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Bipolar Disorder Associated microRNA, miR-1908-5p, Regulates the Expression of Genes Functioning in Neuronal Glutamatergic Synapses

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Bipolar disorder (BD), characterized by recurrent mood swings between depression and mania, is a highly heritable and devastating mental illness with poorly defined pathophysiology. Recent genome-wide molecular genetic studies have identified several protein-coding genes and microRNAs (miRNAs) significantly associated with BD. Notably, some of the proteins expressed from BD-associated genes function in neuronal synapses, suggesting that abnormalities in synaptic function could be one of the key pathogenic mechanisms of BD. In contrast, however, the role of BD-associated miRNAs in disease pathogenesis remains largely unknown, mainly because of a lack of understanding about their target mRNAs and pathways in neurons. To address this problem, in this study, we focused on a recently identified BD-associated but uncharacterized miRNA, miR-1908-5p. We identified and validated its novel target genes including *DLGAP4, GRIN1, STX1A, CLSTN1* and *GRM4*, which all function in neuronal glutamatergic synapses. Moreover, bioinformatic analyses of human brain expression profiles revealed that the expression levels of miR-1908-5p and its synaptic target genes show an inverse-correlation in many brain regions. In our preliminary experiments, the expression of miR-1908-5p was increased after chronic treatment with valproate but not lithium in control human neural progenitor cells. In contrast, it was decreased by valproate in neural progenitor cells derived from dermal fibroblasts of a BD subject. Together, our results provide new insights into the potential role of miR-1908-5p in the pathogenesis of BD and also propose a hypothesis that neuronal synapses could be a key converging pathway of some BD-associated protein-coding genes and miRNAs.

Key words: Bipolar disorder, microRNA, miR-1908-5p, glutamatergic synapse

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INTRODUCTION

Bipolar disorder (BD) is a highly heritable, chronic, and devastating mental illness characterized by recurrent mood swings between depression and mania with intervening euthymic

states. With a lifetime prevalence of 1~2%, BD is recognized as the sixth leading cause of disability worldwide and also incurs huge social and economic costs [1]. Although the underlying pathogenic mechanisms are poorly defined, recent genome-wide molecular genetic studies have identified many single-nucleotide polymorphisms (SNPs) associated with BD in multiple chromosomal regions [2-4]. These loci include several protein-coding genes (e.g. *ANK3, CACNA1C, NCAN*, and *ODZ4*) that are involved in various neuronal processes, such as calcium signaling and synaptic development and function [5, 6]. Moreover, studies on postmortem human brains and animal models of BD show changes in the expression of synaptic proteins as well as abnormalities of synaptic morphology and function [7-10]. Together, these results suggest that 'synaptic pathology (or synaptopathy)' could be one of the major features of BD.

MicroRNAs (miRNAs) are 21~25-nucleotide small non-coding RNAs that regulate the expression of target mRNAs by directly interacting with the 'seed' complementary sequences in 3' untranslated regions (3'UTRs) [11]. miRNA binding downregulates the expression of target mRNAs either by decreasing their stability or by inhibiting translation. Notably, the expression levels of many miRNAs are altered in postmortem brains and blood samples of BD patients, suggesting a potential role for miRNAs in BD pathophysiology [12-14]. In many cases, however, it is unclear whether such changes in miRNA expression have any causative role in BD or simply reflect disease progression or patient medication. Independently, molecular genetic studies have also identified several miRNAs associated with BD, such as miR-499, miR-708, miR-1908, and miR-2113 [4, 15]. Although these miRNAs could be potentially involved in BD pathogenesis, the key target mRNAs that could mediate the pathogenic process remain largely unknown. Importantly, it has been shown that a single miRNA can regulate a specific biological function by modulating the expression of a group of proteins interacting with each other or participating in the same pathway [16]. Therefore, we reasoned that identifying the target genes of BD-associated miRNAs and understanding their enriched functions could provide important insights into the key pathogenic mechanisms of BD.

In this study, we tested this hypothesis by focusing on a recently identified BD-associated, primate-specific but uncharacterized miRNA, miR-1908-5p. We identified and validated its novel target genes including *DLGAP4, GRIN1, STX1A, CLSTN1* and *GRM4*, which all function in neuronal glutamatergic synapses. Moreover, our bioinformatic analyses showed inverse-correlations between the expression levels of miR-1908-5p and its synaptic target genes in multiple human brain regions. From a preliminary experiment, we also found that chronic treatment with valproate, a common medication for BD, could increase the expression of miR-1908-5p in control human neural progenitor cells (NPCs), while it could decrease the level of miR-1908-5p in NPCs derived from dermal fibroblasts of a BD subject. Together, our study identifies the synaptic target genes of BD-associated miR-1908-5p, and thereby provides another piece of evidence supporting the 'synaptic pathology' hypothesis of BD.

MATERIALS AND METHODS

Luciferase assays

The 3'UTR regions of human *CLSTN1* (NM_001009566.2, 1-1,426), *DLGAP4* (XM_005260333.3, 1-1,602), *GRASP* (XM_011537996.1, 1-636), *GRIN1* (NM_007327.3, 1-1,235), *GRM4* (XM_011514531.1, 1-947), and *STX1A* (XM_011516541.1, 1-1,157) were PCR amplified from fetal or adult brain cDNA libraries and subcloned into the psiCHECK-2 vector (Promega). Mutagenesis reactions of the *GRM4* 3'UTR construct were performed using the QuikChange XL II Site-Directed Mutagenesis Kit (Agilent Technologies) to change the three nucleotides of the miR-1908-5p seed match regions (position 4 to 6, CGC) into complementary sequences (GCG). HEK293T cells in 24-well plates were transfected with 30 ng of psiCHECK-2 construct plus 20 pmol of either cel-miR-67 (negative control miRNA) or miR-1908-5p duplex (miRIDIAN Dharmacon) using Lipofectamine 2000 (Invitrogen). After 24 h, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

Human neural progenitor cell culture and drug treatment

The control and bipolar patient iPS cell lines were generated by transfection of integration-free episomal expression vectors containing p53shRNA, Oct3/4, Sox2, Klf4, L-Myc and Lin28 as described in Okita et al. [17]. The iPSC lines were maintained in mTeSR1 medium (Stemcell Technologies). For differentiation of iPSC to NPC, we followed Cho et al. with minor modifications [18]. In brief, iPSC colonies were detached with dispase and cultured with STEMdiff Neural Induction Medium (Stemcell Technologies) in a bacterial dish for 5 days to form EBs. EBs then were plated onto matrigel-coated dishes in STEMdiff Neural Induction Medium for 7 days to form neural rosettes. The neural rosettes were mechanically isolated and cultured in DMEM/F12 plus N2 and bFGF, in order to form Spherical Neural Masses (SNMs). For differentiation of SNMs to NPCs, SNMs were chopped using a stainless steel blade (Dorco) and plated onto matrigel-coated dishes in DMEM/F12 plus N2 and B27. NPCs were chronically

exposed to 1 mM lithium chloride (Sigma-Aldrich) or valproic acid sodium salt (Sigma-Aldrich) for a week.

RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted from human NPCs using a miRNeasy minikit (Qiagen) according to the manufacturer's instruction. From 50 ng of total RNA, cDNAs for *RNU6B* (internal control) and miR-1908-5p were synthesized using TaqMan microRNA reverse transcription kit (Applied Biosystems). *RNU6B* and mature miR-1908-5p were detected and quantified by a real-time PCR instrument (CFX96 Touch, BIO-RAD) using the TaqMan microRNA assays (Applied Biosystems). The experiments were performed in three independent technical repeats.

Bioinformatics analysis

The target genes of miRNAs were predicted by using the TargetScan database (Release 7.0, http://www.targetscan.org) [19]. The predicted target genes were sorted by context++ scores of the binding sites, and low scored target genes were discarded to remove less likely target genes. To further restrict the target genes, low-expressed genes whose expression levels were less than a 50^{th} quantile of the average expression in mouse brain were also discarded. The expression values of mouse brain were obtained from 84 GDS datasets downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO). The gene ontology (GO) analysis for the remaining target genes was carried out using DAVID software (v6.7, https://david.ncifcrf.gov) [20]. GO terms with an adjusted p-value (Benjamini) less than 0.05 were considered significant. Barplots were generated using the R (v.3.2.3) package ggplot2 (v.2.0.0).

The miRNA sequencing data of the developing human brain were downloaded from the BrainSpan database (http://www.brainspan.org). This dataset contains 216 samples spatially covering 16 brain regions and temporally spanning developmental periods from 4 months to 23 years of age. Reads were normalized to reads per million mapped reads (RPM) using the formula:

$$R = \frac{10^6 C}{NL}$$

where C is the number of reads mapped to one miRNA, N is the total number of mapped reads in the sample, and L is the length of the miRNA. The gene expression data of the human brain were downloaded from the NCBI GEO (GEO accession number GSE25219) [21]. This dataset contains 1,340 samples spatially covering multiple brain regions and temporally spanning periods from 4 post-conceptional weeks to 82 years of age. The expression levels of genes were assayed using the Affymetrix GeneChip Human Exon 1.0 ST Array platform, and represented as normalized $\log_2$-transformed signal intensity values. For brain regions with both left and right hemispheres of the same donor profiled, gene expression values were averaged. The samples in the miRNA expression data and those in the gene expression data were matched using the sample mapping table downloaded from the BrainSpan database, and 213 matched samples were used for analyses. To explore the regional expression pattern of miR-1908-5p and its target genes, both the miRNA and gene expression datasets were divided into 16 brain regions, with the samples from the same brain region being grouped together. To investigate correlations between the expression level of miR-1908-5p and those of its target genes, Spearman's correlation coefficient was calculated among the matched samples for each brain region.

RESULTS

Identification and validation of synaptic targets of miR-1908-5p

miR-1908 is an intronic miRNA of the fatty acid desaturase 1 (*FADS1*) gene in human chromosome 11. miR-1908 is transcribed and processed to generate two 21-nucleotide mature miRNAs, miR-1908-3p and miR-1908-5p, which have different sequences and thus potentially different target mRNAs (http://www.mirbase.org/). According to the UCSC genome browser (hg38), the genomic sequence of miR-1908 is poorly conserved. Among 100 animal genomes, the seed sequences of miR-1908-3p and miR-1908-5p are conserved only in humans and other primates, which contrasts with well-conserved miRNAs, such as miR-34a (Additional file 1: Figs. S1 and S2). Recently, Forstner et al. [15] revealed a significant association of miR-1908 with BD and performed pathway analysis of miR-1908 target genes starting from only 67 targets predicted by TargetScan (Release 6.2) [22], which considers the conservation of miRNA binding sites in 3'UTRs. However, this approach might have missed some meaningful human targets of miR-1908, especially considering the human and primate-specific conservation of miR-1908. Moreover, TargetScan (Release 6.2) predicted the targets of only miR-1908-5p and not miR-1908-3p.

To solve these problems and to better understand the genes and biological pathways regulated by miR-1908-3p and miR-1908-5p, we decided to use the newest version of TargetScan (Release 7.0) [19]. We found that TargetScan (Release 7.0) predicted a total of 481 and 2,500 target genes of miR-1908-3p and miR-1908-5p, respectively. In TargetScan (Release 7.0), targets are

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ranked based on the cumulative weighted context++ score, which combines the contribution of 14 features irrespective of binding site conservation [19]. To focus on the high-ranked and more likely targets, we set thresholds for context++ scores and narrowed down the gene list to 303 and 358 genes for miR-1908-3p and miR-1908-5p, respectively (Additional file 1: Fig. S3). We further restricted the targets by selecting brain-expressed genes (184 and 225 genes, respectively) and performed Gene Ontology (GO) analysis (Fig. 1a and b). After applying adjusted p-value (Benjamini, <0.05), we found some significant terms, including 'regulation of cell development' and 'regulation of axonogenesis' in the Biological Process category for miR-1908-3p targets (Fig. 1a and Additional file 1: Fig. S4a). For miR-1908-5p, 'synapse' and 'synaptic vesicle' related terms were significantly enriched in the Cellular Component category (Fig. 1b and Additional file 1: Fig. S4b).

For the validation of putative miR-1908 targets, we decided to focus on the 'synapse' related targets of miR-1908-5p, which was the most significant term in our entire GO analysis (Additional file 1: Fig. S4). Moreover, both human genetic and animal model studies have proposed that abnormalities in neuronal synapses could be one of the major pathogenic mechanisms of BD [8, 23, 24]. We searched the literature and found that most of the putative synaptic targets of miR-1908-5p are located in excitatory glutamatergic synapses, either pre- or post-synaptic side (Fig. 1c). We selected the top 6 synaptic genes based on the target rank from TargetScan (Release 7.0) (Fig. 1c) and cloned their 3'UTRs to generate luciferase constructs. Each 3'UTR of the 6 genes had at least two putative miR-1908-5p binding sites (Fig. 1d). In HEK293T cells, miR-1908-5p overexpression significantly decreased the luciferase activities of constructs with *DLGAP4, GRIN1, STX1A, CLSTN1*, and *GRM4* 3'UTRs (Fig. 1d). However, the expression of the *GRASP* 3'UTR was not affected. It is not uncommon for miRNAs

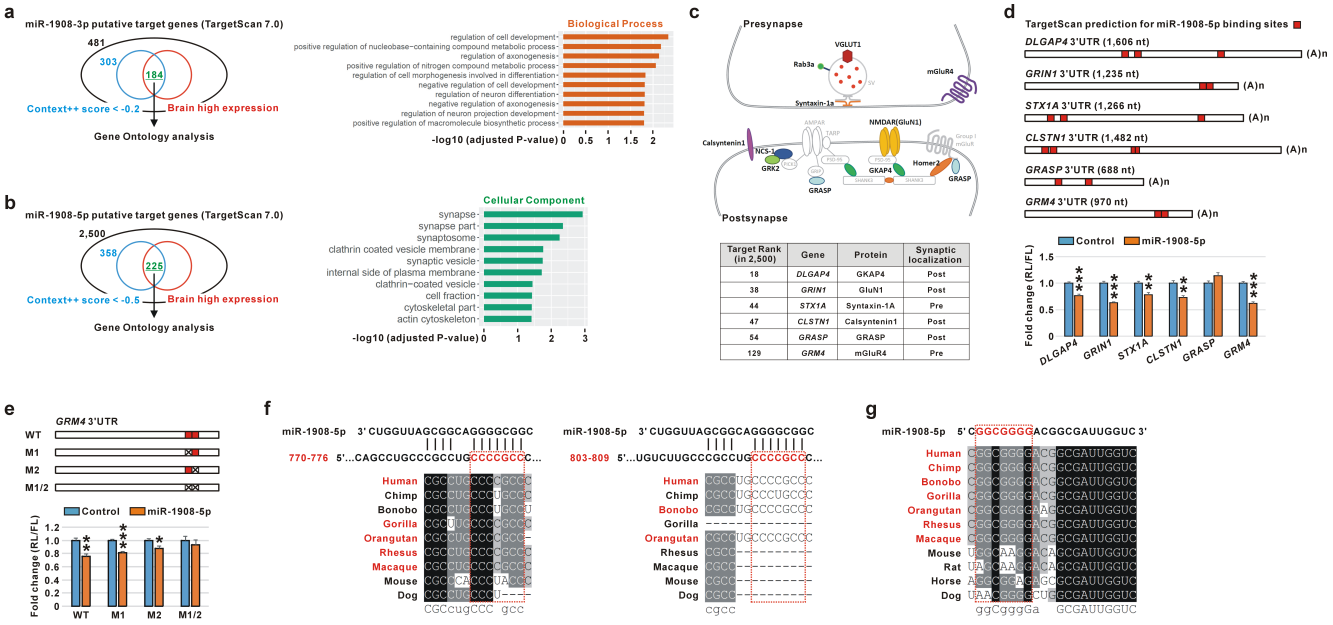

Fig. 1. Identification of synaptic target genes of miR-1908-5p and validation of binding sites with luciferase assays. (a) GO analysis of putative miR-1908-3p target genes. The 481 miR-1908-3p targets predicted by TargetScan were narrowed down to 184 genes by selecting those with both context++ scores less than -0.2 and high brain expression (left panel). GO analysis with the 184 miR-1908-3p targets showed significant terms including 'regulation of cell development' and 'regulation of axonogenesis' in the Biological Process category (right panel). (b) GO analysis of putative miR-1908-5p target genes. The 2,500 miR-1908-5p targets predicted were narrowed down to 225 genes by selecting those with both context++ scores less than -0.5 and high brain expression (left panel). GO analysis with the 225 miR-1908-5p targets revealed 'synapse' as the most significant term in the Cellular Component category (right panel). (c) Pre- and post-synaptic localization of the putative miR-1908-5p target genes (color coded, upper panel). Some of the interacting proteins (e.g. PSD-95) of miR-1908-5p synaptic targets are also shown (gray). We selected six synaptic targets of miR-1908-5p for further analysis (lower panel). (d) Luciferase assays with the 3'UTRs of six synaptic targets of miR-1908-5p. The putative miR-1908-5p binding sites in the 3'UTRs are shown (upper panel). miR-1908-5p decreased the luciferase activities of constructs with *DLGAP4, GRIN1, STX1A, CLSTN1*, and *GRM4* 3'UTRs but not with *GRASP* 3'UTR (lower panel). (n=6 from three independent experiments). (e) Validation of the miR-1908-5p binding sites in the *GRM4* 3'UTR. Mutations of both binding sites (M1/2), but not each binding site alone (M1 or M2), blocked the inhibitory effect of miR-1908-5p on the expression of *GRM4* 3'UTR. (n=6 from three independent experiments). (f) Conservation of the first (770~776) and second (803~809) miR-1908-5p binding sites in the *GRM4* 3'UTR. The species with miR-1908-5p binding seed sequences are red-colored. (g) Conservation of the miR-1908-5p sequence. The species with miR-1908-5p seed sequences are red-colored. All data are presented as mean±SEM. Statistical analyses are in Additional file 1: Table S1.

to not affect the expression of mRNAs even with putative binding sites, which might be explained by various factors including RNA secondary structures [25]. Importantly, in the previous study by Forstner et al., *DLGAP4, GRIN1, STX1A, CLSTN1*, and *GRM4* were missed in the list of 67 targets of miR-1908 because only the miR-1908 targets with conserved binding sites were considered in TargetScan (Release 6.2) [15].

Among those synaptic targets of miR-1908-5p, *GRM4* is the most interesting gene in terms of its known association with mood disorders. *GRM4* encodes metabotropic glutamate receptor 4 (mGluR4), a member of group III mGluRs, which is mainly localized to the pre-synaptic side of glutamatergic synapses and regulates synaptic transmission [26, 27]. *GRM4* has been associated with BD and schizophrenia [28]. Moreover, recent studies showed increased expression of *GRM4* in postmortem brains and blood samples of major depressive disorder patients [29, 30]. Therefore, abnormalities in the tight control of *GRM4* expression might be involved in the pathogenesis of various mood disorders. There are two putative miR-1908-5p binding sites (770-776 and 803-809) in the *GRM4* 3'UTR. To identify the authentic binding site, we generated more luciferase constructs with either singly or doubly mutated binding sites (Fig. 1e). We found that miR-1908-5p did not decrease the luciferase activity only when both sites were mutated, suggesting that both sites are the authentic

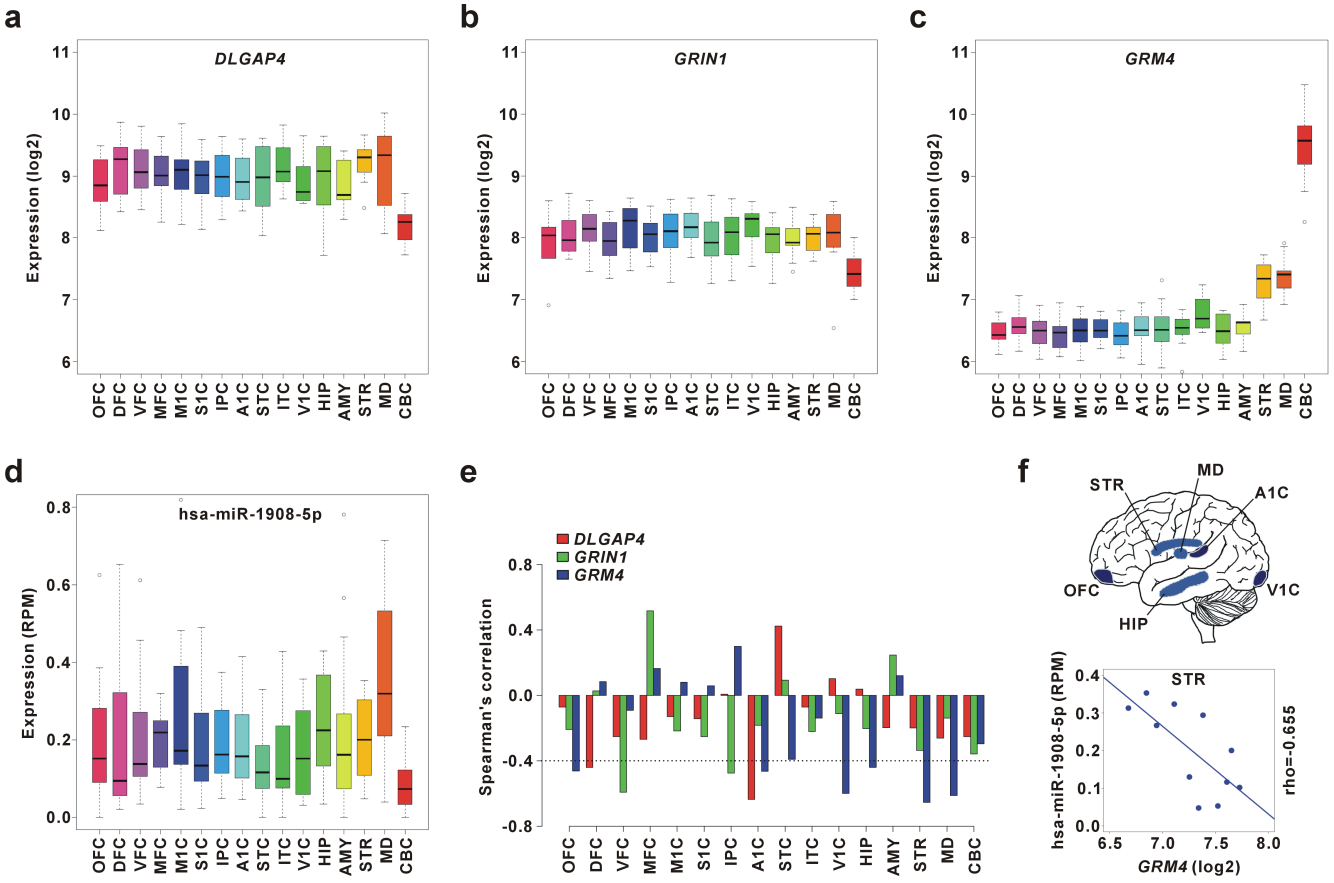

Fig. 2. Human brain expression of miR-1908-5p and its synaptic target genes. (a) Box plots showing the expression distribution of *DLGAP4* in 16 human brain regions. The black line in each box indicates the median value. The lower and upper hinges of each box indicate the lower and upper quartile values, respectively. The whiskers of each box indicate the most extreme data values within 1.5 times the interquartile range. The open circles indicate the data values beyond the whisker limits. OFC, orbital prefrontal cortex; DFC, dorsolateral prefrontal cortex; VFC, ventrolateral prefrontal cortex; MFC, medial prefrontal cortex; M1C, primary motor cortex; S1C, primary somatosensory cortex; IPC, posterior inferior parietal cortex; A1C, primary auditory cortex; STC, superior temporal cortex; ITC, inferior temporal cortex; V1C, primary visual cortex; HIP, hippocampus; AMY, amygdala; STR, striatum; MD, mediodorsal nucleus of the thalamus; CBC, cerebellar cortex. (b) The expression distribution of *GRIN1* in 16 human brain regions. (c) The expression distribution of *GRM4* in 16 human brain regions. (d) The expression distribution of miR-1908-5p in 16 human brain regions. (e) Bar plots showing Spearman's correlations between the expression level of miR-1908-5p and those of *DLGAP4, GRIN1*, and *GRM4* in 16 human brain regions. (f) The brain regions with relatively stronger inverse-correlation (<-0.4) between miR-1908-5p and *GRM4* are shown (upper panel). Scatter plots showing the expression level for miR-1908-5p versus *GRM4* in the striatum with linear regression line fit (Spearman's correlation coefficient, rho=-0.655) (lower panel).

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targets of miR-1908-5p in the *GRM4* 3'UTR (Fig. 1e). Notably, we found that both binding sites are poorly conserved, especially the second binding site (803-809), which exists only in humans and a few primates (Fig. 1f and Additional file 1: Fig. S5). These results, together with poorly conserved expression of miR-1908-5p (Fig. 1g and Additional file 1: Fig. S1), raise an interesting hypothesis that the regulatory interaction between *GRM4* and miR-1908-5p, the two BD-associated components, could occur only in humans and a few primates. Similarly, regulation of *GRM4* expression by a primate-specific miRNA, miR-1202, was recently reported [29]. Notably, most of the putative miR-1908-5p binding sites in the 3'UTRs of *DLGAP4, GRIN1, STX1A* and *CLSTN1* also exist only in humans and other primates (http://targetscan.org/).

Human brain expression profiles of miR-1908-5p and its synaptic target genes

The human and primate-specific interactions of miR-1908-5p and its synaptic target genes prompted us to investigate their expression profiles and correlations in the human brain. We first investigated the regional expression profiles of *DLGAP4, GRIN1, STX1A, CLSTN1, GRM4*, and miR-1908-5p in human brain by performing bioinformatic analyses on the developing human brain miRNA and gene expression data from the BrainSpan database (http://www.brainspan.org). *DLGAP4, GRIN1, STX1A, CLSTN1* and *GRM4* were detected throughout all brain regions with some gene-specific patterns (Fig. 2a~c and Additional file 1: Fig. S6a and b). For example, the lowest median values of $\log_2$ intensity of *DLGAP4* and *GRIN1* were found in the cerebellar cortex (CBC) (Fig. 2a and b), while the highest $\log_2$ intensity of *GRM4* was found in the CBC followed by the mediodorsal nucleus of the thalamus (MD) and striatum (STR) (Fig. 2c). Although the expression of miR-1908-5p was reported in some regions of the human brain, including hippocampus and cerebellar cortex [15, 31], its overall expression pattern has not been characterized. We found that miR-1908-5p was detected in all brain regions, with the highest median value of reads per million mapped reads (RPM) from the MD and the lowest median value of RPM from the CBC (Fig. 2d).

Next, we investigated correlations between the expression level of miR-1908-5p and those of its target genes. We reasoned that if miR-1908-5p functions as an important regulator of *DLGAP4, GRIN1, STX1A, CLSTN1* and *GRM4* in some brain regions, their expression levels are likely to show an inverse-correlation. To identify the brain regions showing such inverse-correlations, we calculated Spearman's correlation among the samples for each brain region. Notably, we found that most of the brain regions show an inverse-correlation between the expression level of miR-1908-5p and those of *DLGAP4, GRIN1, STX1A, CLSTN1* and *GRM4* (Fig. 2e and Additional file 1: Fig. S6c). In the case of *GRM4*, the inverse-correlation was relatively stronger (<-0.4) in the orbital prefrontal cortex (OFC), primary auditory A1 cortex (A1C), primary visual V1 cortex (V1C), hippocampus (HIP), STR, and MD than other brain regions (Fig. 2e and f). The striatum showed the strongest inverse-correlation (Spearman's correlation coefficient, rho=-0.655), suggesting that miR-1908-5p could be, at least partly, involved in regulating *GRM4* expression in this brain region (Fig. 2f).

Preliminary investigation of miR-1908-5p expression in human neural progenitor cells after chronic treatment with lithium or valproate

Lithium and valproate are the two most common medications for BD [32]. Previously, it was shown that treatment with lithium

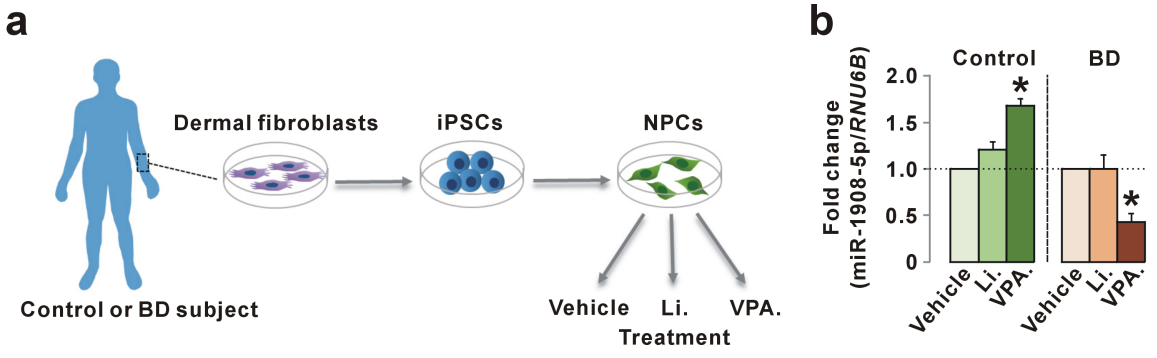

Fig. 3. miR-1908-5p expression in control and BD human NPCs after chronic treatment with lithium or valproate (a) Schematic diagram showing our experimental process. Human NPC lines derived from dermal fibroblasts of either a control or a BD subject were treated with vehicle or 1 mM lithium (Li.) or valproate (VPA.) for a week. (b) qRT-PCR analysis on the miR-1908-5p expression in control and BD NPCs after lithium or valproate treatment. The results were normalized to the vehicle-treated conditions for each NPC line. miR-1908-5p expression in control NPCs was increased by about 70% after valproate treatment, while it was decreased by about 60% in BD NPCs. The experiments were performed in three independent technical repeats (n=3). All data are presented as mean ± SEM. Statistical analyses are in Additional file 1: Table S1.

or valproate could change the expression levels of several miRNAs in the rat hippocampus and human lymphoblastoid cell lines [33, 34]. Therefore, we decided to investigate whether miR-1908-5p expression could also be affected by these drugs in human neural progenitor cells (NPCs). First, we established two human NPC lines derived from dermal fibroblasts of either a control or a BD subject. We then treated the cultured NPCs with vehicle or 1 mM lithium or valproate for a week (Fig. 3a). During this period, no overt difference in growth and morphology of the NPCs was observed among the different treatment conditions (data not shown). After the chronic drug treatment, total RNAs were purified and processed for quantitative real-time reverse transcription PCR (qRT-PCR) to measure the levels of mature miR-1908-5p. We found that valproate, but not lithium, increased miR-1908-5p expression by about 70% in control NPCs (Fig. 3b). In contrast, valproate, but not lithium, decreased miR-1908-5p expression by about 60% in BD NPCs (Fig. 3b). Together, we found that miR-1908-5p expression might be possibly affected in the opposite direction in control and BD NPCs after chronic treatment of valproate. Further experiments with more control and BD NPC lines are necessary to confirm this intriguing but preliminary result.

DISCUSSION

BD is a highly heritable (at least 80%) and polygenic disease, meaning that there are many risk alleles with small effects [6, 35]. Therefore, understanding the common functional pathways of BD-associated genes, rather than focusing on each, might provide better insight into the key pathophysiology of BD. Indeed, abnormalities in calcium signaling have been considered as a potential pathogenic mechanism for BD based on the identification of both common and rare variants in genes encoding calcium channels [6, 36]. In a similar manner, 'synaptic pathology' has been proposed to be involved in the pathogenesis of BD. For example, BD-associated *ANK3* gene encodes ankyrin-G protein that localizes to dendritic spines, tiny protrusions on neuronal dendrites representing excitatory post-synapses, to regulate their structure and function [5]. In addition to the genetic association, altered expression of synaptic proteins, and abnormalities in synaptic morphology and function have been observed in postmortem human brains and animal models of BD [7-10]. Our study provides another piece of evidence supporting this 'synaptic pathology' hypothesis by demonstrating that a recently identified BD-associated and primate-specific miRNA, miR-1908-5p, could regulate the expression of genes functioning in neuronal glutamatergic synapses.

Notably, none of the synaptic target genes of miR-1908-5p identified in this study were listed in the original study by Forstner et al. [15] where they used the previous version of TargetScan (Release 6.2) that considered the conservation of miRNA binding sites in 3'UTRs. Their approach could have missed meaningful targets of miR-1908-5p, especially considering that miR-1908-5p expression itself is limited to humans and other primates. Indeed, we found that the validated miR-1908-5p binding sites in the *GRM4* 3'UTR are also poorly conserved. In addition, Lopez et al. recently demonstrated that a primate-specific miRNA, miR-1202, regulates *GRM4* expression through a poorly conserved binding site in the 3'UTR, and could be involved in major depressive disorder [29]. Therefore, for the poorly conserved and disease-associated miRNAs, investigating the putative targets relevant to the disease, regardless of the binding site conservation, might be a better initial approach [16].

Among the miR-1908-5p synaptic targets, *GRIN1* and *GRM4* were previously shown to be associated with BD [28, 37]. *GRIN1* encodes ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) type subunit 1 (GluN1), an essential subunit of heteromeric NMDA receptors in glutamatergic post-synapses. Our bioinformatic analysis of human brain expression profiles revealed that there is a strong inverse-correlation between miR-1908-5p and *GRIN1* in the ventrolateral prefrontal cortex (VFC), anatomical and functional changes of which have been associated with the abnormal emotional processing in BD [38, 39]. Meanwhile, the striatum showed the strongest inverse-correlation between miR-1908-5p and *GRM4*. The striatum is a critical component of the brain reward circuitry, and its biochemical, anatomical and functional changes have been observed in BD [39-41]. Therefore, it is tempting to speculate that miR-1908-5p might be, at least partly, involved in BD pathogenesis by regulating the expression of *GRIN1* and *GRM4* in these brain regions and thereby affecting their synaptic function.

As a preliminary study, we compared the expression levels of miR-1908-5p in human NPCs derived from dermal fibroblasts of control and BD subjects under vehicle- and drug-treated conditions. Interestingly, we found that chronic treatment with valproate, but not lithium, increased the miR-1908-5p expression in control NPCs. In contrast to the control NPCs, valproate treatment decreased miR-1908-5p expression in BD NPCs. Although it is known that lithium and valproate exert their therapeutic efficacy by targeting both common and specific biological pathways [42-44], the detailed mechanisms that explain how miR-1908-5p expression was selectively affected by valproate, and how NPCs from control and BD subjects showed the opposite changes are not clear at this moment. Recently, Kuang et al. showed

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that miR-1908 expression is not correlated with its host gene, *FADS1*, in human adipocytes [45]. Instead, miR-1908 has its own promoter regions where NF-kappaB activated by tumor necrosis factor α (TNF-α) binds and regulates the transcription of miR-1908 [45]. TNF-α is a pro-inflammatory cytokine and involved in various cellular processes by binding to specific receptors, TNFR1 and TNFR2 [46, 47]. One of the downstream action of these receptors is activation of transcription factors like NF-kappaB [48]. Notably, it has been repeatedly reported that BD patients, especially during manic episodes, show elevated serum levels of inflammatory markers including TNF-α [46, 49, 50]. Therefore, it will be interesting in future studies to investigate the role of miR-1908-5p in mediating the effect of elevated TNF-α during BD pathogenesis and the potential interaction between valproate and TNF-α signaling [51] in regulating miR-1908-5p expression during BD treatment.

In conclusion, we focused on a recently identified BD-associated miRNA, miR-1908-5p, and identified and validated its novel target genes functioning in neuronal glutamatergic synapses. Further studies, such as functional experiments using human induced pluripotent stem cell (iPSC)-derived neurons, will help us better understand the role of miR-1908-5p in synaptic function and its potential implications for BD. We also propose that similar approaches could be applied to other BD-associated miRNAs, such as miR-499, miR-708, and miR-2113 [4, 15] since none of their target mRNAs has been directly validated yet. When combined, these studies will provide more insights into the molecular basis of BD pathophysiology and potentially into better diagnostic and therapeutic approaches for this devastating mental illness.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from the subjects. This study was approved by the Korea University Anam Hospital Institutional Review Board.

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Bipolar disorder associated microRNA, miR-1908-5p, regulates the expression of genes functioning in neuronal glutamatergic synapses

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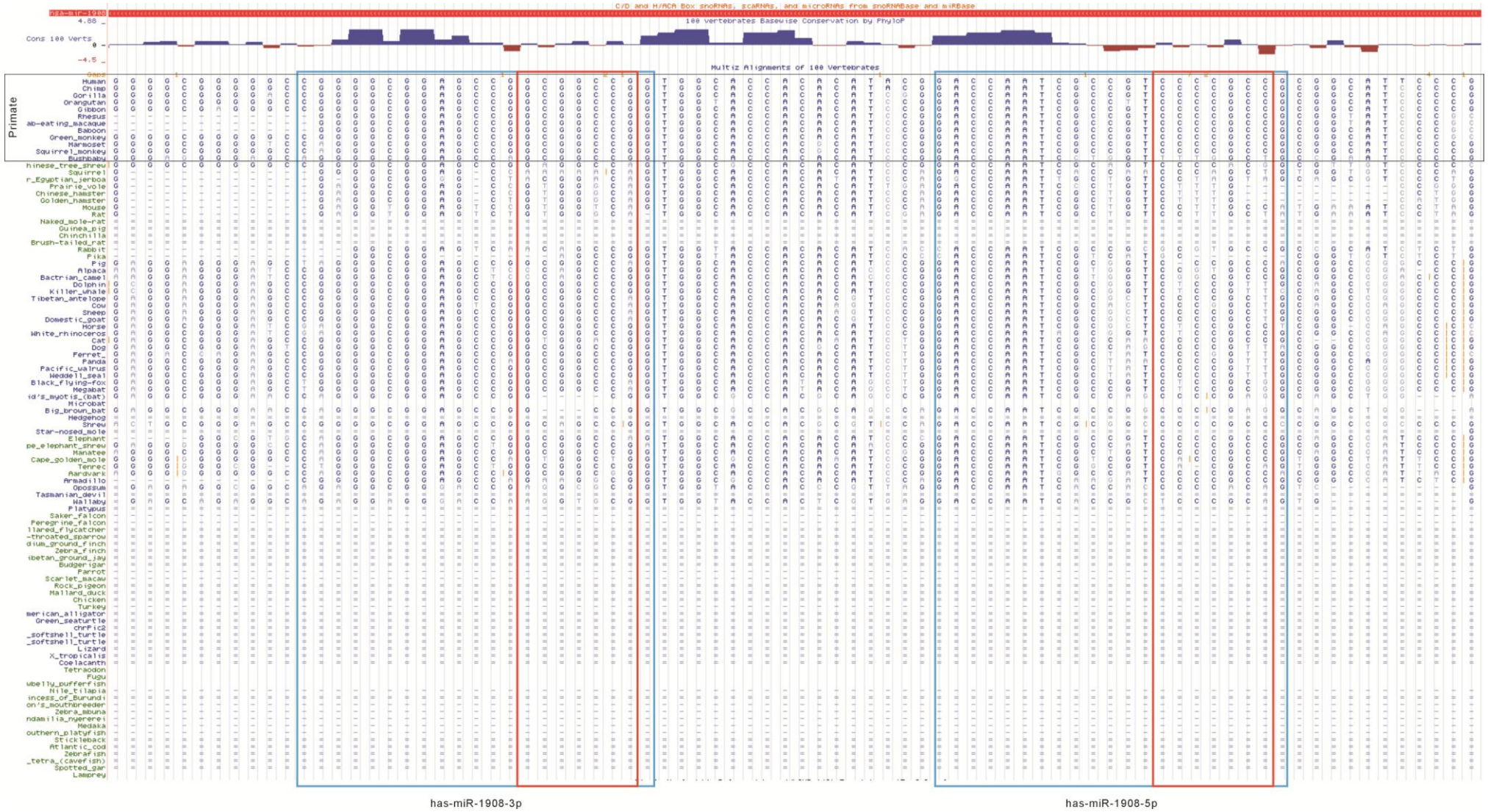
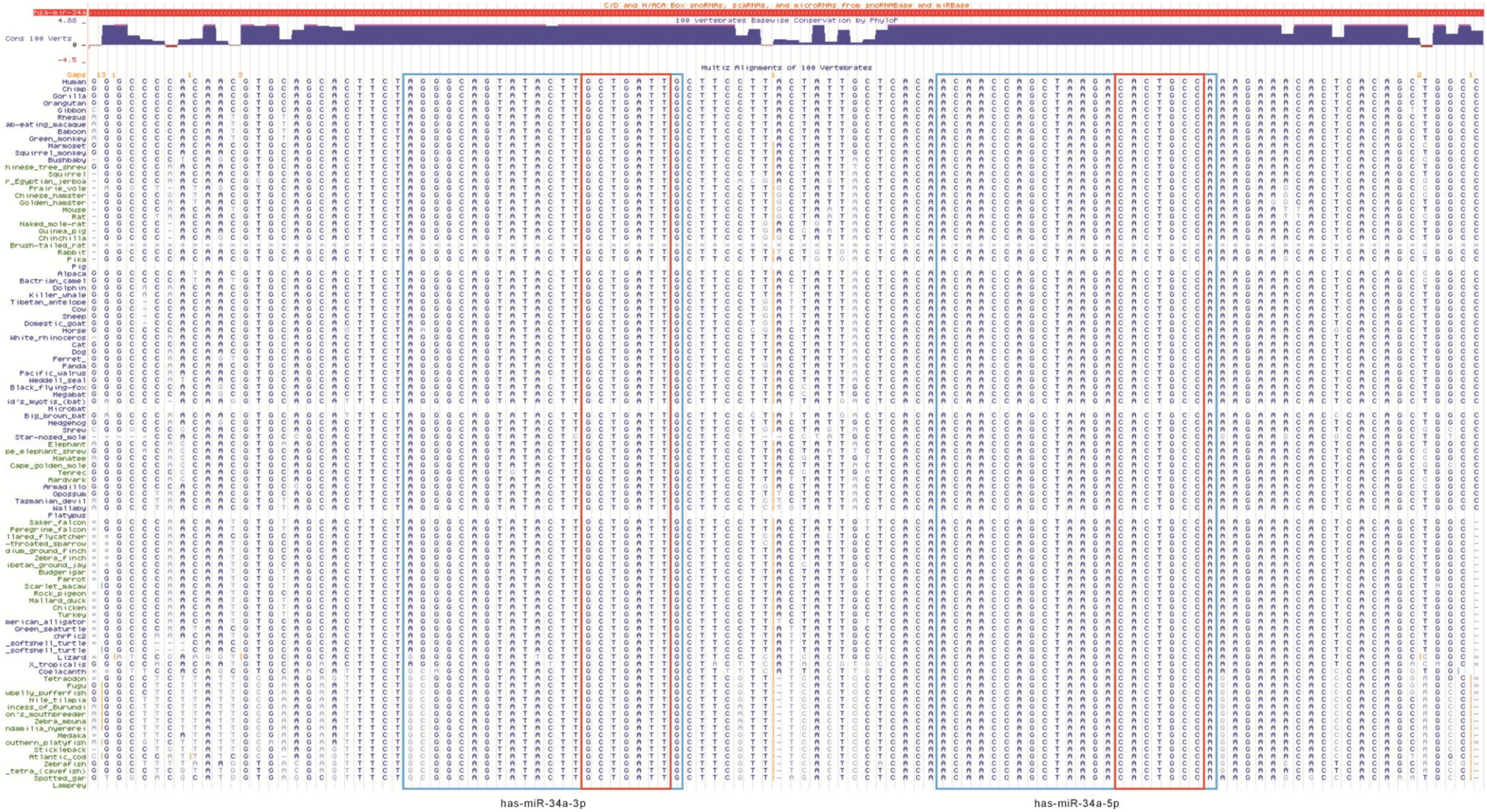
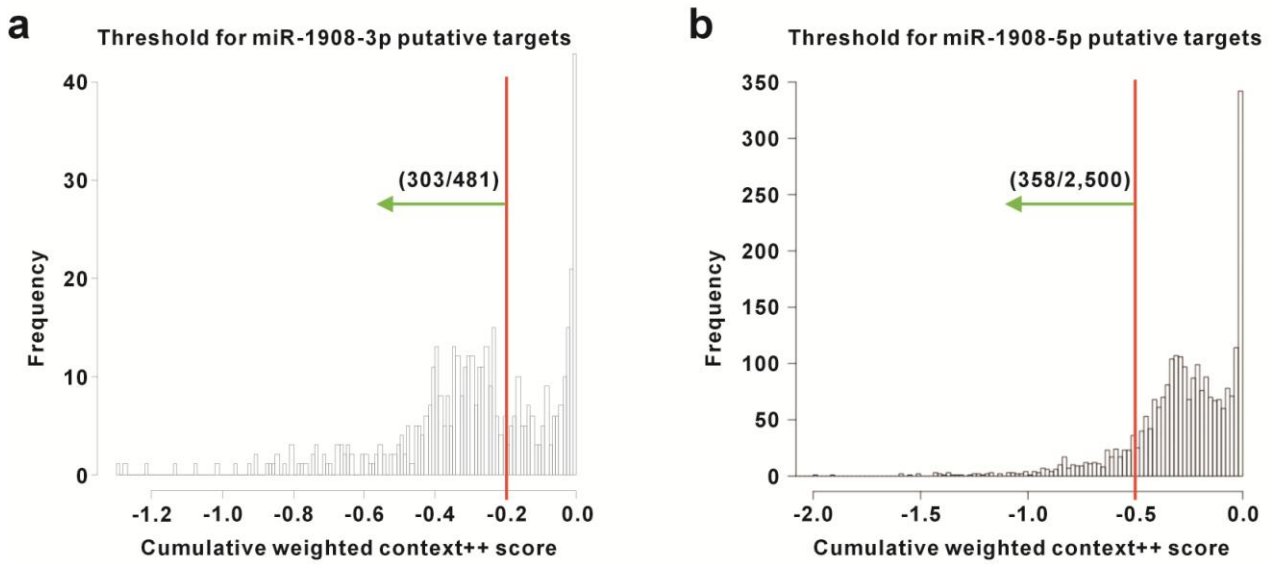
Supplementary Figure 1 Conservation of the miR-1908-3p and miR-1908-5p sequences among 100 aminal genomes. The red and blue boxes indicate seed and full sequences of the mature miRNAs, respectively.



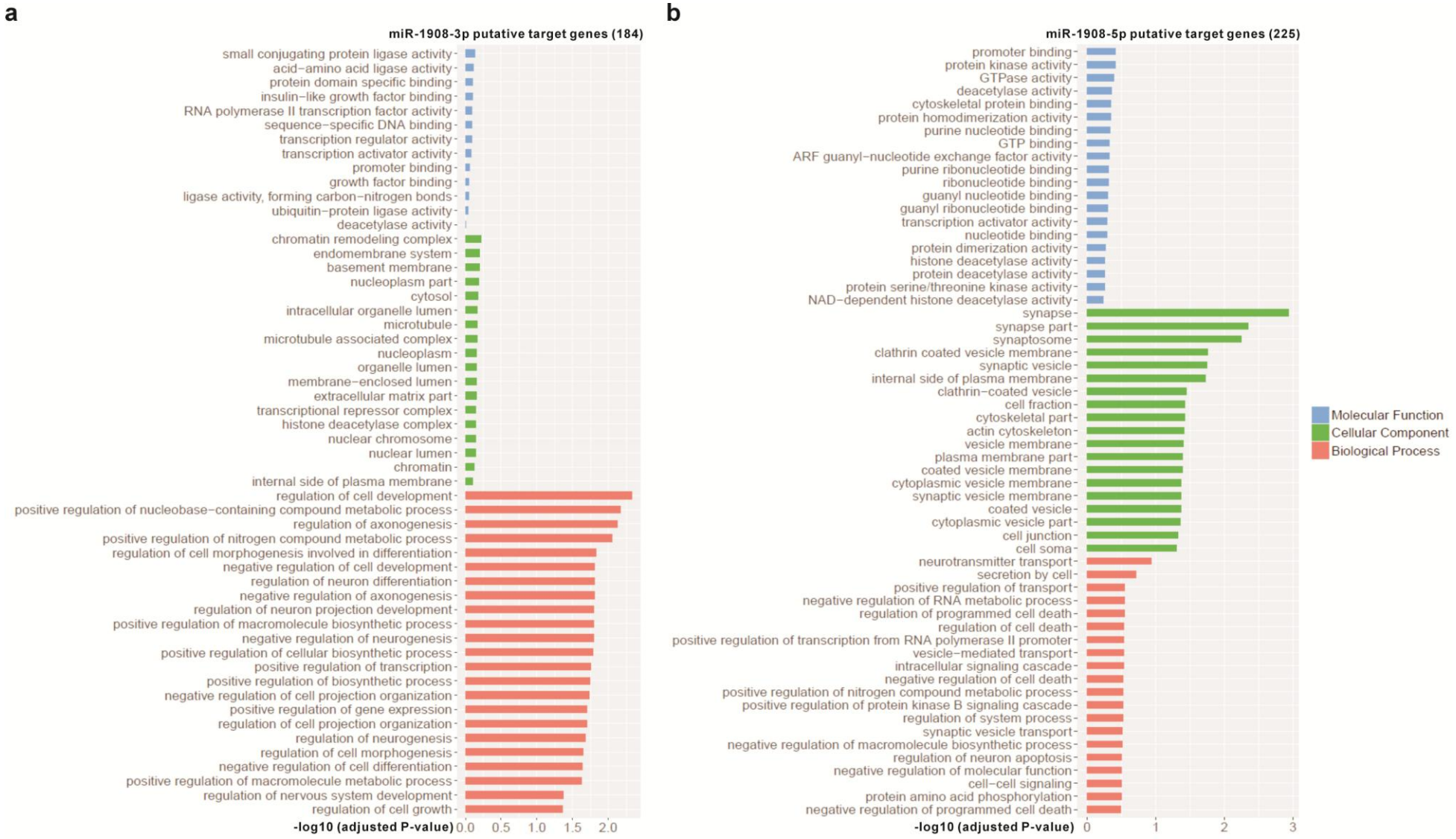
Supplementary Figure 2 Conservation of the miR-34a-3p and miR-34a-5p sequences among 100 aminal genomes. The red and blue boxes indicate seed and full sequences of the mature miRNAs, respectively.



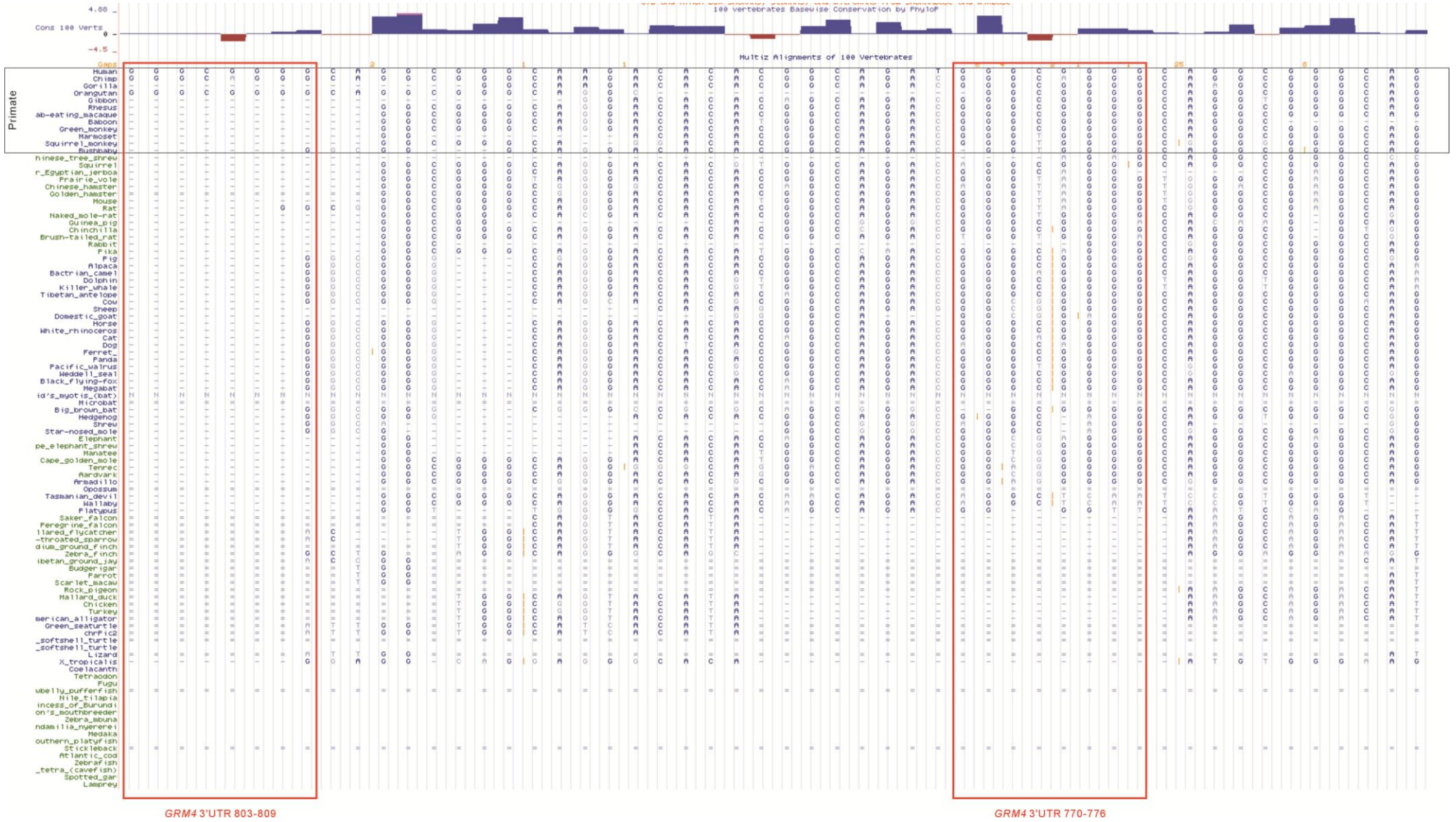
Supplementary Figure 3 The thresholds of context++ scores for miR-1908-3p and miR-1908-5p putative target genes predicted by TargetScan. The thresholds of -0.2 and -0.5 were selected for miR-1908-3p and miR-1908-5p to narrow down the target gene list to 303 and 358, respectively.



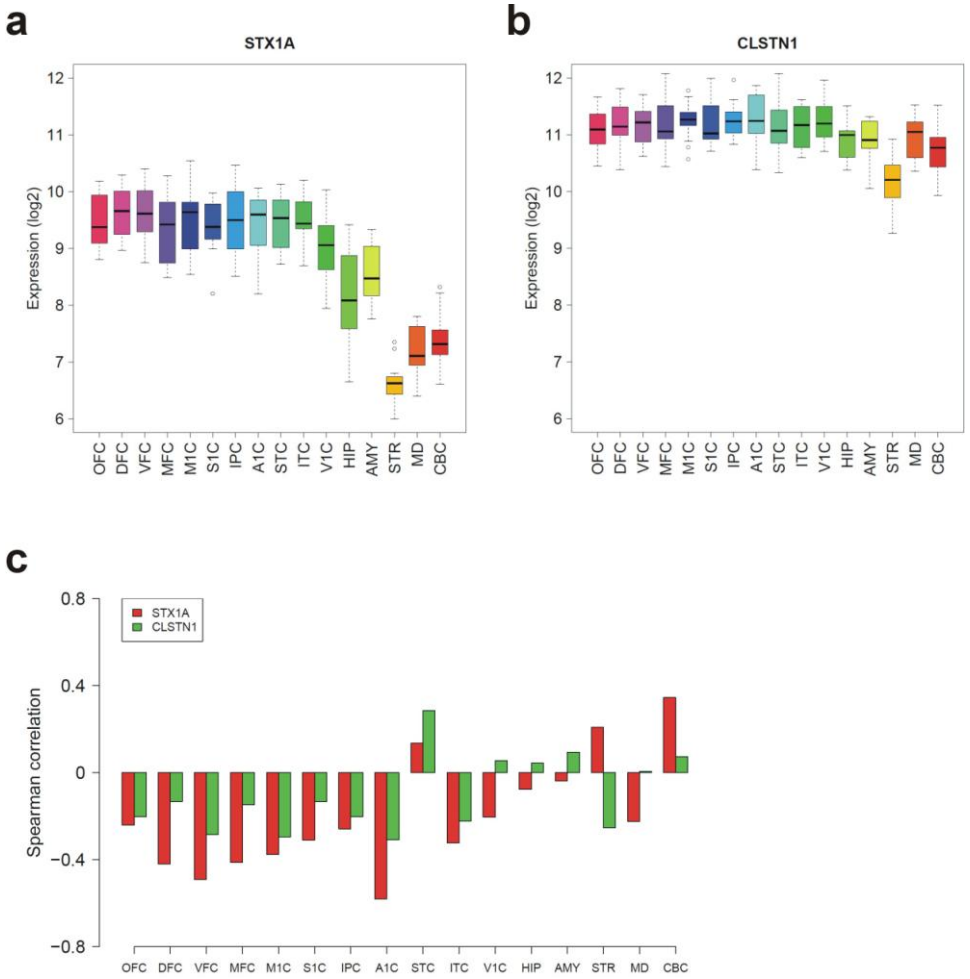
Supplementary Figure 4 GO analysis of miR-1908-3p and miR-1908-5p target genes. There was no significant term in the Molecular Function and Cellular Component categories from miR-1908-3p targets (a). There was no significant term in the Molecular Function and Biological Process categories from miR-1908-5p targets (b).



Supplementary Figure 5 Conservation of the first (770-776) and second (803-809) miR-1908-5p binding sites in the *GRM4* 3'UTR among 100 aminal genomes. The red boxes indicate miR-1908-5p seed binding sequences.



Supplementary Figure 6 Human brain expression of *STX1A* and *CLSTN1*, and their Spearman's correlations with miR-1908-5p. The expression distribution of *STX1A* (a) and *CLSTN1* (b) in 16 human brain regions. (c) Bar plots showing Spearman's correlations between the expression level of miR-1908-5p and those of *STX1A* and *CLSTN1* in 16 human brain regions.



Supplementary Table 1 Summary of statistical analyses for the experiments.

Assay/Measurement	Values (mean±SEM, n)	Statistical test and P values	Figure
Luciferase assay for miR-1908-5p synaptic target genes	*DLGAP4*, control miR (1±0.03, 6) *DLGAP4*, miR-1908-5p (0.76±0.03, 6) *GRIN1*, control miR (1±0.04, 6) *GRIN1*, miR-1908-5p (0.63±0.02, 6) *STX1A*, control miR (1±0.04, 6) *STX1A*, miR-1908-5p (0.78±0.04, 6) *CLSTN1*, control miR (1±0.05, 6) *CLSTN1*, miR-1908-5p (0.73±0.04, 6) *GRASP*, control miR (1±0.04, 6) *GRASP*, miR-1908-5p (1.14±0.06, 6) *GRM4*, control miR (1±0.03, 6) *GRM4*, miR-1908-5p (0.62±0.03, 6)	Unpaired two-tailed Student's t-test, **P<0.01, ***P<0.001	Figure 1d
Luciferase assay for *GRM4* binding sites	*GRM4* WT, control miR (1±0.04, 6) *GRM4* WT, miR-1908-5p (0.76±0.04, 6) *GRM4* M1, control miR (1±0.02, 6) *GRM4* M1, miR-1908-5p (0.82±0.03, 6) *GRM4* M2, control miR (1±0.03, 6) *GRM4* M2, miR-1908-5p (0.88±0.04, 6) *GRM4* M1/2, control miR (1±0.06, 6) *GRM4* M1/2, miR-1908-5p (0.93±0.09, 6)	Unpaired two-tailed Student's t-test, *P<0.05, **P<0.01, ***P<0.001	Figure 1e
qRT-PCR for miR-1908-5p in human NPCs	Control, vehicle (1±0.00, 3) Control, lithium (1.21±0.09, 3) Control, valproate (1.68±0.08, 3) BD, vehicle (1±0.00, 3) BD, lithium (1.00±0.15, 3) BD, valproate (0.43±0.09, 3)	Unpaired two-tailed Student's t-test, *P<0.05	Figure 3b