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ARTICLE

Rare variants of the gene encoding the potassium chloride co-transporter 3 are associated with bipolar disorder

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Abstract

Recessive mutations of the potassium chloride co-transporter 3 gene (*SLC12A6*, *KCC3*) cause severe peripheral neuropathy frequently associated with agenesis of the corpus callosum and psychoses (ACCPN). *SLC12A6* is localized on chromosome 15q14, a region where linkage to schizophrenia and bipolar disorder has previously been shown. Mutation analysis of *SLC12A6* was carried out by direct sequencing of PCR-generated DNA fragments in two affected members of a multiplex family, and three non-affected individuals. A case-control study was performed to assess association of variants with bipolar disorder and schizophrenia in a large sample. Several variants including two rare single nucleotide polymorphisms (G/A, G/A) in the promoter and 5′-UTR, and a thymidine insertion in intron 4 were found. The two G variants and the insertion variant were co-inherited with chromosome 15-related schizophrenia in a large family that strongly supports the region on chromosome 15q14-15 between markers D15S144 and D15S132. Furthermore, they are in linkage disequilibrium with each other, and significantly associated with bipolar disorder in a case-control study. Our data strongly suggest that rare variants of *SLC12A6* may represent risk factors for bipolar disorder.

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Key words: Association study, bipolar disorder, KCC3, potassium chloride co-transporter 3, mutation analysis, *SLC12A6*.

Introduction

Cation-chloride co-transporters, including the potassium chloride co-transporters, are involved in the electroneutral movement of ions across the plasma membrane, thus controlling ionic and osmotic homeostasis of various cell types. Recently, Shen and co-workers demonstrated that expression of the potassium chloride co-transporter 3 (formerly KCC3; the approved symbol provided by the HUGO Gene Nomenclature Committee is SLC12A6 for solute carrier family 12, member 6) increases the cell proliferation rate (Shen et al., 2001). Two isoforms of the co-transporter generated by alternative splicing, and designated KCC3A and KCC3B, have been described (Mount et al., 1999). Of these, KCC3A is predominantly expressed in the brain, whereas KCC3B was found in peripheral tissues (Pearson et al., 2001). The gene *SLC12A6* has been assigned to chromosome

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15q13-14 (Hiki et al., 1999; Mount et al., 1999), a region that shows a linkage peak with the recessively inherited Andermann syndrome (peripheral neuropathy with or without agenesis of the corpus callosum, ACCPN, OMIM 218000). ACCPN is linked to the marker D15S971, which is localized in the vicinity of the connexin 36 gene (*CX36*) and *SLC12A6* (Casaubon et al., 1996, Lander et al., 2001). Delpire and Mount (2002) suggested *SLC12A6* as a candidate gene for both ACCPN and chromosome 15-related schizophrenia (SCZD10, periodic catatonia, OMIM 605419), and more recently, Howard and colleagues (2002) demonstrated that recessive mutations of *SLC12A6* in fact cause ACCPN. Moreover, electrophysiological, morphological, and neuropsychological studies over the last years point to altered callosal connectivity in schizophrenics (Narr et al., 2003), and monozygotic twins with schizophrenia associated with agenesis of the corpus callosum have been described (Motomura et al., 2002; for review, see Innocenti et al., 2003).

The chromosome 15q13-15 region has recently been suggested to contain a susceptibility gene for hereditary schizophrenia (Gejman et al., 2001; Riley et al., 2000), chromosome 15-related schizophrenia (SCZD10, periodic catatonia, OMIM 605419) (Meyer et al., 2002a; Stöber et al., 2000, 2002), schizophrenia combined with a neurophysiological trait, namely P50 auditory event-evoked potential inhibition deficit (Freedman et al., 1997, 2001), and lithium-responsive bipolar disorder (Turecki et al., 2001). Previously, *SLC12A6* has been considered as a candidate gene for Rolandic epilepsy and juvenile myoclonic epilepsy (EJM2, OMIM 604827) both mapping to chromosome 15q14. However, attempts to detect variants of *SLC12A6* responsible for epilepsy were not successful (Steinlein et al., 2001).

We have recently reported confirmed linkage based on a dominant model of inheritance (maximum GENEHUNTER-PLUS lod score 3.57, $p = 0.000026$, at coordinate 35.3 cM) of polymorphic markers between D15S144 and D15S1028 with periodic catatonia (Meyer et al., 2002a; Stöber et al., 2000). Periodic catatonia is a disorder characterized by recurrent episodes of psychosis and psychomotor disturbances. Patients with periodic catatonia express a fluctuating phenotype combining akinetic negativism, immobility and mutism, or hyperkinesia with stereotypies and parakinetic movements as well as increased anxiety, impulsivity and aggressiveness. It is currently conceptualized as a subtype of schizophrenia, however, it also can occur in mood disorders, especially in bipolar disorder (Taylor and Fink, 2003).

Based on our positive linkage findings, the positional candidate *CX36* (Meyer et al., 2002c) as well as the gene encoding the nicotinic $\alpha 7$ receptor (*CHRNA7*; Meyer et al., 2002a) were subsequently excluded as risk factors in the families with periodic catatonia. Here, we report the results of a mutation analysis of *SLC12A6*. We sequenced all exons, exon/intron boundaries, and the putative transcriptional control region of the gene. The aim of present study was to clarify whether a *SLC12A6* mutation is involved in SCZD10, schizophrenia spectrum and bipolar disorder in a large family and other patients.

Material and methods

Patients

Members of large families were ascertained as previously described in detail by our group and others (Meyer et al., 2002a; Stöber et al., 2000, 2001, 2002). Family 11 (Figure 1) consists of three generations with 10 healthy and seven members affected with periodic catatonia as depicted in Stöber et al. (2001) and Meyer et al. (2002a). This family strongly supports the chromosome 15q14 locus (Meyer et al., 2002a; Stöber et al., 2001). Consistent diagnostic classification according to ICD-10 and the Leonhard classification (Leonhard, 1999) was achieved by extensive clinical evaluation, and additional information was collected from different sources, including case history, medical records and/or family informants.

A total of 186 patients from the Lower Franconia area in Germany participated in the association study. These subjects were ascertained either at the Department of Psychiatry and Psychotherapy, University of Wuerzburg, or at the Psychiatric District Hospital Werneck. A total of 114 patients suffered from schizophrenia spectrum disorders according to ICD-10 criteria (31 paranoid type, 14 hebephrenic type, 4 catatonic type, 5 undifferentiated type, 23 residual type, 4 schizophrenia simplex, 1 schizotypical disorder, 9 delusional disorder, 23 schizoaffective disorder). All schizophrenia spectrum disorder patients were in-patients and had been seen by an experienced psychiatrist (A.R. and/or I.T.), and extensive semi-structural interviews had been conducted along with chart reviews and, if possible, further information was retrieved from family informants and case records from other hospitals to ensure consistent diagnoses. Furthermore, one affected patient from each of the 12 families investigated in our genome scan (Stöber et al., 2000, 2001) was genotyped. An additional sample of 72 in-patients suffering from bipolar disorder

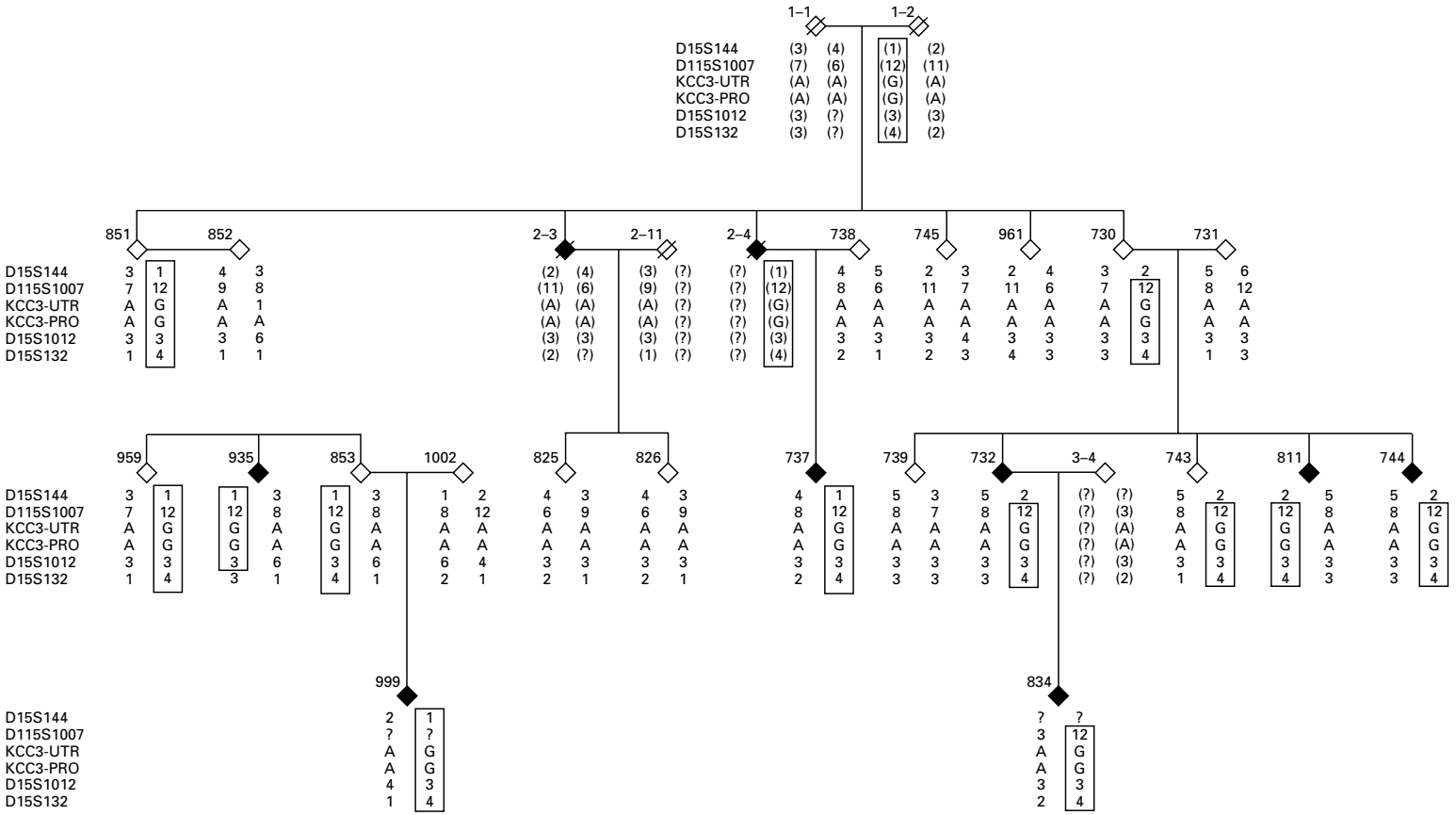

Figure 1. Haplotypes of polymorphic chromosome 15 markers including variants of *SLC12A6* segregating in family 11. The region of interest is indicated by boxed haplotypes. Affected persons are indicated by black diamonds. Assumed haplotypes (of deceased persons) are given in parentheses.

(66 patients) or severe depressive disorder with psychotic symptoms (six patients) according to ICD-10 criteria, were ascertained at the Department of Psychiatry, University of Wuerzburg. Bipolar patients had at least one manic and one depressive episode, and patients suffering from depression had at least two depressive episodes, and showed psychotic symptoms during these episodes. None of the subjects showed significant neurological comorbidity, epilepsy, mental retardation, or a history of substance abuse.

A sample of DNA probes derived from of 350 control subjects has been collected by staff of the Departments of Psychiatry and Psychotherapy, and Transfusion Medicine of the University of Wuerzburg. The control sample consists of healthy blood donors from the same area as the patient group, which were not screened for a history of psychiatric disorders. Mean age of patients was 46 ± 15 yr, of controls 32 ± 10.5 yr. Only patients and healthy volunteers who gave written informed consent after oral and written explanation about the aim and scope of the investigation were enrolled in this study. The study was approved by the Ethics Committee of the University of Wuerzburg.

Mutation analysis

The putative transcriptional control region and all exons of *SLC12A6* from two affected members of family 11 (patients 732 and 737; Meyer et al., 2002a) and three healthy controls were amplified in a T-Gradient thermocycler (Biometra, Göttingen, Germany). Annotation of the gene and primer selection was based on the sequence provided by the International Human Genome Sequencing Consortium (Lander et al., 2001). For these PCRs we have used 37 primer pairs; primers and methods used for individual SNP verification not described here are available on request. PCR was performed in a final volume of 25 μl containing 40–60 ng genomic DNA, 10 pmol of each primer, 200 μM of each NTP, 0.75 or 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3 at 25 °C), 0.025 mg/ml BSA, 0.025% Tween-20, and 0.5 U *Taq* DNA polymerase (Eurogentec, Seraing, Belgium). Resulting PCR products were sequenced using an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). Subsequently, all family members and control subjects were tested for variants that were found to be restricted to the patients.

Genotyping

The following primer pairs were used for genotyping: For 32416574G/A, forward primer PromG/A-f: 5′-CCTGAATCAAGAAACCCAGAC-3′, reverse primer PromG/A-r: 5′-ATCCATTCCATGTTTTCACCA-3′. PCR conditions were as described above, with 0.75 mM $MgCl_2$, annealing temperature 60.6 °C, product size 424 bp. For 32418760G/A, forward primer was UTR-f: 5′-TGTGGGGTATTCACCTG-3′. Variant-specific primers, ending with either C or T, were selected for the reverse strand: UTR-C-r, 5′-TTTCCTGTCGAGGTAGC-3′, UTR-T-r, TTTCCTGTCGAGGTAGT-3′. PCR conditions were as described above, with 0.75 mM $MgCl_2$, annealing temperature 52.2 °C, product size 157 bp. For IVS4 + 1008ins(T)/del(T), primers INS-T-f: 5′-TTTCTGCTTGGAGGTTAATATGG-3′, and INS-T-r: 5′-GAGGTAGACACCCATGAAGG-3′ were used. PCR conditions were as described above, with 0.75 mM $MgCl_2$, annealing temperature 59.2 °C, product sizes 145 and 146 bp.

Statistical analysis

Single marker-disease associations were calculated by exact tests, χ^2 tests, and Armitage trend tests (Sasieni, 1997) using the SAS statistical package SAS/STAT, version 8.1 (SAS Institute Inc., Cary, NC, USA). Haplotype analysis was performed with the program FastEH (Zhao et al., 2000; Zhao and Sham, 2002). To assess Hardy–Weinberg equilibrium of the genotypes in cases and controls, the program FINETTI (T. F. Wienker, unpublished observations) was used. Hardy–Weinberg equilibrium was confirmed for all variants in patients and controls. Pairwise haplotypes and the complete haplotype block for the three variants in the combined sample and in the two subgroups were assessed first. Subsequently, the single variants were explored for association, the p values were corrected for 12 times multiple testing. A p value of < 0.0042 is, therefore, considered to be significant.

Results

The putative promoter region, a newly defined 5′-untranslated region (5′-UTR), and the coding sequence of *SLC12A6* were compared between patients and controls. Variants of the predicted promoter, 5′-UTR, and the coding region are summarized in Table 1.

Promoter and 5′-UTR

The putative promoter region of *SLC12A6* as predicted by PROSCAN software version 1.7 (http://bimas.dcrt.nih.gov/molbio/proscan/) was compared between patients and controls by sequencing. A newly defined 5′-UTR of 1185 nucleotides of exon 1A was assembled by annotation of several novel expressed sequence tag

Table 1. Variants of *SLC12A6*

Nucleotide position[a]	Position in gene	Allele frequencies
32418760[AC(A/G)TT]	Promoter	(see Table 3)
32417688[GC(C/T)GG]	Promoter	C: 7/10; T: 3/10
32416574[GG(A/G)CT]	5′-UTR	(see Table 3)
32416459[TT(C/T)TG]	5′-UTR	C: 7/10; T: 3/10
32416388 [AA(A/T)AT]	5′-UTR	A: 7/10; T: 3/10
32416316[TT(G/A)GG]	5′-UTR	G: 7/10; A: 3/10
32340523ins(T)/del(T)	IVS2 + 14311	del(T): 5/10
32337302ins(T)/del(T)	IVS4 + 1008	(see Table 3)
32330591ins(T)/del(T)	IVS9 + 1063	del(T): 5/10
32330347T/C	IVS10 + 45T/C	T: 7/10; C: 3/10
32329898A/G	IVS11 + 226A/G	A: 8/10; G: 2/10
32324727G/A	IVS14 + 51G/A	G: 0/10; A: 10/10*
32323591C/T	IVS14 + 1187C/T	C: 8/10; T: 2/10
32318756ins(T)	IVS18 + 1387	ins(T): 3/10
32317068T/C	IVS20 + 657T/C	T: 7/10; C: 3/10
32338331CTG/TTG (159Leu)	Exon 4	C: 7/10; T: 3/10
32331762TCG/TCA (412Ser)	Exon 9	G: 7/10; G: 3/10
32330487TTT/TCT (Phe466Ser)	Exon 10	T: 0/70; C: 70/70
32330165CCG/CCC (517Pro)	Exon 11	G: 7/10; C: 3/10
32320822GAA/GAG (723Glu)	Exon 17	A: 0/10; G: 10/10**
32316240CTC/CTT (1001Leu)	Exon 22	C: 3/10; T: 7/10

Results are shown for two affected members of family 11, and three controls (in total 10 chromosomes) studied by mutational analysis in the first step. Of the two polymorphisms already present in the databases, and indicated by asterisks (*rs4371122, **rs2705357), only one allele was found in this sample.
[a] Nucleotide positions according to UCSC browser (Golden Path) of May 2004, Freeze.

clones (ESTs) deposited in the GenBank database (GenBank accession nos. BI561477, AF477977, AF105366, BI032773, BI032775, BI463275, and *Macaca fascicularis* cDNA clone QtsA-15786 (GenBank accession no. AB070168) to the human genomic sequence (Lander et al., 2001). Due to alternative splicing of exon 1A, parts of these sequences may either represent exonic 5′-UTR or intronic sequence. Two rare variants, 32416574G ([CTTTAC(A/G)TTAGGA], 5′-UTR) and 32418760G ([TAAAGG(A/G)CTACCT], promoter), were found in all affected members of family 11 (numbering according to UCSC Browser Golden Path of May 2004, Freeze). Additionally, one affected member of each of the remaining 11 families involved in our initial genome scan (Stöber et al., 2000, 2001) was genotyped for these two variants, but neither was detected in these families.

Coding region

No variants of the coding sequence of *SLC12A6* have been described so far, however, we found five coding-synonymous variants, and all five subjects investigated in the first step and an additional 30 caucasian subjects of German ($n = 26$) and American ($n = 4$) origin being homozygous for codon 466 TCT encoding serine (Table 1). This codon is in accordance with the sequence (contig accession no. NT_024680) provided by the International Human Genome Sequencing Consortium (Lander et al., 2001). A TTT triplet encoding phenylalanine was present in three entries deposited in the GenBank database at amino acid positions 466 (*KCC3A*, GenBank accession no. XM_007749 and XM_036778) and 327 (*KCC3B*, GenBank accession no. XM_036777) respectively. However, these putatively erroneous entries have since been removed from GenBank.

Introns

We found a T/C polymorphism [5′-GCTCTAATT-TT(T/C)CA-3′] adjacent to a putative branch site (CTCTAAT) of intron 20. The novel C variant (IVS20 + 657C) is not co-segregating with disease in

Table 2. Pair-wise linkage disequilibrium (LD) between three polymorphisms of *SLC12A6*

Marker	In LD with	Number of individuals (cases and controls)	Standardized measure for LD D' (S.E.)	Test for LD χ^2, d.f., p value	χ^2 statistic for marker–marker association LR χ^2, d.f., p value
32418760(G/A)	32416574(G/A)	503	1.0000 ($\pm$0.0002)	$\chi^2=706.55$, 1 d.f., $p=0.0000$	$\chi^2=92.92$, 1 d.f., $p=0.0000$
32418760(G/A)	IVS4+1008ins(T)	410	0.7884 ($\pm$0.1061)	$\chi^2=146.79$, 1 d.f., $p=0.0000$	$\chi^2=41.66$, 1 d.f., $p=0.0000$
32416574(G/A)	IVS4+1008ins(T)	416	0.9056 ($\pm$0.0875)	$\chi^2=143.88$, 1 d.f., $p=0.0000$	$\chi^2=40.52$, 1 d.f., $p=0.0000$

D', Standardized measure for LD (40); LR, likelihood ratio.
χ^2 statistic for marker association of all three markers: LR $\chi^2=135.13$, d.f. $=4$, $p=0.0000$.

family 11. However, an additional thymidine residue was found in a thymidine stretch adjacent to an intronic TATCAAT motif [5′-GATTTTTT(insT)ATC-AAT-3′], another putative branch site that is localized 22 bp upstream of the splice acceptor site of the intron 4 intervening sequence (IVS4) of the gene. The relatively rare IVS4+1008ins(T) variant was present in all seven affected members of the large pedigree 11 (Figure 1) and was studied in more detail due to its putative functional relevance, as were both putatively functional G variants in promoter and 5′-UTR (see Tables 1–4)

To assess the possible effects of these variants in the pathogenesis of schizophrenia and affective disorders, we conducted a case-control association study. For 32418760G, 186 cases and 318 controls were genotyped, for 32416574G, 186 cases and 346 controls were genotyped, and for IVS4+1008ins(T), 181 cases and 266 controls were genotyped.

As the promotor and the 5′-UTR variants (32418760G and 32416574G) and the intron variant IVS4+1008ins(T) showed strong linkage disequilibrium (LD; Table 2), first, the association of the haplotypes with both disorders and the combined sample was assessed. The haplotype of the three variants 32418760G, 32416574G and IVS4+1008ins(T) was associated with the bipolar subsample. For the haplotypes containing only two variants, association was found for the ones including 32418760G in the bipolar subsample. A trend for association of these haplotypes with the combined sample was also found. As shown in Table 4, the two G variants were less abundant compared to IVS4+1008ins(T), thus rarely leading to the occurrence of the combined haplotype. As genotyping rates differed between the variants, and LD was least strong with the intron variant IVS4+1008ins(T), an additional single marker analysis was conducted, which showed association of 32418760G in the bipolar disorder subsample and the combined sample and also a trend for an association of 32416574G in those samples (Table 4). No clinical differences were found in patients carrying the rare variants, and those who were not carriers.

Discussion

Based on the hypothesis that specific genes may cause both neuropathies and psychoses (Delpire and Mount, 2002; Meyer et al., 2002b), we have selected variants of *SLC12A6* for association studies with schizophrenia spectrum and bipolar disorder. This gene was chosen as a candidate, since it is located in a candidate region for schizophrenia and bipolar affective disorder on chromosome 15q14. Furthermore, recessive mutations in *SLC12A6* were shown to cause severe peripheral neuropathy with or without agenesis of the corpus callosum (Andermann syndrome, ACCPN, OMIM 218000) (Howard et al., 2002), which is frequently associated with psychoses (Filteau et al., 1991). Previously, a considerable amount of overlapping chromosomal loci for both bipolar disorder and schizophrenia has been shown (for review, see Berrettini, 2000; Klar, 2001). Additionally, periodic catatonia (SCZD10) and manic-depressive disorder share bipolarity as a typical feature (Fink and Taylor, 2001).

We have detected three variants of *SLC12A6* co-segregating with disease in a multiplex family with schizophrenia of periodic catatonia type. These were further assessed for association in two patient samples with either schizophrenia or bipolar disorder, and the combined patient sample, diagnosed according to ICD-10, of German origin. Our finding of associated three and two variant haplotypes in bipolar disorder

Table 3. Association of haplotypes of *SLC12A6* variants with bipolar disorder and schizophrenia

Haplotypes	Association with schizophrenia spectrum test for heterogeneity, empirical *p* value	Association with bipolar disorder test for heterogeneity, empirical *p* value	Association with schizophrenia spectrum and bipolar disorders combined test for heterogeneity, empirical *p* value
32418760(G/A)– 32416574(G/A)	Cases ($n=114$) Controls ($n=317$) $p=0.2467$	Cases ($n=72$) Controls ($n=317$) $\mathbf{p=0.0016}$	Cases ($n=186$) Controls ($n=317$) $p=0.0064$
32418760(G/A)– IVS4+1008ins(T)/del(T)	Cases ($n=111$) Controls ($n=229$) $p=0.0751$	Cases ($n=70$) Controls ($n=229$) $\mathbf{p=0.0017}$	Cases ($n=181$) Controls ($n=229$) $p=0.0042$
32416574(G/A)– IVS4+1008ins(T)/del(T)	Cases ($n=111$) Controls ($n=235$) $p=0.2176$	Cases ($n=70$) Controls ($n=235$) $p=0.0175$	Cases ($n=181$) Controls ($n=235$) $p=0.0266$
32418760(G/A)– 32416574(G/A)– IVS4+1008ins(T)/del(T)	Cases ($n=111$) Controls ($n=228$) $p=0.1382$	Cases ($n=70$) Controls ($n=228$) $\mathbf{p=0.0022}$	Cases ($n=181$) Controls ($n=228$) $p=0.0118$

Cases, n = number of affected individuals; controls, n = number of controls.

supports the hypothesis of a close relationship of periodic catatonia and bipolar disorder. Because of the fluctuating phenotype with periods of so-called 'positive' and 'negative' symptoms, periodic catatonia might be more related to the bipolar spectrum disorders than to schizophrenia (Taylor and Fink, 2003). However, these findings do not allow a final judgement as to whether periodic catatonia is a true nosological entity or not.

Due to the association findings, the promoter polymorphism itself, or haplotypes containing this polymorphism may influence pathogenesis directly. As the 5′-UTR SNP also showed a trend towards association with bipolar disorder, this polymorphism may either influence pathogenesis, or the association may be due to strong LD between both G variants. Our finding of an association of *SLC12A6* implicated in agenesis of the corpus callosum further supports the developmental hypothesis of mental disorders.

A few limitations of our study should be addressed. Our finding may represent a false-positive result due to population stratification (Lander and Schork, 1994). However, stratification seems unlikely since both patients and controls originate from the same narrow area around Wuerzburg, Germany, which is inhabited by a homogenous Franconian population.

Furthermore, both rare G variants may be in LD with the 'true' disease causing mutation, which could then be expected distantly to the *SLC12A6* locus, since another large family (family 9; Stöber et al., 2002) with five affected members supports a region of 7.7 cM between markers D15S1042 and D15S182 distally of *SLC12A6* (Meyer et al., 2003). Alternatively, a second susceptibility gene for SCZD10 may be located on chromosome 15, and be responsible for the disease in family 9. However, the distance between *SLC12A6* and marker D15S1042 representing the upper limit for linkage in family 9 spans ~1.6 Mb, whereas LD in the general population could be expected for intervals from 3 to 100 kb (Kruglyak, 1999; Ott, 2000; Reich et al., 2001).

Our finding supports the view of Liu and colleagues (2005), that rare variants should not be ignored in genetic studies of complex diseases. However, since our sample size is relatively small, replication of the results of the association study is needed. In particular, the borderline association found with the schizophrenia subsample is worthy of being evaluated in an independent study.

Taken together, our data combined with the results of others provide evidence that *SLC12A6* should be considered as a risk gene for developing manic-depressive disorder. In the single marker analysis, the strongest association was found for the promoter 32418760G/A polymorphism. Preliminary results indicate that the CpG site created by the

Table 4. Association of alleles and genotypes of *SLC12A6* polymorphisms with bipolar disorder and schizophrenia

Variants	Controls *n* (%)	Schizophrenia *n* (%)	Statistic Schizophrenia vs. controls	Bipolar disorder *n* (%)	Statistic Bipolar disorder vs. controls	All cases *n* (%)	Statistic All cases vs. controls
1. Alleles							
32418760A	632 (99.4%)	223 (97.8%)	Exact test, $p=0.0596$	136 (94.4%)	Exact test, $\mathbf{p<0.0001}$	359 (96.5%)	χ^2 test, 1 d.f., $\chi^2=11.63$, $\mathbf{p=0.0007}$
32418760G	4 (0.6%)	5 (2.2%)	Odds ratio 3.54 (0.94–13.31)	8 (5.6%)	Odds ratio 9.29 (2.76–31.31)	13 (3.5%)	Odds ratio 5.72 (1.85–17.68)
32416574A	689 (99.6%)	224 (98.2%)	Exact test, $p=0.0678$	139 (96.5%)	Exact test, $p=0.0051$	363 (97.6%)	Exact test, $p=0.0053$
32416574G	3 (0.4%)	4 (1.8%)	Odds ratio 4.10 (0.91–18.46)	5 (3.5%)	Odds ratio 8.26 (1.95–34.97)	9 (2.4%)	Odds ratio 5.69 (1.53–21.16)
IVS4+1008ins(T)	27 (5.1%)	17 (7.7%)	χ^2 test, 1 d.f., $\chi^2=1.90$, $p=0.1680$	15 (10.7%)	χ^2 test, 1 d.f., $\chi^2=6.02$, $p=0.0142$	32 (8.8%)	χ^2 test, 1 d.f., $\chi^2=4.95$, $p=0.0260$
IVS4+1008del(T)	505 (94.9%)	205 (92.3%)	Odds ratio 1.55 (0.83–2.91)	125 (89.3%)	Odds ratio 2.24 (1.16–4.35)	330 (91.2%)	Odds ratio 1.81 (1.07–3.08)
2. Genotypes							
32418760A/A	314 (98.7%)	109 (95.6%)	Exact test, $p=0.0586$	64 (88.9%)	Exact test, $\mathbf{p=0.0000}$	173 (93.0%)	Armitage trend test, $Z=-3.44$
32418760A/G	4 (1.3%)	5 (4.4%)	Odds ratio	8 (11.1%)	Odds ratio	13 (7.0%)	$\mathbf{p=0.0006}$
32418760G/G	0 (0.0%)	0 (0.0%)	3.60 (0.95–13.65)	0 (0.0%)	9.81 (2.87–33.57)	0 (0.0%)	Odds ratio 5.90 (1.89–18.37)
32416574A/A	343 (99.1%)	110 (96.5%)	Exact test, $p=0.0671$	67 (93.1%)	Exact test, $p=0.0049$	177 (95.2%)	Exact test, $p=0.0051$
32416574A/G	3 (0.9%)	4 (3.5%)	Odds ratio	5 (6.9%)	Odds ratio	9 (4.8%)	Odds ratio
32416574G/G	0 (0.0%)	0 (0.0%)	4.12 (0.92–18.86)	0 (0.0%)	8.53 (1.99–36.56)	0 (0.0%)	5.81 (1.55–21.74)
IVS4+1008 ins(T)/ins(T)	0 (0.0%)	0 (0.0%)	Armitage trend test, $Z=-1.42$	0 (0.0%)	Armitage trend test, $Z=-2.54$	0 (0.0%)	Armitage trend test, $Z=-2.31$
IVS4+1008 ins(T)/del(T)	27 (10.1%)	17 (15.3%)	$p=0.1546$	15 (21.4%)	$p=0.0111$	32 (17.7%)	$p=0.0210$
IVS4+1008 del(T)/del(T)	239 (89.9%)	94 (84.7%)	Odds ratio 1.60 (0.83–3.07)	55 (78.6%)	Odds ratio 2.41 (1.20–4.84)	149 (82.3%)	Odds ratio 1.90 (1.10–3.30)

Odds ratio, heterozygosity as risk vs. homozygosity; 95% confidence interval in parentheses.

G allele is methylated in peripheral blood lymphocytes, thus suggesting a putative regulatory function of the 32418760G promoter variant (Moser et al., unpublished observations). Therefore, *SLC12A6* should be regarded as one of the susceptibility genes in this chromosomal region responsible for the pathogenesis of bipolar disorder, and, perhaps, schizophrenia with bipolar features.

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Statement of Interest

None.

References

Berrettini WH (2000). Are schizophrenic and bipolar disorders related? A review of genetic and molecular studies [Review]. *Biological Psychiatry 48*, 531–538.

Casaubon LK, Melanson M, Lopes-Cendes I, Marineau C, Andermann E, Andermann F, Weissenbach J, Prevost C, Bouchard JP, Mathieu J, Rouleau GA (1996). The gene responsible for a severe form of peripheral neuropathy and agenesis of the corpus callosum maps to chromosome 15q. *American Journal of Human Genetics 58*, 28–34.

Delpire E, Mount DB (2002). Human and murine phenotypes associated with defects in cation-chloride cotransport [Review]. *Annual Reviews of Physiology 64*, 803–843.

Filteau MJ, Pourcher E, Bouchard RH, Baruch P, Mathieu J, Bedard F, Simard N, Vincent P (1991). Corpus callosum agenesis and psychosis in Andermann syndrome. *Archives of Neurology 48*, 1275–1280.

Fink M, Taylor MA (2001). The many varieties of catatonia. *European Archives of Psychiatry and Clinical Neuroscience 251* (Suppl. 1), I/8–I/13.

Freedman R, Coon H, Myles-Worsley M, Orr-Urtreger A, Olincy A, Davis A, Polymeropoulos M, Holik J, Hopkins J, Hoff M, et al. (1997). Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. *Proceedings of the National Academy of Sciences USA 94*, 587–592.

Freedman R, Leonard S, Gault JM, Hopkins J, Cloninger CR, Kaufmann CA, Tsuang MT, Farone SV, Malaspina D, Svrakic DM, Sanders A, Gejman P (2001). Linkage disequilibrium for schizophrenia at the chromosome 15q13-14 locus of the alpha7-nicotinic acetylcholine receptor subunit gene (CHRNA7). *American Journal of Medical Genetics 105*, 20–22.

Gejman PV, Sanders AR, Badner JA, Cao Q, Zhang J (2001). Linkage analysis of schizophrenia to chromosome 15. *American Journal of Medical Genetics* (*Neuropsychiatric Genetics*) *105*, 789–793.

Hiki K, D'Andrea RJ, Furze J, Crawford J, Woollatt E, Sutherland GR, Vadas MA, Gamble JR (1999). Cloning, characterization, and chromosomal location of a novel human K+-Cl-cotransporter. *Journal of Biological Chemistry 274*, 10661–10667.

Howard HC, Mount DB, Rochefort D, Byun N, Dupre N, Lu J, Fan X, Song L, Riviere JB, Prevost C, et al. (2002). The KCl cotransporter KCC3 is mutant in a severe peripheral neuropathy associated with agenesis of the corpus callosum. *Nature Genetics 32*, 384–392.

Innocenti GM, Ansermet F, Parnas J (2003). Schizophrenia, neurodevelopment and corpus callosum. *Molecular Psychiatry 8*, 261–274.

Klar AJS (2001). The 1;11 translocation provides the best evidence supporting genetic etiology for schizophrenia and bipolar affective disorders [Review]. *Genetics 160*, 1745–1747.

Kruglyak L (1999). Prospects for whole-genome linkage disequilibrium mapping of common disease genes. *Nature Genetics 22*, 139–144.

Lander ES, Schork NJ (1994). Genetic dissection of complex traits. *Science 266*, 353.

Lander ES, et al. (International Human Genome Sequencing Consortium) (2001). Initial sequencing and analysis of the human genome. *Nature 409*, 860–921.

Leonhard K (1999). *Classification of Endogenous Psychoses and their Differentiated Etiology* (2nd edn). Vienna, New York: Springer.

Liu PY, Zhang YY, Lu Y, Long JR, Shen H, Zhao LJ, Xu FH, Xiao P, Xiong DH, Liu YJ, Recker RR, Deng HW (2005). A survey of haplotype variants at several disease candidate genes: the importance of rare variants for complex diseases. *Journal of Medical Genetics 42*, 221–227.

Meyer J, Mai M, Ortega G, Mössner R, Lesch KP (2002c). Mutational analysis of the connexin 36 gene (*CX36*) and exclusion of the coding sequence as a candidate region for catatonic schizophrenia in a large pedigree. *Schizophrenia Research 58*, 87–91.

Meyer J, Ortega G, Schraut K, Nürnberg G, Rüschendorf F, Saar K, Mössner R, Wienker TF, Reis A, Stöber G, Lesch KP (2002a). Exclusion of the neuronal nicotinic acetylcholine receptor $\alpha 7$ subunit gene as a candidate for catatonic schizophrenia in a large family supporting the chromosome 15q13-22 locus. *Molecular Psychiatry 7*, 220–223.

Meyer J, Rüschendorf F, Lesch KP (2003). A second large family with catatonic schizophrenia supports the region distally of CHRNA7 on chromosome 15q14-15 [Letter]. *Molecular Psychiatry 8*, 259–260.

Meyer J, Scherer S, Mössner R, Lesch KP (2002b). Reply concerning KIAA0027 (WKL1, MLC1) and

psychosis: white matters [Letter]. *Molecular Psychiatry 7*, 1037–1038.

Motomura N, Satani S, Inaba M (2002). Monozygotic twin cases of the agenesis of the corpus callosum with schizophrenic disorder. *Psychiatry and Clinical Neuroscience 56*, 199–202.

Mount DB, Mercado A, Song L, Xu J, George Jr. AL, Delpire E, Gamba G (1999). Cloning and characterization of KCC3 and KCC4, new members of the cation-chloride cotransporter gene family. *Jounal of Biological Chemistry 274*, 16355–16362.

Narr KL, Cannon TD, Woods RP, Thompson PM, Kim S, Asunction D, van Erp TGM, Poutanen V-P, Huttunen M, Lönnqvist J, Standerksjöld-Nordenstam CG, Kaprio J, Mazziotta JC, Toga AW (2003). Genetic contributions to altered callosal morphology in schizophrenia. *Journal of Neuroscience 22*, 3720–3729.

Ott J (2000). Predicting the range of linkage disequilibrium. *Proceedings of the National Academy of Sciences USA 97*, 2–3.

Pearson MM, Lu J, Mount DB, Delpire E (2001). Localization of the K(+)-Cl(-) cotransporter, KCC3, in the central and peripheral nervous systems: expression in the choroid plexus, large neurons and white matter tracts. *Neuroscience 103*, 481–491.

Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Ritcher DJ, Lavery T, Kouyoumjian R, Farhadian SF, Ward R, Lander ES (2001). Linkage disequilibrium in the human genome. *Nature 411*, 199–204.

Riley BP, Makoff A, Mogudi-Carter M, Jenkins T, Williamson R, Collier D, Murray R (2000). Haplotype transmission disequilibrium and evidence for linkage of the CHRNA7 gene region to schizophrenia in Southern African Bantu families. *American Journal of Medical Genetics 96*, 196–201.

Sasieni PD (1997). From genotypes to genes: doubling the sample size. *Biometrics 53*, 1253–1261.

Shen MR, Chou CY, Hsu KF, Liu HS, Dunham PB, Holtzman EJ, Ellory JC (2001). The KCl cotransporter isoform KCC3 can play an important role in cell growth regulation. *Proceedings of the National Academy of Sciences USA 98*, 14714–14719.

Steinlein OK, Neubauer BA, Sander T, Song L, Stoodt J, Mount DB (2001). Mutation analysis of the potassium chloride cotransporter KCC3 (SLC12A6) in rolandic and idiopathic generalized epilepsy. *Epilepsy Research 44*, 191–195.

Stöber G, Pfuhlmann B, Nürnberg G, Schmidtke A, Reis A, Franzek E, Wienker TF (2001). Towards the genetic basis of periodic catatonia: pedigree sample for genome scan I and II. *European Archives of Psychiatry and Clinical Neuroscience 251* (Suppl. 1), I/25–I/30.

Stöber G, Saar K, Rüschendorf F, Meyer J, Nürnberg G, Jatzke S, Franzek E, Reis A, Lesch KP, Wienker TF, Beckmann H (2000). Splitting schizophrenia: periodic catatonia-susceptibility locus on chromosome 15q15. *American Jounal of Human Genetics 67*, 1201–1207.

Stöber G, Seelow D, Rüschendorf F, Ekici A, Beckmann H, Reis A (2002). Periodic catatonia: confirmation of linkage to chromosome 15 and further evidence for genetic heterogeneity. *Human Genetics 111*, 323–330.

Taylor MA, Fink M (2003). Catatonia in psychiatric classification: a home of its own. *American Journal of Psychiatry 160*, 1233–1241.

Turecki G, Grof P, Grof E, D'Souza V, Lebuis L, Marineau C, Cavazzoni P, Duffy A, Betard C, Zvolsky P, Robertson C, Brewer C, Hudson TJ, Rouleau GA, Alda M (2001). Mapping susceptibility genes for bipolar disorder: a pharmacogenetic approach based on excellent response to lithium. *Molecular Psychiatry 6*, 570–578.

Zhao JH, Curtis D, Sham PC (2000). Model-free analysis and permutation tests for allelic associations. *Human Heredity 50*, 133–139.

Zhao JH, Sham PC (2002). Faster allelic association analysis using unrelated subjects. *Human Heredity 53*, 36–41.