Genome-wide Association Study Identifies Genetic Variation in Neurocan as a Susceptibility Factor for Bipolar Disorder

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We conducted a genome-wide association study (GWAS) and a follow-up study of bipolar disorder (BD), a common neuropsychiatric disorder. In the GWAS, we investigated 499,494 autosomal and 12,484 X-chromosomal SNPs in 682 patients with BD and in 1300 controls. In the first follow-up step, we tested the most significant 48 SNPs in 1729 patients with BD and in 2313 controls. Eight SNPs showed nominally significant association with BD and were introduced to a meta-analysis of the GWAS and the first follow-up samples. Genetic variation in the neurocan gene (*NCAN*) showed genome-wide significant association with BD in 2411 patients and 3613 controls (rs1064395, $p = 3.02 \times 10^{-8}$; odds ratio = 1.31). In a second follow-up step, we replicated this finding in independent samples of BD, totaling 6030 patients and 31,749 controls ($p = 2.74 \times 10^{-4}$; odds ratio = 1.12). The combined analysis of all study samples yielded a p value of 2.14×10^{-9} (odds ratio = 1.17). Our results provide evidence that rs1064395 is a common risk factor for BD. *NCAN* encodes neurocan, an extracellular matrix glycoprotein, which is thought to be involved in cell adhesion and migration. We found that expression in mice is localized within cortical and hippocampal areas. These areas are involved in cognition and emotion regulation and have previously been implicated in BD by neuropsychological, neuroimaging, and postmortem studies.

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Bipolar disorder (BD [MIM 125480]) is a highly heritable disorder of mood, characterized by recurrent episodes of mania and depression that are often accompanied by behavioral and cognitive disturbances. Linkage and candidate-gene studies were the only available approaches for unraveling the genetic underpinnings of the disorder until the recent introduction of genome-wide association studies (GWAS). To date, six GWAS using common SNPs have been published.[1–6] Although there has been only limited consistency across studies regarding the top associated genomic regions,[1–3,5,6] meta-analyses of some of these studies have revealed common association signals: a meta-analysis[7] of the Baum et al.[2] and Wellcome Trust Case Control Consortium (WTCCC)[1] data sets found evidence of a consistent association between BD and variants in the genes *JAM3* (MIM 606871) (rs10791345, $p = 1 \times 10^{-6}$) and *SLC39A3* (MIM 612168) (rs4806874, $p = 5 \times 10^{-6}$). A combined analysis[4] of the Sklar et al.[3] and WTCCC[1] studies, which included a total of 4387 patients and 6209 controls, identified a genome-wide significant association signal for BD in *ANK3* (MIM 600465) (rs10994336, $p = 9.1 \times 10^{-9}$). The second strongest finding was for rs1006737 in *CACNA1C* (MIM 114205) ($p = 7 \times 10^{-8}$). Further independent support for *ANK3* rs10994336 has recently been found by Schulze et al.[8] in samples from Germany (overlapping with samples used in the present GWAS; see Table S1 available online) and the USA; the same study[8] reported evidence for allelic heterogeneity at the *ANK3* locus. Although GWAS studies of BD have identified a number of potentially relevant genetic variants, the widely acknowledged formal threshold for genome-wide significance of $p = 5 \times 10^{-8}$ has been surpassed only for variation in *ANK3* so far.

In the present study, we performed a GWAS and a two-step follow-up study of clinically well-characterized BD samples from Europe, the USA, and Australia. The GWAS and replication I included only European BD samples and produced genome-wide significant evidence for association in the neurocan gene (*NCAN* [MIM 600826]) (rs1064395, $p = 3.02 \times 10^{-8}$; odds ratio [OR] = 1.31). We then replicated this finding in large, independent samples from Europe, the USA, and Australia ($p = 2.74 \times 10^{-4}$; OR = 1.12). A combined analysis across all samples, adding up to 8441 patients with BD and 35,362 controls, gave $p = 2.14 \times 10^{-9}$ (OR = 1.17). Further support for an involvement of this gene in BD comes from our observation that *Ncan* expression in mice is localized within cortical and hippocampal areas. These regions have previously been implicated in BD by neuropsychological, neuroimaging, and postmortem studies.

In the following text, we provide a phenotype description of the samples used in each step of our study (GWAS, replication I, replication II), specifications of the SNP genotyping, and the quality control (QC) measures applied to the raw genotyping data:

The patients included in our GWAS and replication I step received a lifetime diagnosis of BD according to the DSM-IV[9] criteria on the basis of a consensus best-estimate procedure[10] and structured diagnostic interviews.[11,12] Protocols and procedures were approved by the local ethics committees. Written informed consent was obtained from all patients and controls. They were recruited from consecutive admissions to psychiatric inpatient units at (1) The Central Institute of Mental Health, Mannheim (n = 1081), (2) Department of Psychiatry, Poznan University of Medical Sciences, Poznan, Poland (n = 446), (3) Alexandru Obregia Clinical Psychiatric Hospital, Bucharest, Romania (n = 237), (4) Civil Hospital Carlos Haya, Málaga, Spain (n = 298), (5) Russian State Medical University, Moscow (n = 329), and (6) Kosevo Hospital, Sarajevo, Bosnia and Herzegovina (n = 125). All controls of replication I were also recruited by the abovementioned institutions. All GWAS controls were drawn from three population-based epidemiological studies: (1) PopGen[13] (n = 490), (2) KORA[14] (n = 488), and (3) the Heinz Nixdorf Recall Study (Risk Factors, Evaluation of Coronary Calcification, and Lifestyle) (HNR,[15] n = 383). Ancestry was assigned to patients and controls on the basis of self-reported ancestry. More detailed sample descriptions are given in Table 1.

Lymphocyte DNA was isolated from ethylenediaminetetraacetic acid anticoagulated venous blood by a salting-out procedure using saturated sodium chloride solution[16] or by a Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany). Genotyping was performed on HumanHap550v3 BeadArrays (Illumina, San Diego, CA, USA). QC was performed with PLINK[17] (version 1.05). In detail, the X-chromosomal heterozygosity rates were used to determine the sex of each subject; subjects with a discrepant sex status were excluded (five patients and three controls). Subjects with a call rate (CR) < 0.97 were also excluded (eight patients and 26 controls). Using identical-by-state (IBS) analysis, cryptically related individuals (IBS > 1.65) were identified, and the DNA sample with the lower CR was removed. For the identification of possible population stratification, a multidimensional scaling (MDS) analysis was performed. In total, seven patients and 32 controls were excluded on the basis of relatedness or outlier detection. SNPs with a CR < 0.98 and monomorphic SNPs were excluded (18,618 SNPs in patients and 14,880 in controls). Additional markers were excluded on the basis of nonrandom differences in missingness patterns with respect to phenotype and for significant results in the "pseudo" patient-control analysis using control samples and a Cochran-Armitage test for linear trend (TREND) (a total of 7101 SNPs in patients and 8076 in controls). SNPs with a minor allele frequency (MAF) < 1% in patients or controls were excluded (11,434 in patients and 12,155 in controls), as were SNPs in Hardy-Weinberg disequilibrium (Hardy-Weinberg equilibrium [HWE], $p_{exact} < 1 \times 10^{-4}$, 351 SNPs in controls; $p_{exact} < 1 \times 10^{-6}$, 132 SNPs in patients). With the use of the remaining patients and controls, a MDS analysis was performed, first with patients and controls from our GWAS sample and then with the GWAS sample

Table 1. Descriptive Data for Patients with Bipolar Disorder and Controls Following Quality Control

Sample	Ancestry	N Patients	N Controls	BD1 (%)	BD2 (%)	SAB (%)	BD-NOS (%)	MaD (%)	Diagnosis	Interview	Controls Screened?
GWAS											
Germany I	German	682	1300	679 (99.56)	2 (0.29)	1 (0.15)	0	0	DSM-IV	SADS-L, SCID	no
Replication I											
Germany II	German	361	755	138 (38.23)	93 (25.76)	130 (36.01)	0	0	DSM-IV	SADS-L, SCID	no
Poland	Polish	411	504	323 (78.59)	88 (21.41)	0	0	0	DSM-IV	SCID	no
Spain	Spanish	297	391	290 (97.64)	7 (2.36)	0	0	0	DSM-IV	SADS-L	no
Russia	Russian	326	329	324 (96.38)	2 (0.61)	0	0	0	DSM-IV	SCID	no
Romania	Romanian	227	221	227 (100)	0	0	0	0	DSM-IV	SCID	yes
Bosnia and Herzegovina / Serbia	Bosnian / Serbian	107	113	107 (100)	0	0	0	0	DSM-IV	SCID	no
Total		2411	3613	2088	192	131	0	0			
Replication II											
GAIN-EA / TGEN1	European	2189	1434	2062 (94.20)	0	127 (5.80)	0	0	DSM-III, DSM-IV, RDC	DIGS	yes
WTCCC-BD / Exp. Ref. Grp.	British	1868	14,311	1594 (85.33)	134 (7.17)	98 (5.25)	38 (2.03)	4 (0.21)	RDC	SCAN	no
Germany III	German	497	857	376 (75.65)	88 (17.71)	2 (0.04)	31 (6.24)	0	DSM-IV	AMDP, CID-S, SADS-L, SCID	yes
France	French	471	1758	360 (76.43)	99 (21.02)	0	12 (2.55)	0	DSM-IV	DIGS	no
Iceland	Icelandic	422	11,487	323 (76.54)	72 (17.06)	0	27 (6.40)	0	DSM-III, DSM-IV, ICD10, RDC	CID-I, SADS-L	no
Australia	European	380	1530	291 (76.58)	87 (22.89)	1 (0.26)	1 (0.26)	0	DSM-IV	DIGS, FIGS, SCID	no
Norway	Norwegian	203	372	128 (63.05)	65 (32.02)	0	10 (4.93)	0	DSM-IV	SCID, PRIME-MD	yes
Total		6030	31,749	5134	545	228	119	4			
Grand Total		8441	35,362	7222	737	359	119	4			

Patients received diagnoses according to the indicated diagnostic criteria and interviews. Protocols and procedures were approved by the local Ethics Committees. Written informed consent was obtained from all patients and controls. Ancestry was assigned to patients and controls on the basis of self-reported ancestry. The samples from Bosnia and Herzegovina / Serbia were merged due to the small number of subjects. The Expanded Reference Group for the WTCCC-BD sample comprised the 1958 British Birth Cohort controls, the UK Blood Services controls supplemented by the other 6 disease sets (coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type 1 and type 2 diabetes) as defined by the WTCCC (2007).[1]
The following abbreviations are used: AMDP, Association for Methodology and Documentation in Psychiatry;[31] BD1, bipolar disorder type 1; BD2, bipolar disorder type 2; BD-NOS, bipolar disorder not otherwise specified; CID-I, Composite International Diagnostic Interview;[32,33] CID-S, Composite International Diagnostic Screener;[34] DIGS, Diagnostic Interview for Genetic Studies;[35] DSM-III / DSM-IV, Diagnostic and Statistical Manual of Mental Disorders;[9] Exp. Ref. Grp., Expanded Reference Group (11,373 controls);[1] FIGS, Family Interview for Genetic Studies;[36] GAIN-EA, BD sample with European ancestry from the Genetic Association Information Network (1001 patients and 1033 controls);[6] ICD10, International Statistical Classification of Diseases and Related Health Problems;[37] MaD, Manic disorder according to RDC; N, number of subjects; PRIME-MD, Primary Care Evaluation of Mental Disorders;[38] RDC, Research Diagnostic Criteria;[39] SAB, schizoaffective disorder (bipolar type); SADS-L, Schedule for Affective Disorders and Schizophrenia;[40] SCAN, Schedules for Clinical Assessment in Neuropsychiatry;[41] SCID, Structured Clinical Interview for DSM disorders;[11] TGEN1, Translational Genomics Research Institute (genotyping wave 1 with 1190 patients and 401 controls).

and unrelated individuals from the CEU (Utah residents with ancestry from northern and western Europe), CHB (Han Chinese in Beijing, China), JPT (Japanese in Tokyo, Japan), and YRI (Yoruba in Ibadan, Nigeria) HapMap panels. On the basis of this analysis, seven controls and zero patients were excluded (Figure S2). At the marker

level, nonrandom missingness patterns were identified with the use of PLINK's "mishap" test, and another 1825 SNPs were excluded. In total, we excluded 20 patients and 68 controls as well as 49,488 SNPs in the course of our QC before association analysis. The effect of our QC on the resulting p values with data sets has been depicted in a quantile-quantile plot (Figure S1). The genomic inflation factor (λ) after QC was 1.071 (1.107 before QC).

The follow-up SNP set (replication I) was genotyped on the MALDI TOF-based MassARRAY system with the use of the iPLEX Gold assay (Sequenom, San Diego, CA, USA). The iPLEX primer sequences and assay conditions may be obtained from the authors upon request. Of the top 79 SNPs from the GWAS, 18 were excluded from the follow-up step on the basis of linkage disequilibrium (LD), failed assay design, or poor genotype clustering in the iPLEX genotyping assay. Of the remaining 61 SNPs, 13 did not pass our QC filters (six SNPs with CR < 0.95, two SNPs with nonrandom differences in missingness patterns between subsamples, one SNP with an MAF $<$ 0.01 in controls, and four SNPs with deviations from HWE [$p_{exact} < 1 \times 10^{-4}$ in controls]). In total, 181 individuals were excluded (n = 31 cryptically related individuals, n = 150 with call rates $< 95\%$). To identify the cryptically related individuals (IBS > 1.65), we had computed an IBS matrix on the basis of the quality-controlled GWAS sample and all six replication samples.

For the replication II step, *NCAN* rs1064395 genotypes were extracted from the following genome-wide data sets: GAIN-EA[6]/TGEN1, WTCCC-BD,[1] Germany III, France, Iceland, Australia, and Norway, which are described in Table 1 and Table S2.

The quality-controlled genotype data were subjected to the following association tests: In the GWAS and replication I steps, analyses were computed with PLINK[17] (version 1.05). Autosomal-wide analysis was performed via the TREND test with 1 degree of freedom (df). The X chromosome (females and males combined) was analyzed via the Wald test with 1 df. In replication I and the meta-analysis (GWAS and replication I), autosomal and X-chromosomal SNPs were investigated via the Cochran-Mantel-Haenszel (CMH) test, stratified for ethnicity with the use of a 2 × 2 × K table in which K = 6, reflecting the six European countries. The CMH test was two-tailed for all analyses. To investigate the homogeneity of ORs for the replicated SNPs, we used the Breslow-Day test. We selected $p < 5 \times 10^{-8}$ as the threshold for genome-wide significance, assuming one million noncorrelated common SNPs in the genome, as proposed by the Diabetes Genetics Initiative[18] and the International HapMap Consortium.[19] In the replication II step, *NCAN* rs1064395 was investigated via logistic regression (assuming an additive effect), which was a two-tailed test. *NCAN* rs1064395 was also investigated with the use of a fixed-effects meta-analysis based on the weighted *Z*-score method,[20] which was two-tailed for replication II samples (n = 7) and for the combined analysis of all study samples (n = 14).

On the basis of the QC specifications and statistical procedures described above, we performed a GWAS of 682 BD patients and 1300 controls (Table 1), using 499,494 autosomal SNPs and 12,484 X-chromosomal SNPs. A genome-wide overview of the GWAS p values is given in Figure 1A. In the first follow-up step (replication I), we genotyped the most significant 48 SNPs (autosomal SNPs: $p \leq 7.57 \times 10^{-5}$; X-chromosomal SNPs: $p \leq 1.89 \times 10^{-4}$; for SNPs in LD ($r^2 \geq 0.8$), only the SNP with the smallest GWAS p value was genotyped in the follow-up) in six European follow-up samples comprising a total of 1729 BD patients and 2313 controls (Table 1). The same set of instruments was used across all centers.[21] In the replication, we used phenotypically more relaxed criteria than in the initial GWAS and also included patients with a diagnosis of BD type II, schizoaffective disorder (bipolar type), and BD not otherwise specified. To account for possible heterogeneity, autosomal and X-chromosomal we investigated SNPs via the CMH test and stratified them with regard to ethnicity. Eight of the 48 SNPs showed nominally significant association in the combined replication samples, all with the same alleles as in the GWAS (Table 2, Table S3). This number of replicated SNPs is significantly higher than would be expected to occur by chance ($n_{expected} = 2.4$; $p = 2.11 \times 10^{-4}$). The strongest evidence for replication was obtained for rs1064395, which is located in the 3′ untranslated region of *NCAN* on 19p13.11 ($p = 4.61 \times 10^{-4}$, OR = 1.23), and for rs11764590, which is located in an intron of *MAD1L1* (MIM 602686) on 7p22.3 ($p = 0.0020$, OR = 1.18). Our top GWAS signal, rs2774339, located in *GNG4* (MIM 604388) on 1q42.3 ($p = 1.02 \times 10^{-6}$; Figure S3) was not replicated ($p = 0.5772$). Given that our follow-up samples were derived from six different European countries, we specifically tested for possible heterogeneity by using the replication data. In the case of the successfully replicated SNPs, there was no evidence of ethnic heterogeneity (Breslow-Day $p_{min} = 0.178$). In support of this was the observation that subtraction of any individual replication sample did not markedly alter the effect sizes (Table S4).

In a subsequent analysis, we applied a meta-analysis approach (CMH test) to combine all investigated samples. SNP rs1064395 in *NCAN* surpassed the threshold for genome-wide significance ($p = 3.02 \times 10^{-8}$, OR = 1.31; Figure 1B); the minor allele (A) was overrepresented in patients in comparison to controls (19.5% versus 15.3%). The second-best result was found for *MAD1L1* rs11764590 ($p = 1.28 \times 10^{-7}$, OR = 1.26; Figure 1B): an excess of T alleles was observed in patients (26.0% versus 21.6%). Another *MAD1L1* marker, rs10278591, which was in moderate LD with rs11764590 ($r^2 = 0.70$), showed $p = 1.81 \times 10^{-5}$. HapMap-based imputations of our BD GWAS data provided additional support for the *NCAN* and *MAD1L1* regions (Figures S4a and S4b). After QC of real genotypes, locus-targeted imputations were performed in MACH (version 1.0.16) (Y. Li and G.R. Abecasis, 2006, Am. Soc. Hum. Genet. abstract) with

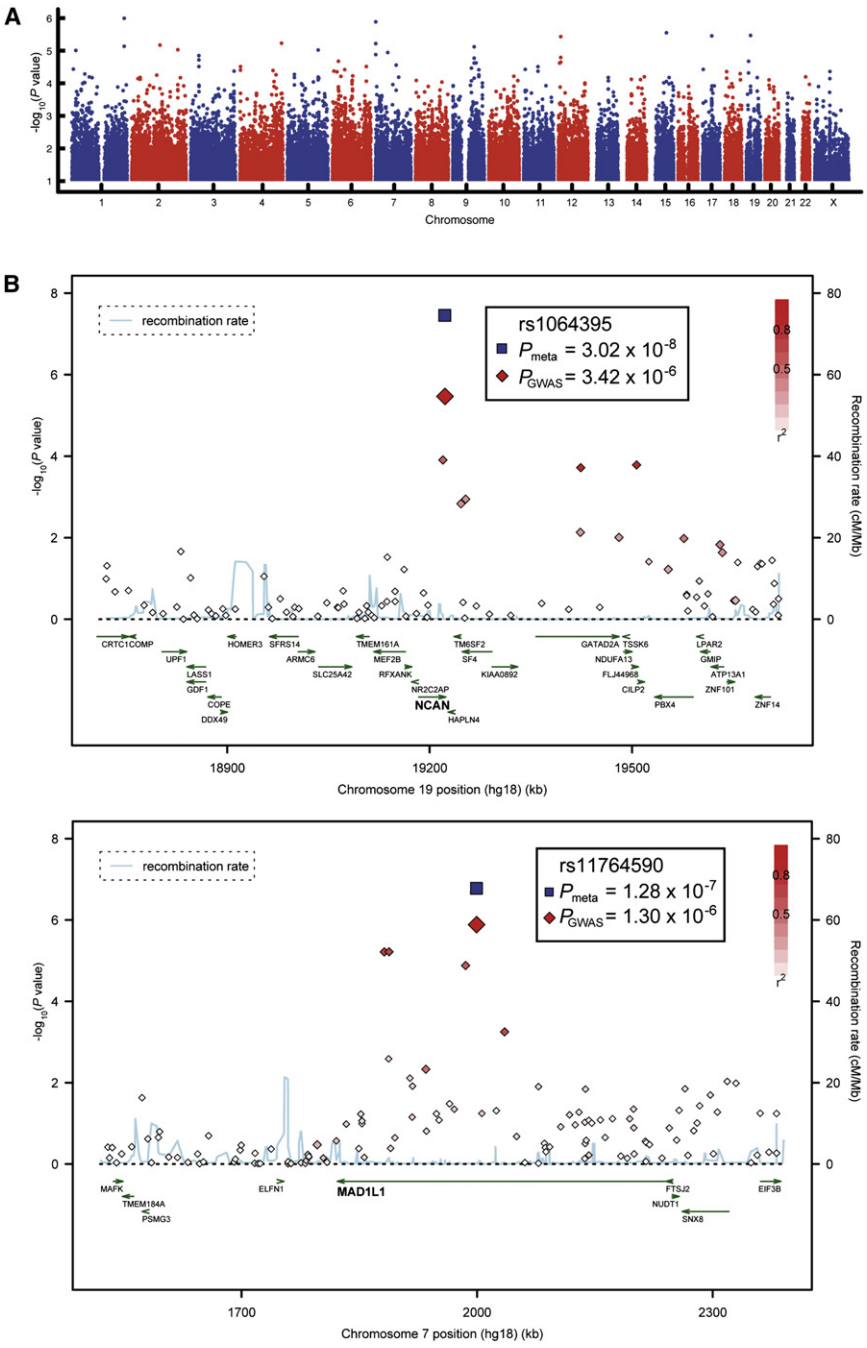

Figure 1. Association Results for the GWAS and the Two Best-Supported Genes from the Follow-Up Study
(A) Manhattan plot.
(B) Regional association plots (RAPs) displaying *NCAN* and *MAD1L1*. The most associated marker from the GWAS (enlarged red diamond) is centered in a genomic window of 1 Mb (hg18, RefSeq genes); its p value from the combined analysis (meta) is shown (enlarged blue diamond). The LD strength (r^2) between the sentinel SNP from the GWAS and its flanking markers is demonstrated by the red (high) to white (low) color bar. The recombination rate (cM/Mb; second y axis) is plotted in blue, according to HapMap-CEU. RAPs were generated with SNAP.[42]

phased haplotypes from the 60 HapMap CEU founders (release 22) used as a reference. The imputations were restricted to windows of 2 Mb on either side of the sentinel SNP. Imputed SNPs with a MACH quality score less than 95% were excluded before the association analysis. Regional association plots for the other five replicated SNPs are provided in Figures S4c–S4g and Figures S5a–S5e.

In a second replication step, we sought further support for the genome-wide significant finding in *NCAN* and tested rs1064395 in BD samples from Europe (WTCCC-BD,[1] Germany III, France, Iceland, Norway), from the USA (combined GAIN-EA[6]/TGEN1), and from Australia. In all seven patient samples, the A allele was overrepresented (Table 3). Through logistic regression assuming an additive effect, the GAIN-EA/TGEN1 samples showed p = 0.2047 (OR = 1.09), WTCCC-BD/Expanded Reference Controls showed p = 0.0510 (OR = 1.09), Germany III samples showed p = 0.2129 (OR = 1.15), those from France showed p = 0.0066 (OR = 1.28), those from Iceland showed p = 0.3385 (OR = 1.10), those from Australia showed p = 0.3163 (OR = 1.11), and those from Norway showed p = 0.7205 (OR = 1.06). The fixed-effects meta-analysis of these samples, totaling 6030 patients and 31,749 controls, resulted in $p = 2.74 \times 10^{-4}$ (OR = 1.12).

The combined analysis of all study samples (GWAS + replication I + replication II), with 8441 patients and 35,362 controls, resulted in $p = 2.14 \times 10^{-9}$ (OR = 1.17). An overview of the significance levels and genetic effect sizes of *NCAN* rs1064395 at all steps of analysis is provided in Figure 2 and Table 3. It is interesting to note that there is no improvement of the OR when we perform an analysis of BD type I only (OR = 1.16), providing no strong evidence that genetic variation in *NCAN* would have a much stronger effect in BD type I compared to the other diagnoses included in our study (BD type II, schizoaffective disorder [bipolar type], and BD not otherwise specified).

Neurocan was originally described in the rat brain,[22] where its expression decreases significantly in the first week after birth.[23] To validate whether *Ncan* is expressed in brain areas previously implicated in BD,[24] we performed RNA in situ hybridization with whole mounts and sections

Table 2. Eight GWAS SNPs Showing Evidence for Association with BD In Six Independent Samples of BD

		Association Data (BD)								
		GWAS			Replication I			Combined		
SNP Data		TREND		MAF	CMH (K = 6)		MAF	CMH (K = 6)		Gene Data
Band	SNP, MA	p Value	OR	Pat / Con (682 / 1300)	p Value	OR	Pat / Con (1729 / 2313)	p Value	OR	Nearest Gene or Transcript
19p13.11	rs1064395, A	3.42×10^{-6}	1.53	0.19 / 0.14	4.61×10^{-4}	1.23	0.20 / 0.16	3.02×10^{-8}	1.31	*NCAN*, 3′ UTR
7p22.3	rs11764590, T	1.30×10^{-6}	1.47	0.27 / 0.20	2.01×10^{-3}	1.18	0.26 / 0.22	1.28×10^{-7}	1.26	*MAD1L1*, intron
7p22.3	rs10278591, T	6.05×10^{-6}	1.43	0.27 / 0.21	0.0348	1.12	0.26 / 0.23	1.81×10^{-5}	1.21	*MAD1L1*, intron
2p23.2	rs6547829, T	7.21×10^{-5}	1.59	0.11 / 0.07	0.0134	1.22	0.09 / 0.08	2.50×10^{-5}	1.32	*BRE*, intron
7q22.1	rs985409, G	6.52×10^{-5}	1.31	0.45 / 0.38	0.0206	1.11	0.47 / 0.44	3.89×10^{-5}	1.17	*LHFPL3*, intron
9q21.31	rs2209263, A	3.44×10^{-5}	0.73	0.24 / 0.30	0.0436	0.90	0.26 / 0.28	5.58×10^{-5}	0.84	*TLE4; TLE1*
3q28	rs779279, A	4.25×10^{-5}	0.76	0.41 / 0.48	0.0402	0.91	0.46 / 0.48	6.39×10^{-5}	0.86	*FGF12; PYDC2*
14q21.1	rs9322993, T	7.56×10^{-5}	1.75	0.07 / 0.04	0.0382	1.23	0.06 / 0.05	7.54×10^{-5}	1.37	*SIP1*, intron

In the six different clusters (countries), all SNPs were in HWE in patients and controls ($p > 0.05$).
The following abbreviations are used: MA, minor allele, refers to dbSNP build 130; TREND, Cochran-Armitage test; OR, odds ratio referring to the minor allele; MAF, minor allele frequency; Pat, patients; Con, controls; CMH, Cochran-Mantel-Haenszel test; K, CMH's cluster variable; UTR, untranslated region.

from embryonic and postnatal wild-type mice, using procedures described previously.[25] We amplified *Ncan* probes from postnatal day 46 (P46) mouse brain cDNA (strain C57BL/6), covering nucleotides 5715–6515 of GenBank accession NM_007789.3. This was cloned into pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany). Animals

Table 3. Association Results for NCAN rs1064395 at All Steps of the Analysis: GWAS, Replication I, and Replication II

Sample	N Patients	N Controls	Test, p Value	OR	MA	MAF: Patients	MAF: Controls
GWAS	**682**	**1300**	**TREND, 3.42×10^{-6}**	**1.53**	**A**	**0.19**	**0.14**
Germany I							
Replication I	**1729**	**2313**	**CMH (K = 6), 4.61×10^{-4}**	**1.23**			
Germany II			TREND, 0.0490	1.28	A	0.17	0.14
Poland			TREND, 0.1354	1.21	A	0.17	0.15
Spain			TREND, 0.0317	1.32	A	0.26	0.21
Russia			TREND, 0.9010	1.02	A	0.18	0.17
Romania			TREND, 0.0484	1.38	A	0.21	0.17
Bosnia and Herzegovina / Serbia			TREND, 0.4427	1.20	A	0.21	0.18
GWAS + Replication I	**2411**	**3613**	**CMH (K = 6), 3.02×10^{-8}**	**1.31**			
Replication II	**6030**	**31,749**	**FEM1 (n = 7), 2.74×10^{-4}**	**1.12**			
GAIN-EA / TGEN1	2189	1434	L, 0.2047	1.09	A	0.17	0.15
WTCCC-BD / Exp. Ref. Grp.	1868	14,311	L, 0.0510	1.09	A	0.17	0.16
Germany III	497	857	L, 0.2129	1.15	A	0.17	0.14
France	471	1758	L, 0.0066	1.28	A	0.22	0.19
Iceland	422	11,487	L, 0.3385	1.10	A	0.16	0.15
Australia	380	1530	L, 0.3163	1.11	A	0.18	0.17
Norway	203	372	L, 0.7205	1.06	A	0.15	0.14
GWAS + Replication I + Replication II	**8441**	**35,362**	**FEM2 (n = 14), 2.14×10^{-9}**	**1.17**			

The following abbreviations are used: TREND, Cochran-Armitage test; CMH, Cochran-Mantel-Haenszel test; FEM, fixed-effects meta-analysis based on the weighted z-score method;[20] L, logistic regression assuming an additive effect; OR, odds ratio referring to the minor allele (MA); n, number of analyzed study samples; MAF, MA frequency; Pat, patients; Con, controls.

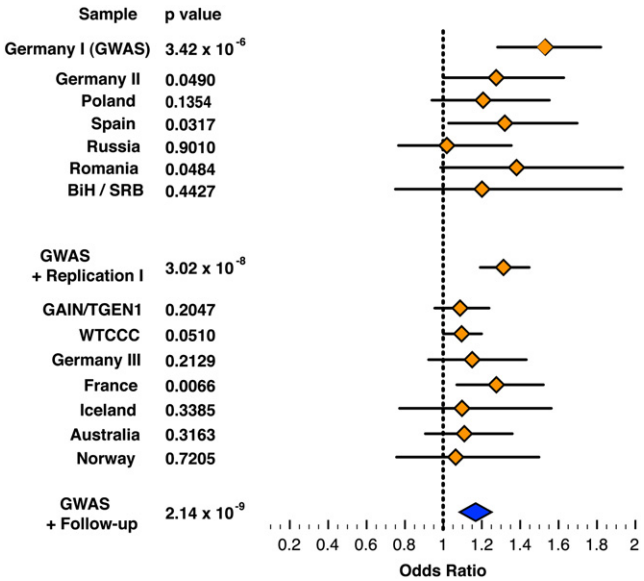

Figure 2. Genetic Effect Sizes and Significance Levels for *NCAN* rs1064395 at All Steps of Analysis

Forest plot shows odds ratios (orange diamonds) and their 95% confidence intervals (horizontal lines) of individual study samples. The odds ratio referring to the meta-analysis of all study samples is represented by the enlarged blue diamond. BiH / SRB, Bosnia and Herzegovina / Serbia.

were handled according to European and German laws. At embryonic day 14.5 (E14.5), *Ncan* expression was confined to the central nervous system (Figure 3A). However, *Ncan* transcripts have also been described in the peripheral nervous system in later developmental stages.[26] Between E14.5 and E16.5, *Ncan* was highly expressed in the cortical plate, as well as in the ventricular zone of the basal ganglia (Figures 3A–3C). In the subventricular zone of the neocortex, the transcripts were located mainly in the caudal region (Figure 3C), where neurocan proteins may be involved in axon guidance.[27] *Ncan* was also present in the developing hippocampus (data not shown). After birth, its general expression was found to be decreased. It was detected in the dentate gyrus and CA1 of the hippocampus (Figure 3E), as well as in the cortical layer II (Figure 3D) at P46. Its expression in the cortical layer II remained unabated, at least up to P18, with higher expression in the frontal cortex (data not shown). On the contrary, *Ncan* expression was not detected in any hippocampal area at this age.

To investigate whether *NCAN* and *MAD1L1* are expressed in the human brain, we analyzed the transcriptional expression of both genes in human hippocampus tissue samples (n = 148), using data from whole-genome HumanHT-12 Expression BeadChips (Illumina, San Diego, USA). Tissues were obtained from surgery on patients with pharmaco-resistant epilepsy. Total RNA was isolated from fresh frozen tissues and underwent QC via BioAnalyzer measurements (Agilent Technologies, Waldbronn, Germany). The background of expression profiles was subtracted and signals were normalized (average-normalization) with Illumina's GenomeStudio software. The analysis showed that *NCAN* and *MAD1L1* were expressed in the hippocampus (Figure S6). For *NCAN*, an average signal-intensity $\log_2$ ratio of 7.99 with a standard deviation of 0.8 (intensities, min = 5.82 and max = 10.4) was detected, and for *MAD1L1*, an average signal-intensity $\log_2$ ratio of 5.21 with a standard deviation of 0.46 (intensities, min = 3.55 and max = 6.1) was detected.

Our GWAS and follow-up study of BD samples, including a total of 43,803 individuals (8441 patients and 35,362 controls), provided a significant level of statistical support for the idea that common genetic variation in *NCAN* is involved in the etiology of this common and severe neuropsychiatric disorder. The overall p value for the top-associated SNP, rs1064395, was 2.14×10^{-9} (OR = 1.17). *NCAN* encodes neurocan (MIM 600826), an extracellular matrix glycoprotein. The gene is highly expressed in the brain, and is thought to be involved in cell adhesion and migration. To map its spatiotemporal expression, we performed in situ hybridizations in embryonic and postnatal wild-type mice. We observed that murine *Ncan* was expressed in cortical and hippocampal areas and could confirm that *NCAN* transcripts are highly abundant in the human hippocampus. These brain regions,

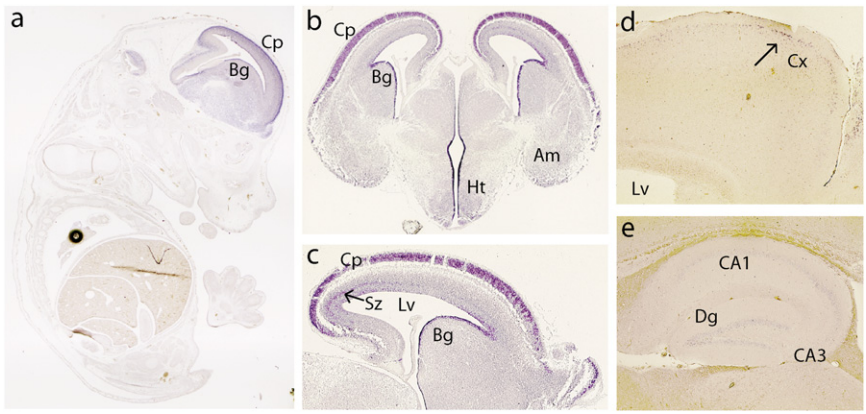

Figure 3. Expression Patterns of Neurocan in Mouse Brain Areas Previously Implicated in BD

RNA in situ hybridization of *Ncan* in sagittal (A, C–E) and coronal (B) sections at E14.5 (A), E15.5 (B), E16.5 (C), and P46 (D and E). In the embryo, *Ncan* is observed exclusively in the CNS, with high expression in the cortical plate (Cp) and subventricular zone (Sz, arrow in C) of the neocortex and in the ventricular zone of the basal ganglia (Bg, A–C). *Ncan* was also detected in the hypothalamus (Ht) and amygdala (Am, B). In postnatal mice, *Ncan* expression in the cortex (Cx) is restricted to layer II (arrow in D). In the hippocampus (E), it was detected at lower intensity in the dentate gyrus (Dg) and CA1. Lv indicates the lateral ventricle.

which are involved in cognition and regulation of circuits involved in emotion, have previously been implicated in BD by neuropsychological, neuroimaging, and post-mortem studies.[28] *Ncan*-deficient mice show no obvious defect in brain morphology, and basic synaptic transmission appears to be normal.[26] However, the maintenance of late-phase long-term potentiation in the hippocampal CA1 region in null mutants is reduced, which could lead to mild deficits in learning and memory;[26] i.e., there are disturbances in the mechanisms that underlie the cognitive deficits observed in BD. This suggests that *Ncan*-deficient mice should be reexamined for more subtle changes in the brain, such as synaptic plasticity.

MAD1L1 (mitotic arrest deficient-like 1 [MIM 602686]) is a component of the chromosome spindle-assembly checkpoint in mitosis. Defects in mitotic checkpoints can lead to aneuploidy, which may play a role in carcinogenesis and aging.[29] Homozygous knockout of *Mad1l1* in mice confers embryonic lethality, which indicates that it has an essential function in development.[30] *MAD1L1* is expressed in the human hippocampus (Figure S6), although its neurobiological effects have not been established.

In summary, the present study has identified a susceptibility factor for BD, *NCAN,* and a potential BD susceptibility factor, *MAD1L1*. Expression studies in mice provide strong support for a role of *NCAN* in BD, because its expression is localized to brain areas (cortex, hippocampus) in which abnormalities have been identified in BD. These abnormalities may be indicative of disturbances in key neuronal circuits.

Supplemental Data

Supplemental Data include a list of members of the Bipolar Disorder Genome Study (BiGS) Consortium, four tables, and six figures and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

The International HapMap Project, http://www.hapmap.org/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PLINK: Whole Genome Association Analysis Toolset, http://pngu.mgh.harvard.edu/~purcell/plink/
Single Nucleotide Polymorphism database (dbSNP), http://www.ncbi.nlm.nih.gov/projects/SNP/
SNAP: A web-based tool for identification and annotation of proxy SNPs using HapMap, http://www.broadinstitute.org/mpg/snap/

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