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Evaluation of *5-HTTLPR* (insertion/deletion) and *BDNF* (rs6265) genetic variations in the Slovakian individuals suffering from affective disorders

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Abstract. The pathophysiology of affective disorders (AD), including depressive disorders (DD) and anxiety disorders (ANXD), is still unclear. To understand risk factors of the disorders, we evaluated genetic variations of the serotonin reuptake transporter (*5-HTTLPR*, ins/del) and the brain-derived neurotrophic factor (*BDNF*, rs6265) in Slovak patients suffering from AD. After genotyping we observed a significantly increased frequency of LS and LL genotypes (*5-HTTLPR*) in individuals diagnosed with AD compared to controls (OR = 1.99, 95% CI = 1.21–3.27, $p = 0.006$). There was also a significant relationship between TT (*BDNF*) genotype and the risk of AD in males (OR = 5.93, 95% CI = 1.42–27.07, $p = 0.011$). In gene-gene analysis, the LL or LS (*5-HTTLPR*) and CT or TT (*BDNF*) genotype combinations had a risk-enhancing effect on AD susceptibility (mainly ANXD in males), while SS (*5-HTTLPR*) and TT (*BDNF*) combination had a protective effect on AD risk (mainly ANXD). However, larger prospective studies are needed to confirm our findings.

Key words: *5-HTTLPR* — *BDNF* — Affective disorder — Depressive disorder — Anxiety disorder

Abbreviations: AD, affective disorder; ANXD, anxiety disorder; BD, bipolar depression; BDNF, brain-derived neurotrophic factor; DD, depressive disorder; GAD, generalized anxiety disorder; 5-HT, serotonin; *5-HTTLPR,* genetic variation of the serotonin reuptake transporter; HWE, Hardy-Weinberg equilibrium; OCD, obsessive-compulsive disorder; PTSD, post-traumatic stress disorder; *SLC6A4,* gene encoded serotonin reuptake transporter; SERT, serotonin reuptake transporter; UD, unipolar depression.

Introduction

Affective disorders (ADs) are serious, mostly recurrent, or chronic mental disorders that affect the general emotional state of a patient. Although they are not clearly defined diagnostic entities, according to the International Society for Affective Disorders (ISAD, 2021, https://www.isad.org.uk/), they include unipolar depression (UD) and bipolar depression (BD), generalized anxiety disorder (GAD), and more specific anxiety disorders, such as social phobia, agoraphobia, panic disorder, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD). Moreover, depressive and anxiety conditions can occur simultaneously in the same patient. Generally, the ISAD recommends considering all these disorders as a single group because of a high level of similarity between them. It is estimated that over 4.4% of the world's population suffers from any type of depressive disorders (DDs) and 3.6% of the global population from

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anxiety disorders (ANXDs) (World Health Organization 2017). On the other hand, according to the International Statistical Classification of Diseases and Related Health Problems (ICD), usually only unipolar affective disorder and bipolar affective disorder are considered as ADs.

The precise pathophysiology of ADs is still unclear despite a substantial research effort in this field. Thus, many potential biomarkers for AD risk, as well as genetic variations of such molecules are studied as possible causal factors of the disorder.

The hypothesis of serotonin (5-HT) dysregulation in the development of depression and anxiety has been attracting attention for several decades (Liu et al. 2018). There has also been a growing interest in the study of the serotonin reuptake transporter (SERT) which is considered the main molecular target of selective serotonin reuptake inhibitors, the most widely utilized antidepressants. This transporter, also known as solute carrier family 6, member 4, is encoded by the *SLC6A4* gene located on chromosome 17. Concerning ADs, considerable attention has been mainly paid to its functional *5-HTTLPR* genetic variation that represents the insertion (ins) generating the long ("L") allele or deletion (del) generating the short ("S") allele in the promoter region of the gene (Iurescia et al. 2016). The data appear to suggest that the S allele variant can result in reduced transcriptional efficiency of the promoter, leading to lower availability and decreased reuptake activity of the transporter in comparison with L allele (Lesch et al. 1996). Many studies monitoring the role of ins/del genetic variation in ADs risk have been published, however, with inconsistent results (Mohamed Saini et al. 2012; Jiménez et al. 2019; Miozzo et al. 2020).

Much attention is also paid to the brain-derived neurotrophic factor (BDNF). This protein, widely expressed in the central nervous system, is involved in the control of neuronal growth, survival, neurogenesis, and differentiation, neurotransmitter signalling, and synaptic plasticity (Park and Poo 2013). Several publications indicate that its dysregulation might have a role in the etiology of diverse neuropsychiatric conditions, including DDs and ANXDs (Lin and Huang 2020). For instance, lower plasma and serum BDNF levels were found in patients with depression than in controls (Bocchio-Chiavetto et al. 2010). Similarly, much research on the most common single nucleotide *BDNF* genetic variation 196C>T (rs6265; MAF = 0.20) in exon 5 located on chromosome 11p13 has been done. This genetic variation results in an amino acid substitution of valine (Val) for methionine (Met) at codon 66 (Val66Met). Although the Met allele was associated with decreased cellular trafficking, processing, and activity-dependent BDNF secretion, impairment of synaptic transmission, and cortical plasticity, as well as with changes in an extracellular level of BDNF (Egan et al. 2003; Pattwell et al. 2012), its role in AD susceptibility is controversial (Verhagen et al. 2010; Gyekis et al. 2013; Zhao et al. 2018; Border et al. 2019).

Although some data indicated an interaction between BDNF and serotonin signalling in mood control (Martinowich and Lu 2008), only several studies were aimed at the evaluation of gene-gene interaction of the described genetic variations with AD susceptibility (Grabe et al. 2012; Kostic et al. 2016; Nestor et al. 2019; Wang et al. 2020).

Based on the inconsistency of data published earlier, our study focused on determining the relevance of the *5-HTTLPR* (ins/del) and *BDNF* (Val66Met), including their gene-gene interactions, to the risk of development of ADs in the population sample of Slovakian individuals.

Material and Methods

Participants

The current case-control study was performed in two groups of adult participants of Slovak origin (Caucasians). The case (patient) group comprised individuals aged 18 and above at the time of onset of any type of AD who were treated or monitored at the 2nd Department of Psychiatry of Pavol Jozef Safarik University in Kosice, Slovakia. All patients were on antidepressant treatment in the acute phase of the disorder.

Exclusion criteria included: organic mental disorders, mental retardation, personality disorders, drug or alcohol abuse, psychotic symptoms, suicidal behavior, and pregnancy.

The control group with adult volunteers had no relation to the cases and was without any known psychiatric disorder.

Blood samples from both groups were collected from November 2018 to January 2021 and participation in testing was voluntary and could be cancelled by any individual at any time during the study.

The clinical diagnosis was made or confirmed by two certified psychiatrists who were kept blind to the diagnosis made by one to another and sample genotyping.

This study was approved by the local ethics committee, and all subjects provided written informed consent.

Genotyping

DNA from the whole blood was extracted with a Wizard Genomic DNA Isolation kit (Promega, Co, Ltd, USA). Genotyping of the *BDNF* rs6265 was performed using asymmetric real-time polymerase chain reaction and subsequent high-resolution melting analysis in the presence of an unlabelled probe on the Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The oligonucleotides (forward limit: 5'-GCCGAACTTTCTGGTCCTCATCC-3', reverse excess: 5'-AAGGCAGGTTCAAGAGGCTTG-3', and probe: 5'-GCTCTTCTATCACGTGTTCGAAAGTGTC-Phos) were designed in our laboratory. Genotypes were identified using Eco^{TM} Software 4.1.

The *5-HTTLPR* genetic variation was genotyped using forward 5'-GGCGTTGCCGCTCTGAATGC-3' and reverse 5'-GAGGGACTGAGCTGGACAACCAC-3' primers according to Murakami et al. (1999). The PCR products were separated by electrophoresis on a 2% agarose gel.

Statistical analysis

SPSS software for Windows (version 16.0, USA) and GraphPad Prism 9 (GraphPad Software, Inc., USA) were used for all statistical analyses. A p-value of <0.05 was taken as statistically significant. The Chi-square or Fisher's exact tests were conducted to compare contingency tables, while the strength of the relative associations was assessed *via* odds ratios (ORs) with their corresponding 95% confidence intervals (CIs). Quantitative values were evaluated using the Mann-Whitney U test. The Hardy-Weinberg equilibrium (HWE) assumption was assessed for the tested groups by comparing the observed numbers of each genotype with those expected under the HWE for the estimated allele frequency. Codominant, dominant, recessive, and overdominant genetic models were used to analyze the association between a genetic variation and phenotype. Likewise, we used binary logistic regression analysis to determine the role of tested genetic variations as independent risk factors for affective disorder susceptibility.

Results

Characteristics of the participants

A total of 500 subjects were included in the current study. Out of 280 patients, 183 (65.4%) were females and 97 (34.6%) were males (female:male ratio: 1.9:1). The mean age of the cases was 50.3 ± 13.9 years (range: 18–83 years).

Overall, 222 (79.3%) patients suffered from depressive disorders – DDs (UD: 142 females and 62 males; BD: 9 females and 9 males), and 58 (20.7%) patients were diagnosed with anxiety disorders – ANXDs (GAD: 26 females and 18 males; OCD: 4 females and 4 males; panic disorder and social phobia: 1 male; PTSD: 2 females and 3 males).

Age-, gender-, and ethnicity-matched healthy adults served as the control group. Out of 220 individuals, 134 (60.9%) were females and 86 (39.1%) were males.

All collected blood samples were successfully genotyped for both genetic variations. The relevant genotype and allele frequencies of patients and controls are shown

Table 1. Genotype and allele frequencies of *5-HTTLPR* (ins/del) and *BDNF* (Val66Met, rs6265)

Variable	*5-HTTLPR* (ins/del)					*BDNF* (rs6265)				
	Genotypes			Alleles		Genotypes			Alleles	
	LL (%)	LS (%)	SS (%)	L (%)	S (%)	CC (%)	CT (%)	TT (%)	C (%)	T (%)
Controls										
Total	68 (30.9)	107 (48.6)	45 (20.5)	243 (55.2)	197 (44.8)	144 (65.5)	64 (29.1)	12 (5.4)	352 (80.0)	88 (20.0)
Females	46 (34.3)	64 (47.8)	24 (17.9)	156 (58.2)	112 (41.8)	82 (61.2)	42 (31.3)	10 (7.5)	206 (76.9)	62 (23.1)
Males	22 (25.6)	43 (50.0)	21 (24.4)	87 (50.6)	85 (49.4)	62 (72.1)	22 (25.6)	2 (2.3)	146 (84.9)	26 (15.1)
AD										
Total	102 (36.4)	146 (52.1)	32 (11.4)	350 (62.5)	210 (37.5)	178 (63.6)	78 (27.9)	24 (8.6)	434 (77.5)	126 (22.5)
Females	72 (39.3)	90 (49.2)	21 (11.5)	234 (63.9)	132 (36.1)	124 (67.8)	47 (25.7)	12 (6.6)	295 (80.6)	71 (19.4)
Males	30 (30.9)	56 (57.7)	11 (11.3)	116 (59.8)	78 (40.2)	54 (55.7)	31 (32.0)	12 (12.4)	139 (71.6)	55 (28.4)
DD										
Total	79 (35.6)	114 (51.4)	29 (13.1)	272 (61.3)	172 (38.7)	147 (66.2)	55 (24.8)	20 (9.0)	349 (78.6)	95 (21.4)
Females	59 (39.1)	74 (49.0)	18 (11.9)	192 (63.6)	110 (36.4)	104 (68.9)	36 (23.8)	11 (7.3)	244 (80.8)	58 (19.2)
Males	20 (28.2)	40 (56.3)	11 (15.5)	80 (56.3)	62 (43.7)	43 (60.6)	19 (26.8)	9 (12.7)	105 (73.9)	37 (26.1)
UD										
Total	76 (37.3)	101 (49.5)	27 (13.2)	253 (62.0)	155 (38.0)	134 (65.7)	52 (25.5)	18 (8.8)	320 (78.4)	88 (21.6)
Females	56 (39.4)	68 (47.9)	18 (12.7)	180 (63.4)	104 (36.6)	96 (67.6)	35 (24.6)	11 (7.7)	227 (79.9)	57 (20.1)
Males	20 (32.3)	33 (53.2)	9 (14.5)	73 (58.9)	51 (41.1)	38 (61.3)	17 (27.4)	7 (11.3)	93 (75.0)	31 (25.0)
ANXD										
Total	23 (39.7)	32 (55.2)	3 (5.2)	78 (67.2)	38 (32.8)	31 (53.4)	23 (39.7)	4 (6.9)	85 (73.3)	31 (26.7)
Females	13 (40.6)	16 (50.0)	3 (9.4)	42 (65.6)	22 (34.4)	20 (62.5)	11 (34.4)	1 (3.1)	51 (79.7)	13 (20.3)
Males	10 (38.5)	16 (61.5)	0 (0.0)	36 (69.2)	16 (30.8)	11 (42.3)	12 (46.2)	3 (11.5)	34 (65.4)	18 (34.6)

AD, affective disorder; ANXD, anxiety disorder; DD, depressive disorder; UD, unipolar depression.

in Table 1. The L (5-*HTTLPR*) and C (*BDNF*) alleles were found to be more frequent in screened groups of participants.

The genotype distribution among controls did not deviate from HWE ($p \geq 0.05$) for both tested genetic variations. Similarly, the HWE was satisfactory in the observed genotype frequencies for the *5-HTTLPR* in the patient group. However, the distribution of the *BDNF* genotypes among the cases showed a deviation from HWE ($p < 0.01$).

Impact of 5-HTTLPR (ins/del) and BDNF (Val66Met) on the risk of AD

Firstly, we assessed genotype and allele distributions between all patients with ADs (involvement of both DDs and ANXDs, $n = 280$) compared to control individuals.

We detected a statistically significant association between *5-HTTLPR* and the disorder development in the codominant (LL *vs.* SS: reciprocal of OR = 2.11, 95% CI = 1.23–3.71, $p =$

Table 2. Distribution of genotypes of *5-HTTLPR* (ins/del) among cases (patients with AD) and controls

Genotypes of *5-HTTLPR* (ins/del)	Case *n* (%)	Control *n* (%)	OR (95% CI)	*p* value
AD – all patients				
LL	102 (36.4)	68 (30.9)	1.00[a]	
LS	146 (52.1)	107 (48.6)	0.91 (0.61–1.37)	0.639
SS	32 (11.4)	45 (20.5)	0.47 (0.27–0.81)	0.007*,χ/0.009*,f
			2.11 (1.23–3.71)[r]	
LL+LS	248 (88.6)	175 (79.5)	1.00[c]	
SS	32 (11.4)	45 (20.5)	0.50 (0.31–0.83)	0.006*,χ/0.006*,f
			1.99 (1.21–3.27)[r]	
AD – males				
LL	30 (30.9)	22 (25.6)	1.00[a]	
LS	56 (57.7)	43 (50.0)	0.95 (0.48–1.88)	0.894
SS	11 (11.3)	21 (24.4)	0.38 (0.15–0.98)	0.038*,χ/0.045*,f
			2.60 (1.02–6.68)[r]	
LL+LS	86 (88.7)	65 (75.6)	1.00[c]	
SS	11 (11.3)	21 (24.4)	0.40 (0.18–0.86)	0.020*,χ/0.031*,f
			2.53 (1.16–5.41)[r]	
DD – all patients				
LL	79 (35.6)	68 (30.9)	1.00[a]	
LS	114 (51.4)	107 (48.6)	0.92 (0.61–1.39)	0.685
SS	29 (13.1)	45 (20.5)	0.56 (0.32–0.99)	0.041*,χ/0.047*,f
			1.80 (1.01–3.17)[r]	
LL+LS	193 (86.9)	175 (79.5)	1.00[c]	
SS	29 (13.1)	45 (20.5)	0.58 (0.35–0.96)	0.037*,χ/0.042*,f
			1.71 (1.04–2.82)[r]	
UD – all patients				
LL	76 (37.3)	68 (30.9)	1.00[a]	
LS	101 (49.5)	107 (48.6)	0.85 (0.55–1.28)	0.436
SS	27 (13.2)	45 (20.5)	0.54 (0.30–0.97)	0.034*,χ/0.043*,f
			1.86 (1.03–3.36)[r]	
ANXD – all patients				
LL	23 (39.7)	68 (30.9)	1.00[a]	
LS	32 (55.2)	107 (48.6)	0.88 (0.47–1.64)	0.695
SS	3 (5.2)	45 (20.5)	0.20 (0.06–0.65)	0.006*,χ/0.006*,f
			5.07 (1.55–16.66)[r]	
LL+LS	55 (94.8)	175 (79.5)	1.00[c]	
SS	3 (5.2)	45 (20.5)	0.21 (0.07–0.65)	0.006*,χ/0.006*,f
			4.71 (1.53–14.93)[r]	

AD, affective disorder; ANXD, anxiety disorder; CI, confidence interval; DD, depressive disorder; OR, odds ratio; UD, unipolar depression; * significant association; [a] codominant model; [c] recessive model; [f] Fisher's exact test; [r] reciprocal of OR with the relevant CI; [χ] Chi-square test.

Table 3. Distribution of genotypes of *BDNF* (Val66Met, rs6265) among male cases (patients with AD) and controls

Genotypes of *BDNF* (rs6265)	Case *n* (%)	Control *n* (%)	OR (95% CI)	*p* value
AD – males				
CC	54 (55.7)	62 (72.1)	1.00[a]	
CT	31 (32.0)	22 (25.6)	1.61 (0.83–3.03)	0.150
TT	12 (12.4)	2 (2.3)	6.89 (1.58–31.64)	0.006[*,χ]/0.009[*,f]
CC	54 (55.7)	62 (72.1)	1.00[b]	
CT+TT	43 (44.3)	24 (27.9)	2.06 (1.10–3.84)	0.021[*,χ]/0.031[*,f]
CC+CT	85 (87.6)	84 (97.7)	1.00[c]	
TT	12 (12.4)	2 (2.3)	5.93 (1.42–27.07)	0.011[*,χ]/0.012[*,f]
DD – males				
CC	43 (60.6)	62 (72.1)	1.00[a]	
CT	19 (26.8)	22 (25.6)	1.25 (0.62–2.50)	0.554
TT	9 (12.7)	2 (2.3)	6.49 (1.54–30.69)	0.010[*,χ]/0.012[*,f]
CC+CT	62 (87.3)	84 (97.7)	1.00[c]	
TT	9 (12.7)	2 (2.3)	6.10 (1.49–28.66)	0.011[*,χ]/0.024[*,f]
UD – males				
CC	38 (61.3)	62 (72.1)	1.00[a]	
CT	17 (27.4)	22 (25.6)	1.26 (0.59–2.61)	0.545
TT	7 (11.3)	2 (2.3)	5.71 (1.14–27.90)	0.020[*,χ]/0.031[*,f]
CC+CT	55 (88.7)	84 (97.7)	1.00[c]	
TT	7 (11.3)	2 (2.3)	5.35 (1.11–25.94)	0.024[*,χ]/0.035[*,f]
ANXD – males				
CC	11 (42.3)	62 (72.1)	1.00[a]	
CT	12 (46.2)	22 (25.6)	3.07 (1.18–8.30)	0.018[*,χ]/0.024[*,f]
TT	3 (11.5)	2 (2.3)	8.46 (1.52–49.68)	0.011[*,χ]/0.038[*,f]
CC	11 (42.3)	62 (72.1)	1.00[b]	
CT+TT	15 (57.7)	24 (27.9)	3.52 (1.36–8.87)	0.005[*,χ]/0.009[*,f]

AD, affective disorder; ANXD, anxiety disorder; CI, confidence interval; DD, depressive disorder; OR, odds ratio; UD, unipolar depression; * significant association; [a] codominant model; [b] dominant model; [c] recessive model; [f] Fisher's exact test; [χ] Chi-square test.

0.007) and recessive (LL+LS *vs.* SS: reciprocal of OR = 1.99, 95% CI = 1.21–3.27, $p = 0.006$) models (Table 2). In brief, individuals carrying at least one L allele had a significantly higher risk of AD development. The difference in allele frequencies between patients suffering from AD and controls was also found to be statistically significant (L *vs.* S: OR = 1.35, 95% CI = 1.05–1.74, $p = 0.020$). No differences in *BDNF* genotype distributions were observed between cases and controls.

Secondly, the comparative analyses were gender-stratified. The significant values were observed only in male samples. The difference in genotype frequencies of the *5-HTTLPR* between controls and cases was found to be statistically significant in the codominant (LL *vs.* SS: reciprocal of OR = 2.60, 95% CI = 1.02–6.68, $p = 0.038$) and recessive (LL+LS *vs.* SS: reciprocal of OR = 2.53, 95% CI = 1.16–5.41, $p = 0.020$) models (Table 2).

A significant link between AD susceptibility in males and *BDNF* genotypes in the codominant (TT *vs.* CC: OR = 6.89, 95% CI = 1.58–31.64, $p = 0.009$), dominant (CT+TT *vs.* CC: OR = 2.06, 95% CI = 1.10–3.84, $p = 0.021$) and recessive (TT *vs.* CC+CT: OR = 5.93, 95% CI = 1.42–27.07, $p = 0.012$) models (Table 3) was observed. Additionally, a higher frequency of T allele was detectable in cases compared to controls (OR = 2.22, 95% CI = 1.34–3.79, $p = 0.002$). A significantly higher frequency of T allele was also found in males with AD than in females ($p = 0.017$).

In women, statistical analyses did not reach significance and the risk of AD was not associated with the studied genetic variations.

Furthermore, binary logistic regression revealed LS and LL genotypes (*5-HTTLPR*) as independent predictive factors of AD (LS genotype: OR = 1.81, 95% CI = 1.07–3.07, $p = 0.027$; LL genotype: OR = 1.96, 95% CI = 1.12–3.42, $p = 0.018$) (Table 4). In male subgroup of cases, only *BDNF* TT genotype significantly increased the risk of AD (OR = 6.67, 95% CI = 1.39–31.96, $p = 0.018$). In females, no significant values were obtained.

Impact of 5-HTTLPR (ins/del) and BDNF (Val66Met) on the risk of DD

The *5-HTTLPR* analysis identified a significantly higher frequency of LL genotype or a combination of LL and LS genotypes in patients suffering from DD (UD, BD) than in donors collected as controls (LL *vs.* SS: reciprocal of OR = 1.80, 95% CI = 1.01–3.17, $p = 0.041$, codominant model; LL+LS *vs.* SS: reciprocal of OR = 1.71, 95% CI = 1.04–2.82, $p = 0.037$, recessive model) (Table 2). A similar trend was observed in a separate analysis performed for the risk of UD. However, a significant association was reached only in the codominant model (LL *vs.* SS: reciprocal of OR = 1.86, 95% CI = 1.03–3.36, $p = 0.034$).

When *BDNF* genetic variation was analyzed, only gender stratified analysis revealed a significant association (Table 3). It can be concluded that TT genotype and T allele were more prevalent in male cases with DD than in controls (TT *vs.* CC: OR = 6.49, 95% CI = 1.54–30.69, $p = 0.012$, codominant model; TT *vs.* CC+CT: OR = 6.10, 95% CI = 1.49–28.66, $p = 0.024$, recessive model; T *vs.* C: OR = 1.98, 95% CI = 1.13–3.45, $p = 0.016$). Equally important, the presence of TT genotype or T allele was associated with a significantly higher risk of UD (TT *vs.* CC: OR = 5.71, 95% CI = 1.14–27.90, $p = 0.031$, codominant model; TT *vs.* CC+CT: OR = 5.35, 95% CI = 1.11–25.94, $p = 0.035$, recessive model; T *vs.* C: OR = 1.87, 95% CI = 1.06–3.39, $p = 0.033$).

The results indicated no statistically significant difference between the tested genetic variations and disorder susceptibility in the subgroup of female participants.

In case of binary logistic regression, only TT (*BDNF*) genotype was found as an independent risk factor for DD (OR = 6.10, 95% CI = 1.23–30.24, $p = 0.027$) and for UD (OR = 5.57, 95% CI = 1.06–29.31, $p = 0.043$) in male subgroup of cases.

Impact of 5-HTTLPR (ins/del) and BDNF (Val66Met) on the risk of ANXD

A preliminary analysis was carried out in the subgroup of patients with ANXDs ($n = 58$). The statistical assessment

Table 4. Binary logistic regression analysis to identify risk genotypes of *5-HTTLPR* (ins/del) and *BDNF* (Val66Met, rs6265) associated with AD susceptibility

Variable	B	OR	95% CI	*p* value
AD – all patients				
5-HTTLPR (LS genotype)	0.593	1.81	1.07–3.07	0.027*
5-*HTTLPR* (LL genotype)	0.673	1.96	1.12–3.42	0.018*
BDNF (CT genotype)	0.029	1.03	0.69–1.54	0.888
BDNF (TT genotype)	0.452	1.57	0.74–3.34	0.241
AD – males				
5-HTTLPR (LS genotype)	0.843	2.32	0.99–5.44	0.052
5-HTTLPR (LL genotype)	0.818	2.27	0.89–5.79	0.088
BDNF (CT genotype)	0.400	1.49	0.76–2.92	0.243
BDNF (TT genotype)	1.898	6.67	1.39–31.96	0.018*
AD – females				
5-*HTTLPR* (LS genotype)	0.414	1.51	0.75–3.05	0.247
5-HTTLPR (LL genotype)	0.479	1.62	0.78–3.34	0.197
BDNF (CT genotype)	−0.202	0.82	0.49–1.37	0.445
BDNF (TT genotype)	−0.272	0.76	0.30–1.92	0.565
DD – all patients				
5-HTTLPR (LS genotype)	0.414	1.51	0.88–2.61	0.138
5-HTTLPR (LL genotype)	0.477	1.61	0.90–2.88	0.107
BDNF (CT genotype)	−0.141	0.87	0.56–1.34	0.524
BDNF (TT genotype)	0.501	1.65	0.76–3.59	0.207
DD – males				
5-HTTLPR (LS genotype)	0.532	1.70	0.72–4.06	0.230
5-HTTLPR (LL genotype)	0.402	1.49	0.56–3.99	0.423
BDNF (CT genotype)	0.166	1.18	0.56–2.48	0.661
BDNF (TT genotype)	1.808	6.10	1.23–30.24	0.027*
DD – females				
5-HTTLPR (LS genotype)	0.336	1.40	0.68–2.89	0.365
5-HTTLPR (LL genotype)	0.397	1.49	0.70–3.16	0.302
BDNF (CT genotype)	−0.304	0.74	0.43–1.27	0.275
BDNF (TT genotype)	−0.151	0.86	0.34–2.20	0.754
UD – all patients				
5-HTTLPR (LS genotype)	0.366	1.44	0.82–2.52	0.200
5-HTTLPR (LL genotype)	0.510	1.67	0.92–3.00	0.090
BDNF (CT genotype)	−0.109	0.90	0.58–1.39	0.628
BDNF (TT genotype)	0.479	1.61	0.73–3.57	0.238
UD – males				
5-HTTLPR (LS genotype)	0.591	1.81	0.72–4.55	0.211
5-HTTLPR (LL genotype)	0.619	1.86	0.67–5.16	0.235
BDNF (CT genotype)	0.167	1.18	0.55–2.56	0.671
BDNF (TT genotype)	1.717	5.57	1.06–29.31	0.043*
UD – females				
5-HTTLPR (LS genotype)	0.252	1.29	0.62–2.66	0.497
5-HTTLPR (LL genotype)	0.354	1.43	0.67–3.03	0.356
BDNF (CT genotype)	−0.262	0.77	0.44–1.33	0.350
BDNF (TT genotype)	−0.058	0.94	0.37–2.42	0.903
ANXD – all patients				
5-HTTLPR (LS genotype)	1.479	4.39	1.27–15.23	0.020*
5-HTTLPR (LL genotype)	1.598	4.94	1.38–17.67	0.014*
BDNF (CT genotype)	0.536	1.71	0.90–3.23	0.099
BDNF (TT genotype)	0.261	1.30	0.37–4.51	0.682

AD, affective disorder; ANXD, anxiety disorder; B, binary logistic regression coefficient; CI, confidence interval; DD, depressive disorder; OR, odds ratio; UD, unipolar depression; * significant association.

showed a significantly increased risk for developing ANXD in cases carrying at least one L allele in comparison with SS genotype in the codominant (LL *vs.* SS: reciprocal of OR = 5.07, 95% CI = 1.55–16.66, $p = 0.006$) and recessive (LL+LS *vs.* SS: reciprocal of OR = 4.71, 95% CI = 1.53–14.93, $p = 0.006$) models (Table 2). There was also a significant association between allele frequency and ANXD risk (L *vs.* S: OR = 1.66, 95% CI = 1.07–2.57, $p = 0.020$) observed. No patient with SS genotype was found among male cases when the gender stratified analysis was performed.

In the case of *BDNF*, our findings indicated only significant associations in males. The CT and TT genotypes (Table 3), as well as T allele, were positively associated with an increased risk of ANXD in comparison with the reference CC genotype event. reference C allele (CT *vs.* CC: OR = 3.07, 95% CI = 1.18–8.30, $p = 0.018$ and TT *vs.* CC: OR = 8.46, 95% CI = 1.52–49.68, $p = 0.038$, codominant model; CT+TT *vs.* CC: OR = 3.52, 95% CI = 1.36–8.87, $p = 0.005$, dominant model; T *vs.* C: OR = 2.97, 95% CI = 1.51–6.04, $p = 0.002$).

Binary logistic regression determined LS and LL genotypes (OR = 4.39, 95% CI = 1.27–15.23, $p = 0.020$; OR = 4.94, 95% CI = 1.38–17.67, $p = 0.014$, respectively) as independent predictors of ANXD (Table 4). The gender stratified analysis was not performed due to the small number of cases.

Combined effect of 5-HTTLPR (ins/del) and BDNF (Val66Met) on the risk of AD

Finally, we evaluated the possible combined effects of both tested genetic variations in relation to the development of ADs and their subtypes. Overall, 9 combinations (LL-CC = 20.0%, LL-CT = 9.1%, LL-TT = 1.8%, LS-CC= 32.3%, LS-CT = 13.2%, LS-TT = 3.2%, SS-CC = 13.2%, SS-CT = 6.8%, SS-TT = 0.5%) were found in the control group and 8 combinations (LL-CC = 23.9%, LL-CT = 8.9%, LL-TT = 3.6%, LS-CC = 32.1%, LS-CT = 15.0%, LS-TT = 5.0%, SS-CC = 7.5%, SS-CT = 3.9%) in the total patient sample. The best model obtained in our study group was the comparison of the distribution of combination LL or LS (*5-HTTLPR*) and CT or TT (*BDNF*) among cases and controls (Table 5). A significantly increased risk of developing any type of AD was observed in male cases carrying this genotype combination in comparison with male controls (OR = 2.58, 95% CI = 1.35–4.94, $p^1 = 0.004$). A significantly higher frequency of LL or LS and CT or TT combination was also associated with the risk of anxiety susceptibility, including the male subpopulation (all cases with ANXD *vs.* controls: OR = 2.02, 95% CI = 1.11–3.68, $p^2 = 0.020$; males with ANXD *vs.* controls: OR = 4.81, 95% CI = 1.89–12.19, $p^3 = 0.001$). On the other hand, cases carrying a combination of genotypes SS (*5-HTTLPR*) and TT (*BDNF*) had a significantly lower chance of developing any type of AD (OR = 0.53, 95% CI = 0.29–0.97, $p^4 = 0.036$). In the same way, this genotype combination was associated with a lower risk of ANXD (OR = 0.12, 95% CI = 0.02–0.87, $p^5 = 0.009$). Despite a p^6-value less than 0.05, no statistically significant association was found among the subgroup of male cases with ANXD and control males because of values of 95% CI outside the required interval.

Discussion

Our study was aimed to investigate whether two common genetic variations (*5-HTTLPR*, ins/del and *BDNF* 196C>T) influence susceptibility to ADs or their subtypes. Moreover, the impact of gene-gene interaction on disorder risk was studied. To our knowledge, this is the first study evaluating these relationships in the Slovakian population sample.

Initially, we observed a higher susceptibility to DDs and ANXDs among females than males, which was in line with previous research (World Health Organization 2017). Next, the L allele (*5-HTTLPR*) and C allele (*BDNF*) were found to be the major alleles, the findings being consistent with reports from other European populations (The 1000 Genomes Project Consortium 2015).

Table 5. Analysis of gene-gene interaction of *5-HTTLPR* (ins/del) and *BDNF* (Val66Met, rs6265) between patients and controls

		Combination			
		1	2	3	4
	N	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Controls	220	115 (52.3)	60 (27.3)	29 (13.2)	16 (7.3)
Males	86	46 (53.5)	19 (22.1)	16 (18.6)	5 (5.8)
Females	134	69 (51.5)	41 (30.6)	13 (9.7)	11 (8.2)
AD	280	157 (56.1)	91 (32.5)	21 (7.5)^{p4}	11 (3.9)
Males	97	45 (46.4)	41 (42.3)^{p1}	9 (9.3)	2 (2.1)
Females	183	112 (61.2)	50 (27.3)	12 (6.6)	9 (4.9)
DD	222	127 (57.2)	66 (29.7)	20 (9.0)	9 (4.1)
Males	71	34 (47.9)	26 (36.6)	9 (12.7)	2 (2.8)
Females	151	93 (61.6)	40 (26.5)	11 (7.3)	7 (4.6)
UD	204	116 (56.9)	61 (29.9)	18 (8.8)	9 (4.4)
Males	62	31 (50.0)	22 (35.5)	7 (11.3)	2 (3.2)
Females	142	85 (59.9)	39 (27.5)	11 (7.8)	7 (4.9)
ANXD	58	30 (51.7)	25 (43.1)^{p2}	1 (1.7)^{p5}	2 (3.5)
Males	26	11 (42.3)	15 (57.7)^{p3}	0 (0.0)^{p6}	0 (0.0)
Females	32	19 (59.4)	10 (31.3)	1 (3.1)	2 (6.3)

AD, affective disorder; ANXD, anxiety disorder; DD, depressive disorder; UD, unipolar depression. *N*, total number of participants; n, the number of participants carrying specific combination of genotypes. Combinations of genotypes: 1: LL or LS (*5-HTTLPR*) and CC (*BDNF*); 2: LL or LS (*5-HTTLPR*) and CT or TT (*BDNF*); 3: SS (*5- HTTLPR*) and CC (*BDNF*); 4: SS (*5-HTTLPR*) and CT or TT (*BDNF*); ^{p1} $p = 0.004$ (χ^2-test), ^{p2} $p = 0.020$ (χ^2-test), ^{p3} $p = 0.001$ (χ^2-test), ^{p4} $p = 0.036$ (χ^2-test), ^{p5} $p = 0.009$ (Fisher's exact test), ^{p6} $p = 0.021$ (Fisher's exact test).

Regarding *5-HTTLPR* (ins/del), the S allele was traditionally considered the risk allele for developing a variety of psychiatric disorders (Lesch et al. 1996). On the contrary, our analysis indicated a significantly increased frequency of LS and LL genotypes in individuals diagnosed with AD than in controls. The subsequent disease-stratified analysis confirmed similar significant associations only in relation to anxiety phenotype. Our results are consistent with findings from several other studies, although these evaluated non-Caucasian populations or nonpathological affective symptomatology. To illustrate, Chinese healthy L carriers had significantly higher anxiety scores than S homozygotes (Long et al. 2013). In addition, Columbian young adults with LL genotype had a higher incidence of anxiety symptoms compared with other *5-HTTLPR* genotypes (Jiménez et al. 2019). The L allele was also linked with a higher risk of depression (Peralta-Leal et al. 2012). Another study implicated LL genotype as a predisposing factor for major depression only if individuals had been exposed to negative life events (Zhang et al. 2009). However, a strong relationship between stress events and *5-HTTLPR* genotypes contributing to the risk of depression has not been confirmed in a recently published meta-analysis (Culverhouse et al. 2018). Attention was also paid to the evaluation of *5-HTTLPR*-affective disorder relationships in specific categories of patients suffering from somatic comorbidities. For example, LL genotype was found to be associated with the mean depression score in patients with coronary artery disease (Meyer et al. 2020). Surprisingly, a recent meta-analysis has identified S allele as a predictive factor for developing depression among Asian and White patients with coronary artery disease (Zhang et al. 2020).

Several mechanisms explaining the consequences of L allele as a risk factor for ADs have been suggested. For example, the literature showed reduced activation of the amygdala in L homozygotes compared to S carriers (Hariri et al. 2002). Furthermore, reduced amygdala volume and psychopathy scores were presented with the correlations strongest for affective and interpersonal symptoms (Yang et al. 2009). Other findings related to L allele, such as the impact on blood flow in the amygdala, cortisol response to stress, serotonin transport activity, neuropsychological functions were reviewed by Glenn (2011). Moreover, L allele was associated with increased gene transcription, 5-HT transporter level, or its reuptake activity (Lesch et al. 1996), with lower extracellular 5-HT levels expected. Interestingly, a recent animal study has described an association between increased SERT expression in specific brain regions and anxiety-like behavior reversed by the administration of a selective serotonin reuptake inhibitor (Quah et al. 2020). Unexpectedly, this phenomenon was gene independent. Additionally, no relationship between *5-HTTLPR* (ins/del) and AD risk (Mohamed Saini et al. 2012) was reported or S allele was found as more predictive for disorder development (Miozzo et al. 2020).

Our study also showed a significantly increased frequency of L allele in male cases compared to male controls, but the result was not confirmed by logistic regression. The possible underlying mechanisms conferring on gender-based associations between genetic variations and diverse psychiatric disorders, including ADs were outlined by some authors (Wang et al. 2014; Zhao et al. 2020). For instance, sex-specific differences in 5-HT signalling (e.g., 5-HT synthesis, the number of nerve cells, 5-HTT gene expression, serotonin transporter degradation), different brain morphology or different responsiveness to environmental factors might have a role in this phenomenon.

In our study, the focus was also on the link between *BDNF* (rs6265) and the risk of various ADs. Initially, we found a significant deviation from HWE in the distribution of the *BDNF* genotypes for the cases. However, in case-control studies, it is recommended to test the deviation from HWE only in the control group (Ziegler et al. 2010). Additionally, other researchers reported HWE disequilibrium for rs6265 in patients with major depression (Aldoghachi et al. 2019). Furthermore, there was no deviation from HWE for the 5-*HTTLPR* (ins/del) tested in the same sample of cases in our study. Next, we identified significant associations between rs6265 and disorder risk in gender-stratified analysis. Compared with controls, a significantly higher frequency of TT (MetMet) genotype was found in male cases with any type of AD, as well as with DD and UD. The data obtained are consistent with the hypothesis that T allele could be implicated in the higher risk of psychiatric disorders due to reduction of trafficking and secretion of BDNF, reduced synaptic transmission, cortical plasticity, and decreased extracellular protein levels (Pattwell et al. 2012). Furthermore, low plasma or serum levels of BDNF were identified in patients suffering from ADs (Aldoghachi et al. 2019; Suliman et al. 2013; Schröter et al. 2020). Conversely, a recent twin study has failed to confirm the relationship between low BDNF levels and DD (Ottesen et al. 2020). Our results are consistent with the prior findings from meta-analysis aimed at the study of rs6265 in patients with major depression (Verhagen et al. 2010). The authors implicated T allele and TT genotype as potential risk factors of the disorder only in males. No significant association was found in the total sample of cases with ADs and females, as well as in ethnicity-stratified analysis. We should mention that a higher frequency of T allele was identified in male cases compared to females in our study, but this difference reached significance only in the total sample. In contrast to our study, the meta-analysis demonstrated comparable allele distribution among both genders (Verhagen et al. 2010). The gender-related effect of rs6265 on depression risk might be explained by the action of gonadal hormones.

It has been justified that estrogens have positive regulatory effects on BDNF levels and signalling (Chan and Ye 2017). Thus, we suppose that estrogens can mitigate the negative effect of T allele in females. Overall, sex differences in BDNF signalling and function are elucidated in several articles (Verhagen et al. 2010; Chan and Ye 2017; Tsai 2018). On the other hand, later meta-analyses found no association between rs6265 and major depression (Gyekis et al. 2013) or the effect of the tested genetic variation was moderated by stress events (Zhao et al. 2018). Conversely, a recently published case-control study has demonstrated positive relationships between T allele and the risk of major depression (Aldoghachi et al. 2019). In the other study, T allele was associated only with the severity of depression (Losenkov et al. 2020). Although an animal study identified a relationship between MetMet genotype and increased risk of anxiety-related behavior, we found no impact of rs6265 on ANXD susceptibility. As demonstrated in a meta-analysis, BDNF plays an important role mainly in obsessive-compulsive disorder (Suliman et al. 2013). However, specific analyses aimed at subtypes of ANXD were not conducted because of the small sample size. Overall, inconsistent findings from case-control studies evaluating the role of rs6265 in AD development are usually explained by the existence of gene-environment (Zhao et al. 2018) or gene-gene relationships (Nestor et al. 2019). Next, some studies reported changes in BDNF pathway affected by taking pharmacotherapy (Chen et al. 2010). Other factors have been also outlined, such as the activity of the disorder (acute *vs.* chronic phase), sample size, group differences (e.g., mean age), errors in the selection or implementation of laboratory techniques, even effects of exercise (Ottesen et al. 2020; Szuhany and Otto 2020).

To elucidate the contradictory results from univariate gene association studies, we also examined *5-HTTLPR-BDNF* combination in predicting the risk of ADs. We found that the probability of a male case with AD to be simultaneously a LL or LS (*5-HTTLPR*) and CT or TT (*BDNF*) carrier was significantly higher compared with controls. However, this significant association was subsequently confirmed only in the subgroup of male cases suffering from ANXD. In addition, in the total sample, the combination of SS (*5-HTTLRP*) and TT (*BDNF*) was associated with a lower risk of any type of AD, as well as ANXD. In the same line, a recent gene-gene study has pointed out the importance of specific allelic combinations of 5-*HTTLPR* and *BDNF* to the risk of depression or anxiety symptoms (Nestor et al. 2019). The combined effect of *BDNF*-T allele with S allele of *5-HTTLPR* showed a protective effect, while the interaction of T allele with L allele increased the risk of psychopathology. Moreover, the results were independent of adverse childhood experiences. On the other hand, a prior German analysis supported gene-gene-environment hypothesis. In case of childhood neglect, participants carrying SS and CC genotypes had the highest risk of DD compared to carriers of T and S alleles (Grabe et al. 2012). The L allele increased the risk of the disorder in combination with at least one T allele. In adolescents, the simultaneous presence of several combinations of *5-HTTLPR* and *BDNF* (SS+CT; L allele+CC and SS+CC) but not (L allele+TT, SS+TT and L allele+CT) led to fewer depressive symptoms in case of higher levels of positive parenting (Wang et al. 2020). The importance of the evaluation of gene-gene interactions in relation to ADs was also supported by other researchers (Kostic et al. 2016).

Overall, we suggest that inconsistent, even contradictory findings in univariate or multivariate analyses of the tested genetic variations in relation to AD risk could be a consequence of low sample size, different phenotypic heterogeneity of ADs, different classifications of affective disorders, as well as the diversity of the population sample. Moreover, it seems evident that the effects of genetic variations are likely to be much more complex. The more complex pathogenesis of psychiatric disorders is also supported by the fact that the incidence of any type of AD is lower than the frequency of risk genotypes or alleles.

Although the main strength of our research was that all analyses were performed on the same population sample, several limitations of this study must be underscored. Firstly, the major drawback of the study was the small sample size in the subgroup of patients with ANXD. Thus, our results must be interpreted with caution. Secondly, a lack of analysis of additional genetic variations influencing the functionality of *5-HTTLPR* (ins/del) alleles could be another limitation. Hence, mainly genotyping for the nearby genetic variation rs25531 (1936A>G, MAF in European population = 0.026; "triallelic marker" – S, L_A and L_G) located within the L allele of *5-HTTLPR* must be performed in the next larger studies to elucidate the real role of the long allele in psychiatric disorders. Previous research indicated that the L_G variant functions similarly to the short allele (Praschak-Rieder et al. 2007). However, inconsistent conclusions were observed in gene association studies. Surprisingly, several studies evaluating the Caucasian population revealed that L_AL_A carriers can have higher rates of depressive and anxiety disorders or more depressive symptoms (Laucht et al. 2009; Warnke et al. 2020). On the other hand, a meta-analysis evaluating *5-HTTLPR* in relation to antidepressant response reported a predictive value of biallelic but not triallelic genetic variation (Ren et al. 2020). Furthermore, a meta-analysis suggested an increasing prevalence of depression over time in the general population (Moreno-Agostino et al. 2021). Thus, a lack of gene-environment interactions could be another limitation. In addition, assessing the impact of hormone levels, concurrent diseases, or receiving pharmacotherapy on the gene-AD relationship could be beneficial.

Conclusions

Our findings suggest that both genetic variations *5-HTTLPR* (ins/del) and *BDNF* (rs6265) might have a role in the development of affective disorders. The LS and LL genotypes (*5-HTTLPR*) were predictive risk markers rather for ANXD than for DD, while TT (*BDNF*) genotype mainly increased the risk of AD in male individuals. However, in gene-gene analysis, the effect of T allele was affected by the *5-HTTLPR* background. The LL or LS and CT or TT genotype combinations had a risk-enhancing effect on AD susceptibility (mainly ANXD in males), while SS (*5-HTTLPR*) and TT (*BDNF*) combination had a protective effect on AD risk (mainly ANXD). It seems that the tested genetic variations might play more important role in males than in females.

Overall, the translation of lab data into clinical practice represents a great challenge. It has been hypothesized that the understanding the genetic risk factors for affective disorders could help identify risk patients in the early stages, prevent disorder progression, or even use pharmacogenomics-guided interventions of these disorders, which generally may provide a new, more targeting strategy to tackle this serious socio-economic problem. Thus, larger multicenter prospective studies are needed to elucidate the contradictory implications of gene association studies performed in patients suffering from diverse types of affective disorders. Moreover, the evaluation of additional risk factors could be beneficial in such studies to fully understand the role of the tested genetic variations and their combinations.

Conflict of interest. None.

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