Variants in the *CNR1* gene predispose to headache with nausea in the presence of life stress

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One of the main effects of the endocannabinoid system in the brain is stress adaptation with presynaptic endocannabinoid receptor 1 (CB1 receptors) playing a major role. In the present study, we investigated whether the effect of the CB1 receptor coding *CNR1* gene on migraine and its symptoms is conditional on life stress. In a cross-sectional European population ($n = 2426$), recruited from Manchester and Budapest, we used the ID-Migraine questionnaire for migraine screening, the Life Threatening Experiences questionnaire to measure recent negative life events (RLE), and covered the *CNR1* gene with 11 SNPs. The main genetic effects and the *CNR1* × RLE interaction with age and sex as covariates were tested. None of the SNPs showed main genetic effects on possible migraine or its symptoms, but 5 SNPs showed nominally significant interaction with RLE on headache with nausea using logistic regression models. The effect of rs806366 remained significant after correction for multiple testing and replicated in the subpopulations. This effect was independent from depression- and anxiety-related phenotypes. In addition, a Bayesian systems-based analysis demonstrated that in the development of headache with nausea all SNPs were more relevant with higher *a posteriori* probability in those who experienced recent life stress. In summary, the *CNR1* gene in interaction with life stress increased the risk of headache with nausea suggesting a specific pathological mechanism to develop migraine, and indicating that a subgroup of migraine patients, who suffer from life stress triggered migraine with frequent nausea, may benefit from therapies that increase the endocannabinoid tone.

Migraine is a complex multifactorial neurologic disorder, in which genetic factors explain about 46% of the risk with the remaining 54% of the variability related to environmental factors (Mulder *et al.* 2003). Recent genome wide association studies (GWAS) have successfully identified several genetic susceptibility loci for migraine (Anttila *et al.* 2013; Gormley *et al.* 2016) but much less is known of which genetic variants operate in the presence of a given environmental effect (Eising *et al.* 2013b).

One of the main environmental risk factor for migraine is psychosocial stress (Sauro & Becker 2009), and it has been demonstrated that migraine patients show maladaptive stress responses (Borsook *et al.* 2012; Lipton *et al.* 2014). The endocannabinoid system plays a key role in modulation of stress response (Hill *et al.* 2010; McLaughlin *et al.* 2014). Its two main mediators are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) that are synthesized on demand in the postsynaptic cells and act as retrograde neurotransmitters on GABAergic and glutamatergic neurons to balance inhibitory and excitatory neural activity. In the brain the primary target of endocannabinoids is the endocannabinoid receptor 1 (CB1 receptor), which is predominantly expressed in cortical and limbic areas and is responsible for the regulation of stress response and emotional behaviour (McLaughlin *et al.* 2014).

Activation of the CB1 receptors enhances the activity of serotonergic (5HT) and noradrenergic (NA) neurons in the brainstem (McLaughlin *et al.* 2014) and, through negative feedback inhibition, controls the activity of hypothalamic–pituitary–adrenal (HPA) axis (Hill *et al.* 2010). Thus lack or decreased CB1 signalling results in chronic stress-like state (Lazary *et al.* 2011). More importantly, it has been demonstrated that during chronic stress the endocannabinoid signalling changes gradually leading to stress habituation (Patel & Hillard 2008), which is impaired in migraine patients (Afra *et al.* 2000).

Indeed, it has been previously proposed that migraine is related to endocannabinoid deficiency (Russo 2004), although whether it is the cause or the consequence of

migraine has not been understood yet. In human studies, the metabolism of endocannabinoids is increased in migraineurs (Cupini *et al.* 2006) resulting in decreased endocannabinoid tone (Sarchielli *et al.* 2007; Van der Schueren *et al.* 2012). In addition, animal studies showed that AEA was able to diminish the activation of the trigeminovascular system in nitroglycerin-induced migraine models (Akerman *et al.* 2004; Greco *et al.* 2010; Nagy-Grocz *et al.* 2015) through a CB1 receptor mediated mechanism (Akerman *et al.* 2013).

Indeed, our previous genetic association studies showed that genetic variants in the CB1 receptor coding *CNR1* gene were associated with neuroticism (Juhasz *et al.* 2009a), a personality trait which predisposes to perceive life events as stressful and which is a risk factor for migraine (Ligthart & Boomsma 2012). In addition, *CNR1* genetic variants in interaction with recent negative life events (RLE) increased the risk of depression (Juhasz *et al.* 2009a), and in interaction with the serotonin transporter functional polymorphism (*5HTTLPR)* exerted anxiogenic effects (Lazary *et al.* 2009, 2011). Regarding migraine, we demonstrated that a *CNR1* haplotype increased the risk of migraine headaches (Juhasz *et al.* 2009b). However, recent migraine GWAS studies could not identify risk genetic variants within the endocannabinoid system (Anttila *et al.* 2013; Gormley *et al.* 2016). Although accumulating evidence suggests that impaired CB1 signalling is associated with chronic stress-related hyperalgesia (Jennings *et al.* 2015; Lomazzo *et al.* 2015; Rea *et al.* 2014), the relationship between *CNR1* gene and migraine has not been investigated in interaction with life stressors.

Thus, in the present study we tested the hypothesis that the *CNR1* gene in interaction with life stressors is an important risk factor for migraine type headache, in a European cohort recruited from Budapest and Manchester. Furthermore, we hypothesized that the modulatory effect of life stress - *CNR1* gene interaction is different for specific migraine related symptoms.

Materials and methods

Subjects
Participants aged between 18 and 60 years from Greater Manchester, UK and Budapest, Hungary have been recruited through general practices and advertisements. The studies were part of NewMood (New Molecules in mood Disorders, 2004–2009), an EU funded research programme into pathomechanism of depression and related conditions. Both studies were approved by local Ethics Committees (Scientific and Research Ethics Committee of the Medical Research Council, Budapest, Hungary, ad.225/KO/2005.; ad.323-60/2005-1018EKU and ad.226/KO/2005.; ad.323-61/2005-1018 EKU; North Manchester Local Research Ethics Committee, Manchester, UK REC reference number: 05/Q1406/26) and were carried out in accordance with the Declaration of Helsinki. All participants provided written informed consent to the study. In the present research, we included participants who were European white origin, completed the questionnaires and consented to DNA. Details of the recruitment strategies and response rate were published earlier (Juhasz *et al.* 2009a; Lazary *et al.* 2009).

Questionnaires
Brief standard questionnaires, English and Hungarian versions respectively, were used for the study. The background questionnaire collected information about socio-demographic data, personal and family psychiatric history. Sex, age, ethnicity, reported migraine and reported lifetime depression data were derived from this validated questionnaire (Juhasz *et al.* 2011).

The ID-migraine questionnaire was used to collect information about headaches and especially migraine type headache symptoms in the past 3 months (Lipton *et al.* 2003). The ID-Migraine is a validated screening tool for migraine, which includes 3 items of the main migraine symptoms: nausea, photophobia and disability. In the present study we assigned possible migraine to patients who answered YES to 2 or 3 migraine symptom questions as they have 93% probability of having migraine based on the IHS diagnostic criteria for migraine (Lipton *et al.* 2003). In addition, we investigated the genetic effect on the 3 reported symptoms separately.

The RLE score was based on the validated Life Threatening Experiences questionnaire (Brugha *et al.* 1985) and we calculated the sum of negative life events in the last year for the analysis.

To measure neuroticism the Big Five Inventory (BFI-44) (John & Srivastava 1999) was applied. Current (last week) anxiety symptoms and depressive symptoms were measured by the relevant subscales of the Brief Symptom Inventory (BSI) (anxiety subscale for anxiety, and depression plus additive subscales for depression; (Derogatis 1993). For these variables continuous weighted dimension scores (sum of item scores divided by the number of items completed) were calculated for the analysis.

Genotyping
Genomic DNA was extracted from buccal mucosa cells (Juhasz *et al.* 2009a). After extraction of DNA all samples were normalized and genotyped using the Sequenom® MassARRAY Technology (Sequenom®, San Diego, CA, USA, www.sequenom.com). Genotyping was performed under the ISO 9001:2000 requirements and was blinded for the phenotypic data.

To investigate the effect of the *CNR1* gene we selected possibly functional single nucleotide polymorphisms (SNPs) from previous literature and haplotype tag SNPs (htSNPs) to cover the whole gene. Genetic data were extracted from the International HapMap project (http://hapmap.ncbi.nlm.nih.gov/, Phase I. June 2005, CEPH population and AFD_EUR_18-MAY-2004 panel). Gabriel method implemented in the HaploView software package was used to identify htSNPs with minimum pairwise correlation $r^2 = 0.8$ (Gabriel *et al.* 2002a). The list and minor allele frequencies of the selected SNPs can be seen in Table 1.

Statistical analysis
To calculate Hardy-Weinberg equilibrium *P* values, to run logistic regression analysis with additive genetic models, and to compute haplotypes PLINK v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) analysis programme was used. First, statistical analysis for the main effect of the *CNR1* SNPs and the *CNR1* SNPs × RLE interactions on possible migraine and migraine-related symptoms were carried out in the total sample, according to a recent guideline (Dick *et al.* 2015). Pairwise deletion was used to handle missing data. To adjust *P* values for the multiple testing false discovery rate q values were calculated and were accepted at level of 5% (Q value, http://www.bioconductor.org/packages/release/bioc/html/qvalue.html). To handle possible ancestral differences, ethnicity was determined by the self-reported data derived from the Background questionnaire (see Questionnaires) and subjects with European white origin were included in the analysis. In addition, possible ancestral differences according to study sites were tested by including this factor (namely, Budapest vs. Manchester) into the post hoc tests. Thus we tested the main finding in these subgroups separately, and also ran a post hoc test by including study site into the analysis.

In addition, haplotypes were computed for the nominally significant SNPs in haploblock 1 applying the expectation maximization (EM) algorithm. The effects of the computed haplotypes were similarly tested in the total sample as the SNPs, also as a post hoc test. Age and sex were covariates in all analyses.

Table 1: Details of the investigated populations

A. Phenotypic description	Total population	Manchester	Budapest
Demographics			
Participant number (*n*)	2426	1375	1051
Female (*n*, %)	1690 (70%)	958 (70%)	732 (70%)
Age (mean ± SEM)	32.9 (± 0.22)	34.04 (± 0.28)	31.2 (± 0.34)
Migraine and headache			
Reported migraine (*n*, %)	144 (6%)	104 (8%)	40 (4%)
ID-possible migraine (*n*, %)	668 (28%)	430 (31%)	238 (23%)
ID nausea (*n*, %)	710 (29%)	454 (33%)	256 (24%)
ID photophobia (*n*, %)	688 (28%)	433 (32%)	255 (24%)
ID disability (*n*, %)	673 (28%)	422 (31%)	251 (24%)
Stresses			
Recent negative life events (mean ± SEM)	1.22 (± 0.03)	1.32 (± 0.04)	1.08 (± 0.04)
Recent life event categories (*n*, %)			
No or mild	1619 (67%)	880 (64%)	739 (70%)
Moderate	451 (19%)	258 (19%)	193 (19%)
Severe	352 (14%)	237 (17%)	115 (11%)
Psychiatric measures			
Reported lifetime depression (*n*, %)	989 (41%)	770 (56%)	219 (21%)
BFI neuroticism (mean ± SEM)	3.12 (± 0.02)	3.36 (± 0.03)	2.81 (± 0.03)
BSI current depression score (mean ± SEM)	0.85 (± 0.02)	1.07 (± 0.03)	0.55 (± 0.02)
BSI current anxiety score (mean ± SEM)	0.87 (± 0.02)	1.02 (± 0.03)	0.69 (± 0.02)
B. Genetic data			
Minor allele frequencies (MAF)*			
rs2180619 (G/A)	39.6%	37.8%	42.2%
rs806379 (T/A)	47.4%	49.5%	44.7%
rs1535255 (G/T)	17.3%	18.7%	15.4%
rs2023239 (C/T)	17.6%	19.1%	15.5%
rs806369 (T/C)	30.0%	27.9%	32.8%
rs1049353 (A/G)	25.9%	28.3%	22.7%
rs4707436 (A/G)	26.1%	28.5%	22.9%
rs12720071 (G/A)	8.8%	8.3%	9.6%
rs806368 (C/T)	21.3%	20.0%	23.2%
rs806366 (T/C)	48.4%	49.7%	46.9%
rs7766029 (T/C)	48.1%	46.3%	50.4%

(A) Shows the phenotypic data, (B) summarises the genetic variables.
BFI, Big Five Inventory (30); BSI, Brief Symptom Inventory (31); ID, data derived from the ID-Migraine questionnaire (28); SEM, standard error of mean.
*All SNPs are in Hardy-Weinberg equilibrium with an average callrate of 95%.

To further explore the effects of *CNR1* SNPs in subpopulations defined by the recent life event categories (no or mild = 0–1, moderate = 2, severe = 3 or more) systems-based Bayesian relevance analysis was carried out (Antal *et al.* 2006, 2014; Hullam *et al.* 2012) to determine the strong relevance of predictors (11 *CNR1* SNPs, age and sex) with respect to headache with nausea. This method applies Bayesian model averaging, both at structural and parametric levels, which provides a coherent multivariate solution for the multiple hypothesis problem. For detailed method, see Antal *et al.* (2014) and Juhasz *et al.* (2014, 2015).

Additional statistical analyses were performed with SPSS 21.0 for Windows (IBM). All statistical testing adopted two-tailed $P = 0.05$ threshold. For display purposes, in all three RLE severity categories, we calculated positive likelihood ratios (LR+) for significant genotypes and haplotypes by dividing the genotype/haplotype frequencies in possible migraine cases/symptom carriers by those in control subjects/non-carriers, as previously described (Juhasz *et al.* 2009b).

Quanto 1.2 version (http://biostats.usc.edu/Quanto.html/) was employed to calculate the power of the recruited populations. Assuming a case–control design (3 controls/case) and an additive genetic model with a minor allele frequency between 10% and 50%

in our study ($n = 2426$) we have 45–84% power to detect genetic main effects and 65–95% power to detect a gene × environment interaction ($P = 0.05$ two-tailed) that is associated with 1.2 odds ratio for a disease.

Results

Detailed description of the included study population can be found in Table 1. Two-thirds of the recruited study population was female and about 40% reported lifetime depression.

Despite the fact that only 6% of the subjects reported migraine as a long-standing medical condition in the total study population, about one-third of the subjects had migraine-related symptoms when they experienced headache in the last 3 months. Based on our data in this study the ID migraine questionnaire compared to the
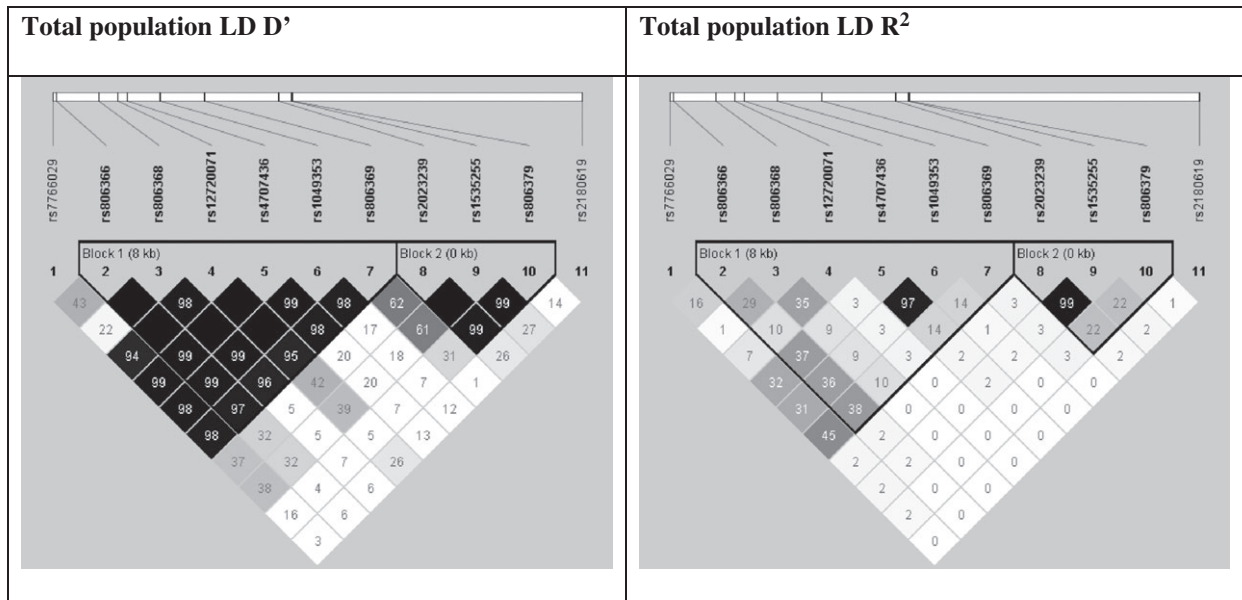
Figure 1: LD block structure of the total population. Linkage analysis showed two main haploblocks (haploblock 1: rs806366, rs806368, rs12720071, rs4707436, rs1049353, rs806369 and haploblock 2: rs2023239, rs1535255, rs806379; while rs7766029 at the 3′ end and rs2180619 at the 5′ end were not in linkage with these haploblocks.

reported migraine had 85% sensitivity and 76% specificity to identify possible migraine, which is in a comparable range with the original validation study (Lipton *et al.* 2003).

CNR1 *gene*

All of the investigated SNPs were in Hardy-Weinberg equilibrium, both in the total population and in the separate populations according to study sites of Budapest and Manchester, respectively. Linkage analysis in HaploView (Barrett *et al.* 2005; Gabriel *et al.* 2002b) showed two main haploblocks (haploblock 1: rs806366, rs806368, rs12720071, rs4707436, rs1049353, rs806369, and haploblock 2: rs2023239, rs1535255, rs806379; while rs7766029 at the 3′ end and rs2180619 at the 5′ end were not in linkage with these haploblocks. This genetic structure was similar in the Budapest and Manchester sample and agrees with the LD pattern of the European white populations. (For LD block structure of the total population see Fig. 1, and of the investigated subpopulations see Fig. S1.)

Main effect of RLEs on headache

Using logistic regression model, as we expected, with increasing number of RLE the odds of having possible migraine or migraine related symptoms were increased [possible migraine: Odds Ratio (OR) = 1.32 (95% CI 1.23–1.41) $P < 0.001$; nausea: OR = 1.24 (95% CI 1.16–1.33) $P < 0.001$; photophobia: OR = 1.24 (95% CI 1.16–1.33) $P < 0.001$; disability: OR = 1.28 (95% CI 1.19–1.37) $P < 0.001$; Fig. 2]. This effect remained significant when lifetime depression, current depression and anxiety score, and neuroticism were included in the regression model [possible migraine: OR = 1.17
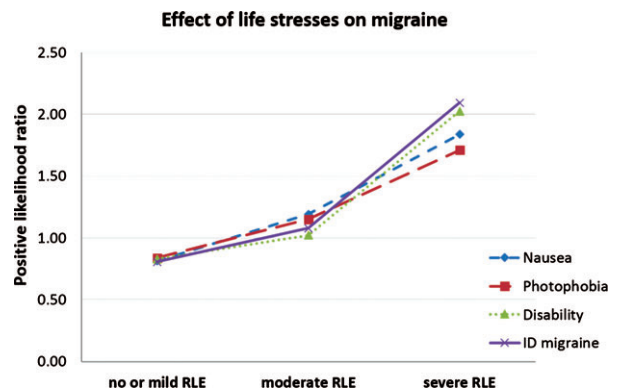

Figure 2: Effect of life stresses on headache. RLEs (recent negative life events) experienced in the last year increased the positive likelihood ratio of having possible migraine or migraine related symptoms with headache, defined by the ID-Migraine screening questionnaire.

(95% CI 1.08–1.26) $P < 0.001$; nausea: OR = 1.10 (95% CI 1.02–1.18) $P = 0.014$; photophobia: OR = 1.15 (95% CI 1.07–1.23) $P < 0.001$; disability: OR = 1.16 (95% CI 1.08–1.25) $P < 0.001$].

Main effect of CNR1 *gene on headache*

None of the investigated SNPs showed significant main genetic effect on possible migraine or migraine related symptoms using logistic regression models with age and sex as co-variants.

Table 2: Statistical results of interaction effect between RLE and *CNR1* gene haplotype tag SNPs in the total study population

		Possible migraine				Headache with nausea				Headache with photophobia				Headache with disability			
SNP	A1	OR	L95	U95	*P*	OR	L95	U95	*P*	OR	L95	U95	*P*	OR	L95	U95	*P*
1 rs2180619	G	1.006	0.908	1.115	0.904	0.922	0.834	1.021	0.118	1.000	0.904	1.106	0.999	1.021	0.923	1.130	0.687
2 rs806379	T	0.969	0.877	1.071	0.537	1.012	0.917	1.117	0.811	*0.910*	*0.825*	*1.004*	*0.061*	1.007	0.912	1.112	0.893
3 rs1535255	G	0.919	0.808	1.044	0.195	0.939	0.828	1.065	0.326	0.963	0.850	1.093	0.562	0.966	0.851	1.097	0.594
4 rs2023239	C	0.931	0.816	1.062	0.287	0.935	0.822	1.063	0.302	0.985	0.867	1.120	0.820	1.000	0.878	1.138	0.994
5 rs806369	T	1.085	0.967	1.217	0.166	**1.123**	**1.002**	**1.259**	**0.045**	1.090	0.974	1.221	0.134	1.086	0.970	1.217	0.154
6 rs1049353	A	**0.885**	**0.789**	**0.993**	**0.037**	**0.846**	**0.755**	**0.948**	**0.004**	0.965	0.863	1.079	0.531	0.915	0.817	1.024	0.123
7 rs4707436	A	*0.892*	*0.796*	*1.001*	*0.051*	**0.860**	**0.767**	**0.963**	**0.009**	0.981	0.877	1.097	0.737	0.928	0.829	1.039	0.196
8 rs12720071	G	1.003	0.833	1.209	0.974	0.980	0.816	1.178	0.832	0.944	0.786	1.132	0.531	0.933	0.780	1.115	0.447
9 rs806368	C	1.010	0.896	1.139	0.873	0.943	0.838	1.061	0.327	0.985	0.876	1.107	0.797	0.974	0.865	1.096	0.661
10 rs806366	T	**0.884**	**0.791**	**0.987**	**0.028**	**0.819**	**0.733**	**0.916**	**0.0005***	0.959	0.862	1.066	0.438	**0.896**	**0.804**	**0.999**	**0.048**
11 rs7766029	T	**1.111**	**1.001**	**1.232**	**0.047**	**1.114**	**1.006**	**1.234**	**0.038**	1.079	0.976	1.194	0.139	*1.103*	*0.996*	*1.222*	*0.060*

Possible migraine: 2 or 3 migraine related symptoms measured by the ID-Migraine screening questionnaire.
Bold, nominally significant effects; italic, trend effects; L95-U95, 95% confidence interval; OR, odds ratio; *P*, significance.
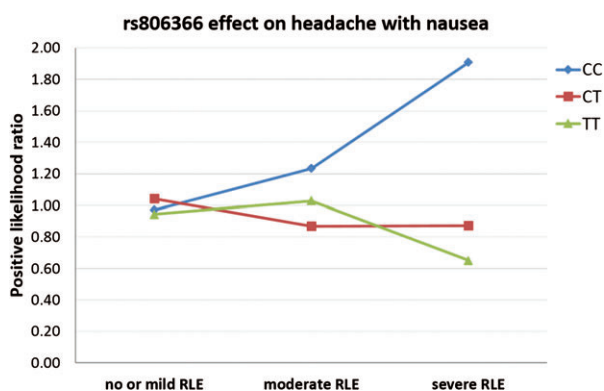*Remained significant after false discovery rate correction of multiple testing.

Figure 3: rs806366 effect on headache with nausea. The interaction effect of recent negative life events (RLE) and the rs806366 within the *CNR1* gene on headache with nausea in the total population. Number of cases in the no or mild RLE group: CC = 361, CT = 684, TT = 315. Number of cases in the moderate RLE group: CC = 103, CT = 183, TT = 95. Number of cases in the severe RLE groups: CC = 74, CT = 147, TT = 69.

CNR1 × *RLE* interaction on headache

Nominally significant interaction effects were identified between rs1049353, rs806366, rs7766029 and RLE on possible migraine, between rs806369, rs1049353, rs4707436, rs806366, rs7766029 and RLE on headache with nausea, and between rs806366 and RLE on headache with disability (Table 2). Taking into account the number of tests we carried out (4 phenotypes, genetic main effect, gene × environment interactions, 11 SNPs) after false discovery rate correction for multiple testing the interaction effect of rs806366 and RLE on headache with nausea remained significant (FDR *q* = 0.040). The minor T allele decreased the risk of having headache with nausea with the increasing number of RLE (Fig. 3).

Post hoc tests of **CNR1** × **RLE** interaction on headache with nausea

Investigating the *CNR1* × RLE interaction on headache with nausea separately according to study sites, 8 out of 11 SNPs showed the same direction of effect in both populations and the effect of rs806366 × RLE was replicated in the Budapest and Manchester samples at nominal significance level (Table 3).

In the total population, the rs806366 × RLE interaction effect remained significant after controlling for the effects of the study site, neuroticism, lifetime depression and current depression and anxiety scores (Table 3).

To demonstrate the cumulative effect of the significant SNPs in haploblock 1 (rs806366, rs4707436, rs1049353, rs806369) in the total population we computed haplotypes and tested the *CNR1* × RLE interaction effects on headache with nausea (for haplotype frequency and results see Table S1, Supporting Information and Fig. 2). The most frequent TGGC haplotype (frequency 30.8%) was a significant risk variant [OR = 1.20 (95% CI 1.06–1.36) *P* = 0.005] whereas the complementary CAAT haplotype (frequency 26.5%) showed significant protective effect [OR = 0.84 (95% CI 0.75–0.95) *P* = 0.006; Fig. 4]. Other frequent haplotypes (frequency > 5%) had no significant interaction effect on headache with nausea.

Bayesian relevance of **CNR1** *SNPs on headache with nausea in different RLE categories*

Using a Bayesian model averaging approach over models consisting of all SNPs plus age and sex, an *a posteriori* probability of strong relevance was calculated for each predictor. Results indicated that all *CNR1* SNPs had higher *a posteriori* probability of strong relevance in subgroups with either moderate or severe RLE (moderate = 2, severe = 3 or more serious life events in the last year) compared to no or mild RLE (no or mild = 0–1 serious life events in the last year) (Fig. 5 and Appendix S1). The *a posteriori* probability of strong relevance

Table 3: Post hoc analysis of *CNR1* × RLE interaction on headache with nausea in the separate study populations, and in the total population after controlling for study sites, neuroticism, lifetime depression and current depression and anxiety scores

			Budapest				Manchester				Total population corrected for study site and depression related variables			
	SNP	A1	OR	L95	U95	*P*	OR	L95	U95	*P*	OR	L95	U95	*P*
1	rs2180619	G	1.055	0.879	1.266	0.564	**0.870**	**0.768**	**0.985**	**0.028**	0.931	0.836	1.036	0.189
2	rs806379	T	0.905	0.759	1.080	0.268	1.083	0.959	1.224	0.199	1.033	0.930	1.146	0.547
3	rs1535255	G	0.977	0.791	1.208	0.830	0.923	0.788	1.081	0.322	0.935	0.820	1.067	0.319
4	rs2023239	C	0.937	0.757	1.160	0.550	0.939	0.798	1.105	0.449	0.925	0.809	1.057	0.253
5	rs806369	T	1.173	0.962	1.430	0.115	1.087	0.942	1.254	0.252	*1.128*	*0.998*	*1.275*	*0.054*
6	rs1049353	A	0.875	0.711	1.077	0.209	**0.834**	**0.726**	**0.959**	**0.011**	**0.840**	**0.744**	**0.948**	**0.005**
7	rs4707436	A	0.869	0.706	1.068	0.181	**0.856**	**0.745**	**0.983**	**0.028**	**0.854**	**0.757**	**0.964**	**0.010**
8	rs12720071	G	1.176	0.853	1.621	0.324	0.910	0.721	1.150	0.431	0.964	0.797	1.164	0.700
9	rs806368	C	0.953	0.783	1.160	0.630	0.943	0.811	1.095	0.440	0.941	0.831	1.065	0.335
10	rs806366	T	**0.818**	**0.677**	**0.988**	**0.037**	**0.829**	**0.720**	**0.955**	**0.009**	**0.814**	**0.722**	**0.917**	**0.0007**
11	rs7766029	T	1.026	0.848	1.240	0.792	**1.149**	**1.013**	**1.303**	**0.030**	*1.104*	*0.990*	*1.232*	*0.076*

Bold, nominally significant effects; italic, trend effects; L95-U95, 95% confidence interval; OR, odds ratio; *P*, significance.
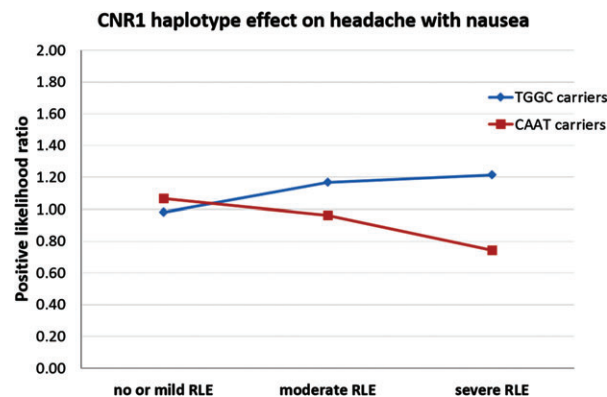

Figure 4: CNR1 haplotype effect on headache with nausea. Significant *CNR1* haplotype × recent negative life events (RLE) interaction effects on headache with nausea in the total population. Number of cases in the no or mild RLE group: TGGC carriers = 733, CAAT carriers = 620. Number of cases in the moderate RLE group: TGGC carriers = 186, CAAT carriers = 177. Number of cases in the severe RLE groups: TGGC carriers = 152, CAAT carriers = 144.

was highest for rs806366 (Pr = 0.8) in the severe RLE sub-population, giving a similar result to the frequentist analysis. This suggests that in 80% of all models the rs806366 was an important factor to determine headache with nausea.

Discussion

Our results show that the *CNR1* gene in interaction with life stresses increases the risk of headache with nausea, suggesting a potential specific pathological mechanism in the development of migraine. This finding emphasises that possible migraine, as a screening questionnaire outcome, is not directly associated with the *CNR1* gene, which is

supported by recent GWAS studies (Anttila *et al.* 2013). However, (1) the *CNR1* gene exerts a significant effect on headache with nausea rather than possible migraine itself, and (2) the *CNR1* gene effect on headache with nausea is conditional on the presence of stressors that might serve as migraine triggers. This latter observation is supported by previous studies, which confirmed that endocannabinoid signalling has a fundamental role in stress adaptation (Hill *et al.* 2010).

A recent large meta-analysis of GWAS studies (Anttila *et al.* 2013) identified several susceptible genetic loci for migraine from which only one (*TRPM8*, encoding the transient receptor potential melastatin 8, which is a cold and menthol-activated ion channel in sensory neurons) has a direct role in pain sensation. The other genetic variants are predominantly expressed in the brain, mainly exert their effects through synaptic and neuronal regulation (Anttila *et al.* 2013; Eising *et al.* 2013a) and thus may contribute to the neuronal hyperexcitability of the migraine brain. In addition, it has been demonstrated that these genetic variants, with the exception of *TRPM8*, show different and unique association patterns with additional migraine features, such as nausea, photophobia or aggravation by physical activity, which suggests that genetic variants play a divergent pathophysiological role in the development of migraine (Chasman *et al.* 2014; Zameel Cader 2013), similar to our finding.

The endocannabinoid system, acting through the *CNR1* coded CB1 receptor, is another key player in synaptic plasticity that has modulatory effects on trigeminovascular activation (Akerman *et al.* 2004, 2013; Nagy-Grocz *et al.* 2015), pain processing (Morena *et al.* 2016) and regulation of nausea and vomiting (Parker *et al.* 2011; Sharkey *et al.* 2014) drawing attention to its potential role in the pathophysiology of migraine.

Our results do not support the hypothesis that in humans genetic variations of the *CNR1* gene are directly associated with migraine type headache through the effect of endocannabinoid system on trigeminovascular activation. This is because we have not seen any significant main effects of
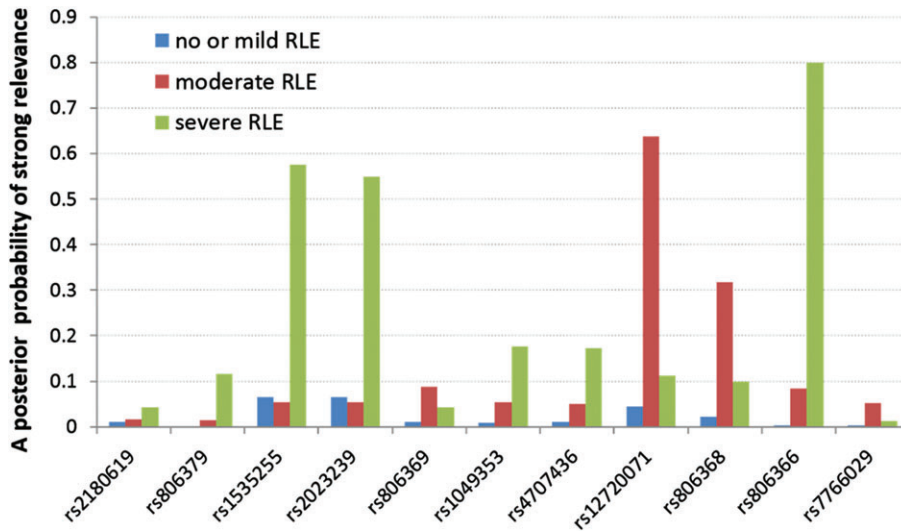
Relevance of *CNR1* SNPs on headache with nausea

Figure 5: Relevance of CNR1 SNPs on headache with nausea. *A posteriori* probability of strong relevance of *CNR1* SNPs on headache with nausea according to the RLE (recent negative life events) exposure.

SNPs on possible migraine headache. Indeed, CB1 receptors are more abundantly expressed in the prefrontal cortical areas compared to the brainstem (McLaughlin *et al.* 2014). In our previous smaller study, we found direct effects of *CNR1* gene on migraine only when using extreme trait combinations (0 symptoms vs. 3 symptoms on ID-Migraine questionnaire) and haplotype analysis of the *CNR1* gene. Even in this case, in the extended sample (including those who has only 1 or 2 symptoms) the distribution of association patterns with other migraine related symptoms were uneven (Juhasz *et al.* 2009b) suggesting that this gene has a selective pathophysiological role.

The endocannabinoid system plays an important regulatory role in pain processing at multiple levels of this pathway. The activation of the CB1 receptors have analgesic effects at the peripheral sensory afferents, in the dorsal horn of the spinal cord, the periaqueductal grey (PAG) and the brainstem (Woodhams *et al.* 2015). In addition, endocannabinoids are indispensable in the development of acute stress induced analgesia (Morena *et al.* 2016), and the downregulation of endocannabinoid signalling is an important mechanism in the development of chronic stress induced hyperalgesia (Jennings *et al.* 2015; Lomazzo *et al.* 2015; Rea *et al.* 2014). However, more and more evidence suggests that endocannabinoids mainly influence the affective component of pain, in which amygdala activity has a pivotal role, compared to the sensory aspects (Lee *et al.* 2013). As such, the strength of life stress × *CNR1* gene interaction on headache with nausea would be expected to decrease after controlling for depression and anxiety related phenotypes, but this was not the case. Thus, our findings suggest that a distinctive mechanism related to stress induced headache with nausea is primarily responsible for the effect of *CNR1* gene in migraine type headache in humans.

Nausea and/or vomiting is a characteristic feature of migraine headache, which has been reported by 70–90% of migraine patients (Lipton *et al.* 2001). The presence of nausea and its intensity correlates significantly with migraine pain severity (Kelman & Tanis 2006). In addition, frequent nausea occurs in about 50% of migraine sufferers where headache is accompanied by nausea more than half of the time, which predicts more disability (Lipton *et al.* 2013), worse quality of life, and transition to chronic migraine (Reed *et al.* 2015). Furthermore, a major problem with nausea is that it can interfere with willingness to take and ingest oral migraine medication causing ineffective pain control (Lipton *et al.* 2013).

Accumulating evidence supports cannabis and endocannabinoids supressing emesis and nausea through the CB1 receptor (Parker *et al.* 2011; Sharkey *et al.* 2014). In animal models, cannabinoid derivatives have a distinctive effect compared to other available antiemetic drugs in being able to suppress not only vomiting but also anticipatory and delayed nausea, probably via the inhibitory effect of CB1 receptors on serotonin release in the insular cortex (Parker *et al.* 2015); this is a possible mechanism in humans as well.

The neural regulator of emesis is integrated at the dorsal vagal complex, which receives peripheral (gut), vestibular, and cerebral inputs and initiates motor response characteristic for vomiting (Parker *et al.* 2011; Sharkey *et al.* 2014). Nevertheless, the neuronal control of nausea is poorly understood but is clearly distinctive from the emesis control. A recent human functional magnetic resonance imaging (fMRI) study suggested that sustained nausea activates an extensive network of limbic, interoceptive and cognitive brain areas including the insular cortex and the anterior cingulate (Napadow *et al.* 2013). Correlation between the activity of the insular cortex and midcingulate predicted the development of strong nausea (Napadow *et al.* 2013). In addition, a recent PET study demonstrated that nausea as a premonitory symptom during nitroglycerin-induced migraine is centrally driven by activation of the PAG and rostral dorsal medulla or regions that control their activity (Maniyar *et al.* 2014).

The headache escalating effect of nausea can be experimentally demonstrated in migraine patients during motion

sickness by applying a painful stimuli on the face (Drummond & Granston 2004). This observation suggests that the dorsovagal complex and the trigeminovascular complex mutually relay information to each other which leads to a vicious circle increasing both symptoms (Cuomo-Granston & Drummond 2010; Drummond & Granston 2004). It is interesting to note, that migraineurs are more susceptible to motion sickness than controls probably due to the ineffective top-down control on relevant brainstem areas in response to excessive or stressful sensory inputs (Cuomo-Granston & Drummond 2010). Regarding motion sickness, it has been demonstrated that those who respond with acute motion sickness to parabola flight have significantly higher stress scores accompanied by increased salivary cortisol concentration, lower whole blood endocannabinoid levels and decreased CB1 mRNA leukocyte expression compared to those with no motion sickness (Chouker et al. 2010).

Limitations

The main advantage of our study is that the systematic investigation of genetic main effect and stress interaction on different migraine related symptoms allowed us to delineate a specific pathomechanism, which may contribute to the development of migraine in a susceptible subgroup of patients. However, the study has some limitations. Although we managed to replicate our findings in two European populations, independent replications will be necessary to confirm our results. Our study had a cross-sectional design, therefore the causative role of life stressors and the temporal relationship between stress and headache could not be investigated. Furthermore, we used a short screening questionnaire to determine the probability of migraine headache and to identify migraine related symptoms without proper medical diagnosis. Nevertheless, this is a usual method in large population based studies and previous epidemiologic and GWAS studies suggest that this method provides trustworthy results (Anttila et al. 2013).

Finally, although we covered the whole CNR1 gene with haplotype tag SNPs to determine its function in migraine, the endocannabinoid system is a very complex network of synthetizing and metabolizing enzymes, receptors and transporters with high potential to adaptive changes. For example, genetic variants in the gene of fatty acid amide hydrolase (FAAH) enzyme, which metabolizes AEA, have been associated not only with pain sensitivity (Cajanus et al. 2016) but with mood symptoms elicited by early life stress (Lazary et al. 2016), probably through neurodevelopmental effects. Indeed, the endocannabinoid system is responsible to regulate divergent physiological processes from metabolic routes through regulation of emotional behaviour to pain modulation, reflected by its broad expression throughout the brain and other parts of the body, making it challenging to specifically target it by therapeutic interventions. Although, a promising approach is to increase the endocannabinoid tone by inhibiting the FAAH enzyme that showed antinociceptive (Greco et al. 2015) and antiemetic (Parker et al. 2015) effects in animal models, serious treatment related adverse events

in a recent human study suggest that tissue and/or receptor specific cannabinoids are warranted (Kaur et al. 2016).

Conclusions

In conclusion, our results suggest that genetic variations in the CNR1 gene increases the risk of developing headache with nausea in life stress exposed subjects most likely through an impaired endocannabinoid system driven top-down cortical control on important brainstem areas. Based on these results a subgroup of migraine patients, who have frequent nausea associated with migraine attacks triggered by moderate or severe life stress, may benefit from therapies that increase the endocannabinoid tone.

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Acknowledgments

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Advantage of the Bayesian system-based analysis and potential biological explanation of the results.

Table S1: Haplotype frequencies in the *CNR1* gene (using the nominally significant SNPs in haploblock 1: rs806366, rs4707436, rs1049353, rs806369), and statistical results of interaction effects between recent negative life events (RLE) and haplotypes on headache with nausea in the total study population.

Figure S1: LD block structure of the investigated subpopulations.

Figure S2: LD block structure of rs806366, rs4707436, rs1049353, rs806369 in the total population.