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Association of purinergic receptor *P2RX7* gene polymorphisms with depression symptoms

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Highlights

- Genetic variants of the *P2RX7* gene can contribute to the susceptibility to depression.
- Present case-control analysis of *P2RX7* polymorphisms in MDD and BD yielded negative results.
- The A-allele of rs1718119 (Ala348Thr) was associated with increased depression score.
- The rs1718119-A~rs2230912-G~rs1653625-A haplotype showed the highest depression score.
- The *P2RX7* 3' UTR haplotype affected gene expression according to predicted miRNA binding efficiency.

Abstract

Introduction: The activation of the ATP-gated *P2RX7* (purinergic receptor P2X, ligand-gated ion channel, 7) produces microglial activation, a process which has been demonstrated in depression, bipolar disorder, and schizophrenia. Emerging data over the last years highlighted the importance of P2X7 cation channel as a potential drug target for these central nervous system disorders. The Gln460Arg (rs2230912) polymorphism of the *P2RX7* gene has been widely studied in mood disorders, however the results are still controversial. Therefore, we aimed to investigate the C-terminal region of this gene in major depressive and bipolar disorders (MDD and BD) by studying possibly functional, non-synonymous polymorphisms within a 7 kb long region around the Gln460Arg, including Ala348Thr (rs1718119), Thr357Ser (rs2230911), and Glu496Ala (rs3751143) variants. Since Gln460Arg is located at the 3' end of the *P2RX7* gene, we included additional, potentially functional single nucleotide polymorphisms (SNPs) from the 3' untranslated region (UTR), which can be in linkage with Gln460Arg. Based on *in silico* search, we chose two SNPs in putative microRNA target sites which are located in consecutive positions: rs1653625 and rs1718106.

Methods: *P2RX7* SNPs from the C-terminal region were selected based on previous functional assays, 3' UTR variants were chosen using PolymiRTS and Patrocles databases. The genotyping of the non-synonymous SNPs was carried out by pre-designed TaqMan® kits, while the 3' UTR variants were analyzed by PCR-RFLP method. Case-control analyses were carried out between 315 inpatients with acute major depressive episode (195 MDD, 120 BD) and 406 healthy control subjects. The two subscales of the Hospital Anxiety and Depression Scale (HADS) self-report questionnaire were used for quantitative analyses, including an additional, "at-risk" population of 218 patients with diabetes mellitus. The *in vitro* reporter gene assays were carried out on HEK and SK-N-FI cells transiently transfected with pMIR vector constructs containing the *P2RX7* 3' UTR downstream of the luciferase gene.

Results: Haplotype analysis indicated a relatively high linkage between the analyzed *P2RX7* SNPs. Our case-control study did not yield any association between *P2RX7* gene variants and depression. However, dimensional analyses showed significant associations of the HADS depression severity scores with Gln460Arg (rs2230912) and Ala348Thr (rs1718119) in the depressed and diabetic patient groups. In the *in vitro* experiments, the *P2RX7* 3' UTR constructs with the lowest predicted binding efficiency to their miRNAs showed the highest expression of the gene. The combination of the depression-associated *P2RX7* C-terminal and 3' UTR SNPs contributed to the highest depression severity score in the haplotype analysis.

Conclusion: Based on our findings, we propose that a *P2RX7* haplotype combination of the Gln460Arg and neighboring SNPs contribute to the observed genetic association with depressive symptoms.

1. Introduction

During the past decades, major depressive disorder (MDD) has been among the ten most prominent causes of disability (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017). This highly prevalent mood disorder, together with bipolar disorder (BD), is characterized by significant loss of quality of life and social functioning, and result in reduced work activity, creating personal and social burdens worldwide (World Health Organization, 2017). Prevalence estimates of MDD indicate that 16.6% of adults have been depressed at some point in their lifetime, whereas BD shows a lifetime prevalence of 1% (Kessler et al., 2005). As complex, polygenic inheritance disorders, MDD has a moderate heritability of 31-42% (Flint and Kendler, 2014), while BD is estimated to have as high heritability as 80% based on twin studies (Craddock and Sklar, 2013). In spite of extensive research, the genetic basis, pathogenesis, and pathophysiology have remained mainly unclear in these depressive disorders. Evidence suggests a shared genetic etiology, although the majority of genetic variance of mania in BD seems to be independent from depression (McGuffin et al., 2003).

Candidate gene studies indicated a few shared susceptibility genes in MDD and BD, such as the serotonin transporter (Battersby et al., 1996), tryptophan hydroxylase 2 (Van Den Bogaert et al., 2006), and Disrupted-in-Schizophrenia-1 (Schosser et al., 2010). Linkage studies also revealed certain overlapping chromosomal regions, e.g. the 12q23-24 region was consistently pointed out in both MDD (Abkevich et al., 2003; McGuffin et al., 2005; Zubenko et al., 2003) and BD (Curtis et al., 2003; Dawson et al., 1995; Degn et al., 2001; Ekholm et al., 2003; Ewald et al., 1998; Maziade et al., 2001; Morissette et al., 1999; Shink et al., 2005). Subsequent studies pointed to the purinergic receptor P2X, ligand-gated ion channel, 7 (*P2RX7*) gene (Lucae et al., 2006) in this chromosome region. This purinergic receptor was an interesting new candidate in depressive disorders' genetics, because immunological studies have repeatedly indicated alterations in the innate immune system and inflammatory responses of patients with mood disorders (Nestler et al., 2002), for example, increased levels of interleukin 1-beta (Maes et al., 1997), and P2X7 receptor was shown to be involved in the secretion of interleukin 1-beta (Ferrari et al., 2006). The frequently comorbid chronic inflammatory diseases, such as rheumatoid arthritis, further strengthen the possible role of inflammation in the development of depression (Anisman et al., 2008; Walker et al., 2014).

On the other hand, through genome-wide association (GWA) studies, several other genetic variants have been associated with MDD to date, showing the polygenic background of MDD

(Major Depressive Disorder Working Group of the PGC, 2018). In addition, the Converge study including 5278 patients with recurrent MDD and 5196 screened controls showed that common single nucleotide polymorphisms (SNPs) explained ~20% of the variance in MDD risk, supporting a common polygenic etiology (Peterson et al., 2017). Importantly, a recent meta-analysis using published MDD GWA studies' results identified 15 genetic regions in individuals of European descent (Hyde et al., 2016). Interestingly, these loci were also implicated in other neuropsychiatric disorders, such as the myocyte enhancer factor 2C gene, which was identified as susceptibility locus for schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014) and Alzheimer's disease (Lambert et al., 2013), and has been previously associated with epilepsy and intellectual disability (Le Meur et al., 2010). Other meta-analytic GWA study identified further polymorphisms associated with depressive symptoms using a continuous phenotype from the UK Biobank data of more than a hundred thousand people (Okbay et al., 2016). GWA studies of BD have also identified several risk loci across the genome (Sklar et al., 2011; Stahl et al., 2017), like at the ion channel subunits CACNA1C, GRIN2A and SCN2A, or genes coding for synaptic components, such as ankyrin 3 and regulating synaptic membrane exocytosis 1. In the most recent BD GWA study almost one third of the 30 identified loci also harbor schizophrenia associations (Stahl et al., 2017). Furthermore, genetic overlap between BD, MDD, along with schizophrenia and borderline personality disorder has been revealed through later GWA studies, e.g. (Witt et al., 2017). Therefore, we aimed to study possibly overlapping genetic risk factors in the background of MDD and BD by using previously indicated polymorphisms of the *P2RX7* gene and continuous scales for symptom severity. An additional sample of diabetic patients was also tested in the quantitative analyses to assess the effects of these genetic variants on depressive symptoms in an "at-risk" population, since patients with diabetes mellitus are at higher risk for developing depression (Holt et al., 2014).

The P2X7 receptors are ATP-gated non-selective cation channels activated by high ATP concentration (Rassendren et al., 1997). P2X7 receptors are primarily localized on immune cells and glia in the central nervous system, but astrocytes and neurons can also express this purinergic receptor (Burnstock, 2007). P2X7 receptors have been implicated in many physiological and pathophysiological mechanisms, such as inflammatory responses (Chessell et al., 2005), neuronal functions (Sperlagh et al., 2006), and were proposed as potential therapeutic targets in neuropsychiatric disorders, like depression (Basso et al., 2009; Boucher et al., 2011; Csolle et al., 2013; Deussing and Arzt, 2018), schizophrenia (Kovanyi et al.,

2016), and BD (Gubert et al., 2016). In addition, animal studies investigating genetic deletion or pharmacological inhibition of P2X7 receptors found antidepressant phenotype in behavioral paradigms modeling depressive-like behavior (Basso et al., 2009; Csolle et al., 2013). Furthermore, gene expression studies associated *P2RX7* transcript changes with MDD: lower *P2RX7* mRNA level was found in peripheral blood mononuclear cells of patients diagnosed with MDD and post-traumatic stress disorder (Zhang et al., 2011); whereas increased *P2RX7* expression was observed in blood samples of women with treatment-resistant depression (Iacob et al., 2013). However, these biomarker study results should be taken cautiously because of the small sample sizes. Interestingly, a large-scale study showed that *P2RX7* mRNA level was increased in healthy controls during sleep-deprivation, which is an important characteristic in the rapid cycling form of BD (Backlund et al., 2012).

After the first positive association studies of *P2RX7* genetic variants (Backlund et al., 2012; Lucae et al., 2006; McQuillin et al., 2009), several studies failed to replicate association with either MDD or BD (Green et al., 2009; Grigoroiu-Serbanescu et al., 2009; Lavebratt et al., 2010; Viikki et al., 2011). A meta-analysis concluded lack of association between the most widely investigated SNP in the *P2RX7* gene, namely the Gln460Arg (A1405G, rs2230912:A>G) and mood disorders based on classical case-control design (Feng et al., 2014). However, a more recent meta-analysis of published case-control studies and a new dataset showed a significant association between rs2230912 and combined mood disorders (MDD or BD together) for allelic, dominant and heterozygous-disadvantage models (Czamara et al., 2018). After stratification by disorder, significant findings were revealed in the allelic model for MDD, however no significant association emerged for BD. Previously we proposed a more sensitive approach using symptom severity scores, and observed genetic associations of depressive symptoms with *P2RX7* Gln460Arg among MDD and BD patients (Halmai et al., 2013; Hejjas et al., 2009), as well as among patients diagnosed with diabetes mellitus (who are at higher risk for developing depression) (Nagy et al., 2008). A similar approach was used by a Finnish group with the ‘time being ill’ in their genetic analyses; they showed association with both Gln460Arg and His155Tyr (rs208294) polymorphisms (Soronen et al., 2011). They also indicated neuroticism as a possible mediator in the His155Tyr genetic effect (Mantere et al., 2012).

Beside the Gln460Arg polymorphism, other *P2RX7* gene variants could affect the genetic predisposition to depressive disorders and/or symptoms. Therefore, in our present association study we selected three additional non-synonymous SNPs from the C-terminal region of the

receptor (focusing on the gene region around Gln460Arg spanning ~7.2 kb): Ala348Thr (G1068A, rs1718119:G>A), Thr357Ser (C1096G, rs2230911:C>G), and Glu496Ala (A1513C, rs3751143:A>C). These genetic variants were indicated to have possible functional effects as gain- or loss-of-function mutations (Denlinger et al., 2006). Importantly, among these SNPs the Glu496Ala was indicated in BD (Gubert et al., 2018), and Ala348Thr has been associated with cognitive manic symptoms (Backlund et al., 2011), as well as with panic and agoraphobia symptom severity (Erhardt et al., 2007). Since Gln460Arg is located at the 3' end of the *P2RX7* gene, SNPs in the 3' untranslated region (UTR) can be potentially in linkage with this candidate polymorphism. Therefore, we analyzed SNPs at microRNA (miRNA) binding sites in the 3' UTR of the *P2RX7* gene, which might affect miRNA binding, thus gene expression. Based on *in silico* search, three A/C SNPs were identified as putative miRNA target sites in consecutive positions in the 3' UTR of the *P2RX7* gene (689-691 bp downstream of the Gln460Arg): rs1653625:A>C, rs1718106:A>C, rs28969482:A>C (Rahman et al., 2010). Our aim was to provide a better understanding on these non-coding *P2RX7* genetic variants, using both genetic and gene expression analyses.

2. Materials and Methods

2.1. Subjects and symptom assessment

In this study two previously recruited patient groups were analyzed: The depressed patient group consisted of 315 inpatients with a current major depressive episode treated at the Department of Psychiatry, Kútvölgyi Clinical Centre (DSM-IV diagnosis: 195 MDD and 120 BD, women 75.2%, average age 47.3 ± 11.5). The inclusion and exclusion criteria were described in details previously (Halmai et al., 2013). Briefly, the clinician-rated Montgomery–Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) was used to measure overall severity of depressive symptoms of the psychiatric patients. All inpatients had a total MADRS score higher than 6, indicating moderate - severe forms of depression. Control subjects without any self-reported psychiatric disorder or current depressive symptoms were recruited at the Eötvös Loránd University and at elderly homes (N=406, women 69.9%, average age 28.0 ± 12.2, mostly student participants). The healthy status was obtained by online questions from the younger control participants (screening for chronic diseases, such as diabetes mellitus, allergy or other chronic inflammatory diseases, and psychiatric disorders), whereas elderly home dwellers were screened for dementia using the Hungarian version of the Mini Mental State Examination (Janka et al., 1988). The control

group's sex ratio was adjusted to the depressive patient group, but controls were not matched for age (see Supplementary Table 1), since previous analyses of an 'age-adjusted' control group (N=163, women 74.2%, average age: 37.8 ± 12.9) did not reveal significant difference from non-matched control group on either symptom scores or *P2RX7* Gln460Arg genotype frequency (Halmai et al., 2013). An additional, "at-risk for developing depression" sample of diabetic patients was recruited from the inpatient and outpatient services of the 2nd Department of Internal Medicine at the Semmelweis University (women 51.8%, average age 58.0 ± 13.6, with exclusion criteria of any severe or acute medical illness that may affect the presence of depressive symptoms, e.g. liver cirrhosis, end stage renal disease, or cancer) (Nagy et al., 2008). The study protocols were in accordance with the principles of the Helsinki Declaration and were approved by the Local Ethical Committee (TUKEB). Every participant provided written informed consent for the genetic study and filled out the self-report Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith, 1983). Subjects of both patient and control groups were of Hungarian origin, thus creating an ethnically homogenous case and control population.

2.2. Non-synonymous SNP selection

When selecting SNPs surrounding the widely studied Gln460Arg polymorphism, we mainly focused on their location and possible functional effects. Both the Thr357Ser (rs2230911) and Glu496Ala (rs3751143) are located in the cytoplasmic C-terminal domain, similarly to Gln460Arg (rs2230912). The Glu496Ala polymorphism was the first functionally described variation (Gu et al., 2001); the adenosine triphosphate (ATP)-induced ethidium uptake was abolished by the 496Ala variant in native and transfected cells, although the immediate ATP-induced opening of the cation-selective channel was unaffected (Boldt et al., 2003). The 357Ser variant showed reduced agonist-induced ionic currents and dye uptake with no alteration to the agonist sensitivity (Roger et al., 2010; Shemon et al., 2005), while the 460Arg variant exhibited increased pore activity (Denlinger et al., 2006). The nearby Ala348Thr (rs1718119) polymorphism was also included, since it is a human specific variation (Jiang et al., 2013). This amino acid change can be found in the transmembrane helix, and the 348Thr variant had gain-of-function effect by increased agonist-induced ionic currents and dye uptake (Bradley et al., 2011; Roger et al., 2010).

2.3. Identification of possible miRNA target sites in the 3' UTR of the *P2RX7* gene

In silico search was carried out using the PolymiRTS (http://compbio.uthsc.edu/miRSNP/) and Patrocles (http://www.patrocles.org/) databases. Out of the three identified SNPs (rs1653625:A>C, rs1718106:A>C, rs28969482:A>C), only the rs1653625 was found in both databases targeted by two different miRNAs (miR-625 in PolymiRTS and miR-1302 in Patrocles). The other two hits were found in the Patrocles database with rs1718106 targeted by miR-1275 and rs28969482 targeted by miR-491-5p. In case of the rs28969482 SNP the minor allele frequency is only ~2% in European populations according to the 1000 Genomes Project data. To check the allele frequency in our cohorts 3% of the samples were sequenced (10 depressed patients and 13 controls), but no C-allele was detected at the rs28969482 position, therefore this SNP was not included in our association study.

2.4. Genotyping

Buccal epithelial cell samples were obtained in duplicate and DNA was extracted by Gentra DNA purification kit (Qiagen). The exonic SNPs were genotyped using pre-designed TaqMan® kits: C__11704039_10 for rs1718119:G>A, C__15853705_20 for rs2230911:C>G, C__15853715_20 for rs2230912:A>G, and C__27495274_10 for rs3751143:A>C (Thermo Fisher Scientific) on 7300 Real-Time PCR System. The rs1653625:A>C SNP was genotyped by Eco24I (BanII) (Fermentas) restriction digestion method described earlier (Rahman et al., 2010) using the 5'-AGGCACAGCAAACTGAGCC-3' forward primer (underlined are the mismatched nucleotides which are used to create the cleavage site for the restriction enzyme) and the 5'-TCAGACACAGAGAGCAACAGAAG-3' reverse primer. The genotyping of the rs1718106:A>C SNP was done by direct haplotype analysis of the rs1653625 and rs1718106 positions. Primers were designed according to the NM_002562.4 (06. June 2010) NCBI Reference Sequence. Based on previously acquired rs1653625 genotypes, one of the allele-specific forward primers (either 5'-AGGCACAGCAAACTGGTCC**A**-3' or 5'-AGGCAC-AGCAAACTGGGCC**C**-3' for the rs1653625 A- or C-allele, respectively) and 5'-AGACAG-TTCATGTGCACTGTCTC-3' reverse primers were used in the PCR amplification (2 separate reactions were carried out for the rs1653625 AC heterozygotes). The HotStar Taq DNA polymerase kit (Qiagen) was used in a total volume of 10 µl PCR reaction consisting of 0.025 U/µl HotStar Taq DNA polymerase, 1x reaction buffer (containing 1.5 mM $MgCl_2$), 1x Q solution (supplied), 1 µM forward and reverse primers, 0.2 mM of dNTP mix, and 10-20 ng genomic DNA. Thermocycling consisted of a 15 min 95°C initial denaturation step, 40 cycles of 94°C 1 min, 54°C 30 sec, 72°C 1 min, and 72°C 10 min final extension in the genotyping process of the rs1653625. The cycles were shortened for the rs1718106 with 94°C 30 sec,

55°C 30 sec, and 72°C 30 sec steps. EcoO109I (DraII) restriction enzyme (Fermentas) was used to detect the rs1718106 alleles in the rs1653625-C-specific PCR fragment (for detailed design see Supplementary Figure 1a) by creating 361 bp and 16 bp fragments in case of rs1718106 C-allele (A-allele was the undigested case), whereas Hpy166II (New England BioLabs) restriction enzyme was used for the digestion of the rs1653625-A-specific PCR fragment (for detailed design see Supplementary Figure 1b). PCR fragments were separated in a mixed 1.5% agarose and 2% metaphor agarose using a horizontal gel electrophoresis system. The final haplotype call of the two *P2RX7* 3' UTR SNPs was based on two parallel reactions per subject. No significant deviation from the Hardy–Weinberg equilibrium was found at any of the SNPs neither in the control nor in the patient groups (all p-values ≥ 0.05).

2.5. Cell culture and transient transfection experiments

Out of the four possible *P2RX7* 3'UTR haplotypes consisting of the rs1653625:A>C and rs1718106:A>C, the three most frequent ones, namely the A~A, C~A and C~C haplotypes were used to check the possible effect of the SNPs on gene expression. Altogether three possible miRNAs' target sites were predicted to be altered by the two *P2RX7* 3' UTR SNPs: miR-625 and miR-1302 in the case of rs1653625:A>C, and miR-1275 in the case of rs1718106:A>C. The HEK and SK-N-FI (ATCC® CRL2142™) cells were transfected with β-galactosidase plasmid (Ambion) and miRNA Expression Reporter Vector System's pMIR-REPORT™ Luciferase plasmid (Thermo Fisher Scientific) containing the *P2RX7* 3' UTR downstream of the luciferase gene using Lipofectamine 2000 (Thermo Fisher Scientific). The *P2RX7* 3' UTR was amplified from a genomic DNA with 5'-AAAAAACGCGTAGCC-AGGCACCGTGGCTCA-3' forward and 5'-AAAAAAAGCTTGAGATGGAGTCTCGCT-CTGTC-3' reverse primer incorporating the MluI and HindIII restriction enzyme sites (underlined in the primer sequences) for the cloning step; T4 ligase (Fermentas) was used at the ligation step. To create the different rs1653625~rs1718106 haplotypes, mutations were introduced using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol; selected clones were checked by Sanger-sequencing (Biomi Ltd., Gödöllő, Hungary). During transfection, cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 2 mM glutamine and 10% foetal bovine serum (Sigma-Aldrich). Luciferase and β-galactosidase activities were measured by VarioSkan system (Thermo Fisher Scientific).

2.6. Statistical analysis

The SPSS program for Windows (22.0 version) was used for the statistical analyses. In the categorical analyses chi-square tests were applied to compare depressed patients to controls. Dimensional analyses within each group were carried out by analyses of covariance using the two HADS symptom severity scales as dependent variables and the *P2RX7* genotypes as the grouping variable with sex and age as covariates. In the conditional analysis, linear regression modeling was used on the depression scale with sex and age in the first block, then the lead *P2RX7* genetic variable, finally the other associated genetic variables in the subsequent blocks in increasing order of their p-values. The linkage disequilibrium between the SNPs was calculated by the Haploview program (Barrett et al., 2005), the case-control haplotype analyses were run with the UNPHASED software (3.1.7 version) (Dudbridge, 2008), whereas quantitative analyses of the estimated haplotypes were performed with the THESIAS program (Tregouet and Garelle, 2007). The p-value threshold for multiple comparisons was calculated by the False Discovery Rate adjustment (Benjamini et al., 2001) according to the 6 genetic variants analyzed, setting the significance level to $p < 0.008$.

3. Results

3.1. Genetic association study of the *P2RX7* polymorphisms

The analyzed *P2RX7* SNPs exhibited relatively high linkage with each other (see high *D'* values at Figure 1a), but none of the SNPs were in perfect linkage (see low r^2 values at Figure 1b), creating 6 common haplotypes (see haplotypes with ≥ 5% frequency at Figure 1c).

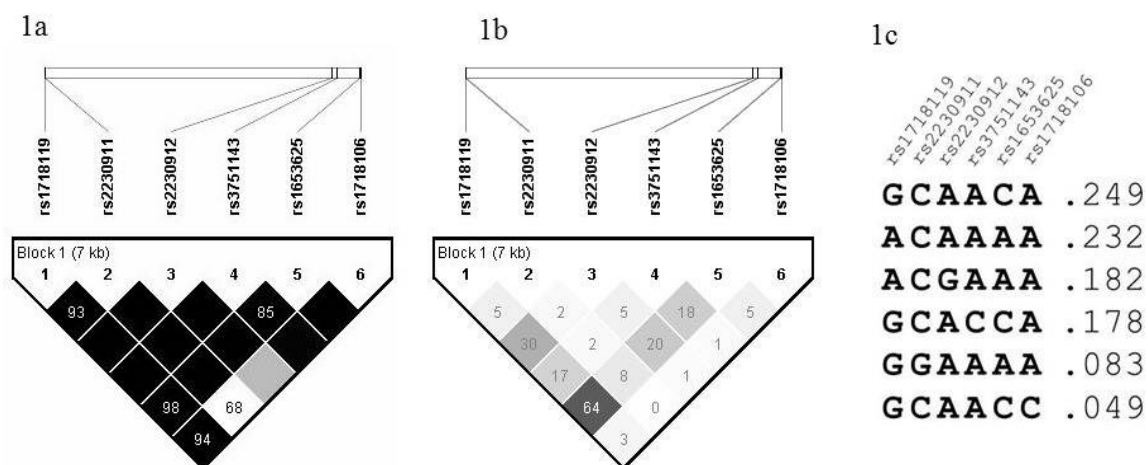

Figure 1: Linkage disequilibrium of the studied *P2RX7* polymorphisms. 1a: Lewontin's D values (cells with dark shade and without number indicates *D'*=1.0), **1b**: r^2 values were calculated from 939 individual genotype data (406 controls, 218 diabetic, and 315 depressed patients) with the Haploview program. **1c:** The estimated frequencies of the most common SNP combinations.

The case-control analyses revealed no significant difference in genotype frequencies at any of the SNPs in the depressed patients vs. controls comparisons (all p-values > 0.05 at the chi-square tests, for sample sizes and genotype frequencies of the 3 genotype categories, see Table 1). Similarly, only few percentage differences have been detected between the estimated haplotype frequencies of the depressed patients vs. controls when using the most common combinations of the assessed *P2RX7* polymorphisms (see UNPHASED calculations at Supplementary Table 2).

Table 1: Sample sizes (N) and genotype frequencies (%) for the analyzed *P2RX7* polymorphisms.

Genotype		Controls N	Controls %	Diabetic N	Diabetic %	Depressed* N	Depressed* %	MDD N	MDD %	BD N	BD %
rs1718119	AA	71	*17.6*	36	*16.5*	51	*16.2*	31	*15.9*	20	*16.7*
Ala348Thr	AG	194	*48.1*	114	*52.3*	157	*49.8*	102	*52.3*	55	*45.8*
G1068A	GG	138	*34.2*	68	*31.2*	107	*34.0*	62	*31.8*	45	*37.5*
				p=0.610		*p=0.852*		*p=0.630*		*p=0.806*	
rs2230911	CC	341	*84.2*	182	*83.5*	265	*84.1*	166	*85.1*	99	*82.5*
Thr357Ser	CG	61	*15.1*	34	*15.6*	45	*14.3*	27	*13.8*	18	*15.0*
C1096G	GG	3	*0.7*	2	*0.9*	5	*1.6*	2	*1.0*	3	*2.5*
				p=0.956		*p=0.545*		*p=0.872*		*p=0.281*	
rs2230912	AA	269	*66.3*	145	*66.5*	218	*69.2*	134	*68.7*	84	*70.0*
Gln460Arg	AG	118	*29.1*	68	*31.2*	87	*27.6*	56	*28.7*	31	*25.8*
A1405G	GG	19	*4.7*	5	*2.3*	10	*3.2*	5	*2.6*	5	*4.2*
				p=0.314		*p=0.507*		*p=0.449*		*p=0.745*	
rs3751143	AA	260	*64.2*	138	*63.3*	211	*67.0*	132	*67.7*	79	*65.8*
Glu496Ala	AC	135	*33.3*	72	*33.0*	92	*29.2*	57	*29.2*	35	*29.2*
A1513C	CC	10	*2.5*	8	*3.7*	12	*3.8*	6	*3.1*	6	*5.0*
				p=0.695		*p=0.331*		*p=0.571*		*p=0.289*	
rs1653625	AA	120	*29.6*	56	*25.7*	87	*27.6*	53	*27.2*	34	*28.3*
	AC	194	*47.8*	113	*51.8*	142	*45.1*	89	*45.6*	53	*44.2*
	CC	92	*22.7*	49	*22.5*	86	*27.3*	53	*27.2*	33	*27.5*
				p=0.541		*p=0.357*		*p=0.472*		*p=0.544*	
rs1718106	AA	200	*90.5*	196	*90.3*	280	*89.5*	176	*90.7*	104	*87.4*
	AC	21	*9.5*	19	*8.8*	33	*10.5*	18	*9.3*	15	*12.6*
	CC	-	*0.0*	2	*0.9*	-	*0.0*	-	*0.0*	-	*0.0*
				p=0.349		*p=0.694*		*p=0.938*		*p=0.375*	

The p-values of chi-square tests analyzing genotype frequency differences of patients vs. controls are shown. *All psychiatric patients grouped together (MDD and BD).

Next, we performed dimensional analyses using the HADS depression and anxiety symptom scores within the three groups separately (i.e. among controls, diabetic and depressed

patients). No significant association was detected with either depression or anxiety score in the control group. However, nominally significant associations were observed in both the diabetic and depressed groups (for depression scores see Table 2, anxiety scores are presented in Supplementary Table 3). The psychiatric patient group was further separated into MDD and BD groups in subsequent analyses.

Table 2: HADS-depression scores of the different genotype groups (mean ± SD).

Genotype		Controls	Diabetic	Depressed*	MDD	BD
rs1718119	AA	3.27 ± 2.38	6.28 ± 3.74	14.02 ± 4.62	12.93 ± 4.62	16.20 ± 3.93
Ala348Thr	AG	3.35 ± 2.40	4.98 ± 3.59	13.17 ± 4.59	13.27 ± 4.70	12.95 ± 4.41
G1068A	GG	2.77 ± 2.14	3.76 ± 2.90	11.87 ± 5.20	11.98 ± 4.63	11.73 ± 5.95
	p	0.091	**0.002**	**0.031**	0.316	**0.008**
rs2230911	CC	3.14 ± 2.27	4.84 ± 3.42	13.07 ± 4.81	12.84 ± 4.80	13.50 ± 4.83
Thr357Ser	CG	3.10 ± 2.48	4.82 ± 3.97	11.55 ± 4.95	12.59 ± 3.87	10.13 ± 5.97
C1096G	GG	5.00 ± 5.00	2.50 ± 3.54	15.00 ± 5.48	13.50 ± 6.36	16.50 ± 6.36
	p	0.835#	0.562#	0.083	0.690#	**0.049#**
rs2230912	AA	3.22 ± 2.35	4.31 ± 3.22	12.48 ± 4.86	12.79 ± 4.69	11.95 ± 5.13
Gln460Arg	AG	2.96 ± 2.23	5.74 ± 3.83	13.37 ± 4.76	12.50 ± 4.67	15.12 ± 4.52
A1405G	GG	3.06 ± 2.65	7.00 ± 4.00	16.10 ± 4.43	16.60 ± 2.88	15.60 ± 5.94
	p	0.623	**0.011**	**0.023**	0.153	**0.013**
rs3751143	AA	3.24 ± 2.43	5.09 ± 3.54	12.57 ± 4.83	12.54 ± 4.54	12.63 ± 5.34
Glu496Ala	AC	3.09 ± 2.13	4.21 ± 3.43	13.43 ± 5.06	13.43 ± 5.11	13.44 ± 5.08
A1513C	CC	1.38 ± 0.92	5.50 ± 3.21	14.60 ± 3.37	13.80 ± 3.70	15.40 ± 3.21
	p	0.088	0.104	0.301	0.609	0.473
rs1653625	AA	3.41 ± 2.56	5.93 ± 3.86	13.75 ± 4.53	13.26 ± 4.56	14.69 ± 4.42
	AC	3.11 ± 2.22	4.72 ± 3.52	12.85 ± 4.95	13.09 ± 4.63	12.44 ± 5.49
	CC	2.87 ± 2.23	3.78 ± 2.64	12.00 ± 4.94	11.89 ± 4.83	12.19 ± 5.20
	p	0.269	**0.013**	0.091	0.348	0.055
rs1718106	AA	3.17 ± 2.40	4.89 ± 3.58	13.01 ± 4.66	12.90 ± 4.51	13.21 ± 4.93
	AC	2.62 ± 1.83	4.00 ± 2.77	11.71 ± 6.37	12.00 ± 6.19	11.33 ± 6.87
	CC	-	3.50 ± 2.12	-	-	-
	p	0.287	0.225#	0.173	0.449	0.230

Nominally significant p-values of the applied analyses of covariance are shown in bold.
*All psychiatric patients grouped together (MDD and BD).
The rare homozygote genotype was grouped together with the heterozygotes because of the low cell count (less than 5), in these analyses the degree of freedom was 1.

Patients carrying the A-allele of rs1718119 (Ala348Thr) showed a step-wise increase at HADS depressive symptoms (diabetic patients: $F(2,218)=6.282$, $p=0.002$, $\eta^2=0.056$, power=0.893; depressed patients: $F(2,258)=3.524$, $p=0.031$, $\eta^2=0.027$, power=0.653). The association at the depressed patients was possibly driven by the BD patients ($F(2,94)=5.170$, $p=0.008$, $\eta^2=0.104$, power=0.815), as there was no significant association found at the MDD

group. Similarly, the risk allele rs2230912-G (coding for 460Arg) showed higher depressive scores in both the diabetic and depressed groups (diabetic patients: $F(2,218)=4.581$, $p=0.011$, $\eta^2=0.041$, power=0.772; depressed patients: $F(2,258)=3.811$, $p=0.023$, $\eta^2=0.029$, power=0.690), the latter association was possibly driven by the BD patients ($F(2,94)=4.569$, $p=0.013$, $\eta^2=0.093$, power=0.762), as MDD cases did not show significant differences according to their genotypes in the separate analysis. In addition, nominally significant association was found at the 3' UTR rs1653625 SNP within the diabetic population as the depression score increased gradually with the presence of the A allele ($F(2,218)=4.448$, $p=0.013$, $\eta^2=0.040$, power=0.759). However, in the psychiatric cohort only a tendentious effect was seen at this SNP ($p<0.1$) with the BD group showing stronger effect ($p=0.055$). After considering the multiple comparisons of the 6 SNPs, only the rs1718119 (Ala348Thr) effect remained significant ($p<0.008$) in case of the diabetic and bipolar patients. In order to check independent genetic effects of the other depression-associated polymorphisms, conditional analyses were carried out, which showed significant effect for the rs2230912 (Gln460Arg) only in the BD group (resulting $p=0.039$). None of the other *P2RX7* gene variants showed statistically significant effect in the diabetic group. At the anxiety symptoms no consistent pattern of the nominally significant associations was found (see Supplementary Table 3).

In the quantitative haplotype analysis using all 6 *P2RX7* SNPs, the ACGAAA haplotype showed significantly higher depression scores in both patient groups (1.73 difference score with 95% CI: 0.71 & 2.75, $p=0.0008$ in the diabetic, 1.98 difference score with 95% CI: 0.74 & 3.22, $p=0.002$ in the depressed patient group) compared to the most frequent GCAACA haplotype (Figure 2). Again, the depressed patient group's association was driven by the BD subgroup (3.89 difference score with 95% CI: 1.66 & 6.13, $p=0.0006$), and no significant difference was observed at the MDD subgroup. The ACAAAA haplotype showed nominally significant difference at both patient groups ($p < 0.05$ but > 0.008), whereas the GCACCA haplotype showed elevated score only in the depressed patient groups ($p=0.03$).

After taking multiple comparisons of the 6 haplotypes into account, only the ACGAAA vs. GCAACA haplotype showed significant and consistent difference, highlighting the importance of rs1718119 (Ala348Thr), rs2230912 (Gln460Arg), and rs1653625 SNPs within the 6 assayed *P2RX7* polymorphisms (their positions are underlined).

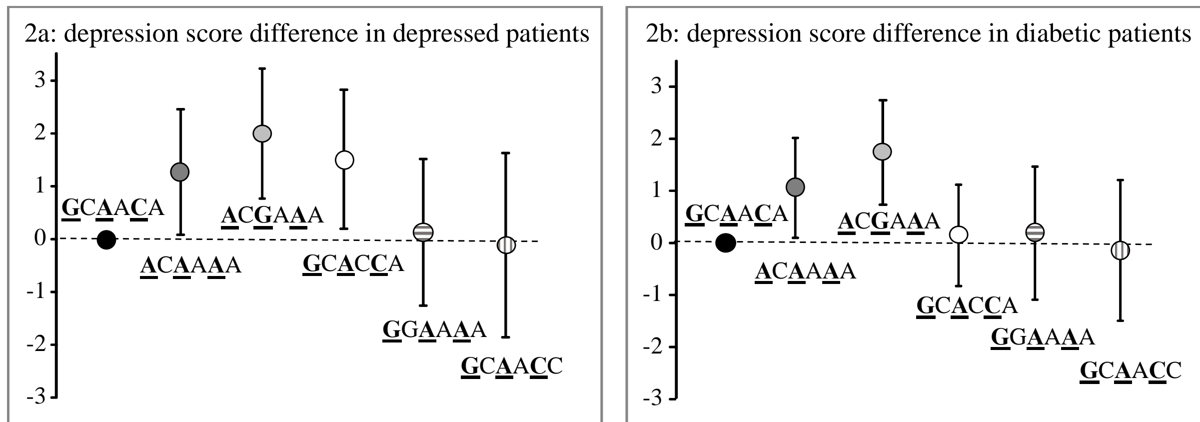

Figure 2. Difference scores of HADS-depression symptom severity at the estimated *P2RX7* haplotype groups constructed from the assayed C-terminal and 3' UTR SNPs. The alleles are presented in the genomic order starting from Ala348Thr = rs1718119:G>A, followed by Thr357Ser = rs2230911:C>G, Gln460Arg = rs2230912:A>G, Glu496Ala = rs3751143:A>C, and rs1653625:A>C, finally rs1718106:A>C. The difference of depression score with 95% CI of each haplotype is shown as compared to the most frequent GCAACA haplotype (~25%, black circle), based on THESIAS calculations. The ACAAAA haplotype (~23%, dark grey) and the ACGAAA haplotype (~18%, light grey) showed higher depression scores in both the depressed (2a) and diabetic (2b) patient groups. Whereas the GCACCA haplotype (~18%, white circle) showed higher score only in the depressed patient group (2a). The less frequent haplotypes (GGAAAA ~8% and GCAACC ~5%, striped symbols) did not show any significant differences.

Follow-up analyses using the combination of these 3 SNPs showed significant differences in depressive scores between the rs1718119-A ~ rs2230912-G ~ rs1653625-A (A~G~A) and G~A~C haplotypes (p=0.0002 in the diabetic, p=0.006 in the depressed, and p=0.002 in the BD patient group). Importantly, these effects were not solely driven by the Gln460Arg variant, because significant differences could be detected when taking out rs2230912 (p-values < 0.008 at the comparison of rs1718119-A ~ rs1653625-A vs. rs1718119-G ~ rs1653625-C haplotypes in both the diabetic and depressed patient groups). These results suggest that other functional polymorphism(s) in the neighborhood of Gln460Arg in the *P2RX7* gene might account for the association with depressive symptoms. Since no experimental data has been reported yet about the rs1653625 in the 3' UTR, we carried out an *in vitro* reporter gene expression analysis.

3.2. Characterization of the *P2RX7* 3' UTR polymorphisms

Considering the seed sequences of the potentially silencing miRNAs (which are essential for their binding to the mRNA 3' UTR) a preliminary prediction on gene expression was made: both rs1653625 and rs1718106 SNPs (their positions are highlighted in bold and underlined) could make an impact on the binding of miR-625 (AGGG**GG**), miR-1275 (AGGG**GG**U), or

miR-491-5p (AGGG**GU**G), whereas miR-1302 (ACAGGG**U**) could be affected only by rs1653625. Since no proof of expression is published on miR-1302 to date, this miRNA was not considered as a possible modulator of gene expression. As the perfect match for both miR-625 and miR-1275 would be the rs1653625 C ~ rs1718106 C haplotype, but for miR-491-5p the rs1653625 C ~ rs1718106 A haplotype, we predicted that the C~C haplotype would show the lowest luciferase activity due to the highest binding efficiency of the miRNAs, having the strongest transcription repression effect. Similar logic can be used for the A~A haplotype, where the lowest miRNA binding efficiency, thus the highest expression is expected.

In the luciferase reporter gene assay all the *P2RX7* 3' UTR constructs showed ~20% repressed activity compared to the empty plasmid in the transfected HEK and SK-N-FI cells, therefore empty plasmid as control is not represented on Figure 3. Among the four theoretically possible 3' UTR haplotypes of the two adjacent A/C SNPs (rs1653625 and rs1718106) we investigated A~A, C~A, and C~C haplotypes, as there was no rs1653625 A ~ rs1718106 C (A~C) combination observed in the studied samples. In both cell lines the C~C haplotype showed the lowest relative luciferase activity, which was in agreement with our prediction regarding efficient miRNA binding and repressed translational efficiency.

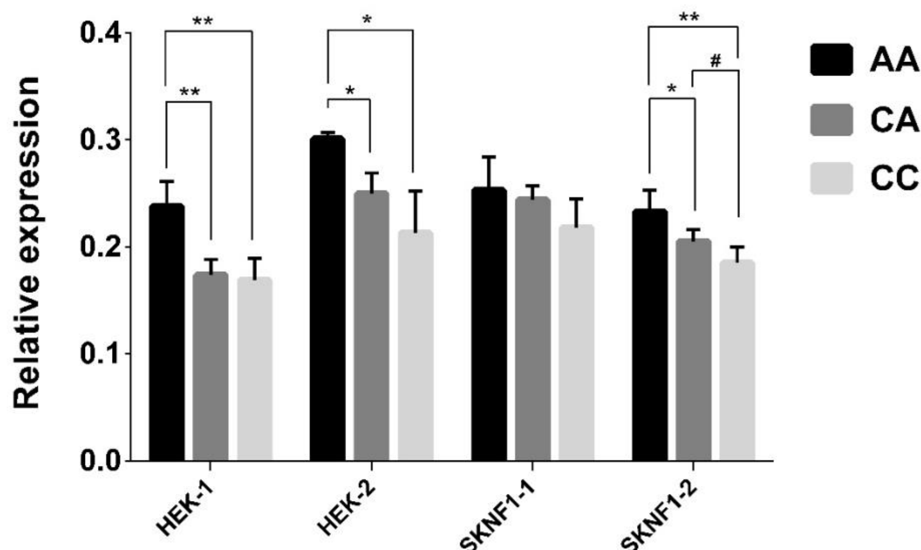

Figure 3: Relative expression levels of the three possible *P2RX7* 3' UTR haplotypes. Two representative transient transfection experiments (measured in triplicates) are shown on HEK and SK-N-FI cell lines. Luciferase activity was normalized to the β-galactosidase activity. Data are presented as fold increments over the pMIR empty vector and shown as mean ± SD. The three haplotypes were compared by analysis of variance (GraphPad InStat).
# $p < 0.1$, * $p < 0.05$, ** $p < 0.001$

4. Discussion

In the present study we focused on genetic and functional effects of four non-synonymous and two 3’ UTR polymorphisms of the *P2RX7* gene previously associated with affective disorders, such as anxiety, major depressive, or bipolar disorder. Since P2X7 receptors play an important role in proinflammatory cytokine release, alteration in the *P2RX7* expression can lead to atypical cytokine production, which have been shown to be deregulated in patients with affective disorders (Liu et al., 2004; Miller et al., 2009; Tonelli et al., 2005). Therefore, the *P2RX7* gene variants are frequently investigated in candidate gene studies (see recent meta-analysis by (Czamara et al., 2018)). Using depressed patients with acute major depressive episode requiring hospitalization as cases and a general population-based sample as a control group, no significant association was observed at the separate case-control analyses when testing each SNP individually (Table 1) or in combinations (Supplementary Table 2). However, the rs1718119 (Ala348Thr) along with the rs2230912 (Gln460Arg) polymorphism was associated with depression symptom severity in two different clinical groups (diabetic and depressed) at our dimensional analyses (Table 2). At both *P2RX7* SNPs a step-wise increase in the severity scores can be observed with respect to the presence of the minor allele: rs1718119-A and rs2230912-G. Interestingly, the subsequent analyses of the two psychiatric patient subgroups showed that the BD group was driving the genetic associations. We have to note that one of the main limitations of our study is the relatively small group size of bipolar patients (N=120). Therefore, we cannot conclude any BD-specific effect until confirmation of the observed associations in independent and larger samples. To date, no genome-wide association has been shown between *P2RX7* gene variants and BD. Nonetheless, among the publicly available results of the recent PGC publication (Bipolar Disorder and Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2018), where 20,129 BD cases and 21,524 controls were investigated, including all the currently analyzed non-synonymous *P2RX7* SNPs, the Ala348Thr (rs1718119) showed nominally significant association with BD (p=0.012). One of the reasons for the difficulty to identify possible *P2RX7* risk variants in GWA studies could be the clinical heterogeneity (such as misdiagnosis), which is a frequent confounding factor in large-scale studies. It has been showed that power is greatly reduced if 10-20% of cases do not have the disorder of interest (Wray et al., 2012) or if genetically quite different disorders are included (Manchia et al., 2013).

The two non-synonymous *P2RX7* polymorphisms highlighted by our present study (rs2230912 and rs1718119) have been associated with emotional dysregulation and mood

instability, including rapid cycling in patients with BD (Arabzadeh et al., 2015; Gurvich et al., 2014; Leboyer et al., 2016). Rapid cycling showed association with a haplotype of rs1718119-A ~ rs2230912-G, which is a gain-of-function variant, as both rs1718119-A (348Thr) and rs2230912-G (460Arg) are reported to cause increased pore activity of P2X7 (Denlinger et al., 2006; Ursu et al., 2014). However, the associations between *P2RX7* gene variants and mood disorders have been inconsistent. A meta-analysis found an association between the rs2230912 gain-of-function allele (G coding for 460Arg) and mood disorder in general, but not with BD specifically (Feng et al., 2014). A recent study on *P2RX7* variants rs2230912 and rs1718119 showed that the minor (gain-of-function) alleles and their combination significantly increased risk for BD in females (Winham et al., 2018). This association is consistent with prior linkage studies that identified *P2RX7* as a BD candidate gene (Shink et al., 2005), as well as with previous case-control studies of bipolar and unipolar affective disorders (McQuillin et al., 2009), see also the studies reported by a recent meta-analysis (Czamara et al., 2018).

The limitations of our genetic association study include the relatively small sample size of the BD subgroup, and the lack of allele transmission data. However, the studied population consisted of ethnically homogenous Caucasian groups, therefore, population stratification is unlikely to pose a problem in our analyses. In addition, the control sample was not age-matched, as it included mostly students. Since the healthy status was obtained only by self-report from the younger control participants, our control group could be regarded as a general population sample (possibly containing individuals who would later develop mood disorders), potentially affecting our case-control analyses. On the other hand, a sample of diabetic patients (with higher mean age) was also tested in our dimensional analyses, providing possibility to check genetic effects in a subgroup of people who are at higher risk to develop depression.

The significance of the rs1718119 (Ala348Thr) in the present study was proven by the fact that after multiple correction, only this variant's effect stayed significant in both the diabetic and depressed cohorts. This non-synonymous SNP has also been associated with an increase in anxiety-related episodes, such as agoraphobia and panic attack (Erhardt et al., 2007). Concerning its possible functionality, *in vitro* studies showed increased ATP-activated ion channel function and pore formation in transfected HEK cells (Roger et al., 2010; Stokes et al., 2010). *Ex vivo* studies using blood cells also reported gain-of-function effect (Denlinger et al., 2006; Stokes et al., 2010). Interestingly, in the later study it was also shown that Gln460Arg had only a minor effect on P2X7-induced ethidium uptake, but in haplotype with

Ala348Thr a net gain-of-function effect was observed, which highlights the major role of Ala348Thr. Stokes et al. (2010) studied the same four *P2RX7* SNPs (Ala348Thr, Thr357Ser, Gln460Arg, Glu496Ala) analyzed in our study in a haplotype block spanning exons 11 to 13. They showed that a haplotype variant containing the 348Thr and 460Arg variants (rs1718119-A ~ rs2230912-G haplotype) displayed increased P2X7 functional responses when compared to the wild-type human P2X7 receptor (rs1718119-G ~ rs2230912-A haplotype), a finding that could be relevant in our understanding to the pathophysiology associated with susceptibility to mood disorders. Our haplotype association analyses also showed the importance of the combination of these two non-synonymous SNPs (see ACGAAA vs. GCAACA haplotype in both patient groups at Figure 2).

Multiple studies have shown that SNPs at miRNA binding sites can affect the risk of developing complex diseases through the alteration of the miRNA binding strength (Dadkhah et al., 2017; Sethupathy and Collins, 2008). Therefore, we also studied SNPs in the *P2RX7* 3' UTR located at miRNA binding sites, potentially influencing *P2RX7* expression. Based on *in silico* search, three SNPs (rs1653625, rs1718106, rs28969482) were predicted to affect miRNA binding in the 3' UTR. However, only rs1653625 and rs1718106 were investigated in our association study, since no variation was observed in 3% of our studied cohorts at the rs28969482 position with Sanger-sequencing.

Based on PolymiRTS and Patrocles databases, binding of miRNAs (miR-625, miR-1302, miR-1275 and miR-491-5p) to their target sequences could be affected by rs1653625 and rs1718106. According to miRNA expression databases, miR-625, miR-1275 and miR-491-5p are expressed in the nervous system, as well as in HEK293 and SK-N-FI cell lines. However, no convincing miR-1302 expression data (negligible RPM values with respect to the other miRNAs) was available to date; therefore, we did not consider this miRNA binding site as of potential functional relevance. Our reporter gene luciferase assays showed altered expression of the different *P2RX7* 3' UTR haplotypes (in the absence of the rs1653625 A ~ rs1718106 C (A~C) haplotype, only the A~A, C~A, and C~C haplotypes were investigated). In these *in vitro* experiments the A~A haplotype showed the highest luciferase activity, which result corresponds to the hypothesis that the lowest binding efficiency of the miRNAs leads to the weakest repression effect, thus to increased protein expression. Importantly, the A~A haplotype of the 3' UTR SNPs can be seen together with the minor alleles of the abovementioned non-synonymous SNPs rs1718119 and rs2230912 in the ACGA**AA** haplotype corresponding to the highest depression severity score (the three depression-associated SNPs are underlined, and the 3' UTR SNPs are in bold).

In summary, our dimensional analyses provided association of the *P2RX7* rs1718119 (Ala348Thr) and rs2230912 (Gln460Arg) with depressive symptoms in two independent patient samples (depressed and diabetic). In addition, a non-coding variant rs1653625 has been indicated by the present study. Once other studies confirm the importance of these gain-of-function *P2RX7* variants, they could be used in genetic tests determining drug responsiveness to anti-inflammatory P2X7 receptor antagonists in order to create individually tailored treatment plan (the so-called personalized medicine).

5. **Conclusions**

Our results highlight the importance of *P2RX7* rs1718119 (Ala348Thr), rs2230912 (Gln460Arg) and rs1653625 polymorphisms in the genetic susceptibility for depression. The minor-allele containing rs1718119-A ~ rs2230912-G ~ rs1653625-A haplotype showed significantly higher depressive symptom score compared to the most frequent G~A~C haplotype among psychiatric patients with a major depressive episode and also among diabetic patients, who are at higher risk for developing mood disorders. This depression-associated *P2RX7* SNP-combination was also linked to increased receptor function by previous studies and the present *in vitro* experiments. Further studies using *in vivo* neurobiological models or patient-derived cells are still needed to reveal the molecular mechanism of the connection between P2X7 receptor functioning and development of depressive symptoms.

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Author contributions: ZN, ASo, and GF designed the study. OAR and ZN performed the *in silico* search and made the plasmid constructs. The *in vitro* experiments were carried out by AV and ZN. Collection of questionnaire data was done by ZH, GN, and ASz. AV and ZN managed the genotyping, analyzed the genetic data, and wrote the manuscript. All authors have read and approved the final version of the manuscript.
Declaration of interest: None of the authors declare competing interest.

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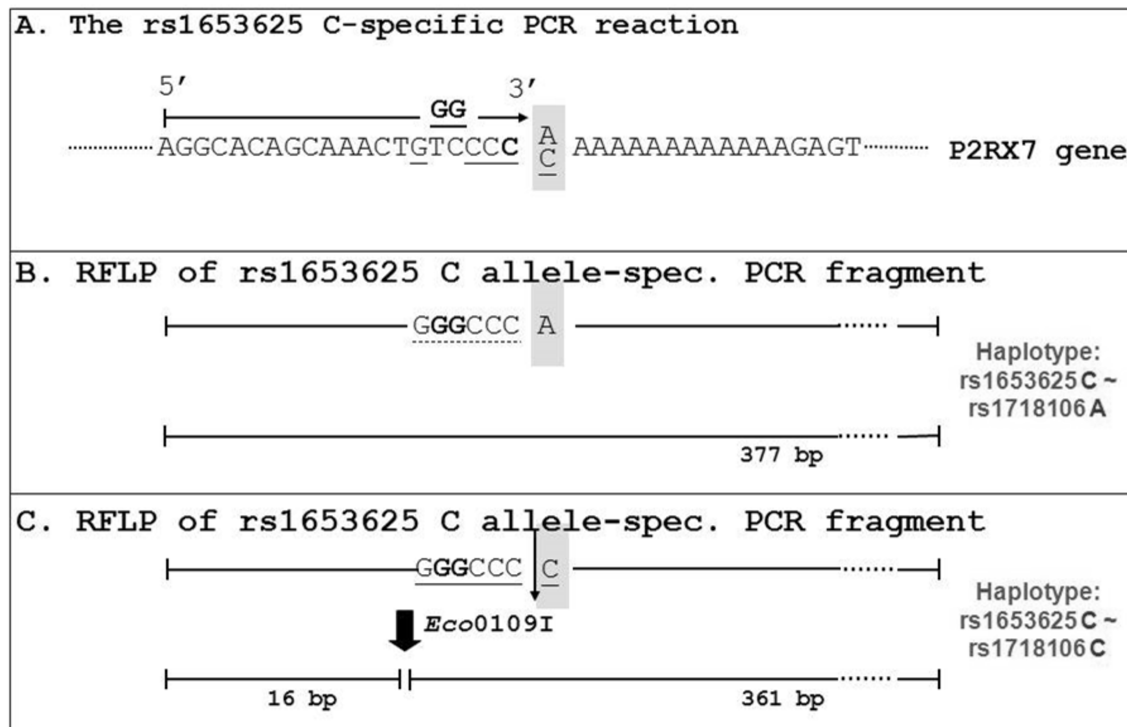
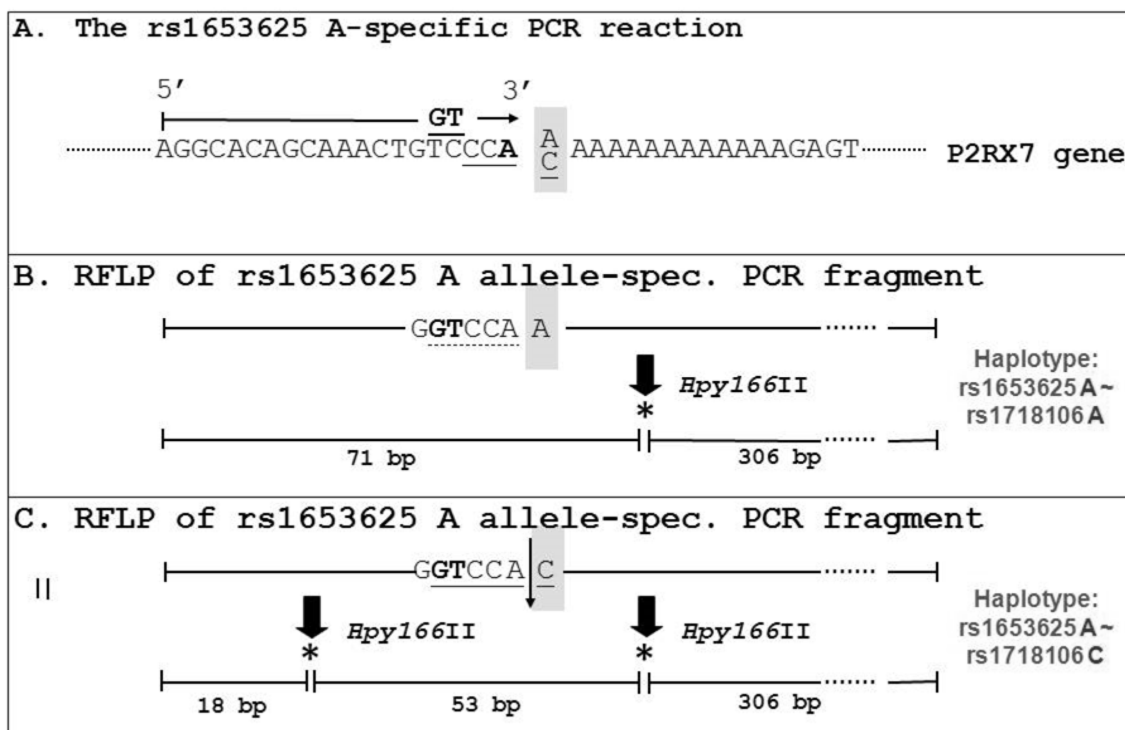
Supplementary Figure 1a: The genotyping principle of rs1718106 with PCR-RFLP in subjects with rs1653625 C-allele. The C-specific forward primer included two mismatches (GG in bold) at the 3' end to obtain a restriction site for Eco0109I (GGGCC**CC** underlined) in the PCR amplicon. Panel A: The rs1653625 C-specific PCR reaction with the allele-specific forward primer. The rs1718106 is indicated by the gray box. Panel B: The restriction site (indicated by dashed line) is incomplete in case of the rs1718106 A-allele, consequently the original 377 bp PCR product cannot be cut by the restriction enzyme. Panel C: Using the primer with two mismatches creates an Eco0109I restriction site (underlined) in case of the rs1718106 C-allele, yielding 16 and 361 bp fragments.



Supplementary Figure 1b: The genotyping principle of rs1718106 with PCR-RFLP in subjects with rs1653625 A-allele. The A-specific forward primer included two mismatches (GT in bold) at the 3' end to obtain a restriction site for Hpy166II (GTCC**AC** underlined) in the PCR amplicon. Panel A: The rs1653625 A-specific PCR reaction with the allele-specific forward primer. The rs1718106 is indicated by the gray box. Panel B: The restriction site (indicated by dashed line) is incomplete in case of the rs1718106 A-allele, consequently the original 377 bp PCR product is cut by the restriction enzyme only at the control restriction site at the other side of the SNP (indicated by an asterisk), resulting in 71 and 306 bp fragments. Panel C: Using the primer with two mismatches creates a Hpy166II restriction site (underlined) in addition to the control cleavage site in case of the rs1718106 C-allele, yielding 18, 53, and 306 bp fragments.



Supplementary Table 1. Demographic characteristics of the control and patient groups. Data is shown in frequency (%) or mean ± SD. Chi-square and t-test statistics are presented for the comparisons between the patients and controls. The self-report Hospital Anxiety and Depression Scale (HADS) questionnaire provided continuous scales for depressive and anxiety symptoms (HADS-depr. and HADS-anx., respectively).

	Controls (N = 406)	Diabetic (N = 218)	Depressed* (N = 315)	MDD (N = 195)	BD (N = 120)
men	30.1%	48.2%	24.8%	25.6%	23.3%
women	69.9%	51.8%	75.2%	74.4%	76.7%
p		**< 0.001**	0.111	0.255	0.148
Age range	18 - 69	22 - 84	19 - 70	19 - 70	21 - 65
mean ± SD	28.0 ± 12.2	58.0 ± 13.6	47.3 ± 11.5	48.6 ± 11.8	45.2 ± 10.7
p		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
HADS-depr.	3.2 ± 2.3	4.8 ± 3.5	12.9 ± 4.9	12.8 ± 4.7	13.0 ± 5.2
p		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
HADS-anx.	6.3 ± 3.4	6.0 ± 3.9	13.7 ± 4.3	13.9 ± 4.3	13.3 ± 4.4
p		0.312	**< 0.001**	**< 0.001**	**< 0.001**

*All psychiatric patients grouped together (MDD and BD)

Supplementary Table 2. Estimated haplotype frequencies of the control and depressed patient groups. The alleles are presented in the genomic order starting from Ala348Thr = rs1718119:G>A, followed by Thr357Ser = rs2230911:C>G, Gln460Arg = rs2230912:A>G, Glu496Ala = rs3751143:A>C, and rs1653625:A>C, finally rs1718106:A>C.

2a: Data is shown in frequency (%) for the 6 most common haplotypes.

P2RX7 haplotype	Controls (N = 406)	Depressed* (N = 315)	MDD (N = 195)	BD (N = 120)
GCAACA	27.3%	26.4%	27.8%	24.0%
ACAAAA	21.8%	23.6%	24.5%	22.3%
ACGAAA	19.2%	16.8%	16.5%	17.2%
GCACCA	19.4%	18.1%	17.3%	19.3%
GGAAAA	6.5%	8.6%	7.7%	10.1%
GCAACC	4.2%	5.1%	4.4%	6.3%

2b: Data is shown in frequency (%) for haplotypes > 5% in the analyzed samples, by leaving out the last SNP rs1718106.

P2RX7 haplotype	Controls (N = 406)	Depressed* (N = 315)	MDD (N = 195)	BD (N = 120)
GCAAC	29.5%	31.4%	32.1%	30.4%
ACAAA	22.3%	23.7%	24.4%	22.5%
ACGAA	19.1%	17.0%	16.9%	17.1%
GCACC	16.8%	18.1%	17.4%	19.2%
GGAAA	7.7%	8.6%	7.7%	10.0%

*All psychiatric patients grouped together (MDD and BD)

Supplementary Table 3. HADS-anxiety scores of the different genotype groups (mean ± SD).

Genotype		Controls	Diabetic	Depressed*	MDD	BD
rs1718119	AA	6.27 ± 3.15	7.56 ± 3.52	13.96 ± 4.26	14.27 ± 3.38	13.33 ± 5.72
Ala348Thr	AG	6.34 ± 3.50	6.25 ± 4.12	13.91 ± 4.03	13.99 ± 4.20	13.76 ± 3.69
G1068A	GG	6.34 ± 3.48	4.78 ± 3.16	13.20 ± 4.81	13.50 ± 4.92	12.78 ± 4.70
	p	0.997	**0.009**	0.431	0.747	0.482
rs2230911	CC	6.35 ± 3.46	6.05 ± 3.85	13.76 ± 4.37	13.88 ± 4.36	13.55 ± 4.41
Thr357Ser	CG	6.17 ± 3.23	5.88 ± 3.86	13.00 ± 4.03	13.95 ± 3.70	11.69 ± 4.21
C1096G	GG	6.33 ± 5.03	4.00 ± 5.66	15.50 ± 6.14	14.00 ± 8.49	17.00 ± 5.66
	p	0.674#	0.522#	0.305	0.931#	0.281#
rs2230912	AA	6.36 ± 3.45	5.53 ± 3.71	13.52 ± 4.29	13.81 ± 4.40	13.03 ± 4.08
Gln460Arg	AG	6.25 ± 3.52	7.00 ± 4.07	13.77 ± 4.28	13.70 ± 4.09	13.92 ± 4.73
A1405G	GG	6.06 ± 2.65	6.40 ± 2.61	15.70 ± 5.54	17.60 ± 1.52	13.80 ± 7.60
	p	0.854	0.065	0.227	0.148	0.656
rs3751143	AA	6.34 ± 3.34	6.24 ± 3.87	13.54 ± 4.30	13.79 ± 4.09	13.08 ± 4.65
Glu496Ala	AC	6.31 ± 3.58	5.57 ± 3.96	13.54 ± 4.51	13.61 ± 4.73	13.40 ± 4.20
A1513C	CC	5.25 ± 3.99	6.00 ± 1.93	17.20 ± 2.20	18.60 ± 1.82	15.80 ± 1.64
	p	0.591	0.427	**0.030**	**0.027**	0.460
rs1653625	AA	6.27 ± 3.13	7.39 ± 3.66	13.99 ± 3.95	14.22 ± 3.42	13.54 ± 4.84
	AC	6.27 ± 3.52	5.72 ± 4.07	13.68 ± 4.42	13.94 ± 4.50	13.24 ± 4.29
	CC	6.47 ± 3.62	5.10 ± 3.11	13.34 ± 4.65	13.43 ± 4.84	13.19 ± 4.42
	p	0.857	**0.011**	0.718	0.701	0.786
rs1718106	AA	6.12 ± 3.49	6.06 ± 3.85	13.82 ± 4.17	14.02 ± 4.10	13.46 ± 4.30
	AC	6.86 ± 2.87	5.47 ± 4.21	12.46 ± 5.60	12.75 ± 5.88	12.08 ± 5.43
	CC	-	5.00 ± 0.00	-	-	-
	p	0.355	0.328#	0.155	0.343	0.302

Nominally significant p-values of the applied analyses of covariance are shown in bold.
*All psychiatric patients grouped together (MDD and BD)
The rare homozygote genotype was grouped together with the heterozygotes because of the low cell count (less than 5), in these analyses the degree of freedom was 1.