A SNAP25 promoter variant is associated with early-onset bipolar disorder and a high expression level in brain.

Bruno Etain, Anne Dumaine, Flavie Mathieu, Fabien Chevalier, Chantal Henry, Jean-Pierre Kahn, Jasmine Deshommes, Frank Bellivier, Marion Leboyer, Stéphane Jamain

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Bruno Etain[1, 3]

Anne Dumaine[1]

Flavie Mathieu[1]

Fabien Chevalier[1]

Chantal Henry[1,2,3]

Jean-Pierre Kahn[4]

Jasmine Deshommes[1, 3]

Frank Bellivier[1,2,3]

Marion Leboyer[1,2,3]

Stéphane Jamain[1]

1 INSERM, U 955, IMRB, Department of Genetics, Psychiatry Genetics, Creteil, F-94000, France;

2 University Paris 12, Faculty of Medicine, IFR10, Creteil, F-94000, France;

3 AP-HP, Henri Mondor-Albert Chenevier Group, Department of Psychiatry, Creteil, F-94000, France;

4 Department of Psychiatry and Clinical Psychology, CHU de Nancy, Jeanne-d'Arc Hospital, 54200 Toul, France

Correspondence: Dr S Jamain, INSERM U 955, Psychiatry Genetics, Hôpital H. Mondor, 51 av. du Mal. de Lattre de Tassigny, CRETEIL, F-94000, France.

Phone: +33-1-4981-3775

Fax: +33-1-4981-3588

E-mail: stephane.jamain@inserm.fr

Keywords: SNAP-25, association study, bipolar affective disorder, attention-deficit hyperactivity disorder, expression study

Running Title: *SNAP25* Gene Variations in Early-Onset Bipolar Disorder

Abstract

Bipolar disorder (BD) is one of the most common and persistent psychiatric disorders. Early-onset BD has been shown to be the most severe and familial form. We recently carried out a whole-genome linkage analysis on sib-pairs affected by early-onset BD and showed that the 20p12 region was more frequently shared in our families than expected by chance. The synaptosomal associated protein SNAP25 is a presynaptic plasma membrane protein essential for the triggering of vesicular fusion and neurotransmitter release, and for which abnormal protein levels have been reported in postmortem studies of bipolar patients. We hypothesised that variations in the gene encoding SNAP25, located on chromosome 20p12, might influence the susceptibility to early-onset BD.

We screened *SNAP25* for mutations and performed a case-control association study in 197 patients with early-onset BD, 202 patients with late-onset BD and 136 unaffected subjects. In addition, we analysed the expression level of the two *SNAP25* isoforms in 60 brains. We showed that one variant, located in the promoter region, was associated with early-onset BD but not with the late-onset subgroup. In addition, individuals homozygous for this variant showed a significant higher *SNAP25b* expression level in prefrontal cortex.

These results show that variations in *SNAP25,* associated with an increased gene expression level in prefrontal cortex, might predispose to early-onset BD. Further analyses of this gene, as well as analysis of genes encoding for the SNAP25 protein partners, are required to understand the impact of such molecular mechanisms in BD.

Introduction

Bipolar disorder (BD) affects 1 to 5% of the general population and is one of the most severe and frequent psychiatric disorders. It is characterised by alternating episodes of major depression and elevated mood (hypo or manic episodes).[1] Twin, family and adoption studies have suggested that genetic factors play a major role in BD, but no causal mutation has yet been identified.[2] The identification of susceptibility genes has been hampered by a lack of consensus concerning the most valid phenotype to investigate and by the unknown genetic validity of the classical clinical classifications. In order to disentangle the genetic and clinical heterogeneity of the disorder, a clinical approach based on candidate symptoms has been proposed.[3] Age at onset (i.e. age at the first mood episode) is one of the most relevant indicators to identify homogeneous subgroups that may reduce the underlying genetic heterogeneity. Three age at onset (AAO) subgroups have been identified for BD[4-6] and there is strong evidence showing that genetic factors make a greater contribution to the disease in the early-onset subgroup than in the other subgroups.[7]

We recently carried out a whole-genome linkage analysis in early-onset BD sib-pairs, and identified six regions with a suggestive multipoint non-parametric lod-score.[8] These regions included the 20p12 region, already reported by three independent studies to contain a gene conferring susceptibility to BD.[9-11] The gene encoding the synaptosomal-associated protein of 25 kDa (SNAP25) is located in this region.

Several arguments suggest that *SNAP25* is a strong candidate gene for BD. First, SNAP25 is a presynaptic plasma membrane protein essential for the triggering of vesicular fusion and neurotransmitter release.[12, 13] Second, postmortem studies have shown modifications of SNAP25 protein levels in some brain regions of bipolar patients.[14, 15] Third, *SNAP25* gene has

been widely associated with attention deficit hyperactivity disorder (ADHD), which is known to share genetic susceptibility with early-onset BD.[16-18]

We analysed *SNAP25* as a candidate gene for susceptibility to bipolar disorder and, more specifically, to early-onset BD. We screened this gene for mutations and performed a case-control association study taking into account the age at onset of the disease. Finally, we analysed the influence of associated susceptibility alleles on the expression level of *SNAP25* in human prefrontal cortices.

Material and methods

Subjects

Patients meeting DSM-IV criteria[1] for type I or II bipolar disorder consecutively admitted to three French university-affiliated psychiatry departments (Paris-Créteil, Bordeaux and Nancy) were interviewed by trained psychiatrists, using the French version of the Diagnostic Interview for Genetic Studies (DIGS version 3.0)[19]. All patients were normothymic at inclusion (i.e. having a Montgomery-Asberg Depression Rating Scale[20] score and a Mania Rating Scale[21] score of no more than five). The healthy controls were recruited from blood donors at the Pitié-Salpêtrière and Henri Mondor Hospitals (France). Controls were interviewed with the DIGS, and asked about family history of psychiatric disorders, using the National Institute for Mental Health Family Interview for Genetic Studies[22]. Only controls, with no personal history of psychiatric disorders and no family history (first-degree) of affective disorders or suicidal behaviour, were included. All patients and controls were of French descent, with at least three grandparents from mainland France. The Research Ethics Board of Pitié-Salpêtrière Hospital reviewed and approved this study. Written informed consent was obtained from all participating subjects.

Definition of age-at-onset of bipolar disorder

For association studies, AAO of bipolar disorder was defined as the age at which the first mood episode (depressive, manic or hypomanic) occurred, as determined by reviewing medical case notes and information obtained with the DIGS. The threshold for early-onset BD (AAO before the age of 22 years) was chosen on the basis of previous admixture analyses, this threshold being defined in four independent samples.[4-6, 23] These studies identified three AAO subgroups: early, intermediate and late onset. In order to have comparable sample size in different subgroups and according to genetic homogeneity,[24] intermediate- and late-onset samples were pooled into a single subgroup, referred to as the "late-onset" subgroup, and compared to early-onset patients.

Brain samples

RNA, cDNA and DNA from 30 individuals affected with bipolar disorder and 30 unaffected control subjects were donated by the Stanley Medical Research Institute, as part of the Array Collection that consisted of samples from the dorsolateral prefrontal cortex (Brodmann's area 46).[25] Diagnoses were made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition.[1] A summary of the demographic and clinical information of subjects used in this study is described in Table S1. The samples were coded and genotypes and disease-status were known only after expression analyses.

Mutation screening and genotyping

Genomic DNA was isolated from blood lymphocytes or B-lymphoblastoid cell lines from independent cases and controls, using the Nucleon BACC3 kit (GE HealthCare, Chalfont St Giles, UK). We first sequenced the whole *SNAP25* genomic region (8 coding exons including one alternative splicing exon, one 5'-untranslated exon, flanking intronic regions and 1,400 bp upstream from the transcription start site) in 31 individuals with early-onset bipolar disorder, to

identify informative single nucleotide polymorphisms (SNPs) in the *SNAP25* gene and to avoid ascertainment bias in the choice of markers to be tested. All the primers used for polymerase chain reaction (PCR) amplification and sequence analysis are available on request. The sequence of the *SNAP25* gene was analyzed by direct sequencing of the PCR products, using the BigDye® terminator v3.1 cycle sequencing kit and a 16-Capillary ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). We used polymorphisms with a minor allele frequency (MAF) greater than 0.05 to convert unphased genotypic data into haplotypes, using the accelerated expectation maximisation algorithm implemented in Haploview v3.32.[26] We evaluated the accuracy of the this algorithm, by carrying out haplotype reconstruction in parallel, using the Bayesian statistical method implemented in Phase v.2.1.1.[27] Equivalent results were obtained with both methods, with high levels of statistical support for all haplotypes. We defined the minimum number of SNPs accounting for the largest proportion of haplotypic diversity, using Haploview v3.32. Seven haplotype-tagging SNPs (htSNPs) were then selected for the genotyping of the entire panel of 545 individuals. DNA samples were genotyped by TaqMan® SNP genotyping assays on a 7000 Real-Time PCR system (Applied Biosystems). Probes were either obtained from commercial sources (*SNP4*, *SNP6*, *SNP8*, *SNP12* and *SNP15*) or were custom-made (*SNP1* and *SNP14*, Applied Biosystems).

Quantitative real-time PCR

Expression levels of the two isoforms of *SNAP25* were determined using TaqMan® gene expression assays (Applied Biosystems), with probes specifically hybridising *SNAP25a* (*Hs00938959_m1*) and *SNAP25b* (*Hs00938964_m1*). Normalisation was performed using an endogenous housekeeping gene encoding the human β-actin (*ACTB*), with limited primers (Applied Biosystems). PCR reaction were performed in a final volume of 20 µl, containing 2.5 ng of cDNA, 1X of probe and 1X of TaqMan Universal Mastermix (Applied Biosystems), and run in a Mastercycler® ep realplex^{2S} (Eppendorf, Hamburg, Germany). PCR cycle parameters were

50°C for 2 min, 95°C for 10 min, 60 cycles of 95°C for 15 s and 60°C for 1 min. Common threshold fluorescence for all the samples was set into the exponential phase of the amplification and determined the C_T, corresponding to the number of amplification cycles needed to reach this threshold. All reactions were performed in triplicate and the mean value of C_T was used for subsequent analysis. Relative gene expression quantification was performed using the $2^{-\Delta\Delta C_T}$ method.[28] For the calibrator, first strand cDNA was generated from 1 µg of human brain total RNA (Clontech Laboratories Inc., Mountain View, CA, USA) using random hexamers and 200 U of SuperScript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) in a final reaction volume of 20 µl. The cDNA was treated with 10 U of ribonuclease H (Invitrogen) to remove bound RNA template and diluted to 1/25.

Statistical analyses

Statistical testing for allelic, genotypic and haplotypic associations was carried out with PLINK v0.99p software (Shaun Purcell *et al*, the Center for Human Genetic Research, Massachusetts General Hospital, http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml). We used the --hap-window options to specify all haplotypes in sliding windows of a fixed number of SNPs, varying from two to seven and shifting by one SNP at a time. Haplotype frequencies were obtained by summing the fractional likelihoods of each individual having each haplotype. We tested for case-control haplotype-specific association, using haplotype-specific tests with one degree of freedom. Analyses of variance (ANOVA) were carried out with StatView v5.0 software (SAS Institute Inc., Cary, North Carolina, USA), to analyse the relationship between age at onset and genotype as well as the mRNA expression level. A potential correlation between *SNAP25a* and *SNAP25b* expression levels and age, postmortem interval (PMI), refrigerator interval (RI) and brain pH has been tested using Spearman's rank correlation test in cases and controls.

Results

Mutation screening

Direct sequencing of samples from 31 patients with early-onset BD led to the detection of one synonymous and fourteen non-coding variants (Table S2 and Figure 1). Three of these variants (*SNP1-3*) were located in the promoter region, one in exon 6 (*SNP9*), seven in introns flanking coding exons, and 3 in the 3'UTR (*SNP13-15*). Ten of these 15 SNPs had a MAF higher than 0.05. We identified htSNPs by calculating the linkage disequilibrium between SNPs. Two linkage disequilibrium blocks had r^2 values>0.8 (*SNP6*, *SNP9* and *SNP10*, and *SNP13* and *SNP15*), resulting in the definition of seven htSNPs (see Table S2 and Figure 1). Three amino-acid changes were reported in public databases, in exons 2, 5a and 6. We therefore screened these exons for mutations in an additional sample of 78 individuals (43 early-onset BD and 35 late-onset BD). No amino-acid change was observed in these individuals.

Association study

The seven htSNPs (*SNP1*, *SNP4*, *SNP6*, *SNP8*, *SNP12*, *SNP14* and *SNP15*) were tested for association with BD subgroups. All SNPs were in Hardy-Weinberg equilibrium in control populations. One of the seven SNPs (*SNP14*) was not in Hardy-Weinberg equilibrium in affected subjects (p=0.017). Patients with early-onset (N=197) and late-onset (N=202) bipolar disorder were compared with unaffected individuals (N=136) in case-control studies (Table S3). Allele distribution for *SNP4* and for *SNP12* differed significantly between the early-onset and control groups (p=0.005 and p=0.04, respectively) (Table 1), whereas no association was observed in the late-onset subgroup (p=0.22 and p=0.63, respectively) (Table S4). For *SNP4*, the result remained significant in early-onset subgroup after correction for multiple testing (corrected empirical p-value for 100,000 permutations p_c=0.03). Significant genotypic association was observed only for *SNP4* (p=0.017), for which the 'CC' genotype was more frequent in early-onset cases (51%) than in controls (39%). Although not significant, a weak

difference was observed for allele frequencies of *SNP4* between subjects affected with early-onset and those affected with late-onset bipolar disorder (p=0.08). We also carried out an overall one-way ANOVA for the whole sample of BD patients, and found significant differences in mean AAO as a function of *SNP4* genotype (F=3.371; Df=2; p=0.035) (Figure 2).

We carried out a haplotype analysis for the early-onset subgroup, using two- to seven-marker haplotype windows, which we slid along the *SNAP25* gene in a 5' $\rightarrow$ 3' direction. Several haplotypes gave significant p-values (not shown). The most significant association was obtained for a four-marker haplotype window (*SNP1-SNP4-SNP6-SNP8* 'GAAA' haplotype, p=0.002, Table 2).

Population stratification

Our control population was ethnically matched to the cases, and we expected only moderate stratification for our population. Nonetheless, we assessed the risk of false positive results due to population stratification, by genotyping 15 unlinked genetic markers randomly distributed in the genome and with allele frequencies similar to those of *SNP4* or *SNP12* (Table S5). The mean χ^2 value (μ) across these 15 loci, representing the level of stratification,[29] was 1.17 (p=0.28), suggesting that the two groups were not genetically different. After direct quantitative correction for stratification, the differences between our cases and controls remained significant for *SNP4* (p=0.009), and were marginal for *SNP12* (p=0.06). Thus, population stratification is unlikely to account for the observed association between *SNAP25* polymorphisms and early-onset bipolar disorder.

Expression analysis

The *SNAP25* promoter region has been previously defined to span 2073 bp upstream to the transcription start site,[30] including the *SNP4*. In order to determine whether this SNP may affect the mRNA expression in patients' brains, we analysed the transcript level of the two isoforms of *SNAP25*, *SNPA25a* and *SNAP25b*, in the prefrontal cortex of patients affected with bipolar disorder (N=30) and unaffected control individuals (n=30). The allelic and genotypic frequencies of *SNP4* were similar in the brain sample and in our populations, for both affected and unaffected subjects (p_{exact}=0.32 and p_{exact}=0.83, respectively for genotypic distributions), showing a higher frequency of the 'CC' genotype in patients than in controls (Table S6). The expression level of *SNAP25b* was higher in individuals homozygous for the 'C' allele of *SNP4*, as compared to those carrying either 'AA' or 'CA' genotypes (one-way ANOVA, F=4.61; Df=1; p=0.04), whereas no significant difference was observed for *SNAP25a* (one-way ANOVA, F=2.17; Df=1; p=0.15), nor for the *SNAP25b*:*SNAP25a* ratio (one-way ANOVA, F=1.50; Df=1; p=0.23). We performed a similar analysis taking into account the disease status (affected or unaffected) in a multivariate model, and showed that the influence of genotypes on the mRNA expression level of *SNAP25b* in prefrontal cortex remained significant (two-way ANOVA, F=4.19; Df=1; p=0.045) (Figure 3). In this analysis, neither an effect of the disease status (p=0.21) nor an interaction between the genotype and the disease status (p=0.87) was observed. Since our genetic results showed a significant increase of the *SNP4* 'CC' genotype only in patients with early-onset BD, we carried out a secondary analysis to compare the three diagnostic groups (controls, late-onset BD and early-onset BD). We did not find any significant influence of these subgroups on the expression level of *SNAP25a* and *SNPA25b* (one-way ANOVA, $F_{SNAP25a}$=0.99; $Df_{SNAP25a}$=2; $p_{SNAP25a}$=0.38, and $F_{SNAP25b}$=0.95; $Df_{SNAP25b}$=2; $p_{SNAP25b}$=0.39), although the small sample size hampered the interpretation of these results. A previous study reported a significant increase of SNAP25 and syntaxin interaction in subjects who died by suicide.[31] Thus, we carried out an additional analysis taking into account the suicide status of subjects. However, no significant effect of suicide status was observed on this

sample (Sudent's t-test, $p_{SNAP25a}$=0.58 and $p_{SNAP25b}$=0.36). Finally, no significant correlation was detected between *SNAP25a* and *SNAP25b* expression levels and age, PMI, RI, and brain pH (data not shown). Altogether, these results showed that the *SNAP25b* expression level was only dependent on the subjects' *SNP4* genotype.

Discussion

We provide here evidence for an association between early-onset BD and a SNP located in the promoter region of the *SNAP25* gene. This association was not observed when considering late-onset BD, suggesting that this susceptibility variant might play a predominant role only in the early-onset subgroup of patients. These results are consistent with those of our previous genome-wide scan for early-onset BD,[8] and strengthened by three other genome-wide scans reporting linkage on chromosome 20p12.[9-11]

The SNP, for which the highest significant association was observed (*SNP4*), is located in a CpG island, spanning the promoter region of the gene, and may affect the transcription level of *SNAP25*. Using quantitative RT-PCR analysis on brain samples, we showed that individuals with 'CC' genotype showed a significant increase in mRNA level of the major isoform of *SNAP25* (*SNAP25b*) in prefrontal cortex. These results are consistent with the significant increase in SNAP25 protein level previously reported in Brodmann's area 9 (dorsolateral prefrontal cortex) in patients with BD,[15] since cellular and animal studies showed that variations in mRNA levels of *SNAP25* correspond to equivalent variation in protein levels.[32, 33]

The SNP4 is located between two AP-1 consensus-binding sequences in a region that contribute to the repression of the *SNAP25* transcription by binding of POU4F2 (also called Brn-3b).[30, 34] This protein is a member of the POU (Pict-Oct-Unc) transcription factor family that play a critical role in the development of the mammalian nervous system and for which an over-expression results in a failure of SNAP25 activation and neurite outgrowth.[35] Thus, *SNP4* might result in a modification in the binding affinity of a transcriptional factor, such as POU4F2, leading

to the increase of *SNAP25b* expression level that we observed in prefrontal cortex of homozygous subjects for the 'C' allele of *SNP4*.

In mice, SNAP25 is regulated during brain growth and synaptogenesis at the level of expression and by alternative splicing between tandem exon 5.[36] This results in a developmental switch between 1 and 3 weeks of age from expression of predominantly *SNAP25a* to *SNAP25b* transcripts that ultimately constitute more than 80% of *SNAP25* mRNA in mouse adult brain. These two isoforms diverge only for 9 amino acids in a domain involved in membrane association and disassembly, after exocytosis, of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex.[37-39] In individuals homozygous for the 'C' allele, we observed that only *SNAP25b* is significantly increased. Interestingly, in absence of this isoform, 75% of mice die before five weeks of age and surviving animals present alteration in synaptic maturation as well as deficit in synaptic transmission.[40] In addition, over expression of SNAP25 in cultured hippocampal neurons resulted also in impaired synaptic transmission.[41] Altogether, these results suggest that an increased *SNAP25b* level might impair synaptic maturation or neurotransmission, which in turns might influence either the risk of developing BD or the age at onset in vulnerable individuals. This increase might also reflect an abnormal switch of *SNAP25a* to *SNAP25b* during adolescence, consistent with the well-documented increase of the susceptibility to BD during and after puberty.

There is compelling evidence demonstrating abnormal serotonergic, dopaminergic and noradrenergic neurotransmission in BD.[42] Theses abnormal patterns of neurotransmission may be underpinned by abnormal exocytosis phenomena and thus linked to SNAP25 dysfunction, since this protein play a crucial role in vesicle docking and exocytosis. Interestingly, coloboma mice have a 2 cM deletion on chromosome 2, including the *SNAP25* gene.[32] Raber *et al*. studied the release of several neurotransmitters in heterozygous mice (Cm/+), expressing 50% of the SNAP25 protein level, and showed that depolarisation failed to induce dopamine release and induced significantly lower than normal amounts of serotonin from the dorsal striatum.[12]

These results were recently confirmed by Fortin *et al.*, who showed that SNAP25 was required for dopamine release from rat neurons in culture.[43] Therefore, polymorphisms in the *SNAP25* gene may influence the susceptibility to BD through the modification of one or several monoaminergic neurotransmission systems in specific brain areas. Further experiments are required to determine which neurotransmission systems are specifically altered in early-onset BD patients, carrying the *SNAP25* susceptibility allele.

Polymorphisms in the *SNAP25* gene have been shown to be associated with ADHD.[16, 44-46] A high comorbidity has been reported between ADHD and BD, more specifically with early-onset BD.[47] Therefore, our results suggest that SNAP25 might be a common susceptibility factors for these psychiatric disorders. These data are strengthened by the recent results obtained by Kim *et al.*, showing that comorbidity with major depressive disorder may enhance detection of the association between *SNAP25* and ADHD.[48] Further association studies on clinically well-defined populations will be necessary to determine how these different phenotypes are influenced by the same gene.

In conclusion, we report here an association between early-onset BD and the -523C/A variant of the *SNAP25* gene promoter, as well as an association between this polymorphism and the expression level of *SNAP25b* isoform in human prefrontal cortex. This raises the hypothesis that the *SNAP25b* expression level in prefrontal cortex, which strongly influences neurotransmitter release, might modify the risk to develop an early-onset BD. These results require confirmation in larger samples to identify more functional variants accounting for the pathophysiology of BD. Furthermore, functional explorations of SNAP25 in bipolar patients and animal models will be necessary to explore, in more details, the role of variations in this gene in bipolar disorder and other psychiatric disorders.

Acknowledgements

This work was supported by INSERM (*Poste d'Accueil* INSERM to B.E.), the National Alliance for Research on Schizophrenia and Affective Disorders (2004 Independent Investigator Award to F.B.), *Assistance Publique des Hôpitaux de Paris*, *Agence Nationale pour la Recherche* (ANR NEURO2006 – Project MANAGE_BPAD), *Fondation pour la Recherche sur le Cerveau* and *Réseau Thématique de Recherche et de Soins en Santé Mentale* (FondaMental®).

We thank bipolar patients and controls for their participation.

Brain specimens were donated by The Stanley Medical Research Institute Brain Collection courtesy of Drs Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken.

We thank M.J. Pereira Gomes, E. Abadie, A. Philippe, the Cochin Hospital cell library (Dr J. Chelly), the Clinical Investigation Centre (CIC) of Mondor Hospital (Dr O. Montagne), and blood donor center (Drs J.L. Beaumont and B. Mignen, EFS, Créteil) for technical assistance.

We thank B. Cochet, O. Elgrabli, A. Raust and L. Zanouy for their participation in the clinical evaluation of patients.

We thank Pr D. Kupfer for helpful discussion and comments on the manuscript.

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Competing Interests

The authors have declared that no competing interest exists.

Abbreviations

AAO, age at onset, ADHD, attention deficit hyperactivity disorder; BD, bipolar disorder; htSNPs, haplotype-tagging single nucleotide polymorphisms; MAF, minor allele frequency, PMI, postmortem interval; RI, refrigerator interval.

Accession Numbers

SNAP25, GeneID:6616, HGNC:11132, Ensembl:ENSG00000132639

SNAP25a, NM_003081

SNAP25b, NM_130811

TABLES

Table 1 Association study between subjects affected with early-onset bipolar disorder (N=197) and healthy controls (N=136)

SNP	Minor allele	Allele frequency in affected individuals	Allele frequency in unaffected controls	χ^2	*p value*	Odds ratio
SNP1	T	0.12	0.11	0.09	0.767	1.08
SNP4	A	0.29	0.39	7.96	**0.005**	0.62
SNP6	T	0.06	0.04	1.20	0.274	1.50
SNP8	C	0.52	0.47	1.46	0.226	1.21
SNP12	A	0.19	0.13	4.22	**0.040**	1.57
SNP14	C	0.25	0.21	1.36	0.244	1.25
SNP15	T	0.37	0.38	0.05	0.817	0.96

Table 2 SNP at-risk haplotypes in early-onset BD as compared to healthy controls

SNP1	SNP4	SNP6	SNP8	SNP12	SNP14	SNP15	Allele frequency in affected individuals	Allele frequency in unaffected controls	*p value*
G	A	A	A				0.19	0.30	**0.002**
	A	A	A	G			0.16	0.24	**0.004**
		A	C	A	C		0.07	0.04	0.06
			C	A	C	C	0.07	0.03	**0.04**

FIGURE LEGENDS

Figure 1. Genomic structure of the *SNAP25* gene and localisation of SNPs identified by sequence analysis. Grey arrows correspond to SNPs with MAF<0.05. Black arrows correspond to SNPs with MAF>0.05. Two blocks of linkage disequilibrium (r^2>0.8, black squares) were identified with Haploview v3.32 software, defining 7 htSNPs (underlined).

Figure 2. Mean age of first mood episode according to SNP4 genotype in the affected population. (ANOVA, F=3.371; Df=2; *p*=0.035). Error bars correspond to ±1 standard error. AAO, age at onset. ** *p*<0.01.

Figure 3. Average level of *SNAP25* isoform mRNA expression in prefrontal cortex of individuals affected with bipolar disorder and unaffected controls, according to SNP4 genotypes. Data are expressed as a mean value of relative mRNA expression level of *SNAP25a* (A), *SNAP25b* (B) and *SNAP25b*:*SNAP25a* ratio (C). AA and AC genotypes were pooled and compared to CC genotype in respect to genotypic data observed in the association study. No significant difference was observed between bipolar patients (black bars) and unaffected controls (white bars) for none of the *SNAP25* isoforms. A significant difference was observed for *SNAP25b* between individuals homozygous for the C allele as compared to those carrying allele A (ANOVA, F=4.61; Df=1; *p*=0.04). Error bars correspond to ±1 standard error. * *p*<0.05.

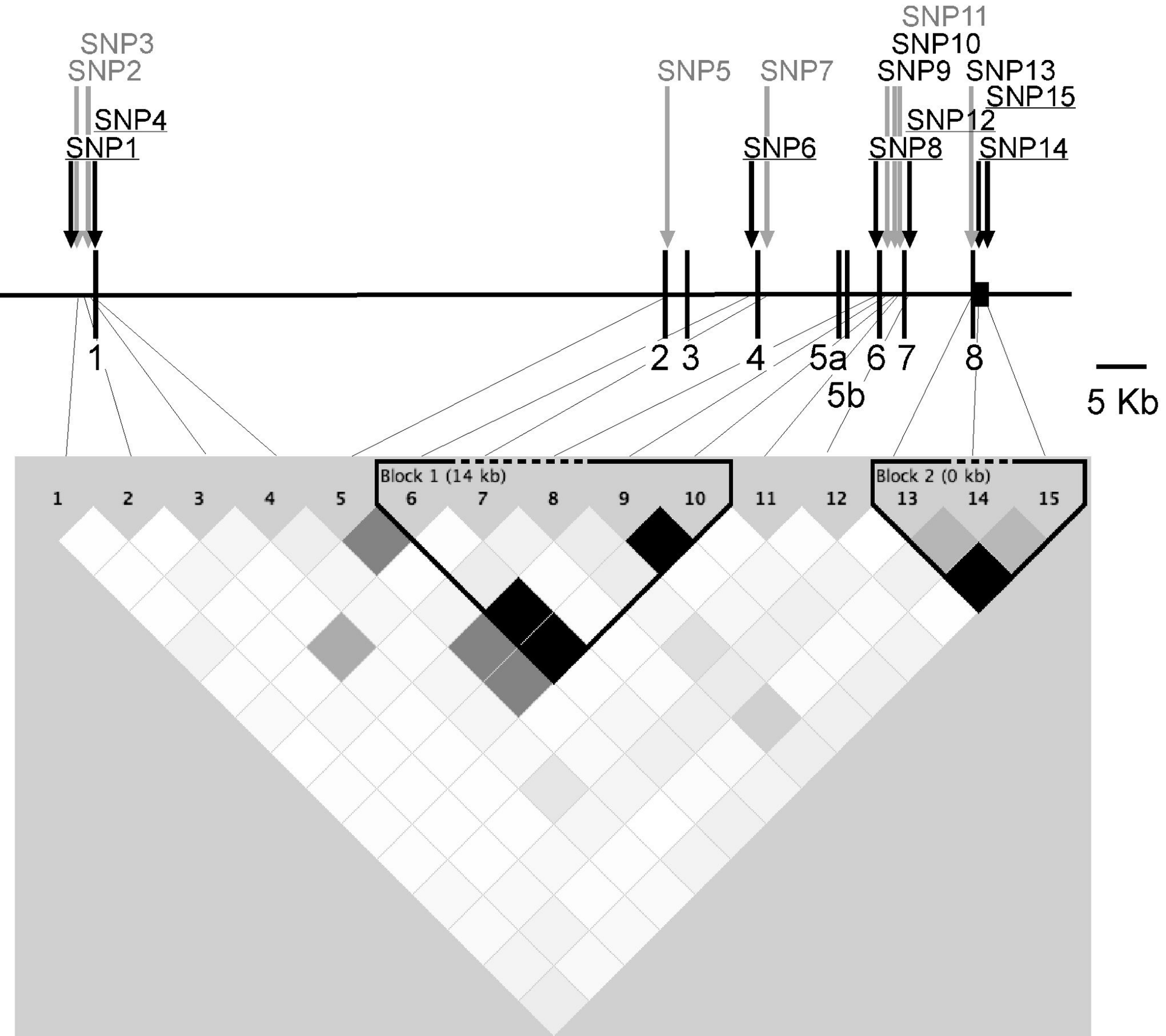
SNP3
SNP2
SNP4
SNP1
SNP5
SNP7
SNP11
SNP10
SNP9
SNP13
SNP15
SNP12
SNP6
SNP8
SNP14
1
2 3
4
5a
5b
6 7
8
5 Kb
Block 1 (14 kb)
Block 2 (0 kb)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 1.

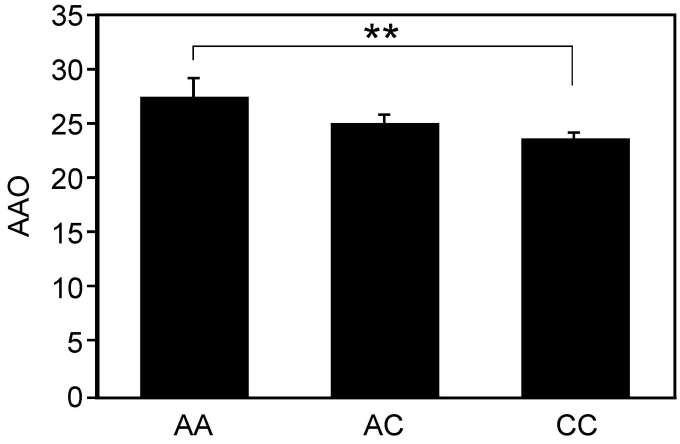

Figure 2.

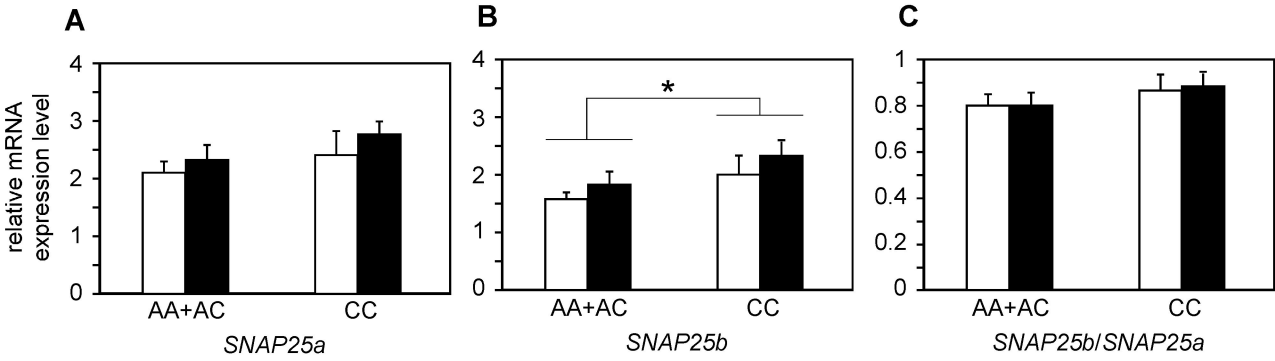

Figure 3.