0	1	0.65	2.53	-0.01									
Akt3	-0.01	0	0	0	0	1	0	0	1	0.54		-0.31									

Table 4

Expression levels of candidate miR-146b targets and non-targets in the HPC of *hBDNF^{Val/Val}* and *hBDNF^{Met/Met}* mice, as compared to *BDNF^{Val/Val}* mice

Genes with miR-146b Binding Site(s)				Genes without miR-146b Binding Site			
gene	<i>hBDNF^{Val/Val}</i> fold change	<i>hBDNF^{Met/Met}</i> fold change	ANOVA <i>P</i> -value	gene	<i>hBDNF^{Val/Val}</i> fold change	<i>hBDNF^{Met/Met}</i> fold change	ANOVA <i>P</i> -value
<i>Per1</i>	1.24	1.28	1.1E-3	<i>B3gat1</i>	0.91	0.97	0.09
<i>Npas4</i>	1.33	1.65	0.03	<i>Kif5c</i>	1.13	1.06	0.10
<i>Robo1</i>	1.10	1.04	0.06	<i>Cdk5r1</i>	1.07	1.09	0.12
<i>Akt3</i>	1.10	1.10	0.14	<i>Ldb2</i>	1.02	0.95	0.18
<i>Traf6</i>	1.03	0.94	0.19	<i>Cpeb2</i>	1.07	1.01	0.31
<i>Syt1</i>	1.13	1.05	0.29	<i>GSK3b</i>	1.03	0.97	0.37
<i>Irak1</i>	1.02	0.96	0.30	<i>Cdk5r2</i>	1.05	1.15	0.39
<i>Sort1</i>	1.10	1.03	0.33	<i>vGlut1</i>	0.99	0.95	0.49
<i>Cask</i>	1.01	0.88	0.36	<i>Agyt2l1</i>	1.10	1.24	0.64
<i>Gria3</i>	0.99	1.11	0.40	<i>Nptx2</i>	0.97	1.05	0.67
<i>Stx3</i>	0.91	1.01	0.41	<i>Hspa8</i>	0.98	0.94	0.71
<i>Srrd</i>	1.09	1.00	0.53	<i>Clec16a</i>	1.01	1.01	0.97
<i>Bsn</i>	1.03	1.05	0.55	<i>Mei2c</i>	0.99	0.99	0.98

Note: While expression levels of two of the selected miR-146b candidate targets (*Per1* and *Npas4*) were significantly altered (upregulated) in HPC due to *Val66Met* SNP ($P < 0.05$, ANOVA), none of the genes without miR-146b binding site shows change in expression levels, as assayed by qRT-PCR.

Table 5

Expression of let-7 family microRNAs in the HPC of *hBDNF^{Met/Met}* Mice, as compared to *hBDNF^{Val/Val}* mice

miRNA ID	FDR <i>P</i> -value	Rank	FC Absolute	Regulation
mmu-let-7a	0.6020	337	1.09	down
mmu-let-7a*	0.1698	66	1.20	up
mmu-let-7b	0.9133	524	1.02	up
mmu-let-7b*	0.6653	389	1.07	up
mmu-let-7c	0.9444	541	1.01	down
mmu-let-7c*	0.8927	506	1.01	up
mmu-let-7d	0.9113	515	1.02	down
mmu-let-7d*	0.2736	116	1.24	up
mmu-let-7e	0.7696	422	1.06	up
mmu-let-7f	0.9067	510	1.02	down
mmu-let-7f*	0.9067	511	1.03	up
mmu-let-7g	0.7345	413	1.02	down
mmu-let-7g*	0.1972	78	1.27	up
mmu-let-7i	0.3366	182	1.04	up
mmu-let-7i*	0.0691	31	1.20	up
mmu-miR-98	0.8246	450	1.11	up
mmu-miR-107	0.5905	326	1.05	down
mmu-miR-143	0.5916	329	1.04	up

Note: None of the let-7 family miRNA showed significantly altered expression (FDR-corrected $P > 0.05$) in HPC in miRNA microarray. See Figure 1 for details of the microarray by LC Sciences.