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SNP Variants Within the Vanilloid *TRPV1* and *TRPV3* Receptor Genes Are Associated With Migraine in the Spanish Population

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The transient receptor potential (TRP) superfamily of non-selective cationic channels are involved in several processes plausibly relevant to migraine pathophysiology, including multimodal sensory and pain perception, central and peripheral sensitization, and regulation of calcium homeostasis. With the aim of identifying single nucleotide polymorphisms (SNPs) in TRP genes that may confer increased genetic susceptibility to migraine, we carried out a case–control genetic association study with replication, including a total of 1,040 cases and 1,037 controls. We genotyped 149 SNPs covering 14 TRP genes with known brain expression. The two-stage study comprised samples of 555 and 485 Spanish, Caucasian patients, selected according to the ICHD-II criteria for the diagnosis of migraine without aura (MO) or migraine with aura (MA). In the discovery sample, 19 SNPs in ten TRP genes showed nominal association ($P < 0.05$) with MO, MA, or overall migraine. In the replication sample, nominal association was confirmed for TRPV3 rs7217270 in MA and TRPV1 rs222741 in the overall migraine group. Risk haplotypes were identified for seven of the genes showing nominal association in the discovery set, but none of them was replicated. The present findings suggest that members of the vanilloid TRPV subfamily of receptors contribute to the genetic susceptibility to migraine in the Spanish population.

Key words: migraine; association study; TRP; vanilloid

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INTRODUCTION

Migraine is a disabling type of primary headache with a global lifetime prevalence of 14% and a female:male ratio of 3:1 [Jensen

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and Stovner, 2008]. The International Headache Society (IHS) has established a set of criteria to define the main migraine phenotypes, most notably the two main subtypes of migraine without aura (MO) and migraine with aura (MA) [International Headache Society, 2004]. These deliberately restrictive definitions have allowed for homogenizing phenotypes, and have thus paved the way for research in migraine genetics. However, to date, the only genes known to cause migraine are those responsible for the rare monogenic form of familial hemiplegic migraine (FHM) [Ophoff et al., 1996; De Fusco et al., 2003; Dichgans et al., 2005; Suzuki et al., 2010], with the addition of the *KCNK18* gene, encoding a two-pore domain potassium channel, in familial MA [Lafreniere et al., 2010]. Even less is known about the genetic basis of common forms of migraine. A recent genome-wide association study (GWAS) in a clinic-based central and northern European sample identified a genetic variant on chromosome 8q22.1 that conferred increased susceptibility to migraine and showed a stronger effect in MA individuals [Anttila et al., 2010].

Migraine's mechanism is only partially understood and may in fact vary among the different migraine subtypes. In MA, cortical spreading depression (CSD) is assumed to underlie the aura phase and to produce brainstem activation via the trigeminovascular system. The molecular underpinnings of CSD are unknown for the most part but, based on functional characterization of the mutations found in FHM patients, ionic channel sensitivity appears crucial in the initiation of the cascade of events that lead to an increased neurotransmitter secretion, the presence of neuronal hyperexcitability and consequently a migraine attack [reviewed in Pietrobon, 2005; de Vries et al., 2009]. Alternatively, it has been hypothesized that the increased brainstem input that results in the headache phase of migraine does not result fully from CSD but also from an impaired nociceptive regulation within the nervous system [Goadsby, 2001]. In this regard, several members of the transient receptor potential (TRP) superfamily of non-selective cationic channels are known to mediate pain perception and processing in various cell types in the peripheral and central nervous system. These cell membrane sensors are capable of integrating both intracellular and extracellular chemical, thermal, osmotic, or mechanical signals. TRP channels are also involved in Mg^{2+} and Ca^{2+} homeostasis, intracellular membrane fusion and fission and, consequently, neuron activation, and neurotransmitter release [reviewed in Cortright et al., 2007]. TRP-related channelopathies are ubiquitous in the organism and are being increasingly reported. As related to neurological disorders, mutations in *TRPV4*, *TRPM1*, *TRPM11*, and *TRPM2*/*TRPM7* have been reported in Charcot–Marie–Tooth type 2C, congenital stationary night blindness, mucolipidosis type IV, and Guamanian amyotrophic lateral sclerosis-Parkinson Dementia Complex, respectively [Nilius and Owsianik, 2010]. TRPV1 channels are mediators of neuropathic pain and have been proposed to play a role in migraineous allodynia and sensitization phenomena [Benarroch, 2008; Meents et al., 2010]. Finally, several TRP channels are functional receptors for algogenic substances [Cortright et al., 2007] and TRPV1 and TRPA1 are expressed in the sensory neurons of the trigeminal ganglia [Story et al., 2003].

Because of their role in nociception and calcium homeostasis, we considered involvement of TRP channels in susceptibility to migraine plausible. We present the findings of a two-stage genetic association study covering 14 TRP channel genes in two samples of Spanish migraineurs.

PATIENTS AND METHODS

Discovery Sample

A total of 555 patients were diagnosed either with MA (n = 232) or MO (n = 323) according to the International Criteria for Headache Disorders 2nd edition (IHS) after being directly interviewed and examined by one of the neurologists in the team. Four hundred thirty-one patients were female (77.6%). For the purpose of analysis, three clinical subgroups were defined: (i) ''all-migraine,'' including all the patients recruited in the study; (ii) ''MO,'' and (iii) ''MA.'' Patients displaying attacks of both MO and MA were classified as MA. Patients with hemiplegic aura, a MA variant showing monogenic inheritance, were excluded. The control sample consisted of 555 unrelated blood donors that lacked any personal or family history of headache and were sex-matched with the cases. Average age at assessment was 36.9 years (SD = 12.67) for patients and 54.8 years (SD = 17.05) for control subjects. All individuals were Caucasian and Spanish and were recruited in three centers: the Fundación Pública Galega de Medicina Xenómica in Santiago de Compostela, the Sant Joan de Déu Hospital in Manresa, and the Pediatric Neurology Section at the Vall d'Hebron Hebron Research Institute in Barcelona.

Replication Sample

The replication cohort consisted of 485 migraine patients (158 MA and 327 MO) and 482 sex-matched unrelated controls. Patients and controls were recruited according to the same criteria used in the discovery set. Three hundred eighty-three patients were female (79%). Average age at assessment was 43.7 years (SD = 16.49) for the patients and 55.3 years (SD = 17.34) for control subjects. All individuals were recruited at the Neurology Service, Hospital Universitari Vall d'Hebron in Barcelona.

The study was approved by the local Ethics Committees and informed consent was obtained from all adult subjects, children, and their parents according to the Helsinki declaration.

DNA Isolation and Quantification

Genomic DNA was isolated either from peripheral blood lymphocytes by a salting-out procedure [Miller et al., 1988] or using a magnetic bead technology with the Chemagic Magnetic Separation Module I and the Chemagic DNA kit (Chemagen, Baesweiler, Germany) or from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Kanata, Ontario, Canada). The DNA concentrations of all samples were measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Gene and Single Nucleotide Polymorphism (SNP) Selection and SNPlex Design

We selected a subset of 14 genes of the TRP channel superfamily that are expressed in the brain, on the basis of their involvement in

nociception and/or inflammatory pain pathways (TRPA1, TRPV1, TRPV2, TRPV3, TRPV4, TRPM4, TRPM8) [Cortright et al., 2007], neurotransmitter release (TRPC4, TRPM7) [Munsch et al., 2003; Krapivinsky et al., 2006], formation of synaptic connections (TRPC3, TRPC5) [Li et al., 1999; Greka et al., 2003], linkage to familial hypomagnesemia with secondary hypocalcemia leading to infantile seizures (TRPM6) [Walder et al., 2002], or possible relevance in neuronal excitability (TRPC1) [Kim et al., 2003]. TRPC7 may form multimeric channels with TRPC1 and TRPC3 [Zagranichnaya et al., 2005]. To ensure full genetic coverage of these genes and minimize redundancy, we used the Haploview v3.32 software and the HapMap phase II database (www.hapmap.org, release #22) to evaluate the LD pattern of the region spanning each candidate gene plus 3–5 kb flanking regions. TagSNPs were selected at an r^2 threshold of 0.85 and minor allele frequency (MAF) > 0.15 for genes with fewer than 15 tagSNPs and MAF > 0.25 for genes with 15 or more tagSNPs. Seven additional non-synonymous or UTR SNPs within the selected genes were also included in the study: rs12583681 and rs3904512 (5′-UTR of *TRPC4*); rs8042919/NP_060142.3:p.Thr1482Ile (TRPM7); rs8065080/NP_061197.4:p.Ile585Val, rs222747/NP_061197.4:p.Met315Ile, and rs222749/NP_061197.4:p.Pro91Ser (TRPV1); rs322937/NP_659505.1:p.Arg117Gly (TRPV3). A total of 202 SNPs were chosen (see Supplementary Table I). Nine SNPs did not pass through the SNPlex design pipeline. To rule out stratification in the discovery sample, we genotyped 48 unlinked anonymous SNPs located at least 100 kb distant from known genes [Corominas et al., 2010]. In the replication study, the 18 SNPs showing nominal association (rs7819749, rs7639403, rs1577007, rs9576354, rs871385, rs11668962, rs9650767, rs11563056, rs6758653, rs222741, rs182637, rs7223530, rs161393, rs733080, rs7217270, rs11078454, rs2455858, and rs10774912) plus the 12 SNPs included in the risk haplotypes identified in the original cohort (rs9851381, rs10161932, rs3904512, rs2151438, rs12339024, rs2151423, rs9547988, rs7858755, rs7867868, rs12465950, rs4790522, and rs10850830) were selected for further analysis. Because of design constrictions, rs758275, rs7218756, and rs4663994 were finally excluded.

Genotyping and Quality Control

Genotyping of the discovery sample was performed using the SNPlex platform (Applied Biosystems, Foster City, CA) at the Barcelona node of the 13 National Genotyping Center (CeGen) as described [Tobler et al., 2005]. Two CEPH samples were included in all genotyping assays and a 100% concordance with HapMap data was obtained. The follow-up, replication sample was genotyped by means of the SNPlex technology as described above and TaqMan Genotyping Assays (Applied Biosystems) with the LightCycler® Real-Time PCR System in 384 multiwell plates following the manufacturer's protocol and with the LightCycler® 480 Software release 1.5 (Roche, Basel, Switzerland) for analysis. Three CEPH samples were included in all TaqMan assays and a 100% concordance with HapMap data was obtained.

Statistical Analysis

Individuals with >40% missing genotypes were excluded from the analysis; SNPs with >20% missing genotypes were considered as failed; SNPs in LD ($r^2 > 0.85$) with any other studied SNP or showing deviation from Hardy–Weinberg equilibrium (HWE; threshold = 0.01) as calculated in our control sample were also excluded. Genetic Power Calculator (pngu.mgh.harvard.edu/~purcell/gpc) [Purcell et al., 2003] was used to calculate post hoc minimal statistical power, assuming an odds ratio (OR) of 1.5, prevalence of 0.14 [Jensen and Stovner, 2008], significance level of 0.05, and the lowest MAF of 0.099 and 0.195 for the first and second population, respectively.

Single-Marker Analysis

The analysis of HWE as well as case–control comparisons of genotype frequencies under a Log-Additive model were performed with the SNPassoc R v2.8.0 library [Gonzalez et al., 2007] adjusting by sex. When a nominal association was identified ($P < 0.05$), dominant and recessive models were also analyzed. In the initial association study the significance threshold under the Bonferroni correction for multiple testing was set at $P < 1.0E{-}04$ upon consideration of 149 SNPs analyzed and three clinical groups (MO, MA, and all-migraine patients).

Multiple-Marker Analysis

In order to minimize type I errors and multiple testing, risk haplotypes were evaluated in the clinical subgroup where the genes showed nominal association in the single-marker analysis (all-migraine, MO, or MA), with the UNPHASED 3.0 software [Dudbridge, 2003]. For each gene, the best two-marker haplotype from all possible combinations was identified. Likewise, additional markers (up to five) were added in a stepwise manner to the initial two-SNP haplotype until the highest OR was achieved. Significance was estimated by a 10,000 permutation procedure. Assignment of specific haplotypes to individuals was performed with the PHASE 2.1 software [Stephens et al., 2001]. The comparisons of risk haplotype carriers versus non-carriers, OR and confidence intervals (CI) were estimated using the SPSS v12.0 Software (SPSS, Inc., IBM Company, Chicago, IL).

Replication Study

For SNPs showing nominal association with migraine in the discovery population, a comparison of genotype frequencies was undertaken in the replication population, under the Log-Additive model. Additionally, risk haplotypes identified in the first sample were tested in the second one.

Interaction

Epistasis analysis was performed on the selected *TRPV1* and *TRPV3* genes by comparing two different regression models with a likelihood ratio test using the statistical package SPSS v12.0. In the first model, we used the affection status as a dependent variable and the two risk alleles as predictive variables (*TRPV3* under the log-additive model, with AA = 2, AG = 1, and GG = 0; *TRPV1* under the dominant model, with TT = 1, CT = 0, and CC = 0) (Affected status = $a + bSNP_1 + cSNP_2$). In the second model, we included the

interaction between SNPs as an independent variable (Affected status = a + bSNP$_1$ + cSNP$_2$ + dSNP$_1$ × SNP$_2$).

RESULTS

Discovery Sample

Among 191 SNPs (tagSNPs, singletons, and missense changes) in 14 candidate genes, we excluded 34 SNPs for genotyping failure (call rate <60%), 2 SNPs that showed deviation from HWE in the control population ($P < 0.01$), and 6 SNPs that were in strong LD with other SNPs ($r^2 > 0.85$). The minimal statistical power for a general two degrees-of-freedom (df) test was 84% for the "all-migraine" group and 73% (MO) or 64% (MA) when clinical subgroups were considered.

Single-marker analysis. No evidence of population stratification was found in the first population, that includes subjects recruited in distant geographical areas in Spain, by applying the STRUCTURE software (posterior probability of a single population approaching one and of two to five populations <2E−137), the Fst coefficient ($\Theta = 0$ with a 99% CI of 0.000–0.001), and the Pritchard and Rosenberg method ($P = 0.268$) as previously reported [Corominas et al., 2010]. The comparison of genotype frequencies between patients and controls under a log-additive model showed nominal association between three genes and MO (*TRPC1* SNP rs7639403, *TRPC7* SNP rs871385, and *TRPM8* SNP rs11563056), nine SNPs within seven genes and MA (*TRPA1* SNP rs7819749, *TPRM4* SNP rs11668962, *TRPM8* SNP rs4663994, *TRPV1* SNPs rs4790522 and rs182637, *TRPV3* SNPs rs7217270 and rs11078454, *TRPC4* SNP rs9576354, and *TRPV4* SNP rs10850830) as well as seven SNPs within four genes and migraine (*TRPC4* SNP rs2151438, rs3904512, and rs1577007; *TRPM8* SNPs rs12465950 and rs6758653; *TRPM6* SNP rs9650767; and *TRPV1* SNP rs222741; Table I). For these SNPs, the comparison under dominant and recessive models was calculated (Supplementary Table I). None of the obtained *P*-values, however, remained significant after applying the stringent Bonferroni correction for multiple comparisons.

Multiple-marker analysis. Genes showing single-marker associations were considered for the multiple-marker analysis in the subgroup(s) of patients where the nominal association was found. However, for *TRPM8*, which showed nominal association with the three clinical categories, that is, MO, MA, and all-migraine, only all-migraine was used in order to enhance statistical power. As for *TRPC4* and *TRPV1*, where all-migraine and a single clinical subgroup showed significant associations, the clinical subgroup was used to favor homogeneity of the sample. Haplotype analyses are shown in Table II. All the positive associations remained significant after adjusting for multiple comparisons by means of correction by permutations.

Migraine without aura. The analysis of all possible SNPs combinations within the *TRPC1* gene revealed a two-marker haplotype (rs7639403/rs9851381) associated with MO (adjusted *P*-value = 0.026), with over-representation of the C-G haplotype in patients (OR = 1.37 [10.5–1.80]). The *TRPC7* gene was not associated with MO in the multiple-marker analysis.

Migraine with aura. The haplotype analysis also showed positive association between MA and four genes: *TRPC4* (rs9547988/rs3904512/rs9576354; adjusted $P = 0.004$, with over-representation of the risk T-A-C allelic combination (OR = 1.87 [1.47–2.46])), *TRPV1* (rs7223530/rs161393/rs733080/rs222741/rs182637; adjusted $P = 0.0033$, with under-representation of the G-A-T-G allelic combination in cases (OR = 1.84 [1.24–2.72])), *TRPV3* (rs7218756/rs11078454/rs2455858; adjusted $P = 0.0171$, with over-representation of the risk G-C-T allele (OR = 1.68 [1.24–2.28])) and *TRPV4* (rs10774912/rs10850830; adjusted $P = 0.0112$, showing over-representation of the T-C haplotype in the clinical sample (OR = 1.51 [1.20–1.90])). No association, however, was found for *TRPA1* and *TRPM4*.

Overall migraine sample. The multiple-marker analysis showed positive association between migraine and the *TRPM6* and *TRPM8* genes. A five-marker haplotype within *TRPM6* was identified in the overall migraine sample (rs9650767/rs12339024/rs2151423/rs7858755/rs7867868; adjusted *P*-value = 0.0013) with over-representation of the G-C-A-A-C haplotype (OR = 2.42 [1.61–3.6]). A three-marker haplotype in the *TRPM8* gene (rs758275/rs11563056/rs6758653; adjusted $P = 0.048$) was also found associated with migraine, showing over-representation of the C-T-G allelic combination (OR = 1.76 [1.19–2.6]) in the clinical sample.

Replication Study

Thirty SNPs were selected for follow-up in a replication cohort. We selected the 18 SNPs nominally associated with the disorder in the discovery population and the SNPs that compose the different risk haplotypes. The minimal statistical power for a general 2-df test in the replication population was 91% for all samples, 83% for MO, and 62% for MA.

Single-marker analysis. The comparison of genotype frequencies between patients and controls was performed for every SNP that had shown association in the discovery sample. The analysis revealed a nominally significant association of the T allele from the *TRPV1* SNP rs222741 ($P = 0.03$) and allele A from the *TRPM6* SNP rs9650767 ($P = 0.01$) with the all-migraine group. Allele A from SNP rs7217270 located in the *TRPV3* gene ($P = 0.02$) was found nominally associated with the MA subgroup. However, after applying Bonferroni correction for multiple comparisons none of them remained significant.

Multiple-marker analysis. None of the haplotypes showing association in the original sample was replicated in the follow-up sample.

Interaction

We then evaluated possible interactions between the SNPs that displayed positive associations in both populations. A pooled association study was performed, including the discovery and the replication populations under the inheritance models and clinical subgroups that displayed the best association signals (Table III). We tested putative interaction in the overall migraine sample, under a log additive model of inheritance for *TRPV1* and a dominant model for *TRPV3*. The difference between the interaction model and the non-interaction model was not significant ($\chi^2 = 0.617$ with 2 df; $P = 0.73455$).

TABLE I. Results of the Case–Control Association Study of 149 SNPs From 14 Genes of the TRP Channels Family in 555 Migraine Patients (323 Migraine Without Aura, 232 Migraine With Aura) and 555 Screened Controls in a Discovery Population. Replication Study of the Nominally Associated SNPs in a Population of 485 Migraine Patients (158 Migraine With Aura and 327 Migraine Without Aura) and 482 Screened Controls

	Discovery population				Replication population			
	MAF	*P*-value			MAF	*P*-value		
	Case–control	Log Add	Dominant	Recessive	Case–control	Log Add	Dominant	Recessive
Migraine without aura								
TRPC1								
rs7639403	0.16–0.21	0.010						
TRPC7								
rs871385	0.45–0.40	0.018						
TRPM8								
rs11563056	0.29–0.36	0.004						
Migraine with aura								
TRPA1								
rs7819749	0.48–0.43	0.039						
TRPC4								
rs9576354	0.47–0.38	0.001						
TRPV3								
rs7217270	0.46–0.39	0.018	NS	0.039	0.43–0.36	0.024	0.043	NS
rs11078454	0.47–0.39	0.005						
TRPV4								
rs10850830	0.44–0.49	0.041						
TRPM4								
rs11668962	0.51–0.46	0.046						
TRPM8								
rs4663994	0.35–0.30	0.049						
TRPV1								
rs4790522	0.38–0.44	0.044						
rs182637	0.52–0.45	0.014						
All-migraine patients								
TRPC4								
rs2151438	0.48–0.44	0.041						
rs3904512	0.45–0.39	0.007						
rs1577007	0.40–0.44	0.038						
TRPM8								
rs12465950	0.31–0.27	0.03						
rs6758653	0.36–0.40	0.033						
TRPV1								
rs222741	0.19–0.23	0.045	NS	NS	0.20–0.24	0.03	0.003	NS
TRPM6								
rs9650767	0.38–0.43	0.010	0.035	0.036	0.48–0.43	0.011	NS	0.009

TRP, transient receptor potential; SNP, single nucleotide polymorphism; MAF, minor allele frequency; Log Add, log-additive model; NS, not significant.

DISCUSSION

In the present study, we performed a comprehensive screen of common genetic variants in genes from the TRP family, to investigate their role in the susceptibility to common forms of migraine. In the discovery stage, 149 SNPs covering 14 TRP genes were genotyped in 555 cases and 555 controls. Nineteen of these SNPs showed nominal association with migraine and were subsequently tested in a follow-up stage. The association was replicated for two intronic SNPs, *TRPV1* rs222741 in the all-migraine group and *TRPV3* rs7217270 in the MA group. The positive associations in the multiple marker analysis could not be replicated.

Association of the Vaniloid TRPV Thermosensors With Migraine

TRPV1 and *TRPV3* lie in close proximity on the 17p13 chromosomal region. They encode two channels pertaining to the polymodal thermo and chemosensitive TRP vaniloid subfamily. TRPV1 and TRPV3 are only modestly permeable to Ca^{2+}, in contrast with

TABLE II. Multiple Marker Analysis of Genes Showing Nominal Association With MO, MA, or All-Migraine Patients Using the UNPHASED Software

Gene/subgroup (haplotype marker[a])	Allelic combination	Cases (%)	Controls (%)	Global *P*-value	Odds ratio [95% CI]	Adjusted *P*-value
TRPC1/MO (4 5)	C G	519 (0.85)	827 (0.80)	0.0206	1.37 [1.05–1.80]	0.026
TRPC4/MA (1 17 22)	T A C	115 (0.29)	170 (0.18)	0.0026	1.87 [1.47–2.46]	0.0002
TRPV1/MA (1 6 9 11)	G A T G	76 (0.26)	312 (0.39)	0.0005	1.84 [1.24–2.72]	0.00079
TRPV3/MA (3 6 13)	G C T	84 (0.20)	131 (0.13)	0.0001	1.68 [1.24–2.28]	0.0171
TRPV4/MA (6 7)	T C	189 (0.44)	352 (0.34)	0.018	1.51 [1.20–1.90]	0.0112
TRPM6/all-migraine (1 5 7 8 14)	G C A A C	84 (0.13)	37 (0.06)	0.0033	2.42 [1.61–3.62]	0.0013
TRPM8/all-migraine (1 9 13)	C T G	72 (0.07)	40 (0.04)	0.0134	1.76 [1.19–2.61]	0.0482

MA, migraine with aura; MO, migraine without aura; CI, confidence interval.
Only significant results are included.
Haplotype markers: *TRPC1*: 4 (rs7639403), 5 (rs9851381); *TRPC4*: 1 (rs9547988), 17 (rs3904512), 22 (rs9576354); *TRPM6*: 1 (rs9650767), 5 (rs12339024), 7 (rs2151423), 8 (rs7858755), 14 (rs7867868); *TRPM8*: 1 (rs758275), 8 (rs12465950), 9 (rs11563056), 13 (rs6758653), 15 (rs11563200); *TRPV1*: 1 (rs7223530), 6(rs161393), 9 (rs733080), 11 (rs182637); *TRPV3*: 3 (rs7218756), 6(rs11078454), 13 (rs2455858); *TRPV4*: 6 (rs10774912), 7(rs10850830).
[a]The *TRPA1*, *TRPC7*, and *TRPM4* genes contained individual markers nominally associated with the disease but none of them displayed an associated haplotype.

TRPV5 and TRPV6, the only highly Ca^{2+} selective channels in the entire TRP superfamily [Nilius and Owsianik, 2010].

TRPV1 was identified as the capsaicin receptor and is activated by noxious temperature, low extracellular pH [Caterina et al., 1997], voltage [Gunthorpe et al., 2000], and diverse lipid derivatives [Zygmunt et al., 1999]. It is abundantly expressed in several neural structures, including sensory C and Aδ fibers, dorsal root ganglia, and trigeminal ganglia [Caterina et al., 1997] and it is thought to play a critical role in the transduction of painful stimuli. TRPV1 channels are stimulated by ethanol [Trevisani et al., 2002] when they mediate neurogenic vasodilatation in the trigeminovascular system and are also involved in the release of calcitonin gene-related peptide (CGRP) [Nicoletti et al., 2008]. It is thus conceivable that this neurogenic inflammatory mechanism might underlie the ability of alcohol to trigger the migraine attack. The analgesic effects of the TRPV1 antagonist SB-705498 on trigeminovascular sensitization and neurotransmission have been studied in an animal model of neurovascular head pain [Lambert et al., 2009]. This compound was able to both reverse and prevent the sensitization to sensory inputs from the trigeminovascular facial and dural receptive fields. TRPV1 might thus be involved in the development of the cutaneous trigeminal allodynia observed in some migraineurs. A phase II clinical trial with SB-705498 in acute migraine has been conducted under GlaxoSmithKline Industry sponsorship (clinicaltrialsfeeds.org/clinical-trials/show/NCT00269022), although to our knowledge the results have not been disclosed. In addition, the observation that continuous exposure to local high-dose capsaicin promotes pain relief through nociceptor inactivation or death of sensory nerve terminals [Jancso et al., 1961] has been followed by the development of high-concentration capsaicin dermal patches for the effective treatment of chronic painful neuropathies [Derry et al., 2009], admittedly a mechanistically dissimilar pain disorder.

A SNP in another member of the vanilloid receptor subfamily, *TRPV3*, was associated with MA. *TRPV3* was identified in 2002 and found expressed in the trigeminal ganglion, dorsal root ganglion, spinal cord, and other brain tissues including the thalamus and striatum [Peier et al., 2002; Smith et al., 2002; Xu et al., 2002]. It is activated by both innocuous and noxious temperatures [Smith et al., 2002; Moqrich et al., 2005], various chemicals such as monoterpenoids [Vogt-Eisele et al., 2007] and is modulated by voltage [Smith et al., 2002]. Like TRPV1, it is a molecule presumably involved in neurogenic vasoregulation and the associated inflammatory pain state.

The evidence that *TRPV1* and *TRPV3* share chromosomal localization, are co-expressed in neurons and display similar

TABLE III. Results of the Pooled Case–Control Association Study of the SNPs From the *TRPV1*, *TRPV3*, and *TRPM6* Genes, Which Showed Nominally Significant Association in the Discovery Sample

Gene SNP	All-migraine patients			MA			MO		
	Log Add	Dominant	Recessive	Log Add	Dominant	Recessive	Log Add	Dominant	Recessive
TRPV3 rs7217270	0.0138	0.0681	0.025	0.0011	0.0074	0.0094	0.2475	0.4958	0.1938
TRPV1 rs222741	0.0033	0.0005	0.8583	0.027	0.0198	0.4959	0.0109	0.0015	0.8284
TRPM6 rs9650767	0.9398	0.6397	0.6617	0.7165	0.3293	0.5858	0.88	0.9719	0.8189

SNP, single nucleotide polymorphism; MA, migraine with aura; MO, migraine without aura; Log Add, log-additive model.
This analysis includes the discovery and the replication sets, with 1,040 migraine patients (650 migraine without aura, 390 migraine with aura) and 1,037 screened controls.

heat-mediated gating, makes them fine candidates for intrafamily heteromerization [Smith et al., 2002]. Assembly of human TRPV1 and TRPV3 has been verified by co-immunoprecipitation experiments and functional assays in heterologous systems, the co-assembly allowing for modulation of the TRPV1 response [Smith et al., 2002]. The two homologous murine thermosensitive subunits have been also detected to co-assemble: spectroscopy-based fluorescence resonance energy transfer (FRET) revealed signals limited to the plasma membrane, and single-channel recordings showed heteromeric channels exhibiting intermediate conductance and gating properties [Cheng et al., 2007]. In the light of this experimental evidence, we analyzed the possible interaction between the replicated SNPs in *TRPV1* and *TRPV3* genes, but found no evidence supporting the existence of epistatic effects between these genes in the risk to develop migraine.

Temperature changes are listed among classical migraine triggers. Weather changes and "heat" have been reported as triggers in a 53.2% and 30.3% of migraineurs, respectively [Kelman, 2007]. Although data on factors triggering migraine attacks was available for most of the thousand patients included in our study, lack of uniformity in data collection at the different participant centers prevented statistical analysis. It is tempting to speculate, however, that genetic variants in TRPV 1–4 might underlie enhanced susceptibility to temperature variations in migraine. In fact, abnormal thermal pain thresholds, indicating interictal cutaneous sensitization, have been found in episodic and chronic migraineurs [Schwedt et al., 2011].

TRPM Subfamily Findings

We also observed association of the intronic rs9650767 in the *TRPM6* gene with migraine. However, the risk allele differed between the discovery and the replication population, thus suggesting the presence of a flip-flop phenomenon. Variation in LD or interlocus correlation in the context of multilocus effects may lead to flip-flop associations [Lin et al., 2007], that are also observed when there is constant LD [Zaykin and Shibata, 2008]. This association could be as well a statistical artifact.

A recent GWAS reported nominal association ($P \leq 5 \times 10^{-5}$) between MA and a locus containing rs17862920 ($P = 1.26\text{E}{-}05$, OR = 1.28 [1.14–1.44]) and several nearby SNPs in the *TRPM8* gene, the cold and menthol receptor [Anttila et al., 2010]. Our experiment was designed under a genetic coverage strategy based on LD patterns. We selected 21 SNPs in the *TRPM8* gene among which 18 were successfully genotyped, but none of them reached significant association values with migraine. The rs17862920 SNP was not included in the design, as our selection was made prior to the publication of the manuscript by Anttila et al. [2010]. One of the SNPs that we have genotyped, rs758275, is in $r^2 = 0.6$ with rs17862920 but does not show association with any of the clinical migraine subgroups (Fig. 1).

Methodological Issues

The present case–control association study raises several methodological questions. First, our limited sample size (555 patients) may have prevented us from detecting susceptibility loci with very subtle effects in the overall population of patients with migraine. Our power decreased further when patients were subdivided in order to reduce clinical heterogeneity according to aura/non-aura clinical subtypes. In this respect, our discovery population had minimal statistical power for general 2 df test of 84% for all-migraine patients, 60% for MO and 45% for MA; and the replication study had 91% for general migraine, 83% for MO and 62% for MA. This power was able to detect a minimum OR of 1.5 for an SNP with an MAF of 0.099 in the first population and a MAF of 0.195 in the second population.

Second, population stratification can lead to false positive signals in a case–control study. For that reason several preventive measures were undertaken: (1) in the discovery population, where patients were recruited in Galicia and Catalonia, population stratification was ruled out; (2) our patient cohort was clinically well defined by neurologists and was ethnically homogeneous, Caucasian with Spanish origin; and (3) control and patient samples were recruited from the same geographical area and were matched for sex.

Third, reducing type I errors in the high-throughput SNP analysis requires correction for multiple comparisons. Under the conservative Bonferroni correction, taking into account 149 SNPs and three clinical groups, the significant threshold was set at $P > 1.0\text{E}{-}04$. No SNP withstood this correction, which on the other hand may be too stringent to identify the subtle genetic factors involved in the etiology of a complex disease such as migraine. The Bonferroni correction assumes independence of all tests performed, whereas many SNPs within the genes studied are not completely independent and the clinical subgroups are also not totally independent.

Fourth, to limit the number of tests, we implemented a systematic approach for the haplotype analysis: (1) in the replication sample we tested the relevant haplotypes only in the clinical group (MO, MA, or all-migraine) where the association was found in the discovery sample; (2) we identified the best SNP combinations in a stepwise manner, always including a SNP showing nominal association in the first combination. As a result, four genes that did not show nominal association with any migraine phenotype and three haplotypes that did not include a nominal SNP in the best two-marker combination were not further analyzed. Therefore, we cannot rule out having missed other haplotypes that contribute to migraine specific traits or modulate the phenotype through interactions with other candidate genes.

Fifth, although we achieved adequate SNP coverage for many genes ($r^2 = 0.85$), gaps still exist in 12 genes, because 21% of the 191 selected SNPs could not be tested. In addition, since uncommon SNPs were not included, we cannot rule out the participation of rare variants in the etiology of the disorder.

Finally, the two variants associated with the disease were intronic: rs222741 (NM_080704.3:c.-34 + 2841C>T) and rs7217270 (NM_145068.2:c.2085 + 395T>C). No known splicing alterations or protein changes are caused by these SNPs. It could be that the identified risk variants may produce by themselves functional alterations or they might lie in LD with other susceptibility loci, in the case of an indirect association. Since both of these SNPs are TagSNPs, we analyzed these variants and those in LD with them by means of the web utility SNP Function Prediction (Xu and Taylor,

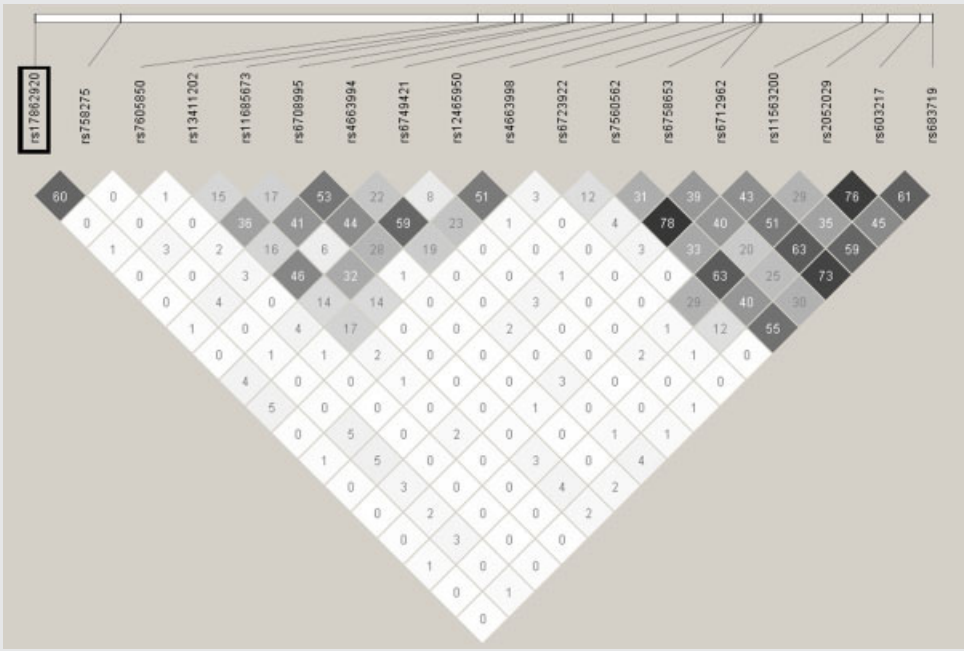

FIG. 1. Linkage disequilibrium patterns within the *TRPM8* gene. Haploview R-square matrix of the *TRPM8* SNPs that were genotyped in the present study and rs17862920 (boxed), the SNP found nominally associated with MA in a previous GWAS [Anttila et al., 2010]. The numbers indicate r^2 values between SNPs.

2009). The predictions of functional effects for transcription factor binding sites, exonic splicing enhancers or silencers, changing of splicing pattern or efficiency by disrupting splice sites, regulation of protein translation by affecting microRNA binding sites and conservation scores were negative. Five SNPs in LD with rs7217270 in the *TRPV3* gene (rs7216486 ($r^2 = 1$), rs7207628 ($r^2 = 1$), rs8079054 ($r^2 = 1$), rs925102 ($r^2 = 0.966$), and rs925101 ($r^2 = 0.932$)) showed marked resemblance to alignment patterns typical of regulatory elements (potential regulatory score above 0.1) (see Supplementary Table II). In *TRPV1*, SNP rs222741 captures rs460716 ($r^2 = 1$) and rs465563 ($r^2 = 0.883$) in the same multiloci bin. Rs460716 is located in the 5′-UTR region of TRPV1 (NM_080704.3:c.-121T>C); this region is shared with the gene encoding sedoheptulokinase (SHPK), the defective enzyme in cystinosis [Touchman et al., 2000], both SNPs being located within the 3′-UTR region of SHPK, rs460716 at NM_013276.2:c. 2046T>C and rs465563 at NM_013276.2:c. 135C>T. The G allele of rs465563 has been associated with carotid intima-media thickness [Lanktree et al., 2009]. Future studies are needed to elucidate whether these positive SNP signals indeed relate to migraine pathophysiology.

CONCLUSIONS

The currently most accepted view envisages migraine as a primary neurogenic disorder, where impaired control of sensory inputs seems to play a central role [Lane and Davies, 2006]. The TRP family of receptors appears crucial for both sensory transduction and intracellular Ca^{2+} homeostasis, thereby becoming suitable candidates for genetic association studies in primary headaches. Our results suggest a role for *TRPV1* and *TRVP3*, two genes belonging to the TRP pain-related pathway, in the susceptibility to migraine. Confirmation of these findings in larger-scale case–control cohorts is warranted, particularly since TRP channels are emerging as novel pharmacological targets.

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