Research Paper

Investigation of the involvement of *MIR185* and its target genes in the development of schizophrenia

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Background: Schizophrenia is a complex neuropsychiatric disorder of unclear etiology. The strongest known genetic risk factor is the 22q11.2 microdeletion. Research has yet to confirm which genes within the deletion region are implicated in schizophrenia. The minimal 1.5 megabase deletion contains *MIR185*, which encodes microRNA 185. **Methods:** We determined *miR-185* expression in embryonic and adult mouse brains. Common and rare variants at this locus were then investigated using a human genetics approach. First, we performed gene-based analyses for *MIR185* common variants and target genes using Psychiatric Genomics Consortium genome-wide association data. Second, *MIR185* was resequenced in German patients (n = 1000) and controls (n = 500). We followed up promising variants by genotyping an additional European sample (patients, n = 3598; controls, n = 4082). **Results:** In situ hybridization in mice revealed *miR-185* expression in brain regions implicated in schizophrenia. Gene-based tests revealed association between common variants in 3 *MIR185* target genes (*ATAT1*,

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SH3PXD2A, *NTRK3*) and schizophrenia. Further analyses in mice revealed overlapping expression patterns for these target genes and *miR-185*. Resequencing identified 2 rare patient-specific novel variants flanking *MIR185*. However, follow-up genotyping provided no further evidence of their involvement in schizophrenia. **Limitations:** Power to detect rare variant associations was limited. **Conclusion:** Human genetic analyses generated no evidence of the involvement of *MIR185* in schizophrenia. However, the expression patterns of *miR-185* and its target genes in mice, and the genetic association results for the 3 target genes, suggest that further research into the involvement of *miR-185* and its downstream pathways in schizophrenia is warranted.

Introduction

Schizophrenia is a complex neuropsychiatric disorder of unclear etiology.[1] The strongest known genetic risk factor is a hemizygous microdeletion in chromosomal region 22q11.2, which causes the phenotypically heterogenous 22q11.2 deletion syndrome (22q11.2DS),[2] also known as velocardiofacial/DiGeorge syndrome. The overall prevalence of 22q11.2DS is 1 in 2000–4000 births.[3,4] Affected individuals have an estimated 30% risk for schizophrenia.[4,5] Since the symptoms of 22q11.2DS-related schizophrenia are largely indistinguishable from those of the idiopathic disease,[4,5] risk genes for 22q11.2DS-related schizophrenia may also be implicated in idiopathic cases.[6]

The size of the 22q11.2 deletion varies. Most are 1.5 megabases (Mb) or 3 Mb in size, and span approximately 35 and 60 known genes, respectively.[7,8] Although the 22q11.2DS phenotype is highly variable, its severity is not correlated with deletion size. This suggests that the minimal 1.5 Mb deletion region is of crucial etiological importance.[3] Several genetic association studies have attempted to identify which genes in this 1.5 Mb region are responsible — through their absence — for the increased risk for schizophrenia.[3] To date, however, no single gene has received consistent independent support.

One known gene within the minimal 1.5 Mb deletion region is *MIR185*,[3] which encodes microRNA 185. MicroRNAs are small noncoding RNAs that control the translation of target messenger RNAs (mRNAs). Accumulating evidence suggests that microRNAs contribute to the basic mechanisms underlying brain development and plasticity,[9,10] thus suggesting their possible involvement in the pathogenesis of several psychiatric disorders,[11] including schizophrenia.[12]

This hypothesis is supported by a large genome-wide association study (GWAS) of schizophrenia conducted by the Psychiatric Genomics Consortium (PGC).[13] Here, a single nucleotide polymorphism (SNP) in an intron of *MIR137* was the second strongest finding. Four other loci with genome-wide significance were predicted targets of *MIR137*, thus providing genetic support for the hypothesis that microRNA-mediated dysregulation is an etiological mechanism in schizophrenia.[13]

Research in mouse-models has also implicated microRNAs in 22q11.2DS. Investigation of *Df(16)A*$^{+/-}$ mice — an engineered mouse strain carrying a chromosomal deficiency spanning a segment syntenic to the human 22q11.2 locus — demonstrated alterations in the biogenesis of brain microRNAs.[11,14] Interestingly, *miR-185* was the top-scoring downregulated microRNA in both the prefrontal cortex (PFC) and the hippocampus,[14] brain areas that are key foci of schizophrenia research.[15] A recent study confirmed a significant reduction in the expression of *miR-185* in the hippocampus and PFC of *Df(16)A*$^{+/-}$ mice and showed that this reduction contributed to deficits in the dendritic and spine development of hippocampal neurons.[16] Furthermore, Earls and colleagues[6] identified *miR-185* as a regulator of sarco(endo)plasmic reticulum Ca(2+) ATPase (SERCA2) in 22q11.2DS mouse models and showed that *miR-185* depletion contributed to SERCA2 upregulation. This mechanism may be implicated in the elevation of SERCA2 protein observed in the postmortem brains of patients with schizophrenia.[6]

Further support for the involvement of *MIR185* in schizophrenia is provided by evidence that 2 of its validated targets, RhoA and Cdc42,[17] are associated with altered expression levels in patients with schizophrenia.[18,19]

We conducted the present study to investigate the role of *MIR185* in schizophrenia using gene expression analyses in mice and human genetics approaches in patients with schizophrenia and controls.

Methods

Animal study of miR-185: in situ hybridization

These experiments involved the wild-type C57BL6 mouse strain. All procedures involving animals followed the guidelines of the German Animal Protection Legislation, and the experiments were approved by the Local Committee for Animal Health (Landesamt für Natur, Umwelt und Verbraucherschutz).

We determined *miR-185* expression profiles to validate microarray-predicted expression in the PFC and hippocampus and to determine *miR-185* expression in brain regions of relevance to 22q11.2DS psychiatric phenotypes. We applied in situ hybridization (ISH) in embryonic and adult mouse brains, as this was the best experimental alternative to microRNA expression profiling at the time of study.[20]

We performed ISH as described previously.[21] Briefly, mouse embryos and isolated young and adult mouse brains for a total of 6 different stages were fixed overnight at 4°C with 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline. Embryos and adult brains were then dehydrated using ethanol/saline solutions. Following Roti-Histol (Roth) treatment, the specimens were embedded in Paraplast sections. The specimens were dewaxed and rehydrated, re-fixed in 4% PFA, and treated with proteinase K (10 µg/mL). An *miR-185* 3′-DIG labelled detection probe (Exiqon, hsa-miR-185:TCAGGAACTGCCTTTCTCTCCA) was used. A sense probe was used as a negative control (NM_001163311, 263 to 1126 base pairs (bp)). After hybridization and immunological detection with antidigoxigenin Fab fragments (Roche), NBT/BCIP staining was performed. The slides were

mounted with CC/Mount aqueous mounting medium (Sigma). Midday of the day on which the vaginal plug appeared was considered embryonic day 0.5 (E0.5).

Expression pattern of miR-185 target genes in the mouse brain

Two publicly available databases were used to determine the expression patterns of *Atat1*, *Sh3pxd2a* and *Ntrk3*: Eurexpress (www.eurexpress.org)[22] for the developing mouse brain, and Mouse Allen Brain Atlas (mouse.brain-map.org)[23] for the adult mouse brain. These genes have the following identifications: T31063 (*Atat1*), T37016 (*Sh3pxd2a*) and T36710 (*Ntrk3*) for Eurexpress, and 68522497 (*Atat1*), 69873720 (*Sh3pxd2a*), and 71234689 (*Ntrk3*) for Mouse Allen Brain Atlas.

Human genetics study of miR-185

The 3-step human genetics study was approved by the respective local ethics committees. Written informed consent was obtained from all participants.

The diagnoses of all German and Dutch patients were made according to DSM-IV criteria; the diagnoses of all Danish patients were made according to ICD-10 (1994–2005) criteria. German controls were screened for schizophrenia, but Dutch controls were not. None of the Danish controls had been assigned a diagnosis of schizophrenia at the time of inclusion according to Danish health registers.[24]

Step 1: gene-based tests for GWAS data

Sample

We used publicly available summary statistics of genotyping data from the PGC study.[13] The PGC data were obtained from 9394 patients with schizophrenia or schizoaffective disorder (66.5% men) and 12 462 controls (48.5% men) of European ancestry. These included individuals from Mannheim/Bonn, Germany (474 patients and 1304 controls); Munich, Germany (434 patients and 351 controls); Utrecht, the Netherlands (704 patients and 631 controls); and Copenhagen, Denmark (482 patients and 457 controls).

Methods

We performed gene-based analyses to investigate whether common variants in the premature *MIR185* sequence increased the risk for schizophrenia and whether common genetic variants associated with schizophrenia were overrepresented in *MIR185* target genes. Gene-based *p* values were computed for *MIR185* and its predicted target genes using FORGE software.[25] The *MIR185* target gene set comprised 124 genes and was derived from the Molecular Signatures Database 3.1 (http://www.broadinstitute.org/gsea/msigdb/index.jsp).[26] Using the Ensembl human gene annotation, SNPs located within the annotated gene coordinates ± 20 000 bp flanking regions were mapped to genes. Gene-wide *p* values were calculated for all 122 target genes in the autosomal data from the PGC using 2 test statistics in the FORGE software: the Sidak correction on minimum *p* value (SIDAK) and the fixed-effect *z*-score statistic (Z FIX). Both statistics are described in detail in the supplemental information of Pedroso and colleagues.[25] While the SIDAK approach is most powerful in situations where only a single functionally relevant variant is observed within the gene, the Z FIX approach is most powerful in situations in which multiple variants are observed. We applied both test statistics, since the true pattern of functionally relevant variants for the presently investigated genes was unknown a priori. Although too conservative (since both tests are not independent), the calculated gene-based *p* values were corrected for multiple testing with twice the number of investigated target genes ($n = 244$).

Further analyses were performed to investigate whether any of the 17 SNPs in the gene-based analysis of *MIR185* showed an individual association with schizophrenia.

Step 2: resequencing

Sample

The resequencing step involved 1000 patients with schizophrenia (57.3% men) of German ancestry and 500 controls (57.4% men) from a population-based sample collected in the Bonn area within the German National Genome Research Network (NGFN).[27] In addition, we investigated 2 patients with schizophrenia with a 22q11.2 microdeletion. These 2 individuals were recruited through the psychiatric services in the German cities of Mannheim and Bonn, respectively. The patient from Bonn is described elsewhere.[28]

Methods

Resequencing was performed to investigate the role of rare variants in idiopathic schizophrenia. In addition, the *MIR185* gene was resequenced in the 2 patients with 22q11.2 microdeletion to investigate the presence of recessive risk alleles on the nondeleted strand.

Prior to resequencing, the presence of a 22q11.2 microdeletion in the 1000 idiopathic patients was excluded using QuantiSNP v1.1[29] and SNP intensity data from HumanHap550v3, HumanHap610v1 and HumanHap660W Bead Arrays (Illumina). These 1000 patients were also screened for small copy number variants (CNVs) pinpointing *MIR185* ± 20 000 bp flanking regions. We retained the CNVs that had a log Bayes factor of 10 or greater and that spanned a minimum of 10 consecutive markers.

Genomic DNA sequences were obtained from the University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway, NCBI build 37.1, hg19; Fig. 1).[30] Using the RefSeq gene definition[31] of *MIR185* (NR_029706.1), primers were designed to amplify a region of around 400 bp, including the target premature microRNA sequence of 82 bp (chr22:20,020,662–20,020,743) and flanking sequences. Primer sequences are available on request.

Resequencing was performed using the Sanger method. The sequencing information was generated and analyzed on the 3130xl Genetic Analyzer (Applied Biosystems). We investigated all obtained nucleotide sequences using SeqMan II (DNASTAR). All patient-specific rare variants were confirmed by sequencing a second, independent amplicon.

We classified variants as "rare" if their minor allele frequency (MAF) in the combined patients/controls was 1% or less. We used a Fisher exact test (2-tailed) to test for association with rare variants. A variant was designated as "novel" if it was not listed in either dbSNP build 135 (http://www.ncbi.nlm.nih.gov/projects/SNP/) or if it was not present in 379 individuals of European ancestry from the 1000 Genomes Project (http://browser.1000genomes.org/index.html).[32] Novel variants were submitted to dbSNP, and identifiers (ss) are provided in the Results section.

Variant nomenclature was assigned according to den Dunnen and Antonarakis.[33] Nucleotide positions correspond to genomic sequence positions (NCBI build 37.1) in human chromosome 22 (NC_000022.10). All variants were investigated for splice site changes, using Human Splicing Finder (http://www.umd.be/HSF/)[34]; for evolutionary conservation, using the Vertebrate Multiz Alignment and Conservation Track; and for localization within transcription factor binding sites, using the HMR Conserved Transcription Factor Binding Sites Track of the UCSC Genome Browser.[30]

Step 3: follow-up genotyping

Sample

This step involved an independent sample of 3598 patients with schizophrenia (61.4% men) and 4082 controls (51.4% men) from 5 centres in Germany (n = 3), the Netherlands (n = 1) and Denmark (n = 1). A subsample of the German control cohort (n = 1097) was drawn from the population-based Heinz Nixdorf Recall Study.[35]

Methods

Genotyping in the 3598 patients and 4082 controls was performed using the iPLEX Gold Sequenom MassARRAY system (Sequenom). The iPLEX primer sequences and assay conditions are available on request. After the exclusion of 66 patients and 62 controls with missing genotypes, the genotyping sample consisted of 3532 patients and 4020 controls.

Results

MiR-185 expression pattern

Table 1 summarizes *miR-185* expression in the mouse brain. At E12.5 in the mouse embryos (Fig. 2A), *miR-185* expression was detected in the cerebral cortex (most

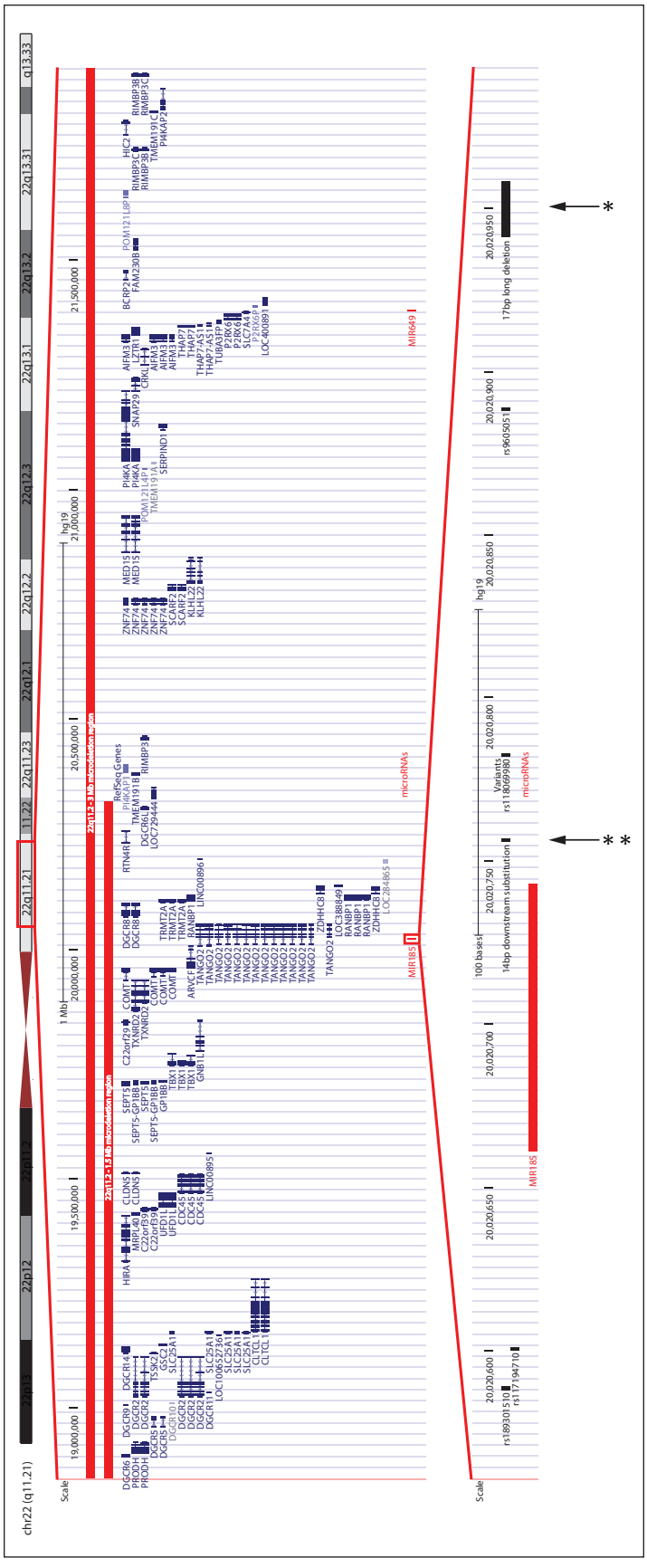

Fig. 1: Overview of the 22q11.2 microdeletion region and the premature *MIR185* sequence obtained from the University of California, Santa Cruz Genome Browser. The extent of the 3 megabase (Mb) and 1.5 Mb deletions are based on the supplementary information in the study by Karayiorgou and colleagues.[3] The locations of the patient-specific novel 14 bp downstream substitution and 17 bp long deletion are indicated by arrows. The number of asterisks indicates the number of observations in patients detected through resequencing.

prominently in the anterior part) and in the basal ganglia. At E14.5, *miR-185* expression appeared to be restricted to the developing central nervous system, in particular the tectum and the spinal cord (Fig. 2B). In the developing cerebral cortex, expression was clearly enriched in the mantle layer (Fig. 2B and D), with low expression being observed in the ventricular zone (Fig. 2D). *MiR-185* expression was also prominent in the septum (Fig. 2E and 4A). At E15.5, *miR-185* expression remained predominant in the cortical plate (Fig. 2F). In the ventricular zone, *miR-185* expression was slightly higher than at E14.5, although it remained low (Fig. 2F). At E16.5, *miR-185* expression was similar to that at E15.5, with prominent expression in the subcortical plate (Fig. 2G). We also observed *miR-185* in the basal ganglia at this time point (Fig. 2G).

In the young (P9) and adult (P46) mouse brains, *miR-185* was observed in the mitral cell and granular layers of the olfactory bulb (Fig. 3A and C). We also detected *miR-185* in the cortex, being enriched at P9 (Fig. 3A) and still detectable at P46 (Fig. 3C and 4M). At P9, *miR-185* was present in the hippocampus (Fig. 3A). At P46, strong expression was present in the cornu ammonis (CA)1 and the dentate gyrus, and lower expression was detected in the CA3 (Fig. 3B and C and Fig. 4E). At P9, *miR-185* was visualized in both the external germinal and internal granular layers of the cerebellum (Fig. 3A). At P46, strong cerebellar expression was restricted to the granular layer (Fig. 3B and C and Fig. 4I).

Table 1: Summary of miR-185 expression in the mouse brain

Ages	Regions	Details	Figure
E12.5	Cerebral cortex	Most prominent rostrally	2A
	Basal ganglia		2A
E14.5	Cerebral cortex	Enriched in mantle layer, low in ventricular zone	2B, 2D, 4A
	Basal ganglia	Septum	2E, 4A
	Tectum		2B
	Spinal cord		2B
E15.5	Cerebral cortex	Predominant in cortical plate, low in ventricular zone	2F
	Basal ganglia		2F
E16.5	Cerebral cortex	Prominent in cortical plate	2G
	Basal ganglia		2G
P9	Olfactory bulb	Mitral cell layer, granular layer	3A
	Frontal cortex	Strong expression	3A
	Hippocampus		3A
	Cerebellum	External germinal and internal granular layers	3A
P46	Olfactory bulb	Mitral cell layer, granular layer	3C
	Frontal cortex		3C, 4M
	Hippocampus	Strong in CA1, dentate gyrus; weak in CA3	3C, 4E
	Cerebellum	Granular layer	3B, 3C, 4I

CA = cornu ammonis.

Gene-based association tests for MIR185 and its predicted target genes

Analysis of the PGC genotyping data[13] revealed no association between *MIR185* and schizophrenia at either the gene level (p_{SIDAK} = 0.53 and $p_{Z\ FIX}$ = 0.11), or the level of individual SNPs (minimal p = 0.09).

With the SIDAK method, 20 of the 122 target genes showed nominally significant p values. With the Z FIX method, 22 target genes showed nominally significant

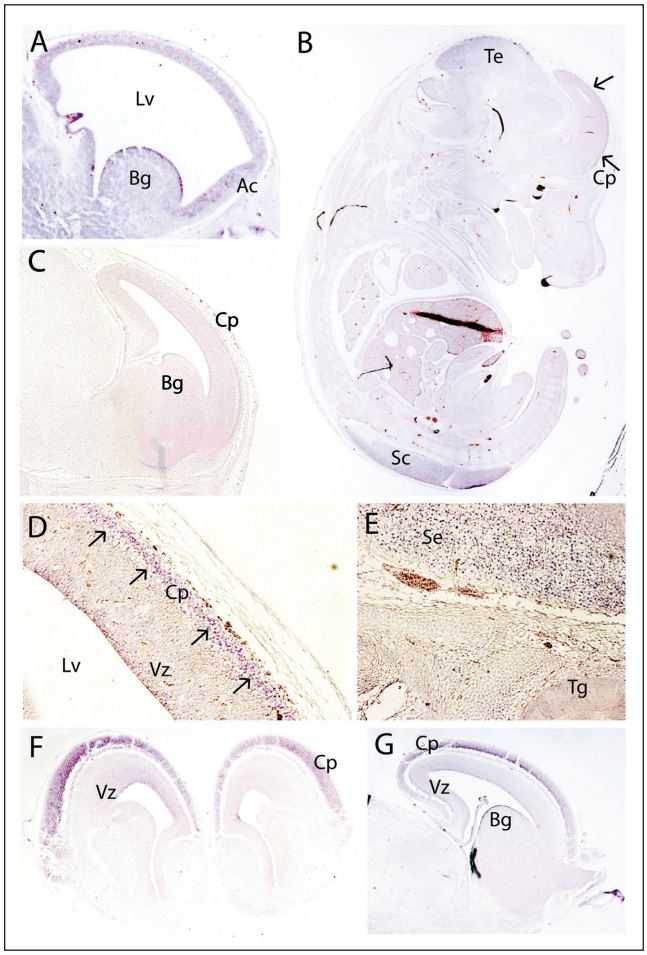

Fig. 2: Expression pattern of *miR-185* in the developing mouse brain at E12.5 (**A**, sagittal), E14.5 (**B, D, E,** sagittal), E15.5 (**F,** coronal) and E16.5 (**G,** sagittal). A negative control with a sense probe is also included (**C,** E14.5, sagittal). At E12.5, *miR-185* is present in the developing cerebral cortex, being most pronounced in the anterior cortex **(A)**; *miR-185* is also present in the basal ganglia **(A)**. At E14.5, *miR-185* expression is prominent in the cortical plate **(B, D)** as well as in the tectum and the spinal cord **(B)**. The septum also shows enriched *miR-185* expression **(E)**, with no expression in the trigeminal ganglion **(E)**. At E15.5 **(F)** and E16.5 **(G)**, *miR-185* is clearly observed in the cortical plate, with low expression in the ventricular zone. Arrows indicate *miR-185* expression in the mantle layer. Ac = anterior cortex; Bg = basal ganglia; Cp = cortical plate; Lv = lateral ventricle; Sc = spinal cord; Se = septum; Te = tectum; Tg = trigeminal ganglion; Vz = ventricular zone.

p values. For both methods, the number of genes with a $p < 0.05$ was significantly higher than expected ($n = 6$), indicating that schizophrenia-associated genes were enriched within the targets of *MIR185* (χ^2 test, $p_{SIDAK} = 0.006$, $p_{Z\ FIX} = 0.002$). Thirty-one genes were nominally associated with schizophrenia according to at least 1 of the 2 methods (Table 2). After correction for multiple testing, 3 genes showed a significant association with schizophrenia: *ATAT1* ($p_{SIDAK} < 0.001$, corrected), *SH3PXD2A* ($p_{SIDAK} = 0.008$, corrected) and *NTRK3* ($p_{Z\ FIX} = 0.002$, corrected).

Mouse brain expression of Atat1, Sh3pxd2a and Ntrk3

The expression patterns of the 3 *miR-185* target genes *ATAT1*, *SH3PXD2A* and *NTRK3* were further investigated in the mouse brain to determine whether they were expressed in the same brain regions as *miR-185* (Fig. 4). Identification of overlap in regions previously implicated in schizophrenia would provide further evidence for the pathogenic role of the *miR-185* regulatory pathway.

At E14.5, all 4 genes were expressed in the cortical plate (Fig. 4A–D). *Atat1*, *Sh3pxd2a* and *Ntrk3* were also detected in the posterior developing cerebral cortex (Fig. 4B–D), where *miR-185* expression was not detected (Fig. 4A). This may indicate differing regulation of the 3 target genes in this brain region. *Atat1* showed broader expression, as it was also observed in the intermediate zone (Fig. 4B).

In the adult mouse brain (P46 for *miR-185*; P56 for the target genes), the 4 genes were observed in the hippocampus (Fig. 4E–H), with lower *miR-185* expression in the CA3 (Fig. 4E). In the cerebellum, all 4 genes were expressed in the granular cell layer (Fig. 4I–L). Overlapping expression of *miR-185* and its 3 target genes was also observed in the frontal cortex (Fig. 4M–P).

Resequencing and array data

No small *MIR185*-spanning CNVs were observed during reinspection of the array intensity data.

Resequencing identified 2 common (MAF > 1%) and 4 rare variants (MAF ≤ 1%). All were located in regions flanking the premature microRNA sequence (Fig. 1). No variants were detected in the premature microRNA sequence per se. Both common variants were known SNPs (rs117194710 and rs9605051). Two of the 4 rare variants were known and 2 were novel, and all individuals with these alterations were heterozygous for the variant allele. The 2 known rare variants (rs189301510 and rs118069980) showed a similar distribution in patients and controls: MAF = 0.46% versus 0.31% (patients v. controls, $p = 0.73$) and MAF = 0.1% (patients and controls, $p > 0.99$), respectively. The 2 novel variants were observed only in patients with

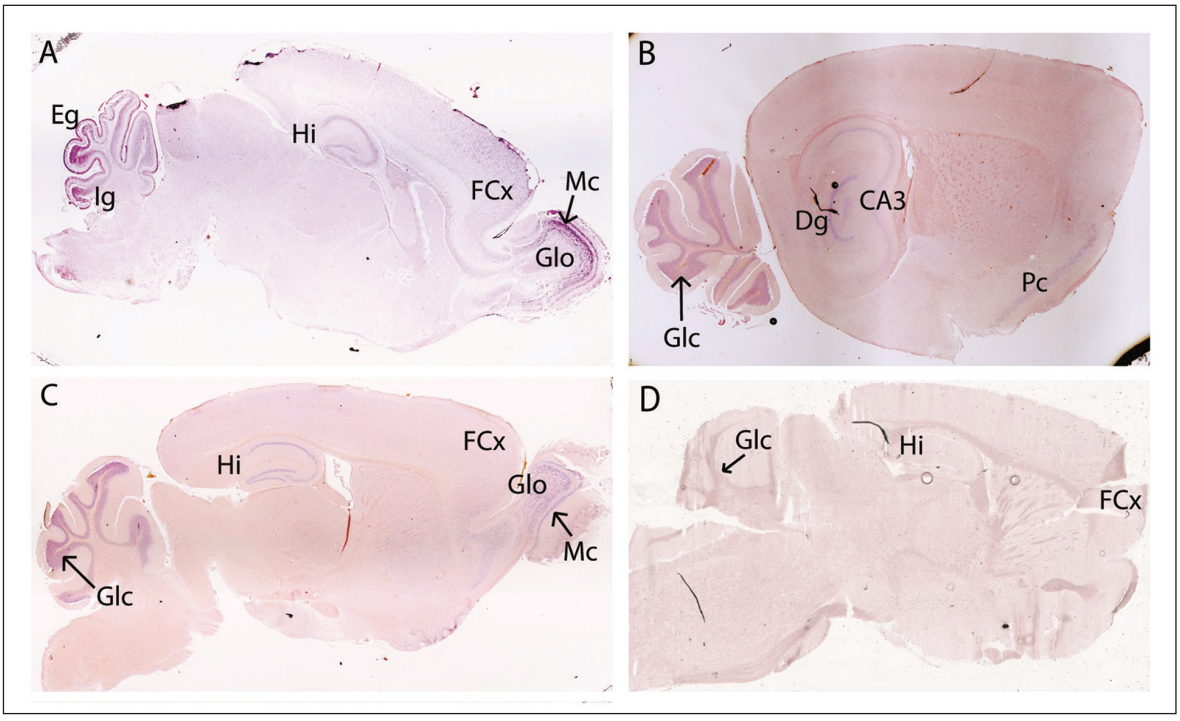

Fig. 3: Expression of *miR-185* in the young (**A,** P9, sagittal) and adult (**B, C,** P46, sagittal) mouse brain. A negative control with a sense probe is also included (**D,** P46, sagittal). At P9, *miR-185* is observed in both mitral cell and granular layers of the olfactory bulb, as well as in the hippocampus **(A)**; *miR-185* is also present in both the external germinal and internal granular layers of the cerebellum **(A)**. At P9 **(A)** and P46 **(C)**, *miR-185* expression is detected in the frontal cortex. At P46, microRNA expression in the hippocampus remains **(B, C)**; *miR-185* expression also persists in the olfactory bulb **(C)** and the piriform cortex **(B)**, and *miR-185* is clearly enriched in the granular layer of the cerebellum **(B, C)**. Dg = dentate gyrus; Eg = external germinal layer of the cerebellum; FCx = frontal cortex; Glc = granular layer of the cerebellum; Glo = granular layer of the olfactory bulb; Hi = hippocampus; Ig = internal germinal layer of the cerebellum; Mc = mitral cell layer; Pc = piriform cortex.

schizophrenia. A cytosine to thymine substitution 14 bp downstream of the premature microRNA sequence (g.20020757C > T, ss748772517) was observed in 2 patients. A 17 bp long deletion located 199 bp downstream was observed in 1 patient (g.20020942_20020958del17, ss748772518).

In silico analysis revealed no potential splice site changes for any of the 4 rare variants. None of the 4 variants was located within transcription factor binding sites. Interestingly, high across-species conservation was observed for the 14 bp downstream substitution (ss748772517). The site was conserved in humans, chimps, rhesus monkeys, mice and dogs.

In the 2 patients with 22q11.2 microdeletion, no rare variants were detected in the analyzed region.

Follow-up genotyping

The 2 patient-specific variants were genotyped in the follow-up sample. Given their similar frequencies in patients and controls, the 2 known rare variants were not followed up. Genotyping revealed that a control individual in the follow-up sample carried the 14 bp downstream substitution (ss748772517). In a combined analysis with the sequencing data, the overall patient and control frequencies for this variant were 0.02% and 0.01%, respectively ($p > 0.99$). None of the follow-up sample participants carried the 17 bp long deletion ($p > 0.99$).

Discussion

The ISH in embryonic and adult mice revealed *miR-185* expression in brain regions implicated in schizophrenia. Of particular interest is the validation of *miR-185* expression in the frontal cortex and hippocampus, with high expression being observed in the dentate gyrus and CA1, since independent studies have reported an association between manifestations of schizophrenia and dysfunction in these regions.[15,36] Furthermore, the *miR-185* expression patterns overlapped with

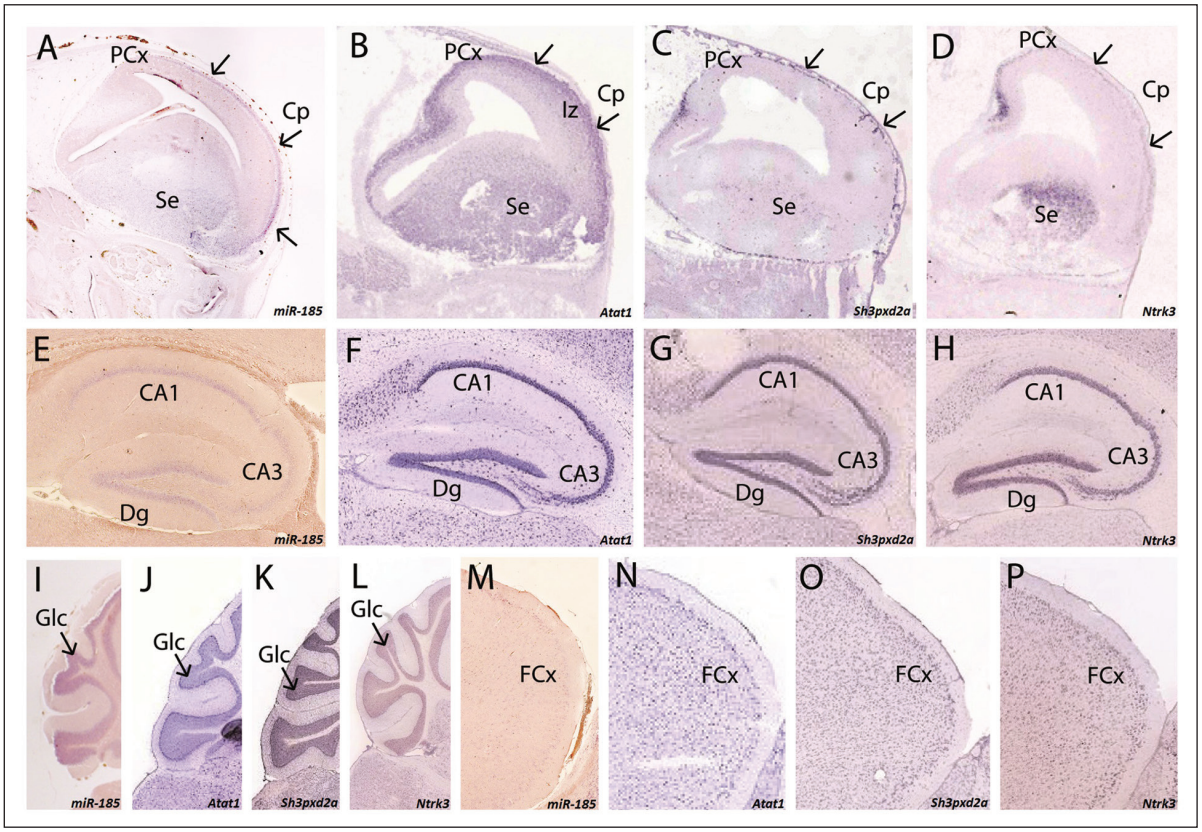

Fig. 4: Comparison of the expression between *miR-185* **(A, E, I, M)** and *Atat1* **(B, F, J, N)**, *Sh3pxd2a* **(C, G, K, O)** and *Ntrk3* **(D, H, L, P)** in both embryonic (**A–D,** E14.5) and adult (**E–P,** P46 for *miR-185*, P56 for the 3 target genes) mouse brain. At E14.5, all 4 genes are detected in the developing cortex, although with differing intensities. While *Atat1* **(B)** has a broad expression, including the intermediate zone, the other genes are restricted to the cortical plate. In addition, the expression of *Atat1* **(B)**, *Sh3pxd2a* **(C)** and *Ntrk3* **(D)** is highlighted in the posterior developing cortex, where *miR-185* **(A)** is not detected. All 4 genes are also detected in the septum **(A–D)**. In the adult mouse brain, all 4 genes are observed in the hippocampus **(E–H)**, the cerebellum **(I–L)** and the frontal cortex **(M–P)**. In the case of the hippocampus, *miR-185* is predominant in the CA1 and the dentate gyrus **(E)**, while the 3 target genes are also clearly seen in CA3 **(F–H)**. In the cerebellum, all 4 genes are present in the granular layer **(I–L)**. Regarding the frontal cortex, *miR-185* **(M)** shows weaker expression than *Atat1* **(N)**, *Sh3pxd2a* **(O)**, and *Ntrk3* **(P)**. Arrows indicate the expression of the 4 genes in the cortical plate. Panels **B–D** were derived from the Eurexpress database.[22] Panels **F–H**, **J–L** and **N–P** were derived from the Allen Mouse Brain Atlas, Allen Institute for Brain Science.[23] Abbreviations: CA = cornu ammonis; Cp = cortical plate; Dg = dentate gyrus; FCx = frontal cortex; Glc = granular layer of the cerebellum; Iz = intermediate zone; PCx = posterior developing cortex; Se = septum.

those of validated *miR-185* targets (RhoA and Cdc42), both of which have been previously associated with schizophrenia.[18,19] In the adult rat brain, RhoA and Cdc42 are expressed in the hippocampus (predominantly in the dentate gyrus and CA1) as well as in the piriform cortex, the cerebral neocortex and the granular layer of the cerebellum.[37] This is consistent with the localization of *miR-185* in the present study.

The *miR-185* expression pattern observed in the present study (Table 1) may provide insight into other 22q11.2DS brain abnormalities. For example, both cortical atrophy[38] and abnormal cortical organization[39] have been associated with 22q11.2DS, in particular polymicrogyria and pachygyria.[40,41] Other structural abnormalities in 22q11.2DS affect the cerebellum. These include hypoplasia of the cerebellar vermis[38] and cerebellar atrophy.[42] Remarkably, *miR-185* expression in the present study was particularly pronounced in these brain regions. As microRNAs play an important role in cortical and cerebellar development,[9] prominent *miR-185* expression in the developing cortical plate and the cerebellum may be related to 22q11.2DS brain abnormalities.[38,39,42]

Gene-based analysis of *MIR185* and its predicted target genes revealed no individual or gene-wide association between schizophrenia and common variants at the *MIR185* locus per se. Two possible explanations for this finding can be proposed. First, common functional variation at this locus, if it exists, may have no effect on schizophrenia susceptibility because *MIR185* is not critically involved in disease-related processes. Second, the lack of association may simply reflect the absence of common functional variation at this locus. This latter explanation is supported by a recent study concerning the genetic regulation of microRNA expression.[43] Here, no SNP had significant cis effects on *miR-185* expression. Lack of common functional variation at the *MIR185* locus would also explain why previous attempts to identify schizophrenia-related genes in the 22q11.2DS region through searches for association with common variants in the region have proven frustrating.

Table 2: Gene-based tests of miR-185 target genes

Gene*	Chromosome	Minimal *p* value†	No. of SNPs	p_{SIDAK} value‡	$p_{Z\ FIX}$ value‡
ADAMTS19	5	0.005	107	> 0.99	> 0.99
ANXA2	15	0.008	50	> 0.99	> 0.99
APBA1	9	< 0.001	109	> 0.99	> 0.99
APP	21	< 0.001	153	0.53	> 0.99
ARID1A	1	0.013	13	> 0.99	> 0.99
ATAT1	6	< 0.001	16	< 0.001	> 0.99
CDC42	1	0.011	22	> 0.99	> 0.99
FAM134C	17	< 0.001	17	> 0.99	> 0.99
FMO2	1	< 0.001	50	> 0.99	> 0.99
FOXN3	14	< 0.001	281	> 0.99	> 0.99
KCNN3	1	0.016	135	> 0.99	> 0.99
LPHN1	19	0.005	12	> 0.99	> 0.99
NFASC	1	< 0.001	151	> 0.99	> 0.99
NTRK3	15	< 0.001	187	0.22	0.002
PAK6	15	< 0.001	36	> 0.99	0.13
PBX1	1	< 0.001	237	> 0.99	> 0.99
PRX	19	0.002	15	> 0.99	> 0.99
PTPRE	10	< 0.001	132	> 0.99	> 0.99
RHOA	3	0.014	17	> 0.99	> 0.99
RIC8B	12	0.012	43	> 0.99	> 0.99
SERF2	15	< 0.001	16	> 0.99	0.91
SH3PXD2A	10	< 0.001	130	0.008	0.06
SLC30A3	2	0.006	13	> 0.99	> 0.99
SLC37A2	11	< 0.001	41	> 0.99	> 0.99
SNX19	11	< 0.001	53	0.18	> 0.99
SYNGAP1	6	0.008	17	> 0.99	> 0.99
SYNM	15	< 0.001	57	> 0.99	0.25
TCF12	15	< 0.001	114	> 0.99	> 0.99
TJP1	15	< 0.001	113	> 0.99	> 0.99
VEZF1	17	0.020	15	> 0.99	> 0.99
ZNF385A	12	< 0.001	7	0.30	> 0.99

SNP = single nucleotide polymorphism.
*Thirty-one miR-185 target genes that showed nominal association with schizophrenia according to at least 1 of the 2 applied methods (SIDAK, Z FIX).
†Single marker.
‡Corrected for multiple testing.

Genetic support for the involvement of *MIR185* and its regulatory pathways is provided by the present association analysis of target genes, which showed that schizophrenia-associated genes were significantly enriched within *MIR185* targets. The genes *ATAT1*, *SH3PXD2A* and *NTRK3* showed individual association with schizophrenia after correction for multiple testing. The 2 validated targets *RHOA* and *CDC42* received nominally significant support (Table 2).

The *ATAT1* (*MEC-17*) gene encodes for an α-tubulin acetyltransferase, which plays a conserved role in several microtubule-based processes.[44] A study in rats showed that *ATAT1* was highly expressed in the cerebral cortex during various developmental stages.[45] In adult rats, relatively high *ATAT1* expression was detected in the cerebral cortex, the hippocampus, and the cerebellum. In addition *ATAT1* deficiency caused defects in the migration of cortical neurons, thus indicating that *ATAT1* and α-tubulin acetylation are important for cortical development.[45] The *ATAT1* gene is located in the extended major histocompatibility complex (MHC) region on chromosome 6. One of the investigated SNPs at the locus (rs9262135) achieved genome-wide significance ($p < 0.001$) in the PGC GWAS.[13] Previous studies have reported strong association between common SNPs in the MHC region and schizophrenia.[13,46–48] However, owing to extensive linkage disequilibrium, the issues of whether the association signal is driven by 1 or several genes in the region and whether immune- or nonimmune-related genes are implicated remain unclear.[48]

The product of the SH3 and PX domains 2A gene (*SH3PXD2A*, *TKS5*) is a tyrosine kinase substrate with 5 Src-homology 3 domains. This is required for podosome and invadopodia formation and for cancer cell invasion.[49] The presence of severe developmental defects (including craniofacial abnormalities and heart malformations) in zebrafish embryos with reduced *SH3PXD2A* expression suggests that this product is implicated in neural crest migration during embryonic development.[50]

The association findings for *ATAT1* and *SH3PXD2A* suggest that alterations in neural and neural crest cell migration are plausible mechanisms in the pathophysiology of schizophrenia. This provides further support for the hypothesis that schizophrenia is a neurodevelopmental disorder.[51]

The *NTRK3* (*TRKC*) gene encodes for a tyrosine protein kinase neurotrophin-3 receptor.[52] *NTRK3* is expressed in various structures within the developing mouse brain, including the caudoputamen, cerebellum and hippocampus.[52] In the adult mouse brain, *NTRK3* is expressed in the cerebral cortex, hippocampus, thalamus and hypothalamus.[52] Research has identified reduced *NTRK3* gene expression in the PFC of patients with schizophrenia[53,54] as well as associations between SNPs in the *NTRK3* gene and hippocampal function and various psychiatric disorders. These include bipolar disorder[55] and schizophrenia.[47,56] Interestingly, Cdc42 and RhoA — the 2 validated schizophrenia-associated targets of *miR-185*[18,19] — are also involved in neurotrophin signalling. This may indicate that *miR-185* influences neurodevelopment through regulation of different levels of the neurotrophin signalling pathway. In the case of *NTRK3*, only the *Z* FIX method generated a significant association, which might indicate that multiple common variants exert functional effects on this gene.

The expression patterns of the 3 schizophrenia-associated target genes were further analyzed to investigate whether they were expressed in the same brain regions as *miR-185*, and whether these overlapping regions have been previously implicated in schizophrenia. This revealed substantial overlap in the expression patterns of the 4 genes in brain regions that included the hippocampus and the frontal cortex. Since both regions have been implicated in schizophrenia,[15,36] this finding provides further support for the involvement of the *miR-185* regulatory pathway in the development of schizophrenia.

In the resequencing step, no genetic variants were identified in the 82 bp long premature *MIR185* sequence. Although the existence of ultrarare variants cannot be excluded, the present observations suggest that this region is highly conserved. If this is the case, a plausible hypothesis is that alterations in the mature microRNA sequence could have more dramatic effects, which is also supported by the high conservation of microRNA sequences between species.[57] This hypothesis is consistent with observations from previous studies of microRNA sequences.[58] The present study identified 4 rare variants flanking the premature *MIR185* sequence for which a possible regulatory role could not be excluded. Although the 2 patient-specific variants were followed up by genotyping large samples of patients and controls, their possible contribution to the risk for schizophrenia could be neither confirmed nor entirely excluded. Furthermore, resequencing data derived from the 2 patients with 22q11.2 microdeletion revealed no evidence for the presence of recessive risk alleles in *MIR185* on the nondeleted DNA strand. The investigation of larger numbers of deletion carriers would be required to determine whether recessive alleles contribute to schizophrenia and 22q11.2DS.[59]

Limitations

Reduced *miR-185* expression in 22q11.2DS is attributable to hemizygosity. Future studies of the role of *miR-185* in schizophrenia must identify rare variants that functionally mimic this effect. Although the present samples included several thousand individuals, the power to detect associations with rare variants was limited,[60] since such variants may be extremely rare.

Conclusion

The present human genetic analyses of common and rare variants generated no evidence for the involvement of *MIR185* in the development of schizophrenia in idiopathic or 22q11.2DS patients. This may reflect the lack of common functional variation at this locus and the limited power to detect rare variant associations. However, the expression patterns of *miR-185* and its target genes (*ATAT1*, *SH3PXD2A*, *NTRK3*) observed in the brains of embryonic and adult mice, as well as the significant association findings for the 3 target genes, suggest that further research into the possible involvement of *miR-185* regulatory pathways in the risk for schizophrenia and (possibly) other neuropsychiatric phenotypes in 22q11.2DS, as well as in idiopathic schizophrenia, is warranted.

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Contributors: A.J. Forstner, F.B. Basmanav, M.M. Mattheisen, M. Rietschel, A. Zimmer, M.M. Nöthen, X. Miró and S. Cichon designed the study. A.J. Forstner, F.B. Basmanav, A.C. Böhmer, M.V. Hollegaard, E. Janson, E. Strengman, W. Maier, R. Mössner, D. Rujescu, R.A. Ophoff, S. Moebus, P.B. Mortensen, A.D. Børglum, D.M. Hougaard, J. Frank, S.H. Witt, M. Rietschel, A. Zimmer and X. Miró acquired the data. A.J. Forstner, F.B. Basmanav, M. Mattheisen, L. Priebe, F. Degenhardt, P. Hoffmann, S. Herms, A. Zimmer, M.M. Nöthen, X. Miró and S. Cichon analyzed the data. A.J. Forstner, F.B. Basmanav, A. Zimmer, M.M. Nöthen, X. Miró and S. Cichon wrote the article, which all other authors reviewed. All authors approved the final version for publication.

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