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MicroRNA 101b Is Downregulated in the Prefrontal Cortex of a Genetic Model of Depression and Targets the Glutamate Transporter SLC1A1 (EAAT3) *in Vitro*

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Abstract

Background: MicroRNAs (miRNAs) are small regulatory molecules that cause translational repression by base pairing with target mRNAs. Cumulative evidence suggests that changes in miRNA expression may in part underlie the pathophysiology and treatment of neuropsychiatric disorders, including major depressive disorder (MDD).
Methods: A miRNA expression assay that can simultaneously detect 423 rat miRNAs (miRBase v.17) was used to profile the prefrontal cortex (PFC) of a genetic rat model of MDD (the Flinders Sensitive Line [FSL]) and the controls, the Flinders Resistant Line (FRL). Gene expression data from the PFC of FSL/FRL animals (GEO accession no. GSE20388) were used to guide mRNA target selection. Luciferase reporter assays were used to verify miRNA targets in vitro.
Results: We identified 23 miRNAs that were downregulated in the PFC of the FSL model compared with controls. Interestingly, one of the identified miRNAs (miR-101b) is highly conserved between rat and human and was recently found to be downregulated in the PFC of depressed suicide subjects. Using a combination of *in silico* and *in vitro* analyses, we found that miR-101b targets the neuronal glutamate transporter SLC1A1 (also known as EAAC1 or EAAT3). Accordingly, both mRNA and protein levels of SLC1A1 were found to be upregulated in the PFC of the FSL model.
Conclusions: Besides providing a list of novel miRNAs associated with depression-like states, this preclinical study replicated the human association of miR-101 with depression. In addition, since one of the targets of miR-101b appears to be a glutamate transporter, our preclinical data support the hypothesis of a glutamatergic dysregulation being implicated in the etiology of depression.

Keywords: epigenetics, miRNA, depression, EAAC1, DCBXA

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Significance Statement

MicroRNAs (miRNAs) are small non-coding RNA molecules that cause messenger RNA silencing. In the present study, we found miRNA changes in an established rodent model of major depressive disorder (MDD). Interestingly, one of the dysregulated miRNA (miR-101) was recently also identified as being affected in postmortem brain samples of human MDD subjects. To further our understanding on miR-101b's function, we used *in vitro* assays and found that it targets the neuronal glutamate transporter SLC1A1 (also known as EAAC1 or EAAT3). These preclinical findings suggest a glutamatergic dysregulation in MDD and support the literature showing efficacy of novel antidepressant compounds that act as glutamate receptor modulators.

Introduction

MicroRNAs (miRNAs) are a family of small, on average 22 nucleotides long, non-coding RNAs that regulate gene expression at the posttranscriptional level (Winter et al., 2009). Most known mammalian miRNAs are expressed in a tissue-specific manner, cause translational repression, and can individually target hundreds of genes (He and Hannon, 2004). A known mechanism, through which miRNAs exert their repressive function, involves their incorporation into the RNA-induced silencing complex (RISC), which uses the miRNA's seed region (nucleotides 2–8 at the 5' end of the miRNA) as a template for recognizing complementary sites in the 3' untranslated region (UTR) of target mRNAs (Winter et al., 2009). The latter mRNA targeting by miRNAs leads to mRNA degradation, destabilization, or translational inhibition (Winter et al., 2009).

The human genome codes for thousands of miRNAs (Londin et al., 2015), and over 60% of protein-coding genes seem to have been under selective pressure to maintain miRNA pairings (Friedman et al., 2009). Not unexpectedly, miRNAs have been found to play an important role in human diseases, including cancer, cardiovascular disease, and other pathological conditions involving stress responsive pathways (Mendell and Olson, 2012). Within the nervous system, miRNAs are thought to contribute to brain development, neural function, and synaptic plasticity (Forero et al., 2010). Dysregulation of specific miRNAs has been observed in both patients and animal models of neuropsychiatric disorders, including addiction, anxiety, autism, and schizophrenia (Forero et al., 2010; Miller and Wahlestedt, 2010; Moreau et al., 2011; Chan and Kocerha, 2012). A critical component necessary for miRNA biogenesis, Dicer1, has also been implicated in posttraumatic stress and anxiolytic responses (Dias et al., 2014; Wingo et al., 2015).

With regard to major depressive disorder (MDD), the evidence supporting a miRNA involvement in the pathophysiology and the treatment of the disorder is increasing (Dwivedi, 2016). More specifically, genetic polymorphisms in different miRNAs (e.g., miR-30e and miR-182) have been associated with MDD (Saus et al., 2010; Xu et al., 2010). In addition, certain miRNAs (e.g., miR-16, miR-135, miR-335, and miR-1202) have been found to contribute to the therapeutic action of antidepressants, including that of selective serotonin re-uptake inhibitors, tricyclics, and ketamine (Baudry et al., 2010; Launay et al., 2011; O'Connor et al., 2013; Issler et al., 2014; Lopez et al., 2014; Li et al., 2015). Separate studies also found that the expression levels of a number of miRNAs were changed in blood samples (Bocchio-Chiavetto et al., 2013; Fan et al., 2014) and fibroblasts (Garbett et al., 2015) of patients with MDD. Finally, a miRNA expression study that profiled the prefrontal cortex (PFC) of antidepressant-free depressed suicide subjects found 21 downregulated miRNAs, including miR-101 (Smalheiser et al., 2012), and one study showed increased miR-511 levels in basolateral amygdala from depressed subjects (Yang et al., 2014).

In the present study, we used a well-established genetic rat model of MDD (the Flinders Sensitive Line [FSL]) and its controls (the Flinders Resistant Line [FRL]) to provide further insights into a possible miRNA dysregulation in depression-like states. FSL rats exhibit some key characteristics of MDD, including anhedonia, emotional memory impairment, and psychomotor retardation, all of which are reversed by antidepressant treatment (Overstreet et al., 2005; Eriksson et al., 2012). At the molecular level, FSL animals have reductions in levels of serotonin receptors (Eriksson et al., 2012) and other key molecules associated with MDD, including BDNF, NPY, and P11 (Melas et al., 2012a, 2012b, 2013; Wei et al., 2015). The FSL also exhibits a dysfunctional regulation of glutamate transmission and has a reduction of both neuronal and glial glutamate receptors and transporters (Eriksson et al., 2012; Gomez-Galan et al., 2013). This is of relevance, given the accumulating evidence that suggests an aberrant glutamatergic signaling in humans with MDD (Sanacora et al., 2012) in combination with the promising role of novel antidepressant compounds that act as glutamate receptor modulators (Caddy et al., 2015).

Methods

Animals

Adult, 3-month-old, male FSL and FRL rats (n = 6–7/group) were used for this study. The number of animals used for each experiment is denoted in the respective methodological section or figure legend. PFC regions and hippocampi of all animals were dissected according to Glowinski and Iversen (1966) and immediately stored at -80°C until subsequent experimental analyses. All experiments were approved by the Danish National Committee for Ethics in Animal Experimentation and the Ethical Committee for protection of animals at the Karolinska Institutet.

RNA Extraction and Reverse Transcription

Total RNA from the PFC and one hippocampus of each FSL/FRL animal were extracted using the miRNA Universal Kit (Qiagen) followed by treatment with DNase I (Qiagen) to digest contaminating DNA. Concentrations were determined using the NanoDrop ND-1000 (NanoDrop Technologies Inc.). For miRNA analyses, complementary DNA (cDNA) was synthesized using the Universal cDNA Synthesis Kit II for RT-PCR (Exiqon) according to the manufacturer's protocol. UniSp6 RNA was spiked in for monitoring conversion efficiency. For mRNA analyses, cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen; Life Technologies) according to the manufacturer's protocol. cDNA and RNA samples were stored at -20°C and -80°C, respectively, until further processing.

miRNA Profiling

The nCounter Rat miRNA Expression Assay (NanoString Technologies, Inc.), which includes 423 rat miRNAs derived from miRBase (version 17), was used to profile miRNA expression in the PFC of FSL/FRL animals. In brief, 100 ng of total RNA in 5 µL (n = 6 FSL, n = 6 FRL) was used for hybridizations of each miRNA assay according to the manufacturer's instructions. The miRNA raw data were normalized for lane-to-lane variation using a dilution series of 6 spike-in positive controls. The normalization factor for each lane was calculated as follows: the sum of the 6 positive controls for a given lane, divided by the average sum across lanes. The normalization factor was then multiplied by the raw counts of each lane to produce a normalized value. The background level of each lane was defined as 2 SD above the signal of the no-target sequence (negative) controls. We included a miRNA probe in the subsequent analyses only if at least 5 of 6 samples, of either FSL or FRL, had levels above the background value.

Gene Expression Microarray Data and Pathway Analysis

In a previous study, Blaveri et al. (2010) used 2 independent cohorts of male FSL and FRL animals to conduct a genome-wide mRNA expression profiling of the hippocampus and PFC. For the purposes of their study, they used the Affymetrix Rat Genome 230 2.0 GeneChips and deposited the microarray data in the GEO microarray database (accession no. GSE20388). To guide our selection of putative miRNA targets, we retrieved the microarray PFC data sets and reprocessed them using a mixed model ANOVA according to the initial publication (Blaveri et al., 2010). Since we found a main downregulation of miRNAs in the PFC of FSL animals, we further filtered the microarray probe-set to include only the mRNAs that showed upregulation in the FSL in both analyzed cohorts with a combined P value of $< .05$. The filtered probe-sets were then imported into the Ingenuity Pathway Analysis (IPA; Qiagen) for miRNA target predictions and also for analyzing pathways, diseases, and biofunctions, including molecular and cellular functions and physiological system development and functions.

Coexpression Analysis

A miRNA coexpression analysis was performed based on the method in Smalheiser et al. (2011) with a minor modification. MiRNA pairs were filtered based on 3 criteria: (1) they showed significant pairwise expression level correlations ($r > 0.8$) across the individual rats in the FSL group; (2) the corresponding pairwise correlations in the FRL group were significantly less than those in the FSL group ($rFSL - rFRL > 0.8$); and (3) the miRNA pairs were not significantly negatively correlated in the FRL group ($rFRL > -0.8$).

Validation of miRNAs and mRNA Expression Levels

Validation experiments of miRNA hits and measurements of putative mRNA targets, in the PFC of FSL/FRL animals, were performed using quantitative real-time PCR (qRT-PCR). All qRT-PCR amplifications, as well as that in hippocampal cDNA, were performed in triplicates using Power SYBR Green (Applied Biosystems) on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the following conditions: 95°C for 10 minutes, followed by 40 repeats of 95°C for 15 seconds (10 seconds for miRNA amplification), 60°C for 1 minute, and a final dissociation stage to monitor amplification specificity. For miRNA expression analyses, LNA PCR primers (Exiqon) were used to amplify miRNAs of interest, and the data were normalized to *Rnu5g*. For mRNA expression analyses, conventional primers were used and data were normalized to 2 reference genes (*Mff*, mitochondrial fission factor; and *Cdipt*, CDP-diacylglycerol--inositol 3-phosphatidyltransferase). Relative gene expression quantifications were calculated using the qBase software (version 1.3.4) (Hellemans et al., 2007). The tested genes and corresponding primer pair sequences are listed in supplementary Table 1.

Protein Expression Levels

Protein expression levels of SLC1A1 (also known as EAAC1, EAAT3, or DCBXA), in the PFC of FSL/FRL animals, were quantified using Western blotting as previously described (Wei et al., 2015). Primary antibody incubations were performed overnight at 4°C with a mouse monoclonal anti-EAAT3 antibody (1:1000 dilution, ab78395; Abcam) and with a mouse monoclonal anti-β-actin antibody (1:10,000 dilution, A5316; Sigma-Aldrich) as loading control. Membranes were exposed to the Amersham ECL Plus Western Blotting Detection reagent (GE Healthcare), and immunoreactive bands were detected using the Amersham Hyperfilm ECL (GE Healthcare). Optical densities were quantified using the NIH ImageJ software (1.47 version). SLC1A1 protein expression levels were normalized to the expression levels of β-actin and the data were presented as relative quantifications.

In Vitro Verification of miRNA Targets

To examine whether SLC1A1 is a direct target of miR-101b, we used LightSwitch 3'UTR luciferase reporter constructs (SwitchGear Genomics). HEK 293 cells were co-transfected with 100 ng of a human SLC1A1 GoClone 3'UTR reporter (S810654, SwitchGear Genomics) and either 50 nM of a miR-101b mimic or 50 nM of a miRNA non-targeting control (Switchgear Genomics) using DharmaFECT Duo transfection reagent (GE Dharmacon). An empty vector (no 3'UTR; SwitchGear Genomics) was used as a control. Luciferase activity was measured on a plate luminometer 24 hours after transfection by adding 100 µL of LightSwitch Assay Reagent (SwitcGear Genomics). In total, the experiment was replicated 3 times with at least 3 biological replicates each time.

Statistical Analyses

Data in the bar graphs are presented as mean values ± 1 SEM. The normal distribution of the data and the homogeneity of variances were tested using the Shapiro-Wilk and Levene's tests, respectively. The miRNA expression levels of FSL vs FRL, generated by the NanoString nCounter Rat miRNA assay, were compared using the Mann-Whitney U-test. The difference of miRNA expression levels, generated by RT-PCR, between FSL and FRL was assessed using the 2-tailed Student's t test if the variances in FRL and FSL were equal or was otherwise assessed using the Mann-Whitney U test. The difference in miRNA expression variation between FSL vs. FRL was assessed by comparing the coefficients of variance of individual miRNAs using Mann-Whitney U-test. The threshold for statistical significance was set at $P < .05$. All analyses were performed using IBM SPSS Statistics version 22 (IBM Corporation). Extreme values are defined as potential outliers using box-plot function from SPSS software that are denoted in the figure legends and were excluded from the analyses.

Results

Downregulation of miRNAs in the PFC of the FSL Animals

To detect putative changes in miRNA expression in the PFC of FSL (vs control FRL) animals, we used a miRNA expression assay that enables multiplex detection of >400 rat miRNAs. After data normalization, only 121 miRNAs were above the background level in both rat strains, in agreement with the fact that most miRNAs are expressed in a tissue-specific manner (He and Hannon, 2004). We found a global miRNA expression decrease in the FSL animals that is characterized by depression-like behavior (supplementary Table 2). By plotting the relationship between the standard deviations and the mean expression values across all expressed miRNAs, we also observed that the miRNA expression in the FSL rats was significantly more variable compared with control FRL ($P<.001$) (Figure 1). Using individual tests of statistical significance, we identified 23 miRNAs with lower (at least 30%) expression levels in the FSL rats ($P<.05$, Benjamini-Hochberg False Discovery Rate < 0.22; for the full list of miRNAs, see supplementary Table 2). To validate these observations, we selected 9 miRNAs that showed differential expression and performed individual qRT-PCR experiments. The latter analyses confirmed that all selected miRNAs had decreased expression levels in the FSL, with 8 of the 9 miRNAs reaching statistical significance (Figure 2). We also performed a coexpression analysis and found that in total, 244 miRNA pairs had correlated expression levels only in the FSL group. Of those, 51 miRNAs were significantly coexpressed with at least 3 other miRNAs in this set (supplementary Table 3), including 7 of the 23 miRNAs that were significantly decreased in the FSL. Several of these 51 miRNAs belong to the let-7 miRNA family (let-7a, 7b, 7c, 7d, 7f, and 7i). Some miRNAs (miR-125a-3p and miR-125a-5p, miR-125b-3p and miR-125b-5p, rno-miR-3563-3p and miR-3563-5p) derive from the same precursor miRNA.

Pathway Analyses and miRNA Target Predictions

Since miRNAs have been shown to correlate negatively with levels of target mRNAs (Winter et al., 2009), the miRNA downregulation observed in the FSL model may lead to an upregulation of their mRNA targets. Among the 23 differentially expressed miRNAs, 16 had information about targets in IPA. In a previous study, Blaveri et al. (2010) used PFC regions of the FSL/FRL model to conduct a genome-wide mRNA profiling study. After retrieving the latter microarray dataset, we reanalyzed the data by focusing on the probe sets that showed a significant upregulation ($P<.05$) in the FSL. Next, we tested for an overlap between the latter mRNA candidates and the targets of the 16 differentially expressed miRNAs using IPA. The analysis revealed 1392 potential mRNA targets that were either predicted *in silico* or had been experimentally observed (supplementary Table 4). The core analysis in IPA suggested that the target genes are involved in canonical pathways involved in protein ubiquitination, Ephrin receptor signaling, and Dopamine-DARPP32 signaling (Table 1). These genes were also associated with neurological diseases, psychiatric and psychological disorders, and monogenic disorders (Table 1).

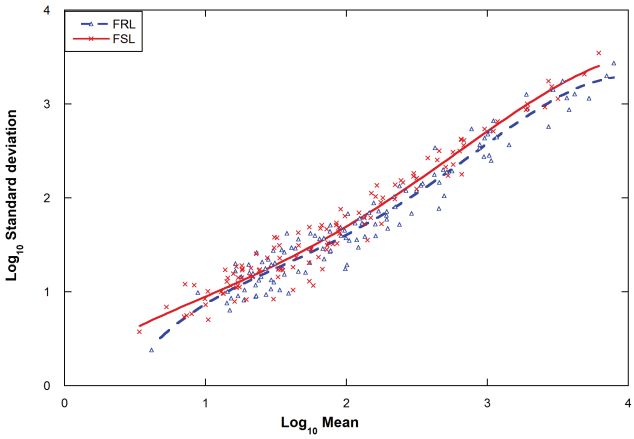

Figure 1. Plot of $\log_{10}$Mean vs $\log_{10}$Standard deviation based on the microRNA (miRNA) assay expression levels in the prefrontal cortex (PFC) of the Flinders Sensitive Line (FSL) vs control-Flinders Resistant Line (FRL) animals. MiRNA expression in the FSL depression model showed significantly more variation compared with controls. Polynomial (order = 2) fitting line was added to each rat strain, with the FSL represented by the solid line and the control-FRL by the dashed line.

Gene Expression Measurements of miR-101b Targets

The human homolog of one of the miRNAs that was confirmed to be downregulated in the FSL, miR-101b, was found to be downregulated in the PFC of depressed suicide subjects (Smalheiser et al., 2012). Therefore, miR-101b was considered to warrant further examination. First, we tested if miR-101b showed different expression levels in the hippocampus, another brain region implicated in MDD, of the FSL/FRL model. We found that hippocampal miR-101b expression levels were not different between FSL and FRL ($P=.86$) (supplemental Figure 1). Further, there is high conservation between the mature rat miR-101b and the mature human miR-101, which share the same seed sequence (Figure 3). A total of 248 genes were predicted to be miR-101b targets (supplementary Table 5), among which 103 genes were also part of the IPA pathway genes. From this list, we identified 8 genes that showed high conservation between human and rat in their 3'UTR and were also implicated in depression-related pathways in IPA (including glutamate, GR or GABA receptor signaling, and NFR2-mediated oxidative stress response). The mRNA levels of these 8 genes (*Slc1a1*, *Gabrb2*, *Adcy5*, *Gclc*, *Pbrm1*, *Prkaa1*, *Rac1*, and *Smardca4*) were therefore measured in the PFC of the FSL/FRL animals. We found that 2 genes, *Slc1a1* and *Rac1*, showed significantly increased mRNA levels in the FSL (Figure 4a), in line with the decreased levels of miR-101b.

MiR-101b Targets SLC1A1 in Vitro and Correlates Negatively with SLC1A1 Protein Levels in Vivo

Next, we chose to focus on SLC1A1 (also known as EAAC1 or EAAT3), since it is a neuronal glutamate transporter that is highly enriched in the cortex (Rothstein et al., 1994). In addition, the FSL model is known to exhibit a dysfunctional regulation of glutamate transmission (Gomez-Galan et al., 2013). It was therefore of interest to verify that the elevated mRNA levels of *Slc1a1* in the FSL rats were due to a specific miR-101b targeting of the *Slc1a1* 3'UTR. For this purpose, HEK 293 cells were transfected with a luciferase reporter construct containing the human *SLC1A1* 3'UTR, which contains the same sequence used for miRNA seed complementarity as the rat *Slc1a1* 3'UTR (Figure 3). There was a significant reduction in luciferase activity when the *SLC1A1*-3'UTR luciferase construct was cotransfected with the miR-101b mimic compared with the *SLC1A1*-3'UTR luciferase construct alone ($P=.004$) (Figure 4b) or when the *SLC1A1*-3'UTR luciferase construct was cotransfected with a nontargeting

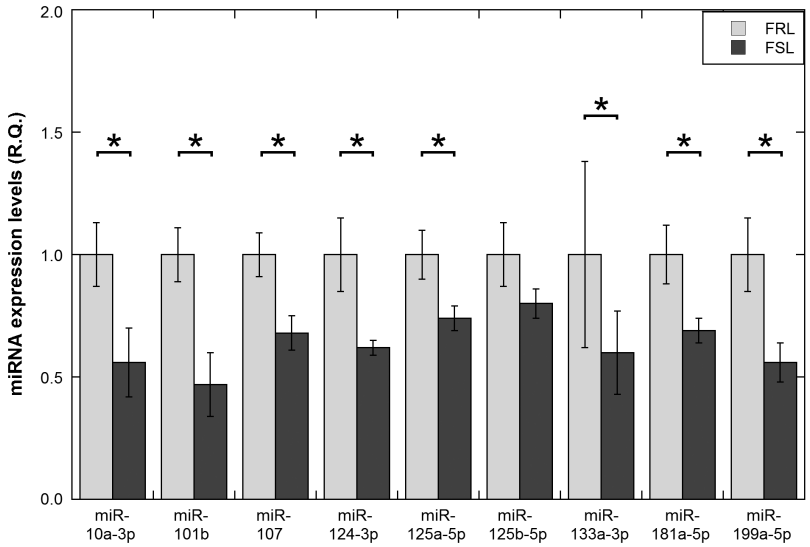

Figure 2. The microRNA (miRNA) assay identified 23 miRNAs with lower expression levels in the prefrontal cortex (PFC) of the Flinders Sensitive Line (FSL) depression model (supplementary Table 2), and we selected 9 of them for individual validation experiments. Eight of the 9 miRNAs reached statistical significance (miR-10a, $P = .046$; miR-101b, $P = .011$; miR-107, $P = .02$; miR-124, $P = .03$; miR-125a, $P = .048$; miR-125b, $P = .2$; miR-133a, $P = .03$; miR-181a, $P = .032$, and miR-199a, $P = .029$). Data are presented as relative quantifications (R.Q.) ± SEM. Levels of *Rnu5g* were used as reference for normalization of mature miRNA expression levels (n = 6–7 animals/group; n = 1 outlier/group),*$P < .05$.

Table 1. Core Analysis Result from IPA

Top Pathways, Diseases, and Biofunction	P-Value/P-Value Range
Top Canonical Pathways	
Protein ubiquitination pathway	4.48E-13
Ephrin receptor signaling	8.56E-07
Dopamine-DARPP32 feedback in cAMP signaling	1.64E-06
Ephrin B signaling	3.26E-06
Axonal guidance signaling	3.42E-06
Top Diseases	
Neurological disease	3.19E-03 - 1.41E-16
Psychological disorders	2.72E-03 - 1.41E-16
Skeletal and muscular disorders	2.72E-03 - 1.99E-16
Hereditary disorder	2.97E-03 - 5.43E-16
Molecular and Cellular Functions	
Cellular assembly and organization	3.26E-03 - 6.36E-24
Cellular function and maintenance	3.26E-03 - 6.36E-24
Molecular transport	2.72E-03 - 8.20E-18
Protein trafficking	5.89E-05 - 1.11E-16
Posttranslational modification	3.31E-03 - 7.54E-16
Physiological System Development and Function	
Organismal survival	9.34E-04 - 2.52E-14
Nervous system development and function	3.22E-03 - 1.78E-13
Tissue development	3.28E-03 - 1.78E-13
Behavior	3.28E-03 - 4.57E-08
Top Networks	
Embryonic development, organismal survival, cell death and survival	
neurological disease, psychological disorders, organismal injury and abnormalities	
free radical scavenging, small molecule biochemistry, hereditary disorder	
cellular assembly and organization, cellular movement, nervous system development and function	

Abbreviation: IPA, Ingenuity Pathway Analysis.

control ($P = .034$) (Figure 4b). The latter results supported the notion of miR-101b directly targeting *Slc1a1*, in line with the miR-101b downregulation and the elevated *Slc1a1* mRNA levels observed in the PFC of the FSL. Finally, since mRNA levels do not always correlate with protein levels (Vogel and Marcotte, 2012), we verified that SLC1A1 protein levels were also upregulated in the PFC of the FSL rats compared with the FRL ($P = .004$) (Figure 4c).

Discussion

There is evidence supporting the miRNA involvement in the pathophysiology and the treatment of MDD (Dwivedi, 2016). In this study, we used the FSL rat model of depression to provide further insights into a possible miRNA dysregulation in depression-like states. By using a miRNA expression-profiling assay, which covers >400 known miRNAs, we first observed that the overall miRNA expression in the PFC of the FSL model was downregulated. This finding coincides with a recent human study that showed a 17% global downregulation of miRNAs in the PFC of depressed suicide subjects (Smalheiser et al., 2012). However, in another rat model of depression, both up- and downregulation of miRNA expression was observed, suggesting an exposure protocol and strain-specific miRNA expression difference (Dwivedi et al., 2015). In contrast, an increase in global miRNA expression was observed in postmortem cortical regions of subjects with schizophrenia, which was found to correlate with an increase in the RNase III Drosha that is involved in the processing of miRNAs (Beveridge et al., 2010). Drosha has been shown to account for widespread downregulation of miRNAs in cancer (Thomson et al., 2006), and our data (Wei et al., 2016) show that the global downregulation of miRNAs observed in the FSL model is also associated with a basal reduction in Drosha levels.

Using individual tests of statistical significance, we identified 23 specific miRNAs that were decreased in the PFC of the FSL model compared with controls. In general, miRNAs are known to exert a translational repressive function that involves their

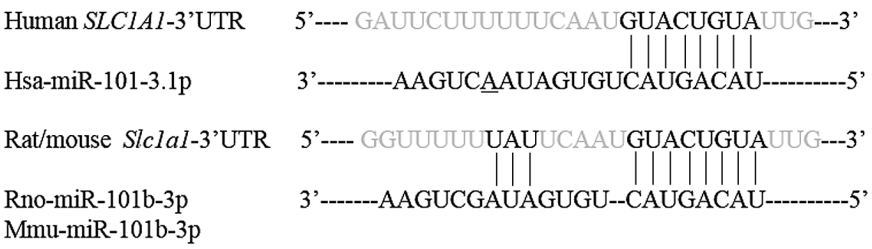

Figure 3. The sequences of the mature miR-101 and the 3' untranslated region (UTR) of the *SLC1A1* gene in human, rat, and mouse is shown. The mature miR-101 sequence is highly conserved between species, with only one nucleotide difference between human and rat/mouse (the nucleotide difference is underlined in the human sequence). The target site in the 3′-UTR of the *SLC1A1* gene was predicted by TargetScan and shows that both the miR-101 seed region (5'-UACAGUAC-3') and the mRNA target site are conserved between human and rat/mouse.

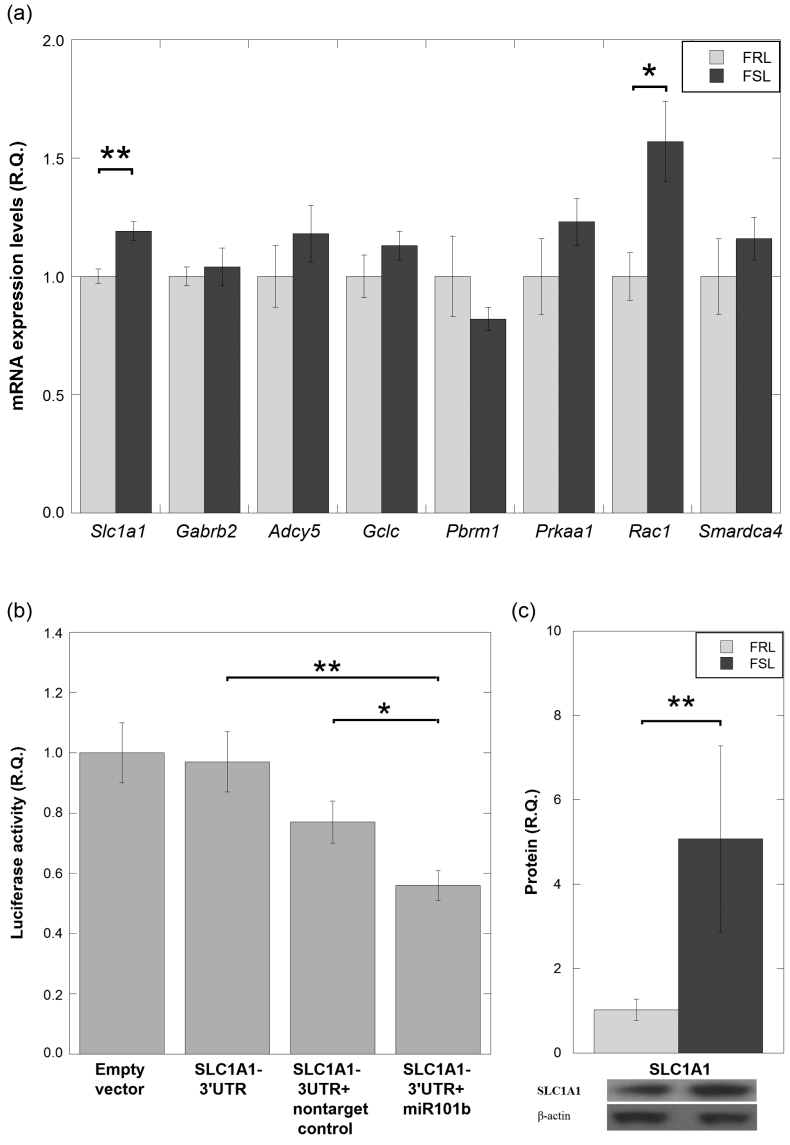

Figure 4. (a) From a list of >200 genes that were predicted to be miR-101b targets (supplementary Table 5), we identified 8 genes that showed high conservation between human and rat in their 3' untranslated region (UTR) and were also implicated in depression-related pathways. The mRNA expression of these 8 genes was quantified in the prefrontal cortex (PFC) of the Flinders Sensitive Line (FSL) and control-Flinders Resistant Line (FRL) animals. Only *Slc1a1* and *Rac1* mRNA levels were significantly increased in the FSL compared with controls (*Slc1a1*: $P = .001$; *Rac1*: $P = .023$; *P*-values of the other 6 genes >.2). (b) Luciferase activity was measured in HEK293 cells transfected with either an empty vector (first bar; positive control), an *SLC1A1*-3'UTR luciferase construct (second bar), an *SLC1A1*-3'UTR luciferase construct cotransfected with a nontargeting control (third bar; negative control), or an *SLC1A1*-3'UTR luciferase construct cotransfected with a miR-101b mimic (fourth bar). MiR-101b significantly inhibited the luciferase activity of the reporter construct containing the 3'UTR (fourth bar) compared with both *SLC1A1* alone (second bar) and *SLC1A1*-3'UTR cotransfected with a nontargeting control (third bar). (c) In line with the increased levels of *Slc1a1* mRNA, the FSL also showed increased protein levels of SLC1A1. Representative immunoblotting images of SLC1A1 and β-actin (loading control) are shown below the graph. Data are presented as relative quantifications (R.Q.) ± SEM. Two reference genes (*Cdipt* and *Mff*) were used for normalization in 4a (n = 5–7 animals/group). *P < .05, **P < .01.

incorporation into the RISC, which uses the miRNA's seed region as a template for recognizing complementary sites in the 3' UTR of target mRNAs (Winter et al., 2009). When we tested for predicted mRNA targets of the significant miRNAs and analyzed the data using pathway analysis software, we found that the putative target genes are involved in protein ubiquitination, Ephrin receptor signaling, and Dopamine-DARPP32 signaling. Protein ubiquitination is known to play an important role in neuronal function (Tai and Schuman, 2008), Ephrin ligands and their receptors regulate synapse formation and neuronal plasticity (Klein, 2009), and Dopamine-DARPP32 is extensively involved in intracellular signaling pathways and integrating dopaminergic and glutamatergic signaling (Girault and Greengard, 2004). Accordingly, the same genes were associated with both neurological and psychiatric disorders, for example MDD, in the pathway analysis. However, besides the overall analysis of the miRNA list, one specific miRNA (miR-101) was of particular interest, since it was found to be downregulated in the PFC of depressed suicide subjects (Smalheiser et al., 2012). Using a combination of *in silico* and *in vitro* analyses, we found that miR-101 targets the 3'UTR of the neuronal glutamate transporter SLC1A1 (also known as EAAC1 or EAAT3). In agreement with this result, both mRNA and protein levels of SLC1A1 were upregulated in the PFC of the FSL model. Whereas decreased levels of SLC1A1 have been previously reported in stress-induced offspring depression (Zhang et al., 2013), this is to our knowledge the first report to show elevated SLC1A1 levels in a depression-like state. Several approaches have been successfully reported to manipulate specific miRNA expression levels that provide possibilities for future *in vivo* examination of miR-101b function in the FSL/FRL model (Issler and Chen, 2015; Li et al., 2016).

In addition to having an important role in transporting glutamate across neuronal membranes, SLC1A1 is also essential for rapidly binding released glutamate to shape synaptic transmission (Tzingounis and Wadiche, 2007). SLC1A1 has also been found to interact with NMDA receptor subunits NR1, NR2A, and NR2B (Waxman et al., 2007). Interestingly, in the FSL model, NR2A and NR2B subunits are decreased in both the PFC and the hippocampus (Eriksson et al., 2012), which may suggest a novel coregulatory mechanism. Whereas the FSL animals display reductions in serotonin receptors (Eriksson et al., 2012), an independent group recently also confirmed an aberrant glutamatergic transmission in this model of depression (Gomez-Galan et al., 2013). In the latter study, the authors examined the hippocampal region of the FSL and found a dysfunctional astrocytic glutamate regulation that was accompanied by a downregulation of the glial glutamate transporter GLAST (also known as EAAT1). Obviously, our finding of increased SLC1A1 (EAAT3) in the PFC may represent just one of many depression-related glutamatergic and/or serotonergic genes that are being targeted by our validated miRNAs. This is supported by the fact that besides SLC1A1, one other gene that was confirmed to be upregulated in the PFC of the FSL was *Rac1*, which can be activated by serotonin through 5-HT2A receptor stimulation (Dai et al., 2008) and is a confirmed miR-101 target (Lin et al., 2014). RAC1 is a small Rho family GTPase that acts as a key regulator of actin cytoskeletal dynamics. It plays an important role in dendritic development and spinogenesis. Dysregulation of RAC1 has been associated with cognitive impairment, memory disturbance, and neurodevelopmental disorders (Govek et al., 2005; Martinez and Tejada-Simon, 2011; Stankiewicz and Linseman, 2014), which may support a role of RAC1 in the pathophysiological process of depression. Taken together, these preclinical data support the shift from a solely monoamine-centered hypothesis in the pathophysiology of depression to one that includes the involvement of the glutamatergic system (Sanacora et al., 2012).

The traditional approach to identify compounds targeting single molecules (usually receptors or their ligands) has been a hinder for the development of effective psychiatric drugs, and an approach targeting the defective components of an entire biological pathway is likely more efficient. Therefore, compared with single-target drugs, miRNAs possess an advantage that is attributed to their pleiotropic nature, which allows them to individually regulate hundreds of genes.

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

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Statement of Interest

None.

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