Human Molecular Genetics, 2006, Vol. 15, No. 16 **2438–2445**
doi:10.1093/hmg/ddl166
Advance Access published on July 5, 2006

P2RX7, a gene coding for a purinergic ligand-gated ion channel, is associated with major depressive disorder

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Received March 21, 2006; Revised and Accepted June 28, 2006

The *P2RX7* gene is located within a region on chromosome 12q24.31 that has been identified as a susceptibility locus for affective disorders by linkage and association studies. P2RX7 is a purinergic ATP-binding calcium channel expressed in neurons as well as in microglial cells in various brain regions. We investigated 29 single nucleotide polymorphisms (SNPs) within the *P2RX7* gene and adjacent genes in a sample of 1000 German Caucasian patients suffering from recurrent major depressive disorder (MDD). These were contrasted with diagnosed healthy Caucasian controls from the same population ($n = 1029$). A non-synonymous coding SNP in the *P2RX7* gene (rs2230912), previously found to be associated with bipolar disorder, was significantly associated ($P = 0.0019$) with MDD. This polymorphism results in an amino acid exchange in the C-terminal cytosolic domain of the P2RX7 channel protein, suggesting that the observed *P2RX7* polymorphism might play a causal role in the development of depression.

INTRODUCTION

Major depressive disorder (MDD) and bipolar disorder (BP) are common psychiatric diseases with lifetime prevalence rates of 16–17% for MDD (1,2) and 1–3.3% for BP (3,4). Although both, MDD and BP patients, suffer from recurrent episodes with symptoms such as sad mood, loss of interest and energy, cognitive impairment, insomnia and loss of appetite, bipolar patients in addition encounter episodes of mania (bipolar I, BPI) or hypomania (bipolar II, BPII), characterized by excessive elation, increased energy, decreased need for sleep, increased sexual desire and grandiose notions. Both BP and MDD have a high heritability, with 83–93% for BP (5,6) and 34–75% for MDD (7–9).

Genome-wide linkage analyses with BP patients yielded several regions of interest (10). Two genome-wide scans on pedigrees from the Saguenay-Lac-St-Jean (SLSJ) region of Quebec demonstrated the presence of a susceptibility locus on 12q24.31 administering both parametric and non-parametric analyses and using a broad affection status model (ASMII) that includes BPI, BPII and recurrent MDD. Four consecutive markers gave maximum sibpair LOD scores close to or above 5, with empirical *P*-values of <0.0001 (11,12).

Linkage analysis using tightly spaced microsatellite markers gave a LOD score >3.7 (*P*-value 0.0001) at marker NBG6 under ASMII, and a case–control association analysis with the same marker showed positive allelic association with BP (*P*-value $= 0.008$) (13). As this marker is located within intron 9 of the *P2RX7* gene, coding for a member of the purinergic ligand-gated ion channels of the P2X family, 24 single nucleotide polymorphisms (SNPs) in *P2RX7* and the neighbouring genes, e.g. *P2RX4* and *CAMKK2*, were genotyped in a bipolar case–control sample and 12 SNPs in the pedigrees

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used for the linkage studies. The strongest association ($P = 0.000708$) was observed in bipolar families at the non-synonymous SNP P2RX7-E13A (rs2230912, Gln460Arg) lying in exon 13 of *P2RX7* (14).

The linkage results in the SLSJ population were the strongest under a broad affection model including MDD patients. The significance of this chromosomal region in the susceptibility for MDD has been strengthened by further studies that reported linkage of this region in pedigrees consisting of BP and MDD patients (15–17).

Given the fact that there is a constant diagnostic conversion from MDD to BP of 1.25% per year throughout the lifespan (18), we were interested if these heritable disorders share at least some genetic commonalities. Therefore, in our study, we concentrated on *P2RX7* as a candidate gene for MDD.

ATP-gated P2X-receptors are cation selective ion channels with high calcium permeability, which open upon binding of extracellular ATP (19,20). In the brain, P2RX7 has been shown to be expressed in glial cells (21); immunohistochemical studies have suggested that P2RX7 to be also expressed in central and peripheral neurons and may regulate immune function and neurotransmitter release (22,23). However, expression studies of P2RX7 in neurons have been inconsistent because of a limited quality of available antibodies (22,24). Polymorphisms in the gene are discussed to be relevant in the survival of chronic lymphocytic leukaemia (25) and *P2RX7* is considered to be a candidate gene for systemic lupus erythematosus and type I diabetes (26,27).

To investigate the implication of P2RX7 in MDD, we performed a case–control study in a sample of German Caucasian patients with recurrent MDD and diagnosed healthy controls from the same population. We report genotypic association in the *P2RX7* gene providing evidence that *P2RX7* might indeed be a susceptibility gene for MDD.

RESULTS

Allele frequencies and quality control

Twenty-three of 29 genotyped SNPs were polymorphic. Eighteen of these SNPs had a minor allele frequency (MAF) greater than 1% and were included in the analysis. Reference numbers for SNPs are given when available; the remaining SNPs were named according to their position on the genomic reference sequence (Table 1). Information about genes, location of polymorphisms within genes, function and MAF are presented in Table 1. Genotyping was successful in 98% of cases and controls.

We separately calculated the *P*-value for deviation from the Hardy–Weinberg equilibrium (HWE) in diagnosed controls and patients. One SNP in the patient group (rs3815990) and three SNPs in controls (29364 G→A, rs6489795 and rs2230912) showed nominally significant deviations from the HWE (Supplementary Material, Table 1). After correcting for multiple testing, none of these deviations remained significant.

Case–control association

The confirmatory analysis of the three SNPs that previously turned out to be associated with BP showed a significant association with the exonic SNP, rs2230912 (Gln460Arg), of the *P2RX7* gene with a nominal *P*-value equal to 0.0019 (Fig. 1), remaining significant after correcting for multiple comparison. Homozygote A allele carriers were more frequent among controls, whereas heterozygous carriers were over-represented among MDD (Fig. 2). We tested different genetic models underlying this association. In the case of a dominant model, the odds ratio was equal to 1.301 with a nominal *P*-value of 0.0081 (Fig. 2). Under the assumption of a 'heterozygote disadvantage' model, i.e. contrasting both homozygous genotypes with the heterozygote AG genotype, we observed an odds ratio of 1.402 with a nominal *P*-value of 0.0009938. The power to detect an effect of this type in our study in the confirmatory analysis was 58%, thus our finding is in keeping with the expectations. The 'heterozygote disadvantage' model appeared slightly better, but the difference was not significant ($P = 0.1407$). The exploratory analysis of the other 15 polymorphic SNPs did not show further significant case–control associations.

The distribution of the observed genotypes of the associated SNP rs2230912 (Gln460Arg) showed a (nominally) significant deviation from the HWE. This could be a consequence of genotyping error, but we can exclude this possibility by verifying the data using different genotyping methods, namely, direct sequencing and pyrosequencing. All cases ($n = 1000$) and controls ($n = 1029$) were genotyped by both MALDI-TOF (at the Genetic Research Center in Munich, overall call rate 95.4%) and pyrosequencing (at the Max Planck Institute of Psychiatry in Munich, overall call rate 99.5%) for rs2230912. Of these genotypes, there were 12 discrepancies out of 974 valid genotypes for both assays in the controls (1.17%) and eight discrepancies out of 952 valid genotypes for both assays in the cases (0.80%). In addition, 500 randomly selected cases and 500 randomly selected controls were genotyped by direct sequencing (overall call rate 99.6%) in the laboratory of Nicholas Barden at Laval University, Quebec, Canada. In these data, there were four discrepancies with the pyrosequencing data and seven discrepancies with the MALDI-TOF data (discrepancy rates of 0.40 and 0.73%, respectively). These discrepancies did not seem directed in any of the cases, and the influence on the various genotyping methods on the results of the analysis was minimal. To give an example, the result of the genotypic test in those samples, where both the MALDI-TOF and the pyrosequencing genotypic data agreed, was 0.001406 for the genotypic model, 0.002611 for the dominant model and 0.0003865 for the heterozygote disadvantage model. For the analysis presented here, we used the pyrosequencing data with missing values supplemented by the MALDI-TOF data. This gave *P*-values of 0.0019, 0.008068 and 0.0009938 for the respective genetic models. *P*-values when using the MALDI-TOF data were 0.001294 for the genotypic test, 0.003265 for the dominant model and 0.0004143 for the heterozygote disadvantage model. *P*-values based on the pyrosequencing data alone were 0.002844, 0.009417 and 0.001227, respectively. Thus, the results of the analysis do not or only in a very minor fashion depend on the genotyping method used.

Another explanation for this deviation could be the fact of investigating a control sample selected for being negative

Table 1. Information on genotyped SNPs

Gene	SNP ID	Position on hg17	Location within gene	Function	MAF
OASL	rs12819210	119921120	Exon 6	Ser503Ser	0.1951
OASL	−14989 G→A	119924518	Exon 5	Val348Met	0.0039
OASL	rs3213545	119934057	Exon 2	Leu136Leu	0.2935
OASL	259 A→G	119939766	5′-UTR	Unknown	0.2465
OASL	2529 C→T	119942036	5′-UTR	Unknown	n.p.
P2XR7	−2575 T→C	120030921	5′-UTR	Unknown	0.0948
P2XR7	−1300 G→A	120032196	5′-UTR	Unknown	0.0068
P2XR7	21749 C→T	120055245	Intron 1	Unknown	0.0024
P2XR7	rs17525809	120055409	Exon 2	Val76Ala	0.0858
P2XR7	23160 C→T	120056656	Exon 3	Arg117Trp	0.0020
P2XR7	29364 G→A	120062860	Intron 4	Unknown	0.0288
P2XR7	29463 G→A	120062959	Exon 5	Gly150Arg	n.p.
P2XR7	32406 G→A	120065902	Exon 6	Glu186Lys	n.p.
P2XR7	32422 G→A	120065918	Exon 6	Leu191Pro	n.p.
P2XR7	rs7958316	120068093	Exon 8	Arg276His	0.0260
P2XR7	rs1718119	120077823	Exon 11	Ala348Thr	0.4075
P2XR7	rs6489795	120077851	Exon11	Thr357Ser	0.0698
P2XR7	rs2230912	120084916	Exon 13	Gln460Arg	0.1472
P2XR7	rs3751143	120085024	Exon 13	Glu496Ala	0.2055
P2XR7	rs2230913	120085100	Exon 13	His521Gln	n.p.
P2XR7	52857C→T	120086353	3′-UTR	Unknown	n.p.
P2XR4	−643 C→T	120110044	5′-UTR	Unknown	0.1262
P2XR4	rs1653622	120110155	5′-UTR	Unknown	0.3330
P2XR4	rs2303998	120117783	Exon 2	Ala87Ala	0.0060
P2XR4	rs7298368	120122404	Intron 2	Unknown	0.1649
P2XR4	rs25644	120129366	Exon 7	Ser242Gly	0.1067
P2XR4	rs11065501	120134690	3′-UTR	Unknown	0.3330
CAMKK2	rs3815990	120153808	Exon 9	Ile365Ile	0.0614
CAMKK2	rs3817190	120174797	Exon 1	Thr85Ser	0.4099

for mental disorders. Provided that heterozygotes represent the high-risk genotype for depression, a lack of heterozygotes in the control group is expected. In that case, the control group would not be in the HWE, in contrast to the combined case and control samples. In fact, if we merge diagnosed controls and depressive patients, they are in the HWE (nominal $P = 0.358$). To prove the statistical significance of the latter hypothesis, we used the goodness-of-fit procedure of Wittke-Thompson *et al*. (28), testing the general, additive, dominant and recessive models. The data were found to be in agreement with the general model ($P = 0.177$). The estimated MAF for the susceptibility locus was equal to 0.157 and, therefore, nearly equal to the observed population frequency of 0.147 for rs2230912 (Table 1). The relative risk for heterozygotes estimated from this model is 1.19, also in agreement with the results presented in Figure 2.

Testing for LD

To get more insight into the pattern of LD between alleles at polymorphic loci, pairwise disequilibrium measures (D' and r^2) in controls were calculated using all SNPs with a MAF over 1%. We detected three independent blocks of LD within the investigated region defined by the Gabriel method (Fig. 3). The first block spans two SNPs in the *OASL* gene, the second contains four SNPs in exons 11 and 13 of *P2RX7* and the third block spans five SNPs in *P2RX4* and *CAMKK2* genes. In the first and third blocks, no SNP was associated with MDD. D' within the second block was equal to 1, albeit r^2 was rather low (0.2, 0.01, 0.05 for pairs of the highest associated SNP rs2230912 with rs1718119, rs6489795 and rs3751143, respectively). The presence of a single associated SNP within the second block is due to the low r^2 despite high D' and somewhat similar MAFs (0.1472 for rs2230912 and 0.2055 for rs3751143) and is coherent with the general recognition of r^2 as important for determining the power of association analyses.

Association of haplotypes

Using the SNPHAP programme, we reconstructed haplotype alleles for each subject on the basis of the four SNPs in the LD block 2 (rs1718119, rs6489795, rs2230912 and rs3751143), because one of these SNPs showed the highest association with MDD. We observed five frequent (>5% population frequency) and two less frequent haplotype alleles (data not shown). The five frequent haplotypes accounted for 99.1% of the chromosomes in our sample. No differences in frequencies were observed between cases and controls. Only one haplotype allele 'ACGA' showed a trend for association with a *P*-value of 0.0753, but that is a reflection of association of the 'G' allele in SNP rs2230912 with MDD.

Explorative genotype–phenotype correlation

Differences in genotype distribution between cases and controls were independent of gender and age. Genotype variation had no influence on age at onset or the number of previous depressive episodes (data not shown).

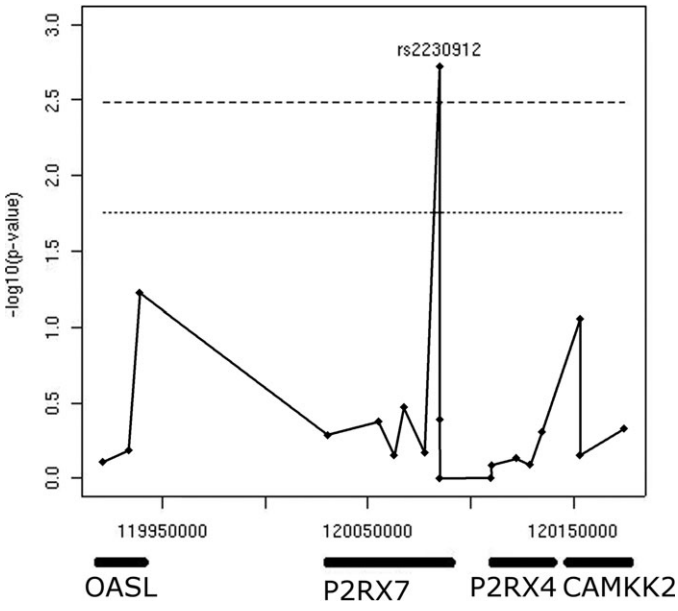

Figure 1. Association of investigated SNPs with MDD. Chromosomal positions are given on the *x*-axis and −log 10 (*P*-values) are on the *y*-axis. The dotted and dashed lines represent the permutation-based 5% type I error rate for the three SNPs implicated in BP in the SLSJ sample (14) and the 17 remaining SNPs, respectively.

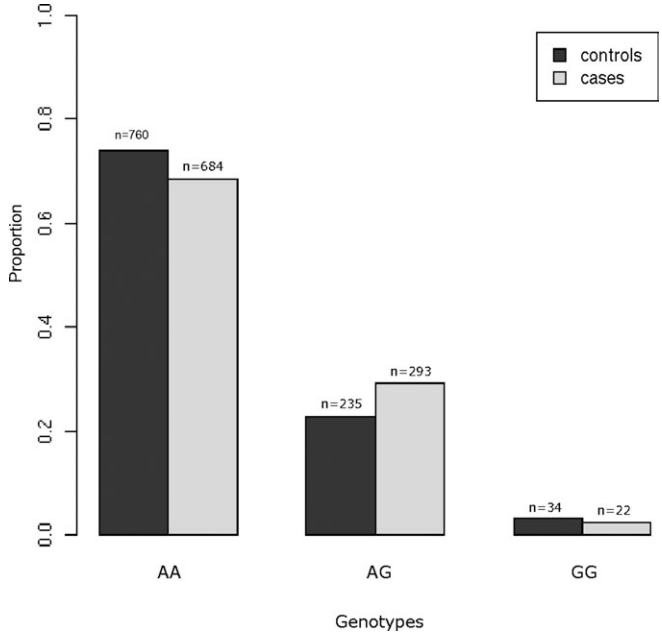

Figure 2. Distribution of genotypes of SNP rs2230912 in cases and controls. The odds ratio for a dominant model (genotypes AG and GG versus AA) was equal to 1.30 (CI = 1.07–1.59; $P = 0.008068$) and for a heterozygote disadvantage model (AG versus AA and GG) 1.40 (CI = 1.14–1.72; $P = 0.0009938$).

DISCUSSION

In this article, we report evidence that SNP rs2230912 in the *P2RX7* gene is associated with MDD. This functional polymorphism rs2230912 is located in exon 13 of the *P2RX7* gene, resulting in a change of the amino acid glutamine to arginine at position 460 (Gln460Arg). Gln460Arg is positioned in the long intracellular C-terminal domain. This domain is unique among P2X receptors and is thought to be responsible for functions that are different from those observed in other ion channels, including alterations in cell morphology (29), intracellular signaling and cytolysis/apoptosis (30). In this region, several loss-of-function polymorphisms have been reported (31–33). The Gln460Arg residue has been described to lead to a functional decrease, albeit minor, when measuring Ca^{2+} influx in peripheral blood lymphocytes of patients affected with chronic lymphocytic leukaemia and in transfected recombinant human embryonic kidney cells (34). Owing to its position in the intracellular domain (35) and the fact that Gln460Arg residue is conserved in mammals, this residue is likely to be involved in P2RX7 dimerization as well as in other protein–protein interactions having effects upon P2RX7-mediated signalling.

Although so far little is known about the functional implications of the Gln460Arg variant, P2RX7 receptors might well play a pivotal role in antidepressant action and the causality of mood disorders through their role in neuroprotection (36) and in neuroinflammatory responses (37) as well as through their influence on neurotransmission in the hippocampus (38).

In our study, we noted a nominally significant deviation from the Hardy–Weinberg (DHW) equilibrium for the associated SNP rs2230912. Because laboratory error is one of the most common reasons for the DHW, we verified our genotyping by pyrosequencing as well as by direct sequencing and found no discrepancies between results from different genotyping methods. This deviation is also unlikely to be due to hidden population structure as our study was restricted to Caucasians with 92% of people originating from Germany. Formal assessment of population structure using STRUCTURE (http://pritch.bsd.uchicago.edu/software.html) gave no evidence of population admixture (data not shown).

Apart from genotyping errors, the DHW in a case–control study may also occur as a consequence of a positive association. Wittke-Thompson *et al.* (28) in their theoretical investigation showed that under certain conditions, the DHW in controls is to be expected in association studies. In that case, the direction of differences between expected and observed genotype frequencies in cases and controls should be opposite, as was the case in our sample. Of course, an alternative explanation for the DHW in our controls sample could simply be chance, especially keeping in mind that after a correction for multiple testing, the DHW appears no longer significant.

In our sample, a heterozygote disadvantage model was the most suitable mode of inheritance, possibly being a reflection of P2RX7 receptors having an oligomeric structure in the plasma membrane based on complexes of identical subunits (39,40). The same variant we found associated, rs2230912, was also the most highly associated SNP in Canadian bipolar families with an over-transmission of the (minor) G-allele in the affected offspring ($P = 0.000708$) (14). In our sample, we observed the same direction of association, with the G-allele being more frequent in patients. SNPs rs3817190 (exon 1 in

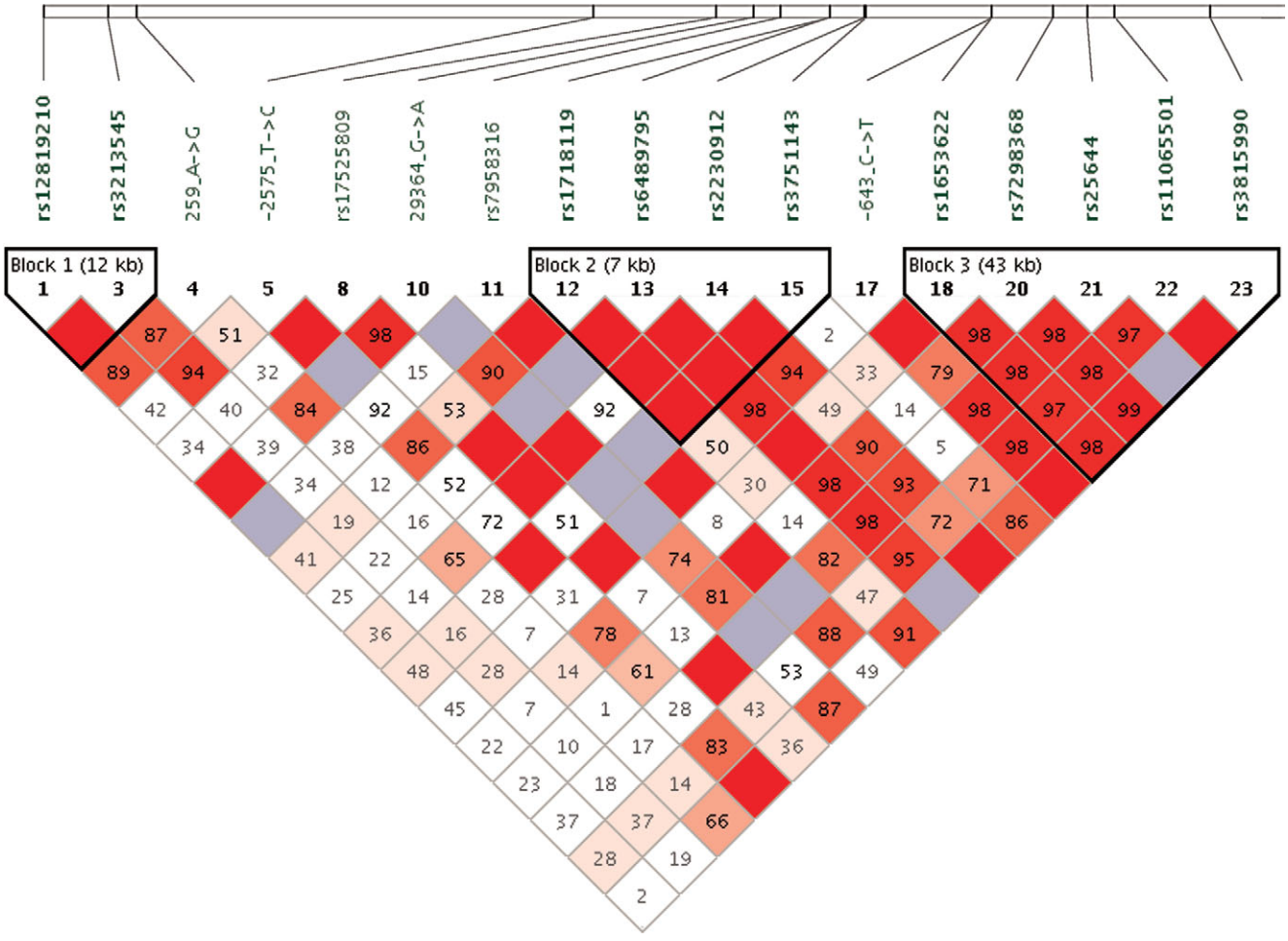

Figure 3. Linkage disequilibrium and block structure (D') of the region. SNPs lying within blocks are depicted in bold type.

CAMKK2) as well as rs1718119 (exon 11 in *P2RX7*) and rs11065501 (3′-UTR in *P2RX4*) were associated in Canadian bipolar families and in the case–control study, respectively, but showed no association in our MDD sample. These findings could reflect the clinical observation of MDD and BP being two different disorders that share some causal factors, with the variances in common conveying susceptibility to both disorders. Polymorphisms in other genes are likely to represent the discriminating factors, determining the ultimate clinical phenotype, unipolar or bipolar. This discrepancy might also be due to differing patterns of LD in the more isolated SLSJ when compared with the non-isolated Munich population. LD seems to have a longer reach in the SLSJ population (14) when compared with the Munich population, which is in keeping with general knowledge about isolated versus non-isolated populations (41).

In addition, epidemiological data indicate that there is a constant diagnostic conversion from MDD to BP of 1.25% per year throughout the lifespan (18). Accordingly, in a sample of MDD patients, a substantial number of hidden bipolar cases are to be expected. However, when calculating odds ratios stratified by age, we observed no linear influence of age or age at onset on the association, which would be expected under the hypothesis that the association in the sample of MDD patients would be due to the hidden bipolar cases (data not shown). Therefore, the data suggest that the Gln460Arg variant might be a susceptibility factor for both disorders.

Our data, in combination with the association data in BP patients, suggest the implication of P2RX7 in affective disorders and are consistent with the possibility that various mood disorders share some genetic commonalities. Being localized in the plasma membrane, P2RX7 is a potential drug target and thus represents an example for a possible pharmacological drug discovery strategy emerging from an unbiased genetic approach.

MATERIALS AND METHODS

Sample description

One thousand patients (326 males and 674 females) with recurrent unipolar depression were recruited from in- and outpatients at the Max Planck Institute of Psychiatry in Munich and psychiatric hospitals in Augsburg and Ingolstadt, located close to Munich. Each hospital contributed one-third of the patients. Patients were diagnosed by WHO-certified raters according to DSM-IV using the Schedule for Clinical

Assessment in Neuropsychiatry. Only Caucasian patients over 18 years with at least two moderate-to-severe depressive episodes were included. Exclusion criteria were the presence of manic or hypomanic episodes, mood incongruent psychotic symptoms, the presence of a lifetime diagnosis of intravenous drug abuse and depressive symptoms only secondary to alcohol or substance abuse or dependence or to a medical illness or medication. Ethnicity was recorded using a self-report sheet for perceived nationality, first language and ethnicity of the subject himself, parents and all four grandparents. All included patients were Caucasian and 91.2% were of German origin. Mean age was 49.35 ± 14.09 years (males: 48.49 ± 13.57 years and females: 49.86 ± 14.38 years).

Thousand twenty-nine controls (336 males and 693 females) matched for ethnicity (using the same questionnaire as for patients), sex and age (to 5-year intervals) were recruited at the Max Planck Institute of Psychiatry. Controls were randomly selected from a Munich-based community sample and screened for the presence of anxiety and affective disorders using the Composite International Diagnostic Screener (42). Only individuals negative for the above mentioned disorders were included in the sample. All included controls were Caucasian and 93.04% were of German origin. These subjects thus represent a group of healthy individuals with regard to depression and anxiety.

The study was approved by the Ethics Committee of the Ludwig Maximilians University in Munich, Germany, and written informed consent was obtained from all subjects.

DNA preparation

On enrolment in the study, 40 ml of EDTA blood was drawn from each patient and each healthy control. DNA was extracted from fresh blood using the Puregene® whole blood DNA-extraction kit (Gentra Systems Inc., MN, USA).

SNP selection and genotyping

We genotyped 29 SNPs in *P2RX7* and in the neighbouring genes *P2RX4*, *OASL* and *CAMKK2*. Polymorphisms were detected by direct sequencing in a Canadian bipolar population, where *P2RX7* was first implicated (14). Some of the identified SNPs are available in the UCSC genome browser (http://www.genome.ucsc.edu/cgi-bin/hggateway).

Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray® system) employing the Spectrodesigner software (Sequenom^{TM}, CA, USA) for primer selection and multiplexing and the homogeneous mass-extension process for producing primer extension products (43). All primer sequences are available upon request.

Genotyping for some SNPs, e.g. rs2230912, was verified by pyrosequencing (Biotage, Uppsala, Sweden).

Statistical analysis

Testing for deviation from the HWE. Deviations from the HWE for each SNP were assessed in both samples, patients and controls, applying the exact test by Wigginton *et al.* (44). For an adjustment for multiple testing, the false discovery rate correction was applied (Supplementary Material, Table 1) (45). All functions used are available in R (http://www.r-project.org/). For investigating whether deviations from the HWE could be explained by an underlying genetic model, we used the goodness-of-fit test by Wittke-Thompson *et al.* (28) (http:/hg-wen.uchicago.edu/dhw2.html).

Case–control analysis. Case–control analysis was performed using Fisher's exact test. We used 2×2 and 2×3 contingency tables to perform genotype-wise analysis. To test for significance under different genetic models (e.g. dominant and heterozygote disadvantage models) and to calculate odds ratios with standard deviations, we used 2×2 contingency tables. Comparison of different genetic models was performed on the basis of the method by Chiano and Clayton (46).

Correction for multiple testing. In the confirmatory part of the study, we analysed three SNPs (rs1718119, rs11065501 and rs2230912) of the *P2RX7* and *P2RX4* genes that have previously been reported to be associated with BP (14). In the exploratory part of the study, we considered the remaining 15 SNPs not supposed to be associated with BP, but turned out to be polymorphic with an MAF of $>1\%$. Correction for multiple testing was performed separately for the confirmatory and exploratory parts of the study using the minimum-P method of Westfall and Young (47), allowing for the linkage disequilibrium between genetic markers. After performing 30 000 permutations, we determined the required threshold for controlling the type I error rate at 2.5% to be equal to 0.0085 for the confirmatory analysis and to be 0.0019 for the exploratory part of the study. The type I error rate was set to 2.5% to allow for the two rounds of testing, confirmatory and exploratory, within this study. We compared the nominal *P*-values, given throughout the text with this threshold, instead of comparing it with 0.05.

Testing for LD. For the LD structure examination, we used D' and r^2measures (48). Visualization of LD measures was performed using HAPLOVIEW (http://www.broad.mit.edu/mpg/haploview/index.php). Blocks were defined using the confidence interval method described by Gabriel *et al.* (49).

Haplotype analysis. We estimated haplotypes using the SNPHAP programme (http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt) for the analysis of the highest associated SNP and for the three neighbouring SNPs that were in one LD block. We tested all haplotypes with frequencies $>5\%$ and a certainty of individual assignment of $>95\%$. For association tests of each haplotype variant, we used Fisher's exact tests on 2×2 contingency tables.

Explorative genotype–phenotype correlation. To determine a possible association of age at onset with the number of previous depressive episodes, we performed logistic regression analysis with genotypes treated as the independent variable and phenotypes as dependent variable. Age at onset was defined as the age at which the diagnostic criteria for MDD were met for the first time.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Ruppert and his team at the Genetic Research Center, Munich for genotyping and Benno Pütz for his help with the figures. This work has been funded by the Bavarian Ministry of Commerce and by the Federal Ministry of Education and Research in the framework of the National Genome Research Network, Förderkennzeichen 01GS0481 and the Bavarian Ministry of Commerce. The authors are responsible for the contents of this publication.

Conflict of Interest statement. None declared.

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