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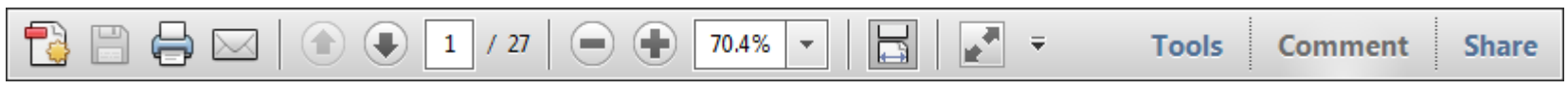

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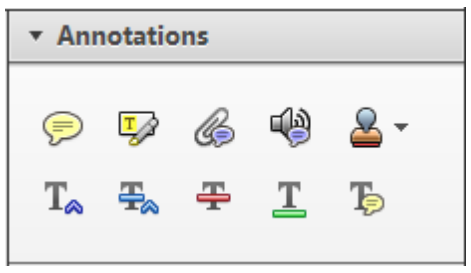

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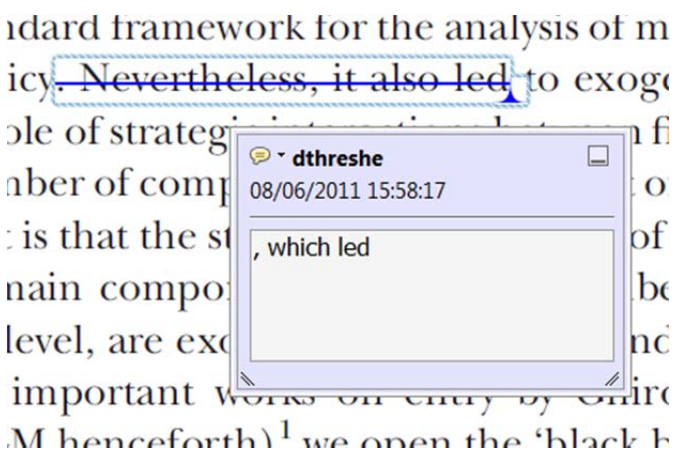

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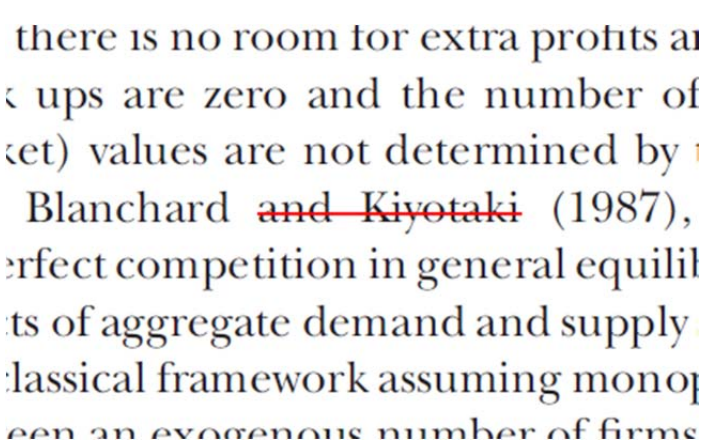

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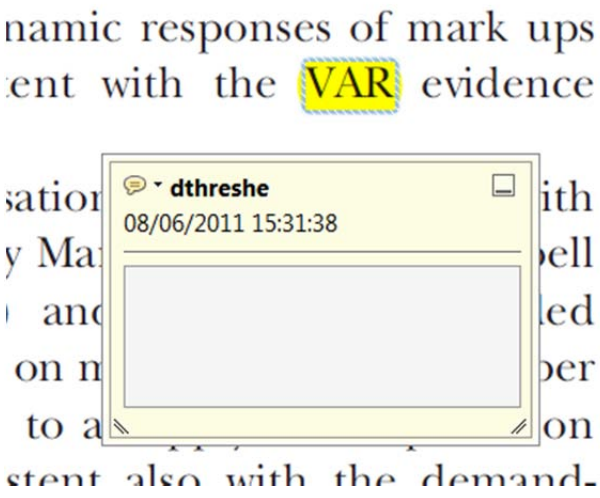

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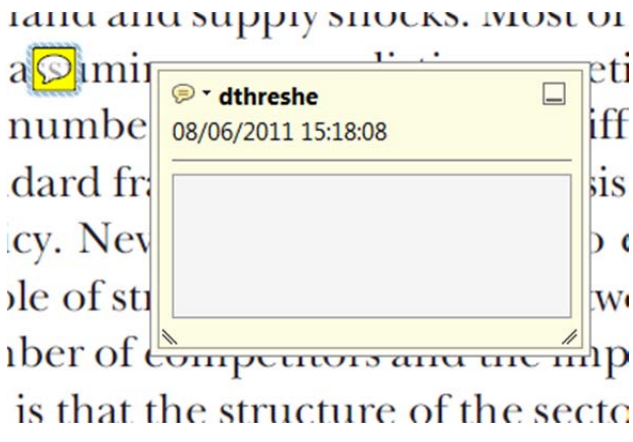

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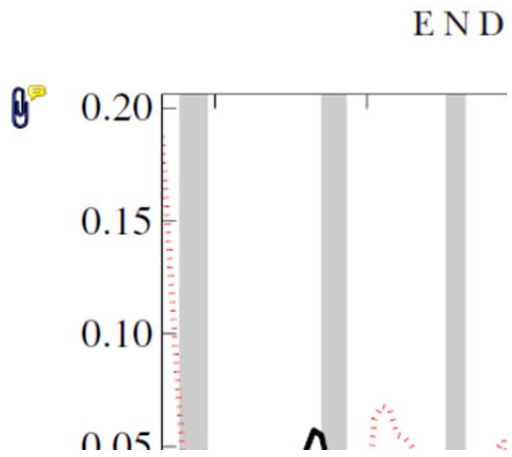

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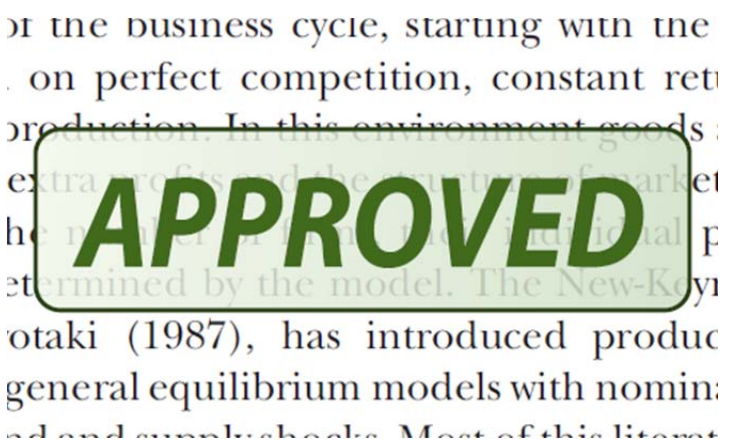

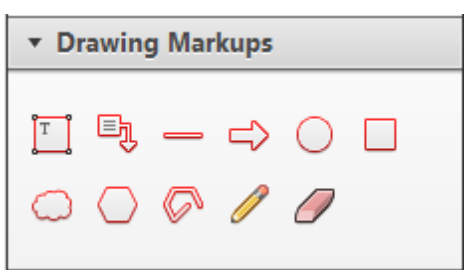

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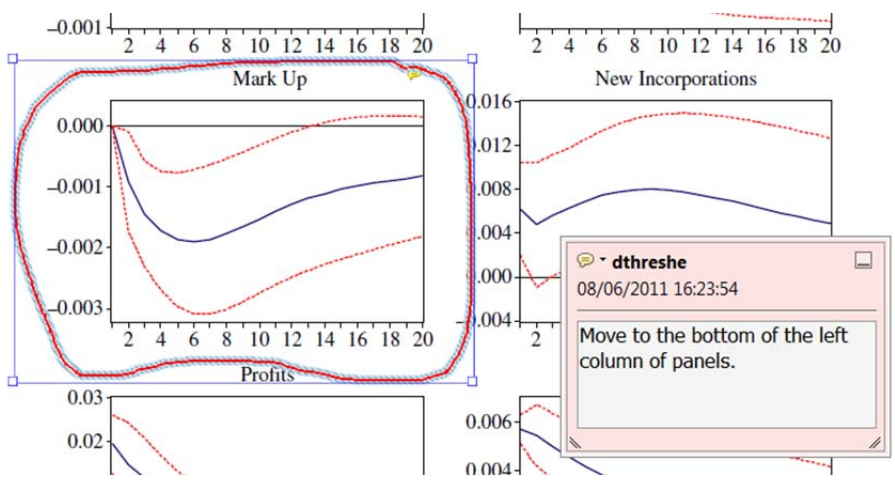

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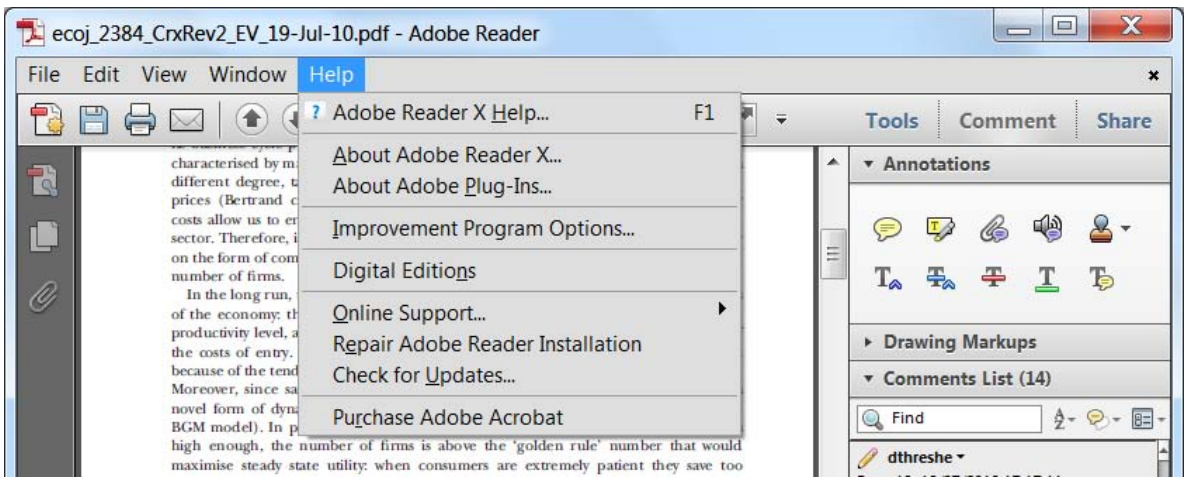

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Research Submissions

Possible Involvement of the *Cacna1e* Gene in Migraine: A Search for Single Nucleotide Polymorphism in Different Clinical Phenotypes

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Objective.—To search for differences in prevalence of a *CACNA1E* variant between migraine without aura, various phenotypes of migraine with aura, and healthy controls.

Background.—Familial Hemiplegic Migraine type 1 (FHM1) is associated with mutations in the *CACNA1A* gene coding for the alpha 1A ($Ca_v2.1$) pore-forming subunit of P/Q voltage-dependent Ca^{2+} channels. These mutations are not found in the common forms of migraine with or without aura. The alpha 1E subunit ($Ca_v2.3$) is the counterpart of $Ca_v2.1$ in R-type Ca^{2+} channels, has different functional properties, and is encoded by the *CACNA1E* gene.

Methods.—First, we performed a total exon sequencing of the *CACNA1E* gene in three probands selected because they had no abnormalities in the three FHM genes. In a patient suffering from basilar-type migraine, we identified a single nucleotide polymorphism (SNP) in exon 20 of the *CACNA1E* gene (Asp859Glu – rs35737760; Minor Allele Frequency 0.2241) hitherto not studied in migraine. In a second step, we determined its occurrence in four groups by direct sequencing on blood genomic DNA: migraine patients without aura ($N = 24$), with typical aura ($N = 55$), complex neurological auras ($N = 19$; hemiplegic aura: $N = 15$; brain stem aura: $N = 4$), and healthy controls ($N = 102$).

Results.—The Asp859Glu – *rs35737760* SNP of the *CACNA1E* gene was present in 12.7% of control subjects and in 20.4% of the total migraine group. In the migraine group it was significantly over-represented in patients with complex neurological auras (42.1%), OR 4.98 (95% CI: 1.69-14.67, uncorrected $P = .005$, Bonferroni $P = .030$, 2-tailed Fisher's exact test). There was no significant difference between migraine with typical aura (10.9%) and controls.

Conclusions.—We identified a polymorphism in exon 20 of the *CACNA1E* gene (Asp859Glu – *rs35737760*) that is more prevalent in hemiplegic and brain stem aura migraine. This missense variant causes a change from aspartate to glutamate at position 859 of the $Ca_v2.3$ protein and might modulate the function of R-type Ca^{2+} channels. It could thus be relevant for migraine with complex neurological aura, although this remains to be proven.

Key words: migraine, migraine aura, genetics, $Ca_v2.3$ channels

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INTRODUCTION

Migraine is a frequent disorder, characterized by headache attacks that may be preceded or accompanied by neurological symptoms in about 30% of patients.[1] These include visual, sensory, and motor disturbances and are globally defined as "migraine auras." In the International Classification of Headache Disorders-3 beta[2] migraine with aura is subdivided into nine subtypes, according to the presence or not of headache and the clinical features of the aura, in particular the presence of motor or basilar-type symptoms and the familial occurrence of the disease. Migraine is known to run in families and great efforts have been made in the last two decades to identify its genetic determinants.

At present the only monogenic forms of migraine with functionally relevant mutations in a single gene are Familial Hemiplegic Migraine (FHM),[3-5] Sporadic Hemiplegic Migraine (SHM),[6-8] and Migraine with Brainstem aura (BM).[9,10] These are phenotypically similar subtypes of migraine with aura, differentiated by familial occurrence or not, the presence of a unilateral motor deficit or of symptoms attributable to brain stem dysfunction.[2]

Familial Hemiplegic Migraine type 1 (FHM1) (ICHD-3beta 1.2.3.1.1), as well as some cases of SHM (1.2.3.2)[6-8] and BM (1.2.2),[9] are caused by mutations in the *CACNA1A* gene (Chr 19p13), coding for the alpha 1A ($Ca_v2.1$) pore-forming subunit of P/Q voltage-dependent Ca^{2+} channels.

Mutations in the *ATP1A2* gene (Chr 1q23), coding for the main subunit of the Na/K ATPase pump have been found in FHM2,[4] SHM2,[6,8] and BM.[10] FHM has also been associated with mutations on the *SCN1A* gene (Chr 2q24), coding for the α1 subunit of the neuronal $Na_V1.1$ sodium channel (FHM3).[5] However, some FHM families do not bear mutations on these genes, so that other FHM genes are to be expected. The FHM mutations are not found in the common forms of migraine with or without aura.

Linkage analyses and particularly genome-wide association studies have identified multiple susceptibility loci as single nucleotide polymorphisms (SNPs).[11,12]

A locus on chromosome 1 (1q31) was initially found to be associated with FHM[13] and later with the common migraine types with and without aura.[14] This locus contains the *CACNA1E* gene, coding for the alpha1E subunit of $Ca_v2.3$ (R-type) Ca2+ channels.[15] $Ca_v2.3$ channels are the counterparts of $Ca_v2.1$ (P/Q Ca^{2+}) channels mutated in FHM1, have a similar anatomical distribution[16] but different functional properties. The *CACNA1E* gene could thus be an interesting candidate gene in migraine. In fact, SNPs in this gene have already been investigated in two large cohorts of migraineurs and controls.[17,18] No significant difference was found between patients and controls, but in the first study[17] only one *CACNA1E* SNP marker was studied compared to four in the second[18] and all dbSNP markers had a rather high minor allele frequency ranging from 0.27 to 0.47.

We decided therefore to explore more extensively the *CACNA1E* gene by using different markers and by studying various clinical phenotypes of migraine with aura. Our aim was to search for differences in prevalence of *CACNA1E* variants between migraine phenotypes and healthy controls.

METHODS

Patients' Enrollment.—Patients and healthy controls were recruited at the Headache Centre of the IRCCS Neuromed (Pozzilli, IS, Italy) and the Headache Research Unit of the University of Liège (Belgium) between 2001 and 2006. All migraineurs were out-patients followed in both Headache Centers; healthy controls were recruited among the medical and administrative staff, as well as students and research fellows attending both hospitals. The study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committees. Blood sampling (10 mL) from cubital

Conflicts of interests: No conflict

Funding Information: Dr Ambrosini was the recipient of the IHS-GSK ~~Pat~~ Humphrey Research Fellowship 2002, aimed to this research project. JS was supported by research convention 3.4.650.09 of the National Fund for Scientific Research-Belgium, Special Research Funds of the University of Liège and grants from the Fonds Frédéricq of the Faculty of Medicine-ULg. MD was supported by PAINCAGE FP7 Collaborative Project num. 603191.

veins, and genetic testing were performed with the written informed consent of the subjects. Non-Caucasian subjects were excluded in order to reduce genetic variability. None of the patients and healthy volunteers had a familial relationship with other participants in the study. The blood samples were collected by the Laboratory of Neuropharmacology of the IRCCS Neuromed, where the DNA extraction and gene screening was performed.

We recruited 98 migraine patients (71 females and 27 males) and 102 healthy controls (72 females and 30 males). When the first genetic analyses were performed, patients were diagnosed according to ICHD-II criteria[19] that did not allow a distinction between FHM subtypes. As soon as ICHD3beta[2] was published with the subdivision of FHM into three genetically distinct subtypes, we rescreened all FHM and SHM patients for mutations in *CACNA1A* (FHM1), *ATP1A2* (FHM2), and *SCN1A* (FHM3) genes known up to 2013. Consequently, using ICHD3beta criteria,[2] patients were diagnosed as suffering from either:

- Migraine without aura – MO (code 1.1) $N = 24$ (F = 20; M = 4)
- Migraine with typical aura with headache – MTA (code 1.2.1.1) $N = 55$ (F = 39: M = 16)
- Familial Hemiplegic Migraine, other loci – FHM (code 1.2.3.1.4) $N = 4$ (F = 2: M = 2)
- Sporadic Hemiplegic Migraine-SHM (code 1.2.3.2) $N = 11$; (F = 7: M = 4)
- Migraine with Brainstem Aura-BM (code 1.2.2) $N = 4$; (F = 3: M = 1)

The latter three groups of patients were globally called migraine with complex neurological aura (MAplus).

Mutation Screening.—Genetic testing was performed by direct sequencing of blood genomic DNA. In a first step, three probands (2 BM and 1 FHM) with a strong family history were chosen for a total screening of exons in the *CACNA1A*, *ATP1A2*, and *SCN1A* genes. UTRs were not taken into consideration. They underwent a total screening of the *CACNA1E* gene by sequencing on an ABI PRISM 3700 capillary sequencer (Applied Biosystem, Foster City, CA). We identified a dbSNP rs35737760 (Asp859Glu in exon 20) in one proband. The dbSNP rs35737760 has genomic coordinates chr1:181732663 in Human Genome version GRCh38/hg38; it is located on exon 20 of NM_000721.3 transcript and corresponds to variant Asp859Glu. Following the identification of the Asp859Glu substitution, we extended the study to migraineurs and controls by restriction fragment length polymorphism (RFLP) analysis. The Asp859Glu polymorphism was analyzed by PCR-RFLP analysis, using the restriction endonuclease FoKI (New England Biolabs). The PCR reactions were performed using the following primers:

Forward: CTGAGGAAGCACATGCAGAT (Sense) Hairpin Blast.

Reverse: ATCCTGGGCTCTCTCTTCTT (AntiSense) Hairpin Blast.

An amplicon of 588 bp was obtained at standard PCR conditions ($T_m = 62°C$).

Statistical Analyses.—No power analysis was made before starting this study, which was aimed to recruit as many migraine with aura patients as possible. Due to the low prevalence of patients with complex auras and the involvement of only two tertiary headache centers, we were not able to predict how many patients we would be able to enroll for analyses. The frequencies of (Asp859Glu – rs35737760) variations were calculated from the observed variation counts. The association of frequencies to the different migraine subtypes was investigated by inserting dichotomized data (wild type and mutated) for the analyzed migraine groups (controls, MO, MTA, MAPlus) in 2 × 2 contingency tables for patients and controls. The association between the selected genetic variation and the migraine type was analyzed with the 2-tailed Fisher's exact test using R-Bioconductor package. The significance level was set to 0.05. The computed *P* values were corrected for multiple comparisons using both the classical Bonferroni procedure to control the Family Wise Error Rate (FWER), and the Benjamini & Hochberg procedure to control for False Discovery Rate (FDR). *P* values corrected with both methods are shown in Table 1. T1

RESULTS

We identified a single nucleotide polymorphism (variation Asp859Glu – *rs35737760*) in exon 20 of

Table 1.—Synopsis of Subjects Recruited for *CACNA1E* Screening and Prevalence of the (Asp859Glu – *rs35737760*) Polymorphism in Healthy Controls and in Migraine Patients According to Clinical Subtypes

		Prevalence		OR (Mutant allele in migraine/vs controls) OR 95% CI			2-tailed Fisher's Test, Mutant allele in migraine subtypes vs controls		
Group	Ntot	Wild type *N*	Mutant allele *N* (%)	OR	From	To	Uncorrected P_{val}	Corrected P_{val} (Bonferroni)	Corrected P_{val} (FDR-BH)
Controls	102	89	13 (12.7%)						
MO	24	18	6 (25.0%)	2.28	0.77	6.80	.200	1.000	.300
MTA	55	49	6 (10.9%)	0.84	0.30	2.34	.803	1.000	.803
MAplus	19	11	8 (42.1%)	4.98	1.69	14.67	.005 (*)	.030 (*)	.017 (*)
				OR (Mutant allele in MAplus vs MTA) OR 95% CI					
				OR	From	To			
MAplus	19	11	8 (42.1%)	5.76	1.43	24.88	.006 (*)	.034 (*)	.017 (*)

Odds ratios (OR) and their 95% Confidence Intervals (CI) are shown for the comparison of mutated in migraine subtypes vs controls, and in MAplus vs MTA.
*Statistical significance was assessed by 2-tailed Fisher's exact test. Both Bonferroni and FDR (Benjamini & Hochberg procedure) *P* value corrections were used. A total of 98 migraine patients were analyzed, 20 of them carrying the mutant allele. This contingency table is divided into: controls ($n = 102$); MO, Migraine without aura; MTA, Migraine with typical aura; MAplus, migraine with complex neurological auras (Brainstem aura, Sporadic or Familial Hemiplegic Migraine).

the *CACNA1E* gene, not previously recognized to link to migraine.

This variation was found in 12.7% of control subjects and in 20.4% of the total group of migraine patients, a difference that did not reach statistical significance (see Table 1).

However, the Asp859Glu variant was significantly more represented (42.1%) in FHM, SHM, and BM patients – called here MAplus subgroup – than in control subjects, with an odds ratio (OR) of 4.98 (95% CI: 1.69-14.67, uncorrected $P = .005$, Bonferroni $P = .030$, 2-tailed Fisher's exact test). The prevalence of the polymorphism was larger in the MAplus subgroup than in MTA patients (OR = 5.76: 95% CI: 1.3-24.88), which was statistically significant (uncorrected $P = .006$, Bonferroni $P = .034$, 2-tailed Fisher's exact test).

In migraine without aura (MO), but not in migraine with typical aura (MTA), there was a slight numerical, but non-significant, overrepresentation of the Asp859Glu variant compared to controls (25.0%; OR = 2.28, 95% CI: 0.82-3.76, uncorrected $P = .200$, Bonferroni $P = 1.000$, 2-tailed Fisher's exact test).

DISCUSSION

We report on a single nucleotide polymorphism (SNP) in exon 20 of the *CACNA1E* gene

(Asp859Glu – *rs35737760*) not studied in migraine up to now. This SNP was overrepresented in the subtype of migraine with aura characterized by complex neurological symptoms such as Familial or Sporadic Hemiplegic and Brainstem aura Migraine not associated with mutations in the known FHM genes. The association was statistically significant, but we are aware that our sample size was small and that replication studies are necessary in an independent, and if possible larger, cohort, to confirm our results. Compared to healthy controls, it tended to be numerically more frequent in migraine without aura patients, but surprisingly had a low prevalence in migraine with typical aura.

The *CACNA1E* gene is composed of 49 exons and encodes the alpha1E subunit of R-type ($Ca_v2.3$) Ca^{2+} channels. The substitution of aspartate by glutamic acid in $Ca_v2.3$ determined by the *rs35737760* variant is likely to produce only minor functional changes that have not yet been studied. Given the functional neuroanatomy of $Ca_v2.3$ channels and certain pathophysiological aspects of migraine and its subtypes, one may nonetheless speculate on its possible role in migraine.

R-type ($Ca_v2.3$) channels have a widespread distribution in the nervous system and share many localizations with P/Q channels.[20] Currents mediated by $Ca_v2.3$ are found in most neurons, such as neocortical and striatal neurons,[21] CA1 neurons,[22] dentate granule cells, cerebellar granule neurons,[23] neurons of the reticular thalamic nucleus,[24] and trigeminal ganglion neurons.[25] $Ca_v2.3$ channels also share many functional properties with P/Q ($Ca_v2.1$) Ca^{2+} channels. Interestingly, in a recent study of patients with a post-concussion syndrome, the CACNA1E SNP was found to be associated with increased balance deficits, which are also common in FHM patients,[26] arguing in favor of its functional significance. We will limit this discussion to four neural phenomena in which a modulation by $Ca_v2.3$ channels might be relevant for migraine pathophysiology: (1) cortical spreading depression (CSD); (2) neuromuscular transmission and cerebellar function; (3) thalamocortical rhythms; (4) trigeminal nociception.

First, CSD is likely the culprit for the migraine aura. FHM1 *CACNA1A* mutations facilitate CSD and glutamate release in knock in mice.[27] After blockade of P/Q-type Ca2+ channels CSD cannot be induced in wild-type mouse cortical slices. By contrast, blockade of R-type Ca2+ channels has only a minor inhibitory effect on CSD.[28] Consequently, if the Asp859Glu – *rs35737760* SNP in patients with complex neurological auras changes the functional properties of R-type Ca2+ channels, this change is not likely to have a major effect on CSD. However, while P/Q Ca2+ channels are seminal in action potential-induced exocytosis, R-type Ca2+ channels play a greater role in spontaneous glutamate release.[29] It remains to be determined whether R-type Ca2+ channels may favor spreading depression in subcortical areas[30] that are relevant for aura symptoms in BM.[31] Lamotrigine is known to inhibit CSD in rat after chronic treatment[32] and to be effective in preventing attacks of MTA,[33,34] FHM, SHM, and BM.[35,36] Interestingly, lamotrigine is able to inhibit $Ca_v2.3$ (R-type) calcium currents.[37] Whether this contributes to its therapeutic effect in migraine with aura remains to be proven. Along the same line, topiramate, another inhibitor of CSD during long-term treatment in rats[38] with preventive action in both migraine with and without aura, was shown to depress R-type Ca2+ channels in hippocampal neurons.[39]

Second, we have described subtle abnormalities in transmission at the neuromuscular junction (NMJ) in migraine patients that were confirmed by others.[40-44] These abnormalities are restricted to migraine with aura patients and most pronounced in patients with prolonged[42] and complex neurological auras,[40,41,43,44] precisely in the present study the subgroup of patients with the highest prevalence of the Asp859Glu polymorphism. The carbonic anhydrase inhibitor acetazolamide that is able to normalize the impairment of neuromuscular transmission[45] inhibits by 30% $Ca_v2.3$ currents in vitro.[46] R-type $Ca_v2.3$ Ca^{2+} channels play a role at the neuromuscular junction and can compensate for defective acetylcholine release due to mutated P/Q $Ca_v2.1$ $Ca2^{+}$ with a loss of function in tottering[47] and lethargic mice mutants.[48] One cannot exclude therefore that a dysfunction of R-type channels might contribute to the NMJ

abnormalities found in subgroups of MA patients. Another subclinical abnormality reported in migraine patients concerns the vestibulo-cerebellar system. Subtle cerebellar dysfunctions were found with various methods in migraine patients, especially those suffering from MA.[49-51] We have shown a positive correlation between abnormal cerebellar tests and abnormal neuromuscular transmission.[52] R-type Ca2+ channels are present in Purkinje cells, where they play a role in presynaptic long-term potentiation.[53] If these channels are dysfunctioning, they could contribute to these subclinical cerebellar abnormalities.

A third argument favoring a possible role of $Ca_v2.3$ channels in migraine pathophysiology is their involvement in activity control of the reticular thalamic nucleus. Thalamo-cortical rhythmicity is altered in $Ca_v2.3(-/-)$ mice[54] and hence $Ca_v2.3$ channels are relevant for the control of thalamocortical loops. The latter are thought to be malfunctioning in migraine and to be responsible for abnormal sensory processing.[55] More specifically, thalamocortical dysrhythmia is likely responsible for the most prevalent electrophysiological biomarker found in migraine between attacks, ie, deficient habituation of cortical evoked potentials. Whether subtle abnormalities in $Ca_v2.3$ channels may play a role in other disorders associated like migraine with thalamo-cortical dysrhythmia remains to be investigated.

Finally, migraine headache is thought to be generated in the trigeminovascular system.[56] Trigeminal nociception is abnormal during and between attacks.[57,58] $Ca_v2.3$ Ca^{2+} channels have a role in nociception, since $Ca_v2.3$ knockout mice display abnormal responses to somatic ($Ca_v2.3-/-$ & $+/-$) and visceral ($Ca_v2.3+/-$) inflammatory pain suggesting that these channels can control pain behavior through both spinal and supraspinal mechanisms.[59] Of interest for trigeminal nociception is that one of the six known isoforms of $Ca_v2.3$ in mammals, $Ca_v2.3_E$, is mainly represented in nociceptive neurons of the trigeminal ganglion.[60] The insert II in the I-II loop that characterizes this isoform is located in exon 20, where the Asp859Glu variant is located. Moreover, zolmitriptan, one of the 5HT1B/D agonists effective in acute migraine treatment, was reported to inhibit R-type Ca2+ channels in dissociated rat trigeminal neurons.[61]

CONCLUSIONS

Up until now the *CACNA1E* gene that encodes the main subunit of R-type ($Ca_v2.3$) voltage-gated calcium channels has received little attention in migraine. Given the distribution and functional roles of $Ca_v2.3$ channels, mutations in the *CACNA1E* gene could in theory be responsible for some aspects of migraine pathophysiology. We report here that a single nucleotide polymorphism in this gene leading to the substitution of aspartate by glutamate in exon 20 is overrepresented in patients suffering from migraine with complex neurological auras. The functional consequences of this substitution are not yet known and probably minor, but this polymorphism could interplay with other genetic abnormalities to modify ion channel function, as previously shown for specific CACNA1A polymorphisms in migraine.[62] While awaiting the results of functional studies, available data on the functional neuroanatomy of $Ca_v2.3$ channels set the scene for a possible role in spreading depression, neuromuscular transmission and cerebellar function, thalamocortical loops, and trigeminal nociception, all of which can be impaired in migraine and its subtypes. Needless to say that further studies are necessary to prove these hypotheses.

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