

## **The role of genetic variability in the GABRA6, 5-HTT and BDNF genes in anxiety-related traits.**

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## **Abstract**

**Objective:** The aims of this study were to test the individual association of the 5-HTT, BDNF, and GABRA6 genes with anxiety-related traits and to explore putative GxG interactions in a healthy sample. **Method:** A sample of 937 individuals from the general population completed the TCI questionnaire; a subsample of 553 individuals also filled in a brief version of the NEO inventory. The whole sample was genotyped for the 5-HTTLPR polymorphism (5-HTT gene), the Val66Met polymorphism (BDNF gene) and the T1521C polymorphism (GABRA6 gene). **Results:** Individuals carrying the TT genotype of the T1521C polymorphism presented slightly higher scores for Harm Avoidance ( HA ) than C allele carriers ( $F=2.96$ ,  $p=0.051$ ). In addition, there was a significant GxG interaction on HA between the 5-HTTLPR and Val66Met polymorphisms ( $F=3.4$ ,  $p=0.009$ ). **Conclusion:** GABRA6 emerges as a putative gene may be involved in the variability of HA. The effect of a significant GxG interaction between the 5-HTT and BDNF genes on HA could explain part of the genetic basis underlying anxiety-related traits.

**Key Words:** harm avoidance, 5-HTT gene, BDNF gene, GABRA6 gene.

### **Significant outcomes**

- A complex gene–gene interaction between the 5-HTT and BDNF genes explained some of the variability in the harm avoidance dimension.
- Genetic variability related to the gabaergic system is associated with the harm avoidance dimension in a sample of the Spanish general population.
- The harm avoidance dimension of the TCI questionnaire is more strongly supported by genetic components than the neuroticism dimension of the NEO inventory.

### **Limitations**

- The two Spanish populations analyzed presented differences with respect to the genotype distribution of the Val66Met polymorphism (BDNF gene).
- Neuroticism data from the NEO inventory were only available for the sample from Castelló.

## Introduction

Anxiety-related traits that are continuously distributed in the normal human personality (1) have been described as individual differences in emotional reactivity, proneness to worry and susceptibility to negative affect. They are mainly captured by the presence of neuroticism (2, 3) or harm avoidance (4). Neuroticism (N) includes traits such as anxiety, anger, hostility, depression, self-consciousness, impulsiveness and vulnerability, while harm avoidance (HA) characterizes individuals with high scores for being cautious, tense, apprehensive, fearful, inhibited, shy, easily fatigued and worried. Both N and HA have been considered as markers for vulnerability to depressive disorders (5-8).

Twin studies have suggested that the estimated heritability for personality traits ranges between 30 and 50% (9). Specifically, these studies on the genetics of personality have demonstrated that the genetic component of anxiety-related traits accounts for 40 to 60% of the observed variance (10, 11).

There is growing interest in the idea that neurotransmitter functions relate to normal variations in personality traits (12, 13). In this sense, serotonin neurotransmission (5-HT) has a fundamental role in the modulation of emotional behaviour and in brain development. Genetic variability associated with 5-HT function is likely to influence behavioural predispositions such as anxiety-related traits (14, 15). The serotonin transporter (5-HTT) has a key role in 5-HT neurotransmission, since it is the main reuptake mechanism and the main biological target for antidepressant drugs. 5-HTT is encoded by the SLC6A4 gene, which contains an insertion/deletion in the 5' promoter region of 44bp (5-HTTLPR), with reduced transcription of the 5-HTTLPR short (S) allele in comparison with the long L allele (16, 17).

The seminal study carried out by Lesch and collaborators (1996) showed an association between anxiety-related traits and the 5-HTT gene (17). Although inconsistencies have emerged as shown in a recent meta-analysis (see review (18, 19)), one recent meta-analysis has shown the definite effect of variability at 5-HTT gene on depression and stress sensitivity (20).

Besides the 5-HTT gene, the brain-derived neurotrophic factor (BDNF) from the neurotrophin family could be of special interest because of its critical role in normal adaptive responses to stress as well as in the response to antidepressant treatment (21, 22). The BDNF gene (chromosome 11p14) presents an SNP (196 G/A -Val 66 Met) located in the 5' pro-BDNF precursor peptide sequence that may affect intracellular processing and secretion of the mature protein (23). Sen et al. (2003) reported that the Met allele was associated with lower levels of neuroticism (24), although later studies failed to replicate such findings (25-27). Recently, the Met allele of the Val66Met polymorphism at the BDNF gene has been shown to play a putative role in anxiety disorders. Met carriers showed impaired learning of cues that signal safety versus threat that rely on extinction mechanisms (28).

Another neurotransmitter that might be involved in the propensity for a fearful or anxious temperament is  $\gamma$ -aminobutyric acid (GABA), the main inhibitory neurotransmitter in the CNS (29, 30). The inhibitory effect of GABA is mediated by GABAA receptors, which are ionotropic GABA-gated chloride channel receptors.

GABA acts as an agonist by inducing conformational changes in the GABA<sub>A</sub> receptor, increasing the permeability of the central pore to chloride ions. The chloride influx hyperpolarizes the neuron, reducing its excitability and having a general inhibitory effect on neuronal activity. In addition, benzodiazepines, one of the pharmacological treatments for anxiety disorders, increase the efficiency of inhibitory GABAergic neurotransmission by means of agonists binding to the receptor GABA<sub>A</sub>.

It has been suggested that individual differences in the frontal cortical GABA<sub>A</sub> receptor complex and GABA systems could modulate patterns of brain activity associated with individual differences in threat-related responses (29). Uhart and collaborators (2004) analysed a single-nucleotide polymorphism (SNP) consisting of a T-to-C substitution (position 1521) in the 3' non-coding region of the GABA<sub>A</sub>α6 receptor subunit gene GABRA6 (cr. 5q34) in a healthy population (31). The results revealed that homozygotes for the C allele showed an attenuated hormonal and physiological response to acute psychological stress. Therefore, this polymorphism could be involved in the individual differences underlying part of the biological basis of anxiety-related traits.

## Materials and Methods

### Sample

Our sample consisted of 937 subjects from the general population (47.8% males; total mean age=30.5, SD=12.2) who were recruited from the campus of the University Jaume I (Castelló, Spain) and from primary care settings from Oviedo (Asturias, Spain).

In terms of education 12.2% of individuals had completed elementary school, 56.8% had completed high school, and 25% had received a university education. Sociodemographic data divided by the geographic origin of the samples (Castelló/Asturias) are displayed in Table 1.

**Table 1.** Sociodemographic data according to geographic origin

Geographic origin	Gender distribution *	Mean age (SD; range)**	Education ***
Castelló (Comunitat Valenciana) (n=533)	242 males 291 females	22.9 (5.4; 18-55)	Elementary 14 (3%) High School 424 (84%) University 67 (13%)
Oviedo (Asturias) (n=404)	202 males 202 females	40.5 (11.3; 20-60)	Elementary 104 (26%) High School 125 (31%) University 175 (43%)

\* $\chi^2=1.95$   $p=0.163$ ; \*\* $F=996.8$   $p<0.0001$ ; \*\*\* $\chi^2=271.8$   $p<0.0001$

Exclusion criteria were the presence of any past or present major psychiatric disorder and/or a history of any severe mental disorder in first-degree relatives. These aspects were screened by means of a short interview designed ad hoc for this study on the basis of selected items of structured scales such as SCID-I (32) and FIGS (33). All participants were of Spanish (Caucasian) ancestry to reduce the possibility of

confounding genetic differences by population stratification (34).

Ethical approval was obtained from local Spanish research ethic committees in each Institution. All participants provided written informed consent before inclusion in the study.

## ***Measurements***

### **Personality assessment**

All participants filled out the self-reported Temperament and Character Inventory (TCI;(35)) composed of 240 items. Given the aims of this study, the analyses focused on the dimension of Harm Avoidance (HA), which has four subscales: Anticipatory worry (HA1), Fear of uncertainty (HA2), Shyness with strangers (HA3) and Fatigability (HA4). Good psychometric properties have been described for TCI (35).

In addition, 533 individuals completed the Brief Big Five Questionnaire (BFQ; (36)). This test has high test-retest reliability and longitudinal stability (37). The analyses focused on the dimension of Neuroticism (N). Subscales corresponding to the N dimension were not available.

### **Laboratory methods**

Genomic DNA from the Castelló subsample was extracted from saliva samples using the Collection Kit BuccalAmp DNA extraction kit (Epicentre® Biotechnologies, Madison, WI) and that from the Oviedo subsample was extracted from blood samples using the salting-out technique (38). The 5-HTTLPR polymorphism of the serotonin transporter gene was analyzed using the protocol previously described by Lesch and collaborators (1996) (17). The SNP rs6265 (Val66Met) of the BDNF gene and the SNP rs3219151 (T1521C) of the GABRA6 gene were genotyped using Applied Biosystems (AB) TaqMan technology. An AB assay-on-demand service supplied the probes.

Those individuals who were not genotyped were not included in the final statistical analyses (see Table 2 for final sample).

Random individuals were re-genotyped in order to confirm the reproducibility of the pattern.

### **Statistical analyses**

Analyses were performed using STATA 9.1 (39) and EpiInfo (40).

The main effects of polymorphisms on harm avoidance and neuroticism were analyzed separately for each of the following polymorphisms: the 5-HTTLPR genotype, BDNF genotype, and GABRA6 genotype using linear regression analysis. Regressions were performed for the harm avoidance dimension and separately for each individual subscale of harm avoidance. The Wald test was performed to test the overall main effect of each polymorphism on each dimension and subscale.

The xi3 command was then used to test interactive effects with categorical predictors

(41). The interactive effects between SLC6A4 5-HTTLPR and BDNF Val66Met, between SLC6A4 5-HTTLPR and GABRA6, and between BDNF Val66Met and GABRA6 T1512C were fitted in models of harm avoidance and neuroticism. The Wald test was used to assess the interaction effect. When a two-way interaction was significant, further simple effects were assessed, that is, the effect of VI1 at each level of VI2.

All regression analyses were controlled for age, gender and demographic origin.

## Results

The genotype, allele distribution and Hardy-Weinberg equilibrium of each population (Castelló and Oviedo) as well as those of the total sample are shown in Table 2.

**Table 2.** Genotype distribution of the three polymorphisms analysed according to geographic origin

<b>5-HTTLPR polymorphism (SLC6A4 gene)</b>					
	<i>Genotype distribution</i>			<i>Allele distribution</i>	
	L/L	L/S	S/S	L allele	S allele
<b>Castelló (n=475)</b>	119 (25.1%)	230 (48.4%)	126 (26.5%)	468 (49.2%)	482 (50.8%)
<b>Oviedo (n=404)</b>	122 (30.2%)	192 (47.5%)	90 (22.3%)	436 (53.9%)	372 (46.1%)
<b>Total sample (n= 879)</b>	241 (27.4%)	422 (48%)	216 (24.6%)	904 (51.4%)	854 (48.6%)
<b>**</b>	$\chi^2=3.75, df=2, p=0.15$			$\chi^2=3.86, df=1, p=0.05$	
<b>Val66Met polymorphism (BDNF gene)</b>					
	<i>Genotype distribution</i>			<i>Allele distribution</i>	
	Val/Val	Val/Met	Met/Met	Val allele	Met allele
<b>Castelló (n=470)</b>	282 (60%)	159 (33.8%)	29 (6.2%)	723 (76.9%)	217 (23.1%)
<b>Oviedo (n=385)</b>	269 (69.9%)	106 (27.5%)	10 (2.6%)	644 (83.6%)	126 (16.4%)
<b>Total sample (n=855)</b>	551 (64.4%)	265 (31%)	39 (4.6%)	1367 (79.9%)	343 (20.1%)
<b>**</b>	$\chi^2=11.83, df=2, p<0.01$			$\chi^2=11.93, df=1, p<0.01$	
<b>T1512C polymorphism (GABRA6 gene)</b>					
	<i>Genotype distribution</i>			<i>Allele distribution</i>	
	T/T	T/C	C/C	T allele	C allele
<b>Castelló (n=481)</b>	161 (33.5%)	236 (49.1%)	84 (17.5%)	558 (58.1%)	404 (41.9%)
<b>Oviedo (n=370)</b>	120 (32.4%)	193 (52.2%)	57 (15.4%)	433 (58.5%)	307 (41.5%)
<b>Total sample (n=851)</b>	281 (33%)	429 (50.4%)	141 (16.6%)	991 (58.2%)	711 (41.8%)
<b>**</b>	$\chi^2=1, df=2, p=0.61$			$\chi^2=0.04, df=1, p=0.83$	

\* Frequencies were in Hardy-Weinberg equilibrium for the Castelló subsample (5-HTTLPR polymorphism  $\chi^2=0.24, p=0.88$ ; Val66Met polymorphism  $\chi^2=0.52, p=0.77$ ; T1512C polymorphism  $\chi^2=0.02, p=0.99$ ); the Oviedo subsample (5-HTTLPR polymorphism  $\chi^2=0.36, p=0.83$ ; Val66Met polymorphism  $\chi^2=0.00, p=0.99$ ; T1512C polymorphism  $\chi^2=1.06, p=0.59$ ); and for the total sample (5-HTTLPR polymorphism  $\chi^2=0.70, p=0.70$ ; Val66Met polymorphism  $\chi^2=0.51, p=0.76$ ; T1512C polymorphism  $\chi^2=0.60, p=0.74$ ).

\*\* Statistical results derived from the comparison between the Castelló and Oviedo subsample distributions.

The genotypic frequencies that we observed in these Spanish populations were similar to those frequencies described for European Caucasians (26, 31, 42, 43).

The two populations presented similar genotype and allele distributions for the 5-HTTLPR polymorphism (5-HTT gene) and for the T1512C polymorphism (GABRA6 gene). However, the genotype and allele distribution differed between Castelló and Oviedo for the Val66Met polymorphism (BDNF gene) (genotype frequencies:  $\chi^2=11.83, df=2, p=0.003$ ; allele frequencies:  $\chi^2=11.93, df=1, p<0.001$ ). These differences were taken into account when we merged the samples for subsequent statistical analyses.

We found no significant associations between the 5-HTTLPR (5-HTT gene) or Val66Met (BDNF gene) polymorphisms and HA or its subscales.

The effect of GABRA6 T1512C polymorphisms on HA approached statistical significance, that is, the TT group had higher scores for HA than TC or CC subjects ( $F=2.96$ ,  $p=0.051$ ). In addition, a significant main effect of the GABRA6 T1512C polymorphism was found on HA1 (Anticipatory worry) ( $F=4.07$ ;  $p=0.017$ ). Specifically, the TT group had higher scores compared to TC or CC. No significant effect was found on the other subdimensions (see Table 3).

**Table 3.** Mean scores (SD) and results (Wald test) per polymorphism analysed in the whole sample for Harm Avoidance and its subscales (n=937).

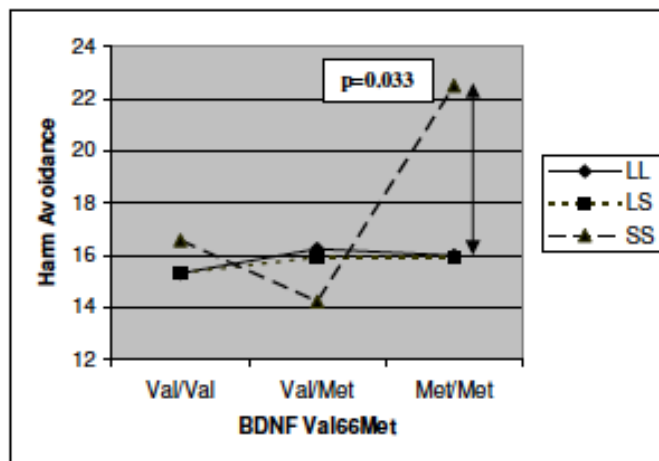
	5-HTTLPR polymorphism				BDNF Val66Met polymorphism				GABRA6 T1512C polymorphism			
	LL (n = 241)	LS (n = 422)	SS (n = 216)	F	Val/Val (n = 551)	Val/Met (n = 265)	Met/Met (n = 39)	F	TT (n = 281)	TC (n = 429)	CC (n = 141)	F
<b>Harm Avoidance</b>	15.6(6.25)	15.5(6.06)	15.9(6.51)	0.52	15.57(6.09)	15.47(6.44)	17.07(7.33)	0.13	16.29(6.60)	15.31(6.34)	15.21(6.33)	<b>2.96*</b>
<b>HA1</b>	4.24(2.49)	4.19(5.59)	4.16(2.69)	0.00	4.17(2.57)	4.14(2.67)	4.76(2.95)	1.38	4.58(2.75)	4.05(2.59)	4.07(2.66)	<b>4.07**</b>
<b>HA2</b>	4.22(1.91)	4.16(1.89)	4.25(1.89)	0.38	4.22(1.89)	4.17(1.91)	4.32(1.96)	0.34	4.34(1.99)	4.18(1.93)	4.08(1.87)	2.10
<b>HA3</b>	3.58(2.14)	3.55(2.06)	3.82(2.00)	1.71	3.59(2.08)	3.63(2.10)	4.12(2.17)	2.01	3.69(2.05)	3.56(2.23)	3.60(2.08)	0.39
<b>HA4</b>	3.52(1.92)	3.61(1.89)	3.69(1.89)	0.14	3.59(1.89)	3.54(1.86)	3.88(1.79)	0.60	3.69(1.84)	3.52(1.96)	3.46(1.96)	1.23

\* $p=0.051$ ; \*\* $p=0.017$  (All analyses controlled for age, gender and geographic origin ) **Abbreviations:** HA1 (Anticipatory worry); HA2 (Fear of uncertainty); HA3 (Shyness with strangers); HA4 (Fatigability)

### Gene and anxiety-related trait interactions

We found a significant two-way interaction in relation to HA. An interaction between the 5-HTTLPR and BDNF Val66Met polymorphisms modulated the HA scores ( $F=3.4$ ,  $p=0.009$ ). Analysis of simple effects showed that subjects with Met/Met and S/S genotypes had higher scores on HA compared to Met/Met-LS or Met/Met-LL ( $F=3.43$ ,  $p=0.033$ ) (see Figure 1). No other two-way interactions were significant with regards to HA.

**Figure 1.** The effect of the BDNF Val66Met polymorphism on HA is moderated by the 5-HTTLPR polymorphism. Met-carriers with the S/S genotype (n=6) showed higher scores for HA compared to LS (n=23) or LL (n=16).





We found no association between either of the polymorphisms analyzed and N, or any gene–gene interaction effect on N.

## Discussion

With regards to the main effect of the 5-HTTLPR polymorphism (5-HTT gene) on anxiety-related traits, we did not succeed in replicating the previously reported association between the S allele and high anxiety-related traits reported by Lesch et al. (1996) (17). In contrast, our results agree with most of the studies that have reported negative results in Caucasian populations (44-51).

It has been suggested that important sources of heterogeneity between studies could be due to the use of slightly different personality constructs, the inclusion of psychiatric populations, or the use of small sample sizes (52). In the present study, both neuroticism and harm avoidance were explored; in addition, the whole sample was screened for any current or lifetime psychiatry disorder and, finally, the sample size was larger than in previous studies (44-51).

The Val66Met polymorphism (BDNF gene) has previously been associated with anxiety-related traits by Sen et al. (2003) (24). The study revealed that Val allele carriers presented higher levels of neuroticism compared to Met allele carriers. However, later studies, in agreement with our findings, did not detect this independent effect in relation to those personality traits (25-27).

Interestingly, a gene–gene interaction was detected between the 5-HTTLPR polymorphism (5-HTT gene) and Val66Met polymorphism (BDNF gene): among individuals with the Met/Met genotype, carriers of the SS genotype exhibited significantly higher scores for harm avoidance than L allele carriers. This gene–gene interaction may underlie the inconsistencies found in studies exploring only the main effects of one of these genes and anxiety-related traits.

The results of our study provide evidence for a role of the GABRA6 T1512C polymorphism in harm avoidance variability in a large sample from the general population. Specifically, the TT genotype of this polymorphism seemed to be strongly associated with high scores on the subscale “anticipatory worry” (HA1). These results together with the results reported by Uhart et al. (2004)(31) suggest that the GABRA6 T1512C polymorphism, in accordance with our hypothesis, is strongly associated with individual differences in anxiety-related traits, specifically in those traits that are closely related to the anxiety spectrum, such as anticipatory worry. However, we know that this polymorphism located at 3' region do not seem to display functional relevance. As  $\alpha 6$  subunit is included into a cluster in chromosome 5q with  $\alpha 1$ ,  $\beta 2$  and  $\alpha 2$  subunit genes (53), the analyzed polymorphism may be in linkage disequilibrium with another site within the GABRA6 gene or these genes located in close proximity. Moreover, GABA<sub>A</sub> receptors are classified as ionotropic receptors which are involved in fast events in the neuron, as opposite to other type of receptors (such 5-HTT transporter) that mediate more long-term effects modifying the responsiveness and plasticity of the neuron (54). We could hypothesized that subtle genetic changes in this fast-response receptors such as this SNP, may alter the signal transmission in the neuron impacting, then, in the final anxiety related-phenotype.

Finally, our results seem to identify genetic components involved in harm avoidance, derived from Cloninger's tridimensional theory of personality. However, negative results were found for the neuroticism dimension derived from the Big Five model developed by Costa and McCrae. These results are consistent with a recent metaanalysis reporting the existence of a small, but significant genetic background for the harm avoidance dimension but not for the neuroticism dimension (19). Taken together, these findings suggest that the HA construct seems to be more strongly associated with genetically related traits than the neuroticism construct. Nevertheless, further research is needed to elucidate the complex biological and genetic architecture of anxiety-related traits.

Our study has some limitations. Firstly, we found differences between the Castelló and Oviedo populations with respect to the genotype distribution of the Val66Met polymorphism (BDNF gene); these differences were basically related to an increase in the frequency of the Val/Val genotype in the subsample from Oviedo. However, when we merged the two populations, the genotype frequencies were similar to those expected in a Caucasian population (26). Thus, it is unlikely that the significant results obtained were due to a bias in the genotype distribution. In fact, the effect of interaction between 5-HTT and BDNF on HA was found with the Met allele and not with the Val allele, as might be expected due to the overrepresentation of this allele in the Oviedo sample.

With a sample of almost a thousand individuals, our study was larger than previous studies (44-51), and therefore the likelihood of Type I or II errors was reduced. However, some of our findings might not have been statistically significant if multiple testing corrections had been carried out. These corrections are likely to be excessively exclusive in the context of the present study since the selection of the genetic polymorphisms, the sample size and the analyses performed had a directional hypothesis based on previous findings (55).

In conclusion, the GABRA6 T1512C polymorphism has emerged as a putative genetic factor involved in human anxiety-related trait variability, specifically HA. Moreover, the effect of a significant GxG interaction between the 5-HTT gene and BDNF gene on HA could partly explain some of the inconsistencies found in previous studies and should be taken into account in the understanding of individual differences in complex constructs such as anxiety-related traits.

### **Disclosure/Conflicts of interest**

The author(s) declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest

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