Variations in the cannabinoid receptor 1 gene predispose to migraine

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ARTICLE INFO

ABSTRACT

In animal models endogenous cannabinoids have an inhibitory effect on trigeminovascular activation through the cannabinoid receptor 1 (CB1), although there is no evidence of the potential role of CB1 in human migraine. In this study we applied single marker association and haplotypic trend regression analysis to investigate the relationship between the CB1 gene (*CNR1*) and headache with migraine symptoms (nausea, photophobia and disability, measured by the ID-migraine questionnaire). We identified our controls (CO = 684) as those who have not reported ID-migraine symptoms at all and defined migraine headache sufferers (*M* = 195) as those who reported all three symptoms. The *CNR1* was covered by 10 SNPs located throughout the gene based on haplotype tagging (htSNP) and previous literature. Our results demonstrated a significant haplotypic effect of *CNR1* on migraine headaches ($p = 0.008$, after permutation $p = 0.017$). This effect was independent of reported depression or drug/alcohol abuse although using neuroticism in the analysis as covariant slightly decreased this association ($p = 0.027$, permutated $p = 0.052$). These results suggest a significant effect of *CNR1* on migraine headaches that might be related to the alteration of peripheral trigeminovascular activation. In addition, this is the first study to demonstrate the effectiveness of using trait components combinations to define extreme phenotypes with haplotype analysis in genetic association studies for migraine. However, further studies are needed to elucidate the role of *CNR1* and the cannabinoid system in migraine.

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Endogenous cannabinoids are specific lipids (anandamide, 2-arachidonoylglycerol) that activate cannabinoid receptors [9]. The CB1 receptor, which is expressed predominantly in the neurons of the central and peripheral nervous systems, is G-protein coupled and able to inhibit adenylyl cyclase, voltage-gated calcium channels, extracellular signal-related kinase and activate G-protein-activated inwardly rectifying potassium channels [9]. Through these molecular mechanisms, cannabinoids decrease the release of several neurotransmitters, e.g. acetylcholine, GABA, glutamate, noradrenalin and serotonin. Of relevance to migraine, CB1 receptors have been shown to be located in trigeminal ganglion, in spinal trigeminal tract and nucleus, and in other pain processing areas, such as the periaqueductal grey matter, the thalamus and limbic areas of the cerebral cortex, especially the cingulate and frontal cortices, and the amygdala [2,3,9]. Limbic areas are also responsible for the emotional component of pain and it has been demonstrated previously that migraine and pain frequently are co-morbid with depression and anxiety [6].

Animal studies have shown that the CB1 receptor agonist anandamide has a migraine-preventive action on the peripheral side of the trigeminal nerve, as measured by dural vessel dilatation [3]. Cannabinoid receptor activation also inhibited trigeminal firing in the trigeminocervical complex. This effect was reversed by administration of a specific CB1 receptor antagonist, indicating that the central effects of cannabinoids are also CB1 receptor-mediated [2]. However, this observation may arise from direct effects on the trigeminocervical relay neurons or effects on the descending pain modulation systems [2]. All these data suggest that the CB1 receptor plays a pathophysiological role in migraine and therefore is a potential target for migraine treatment. However, as far as we are aware, the CB1 receptor gene (*CNR1*) has not been investigated in relation to migraine, until now.

To investigate the genetic background of common forms of migraine (migraine with and without aura) is a challenging task

as they are multifactorial and polygenic disorders. Although several different neurotransmitter mechanisms have been implicated in the pathomechanism of migraine [11,18], studies that used the International Headache Society classification [12], as phenotypes have not identified migraine genes yet probably because the diagnosis does not represent biological pathways influenced by specific genetic variations [4,17,28,30,31]. Therefore we collected information about three traits that are frequently associated with headaches (nausea, photophobia, and disability) and have a strong predictive value for migraine. Trait combinations do not necessarily result in migraine diagnosis but classify more subjects with the same type of headache and thus have the potential to increase the power of genetic studies aimed at identifying genes contributing to migraine pathogenesis [4]. This design has been applied previously for linkage studies and resulted in the identification of susceptibility loci for migraine that were not found using an end diagnosis of migraine [4,31]. Using extreme phenotypes, instead of random sampling, further increases the statistical power of genetic association studies [32]. To optimize our study we combined the two above-mentioned methods and we investigated those who reported no ID-migraine symptoms at all as controls (CO) and those who reported all three symptoms as migraine headache sufferers (M). Our hypothesis was that genetic association exists between M and the CNR1. We also measured neuroticism and asked about depression and drug/alcohol use by self-completed questionnaires in order to investigate their effects on any genetic association.

This study was part of the EU funded NewMood (New Molecules for Mood Disorders) research program that aims to identify new molecular mechanisms of vulnerability to depression and co-morbid complaints, especially pain and headache, in an effort to understand how the genetic basis of depression is expressed. Participants aged 18–60 years were recruited through general practices and a website. From a total of 1547 subjects who were willing to take part in this genetic study, we included 1354 participants who were Caucasian origin, returned the completed questionnaire and a genetic sampling kit by post. Of the excluded subjects, 171 were non-Caucasian and 22 subjects sent back an incomplete questionnaire. The study was approved by the local Ethics Committees and was carried out in accordance with the Declaration of Helsinki. All participants provided written informed consent.

We used brief standard questionnaires that were easy for participants to complete and return to us by post. We collected data covering background information (age and ethnicity), together with personal and family psychiatric history. To assess personality we applied the Big Five Inventory (BFI-44) [15]. Neuroticism was calculated using a continuous weighted dimension score (sum of items scored divided by the number of items completed). To collect information about headaches and especially migraine we asked our subjects whether they suffered from migraine in the background section. In a separate section, we also applied the ID-migraine questionnaire which was designed as a screening tool, and included three items that are most strongly associated with the gold-standard migraine diagnosis: nausea, photophobia and disability [19]. As previous studies suggested that a continuum exists between migraine with and without aura, we did not collect information about aura symptoms [22,31]. Also, because of size constraints (to make the booklet acceptable to participants), we did not use further self-administered headache diagnostic questionnaires. Given these limitations we conducted psychiatric interviews in a subgroup of NewMood subjects to validate our data [16] (the website www.newmood.co.uk has more details of the population study's multi-level design). Our interviewers were not trained to diagnose headaches so we could not validate the ID-migraine questionnaire against IHS migraine diagnosis, but this questionnaire has been validated previously in other populations and has good test–retest reliability [26].

From the participants, buccal mucosa cells were collected and genomic DNA was extracted according to a protocol previously described [16]. Based on the AFD_EUR_18-MAY-2004 population data of PERLEGEN (http://genome.perlegen.com/) [13] and the CEPH population data of the International HapMap Project (http://www.hapmap.org, Phase I, June 2005) we employed the HaploView software package (http://www.broad.mit.edu/haploview/haploview) to identify haplotype tag SNPs [5,10] (htSNPs). We also examined possibly functional htSNPs previously identified [33]. The chosen SNPs were genotyped using the Sequenom® MassARRAY technology (Sequenom®, San Diego). The Iplex™ assay was followed according to manufacturers instructions (http://www.sequenom.com) using 25 ng of DNA. Genotyping was blinded with regard to phenotype. All laboratory work was performed under the ISO 9001:2000 quality management requirements.

HaploView program was used to explore the haplotype structure of CNR1 in our populations [5,10], and Quanto 1.2 Version (http://hydra.usc.edu/gxe) to calculate the power of the recruited populations. PLINK v1.04 (http://pngu.mgh.harvard.edu/purcell/plink/) was used for testing Hardy–Weinberg equilibrium, allelic/genotypic association and interaction with gender. Haplotype analyses were performed using HelixTree™ 6.4.1 (Golden Helix, USA). Haplotypes with a frequency equal to or greater than 5% were used in the analysis. Logistic regression model was used to identify variance in the dependent variable explained by sex (the "reduced model"). We then determined whether adding haplotype probabilities as the regression matrix to the model (now the "full model") explained significantly more variance than the reduced model using a likelihood ratio statistic. In case of the full model the rare haplotypes served as left out regressors. Multiple testing issues were addressed by permuting the dependent variable 10,000 times. Other statistical analyses were performed with SPSS for Windows Statistical Analysis Software, Version 15.0. All statistical testing used $p < 0.05$ and all reported p values are two-tailed. Positive likelihood ratios (LR+) were calculated as follows: haplotypes (HT) with greater probability than 70% have been assigned for each subject and cross-tabulated against migraine status. The calculation of LR+ for each HT = ratio of HT frequency in cases compared to HT frequency in controls, as previously described [34].

In the background questionnaire females more frequently reported migraine than males (females 9.5%, males 2.4%; $p < 0.001$). Based on previous studies the migraine prevalence in European countries is around 14% (in females 18%, in males 7%) and about half of the migraineurs have never received a diagnosis [14]. Thus our results, in line with the previous literature, suggest that migraine is under-reported in our population with the simple background question. Using the ID-migraine questionnaire, females more frequently than males reported all three-migraine symptoms (females 28%, males 9%; $p < 0.001$). Five (0.4%) subjects reported migraine in the background questionnaire but no ID-migraine symptoms which mean that the sensitivity of the ID-migraine questionnaire is 92.4% in our population. In contrast, 134 subjects (9.9%) did not report migraine but had all three ID-migraine symptoms. Based on the ID-migraine validation study we can assume that 93% of these subjects meet the criteria of migraine diagnosis and the remaining 7% might have probable migraine or some other headache [26]. Those subjects who reported two symptoms were likely also to suffer from migraines and/or other severe headache problems [19,26]. However, their symptoms were heterogeneous: 71 (31.5%) reported nausea and photophobia, 71 (31.5%) reported photophobia and disability, and 83 (37%) reported nausea and disability. Thus we created two homogenous groups for the genetic association analysis: CO (n = 684) – who have not experienced migraine symptoms in the last 3 months and did not report migraine; M (n = 195) – who suffered from headache with all of the three highly predictive symptoms

Table 1
Haplotypic association results.

Haplotypes			Migraine		
No	Sequences	Frequencies	Coefficient	p	Odds ratio (95% CI)[a]
HT1	A,T,T,T,T,G,G,A,T,C,T	17.53%	−0.265	0.617	1.00 (0.73–1.37)
HT2	A,T,T,C,A,A,A,T,T,C	15.10%	−0.003	0.996	1.02 (0.73–1.43)
HT3	T,T,T,C,G,G,A,T,C,C	8.98%	−5.951	0.045	0.89 (0.59–1.34)
HT4	T,T,T,T,G,G,A,T,C,T	8.28%	−0.066	0.940	0.83 (0.49–1.42)
HT5	T,G,C,C,A,A,A,T,T,C	7.69%	0.848	0.400	0.93 (0.59–1.46)
HT6	A,T,T,C,G,G,A,C,T,T	7.05%	15.391	0.002	1.58 (0.98–2.56)
HT7	T,T,T,C,A,A,A,T,T,C	5.00%	−14.326	0.068	0.50 (0.21–1.20)
	{RareHaps}	30.37%			1.07 (0.81–1.41)
Full reduced model			p	0.008	
			Permutated p	0.017	

Haplotype variants in the *CNR1* are significantly associated with migraine (*M*) (logistic regression, covaried for gender).

[a] Odds ratios calculated for subjects with haplotype probability greater than 70% using Mantel-Haenszel common odds ratio estimate.

for migraine (nausea, photophobia and disability) independent of whether they had migraine diagnosis or aura symptoms.

There was no significant difference between the ages of those in the CO and *M* groups (mean ± S.E.M.: CO = 34.6 ± 0.39, *M* = 33.7 ± 0.72; *p* = 0.289). As expected, there were significantly more females than males in the *M* group compared to CO group (CO: female = 60%, male = 40%; *M*: female = 86%, male = 14%; *p* < 0.001). Also in accordance with the previous literature, *M* subjects had significantly higher neuroticism scores (mean ± S.E.M.: CO = 3.1 ± 0.03, *M* = 3.6 ± 0.06; *p* < 0.001; co-varied for gender) and reported depressive episodes more frequently (CO = 46%, *M* = 69%; *p* < 0.001). Indeed, during the psychiatric interview we found that SCID lifetime major depression criteria were met in 47% of CO subjects (interviewed *n* = 78), but 79% of *M* participants (interviewed *n* = 24), supporting some co-morbidity between these disorders. Furthermore, *M* subjects reported drug and/or alcohol abuse more frequently (CO = 4.8%, *M* = 10.3%; *p* = 0.005).

Seven htSNPs covered the known *CNR1* (rs806369, rs1049353, rs4707436, rs12720071, rs806368, rs806366, rs7766029) and we additionally genotyped three other SNPs (rs806379, rs1535255, rs2023239) that were reported to have a significant effect on the mRNA expression of this gene [33]. All of the genotyped SNPs were in Hardy–Weinberg equilibrium in both COs and Ms. Linkage disequilibrium data (LD R squared values) can be seen in Supplementary Fig. 1. Using single marker association analysis there was a trend that the rs7766029 T allele (*p* = 0.063) was in association with *M*. *M* was significantly associated with the rs806368 C/C genotype (*p* = 0.008) using the recessive model and this association was still significant after adjusting for multiple testing (permutated *p* = 0.0496; Supplementary Table 1). None of the SNPs showed significant interaction with gender. Our power calculation showed that we have a power of between 60% and 93% (minor allele frequency 10–40%) to detect a significant effect of a polymorphism. Thus we have a reasonably good power in our study especially as we used extreme phenotypes instead of a classical case–control design [32].

CNR1 haplotypes were significantly associated with *M* (*p* = 0.008), and the *p*-value remained significant after the permutation test (*p* = 0.017; Table 1). There were seven common haplotypes that involved 69.63% of the tested population. The strongest risk haplotype was HT6 (*p* = 0.002; LR+ = 1.53), while HT3 (*p* = 0.045; LR+ = 0.90) and HT7 (*p* = 0.068; LR+ = 0.51) showed lowest likelihood for *M*.

In our sample, Chi-squared changes caused by genetic effect decreased from 19.09 to 15.80 when we used neuroticism as a covariate in the full model, therefore the *p*-values were also weakened (*p* = 0.027, permutated *p* = 0.052). Using depression as a covariate, we found similar effects (Chi-squared changes decreased from 19.09 to 18.26, *p* = 0.011, permutated *p* = 0.022). Furthermore, repeating the haplotype trend regression after excluding all sub-

jects with drug/alcohol abuse, we found only minor changes in genetic effect: Chi-squared decreased from 19.09 to 17.54 (*p* = 0.014, permutated *p* = 0.026).

In the whole study population (*n* = 1354, not excluding subjects with 1 or 2 symptoms) the overall effect of *CNR1* was not significant for having a headache with any one of the three migraine symptoms (i.e. headache with nausea, *n* = 446; headache with photophobia, *n* = 424; or headache with disability, *n* = 410). However, HT6 carriers have increased risk of suffering from headaches with photophobia (*p* = 0.023, LR+ = 1.41) or headaches with nausea (*p* = 0.074, LR+ = 1.20). HT6 carriers also reported disability more frequently with their headaches but this did not reach a significant level (*p* = 0.235, LR+ = 1.30; Fig. 1).

Our study provides the first evidence in humans that variations in the *CNR1* are associated with migraine. We demonstrated a significant haplotypic association between *CNR1* and headaches with three highly predictive symptoms for migraine (nausea, photophobia and disability). By utilising trait components combinations to define extreme headache phenotypes in a population sample, we were able to identify a fairly homogenous group of headache sufferers (independently of whether or not they were diagnosed with migraine) and consequently increase the statistical power to identify a risk haplotype. As we mentioned above, about half of the migraineurs never receive a diagnosis in their life [14] therefore
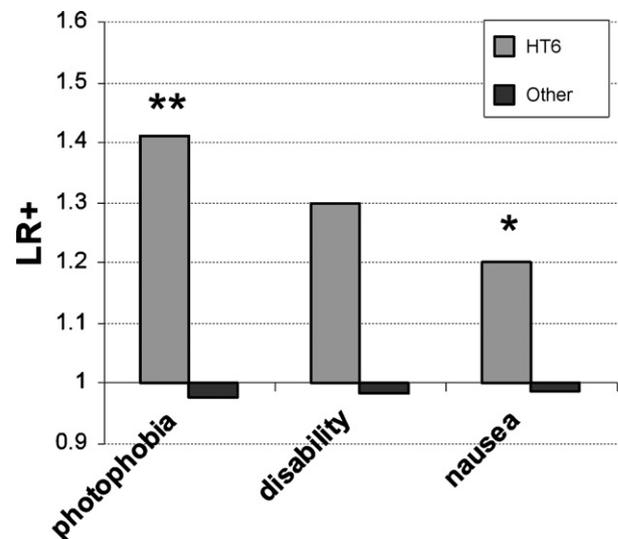
Fig. 1. Positive likelihood ratios (LR+) of HT6 and other (HT1-5, HT7 and rare HT together) haplotypes for migraine headache symptoms in the total recruited population (*n* = 1354). * Trend in haplotype trend association (0.05 < *p* < 0.1); ** significant haplotype trend association (*p* < 0.05).

clinic-based studies have selected populations [30] and selection might bias the results of association studies.

Although the *CNR1* has not been investigated previously as a candidate gene for migraine, linkage studies repeatedly found typical-migraine susceptibility loci on chromosomes 6 [4,7,23]. The human *CNR1* has been mapped to chromosome 6q14–15 (91.8–96.1 cM), which is situated within the region that showed linkage with migraine (71–101 cM on chr6) [23]. This region has been associated with photophobia and disability using latent class analysis method for identifying phenotypes [23] and with vomiting/nausea using trait components method [4]. In our study we used the combination of these three symptoms to classify *M* and determine the risk haplotype. In addition, this risk haplotype increased the likelihood of having these symptoms with headaches in a broader population with less clear headache phenotypes. Data from both our current study and the linkage studies mentioned above, strongly suggest that the *CNR1* is a candidate gene for migraine although replication studies in larger and better-characterised populations are essential to confirm this hypothesis.

CB1 receptors can be detected in periaqueductal gray matter, rostral ventromedial medulla and trigeminal nucleus, which are potential migraine generators and pain modulators [1,9]. In CB1 knockout mice, the antinociceptive response to cannabinoids is absent or attenuated demonstrating the important role of CB1 receptors in mediating analgesia [1]. Based on these data we can assume that the risk haplotype results in attenuated CB1 receptor expression or function, therefore making the carriers more vulnerable to migraine. Migraineurs and patients with medication-overuse headaches showed decreased anandamide levels in cerebrospinal fluid (CSF) and in platelets [27,29]. In addition, lower anandamide and 2-arachidonoylglycerol levels were found to be positively correlated with peripheral serotonin concentration [27] and inversely correlated with calcitonin gene-related peptide (CGRP) and nitric oxide (NO) concentration in the CSF [29], this further emphasises the role of the endocannabinoid system in migraine pathophysiology.

However, CB1 receptors are expressed not only in the central nervous system but also on axon terminals of primary sensory neurons, such as the nociceptive areas of spinal cord, dorsal root ganglia and trigeminal ganglia and CB1 expression is partially co-localised with CGRP and substance P expressing neurons [9,24,25]. Therefore it is possible that cannabinoids exert their antinociceptive effect through peripheral CB1 receptors. Indeed, using knockout mice it has been demonstrated that CB1 receptors in the GABAergic or cortical glutamatergic neurons are not essential for the analgesic effect of Δ9-tetrahydrocannabinol [21], but deletion of CB1 receptors in the peripheral nociceptors considerably reduced the analgesic effect of local and systemic cannabinoids [1]. Thus it is feasible that synthetic cannabinoids, that do not cross the blood-brain barrier, are effective in several pain syndromes [1], and also in migraine. However, this hypothesis has not been tested in relation of trigeminovascular system, and no human data are available to our knowledge.

CB1 receptor stimulation causes activation of $G_{i/o}$-type G-proteins that mediate different inhibitory mechanisms, especially inhibition of voltage-gated calcium channels and activation of potassium channels, and also direct inhibition of the vesicle fusion process [9]. It is interesting to note that all genetic mutations identified in familial hemiplegic migraine (FHM) are related to ion channels or ion transporters [30]. However, the contribution of these genes to common forms of migraine is not yet understood. Based on our results, it is possible that mutations in the ion channel genes cause severe forms of migraine with additional neurological symptoms, while the common forms of migraine are associated with variation in the regulatory mechanisms of ion channels (e.g. CB1 receptor expression).

To further elucidate the possible role of cannabinoids in migraine we tested whether psychiatric co-morbidities, which have been related to abnormalities in the endocannabinoid system, have any confounding effects on the association of *CNR1* with migraine. In humans, rimonabant, a CB1-receptor antagonist that has been tried as an anti-obesity agent, shows significant psychiatric side effects such as depression, anxiety and increased risk of suicide [8]. Our group previously demonstrated that variations in the *CNR1* are associated with neuroticism, namely HT2 carriers showed significantly higher neuroticism scores presumably by reduction in CB1 receptor signalling. CNR1 also influences the effect of recent negative life events on depressive symptoms [16]. Furthermore, CB1 receptors are densely represented in the brain reward circuitry and have a major role in addictive behaviour induced by different drugs [20,33,34]. However, we demonstrated that although neuroticism scores, self-reported depression or drug/alcohol use slightly decreased the association between *CNR1* and migraine, it still remained significant or a strong trend. Therefore our results suggest that although some common mechanisms in these conditions might be driven by the *CNR1*, the main effect of *CNR1* on migraine is independent from these co-morbidities.

In conclusion, our study demonstrates that the *CNR1*, and therefore the endogenous cannabinoid system, may have an important role in susceptibility of migraine. This effect to a certain extent is independent of related psychiatric co-morbidities, such as depression and substance abuse, suggesting that variation in the peripheral CB1 receptor function might be responsible for this association. Further research is warranted to test how *CNR1* exerts its effect on the development of migraine headaches and whether synthetic cannabinoids without central side effects are useful in migraine therapy. In addition, we provide the first evidence that using trait components combinations to identify extreme phenotypes and combined with haplotype analysis might be valuable in genetic association studies of migraine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.06.021.

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