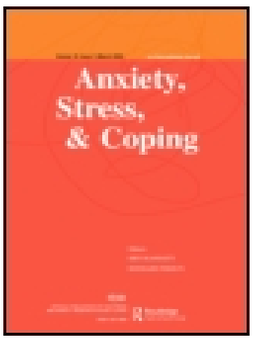

Routledge
Taylor & Francis Group

Anxiety, Stress, & Coping
An International Journal

ISSN: 1061-5806 (Print) 1477-2205 (Online) Journal homepage: https://www.tandfonline.com/loi/gasc20

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To cite this article: Ana M. Peiró, María S. García-Gutiérrez, Beatriz Planelles, Teresa Femenía, Carlos Mingote, Luis Jiménez-Treviño, Sara Martínez-Barrondo, M. Paz García-Portilla, Pilar A. Saiz, Julio Bobes & Jorge Manzanares (2020): Association of cannabinoid receptor genes (*CNR1 and CNR2*) polymorphisms and panic disorder, Anxiety, Stress, & Coping, DOI: 10.1080/10615806.2020.1732358

To link to this article: https://doi.org/10.1080/10615806.2020.1732358

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Association of cannabinoid receptor genes (*CNR1 and CNR2*) polymorphisms and panic disorder

Ana M. Peiró[a], María S. García-Gutiérrez[b,c], Beatriz Planelles[d], Teresa Femenía[b], Carlos Mingote[e], Luis Jiménez-Treviño[f], Sara Martínez-Barrondo[f], M. Paz García-Portilla[f], Pilar A. Saiz[f], Julio Bobes[f] and Jorge Manzanares[b,c]

[a]Clinical Pharmacology Unit and Neuropharmacology on Pain and Functional Diversity (NED), Department of Health of Alicante - General Hospital, ISABIAL, Alicante, Spain; [b]Neuroscience Institute, Alicante, Miguel Hernández University, San Juan de Alicante, Spain; [c]Cooperative Networking in Health Research (RETICS-addictive disorders), Health Institute Carlos III, MICINN and FEDER, Madrid, Spain; [d]Research Unit, Hospital General Hospital, Alicante, Spain; [e]University Hospital 12 de Octubre, Madrid, Spain; [f]Psychiatry Department, Medicine Faculty, University of Oviedo; Biomedical Research Centre in Mental Health Network (CIBERSAM); University Institute of Neuroscience of Asturias, INEUROPA; Health Service of Asturias, SESPA, Asturias, Spain

ABSTRACT

Background and objectives: Panic disorder (PD) is an anxiety disorder characterized by recurrent and unexpected panic attacks along with sudden onset of apprehension, fear or terror. The endocannabinoid system (ECS) has a role in stress recovery, regulating anxiety. The aim of this study was to analyze potential genetic alterations in key ECS targets in patients suffering from panic disorders.

Design and methods: We analyzed single nucleotide polymorphisms (SNPs) of the cannabinoid receptors (*CNR1; CNR2*) and the endocannabinoid hydrolytic enzyme fatty acid amide hydrolase (*FAAH*) genes in 164 Spanish PD patients and 320 matched controls.

Results: No significant differences were observed in the SNPs of the *CNR2* and *FAAH* genes tested. However, when analyzing genotype-by-sex interaction at A592G (rs2501431) and C315T (rs2501432) in the *CNR2* gene, the presence of the G-allele in males was associated with a protective haplotype. Genotyping analysis revealed that variants in *CNR1* confer vulnerability to PD, with a significantly increased risk associated with the G-allele (rs12720071) and C-allele (rs806368). This finding was consistent when analyzing genotype-by-sex interaction, where females presented a greater PD risk.

Conclusions: Polymorphisms at the *CNR1* gene may be a risk factor for PD contributing to sex-specific dysfunction in females.

ARTICLE HISTORY
Received 3 August 2018
Revised 12 February 2020
Accepted 17 February 2020

KEYWORDS
Panic disorder; panic attacks; *CNR1*; *CNR2*; *FAAH*; single nucleotide polymorphism

Introduction

Panic disorder (PD) is a complex, multifactorial, disabling anxiety disorder, with a prevalence of 1% to 4% in the general population. PD is characterized by recurrent and unexpected panic attacks with sudden onset of intense apprehension, fear or terror, and abrupt development of somatic, cognitive and affective symptoms. There is evidence for either genetic heterogeneity or complex inheritance, with interaction between environmental factors and multiple single genes in the aetiology of PD. Linkage analyses have implicated different regions located in several chromosomes (1q, 2q, 4q, 7p, 9q, 12q, 13q, 15q and 22q) and genes of classical candidate

CONTACT Jorge Manzanares jmanzanares@umh.es
Supplemental data for this article can be accessed https://doi.org/10.1080/10615806.2020.1732358

neurotransmitter systems (Jacob, Domschke, Gajewska, Warrings, & Deckert, 2010). Genetic variation in genes of monoamine oxidase A, catechol-*O*-methyltransferase, adenosine receptor (*ADORA2A*) and cholecystokinin B receptor have been inconsistently replicated (Jacob et al., 2010; Watanabe et al., 2017). In several studies including independent samples, only the Val158Met polymorphism of the catechol-O-methyltransferase gene has been associated with increased vulnerability to develop PD. A recent meta-analysis also established an association between the Val158Met polymorphism, emotional and sexual abuse in predicting panic attacks among male carriers of the Val/Val genotype and female carriers of the Val/Met or Met/Met genotype (Asselmann et al., 2018). Despite these promising data, the difficulties of identifying genetic variations and of replicating these results have hampered translation to practice. Moreover, most of these studies have involved limited sample sizes, dominated by white women (Maron, Hettema, & Shlik, 2010). Therefore, additional analyses investigating gender- and ethnicity-dependent interactions and the putative impact on cognitive functions will be necessary.

Several studies support the role of the endocannabinoid system (ECS) in the regulation of stress, anxiety, depression, and addictive disorders, with controversial results (Batista, Haibara, Schenberg, & Moreira, 2017; Wirz, Reuter, Felten, & Schwabe, 2018). Firstly, it seems that the ECS signaling in the amygdala modulates the stress-induced activation of the hypothalamic–pituitary–adrenal (HPA) axis. Indeed, the activation of ECS signaling in the prefrontal cortex is required for the appropriate recovery of the HPA axis to baseline levels following stress offset in songbirds and rats (Anjos-Garcia, Ullah, Falconi-Sobrinho, & Coimbra, & C, 2017; Dickens, Vecchiarelli, Hill, & Bentley, 2015; Hill & Tasker, 2012). In addition, anxiogenic and anxiolytic-like effects have been described after the administration of cannabinoid CB1 receptor (CB1r) agonists in different animal models, including rats and mice. In general, an anxiolytic-like effect was observed following the administration of low doses of cannabinoid agonists, while higher doses induced anxiogenic-like effects (Moreira, Grieb, & Lutz, 2009). Genetic disruption and pharmacological blockade of the CB1r increased anxiety (Marco et al., 2011), whereas the overexpression of the cannabinoid CB2 receptor (CB2r) resulted in an endophenotype resistant to anxiogenic and depressogenic-like stimuli in mice (Garcia-Gutierrez & Manzanares, 2011; Garcia-Gutierrez, Perez-Ortiz, Gutierrez-Adan, & Manzanares, 2010). In contrast, the lack of CB2r increased the vulnerability to both stimuli in mice (Ortega-Alvaro, Aracil-Fernandez, Garcia-Gutierrez, Navarrete, & Manzanares, 2011). Furthermore, CB2r-antagonists and fatty acid amide hydrolase (FAAH) inhibitors induce anxiolytic-like effects in mice, supporting the hypothesis that the ECS has a pivotal role in the pathogenesis of anxiety (Garcia-Gutierrez et al., 2010; Garcia-Gutierrez, Garcia-Bueno, Zoppi, Leza, & Manzanares, 2012; Kathuria et al., 2003).

In humans, genetic variants in cannabinoid receptors (*CNR1,* rs1049353-GG genotype; and *CNR2*, rs2501431-AA genotype) and *FAAH* degradation enzymes *(*rs324420-A allele carriers) have been related to anxiety and emotional response (Conzelmann et al., 2012; Moreira et al., 2009). Also, these variants (*CNR1* rs1049353-GG and *CNR2* rs2501431-G allele) can modify the effectiveness of the antidepressant citalopram (Mitjans et al., 2013; Mitjans, Gasto, Catalan, Fananas, & Arias, 2012) and have been associated with other mental disorders, such as hyperactivity and post-traumatic stress disorder (PTSD) (Lu et al., 2008), and with eating disorders like anorexia and bulimia nervosa (*CNR1* 1359 G/A and *FAAH* cDNA 385C/A) (Monteleone et al., 2009) In fact, genetic dysfunctions in certain key elements of the serotoninergic (serotonin reuptake promoters) and endocannabinoid system (specific constellations of CB1-receptor) have been associated with increased vulnerability to developing anxiety and mood disorders (Lazary et al., 2009).

Little information is currently available from genetic association studies between PD and the ECS genes. The present study was designed to further investigate the potential role of ECS in PD. To this aim, genetic variability in *CNR1, CNR2 and FAAH* genes was evaluated in a Spanish cohort of patients with PD and compared to controls.

Material and methods

The study used a dual case-control design. The Ethics Committees of the Miguel Hernandez University, University Hospital 12 de Octubre, and the University Institute of Neuroscience of Asturias all approved the protocol.

Study population

We included 164 white men and women with PD (mean age, 36 ± 10 years; 71% women), diagnosed by trained psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) criteria. Patients were consecutively recruited from the University Hospital 12 de Octubre (Madrid, Spain) and the outpatient hospital psychiatric departments. None of the patients met criteria for major depression. However, the presence of other anxiety disorders in the sample cannot be ruled out due to the complexity of the diagnosis and the common symptomatology. A total of 320 age- and gender-matched controls with no history of psychiatric disorder or previous psychiatric treatment were chosen from current blood donors. Participants under 18 years of age, currently using any abuse substance, or receiving treatment for addiction, were excluded from the study. All participants signed informed consent. Structural clinical interviews for DSM-IV (SCID) were used in both cases and controls.

DNA extraction

Peripheral blood was obtained from all participants and frozen immediately until DNA extraction. Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells (PBMCs) according to standard procedures. Briefly, following enrollment in the study, 7 mL of blood was collected from each individual and kept in EDTA tubes following standard procedures. PBMCs were separated, and DNA was isolated from the plasmatic fraction according to manufacturer instructions (QIAamp DNA Blood Maxi kit, Qiagen, Hilden, Germany). DNA concentration was measured in Nanodrop 2000 spectrophotometer.

SNP and genotyping analysis

Of the polymorphisms previously identified within *CNR1*, *CNR2* and *FAAH* genes (Lester et al., 2017; Smith, Stanley, Foss, Boles, & McKernan, 2017), we focused on those with a possible impact on a clinical setting. We designed a panel of 12 relevant SNPs selected from public databases (dbSNP; http://www.ncbi.nih.gov). On the *CNR1* gene, these were G1255A (rs1049353), A3777G (rs12720071), T4858C (rs806368), and G843A (rs35057475); on *CNR2*, C1073 T (rs2229579), A592G (rs2501431), C315 T (rs2501432), C7 T (rs28655469), and G524 T (rs41311993); and on *FAAH*, C385A (rs324420), A1191G (rs12094805), and G706A (rs61744669). General information about these SNPs is given in Table 1 and Supplemental Table S1.

Genomic DNA was extracted as described previously. To detect single nucleic acid changes, target regions were initially amplified using multiplex PCR, hybridized to custom-designed primers, and subjected to a single base extension reaction using single mass-modified nucleotides. Products were spotted onto a matrix chip and, after ionization, real-time detection by the MassARRAY mass spectrometer was performed at the National Genotype Centre, CEGEN (Santiago de Compostela, Spain). For each sample, duplicate analyses were performed to confirm genotype concordance.

Statistical analyses

Cases and controls were compared for genotypic and allelic frequencies with the χ^2-test. A linear regression was performed to assess the association between the response variable (binary disease

Table 1. Summary of cannabinoid receptor 1 (*CNR1*), cannabinoid receptor 2 (*CNR2*) and fatty acid amide hydrolase (*FAAH*) gene polymorphisms analyzed.

Gene, locus	mRNA	Protein	Polymorphism		dbSNP
CNR1	NM_033181.3	NP_149421.2	G1323A	420T	rs1049353
6q14-q15	(5387 bp)	(439 aa)	A3777G	3′ UTR	rs12720071
			T4858C	3′ UTR	rs806368
			G843A	260A	rs35057475
CNR2	NM_001841.2	NP_001832.1	C1073T	H316Y	rs2229579
1p36.11	(1789bp)	(360 aa)	A592G	155G	rs2501431
			C315T	Q63R	rs2501432
			C7T	5′ UTR	rs28655469
			G524T	L133I	rs41311993
FAAH	NM_001441.2	NP_001432.2	C467A	P129T	rs324420
1p33	(2105 bp)	(579 aa)	A1191G	H370R	rs12094805
			G706A	W208X*	rs61744669

Note: * *stop codon.*
dbSNP: Single Nucleotide Polymorphism Database.

status) and the analysis of single SNPs by multiple inheritance models (codominant, dominant, recessive, overdominant and log-additive) and of multiple SNPs by haplotype frequency estimation. The association between haplotypes with the response variable was analyzed by SNPStats. Genotypes were coded for the dominant, codominant and recessive effect. Odds ratios (OR) with their 95% confidence intervals (CIs) and the results of the test for interaction are reported. A two-sided *p*-value of less than 0.05 indicated statistical significance. R software (www.r-project.org) was used to calculate haplotype frequencies. Haploview 3.2 (Barrett, Fry, Maller, & Daly, 2005) was used to generate a linkage disequilibrium map and to test for Hardy–Weinberg equilibrium in the haplotype analysis. Statistical analysis was completed at the Research Unit, Institute of Health and Biomedical Research of Alicante (ISABIAL, Alicante, Spain).

Results

A total of 12 SNPs were genotyped in our primary study, including PD cases and controls. Table 2 and Table 3 summarize the results. All subjects were homozygotes for 4 out of 12 SNPs: rs35057475, rs28655469, rs12094805 and rs61744669, suggesting that these are not associated with PD development. No significant deviation from the HWE was observed for polymorphisms except rs12720071, which showed significant deviation from HWE among cases and controls.

Table 2. Summary of associations and analysis between variants of cannabinoid receptor 1 (*CNR1*), cannabinoid receptor 2 (*CNR2*) and fatty acid amide hydrolase (*FAAH*) genes in panic disorder (PD) vs controls.

	Panic disorder ($n = 164$)		**Controls** ($n = 320$)		**Difference in allele frequency**	
SNP (dbSNP)	Genotype 11/12/22	MAF (2) (%)	Genotype 11/12/22	MAF (2) (%)	(Allele 2 vs.1) OR (95%)	*p*
CNR1 G1255A (rs1049353)	95/61/8	23	179/125/16	25	0.91 (0.66-1.24)	0.532
A3777G (rs12720071)	125/39/0	12	275/45/0	7	**1.78 (1.14-2.80)**	**0.012**
T4858C (rs806368)	81/77/6	27	198/108/14	21	**1.38 (1.02-1.88)**	**0.041**
CNR2 C1073 T (rs2229579)	125/39/0	12	253/62/5	11	1.06 (0.70-1.61)	0.764
A592G (rs2501431)	52/76/36	45	106/139/75	45	1.00 (0.76-1.30)	1
C315 T (rs2501432)	52/76/36	45	106/139/75	45	1.00 (0.76-1.30)	1
G524 T (rs41311993)	162/2/0	1	314/6/0	1	0.65 (0.13-3.23)	0.597
FAAH C385A (rs324420)	107/51/6	19	211/102/7	18	1.07 (0.76-1.51)	0.680

Note: SNP: single nucleotide polymorphism; Genotype: 1: Ancestral allele; 2: Minor allele; MAF: Minor allele frequency; HWE: Hardy-Weinberg equilibrium.

Table 3. Summary of associations and analysis between variants of cannabinoid receptor 1 (*CNR1)*, cannabinoid receptor 2 (*CNR2)* and fatty acid amide hydrolase (*FAAH)* genes in panic disorder (PD) vs controls in females and males.

	Female						**Male**					
	PD		**Control**		**Difference in allele frequency**		**PD**		**Control**		**Difference in allele frequency**	
SNP	Genotype		Genotype		**(Allele 2 vs 1)**		Genotype		Genotype		**(Allele 2 vs 1)**	
	1/1	2/2, 1/2	1/1	2/2, 1/2	OR (95%)	*p*	1/1	2/2, 1/2	1/1	2/2, 1/2	OR (95%)	*p*
CNR1												
G1255A (rs1049353)	67	49	107	81	0.97 (0.61-1.54)	0.885	69	63	28	20	1.28 (0.66-2.50)	0.471
A3777G (rs12720071)	85	31	158	30	1.92 (1.09-3.39)	**0.024**	117	15	40	8	0.64 (0.25-1.62)	0.349
T4858C (rs806368)	55	61	114	74	1.71 (1.07-2.73)	**0.025**	84	48	26	22	0.68 (0.35-1.32)	0.25
CNR2												
C1073 T (rs2229579)	91	25	147	41	0.99 (0.56-1.73)	0.958	106	26	34	14	0.60 (0.28-1.27)	0.179
A592G (rs2501431)	42	74	57	131	0.77 (0.47-1.25)	0.288	49	83	10	38	**0.45 (0.20-0.97)**	**0.043**
C315 T (rs2501432)	42	74	57	131	0.77 (0.47-1.25)	0.288	49	83	10	38	**0.45 (0.20-0.97)**	**0.043**
G524 T (rs41311993)	116	0	183	5	0.14 (0.01-2.61)	0.19	131	1	46	2	0.18 (0.02-1.98)	0.16
FAHH												
C385A (rs324420)	76	40	128	60	1.12 (0.69-1.83)	0.643	83	49	31	17	1.08 (0.54-2.14)	0.834

Note: SNP: single nucleotide polymorphism; Genotype: 1: Ancestral allele; 2: Minor allele. OR: Odds ratio.

Genotyping analysis revealed that variants in the *CNR1* gene confer vulnerability to PD, with a significant increased risk associated with the G-allele (A3777G, rs12720071, OR 1.78, 95% CI 1.14–2.80, $P = 0.012$) and C-allele (T4858C, rs806368, OR 1.38, 95% CI 1.02–1.88, P=0.041; Table 2). In men, the G-allele (A3777G) was present in 17% of PD patients vs 11% of controls; the C-allele (T4858C) was observed in 46% vs 36%. Women with PD also showed a higher frequency of the G-allele (A3777G) and C-allele (T4858C) compared to controls (27% vs 16% in A3777G; 46% vs 39% in T4858C, respectively). A genotype-by- sex interaction showed that this *CNR1* haplotype was associated with a greater PD risk in females than males (women: A3777G: OR 1.92, $P = 0.024$ and for T4858C: OR 1.71, $P = 0.025$). Polymorphisms for *CNR2* and *FAAH* were not associated with PD in the total population (Table 2). However, when analyzing genotype-by-sex interaction at A592G (rs2501431) and C315 T (rs2501432) in the *CNR2* gene, the presence of the G-allele in males was associated with a protective haplotype (Table 3).

Discussion

We conducted a case-control association study to evaluate whether genetic variability in ECS genes is a genetic risk factor for PD. The analysis of the SNP variability at the *CNR1* gene revealed a genetic influence on the overall risk of having PD. The G-allele (A3777G, rs12720071) and C-allele (T4858C, rs806368), both in the exonic *CNR1* gene, were overrepresented in PD cases vs controls, with higher frequency in females. Our results confirmed previous evidence suggesting that *CNR1* genetic variants may contribute to underlying risk in emotional dysregulation and anxiety (Haller, Varga, Ledent, Barna, & Freund, 2004; Viudez-Martinez, Garcia-Gutierrez, & Manzanares, 2018) and in determining personality traits (Yao et al., 2018).

The *CNR1* gene is located on chromosome band 6q15 and encodes a seven-transmembrane domain protein of 439 amino acids. Several mutations lead to altered mRNA stability and transcription rate or a reduction of the activity of the encoded protein (Viudez-Martinez et al., 2019). In fact, rs12720071 can serve as an alternative putative promoter region and a potential transcription factor binding site for CCAAT/enhancer-binding protein beta (C/EBPβ), which can promote or repress genes (Haller et al., 2004). Increasing evidence shows an association between rs12720071-G-allele carriers and cognitive impairments. One study has reported that rs12720071-G-allele carriers contributed to white-matter brain volume deficits in patients with schizophrenia and heavy marijuana use (Viudez-Martinez, Garcia-Gutierrez, Fraguas-Sanchez, Torres-Suarez, & Manzanares, 2018). The use of cannabis is an aggravating factor for all psychiatric disorders, and panic attack is the most common complication (Coscas, Benyamina, Reynaud, & Karila, 2013). In addition, the rs12720071-G-allele has been associated with a higher impulsivity index and obesity-related phenotypes in some ethnic groups (Manzanares et al., 2018; Thiele & Navarro, 2014). Some authors argue that impulsivity may play a role in the pathogenesis of neuropsychiatric disorders and may be an important phenotype common to different behavioral disorders, including PD (Llorente-Berzal et al., 2013). In addition, a significant influence of *CNR1* (rs806368, rs806371, and rs2180619) variants in the modulation of personality and psychiatric conditions have been reported in African-Americans (Yao et al., 2018).

CNR1 rs1049353 wild type carriers (GG 58% in our PD group) showed a weaker bilateral amygdala, putamen and pallidum activity, and a left lateralized caudate and thalamus activity. These results could be potentially related to social reward hypo-responsiveness, and increased risk of antidepressant treatment resistance, particularly evident in females presenting a higher degree of anxiety (Ortiz, Oliva, Perez-Rial, Palomo, & Manzanares, 2004). Increased prevalence of the rs1049353-A allele was observed in a case–control study in people with either anorexia or bulimia nervosa. Also, half of the patients with multiple sclerosis present a cognitive impairment, and rs1049353-AA genotype cases could present a more severe phenotype (Zhou et al., 2017). However, the contribution of this variant to the maladaptive mechanism of emotion regulation is yet not defined, and this association was not detected in other psychiatric disorders (Lu et al., 2008; Monteleone et al., 2010). In

another study of co-morbidities associated with attention deficit/hyperactivity disorder, a significant association of rs1049353-A-allele with PTSD diagnosis was identified. However, haplotype analysis indicated that rs806368 could be a far more important risk factor (Lu et al., 2008). Thus, there is a need for further robust clinical trials to validate the potential clinical utility as a candidate biomarker for mental disorders.

In addition, an SNP in the *CNR1* regulatory region, rs806378, could also change the transcription factor binding. This has been studied in anxiety, drug addiction (Ceccarini et al., 2014), and risk of depression (Mitjans et al., 2013), where some haplotypes had a protective role against anxiety. Based on preclinical data demonstrating that *CNR1* activation inhibited serotonin release, hypothesizing that the combination of low *CNR1* expression and low serotonin reuptake contribute through different mechanisms to excessive 5-HTTLPR signaling and, therefore, anxiety (Lazary et al., 2009).

CNR2 is expressed in the central nervous system, but its role in brain disorders is unclear. A recent study in a Japanese population associated increased risk for depression and treatment response to the rs2501432 polymorphism in the *CNR2* gene (Onaivi et al., 2008). In another longitudinal study, AA carriers in the *CNR2* rs2501431 variant presented more severe depression than G carriers (Mitjans et al., 2012). In the same way, the functional presence of *CNR2* in neuronal and glial processes may provide novel targets due to immunological cannabinoid activity (Mitjans et al., 2012; Mitrirattanakul et al., 2007). Cultured CHO cells transfected with the R63 allele of rs2501432 of *CNR2* presented a significantly lower response to CB2 ligands compared to those with the Q63 allele. Furthermore, the ECS is an important neural substrate, signaling response to environmental stress through the modulation of amygdala activity (Ramikie & Patel, 2012). Therefore, the ECS could be relevant to understanding the context-dependent emotional and affective changes in other mental illness such as bipolar disorder (WHO, 2014). In this respect, significantly lower CB2r mRNA and protein levels were observed in the human brain with the CC and CT genotypes of rs12744386 compared with the TT genotype in schizophrenia (Ishiguro, et al., 2010). This is in line with our results, which showed that other SNPs at the *CNR2* gene, A592G (rs2501431) and C315 T (rs2501432), protected males against PD (OR 0.45, 95% CI 0.20–0.97, $P = 0.043$).

An important genetic imaging study identified two phenotypic differences in healthy (white) carriers of the *FAAH* rs324420 polymorphism. People with CA or AA genotypes (65%) exhibited significantly reduced activation in the amygdala compared to those with the CC genotype in response to threatening, fearful and angry facial expressions. In addition, those with the CA or AA genotype showed a diminished relationship between reactivity in the amygdala and trait anxiety compared to CC. These data suggest that individuals with the *FAAH* rs324420A-allele presented reduced sensitivity to potential threats or the possibility of harm. Some authors hypothesize that blunted reactivity to threats in the amygdala contributes to increased risk-taking behavior and vulnerability for addictive disorders in humans (Nikolova, Knodt, Radtke, & Hariri, 2016). In fact, the homozygous CC C385A SNP genotype may contribute to vulnerability to other mood disorders (Monteleone et al., 2010).

The present study has some limitations. First, the size of our sample limits the power to detect very small differences. However, we had enough power to detect small to medium size effects. Moreover, few SNPs were studied (*CNR1*, *CNR2* and *FAAH*), and the possible functional effects of the markers are still under investigation. However, the incidence of significant correlations between *CNR1* polymorphisms and PD supports the need for studies that can elucidate their function. Indeed, SNP studies have a limited ability to establish whether these modifications could be associated with alterations in the gene expression and/or function of the receptors concerned. Further studies are needed to understand if these SNPs may be associated with gene expression and/or functional alterations of CB1/CB2 receptors, and to replicate and confirm the data presented here.

Conclusions

The results suggest that *CNR1* gene SNPs may be an important risk variant in the emotional regulation difficulties underlying PD and, possibly, other co-morbid conditions such as mood disorders.

However, the role of *CNR1* is limited, particularly at the level of a psychiatric diagnosis, so future work using more refined phenotypes is necessary. Further investigation of personality traits associated with PD may improve our understanding of the role that *CNR1* has in this condition and co-morbid disorders. Refinement of putative DNA variants in *CNR1* with functional outcomes is needed to delineate the causal variants contributing to psychiatric illness.

Acknowledgements

We thank all participants in this study. María-del-Mar Inda, PhD, Laura Sánchez Tejada and Pura Ballester Navarro, PhD fellows, supported this work. We thank María Reyes Roca Navarro and Laura Sánchez Tejada for their excellent technical assistance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the [Instituto de Salud Carlos III, Spanish Ministry of Health] under Grant [RETICS, RD12/0028/0019] and [Fondo de Investigación Sanitaria, Spanish Ministry of Economy and Competitiveness] under Grant [FIS PI14/00438].

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