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Dysregulation of miRNA 181b in the temporal cortex in schizophrenia

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Analysis of global microRNA (miRNA) expression in postmortem cortical grey matter from the superior temporal gyrus, revealed significant up-regulation of miR-181b expression in schizophrenia. This finding was supported by quantitative real-time RT–PCR analysis of miRNA expression in a cohort of 21 matched pairs of schizophrenia and non-psychiatric controls. The implications of this finding are substantial, as this miRNA is predicted to regulate many target genes with potential significance to the development of schizophrenia. They include the calcium sensor gene visinin-like 1 (VSNL1) and the ionotropic AMPA glutamate receptor subunit (GRIA2), which were found to be down-regulated in the same cortical tissue from the schizophrenia group. Both of these genes were also suppressed in miR-181b transfected cells and shown to contain functional miR-181b miRNA recognition elements by reporter gene assay. This study suggests altered miRNA levels could be a significant factor in the dysregulation of cortical gene expression in schizophrenia.

INTRODUCTION

Schizophrenia is a severely debilitating psychiatric disorder characterized by a diverse range of symptoms. While extensive research has not determined the definitive cause(s), it is generally accepted that a number of influences including genetic, environmental and developmental factors are involved. Genetics in particular has been known for many years to play a key role in the development of schizophrenia. The pattern of inheritance, however, is far from clear with a complex array of associated loci and putative candidate genes each with a relatively small affect, disbursed across the entire genome (1–3). This picture is further complicated by environmental and epigenetic influences evident by the relatively low rate of concordance in monozygotic twins (~50%) (4,5).

Recent research from a variety of quarters has lent support to the neurodevelopmental hypothesis, which suggests that genetic and environmental influences affect neurodevelopmental processes that cause abnormalities in brain plasticity and connectivity leading to schizophrenia in early adulthood. In this model, regulatory factors that control the stage and tissue expression of molecules involved in neurodevelopmental processes will be particularly important. While transcription factors and protein based signal transduction pathways are usually considered in this context, a new class of gene expression modulators consisting entirely of RNA is also emerging as a significant regulatory factor. These small non-coding RNA molecules known as microRNAs (miRNA) function as guide sequences for the cellular gene silencing pathway to bring about target gene suppression through a process known as post-transcriptional gene silencing (PTGS) (6). These riboregulators are now believed to play a major role in coordinating the regulation of gene expression during the differentiation and development of the brain (7). Indeed, a large proportion of human miRNAs display a brain-specific or brain-enriched expression pattern (8). There is even evidence to suggest they are involved in brain function and neural plasticity via the regulation of molecules involved in long-term potentiation (LTP) (9,10) and in the establishment and maintenance of dendrites (11).

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Considering that each miRNA is potentially manipulating the expression of hundreds of target genes, the clinical implications of an abnormality or disturbance of this system are substantial, particularly if such abnormality occurs during development of the central nervous system. Neurological manifestations of abnormalities in miRNA-mediated regulation have in fact already been identified for fragile X mental retardation (9) and Tourette's syndrome (12). The miRNA pathway is also implicated in DiGeorge syndrome as one of the deleted genes, DGCR8 is associated with the microprocessor complex with Drosha and is essential for processing of the primary miRNA transcript (13). Interestingly, 25% of patients with DiGeorge syndrome also develop schizophrenia (14). In view of these findings, it is reasonable to suspect that alteration in miRNA gene silencing pathways may also be involved in the development of complex psychiatric disorders such as schizophrenia. This is supported by a recent study of miRNA expression in the prefrontal cortex, which identified a number of altered miRNAs (15). In the study reported here, we examined miRNA expression in cortical grey matter from the superior temporal gyrus (STG). The STG contains the primary and secondary auditory cortex thought to be involved in the generation of auditory hallucinations and appears to have reduced volume in schizophrenia (16,17). This analysis revealed significant up-regulation of miR-181b, which was found to correlate inversely with expression of schizophrenia-associated miR-181b target genes GRIA2 and visinin-like 1 (VSNL1) in STG from the same schizophrenia cohort.

RESULTS

miRNA expression in the temporal cortex

High throughput miRNA expression analysis in postmortem cortical grey matter from the STG was accomplished using a custom DNA microarray platform (18). Fluorescence signals observed in duplicate array features at a level 2-fold that of background were considered to be indicative of miRNA expression. This expression profile consisted of 76 miRNAs out of a total of 262 probes corresponding to miRBase version 7.0. Many of these miRNAs have previously been identified as either brain-specific, brain-enriched or constitutively expressed (Supplementary Material, Table S1). This is exemplified by strong expression of miR-125a/b, miR-124a, miR-9/9*, miR-128a/b as well as miR-138, miR-219 and miR-338 which are brain-specific or brain-enriched. The constitutively expressed let-7 group of miRNAs were also well represented.

Differential miRNA expression in schizophrenia

Differential expression of miRNA in postmortem STG from the schizophrenia cohort compared with their matched non-psychiatric controls was determined through two-class unpaired analysis using statistical analysis of microarrays software (Significance Analysis of Microarrays, SAM, version 2.23) (19). This analysis revealed significant up-regulation of two miRNAs, hsa-let-7g and hsa-miR-181b in the schizophrenia group, to levels 1.8-fold ($P = 0.008$) and 2.8-fold ($P = 0.001$), respectively (Fig. 1A).

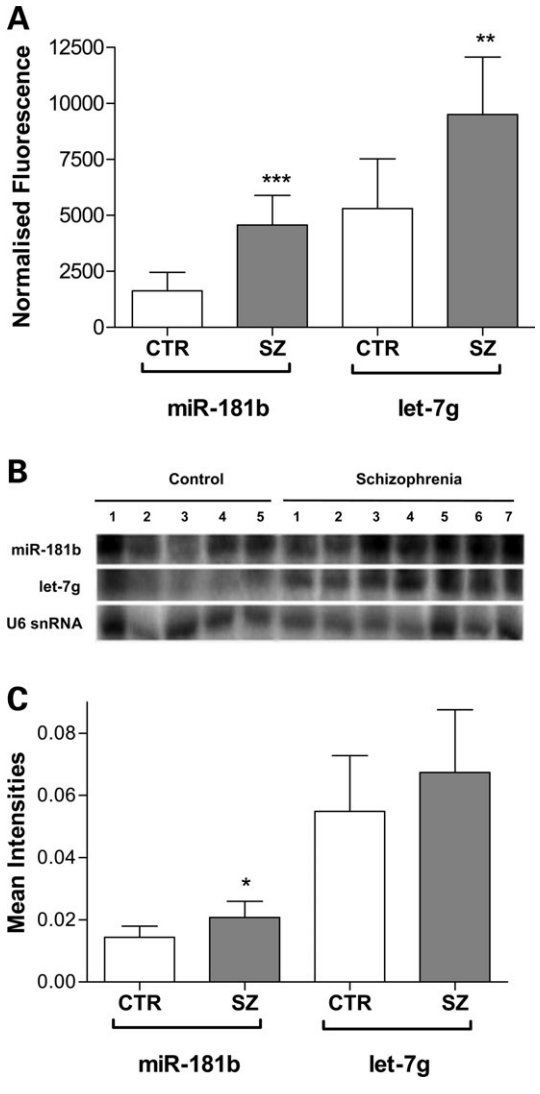

Figure 1. Expression analysis of miR-181b and let-7g by microarray and northern blot. (**A**) Mean normalized microarray fluorescence values for miR-181b (2.8-fold, $P < 0.001$) and let-7g (1.8-fold, $P = 0.008$) in the STG from 7 matched pairs of subjects with schizophrenia and non-psychiatric controls (SZ and CTR 1–7). (**B**) Northern blot analysis of STG RNA comparing schizophrenia samples (SZ 1–7) and non-psychiatric controls (CTR1-5). Each panel consists of phosphorimages of bands probed for miR-181b, let-7g and U6 snRNA, respectively. The intensity of bands corresponding to each miRNA species was then determined using ImageQuant v5.2 and normalized with respect to U6 snRNA. (**C**) Mean normalized intensity for miR-181b and hsa-let-7g miRNAs in the STG of schizophrenia non-psychiatric controls determined by northern blot hybridization. Bars represent mean ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

To confirm the differential expression status of mature let-7g and miR-181b observed by the microarray analysis, RNA from the same tissue was subjected to northern blot analysis with ^{32}P-labelled locked nucleic acid (LNA)-modified probes. As miR-181b expression in the STG was in the low to mid range compared with the entire profile and below that of the constitutively expressed let-7g, the naïve membrane was hybridized first with the miR-181b probe. After stripping, the membrane was re-probed for let-7g and then the U6 snRNA loading control, respectively, with the latter being

used to normalize the expression value of each miRNA (Fig. 1B). Quantitative analysis with normalization to the U6 band intensity confirmed a significantly higher expression of miR-181b in the schizophrenia samples with an average increase of 1.5-fold ($P = 0.041$) compared with the controls. In contrast, the difference in let-7g expression between the two groups was not significant ($P = 0.12$; Fig. 1C).

Validation of miR-181b over-expression in schizophrenia

To further validate the apparent association between altered miR-181b expression in the STG and schizophrenia, tissue from an additional 14 matched pairs was obtained and the RNA extracted to generate a substantially larger cohort (schizophrenia $n = 21$, controls $n = 21$). As less tissue was available from these new cases yielding lower quantities of total RNA, a highly sensitive and specific quantitative real-time RT–PCR-based approach to determining miR-181b expression was established. This method utilized a miR-181b reverse primer consisting of a 9-nucleotide target-specific 3′ segment and an adjoining 9-nucleotide arbitrary tag sequence at the 5′ terminus. While the sequence-specific 9-mer component primed low-temperature cDNA extension on the miRNA target, the adjacent 9-mer tag provided the additional length required for primer recognition during the higher temperature PCR analysis phase. The relative expression of miR-181b from this RT–PCR analysis was consistent with the northern blot analysis confirming a significant up-regulation of this miRNA in the schizophrenia group, with an average increase of 1.24-fold ($P = 0.049$) that was observed in 14 out of 21 matched pairs. However, removal of one control sample, CTR1, that had a higher expression of miR-181b (differed by more than 2.7 SD units from the control mean), elevated the average fold change in miR-181b expression in schizophrenia across the remaining 20 pairs to 1.39-fold ($P = 0.029$).

To determine if the differential expression observed in miR-181b was due to alteration in 181b precursor RNA transcription, primers specific to the pre-181b-1 (transcribed from 1q31.3) and pre-181b-2 (transcribed from 9q33.3) were used for quantitative real-time RT–PCR. These were both normalized against U6 snRNA expression as described for the mature miRNA expression. The results of this analysis (Fig. 2C) indicated that the pre-181b-2 transcript is in much greater abundance in the STG than the pre-181b-1 transcript, with >10-fold higher relative expression. When the expression levels of these precursor miRNA transcripts in the schizophrenia samples were compared with the controls, there was no significant difference (Fig. 2C).

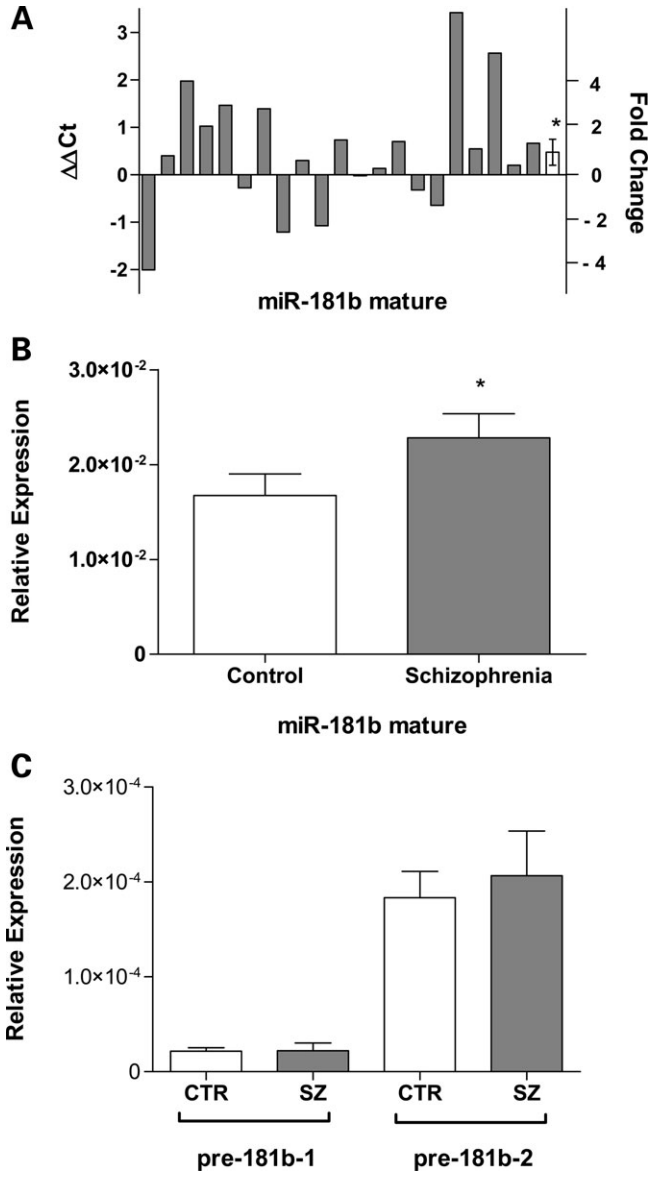

Figure 2. Expression analysis of mature and precursor 181b molecules by quantitative RT–PCR. (**A**) Real-time PCR verification of altered miR-181b expression in the STG. For each sample, the change in cycle threshold (ΔCt) was obtained by subtracting the Ct of U6 snRNA from the Ct of miR-181b. For each pair, the ΔΔCt was obtained by subtracting the ΔCt of each schizophrenia subject from the ΔCt of its corresponding control subject. The pair-wise expression changes are represented for each of the 21 matched pairs (grey bars) and the mean (±SEM) is shown to the far right (white bar). (**B**) Relative expression of miR-181b by RT–PCR in 21 matched pairs of postmortem STG. miR-181b was up-regulated 1.24-fold in the schizophrenia cohort ($P = 0.049$; paired *t*-test). (**C**) Relative expression of precursor 181b molecules. CTR, control samples; SZ, schizophrenia samples. Bars represent mean ± SEM (bar graphs); $^{*}P < 0.05$.

MiR-181b target prediction and pathway analysis

In order to gather some clues about the functional implication of elevated miR-181b concentrations in the cerebral cortex, an extensive list of putative target genes was assembled from a variety of publicly available miRNA data bases using the online TargetCombo web service (http://www.diana.pcbi.upenn.edu/cgi-bin/TargetCombo.cgi) (20). This provides the option of combining target predictions from the leading homology search algorithms miRanda, PicTar, TargetScanS and DIANA-microT (21–24). Each of these programmes screen for motifs with various miRNA homology attributes in the phylogenetically conserved segments of 3′ UTR sequences taken from the genome database. Collectively, these searches identified >800 putative target genes (Supplementary Material, Table S2). A number of these genes are known to play an important role in human brain function and development, and could

conceivably be involved in schizophrenia. This was exemplified by genes involved in synaptic transmission, including gamma amino butyric acid A receptor (GABRA1), glutamate receptors (GRM5, GRM7, GRIK2, GRID and GRIA2), serotonin receptors (HTR1B and HTR2C) and the cannabinoid receptor (CNR1). Some predicted target genes are also thought to be involved in brain development and neurodevelopmental disorders, for example ataxin 1 (ATXN1), zic family member 1 (ZIC1), SLIT and NTRK-like family, member 1 (SLTRK1) and fragile X mental retardation 1 (FMR1). The latter two involved in Tourette's syndrome and fragile X mental retardation, respectively, are already known to be associated with miRNA-related dysfunction.

To further understand the potential of miR-181b regulation, the predicted gene lists were subjected to functional annotation clustering and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the sdatabase for annotation, visualization, and integrated discovery (DAVID; http://david.abcc.ncifcrf.gov) (25). Interestingly, of the 144 conserved target genes predicted by the online version of the miRanda algorithm at the Memorial Sloan-Kettering Cancer Center Computational biology web site (http://www.microrna.org) (21), the most highly enriched gene ontology cluster related to development, nervous system development, neurogenesis and differentiation (Supplementary Material, Table S2). This was followed by a cluster of terms relating more broadly to signalling, signal transduction and transcription. When the combination gene list, consisting of 789 miR-181b target genes was submitted, a similar picture emerged except the ranking of the top two clusters was reversed. The KEGG pathways were also charted and ranked according to their *P*-value. The top five most significant terms for the large list included: LTP, MAPK signalling pathway, axon guidance and neurodegenerative disorders. In the smaller miRanda list developmentally related GO terms again featured highly with the top five consisting of development, neural development, cell differentiation, system development and organ development.

While miRNA target prediction algorithms provide a useful starting point for understanding the function of particular miRNA, they are also prone to a degree of over prediction leading to false positives (23,26). In order to relate the increased miR-181b activity in the context of schizophrenia-associated changes in the STG, the list of putative targets for this miRNA was cross matched with genes already shown in microarray experiments to be down regulated in the same tissue (Supplementary Material, Table S2e; Bowden *et al*., manuscript in preparation). This revealed a number of interesting target genes potentially regulated by miR-181b in the same tissue including the ionotropic glutamate receptor (GRIA2), fragile X- related 2 and VSNL1. Alteration of GRIA2 and VSNL1 expression in the dorsolateral prefrontal cortex and in the hippocampus, respectively, have also been shown to be associated with schizophrenia (27,28).

Validation of schizophrenia associated changes in VSNL1 and GRIA2 expression

To confirm the schizophrenia-associated down-regulation of VSNL1 and GRIA2 from the microarray analysis (Bowden *et al*., manuscript in preparation), STG RNAs (21 matched pairs) were subjected to quantitative real-time RT–PCR (Fig. 3). In the case of VSNL1, a significant decrease in expression was observed in the schizophrenia group averaging 1.91-fold with respect to the control cohort ($P = 0.037$) (Fig. 3A). Similarly, GRIA2 was also decreased by an average of 1.44-fold ($P = 0.047$) in the schizophrenia cohort (Fig. 3B).

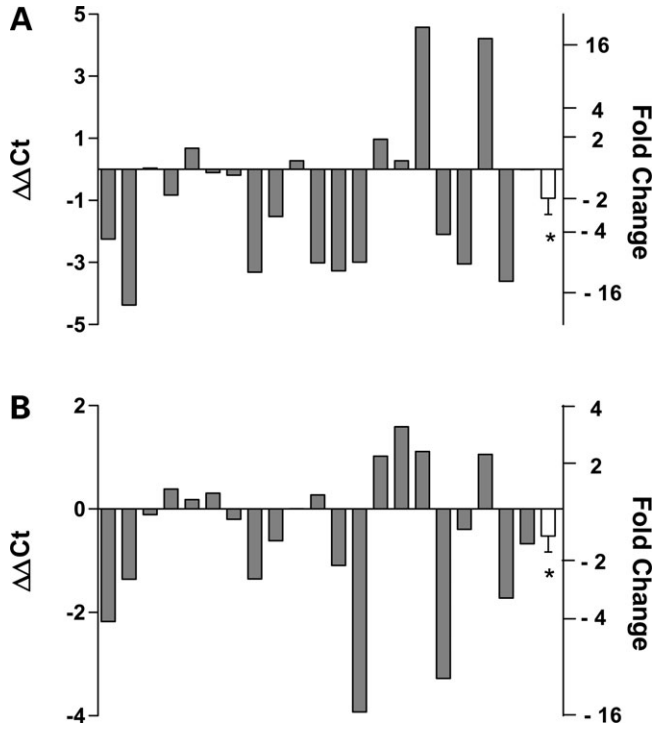

Figure 3. Relative expression of VSNL1 and GRIA2 in the superior temporal gyrus. (**A**) Relative expression of VSNL1 determined by quantitative real-time RT–PCR in 21 matched pairs of STG samples. VSNL1 showed a 1.91-fold down-regulation in the schizophrenia cohort ($P = 0.037$ one-tailed paired *t*-test). (**B**) Relative expression of GRIA2 determined by quantitative real-time RT–PCR in 21 matched pairs of STG samples. GRIA2 showed a 1.44-fold down-regulation in the schizophrenia cohort ($P = 0.047$ one-tailed paired *t*-test). Data is presented as previously described in Figure 2A. Analysis of changes in VSNL1 and GRIA2 expression using unpaired *t*-tests gave *P*-values of 0.027 and 0.073, respectively.

Repression of miR-181b target genes *in vitro*

To gain further biological insight into the miRNA 181b target genes, *in vitro* cell cultures were transfected with synthetic miR-181b (siRNA-181b) and the mRNA expression analyzed using genome-wide Sentrix bead chip arrays (Illumina) (29). Interestingly, both VSNL1 and GRIA2 expression was suppressed by 67 and 41%, respectively, in response to elevated miR-181b in HEK293 cells and SH-SY5Y cells, respectively (Fig. 4).

Biological activity of miR-181b elements in VSNL1 and GRIA2

While circumstantial evidence supported an association between miR-181b expression and the repression of predicted

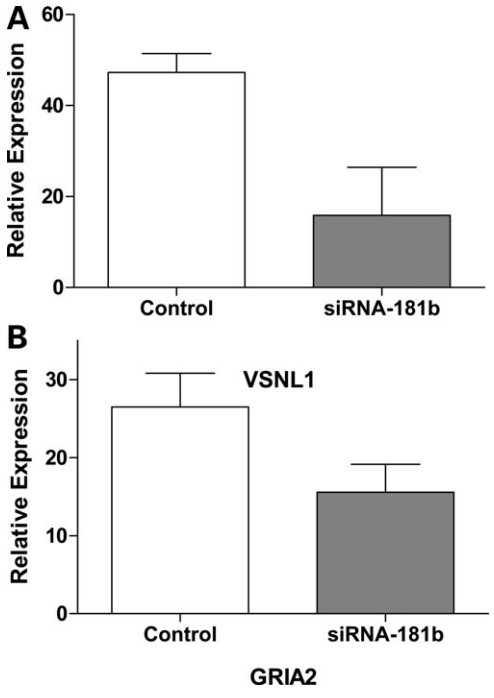

Figure 4. Relative expression of VSNL1 and GRIA2 in response to miRNA 181b transfection. (**A**) Relative VSNL1 expression in untransfected HEK293 cells (control) and HEK293 cells transfected with synthetic miR-181b. (**B**) Relative expression of GRIA2 in untransfected SH-SY5Y cells and those transfected with synthetic miR-181b. Expression was determined in each case using Illumina Sentrix bead arrays. Decreased expression of both VSNL1 and GRIA2 in both cases corresponded to elevated miR-181b levels *in vitro*. Bars represent mean + SEM.

target genes VSNL1 and GRIA2, both in the STG in schizophrenia and *in vitro*, a reporter gene assay was established to further investigate the biological validity of this link. For this purpose, miRNA recognition elements (MRE) from each of the target genes predicted to bind miR-181b were cloned into the 3′UTR of the firefly luciferase gene of the pMIR-REPORT vector (Fig. 5A). Mutant versions of these MREs containing single nucleotide polymorphisms in the seed-pairing region were also cloned into the pMIR-REPORT vector (Fig. 5B and C). Each of these reporter constructs and the control plasmid (pRNL-TK) encoding renilla luciferase were co-transfected (10 and 2.5 ng, respectively) with either siRNA-181b or LNA-modified antisense 181b (250 ng), into HEK-293 cells and their relative luciferase activity measured. In accordance with expectation, relative luciferase expression from reporter constructs containing the VSNL-1 MRE was found to be significantly reduced with respect to the control (average −20%, $P = 0.007$) when co-transfected with si181b, while being significantly increased in the presence of antisense 181b (average 28%, $P = 0.012$) (Fig. 5D). The constructs containing the mutant VSNL1 MRE displayed a smaller response to transfection, with suppression of 8% after co-transfection with si181b ($P = 0.033$), whereas it did not show a significant change in response to antisense 181b (Fig. 5D). The GRIA2 reporter gene displayed a similar response to siRNA-181b transfection, with an average suppression of 21% ($P = 0.006$), whereas antisense 181b transfection increased its expression 24% ($P = 0.038$). Surprisingly, luciferase activity from the construct containing the mutant GRIA2 MRE was also suppressed by si181b transfection (down 24%, $P = 0.003$), though the antisense 181b did not induce a significant change in its expression (Fig. 5E).

DISCUSSION

STG, miRNA expression and schizophrenia

In recent years, a number of genome-wide expression studies in schizophrenia including those from our own laboratory, have shown alterations in large numbers of genes (30,31). In view of the breadth of changes in the neural genomes activity in schizophrenia, it is reasonable to speculate that these could at least in part be due to changes in post-transcriptional gene silencing. To test this hypothesis, we initiated an investigation of miRNA expression patterns in the normal human cerebral cortex and in a matched cohort of tissue derived from subjects with schizophrenia. Postmortem grey matter from the STG of both normal and schizophrenia groups were dissected and the RNA extracted and subjected to high throughput analysis on custom miRNA-specific microarrays (18). While many postmortem expression studies of schizophrenia have focused on the prefrontal cortex, there is evidence to suggest the STG is also a participant in the pathophysiology of schizophrenia. Genome-wide analysis of schizophrenia-associated gene expression in the STG recently reported a large number of altered genes (Bowden *et al*., manuscript in preparation). In this study, we used the exact same tissue to enable a direct comparison between the schizophrenia-associated changes in gene and miRNA expression. The microarray analysis revealed up-regulation of let-7g and miR-181b, and this was also supported for miR-181b by northern blot hybridization and further validated by quantitative real-time RT–PCR in a larger cohort of 21 matched pairs.

miRNA 181b is encoded at two genomic loci, which give rise to primary transcripts for pre-miRNAs 181b-1 (MIRN181B1; 1q31.3) and pre-miRNA 181b-2 (MIRN181B2; 9q33.3). As the 181b-1 locus resides in the vicinity of a schizophrenia linkage region (1q23.3-1q31.1) (32) and the schizophrenia-associated genes for interleukin 10 (IL10; 1q31-32) and plexin A2 (PLXNA2; 1q32.2) (33–35), it is conceivable that its transcription could have been affected by schizophrenia-associated genetic alterations present in this region. To determine the relative contributions of each 181b transcript in the context of schizophrenia, we performed precursor-specific quantitative real-time RT–PCR. This analysis showed that the MIRN181B2 locus on chromosome 9 was actually far more active (∼10-fold) than its counterpart on chromosome 1. While there was some increase in the average pre-miR-181b-2 expression in the schizophrenia group, this was not statistically significant. One interpretation of this finding is that the up-regulation of mature miR-181b expression was not due to changes at the level of transcription, but rather changes that affected its processing or maturation. This would be consistent with the hypothesis developed to explain miRNA expression changes observed in the dorsolateral prefrontal cortex (DLPFC) in

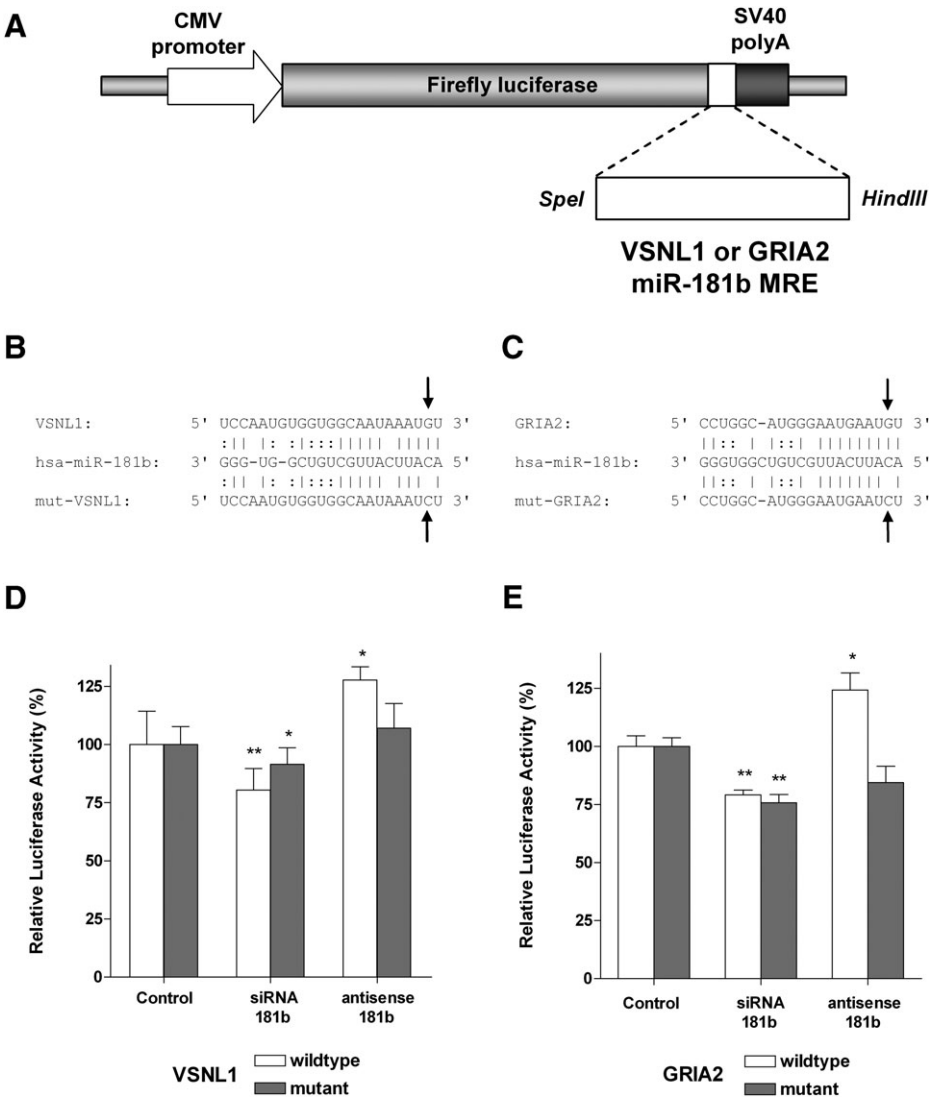

Figure 5. VSNL1 and GRIA2 MRE reporter assay. (**A**) The pMIR-REPORT™ miRNA expression reporter system contains a firefly luciferase gene under the control of CMV promoter. The putative miR-181b MRE for VSNL1 or GRIA2 were inserted into the multiple cloning site in the 3′-UTR of the luciferase gene. (**B** and **C**) Mutant versions of each MRE were also prepared such that a single base change (indicated by arrows) was introduced to the miRNA seed pairing region. (**D**) Using the wild-type MRE derived from VSNL1, expression was suppressed 20% by siRNA 181b ($P = 0.007$) and increased 28% by antisense 181b ($P = 0.012$). Whereas the mutant version was suppressed 8% by siRNA 181b ($P = 0.033$), and not significantly altered by antisense 181b. (**E**) The wild-type MRE from GRIA2 was suppressed 21% by siRNA 181b ($P = 0.006$) and increased 24% by antisense 181b ($P = 0.038$). The GRIA2 mutant MRE was suppressed 24% by siRNA 181b ($P = 0.003$), but was not significantly altered by antisense 181b. Bars represent mean + SD.

schizophrenia (15). In this study, a subset of down-regulated miRNAs (which incidentally did not include miR-181b) shared an upstream consensus sequence, which was suggestive of a role in miRNA maturation. This concept is also supported by the loss of the DiGeorge critical region 8 (DGCR8; an accessory for the primary miRNA processing protein Drosha) (13) in the 21q11 micro deletion responsible for DiGeorge or Velo Cardio Facial syndrome, which is known to be the highest risk factor for schizophrenia (∼25%) (14) apart from having a monozygotic twin with the disorder (∼50%). However, a more specific effect could be operating in respect to the changes in miR-181b expression, observed here, perhaps through changes to pre-miRNA sequence elements involved in maturation of the miRNA. More general deficits in pri-miRNA processing suggested for the DLPFC were not supported here as pre-miR-181b was not altered in the STG and it does not contain the consensus sequence reported for miRNA altered in the DLPFC (15).

MiR-181b target genes

The implications of even a small increase in miR-181b expression in the cerebral cortex could be profound, particularly during development as it is capable of extending a repressive influence over hundreds of different target genes. While miRNA 181b is known to be expressed in a variety of

mammalian tissues, a number of studies have shown brain enrichment (8,36,37) with expression in both neurons and neuroglia (38,39). It is also highly expressed in the thymus and lungs, and has been shown to be directly associated with B-cell lineage differentiation of hematopoietic progenitor cells (40). miR-181b was also found to be up-regulated in acute promyelocytic leukaemia cells in response to retinoic acid-induced differentiation (41). It was also up in thyroid papilliary carcinoma cells compared with those from normal thyroid tissue (42). Conversely, miR-181b was observed to be down-regulated in glioblastoma cells (39). Most of these findings, however, are consistent with a role for miR-181b in differentiation and development. This is broadly supported by miR-181b target gene predictions, which implicate an apparent bias towards genes involved in development, brain development and brain function.

While it is interesting to consider the potential of genome wide interactions, we were able to examine miRNA targets specifically in the context of genes shown previously to be down-regulated in schizophrenia and in the same tissue from the same cohort of controls and schizophrenia subjects (Supplementary Material, Table S2e; Bowden *et al*., manuscript in preparation). Among the 23 genes in this category, two targets including GluR2, the ionotropic glutamate/AMPA receptor gene (GRIA2) and the calcium sensor/trkB mRNA binding protein known as VSNL1 were particularly interesting in the context of schizophrenia. GRIA2 is a major ionotropic glutamate receptor subunit involved in fast excitatory neurotransmission. It has been shown to have an important function in the development of synaptic plasticity because of its involvement with NMDA receptors in the establishment of LTP, long-term depression (43) and by directly stimulating increased growth and density of dendritic spines (44). GRIA2 has also shown a consistent association with schizophrenia and is compatible with the glutamate hypofunction hypothesis, with an observed decrease in both mRNA and protein levels in postmortem samples from a number of brain regions including the medial temporal lobe, hippocampus and dorsolateral prefrontal cortex (27,45,46). The calcium sensor protein VSNL1 has also been shown to be differentially expressed in schizophrenia (28). Specific changes in VSNL1 expression have been reported in the rat brain in phencyclidine (PCP) and ketamine models of schizophrenia (47,48). VSNL1 is thought to be a calcium sensitive signal transduction molecule. Its location within hippocampal neurons has also been shown to be altered in response to stimulation by glutamate (49). Interestingly, VSNL1 is also a calcium-dependent double-stranded RNA-binding protein, that may provide activity-dependent trafficking of certain neuronal mRNAs to the dendrites and more specifically is known to bind the 3′ UTR of mRNA for the neurotrophin receptor (trkB) (50).

To further support the plausibility of a relationship between GRIA2 and VSNL1 expression in the STG and miR-181b, we established a synthetic miRNA 181b transfection system and monitored the expression of theses genes in response to changes in miRNA 181b concentration *in vitro*. The expression of both of these genes responded in accordance with expectation by showing a substantial drop in expression after the addition of the miRNA (Fig. 4). However, in order to directly characterize the miR-181b target status of GRIA2 and VSNL1, the putative MRE from their respective 3′ UTR were cloned downstream of the firefly luciferase gene to measure their response to miR-181b *in vitro*. The presence of either of these MRE and si181b was observed to significantly reduce expression of luciferase expression compared with the control siRNA. In both cases reporter gene expression was also elevated in response to antisense 181b transfection, presumably as a result of depleting the bioavailability of endogenous miR-181b. In most cases, these responses were absent or diminished substantially in the mutant MRE constructs, which contained point mutations in the miRNA seed regions. The one exception to this was the reporter gene carrying the mutant version of the GRIA2 MRE, which was also silenced significantly by si181b. This was not surprising as the mutant was predicted to be a viable miR-181b target gene (albeit a weaker one) and thus capable of responding to saturating levels of the cognate miRNA. In contrast, at lower endogenous concentrations it did not display any signs of the silencing observed with the wild-type reporter gene, evident by the absence of response to the antisense miR-181b. From these experiments, we were able to conclude that both VSNL1 and GRIA2 3′UTR segments are sufficient to support PTGS by miR-181b *in vitro*, enhancing their status as functioning targets of this miRNA.

In summary, we have considered the possibility that alteration of miRNA-mediated PTGS is associated with the pathophysiology of schizophrenia. In support of this hypothesis, miR-181b was shown to be up-regulated in grey matter from the STG. In a similar approach, miRNA expression was also shown to be altered in the DLPFC in schizophrenia, although changes in miRNA 181b expression was not reported in this tissue (15). Genetic analysis of polymorphisms in the vicinity of brain expressed miRNA genes hsa-miR-206 and hsa-miR-198, was recently determined to be weakly associated with schizophrenia (51). There has also been some interest in the analysis of hsa-miR-103b in the context of schizophrenia due to its location in the 22q11 locus, however, no association was observed (52). Time will tell if schizophrenia-associated changes in miRNA expression are due to direct genetic influence or some other upstream regulatory, epigenetic or mechanistic factors affecting miRNA maturation. In either case it has important implications for understanding the neurodevelopmental origins of the schizophrenia, particularly as changes in a given miRNA can affect the expression of hundreds of target genes. In the case of miR-181b there are up to 800 conserved targets predicted in the human genome that have the potential to interact with the miRNA. The true extent of its influence, however, will depend on the biological context and other so far unidentified factors. In respect to schizophrenia and changes in the STG, we have identified two important candidate target genes for miR-181b (GRIA2 and VSNL1) that are suppressed in the same tissue, and may be responding to changes in the local miRNA environment. If these specific effects are a manifestation of the observed changes in miRNA expression, they probably represent the ‘tip of the iceberg’ in terms of their global regulatory influence. The full impact of this and other changes in miRNA expression will no doubt take some time to unravel and appreciate fully.

MATERIALS AND METHODS

Tissue collection

Fresh frozen postmortem STG grey matter tissue from 21 subjects with schizophrenia and 21 non-psychiatric controls was obtained through the NSW Tissue Resource Centre, The University of Sydney, Australia. The grey matter tissue was taken from the outer edge of blocks of STG tissue from the most caudal coronal brain slice containing the STG (Brodmann's Area 22). In all cases, a diagnosis of schizophrenia in accordance with DSM-IV criteria was confirmed by medical file review using the Item Group Checklist of the Schedules for Clinical Assessment in Neuropsychiatry and the Diagnostic Instrument for Brain Studies. Consent was obtained from the next of kin and subjects with a significant history of drug or alcohol abuse, or other condition or gross neuropathology that might have influenced agonal state were excluded. In addition, control subjects were excluded if there was a history of alcoholism or suicide. All subjects were of Caucasian descent. Subjects with schizophrenia were matched for gender, age, brain hemisphere, postmortem interval and pH (Table 1). This cohort of tissue contained 13 matched pairs that were previously analyzed for mRNA expression using microarray analysis (Bowden *el al*., manuscript in preparation).

Tissue dissection and RNA extraction

Postmortem cortical grey matter was dissected from the outer edge of frozen coronal sections (1 cm) using a fine diameter hole punch and scalpel. In each case, ~50–60 mg grey matter was removed and immediately homogenized in 1 ml of Trizol reagent and the total RNA extracted according to the manufacturer's instructions (Invitrogen). The RNA concentration and integrity was determined using an Experion bioanalyser (BioRad).

miRNA expression arrays

miRNAs were labelled directly using a ligation approach consisting of 9 μg of total RNA, in 50 mM HEPES, pH 7.8, 3.5 mM DTT, 20 mM MgCl2, 0.1 mM ATP, 10 μg/ml BSA, 10% DMSO, 500 ng 5′-phosphate-cytidyl-uridyl-Cy3-3′ (Dharmacon) and 20 units T4 RNA ligase (Fermentas) (18,53). After incubating for 2 h on ice the labelled RNA was precipitated with 0.3 M sodium acetate, 2 volumes 100% ethanol and 20 μg glycogen at −20°C overnight. A synthetic reference library consisting of DNA oligonucleotides (representing the entirety of miRBase version 7.0) was labelled with Ulysis platinum conjugated AlexaFluor 647 (equivalent to Cy5) for detection in the control channel, using the labelling kit, according to the manufacturer's instructions (Invitrogen). Unconjugated label was then removed by gel filtration through a Sephadex G-25 spin column (GE Healthcare). The labelled reference library was used at a 1/700 dilution, along side the Cy3 labelled miRNAs, in each array hybridization.

Microarrays were prepared using anti-sense DNA oligonucleotides corresponding to the miRBase Version 7.0 (Sanger Institute, UK) containing 261 human miRNAs sequences. The oligonucleotide probes were printed in duplicate onto GAPS-2 glass slides (Corning). The slides were then prepared and hybridized with the labelled miRNA and synthetic controls as described previously (18). Briefly, slides were prehybridized in 3× SSC, 0.1% SDS and 0.2% BSA for 1 h at 65°C and washed four times with RNAse-free water, once with 100% ethanol, and dried by centrifugation at 150*g* for 5 min. Hybridization chambers were created around each array using 17 mm × 28 mm disposable frame seals and cover slides (Bio-Rad). The labelled RNA sample was added to 100 μl hybridization buffer (400 mM Na_2HPO_4, pH 7.0, 0.8% BSA, 5% SDS, 12% formamide) and heated for 4 min at 95°C (in the dark). The mixture was injected into the chamber and hybridized for 2 h at 37°C in a rotary hybridization oven. The coverslips and frames were removed and the slides washed once in 2× SSC, 0.025% SDS at room temperature, three times in 0.8× SSC at room temperature and three times in ice-cold 0.4× SSC. Each slide was then dried by centrifugation for 10 min at 60*g*. Arrays were then scanned with a Genepix 4000B Scanner (Axon Instruments) and raw pixel intensities extracted with Genepix Pro 3.0 software (Axon Instruments).

A miRNA was considered expressed if its raw Cy3 pixel intensity was at least 200% above background. Raw Cy3 median pixel intensity values were background subtracted and normalized by median centring with respect to arrays using Cluster version 2.2 (Stanford University). Differential miRNA expression was analyzed using Significance Analysis of Microarrays SAM version 2.23 (Stanford University) (19) (available from http://www-stat.stanford.edu/~tibs/SAM/). The threshold for significance was set at 5% and a two-class comparison was performed using 5000 permutations of the data. A list of significantly altered miRNAs was compiled (false-discovery rate <5%).

Northern hybridization

Total RNA from each case (30 μg) was combined with equal volumes of loading dye (0.01% bromophenol blue, 10 mM EDTA and formamide), pre-heated at 95°C for 5 min then electrophoresed on a 16% denaturing (8.3 M urea) polyacrylamide sequencing gel. The RNA was then electro-transferred in a semi-dry blotter at 400 mA for 1 h to GeneScreen Plus nylon membrane (Perkin Elmer NEN) and immobilized by UV-cross linking, followed by baking at 80°C for 1 h. The membrane was then pre-hybridized and hybridized in PerfectHyb Plus hybridization buffer (Sigma) for 3 and 16h, respectively. The later was carried out with the addition of 50 pmol of ^{32}P-labelled unmodified or LNA-modified antisense oligonucleotide prepared earlier using [γ-^{32}P] ATP (Perkin Elmer) and polynucleotide kinase (Fermentas) as described by previously (54). After low and high stringency washes, the radiolabelled membranes were imaged using a typhoon phosphorimager (Amersham) and analyzed using ImageQuant software (Amersham). The sequences of antisense probes for the detection of miR-181b, let-7g and U6 snRNA are presented in Table 2. Statistical analysis, consisting of a one-tailed *t*-test, was performed on the normalized intensity values to determine the significance of observed differences in average expression.

Table 1. Demographic data for schizophrenia and non-psychiatric control subjects

Pair	Diagnosis	Sex	Age	Hemi	PMI	pH	COD	Toxicology	DOI	CPE[i]
1	CRS	M	51	L	21	6.02	IHD[c,f,g]	Thioridazine 2.2 mg/l (fatal), Mesoridazine 2.4 mg/l (toxic/fatal)	24	100–700
2	CPS	M	57	L	33	6.40	Coronary artery thrombosis[a,e]	Thioridazine 0.6 mg/l, Seraline <0.1 mg/l	26	100–400
3	CPS	M	52	R	8	6.10	IHD[a,f]	Temazepam <0.1 mg/l	31	260–600
4	CUS	M	44	L	35	6.55	Hanging suicide[a]	Urine THC detected	17	500–1000
5	CPS	M	30	L	24	6.60	CO poisoning[d,h]	Carbon Monoxide 74% saturation, Clozapine 0.7 mg/l	3.5	130–975
6	CDS	M	32	L	25	6.24	Hanging suicide[a]	N/A	13	780
7	CPS	M	51	R	18	6.62	IHD[a,f]	N/A	30	300–1300
8	CRS	F	51	L	12	5.40	Emphema[a]	Lithium 20 mg/l (fatal), Midazolam 0.02 mg/l	16	112–1000
9	CDS	F	67	R	27	6.20	IHD[c,f]	Benztropine, Mesoridazine, Thioridazine and Paracetamol detected	46	150–1100
10	CPS	M	75	L	36	6.40	IHD[a,f]	Olanzapine - 0.2 mg/l, Fluvoxamine - 0.7 mg/l	44	200–1200
11	CPS	M	54	R	27	6.20	Coronary artery thrombosis[a,f]	Chlorpromazine: 0.7 mg/l, Diazepam: <0.1 mg/l, Nordiazepam: 0.1 mg/l, Insulin: 2 uU/ml	35	50–600
12	CPS	F	61	R	49	6.70	Ischaemic heart disease[c,f]	Clozapine 1.1 mg/l; Diazepam 0.2 mg/l; Laudanosine 0.4 mg/l; Nordiazepame 0.4 mg/l; Olanzapine 0.2 mg/l	42	800–1500
13	CUS	M	67	R	5	6.40	Cardiovascular disease[c,g]	Negative	41	200–2400
14	CPS	M	57	R	48	6.70	ASCVD[a,e]	Carbamazepine 10 mg/l, Citalopram 0.2 mg/l, Quetiapine <0.1 mg/l	17	225–975
15	CPS	M	40	R	21.5	6.20	Dihydrocodeine toxicity and obstructive sleep apnoea[a]	Valproic acid 20 mg/l, Dihydrocodeine 0.7 mg/l, Quetiapine 0.3 mg/l, Sertraline 0.3 mg/l	23	225–1800
16	CPS	F	66	R	12.5	6.30	Faecoloid peritonitis[a,f]	Negative	30	1200–2500
17	CPS	F	61	R	39	6.60	Undetermined[a,e]	Thioridazine and Mesoridazine detected	32	100–600
18	CPS	F	61	L	19	6.10	Sepsis and chronic renal failure[a]	Morphine: 0.06 mg/l, Codeine: 0.05 mg/l, Carbamazepine: 7 mg/l, Pethidine: 0.1 mg/l, Paracetamol: 6 mg/l, Metoclopramide 0.1 mg/l, Diazepam: <0.1 mg/l	39	300–400
19	CPS	M	33	L	48	6.70	Hanging suicide[a]	Doxylamine: 0.9 mg/l, Olanzapine: 0.2 mg/l, Paracetamol: 3 mg/l	10	222
20	CUS	M	52	R	46	6.40	Cardiomegaly[b,f]	N/A	32	17–1165
21	CPS	F	54	R	29	6.50	Asthma[a,f]	Citalopram 0.6 mg/l	35	15–600
Mean (SD)			52.7 (11.7)		28.9 (13.4)	6.4 (0.3)				
1	CON	M	50	L	19	6.26	IHD[e]	Negative		
2	CON	M	58	L	38	6.50	IHD[e]	N/A		
3	CON	M	59	R	20	6.56	Coronary thrombosis[f]	Negative		
4	CON	M	43	L	13	6.43	Thrombotic coronary artery occlusion[f]	Negative		
5	CON	M	34	L	20.5	6.73	Asthma[e]	N/A		
6	CON	M	38	L	13.5	6.00	ASCVD[e]	Negative		
7	CON	M	46	R	25	6.70	Cardiac arrest	Negative		
8	CON	F	52	L	9.5	5.80	IHD	N/A		
9	CON	F	70	R	30	6.80	IHD[g]	Blood EtOH: 0.251 g per 100 mL, Paracetamol <3mgL		
10	CON	M	73	L	10	6.20	Cardiac arrest	N/A		
11	CON	M	56	R	37	6.80	Pulmonary thromboembolus	N/A		
12	CON	F	56	R	23	6.70	Pulmonary thromboembolus[e]	N/A		
13	CON	M	69	R	16	6.60	Cardiac atheroma[f, g]	Paracetamol 23 mg/l, 1% blood saturation of CO (low)		
14	CON	M	56	R	24	6.50	Coronary artery atheroma[f]	N/A		
15	CON	M	37	L	21	6.60	IHD	Negative		
16	CON	F	71	L	16	6.20	Adenocarcinoma of the pancreas[e]	N/A		
17	CON	F	52	L	11	6.20	Ischaemic heart disease[f]	N/A		
18	CON	M	46	L	29	6.70	Pulmonary thromboembolus[e]	N/A		
19	CON	M	46	L	29	6.10	MI[g]	N/A		

Continued

Table 1. *Continued*

Pair	Diagnosis	Sex	Age	Hemi	PMI	pH	COD	Toxicology	DOI	CPE[i]
20	CON	M	53	R	27	6.60	MI	N/A		
21	CON	F	49	R	15	6.90	Arrhythmogeneic right ventricular dysplasia	Chloride ions 118 mmol/l		
Mean (SD)			53.2 (11.4)		21.4 (8.5)	6.5 (0.3)				

CRS, chronic residual schizophrenia; CPS, chronic paranoid schizophrenia; CUS, chronic undifferentiated schizophrenia; CDS, chronic disorganized schizophrenia; CON, control subject; Hemi: brain hemisphere; PMI, postmortem interval (h); COD, cause of death; DOI, duration of illness (years); CPE, chlorpromazine equivalent (mg/day); IHD, Ischaemic heart disease; MI, myocardial infarction; ASCVD, atherosclerotic cardiovascular disease.

[a]Schizophrenia subjects medicated with predominately typical antipsychotics over their lifetime.

[b]Medicated with predominately atypical antipsychotics over their lifetime.

[c]Medicated with only typical antipsychotics over their lifetime.

[d]Medicated equally with typical and atypical antipsychotics over their lifetime.

[e]Moderate nicotine consumption.

[f]Heavy nicotine consumption.

[g]Moderate alcohol consumption.

[h]Heavy alcohol consumption.

[i]Calculated using the mean CPE dosage for each subject. All toxicology results are from blood unless otherwise stated (e.g. urine). All subjects are of Caucasian descent. Mean PMI is higher (6.5 h) in the schizophrenia cohort.

Quantitative real-time RT–PCR

Multiplex reverse transcription was performed on 500 ng of DNaseI-treated total RNA using either random hexamers (mRNA analysis), or a combination of reverse primers (miRNA analysis) specific for mature hsa-miR-181b, the U6 snRNA and β-actin, to a final concentration of 40 nM each (for sequences see Table 2). Reactions were performed using Superscript II reverse transcriptase in 1× first-strand buffer according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed essentially as previously described (31), in triplicate on diluted cDNA combined with Power SybrGreen master mix (Applied Biosystems) with 1 μM of the appropriate forward and reverse primers (Table 2), in a final volume of 25 μl using an ABI prism 7500 sequence detection system (PE Applied Biosystems). Relative miRNA expression was determined by the difference between their individual cycle threshold (Ct) value and that produced in the same sample for the U6 snRNA (ΔCt). Similarly, relative mRNA expression ratio was normalized with respect to the geometric mean of β-actin and U6 snRNA expression. Differential expression of a given miRNA or mRNA was determined by the difference between the mean ΔCt for the schizophrenia and control cohorts (ΔΔCt) expressed as a ratio ($2^{-\Delta\Delta Ct}$) (55). To determine the significance of any difference in average expression in a given direction between the two cohorts, a paired one-tailed *t*-test was applied. No significant differences in the expression of normalizing genes (β-actin and U6 snRNA) were observed with respect to each other, between the schizophrenia and control cohorts.

Cell culture and siRNA transfection

HEK-293 and SH-SY5Y cell cultures were maintained as confluent monolayers at 37°C with 5% CO2 and 90% humidity in DMEM with 10% (vol/vol) fetal calf serum, 20 mM HEPES, 0.15% (wt/vol) sodium bicarbonate and 2 mM L-glutamine. HEK-293 cells were seeded into 10 cm petri dishes and transfected 24 h later using Lipofectamine 2000 (Invitrogen). SH-SY5Y cells were harvested and electroporated using the Nucleofector Kit V (Amaxa), before being seeded into 6-well plates at 1×10^6 cells/well. In each case transfections were performed according to manufacturer's instructions with 100 nM siRNA (si181b) oligonucleotide (Table 2).

Target gene expression profiling

RNA was extracted directly from plates 24 h post-transfection using 2 ml of Trizol and a disposable cell scraper (Greiner Bio-One). miR-181b expression levels in transfected cells were analyzed using real-time PCR as described above. RNA was purified further using an RNAeasy MinElute kit (Qiagen) before amplification-labelling with a TotalPrep amplification kit (Ambion) and hybridization on HumanRef-8 whole-genome expression arrays (Illumina) according to the manufacturer's instructions. Data were normalized and analyzed using Illumina Beadstudio 3.0 and GeneSpringGX 7.3.1 (Agilent Technologies, USA).

Table 2. Oligonucleotide sequences

Type	Name	Sequence	Target
Antisense[a]	181b_antisense	C+CCA+CCG+ACA+GCA+ATG+AAT+GT	miRNA 181b
	let7g_antisense	ACTGTACAAACTACTACCTCA	miRNA let-7g
	U6_antisense	GCCATGCTAATCTTCTCTGTATC	U6 snRNA
Primers[b]	ActinB-1F	TGTGGCATCCACGAAACTACC	β-actin
	ActinB-1R	ACATCTGCTGGAAGGTGGACA	β-actin
	U6_F339	CGGCAGCACATATACTAAAATTGG	U6 snRNA
	VSNL1_F1	AAACAACCTGCCACAATGTGATATG	VSNL1
	VSNL1_R2	ATAGTATTTTACAGGAGGGTAGTGA	VSNL1
	GRIA2_F	GTCCCTTACGTGAGTCCTG	GRIA2
	GRIA2_R	TAAACACACAAGAAAACCATT	GRIA2
	181b_1F5	TGCAGAGATTATTTTTTAAAAGG	pre-181b-1
	181b_1R60	TGAGCTTGTCCACACAGTTC	pre-181b-1
	181b_2F1	CTGATGGCTGCACTCAACAT	pre-181b-2
	181b_2R42	TGATCAGTGAGTTGATTCAGACT	pre-181b-2
	181b_F	TTTCTAACATTCATTGCT	miRNA 181b
	181b_R	CAACCTTCTCCCACCGAC	miRNA 181b
Cassettes[c]	VSNL1_181bT	CTAGAAGGCTTCCAATGTGGTGGCAATAAATGTCCCAAAT	VSNL1
	VSNL1_181bB	AGCTATTTGGGACATTTATTGCCACCACATTGGAAGCCTT	VSNL1
	VSNL1_181bmT	CTAGAAGGCTTCCAATGTGGTGGCAATAAATCTCCCAAAT	mutant
	VSNL1_181bmB	AGCTATTTGGGAGATTTATTGCCACCACATTGGAAGCCTT	mutant
	GRIA2_181bT	CTAGCTTACGTGAGTCCTGGCATGGGAATGAATGTCAGTGT	GRIA2
	GRIA2_181bB	AGCTACACTGACATTCATTCCCATGCCAGGACTCACGTAAG	GRIA2
	GRIA2_181bmT	CTAGCTTACGTGAGTCCTGGCATGGGAATGAATCTCAGTGT	mutant
	GRIA2_181bmB	AGCTACACTGAGATTCATTCCCATGCCAGGACTCACGTAAG	mutant
siRNA[d]	siEGFP+	CGGCAAGCUGACCCUGAAGUU	EGFP
	siEGFP-	GACUCCAGUGGUAAUCUACUU	EGFP
	si9*+	UAAAGCUAGAUAACCGAAAGU	miR-9*
	si9*-	UUUCGGUUAUCUAGCUUUCUU	miR-9*
	si181b+	AACAUUCAUUGCUGUCGGUGGG	miR-181b
	si181b-	CACCGACAGCAAUGAAUGUUUU	miR-181b

[a]The positions of LNA modified bases are preceded by a '+' symbol. U6 antisense was used as a probe for northern hybridization and as a reverse primer for quantitative RTPCR.
[b]The direction of primers with respect to the target sequence was denoted in the name as either F or R for forward and reverse respectively. Underlined sequence is not gene specific and was used to increase the amplicons size and primer recognition sequence.
[c]SpeI/HindIII cassettes containing putative target MRE were used to generate recombinant luciferase reporter gene constructs.
[d]siRNA was used to over express miRNA.

Target gene reporter assay

Validation of predicted miR-181b target genes VSNL1 and GRIA2 was accomplished by co-transfecting HEK293 cells with synthetic si181b or an LNA-modified antisense inhibitor and recombinant firefly luciferase reporter gene constructs containing 3′ UTR sequences substituted from the target gene. Oligonucleotides encoding target gene MRE (or mutant controls) were annealed to form *Spe*I and *Hin*dIII restricted overhangs of a ligatable cassette compatible with *Spe*I and *Hin*dIII digested pMIR-REPORT vector (Ambion). Reporter gene silencing in response to miRNA co-transfection was monitored with respect to a 'spiked-in' control plasmid expressing renilla luciferase using the dual luciferase reporter assay (Promega). To control for non-specific effects associated with siRNA transfection, the controls were co-transfected with siEGFP or miR-9* siRNA predicted to have little or no activity against the fusion transcripts tested here. HEK293 cells were cultured and transfected as described above (except in 24-well plates) using Lipofectamine2000 (Invitrogen).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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