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Promoter Variants of the Cannabinoid Receptor 1 Gene (CNR1) in Interaction With *5-HTTLPR* Affect the Anxious Phenotype

Judit Lazary,[1] Aron Lazary,[2,3] Xenia Gonda,[1,4] Anita Benko,[1] Eszter Molnar,[1,5] Laszlo Hunyady,[6] Gabriella Juhasz,[7] and Gyorgy Bagdy[1,5,8]*

[1]Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

[2]1st Department of Internal Medicine, Semmelweis University, Budapest, Hungary

[3]National Center for Spinal Disorders, Buda Health Center, Budapest, Hungary

[4]Kutvolgyi Clinical Center, Semmelweis University, Budapest, Hungary

[5]Department of Pharmacodynamics, Semmelweis University, Budapest, Hungary

[6]Department of Physiology, Semmelweis University, Budapest, Hungary

[7]Neuroscience and Psychiatry Unit, School of Community Based Medicine, Faculty of Medical and Human Sciences, The University of Manchester, England

[8]Group of Neurochemistry and Group of Neuropsychopharmacology, Semmelweis University and Hungarian Academy of Science, Budapest, Hungary

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Anxiety is a polygenic condition, and the recently discovered Endocannabinoid System (ECS) is one plausible candidate. Experimental data suggest that the ECS can modulate several neurotransmitter systems, including the serotonergic system, which itself plays a significant role in anxiety. However, to date there is no evidence of gene–gene interactions; indeed genetic studies focusing separately on the two systems provide conflicting data. Thus, the aim of our study was to analyze the interaction of the promoter regions of the serotonin transporter *(SLC6A4)* and cannabinoid receptor 1 (*CNR1*) genes on anxiety. We genotyped 706 individuals for the *5-HTTLPR* in the *SLC6A4* promoter and 4 SNPs located in the *CNR1* promoter region. Anxiety was measured by the State-Trait Anxiety Inventory (STAI-S, STAI-T), the anxiety subscale of TEMPS-A (TEMPS-Anx), and the Brief Symptom Inventory (BSI-Anx). Significant *5-HTTLPR* x *CNR1* promoter-promoter interaction was observed using STAI-T ($P = 0.0006$) and TEMPS-Anx ($P = 0.0013$). The risk of high anxiety scores on BSI-Anx was 4.6-fold greater in homozygous 'GG' *rs2180619* in combination with homozygous 'SS' *5-HTTLPR* ($P = 0.0005$) compared to other genotypes. The effect of previously described "TGC" haplotype in the alternative promoter of CNR1 depended both on the conventional promoter polymorphism and the 5-HTTLPR. Our haplotype and putative transcription binding profile analyses strongly suggest that certain constellations of CB1-receptor and 5-HTT promoters yield extremely high or low synaptic 5-HT concentrations, and these are associated with an anxious phenotype. In conclusion, genetically determined serotonergic and endocannabinoid dysfunctions could lead to a vulnerability causing anxiety disorders and possibly depression.

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INTRODUCTION

Although anxiety is one of the most common psychiatric disorder with significant heritable components, its exact pathomechanism and genetic background are still not known. There is a large body of data about the anxiolytic effect of low dose exogenous *Cannabis sativa* derivate [Navarro et al., 1997; Haller et al., 2002; Rodgers et al., 2003], and the evidence that cannabinoid use was frequently

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*Correspondence to:
Gyorgy Bagdy, Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Semmelweis University, Nagyvárad tér 4, 1089 Budapest, Hungary. E-mail: bag13638@iif.hu

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motivated by its positive effect on anxiety [Sethi et al., 1986; Stewart et al., 1997; Ogborne et al., 2000].

The recently discovered Endocannabinoid System (ECS) has been implicated in the pathomechanism of anxiety based on pharmacological and genetic studies [Martin et al., 2002; Kathuria et al., 2003; Haller et al., 2004; Bortolato et al., 2006; Moreira et al., 2007].

The cannabinoid receptor-1 (CB1) is abundantly expressed in the regions of the central nervous system involved in anxiety; and due to its constitutive activity and the presence of common ligands, such as 2-arachydonyl-glycerol, the activity of the receptor modulates neuronal function in many physiological regulations including also behavior [Freund et al., 2003; Pertwee, 2005; Turu et al., 2007]. The gene of the CB1 receptor (*CNR1)* located on chromosome 6 (6q14-15) is composed of 4 exons and besides the conventional 5′ promoter region an alternative one was described in the intron 2 [Zhang et al., 2004], but evidence for their role in anxiety is still poorly available [Lu et al., 2008].

Although selective serotonin reuptake inhibitors (SSRIs) are effective in the treatment of anxiety disorders, several details of the role of the serotonin transporter (5-HTT) in the pathomechanism of anxiety are still unclear. Despite the anxiolytic effect of SSRIs after chronic treatment, acute effects, especially in rodents, are anxiogenic [Bagdy, 1998; To and Bagdy 1999; Bagdy et al., 2001] and increased anxiety has been consistently described in 5-HTT knock out mice [Wellman et al., 2007]. The ''S'' allele of *5-HTTLPR*, a well-known functional polymorphism of the serotonin transporter gene (*SLC6A4*) promoter is associated with reduced transcriptional efficiency of the gene, resulting in decreased 5-HTT expression [Lesch et al., 1996]. Association studies between *5-HTTLPR* and anxiety yielded conflicting data [Lesch et al., 1996; Jorm et al., 1998; Mazzanti et al., 1998; Flory et al., 1999; Murakami et al., 1999; Seretti et al., 1999; Greenberg et al., 2000; Osher et al., 2000; Gonda et al., 2007; Wachleski et al., 2008] confirming the general view that anxiety is a multigenetic condition.

Neurotransmitter-induced Ca^{2+} signal generation leads to endocannabinoid release [Freund et al., 2003; Turu et al., 2009], and activation of the ECS negatively modulates the release of different neurotransmitters in multiple brain areas involved in cognition, memory, and mood regulation (e.g., hippocampus and the prefrontal cortex). Nakazi et al. reported that CB1 activation inhibits 5-HT release, and another in vivo study showed that CB1 antagonists increase 5-HT metabolites in the prefrontal cortex [Nakazi et al., 2000; Tzavara et al., 2003]. Several animal studies confirmed this biological interaction between the serotonergic system and ECS [Hermann et al., 2002; Darmani et al., 2003; Gobbi et al., 2005; Haring et al., 2007; Mato et al., 2007], but human evidence is lacking.

Altogether both systems are potential contributors to anxiety, however, studies focusing on them separately provided conflicting data. Since results from animal studies suggest that the two systems interact with each other, we hypothesized that their interaction at the genetic level could influence human anxiety. The aim of our study was to determine the association between the anxious phenotype and the functional polymorphisms of the promoter regions of *SLC6A4* and *CNR1* genes in a large Hungarian general population.

METHODS AND MATERIALS

Sample

Seven-hundred and six unrelated volunteers, 572 women and 134 men were included in the study. Participants were recruited from the practices of general practitioners, adult students participating in a long-distance learning program and community-based population. The inclusion of subjects was independent of any positive psychiatric anamnesis. The mean age of participants was 30.26 ± 10.62 years. We excluded those who were medicated with psychiatric drugs from the study based on background questionnaire. All subjects were Hungarian and of Caucasian origin and they gave written informed consent before entering the study. The study was approved by the Central Ethics Committee. The descriptive data of the study population are shown in Supplementary Table 1.

Phenotype Measures

As anxiety is a complex condition we measured it by four dimensions, state and trait anxiety with State-Trait Anxiety Inventory (STAI-S and STAI-T, respectively), anxious temperament (anxious subscale of the Temperament Evaluation of the Memphis, Pisa, Paris and San Diego-Autoquestionnaire, TEMPS-Anx), and current clinical status of anxiety (anxious subscale of the Brief Symptom Inventory, BSI-ANX).

The State-Trait Anxiety Inventory (STAI) is a 40-item, well-established self-report questionnaire developed by Spielberger [Spielberger, 1970]. It assesses ''trait'' and ''state'' anxiety as separate, dimensional scales. Both scales range from 0 to 4 with increasing intensity. State anxiety is defined as a transitory emotional condition characterized by tension, apprehension, and hyperactivation of the autonomic nervous system. Trait anxiety is characterized by a stable anxious tendency due to the tendency of the subject to perceive daily situations as threatening leading to an increase in the grade of anxiety.

The Brief Symptom Inventory (BSI) is a 26-item self-report symptom inventory developed from its longer parent instrument, the SCL-90-R designed to reflect the psychological symptom patterns of psychiatric and medical patients and non-patients. The 26-item brief version inventory reports profiles of obsessive-compulsive, interpersonal sensitivity, depression, anxiety, and additional items [Derogatis and Melisaratos, 1983]. Each item is scored on a 5-point scale ranging from 0 (not at all) to 4 (extremely). A case-control-like experiment was designed dividing the study population in two groups. Cut-off point was the median + 2SD of the anxious subscale score. Subjects with BSI-ANX score more than 1.8 were grouped in the high anxiety subgroup. The TEMPS-A questionnaire (Temperament Evaluation of the Memphis, Pisa, Paris and San Diego-Autoquestionnaire) measures affective temperaments. This is a 110 item (109 for males) self-report psychological instrument with subscales representing five affective temperament dimensions: depressive, cyclothymic, hyperthymic, irritable, and anxious [Akiskal et al., 2005]. In these studies we used only the anxious subscale (TEMPS-ANX).

The background questionnaire was adapted from the version developed by the Epidemiolgy Unit of the Universiy of Manchester. This well-structured self-rating questionnaire consists of 22 items

and collects detailed information about medical history including psychiatric history and medications, family psychiatric history and socio-economic background.

Genotyping

Buccal mucosa samples were collected from each subjects and genomic DNA was extracted using conventional phenol-chloroform extraction protocol. DNA quality and quantity was determined with NanoDrop B-100 spectrophotometer, and all samples were diluted to a DNA concentration of 20 ng/μl. For genotyping *5-HTTLPR*, the genomic region containing the polymorphism was amplified using 6-FAM labeled forward primer (Table I) as it was previously described [Lazary et al., 2008]. Four SNPs in *CNR1 (rs2180619, rs806379, rs1535255*, and *rs2023239*) were selected for genotyping based on the literature [Zhang et al., 2004]. *rs2180619* is located in the conventional 5′ promoter of the gene and it tags more than a 2 kbp long region. The other three SNPs *(rs806379, rs1535255*, and *rs2023239)* are located in the intron 2 which is described as an alternative promoter region of the gene [Zhang et al., 2004]. SNPs were genotyped at Center for Integrated Genomic Medical Research at The University of Manchester using the Sequenom® MassARRAY technology (Sequenom Inc., San Diego, CA, USA). The iPLEX™ assay, based on post-PCR single base primer extension, was performed according to manufacturer's instructions.

Statistical Analysis

Descriptive statistics like Hardy–Weinberg equilibrium, minimal allele frequency (MAF) and pair-wise linkage disequilibrium (LD) between genotyped polymorphisms were computed using Haploview 4.0 software [Barrett et al., 2005]. Single marker association studies were performed under the three common genetic models (additive, dominant, and recessive) using generalized linear models (GLM) in the "SNPassoc" R-package [Gonzalez et al., 2007]. All analyses were adjusted to age and gender and α-level was corrected with the number of the studied polymorphisms, thus *P*-values less than 0.01 were considered nominally significant.

Gene–gene interaction based on the polymorphisms (G × G) were tested with two different statistical methods. The "SNPassoc" software determines interaction effects performing log-likelihood ratio tests (LRT). The graphical output of this command visualizes the *P*-values of the interactions between each pair of polymorphisms and the examined trait. To avoid false positive results, only highly suggestive interactions characterized by a *P*-value less than 0.01 were selected and G x G interactions were validated under GLMs. Interactions with a $P < 0.01$ in the regression model were considered significant.

Haplotype analysis of the *CNR1* gene was performed in a subsample consisting of 669 subjects without any missing *CNR1* genotype to avoid false results in haplotype estimation. We tested the effect of the haplotype constructed by the three SNPs located in the alternate promoter as well as the haplotype built by the four *CNR1* SNPs using the THESIAS software [Tregouet and Garelle, 2007]. The program is based on the maximum likelihood model (LRT) and linked to the SEM algorithm. Interaction between the *SLC6A4* promoter polymorphism and *CNR1* promoter haplotypes was also investigated by entering the *5-HTTLPR* as a binomial (0 = "SL or LL" and 1 = "SS") covariate into the model. Individual effect of certain haplotypes was estimated (EPM i.e., estimated phenotypic mean was determined) and compared using LRT analysis. Rare haplotypes less frequent than 1% were excluded from the analyses.

Sequence Analysis

Genomic sequences of the regions next to the genotyped polymorphisms were obtained from the NCBI dbSNP database. Two sequences containing the two allelic variants of each SNP were used

TABLE I. Descriptive Statistics of Genotyped Plymorphisms in the *SLC6A4* and *CNR1* Genes

Marker	Alleles	Site	Genotype frequencies	MAF	HWE	Success	Primer sets[a]
5-HTTLPR	S/L	Promoter of *SLC6A4*	LL: 35.7%; SL: 48.6%; SS: 15.7%	40.0%	0.814	100%	f: 5-GCCAGCACCTAACCCCTAAT-3; e: 5-GTAGGGTGCAAGGAGAATGC-3
rs2180619	A/G	Conventional promoter of *CNR1*	AA: 33.6%; AG: 49.6%; GG: 16.2%	41.2%	0.437	99.4%	f: 5-ACAGGCATTTTTAGCCCACC-3; r: 5-AAGCAACAGATGTTGAAGCC-3; e: 5-GGCAGCGCAAGATTCAAA-3
rs806379	A/T	Intron 2 of *CNR1* (alternative promoter)	AA: 20.1%; AT: 48.6%; TT: 20.8%	45.4%	0.704	99.4%	f: 5-CCTAAATCGCAGAACTGATC-3; r: 5-GACTTACTTTTGTGTCAGGC-3; e: 5-CAGAACTGATCTGAAATTAGATGA-3
rs1535255	T/G	Intron 2 of *CNR1* (alternative promoter)	TT: 71.1%; GT: 27.1%; GG: 1.8%	15.4%	0.384	100%	f: 5-GATCAGTTCTGCGATTTAGG-3; r: 5-GATGGTACTTGGGCAATCAG-3; e: 5-CATCATCCTCATCCCC-3
rs2023239	T/C	Intron 2 of *CNR1* (alternative promoter)	TT: 71.6%; TC: 26.6%; CC: 1.8%	15.1%	0.369	96.9%	f: 5-GGGACACAGAAGACAGTCAC-3; r: 5-GGGAGTTGAAAGGCAAAAGC-3; e: 5-TTTATATATGAGAGAGCTGTTCCTTAC-3

P-value of χ^2 tests for Hardy–Weinberg equilibrium (HWE), minimal allele frequency (MAF) and genotyping success rate (success) are represented.
[a]Primer sets mean forward (f), reverse (r) and extension primers (e) used for genotyping

to search for putative transcription factor binding sites in the TRANSFAC database, using the PROMO web-based software [Messeguer et al., 2002].

RESULTS

Descriptive Statistics

All genotyped polymorphisms were in Hardy–Weinberg equilibrium and MAF was more than 5% in each case (Table I). A haploblock constructed by the three SNPs *(rs806379, rs1535255, rs2023239)* in intron 2 of *CNR1* was determined based on confidence intervals [Gabriel et al., 2002]. There were 59 (8.4%) individuals with high anxiety based on BSI-ANX score that matched point-prevalence of anxiety in the Hungarian population [Szadoczky et al., 1997]. Frequency of high anxiety did not differ significantly between men and women ($P = 0.174$). Means of STAI-S and STAI-T were not different significantly between men and women ($\text{STAI-S}_{\text{men}} = 38.08 \pm 11.886$ vs. $\text{STAI-S}_{\text{women}} = 38.03 \pm 11.291$; $P = 0.967$, $\text{STAI-T}_{\text{men}} = 39.22 \pm 10.678$ vs. $\text{STAI-T}_{\text{women}} = 40.45 \pm 10.130$; $P = 0.212$). Women scored significantly higher compared to men on TEMPS-Anx ($\text{TEMPS-Anx}_{\text{men}} = 0.2405 \pm 0.206$ vs. $\text{TEMPS-Anx}_{\text{women}} = 0.2945 \pm 0.199$; $P = 0.005$).

Pearson correlation tests between the independent scales showed that BSI-ANX correlated at fewest level with other scales ($r_{\text{STAI-S}} = 0.500$ $r_{\text{STAI-T}} = 0.597$, $r_{\text{TEPMS-Anx}} = 0.617$). TEMPS-Anx correlated stronger with STAI-T than STAI-S ($r_{\text{STAI-S}} = 0.496$, $r_{\text{STAI-T}} = 0.675$). All correlations were significant ($P < 0.001$).

Single Marker Associations

To assess single marker associations we analyzed the individual effects of each polymorphism on anxious phenotype. *5-HTTLPR* individually did not show any significant associations with STAI-S, STAI-T, TEMPS-Anx, and BSI-ANX in GLM. Significant associations between SNPs (*rs2180619, rs806379, rs1535255*, and *rs2023239*) of the *CNR1* gene and anxiety scores were also not observed (data not shown).

Interaction Analyses

Regarding evidence from animal studies about the potential cross-talk between the ECS and the serotonergic system, we tested the genetic interactions. G × G interaction analyses showed significant interactions between *rs2180619* of *CNR1* and *5-HTTLPR* of *SLC6A4* on STAI-T, TEMPS-Anx and BSI-Anx as measured by likelihood ratio tests (Fig. 1) and validated in regression analyses (Fig. 2). We found that homozygous "GG" genotype of *rs2180619* in interaction with "SS" genotype of *5-HTTLPR* was significantly associated with the highest score of STAI-T (Mean ± SEM was 46.35 ± 3.262, $P = 0.0006$) and TEMPS-Anx (Mean ± SEM was 0.394 ± 0.05, $P = 0.0013$) (Fig. 2/A). The interaction was not significant on STAI-S ($P = 0.065$). The chance to have high anxiety was more than four-fold (OR = 4.64, 95% CI: 1.7-12.71) in the group of "GG" genotype of *rs2180619* in interaction with "SS" genotype of *5-HTTLPR* compared to "A" allele carriers of *rs2180619* and "L" allele carriers of *5-HTTLPR* (OR = 1). Subjects with "GG" genotype of *rs2180619* and "L" allele carriers of *5-HTTLPR* seemed to be protected from the high anxiety (OR = 0.36, 95% CI: 0.11–1.18) (Fig. 2/B).

Effects of interactions between *5-HTTLPR* and *rs2180619* on anxiety phenotypes were weakened, but remained significant after adjustment for history of depression (TEMPS-Anx, $P = 0.007$; BSI-Anx, $P = 0.0001$ and STAI-T, $P = 0.011$), suggesting the significance of these genes in anxiety, although the possible role of depression in these associations cannot be ruled out.

We analyzed associations of the haplotypes of the three SNPs (*rs806379*, *rs1535255*, and *rs2023239*) located in the intron 2 of *CNR1* with anxiety. This haploblock had no significant effect on any anxious scales, neither in interaction with *5-HTTLPR*. However, significant interaction between the *5-HTTLPR* and the four-locus *CNR1* haplotypes was found (Fig. 3 and Supplementary Table 2). "GTGC" haplotype was associated with the highest EPM of STAI-T and TEMPS-Anx in "SS" carriers and it was related to lower scores in "L" carriers. The difference was significant in case of the STAI-T ("GTGC" related $\text{EPM}_{\text{STAI-T}}$ were 26.23 and 19.47 in "SS" and "SL" + "LL" groups respectively, $P_{G \times G} = 0.005$) and showed a trend in case of the TEMPS-Anx ($P_{G \times G} = 0.085$). In "SS" carriers, the effect of "GTGC" haplotype was different from the effect of "ATGC" regarding STAI-T, but the difference did not remain significant after the multiple correction ($P = 0.041$). In the case of "AATT" *CNR1* haplotype the EPM was significantly higher in "SL" + "LL" carriers compared to "SS" carriers for STAI-T ("AATT" related $\text{EPM}_{\text{STAI-T}}$ was 18.35 and 21.73 in "SS" and "SL" + "LL" groups respectively, $P_{G \times G} = 0.009$) as well as for TEMPS-Anx ("AATT" related $\text{EPM}_{\text{TEMP-Anx}}$ was 0.010 and 0.176 in "SS" and "SL" + "LL" groups respectively, $P_{G \times G} = 0.009$).

Sequence Analysis

To find the potential biological link between the haplotypes and phenotypes, we made "in silico" data analyses for putative transcription factor binding sites in the conventional and the alternative promoter regions of the *CNR1* (Supplementary Table 3). Only allele specific binding sites are represented and discussed. We found two allele-specific binding sites in case of the "A" allele of *rs2180619*. TFIID (*transcription factor II D*, binding site: TTCAAAA—*rs2180619* underlined) and GR (*glucocorticoid receptor*, CAAAAGG) can bind to this sequence with a similarity more than 90%. In case of the "G" allele four different TFs have putative binding site at the sequence; SRY (*sex-determining region Y gene product*, GATTCAAAG), TCF-4E (*T-cell factor 4E*, TTCAAAG), LEF-1 (*lymphoid enhancer-binding factor 1*, ATTCAAAG), and TCF -4 (*transcription factor 4*, GATTCAAAGG). The putative TF binding profile of the alternative promoter site is also different depending on the three SNPs. In case of the "A" allele of *rs806379* an additional binding site of GR-b (*glucocorticoid receptor beta*, AATTA) can be found, while TFIID and GATA-1 (*GATA binding protein 1*, ATGATA) can bind the sequence in case of the "T" allele of the polymorphism. In case of "T" alleles of *rs1535255* and *rs2023239* two allele specific binding sites of YY1 (*YY1 transcription factor*, ATGG) can be seen which disappear in case of the other alleles of the two SNPs.

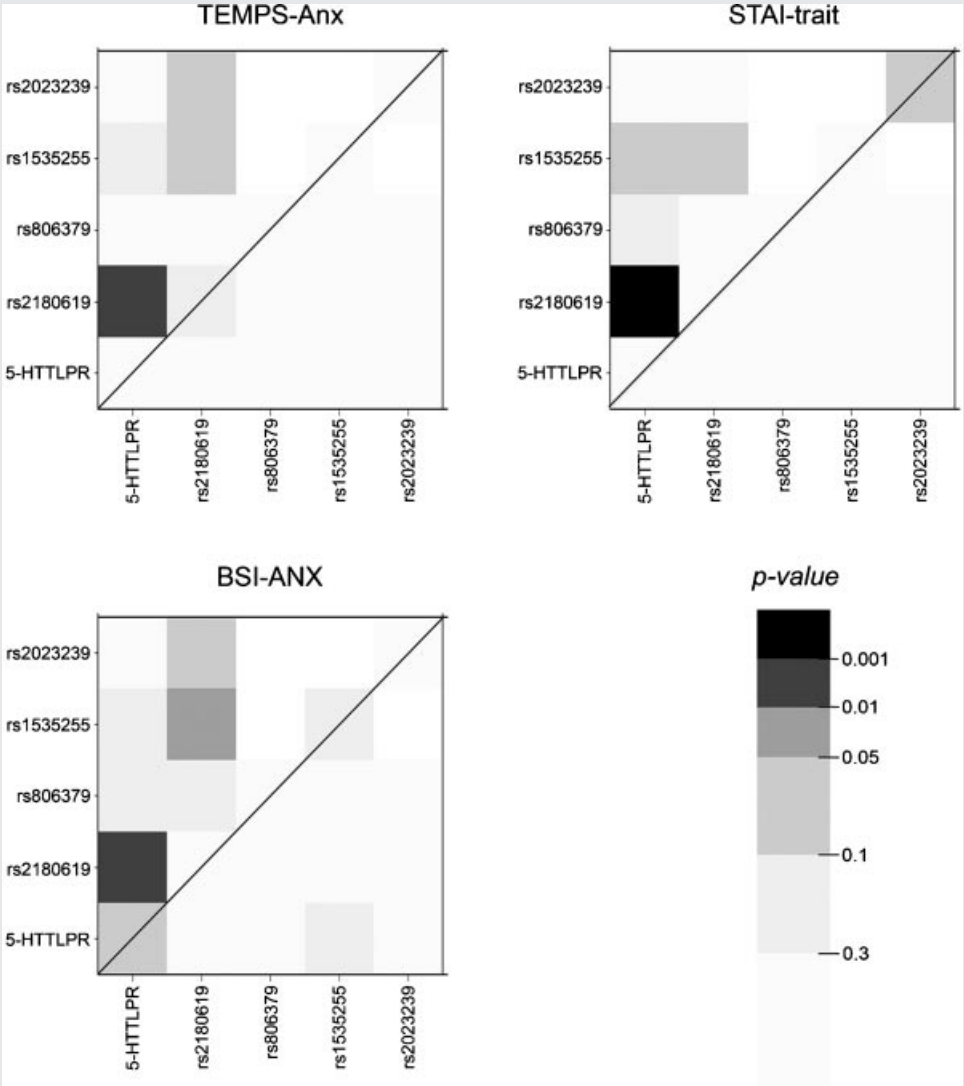

FIG. 1. Gene–gene interaction analyses between *CNR1* and *SLC6A4* promoter polymorphism on anxious phenotype. Visualized results of likelihood ratio tests for interactions between genetic markers (*5-HTTLPR, rs2180619, rs806379, rs1535255*, and *rs2023239*) and TEMPS-Anx, STAI-T and BSI-Anx are generated by "SNPassoc" R-package. Highly suggestive interactions were shown between the *5-HTTLPR* of *SLC6A4* promoter and *rs2180619* of *CNR1* promoter on anxiety ($P < 0.01$).

DISCUSSION

Anxiety is a multifactorial condition influenced by multiple genes. We report the first evidence for significant interaction between the *SLC6A4* and the *CNR1* genes on anxious phenotype. *5-HTTLPR* is a well-known functional length-polymorphism located in the promoter of *SLC6A4*. The "S" allele, resulting reduced 5-HTT expression [Lesch et al., 1996], was previously associated with certain psychiatric disorders [Sen et al., 2004; Gonda et al., 2005; Gonda et al., 2006; Serretti et al., 2006; D'Souza and Craig, 2008; Lazary et al., 2008; Murphy et al., 2008] however, data regarding anxiety are contradictory [Lesch et al., 1996; Ball et al., 1997; Ebstein et al., 1997; Gelernter et al., 1998; Jorm et al., 1998; Ricketts et al., 1998; Deary et al., 1999; Seretti et al., 1999; Sen et al., 2004; Gonda et al., 2007].

To the best of our knowledge, we were the first to publish the genetic interaction between *5-HTTLPR* and *CNR1* on anxiety. Our results showed that *5-HTTLPR* was significantly associated with anxious phenotype only in interaction with *CNR1*. Genotype analyses showed that homozygous "SS" genotype of *5-HTTLPR* in interaction with homozygous "GG" genotype of *rs2180619* was associated with the highest anxiety scores. This gene–gene interaction became more sophisticated testing the effect of the haplotypes of *CNR1* promoter in interaction with *5-HTTLPR*. The highest anxiety score was found in the "SS" subgroup with "GTGC" haplotype. "TGC" sequence of the latter haplotype is an earlier described sequence by Zhang et al. who reported the specific structure of the regulatory elements of *CNR1* [Zhang et al., 2004]. Two genomic regions were associated with significant promoter activities, the 5′ flanking region of exon 1

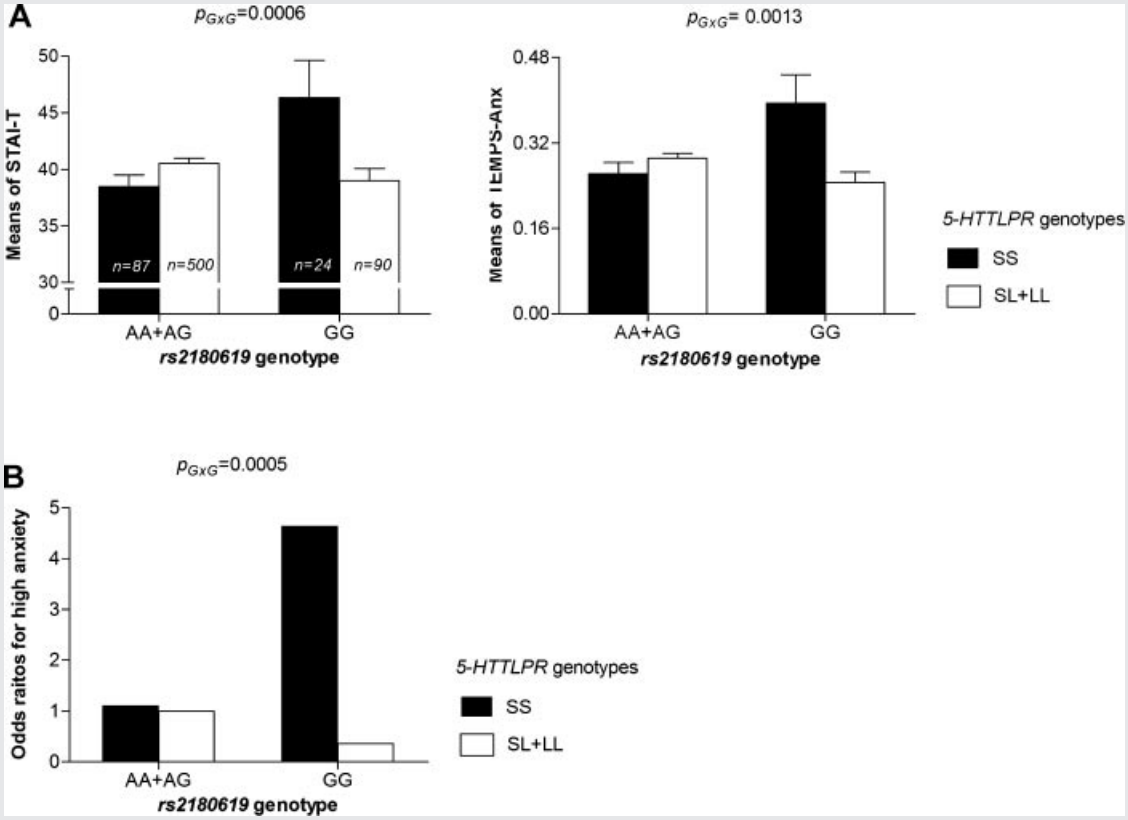

FIG. 2. Significant interaction between *5-HTTLPR* and *rs2180619* on anxious phenotypes. Mean and SEM. of STAI-T and TEMPS-Anx and *P*-values of gene–gene interaction ($P_{G \times G}$) in the regression analyses are presented on (A). The power of the study was 90.8% in case of G × G interaction regarding STAI-T and 87.8% in the case of TEMPS-Anx. Odds ratios for clinical anxiety and *P*-value of gene–gene interaction ($P_{G \times G}$) in different genomic combinations of *5-HTTLPR* and *rs2180619* are demonstrated on (B). Power of the study was 83.4%. Numbers in the bars on the first graph are the same in the two subsequent graphs.

(conventional promoter site) and the 5′ flanking region of exon 3 (intron 2, alternative promoter site). They found that allelic variations of the two promoter regions could be associated with polysubstance abuse and a haplotype of the alternative promoter was associated to lower mRNA expression of *CNR1*. This haplotype (''TAG'' in their study) is matched ''TGC'' sequence in our study according the results of Herman et al. [Hermann et al., 2002]*. Our detailed haplotype analyses suggested that the effect of this ''TGC'' sequence on anxiety was affected not only by the *5-HTTLPR* carrier status but also by the *rs2180619* allele located in the conventional promoter, but this latter was not significant after correction, and thus, further studies are required to confirm this marginally significant result.

*We report the SNP alleles according to the NCBI refSNP marker database corresponding to the plus strand of chromosome 6 in each case. Zhang et al. did not describe the convention they used in representing SNP data but it seems that the genotyping assays they used for *rs1535255* and *rs2023239* were designed for the minus strand of the sequence. Here, according to Herman et al., we report the plus strand minor allele base for the SNPs *rs806379, rs1535255* and *rs2023239* as T, G and C versus that in Zhang et al. of T, A and G.

We hypothesized that phenotypic effects might be associated with altered activity of the *CNR1* promoter depending on the haplotype structure. Certain *CNR1* haplotypes have different transcriptor factor (TF) binding profiles as it was demonstrated by ''in silico'' sequence analyses (see Results and Supplementary Table 3). We found that in case of the ''A'' allele of *rs2180619* two TFs (TFIID, GR) can bind to this sequence with a similarity more than 90%. In case of the ''G'' allele previously associated to polysubstance abuse [Zhang et al., 2004], four different TFs have putative binding site (SRY, TCF-4E, LEF-1, and TCF-4) at the sequence. A negative modulatory role of *rs2180619* can be presumed in the transcription of the gene regarding the ''in silico'' data and that this site was described by Zhang et al. as a likely negative regulatory region [Zhang et al., 2004]. The putative TF binding profile of the alternative promoter site is also different depending on the three-SNP haplotype. Carriers of ''TGC'', previously associated haplotype with lower *CNR1* expression, lose two physical binding sites of YY1 compared to other haplotypes.

Animal studies revealed that presynaptically located CB1 receptors can be found on serotonergic neurons colocalized with the 5-HTT [Haring et al., 2007]. 5-HT release is modulated through CB1

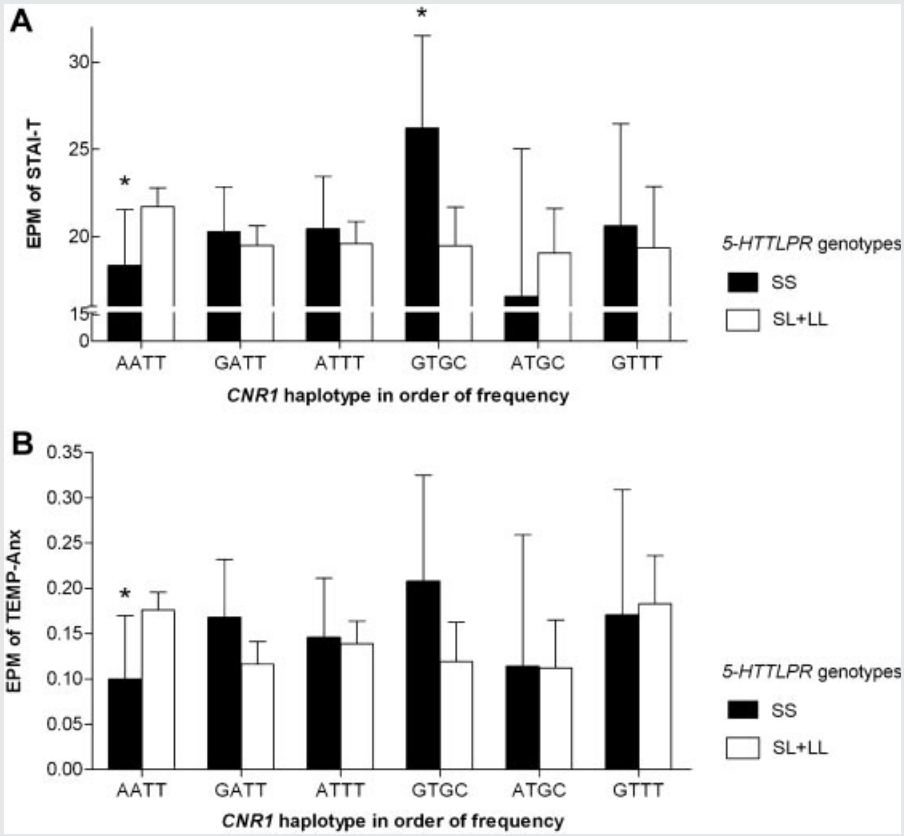

FIG. 3. Effect of *CNR1* × promoter haplotypes in interaction with *5-HTTLPR* on anxious phenotype. Anxiety phenotypes are represented by estimated phenotype mean (EPM) and 95% confidential intervals of STAI-T (A) and TEMPS-Anx (B). *Significant gene–gene interactions were shown on anxiety scales ($P < 0.01$). The highest anxiety score of both scales is associated to the "GTGC" haplotype in "SS" carriers.

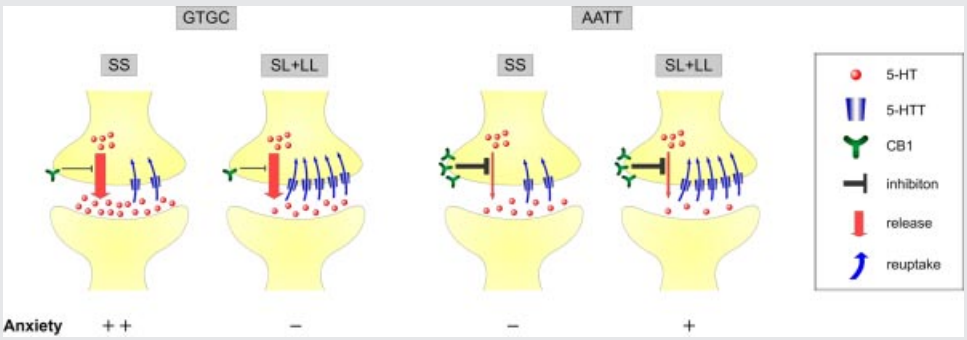

FIG. 4. A model for *SLC6A4* promoter × *CNR1* promoter interaction, synaptic 5-HT concentration and anxiety. Transcription factor binding profile analyses suggest that *CNR1* "GTGC" haplotype is associated with low expression of inhibitory CB1 (A) while "AATT" results high expression of CB1 (B). "SS" genotype of the *5-HTTLPR* is related to reduced serotonin transporter (5-HTT) efficiency compared to "L" carriers. The four genetic constellations, where significant interactions were determined in our model, yield different synaptic 5-HT concentrations. The highest 5-HT concentration is associated with increased release and decreased reuptake ("SS" with "GTGC") yielding the highest anxiety scores in our model. The lowest 5-HT concentration caused by increased reuptake and decreased release mechanism ("SL" + "LL" and "AATT") was also associated with relatively high anxiety. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in mouse brain cortex [Nakazi et al., 2000] especially in prefrontal cortex [Tzavara et al., 2003]. Darmani et al. found that behaviorally active doses of CB1 receptor antagonist (SR141716A) increase brain 5-HT turnover [Darmani et al., 2003]. In light of these data possible explanation can be given for our results about the genetic interaction of the two systems on anxiety (Fig. 4). As described above, in ''SS'' genotype of *5-HTTLPR* reduced 5-HTT efficiency results increased synaptic 5-HT concentration due to decreased reuptake mechanism compared to ''L'' carriers. The lower expression of inhibitory CB1 (''TGC'' haplotype of the alternative promoter and ''G'' allele of *rs2180619*) causes decreased inhibition of 5-HT release. Thus, this genetic constellation (''GTGC'' haplotype with ''SS'' genotype) could result in extremely high synaptic 5-HT concentration in our model. Lack of inhibition of 5-HT release by CB1 receptor can be compensated by higher expression of the 5-HTT and thus increased reuptake in ''L'' carriers. Parsey et al. concluded that high level of extracellular 5-HT influence the regulation of the development of the serotonergic system in an earlier phase of life creating the conditions of a genetic vulnerability for anxiety disorders [Parsey et al., 2006]. Another support of our model is that extremely high serotonin level in the synaptic cleft associated with impaired stress-coping capacity and higher vulnerability for anxiety were found in adult 5-HTT knock out mice [Kim et al., 2005; Carroll et al., 2007; Wellman et al., 2007]. Our results suggest that extreme synaptic 5-HT level can also depend on the genetic interaction between *SLC6A4* and *CNR1* influencing the cellular expression of 5-HTT and CB1. In this model the highest serotonin level (''SS'' genotype with ''GTGC'' haplotype) is associated with the highest anxiety score.

On the other hand, relatively high anxiety was found also in a subgroup of ''L'' carrier subjects. The ''AATT'' haplotype of *CNR1* is supposedly related to a higher cellular expression of CB1 regarding the complete difference in the TF binding profile between the ''AATT'' and ''GTGC'' haplotypes. The higher expression of CB1 yields reduced 5-HT release resulting in low synaptic 5-HT level in interaction with the higher expression of 5-HTT caused by the *5-HTTLPR* ''L'' allele (Fig. 4). The CB1-mediated inhibition of 5-HT release together with the increased reuptake may cause extremely low synaptic serotonin concentration that can also be associated with higher anxiety than average. Decreased 5-HT concentration yielding anxious phenotype was demonstrated by tryptophan depletion studies [Goddard et al., 1995; Klaassen et al., 1998]. Moreover, loss-of-function mutation in tryptophan hydroxylase-2 was identified in unipolar major depression [Zhang et al., 2005]. Data on this topic suggest that serotonergic dysfunction has a crucial role in the development of anxiety and the sensitive balance of the normal regulation can be disturbed by hyper- and hypofunction of the serotonergic system as well.

We investigated the effect of promoter polymorphisms in *SLC6A4* and *CNR1* on four anxiety scales in a large general population. Genetic effects were significant on trait and temperament anxiety but not on state anxiety in our studies. These data suggest that vulnerability for anxiety can be predisposed by genetically determined serotonergic and endocannabinoid dysfunction. Our results support a biologically significant and functionally relevant new model for gene–gene interaction between the serotonergic system and the ECS on anxiety.

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