EVALUATION OF *CACNA1H* IN EUROPEAN PATIENTS WITH CHILDHOOD ABSENCE EPILEPSY

Barry Chioza[a†], Kate Everett[a*†], Harald Aschauer[b], Oebele Brouwer[c], Petra Callenbach[c], Athanasios Covanis[d], Olivier Dulac[e], Martina Durner[f], Orvar Eeg-Olofsson[g], Martha Feucht[h], Mogens Friis[ij], Armin Heils[k], Marianne Kjeldsen[i], Katrin Larsson[g], Anna-Elina Lehesjoki[l], Rima Nabbout[e], Ingrid Olsson[m], Thomas Sander[n], Auli Sirén[o], Robert Robinson[a], Michele Rees[a], R Mark Gardiner[a].

[†]These authors contributed equally to this article.

[a]Department of Paediatrics and Child Health, Royal Free and University College Medical School, University College London, The Rayne Building, 5 University Street, London, WC1E 6JJ, UK. Tel: +44 207 6796101 Fax: +44 207 6796103

[b]Medical University Vienna, Department of Psychiatry, Vienna, Austria. Tel: +43-1-40400-3543 Fax: +43-1-40400-7790

[c]Department of Neurology, University Medical Centre Groningen, Hanzeplein 1/P.O. Box 30001, 9700 RB Groningen, The Netherlands. Tel: +31 50 3612430 Fax: +31 50 3611707

[d]Neurology Department, The Children's Hospital 'Agia Sophia', Thivo & Levadias, 11527, Goudi, Athens, Greece. Tel: +30210 7751637 Fax: +30210 7705785

[e]Hopital St. Vincent de Paul, 74, Avenue Denfert-Rochereau, 75 674 Paris Cedex 14, France. Tel: +331 42192695 Fax: +331 40488046

[f]Division of Statistical Genetics, Columbia University, 722 W 168 Street, New York, NY 10032, USA. Tel: +212-342 0480 Fax: +212-342 0484

[g]Department of Women's and Children's Health, Uppsala University, Sweden. Tel: +46 18 6115899 Fax: +46 18 6115583

[h]Medical University of Vienna, Department of Paediatrics, Vienna, Austria. Tel: +43-1-40400-3805 Fax: +43-1-40400-3041

[i]Department of Neurology (Epilepsy Clinic), Odense University Hospital, DK-5000 Odense C, Denmark. Tel: +45 65411941 Fax: +45 65413389

[j]Department of Neurology, Sygehus Vestsjaelland, DK-4300 Holbaek, Denmark. Tel: +45 65411941 Fax: +45 65413389

[k]Clinic of Epileptology and Institute of Human Genetics, Rheinische Friedrich-Wilhelms-University of Bonn, Bonn, Germany. Tel: +49 228 287 9342 Fax: +49 228 287 6294

[l]Neuroscience Center and Folkhälsan Institute of Genetics, Biomedicum Helsinki, P.O. Box 63 (Haartmaninkatu 8), 00014 University of Helsinki, Finland. Tel: +358-9-191 25072 Fax: +358-9-191 25073

[m]Neuropaediatric Unit, Queen Silvia Children's Hospital, S-416 85 Göteborg, Sweden. Tel: +46-31-343 47 05 Fax: +46-31-25 7960

[n]Gene Mapping Center (GMC), Max-Delbrück-Centrum, Robert-Rössle-Str. 10, 13125 Berlin, Germany. Tel: +49 30 9406 2434 Fax: +49 30 9406 3147

[o]Department of Paediatrics, Tampere University Hospital, Tampere, Finland. Tel: +358 45 6513564 Fax: +358 3 215 7385

Abstract

CACNA1H was evaluated in a resource of Caucasian European patients with childhood absence epilepsy by linkage analysis and typing of sequence variants previously identified in Chinese patients. Linkage analysis of 44 pedigrees provided no evidence for a locus in the *CACNA1H* region and none of the Chinese variants were found in 220 unrelated patients.

Keywords: Childhood absence epilepsy; *CACNA1H*; linkage; sequence variants

1. Introduction

Childhood absence epilepsy (CAE) is characterised by the onset of typical absence seizures in childhood. It has a complex genetic component which has not yet been elucidated. The basic underlying mechanism of generalised absence seizures appears to involve thalamocortical circuitry and the generation of abnormal oscillatory rhythms from that particular neuronal network(Crunelli and Leresche, 2002). There is good evidence that the low-threshold T-type Ca^{2+} channels might be involved in the genesis of absence seizures in the thalamocortical network(Gomora et al., 2001;Kostyuk et al., 1992;Meeren et al., 2005). One member of this family, alpha1G ($Ca_v3.1$), has been shown to have a critical role in the generation of $GABA_B$ receptor-mediated spike-and-wave discharges in the thalamocortical pathway, a defining feature of absence seizures(Kim et al., 2001). The T-type Ca^{2+} channel pore-forming subunit transcripts alpha1G ($Ca_v3.1$), alpha1H ($Ca_v3.2$) and alpha1I ($Ca_v3.3$) have been shown to be widely and differentially distributed throughout the brain(McRory et al., 2001;Perez-Reyes, 1998). These observations render the family of T-type Ca^{2+} channel genes functional candidates for absence epilepsy.

Sequencing of exons one to 37 and the exon-intron boundaries of the *CACNA1G* gene was undertaken in 48 Han Chinese patients with CAE and 48 controls but no exonic mutations were found. Case-control analysis of two SNPs in 192 patients and 192 controls did not demonstrate any significant disease association(Chen et al., 2003b). However, sequencing of exons three to 35 and the exon-intron boundaries of the *CACNA1H* gene in 118 sporadic Han Chinese patients with CAE detected 68 variants(Chen et al., 2003a). Of these, 29 were found in CAE patients but not in 230 unrelated controls. These included 12 missense mutations, altering highly conserved residues, found in 14 patients.

Subsequent *in vitro* functional characterisation in the rat of five of these mutations identified altered channel gating in three (Khosravani et al., 2004). Moreover, a recent paper (Vitko et al., 2005) has reported investigations of these 12 variants in the human $Ca_v3.2$ channel using whole-cell patch-clamp recording. Eleven of the 12 had a functional effect on some aspect of channel gating. However, exons nine to 11 of *CACNA1H*, in which 75% of these mutations occur, were subsequently screened in 192 patients with idiopathic generalised epilepsy or generalised epilepsy with febrile seizures plus. The group included 49 patients with absence epilepsy. None of the Chinese variants were found, but four variants were found in patients but not 96 controls. These occurred in families but did not co-segregate with any specific epilepsy phenotype and were not found in any patient with absence epilepsy(Heron et al., 2004).

In view of these observations, *CACNA1H* was evaluated in our CAE patient resource of 44 nuclear pedigrees and 176 trios. Linkage analysis was undertaken in the pedigrees, and the 29 sequence variants were screened for in the entire resource.

2. Materials and Methods

2.1. Sample

Forty-four nuclear pedigrees (each pedigree contained at least two cases of CAE) and 176 unrelated trios (with one affected offspring with CAE), were all of Caucasian origin ascertained from European populations including the UK, France, Germany, Denmark, Sweden, Finland, Austria, the Netherlands and Greece. Inclusion criteria for CAE were as follows: absence seizures (of any type except myoclonic absences) with onset between two and 12 years; generalised tonic-clonic seizures may also occur; seizures may persist into adulthood; development is normal or with only mild learning difficulties; and the ictal EEG shows bilateral, synchronous, symmetrical discharges of 2.5-4Hz spike-wave complexes on a normal background. Exclusion criteria included: prominent myoclonus; eyelid myoclonus; significant developmental delay; persistent or focal neurological deficit; polyspike-wave complexes in the ictal EEG, or clear evidence of photosensitivity; and clear abnormalities on neuroimaging. Within the 44 pedigrees, DNA from 210 individuals was available including 102 CAE cases.

2.2. Genetic Analysis

Linkage analysis was performed using two fluorescently-labelled microsatellite markers, *D16S521* and *D16S3024*, which flank *CACNA1H* on chromosome 16p13.3. They span a genetic distance of 5.97cM (Marshfield) and a physical distance of 1.56Mb (NCBI sequence map) with *CACNA1H* physically closer

to D16S3024. All members of the 44 pedigrees from which DNA had been obtained were used for linkage analysis.

Genotyping was performed on the ABI 373 Sequence Analyser using the Genescan® and Genotyper® software. All pedigrees were checked for Mendelian inheritance using the PedCheck program(O'Connell and Weeks, 1998). Any pedigrees which failed this test were re-genotyped. Formal linkage analysis was performed using GeneHunter 2.1(Kruglyak et al., 1996). This is a parametric and non-parametric approach which can allow for heterogeneity providing both LOD and HLOD scores as well as an estimate of α, which represents the proportion of pedigrees consistent with linkage at a specific locus. Parametric analysis was performed under the assumption of autosomal dominant inheritance, with a disease allele frequency of 0.005, a penetrance of 0.5 and a phenocopy rate of 0.001. The non-parametric linkage (NPL) statistic, along with the corresponding degree of significance, is also provided.

2.3. Detection of Sequence Variants

Genomic DNA from the trios and the pedigrees was screened for the 29 patient-specific variants reported by Chen *et al*. by KBiosciences (http://www.kbioscience.co.uk) using both the Amplifluor™ and Taqman™ chemistries for genotyping.

3. Results

3.1. Linkage Analysis

Analysis provided no evidence for linkage to the region of 16p flanked by *D16S521* and *D16S3024* and encompassing *CACNA1H* (Table 1).

3.2. DNA Sequence Variants

Assays were successfully designed for 28 of the 29 variants reported and our sample resource was screened with a successful screening rate ranging from 92% to 99% (full details available on request; failed assay was for nucleotide substitution 68036G>A, which was not one of 12 missense mutations). None of the variants reported in the Chinese sample were found in our Caucasian sample.

4. Discussion

CACNA1H has been proposed as a plausible candidate gene for epilepsy primarily because it encodes the pore-forming alpha1H ($Ca_v3.2$) subunit of the T-type voltage-gated calcium channel.

Animal models have provided evidence for the role of T-type voltage-gated calcium channels in epilepsy. Increased T-type currents were first observed in select thalamic nuclei from genetic absence epileptic rats from Strasbourg model (GAERS), an animal model of absence epilepsy (Tsakiridou et al., 1995). Further evidence was provided by a knock-out mouse, where the T-type calcium channel alpha1G ($Ca_v3.1$) subunit was absent (Kim et al., 2001). The thalamocortical relay neurones of these mice were observed to lack the burst mode firing of action potentials but displayed the normal pattern of tonic mode firing. When compared to wild-type mice, the thalami

of the null mice were specifically resistant to the generation of spike-and-wave discharges (SWD) in response to $GABA_B$ receptor activation. The main conclusion of the study was that alpha1G ($Ca_v3.1$) T-type Ca^{2+} channels play a critical role in the generation of $GABA_B$ receptor-mediated SWD in the thalamus, the hallmark of absence seizures.

The linkage analysis of the *CACNA1H* region reported here produced a marginally positive HLOD score with an associated α of 0.15. One interpretation of these results is that the observed α value is due to chance shared inheritances. An alternative explanation is that a small subset of the nuclear pedigrees is linked to *CACNA1H,* but of course significant statistical evidence for this is not possible to attain with the existing resource. Heron *et al.* (Heron et al., 2004) did not detect any of the Chinese variants although only three exons were screened. For so many diverse variants to be private to a population is unusual, although differences have been reported in SNP frequencies between the major human population groups, with the Asian population displaying a large proportion of population-specific SNPs (Schneider et al., 2003; Stephens et al., 2001; Weale et al., 2003). However, there have been no reports of population specific mutations for a common disorder like epilepsy.

The results of Chen *et al.* indicate that rare and diverse alleles in *CACNA1H* may contribute to the CAE phenotype in approximately 12% of the sporadic Chinese cases. Our results do not completely exclude *CACNA1H* as a susceptibility locus in either familial or sporadic CAE cases in a European population. They demonstrate that the Chinese variants are not found in either familial or sporadic European patients, but only full re-sequencing of the entire gene would establish whether or not different rare variants are present in *CACNA1H* in our resource.

The existence of multiple non-synonymous variants specific to cases within a particular ethnic group is not consistent with the common-disease/common-variant (CDCV) hypothesis (Reich and Lander, 2001; Zondervan and Cardon, 2004). This predicts that complex traits will be due to several loci each of which will possess just a few disease alleles at relatively high frequency defining a simple allelic spectrum (e.g. the APOE ε4 allele in Alzheimer's disease (Corder et al., 1993)). Instead, these observations are consistent with the contribution of low frequency, population-specific alleles to a complex trait. These observations indicate that the CD/CV hypothesis may not be comprehensively applicable and that inter-population variability may have a greater disease impact in complex traits than previously thought.

Acknowledgements

This work was supported by the MRC (UK), Wellcome Trust, Action Medical Research and Epilepsy Research Foundation. We are very grateful to the families for participating in this study and to all our 142 collaborating clinicians, including Dr Lina Nashef and Dr Andrew Makoff from the Institute of Psychiatry, Kings College, London. We would like to thank Généthon for their assistance in collecting the French samples. Austrian financial support came from the Austrian Research Foundation (awarded to Harald Aschauer, MD), grant number 10460 and from the Austrian Fund of Scientific Research (grant number P10460-MED). We would also like to thank Wu Xi-Ru and co-workers for their cooperation and assistance in providing DNA samples from their patients.

Table 1: Summary of the GeneHunter 2 linkage analysis results for *D16S521* and *D16S3024*

	POSITION (CM)	*LOD*	*α*	*HLOD*	*NPL SCORE*	*P-VALUE*	*INFORMATION*
D16S521	0	-11.75	0.12	0.08	0.83	0.19	0.75
	1.19	-8.35	0.14	0.09	0.81	0.20	0.65
	2.39	-7.87	0.15	0.11	0.79	0.21	0.62
	3.58	-8.16	0.15	0.11	0.77	0.21	0.63
CACNA1H							
	4.78	-9.26	0.14	0.11	0.76	0.22	0.68
D16S3024	5.97	-13.24	0.13	0.10	0.75	0.22	0.80

Reference List

Chen,Y., Lu,J., Pan,H., Zhang,Y., Wu,H., Xu,K., Liu,X., Jiang,Y., Bao,X., Yao,Z., Ding,K., Lo,W.H., Qiang,B., Chan,P., Shen,Y., Wu,X., 2003a. Association between genetic variation of CACNA1H and childhood absence epilepsy. Ann Neurol 54, 239-243.

Chen,Y., Lu,J., Zhang,Y., Pan,H., Wu,H., Xu,K., Liu,X., Jiang,Y., Bao,X., Zhou,J., Liu,W., Shi,G., Shen,Y., Wu,X., 2003b. T-type calcium channel gene alpha (1G) is not associated with childhood absence epilepsy in the Chinese Han population. Neurosci Lett 341, 29-32.

Corder,E.H., Saunders,A.M., Strittmatter,W.J., Schmechel,D.E., Gaskell,P.C., Small,G.W., Roses,A.D., Haines,J.L., Pericak-Vance,M.A., 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261, 921-923.

Crunelli,V., Leresche,N., 2002. Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci 3, 371-382.

Gomora,J.C., Daud,A.N., Weiergraber,M., Perez-Reyes,E., 2001. Block of cloned human T-type calcium channels by succinimide antiepileptic drugs. Mol Pharmacol 60, 1121-1132.

Heron,S.E., Phillips,H.A., Mulley,J.C., Mazarib,A., Neufeld,M.Y., Berkovic,S.F., Scheffer,I.E., 2004. Genetic variation of CACNA1H in idiopathic generalized epilepsy. Ann Neurol 55, 595-596.

Khosravani,H., Altier,C., Simms,B., Hamming,K.S., Snutch,T.P., Mezeyova,J., McRory,J.E., Zamponi,G.W., 2004. Gating effects of mutations in the Cav3.2 T-type calcium channel associated with childhood absence epilepsy. J Biol Chem 279, 9681-9684.

Kim,D., Song,I., Keum,S., Lee,T., Jeong,M.J., Kim,S.S., McEnery,M.W., Shin,H.S., 2001. Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type Ca(2+) channels. Neuron 31, 35-45.

Kostyuk,P.G., Molokanova,E.A., Pronchuk,N.F., Savchenko,A.N., Verkhratsky,A.N., 1992. Different action of ethosuximide on low- and high-threshold calcium currents in rat sensory neurons. Neuroscience 51, 755-758.

Kruglyak,L., Daly,M.J., Reeve-Daly,M.P., Lander,E.S., 1996. Parametric and nonparametric linkage analysis: a unified multipoint approach. Am J Hum Genet 58, 1347-1363.

McRory,J.E., Santi,C.M., Hamming,K.S., Mezeyova,J., Sutton,K.G., Baillie,D.L., Stea,A., Snutch,T.P., 2001. Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276, 3999-4011.

Meeren,H., van Luijtelaar,G., Lopes,d.S., Coenen,A., 2005. Evolving concepts on the pathophysiology of absence seizures: the cortical focus theory. Arch Neurol 62, 371-376.

O'Connell,J.R., Weeks,D.E., 1998. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. Am J Hum Genet 63, 259-266.

Perez-Reyes,E., 1998. Molecular characterization of a novel family of low voltage-activated, T-type, calcium channels. J Bioenerg Biomembr 30, 313-318.

Reich,D.E., Lander,E.S., 2001. On the allelic spectrum of human disease. Trends Genet 17, 502-510.

Schneider,J.A., Pungliya,M.S., Choi,J.Y., Jiang,R., Sun,X.J., Salisbury,B.A., Stephens,J.C., 2003. DNA variability of human genes. Mech Ageing Dev 124, 17-25.

Stephens,J.C., Schneider,J.A., Tanguay,D.A., Choi,J., Acharya,T., Stanley,S.E., Jiang,R., Messer,C.J., Chew,A., Han,J.H., Duan,J., Carr,J.L., Lee,M.S., Koshy,B., Kumar,A.M., Zhang,G., Newell,W.R., Windemuth,A., Xu,C., Kalbfleisch,T.S., Shaner,S.L., Arnold,K., Schulz,V., Drysdale,C.M., Nandabalan,K., Judson,R.S., Ruano,G., Vovis,G.F., 2001. Haplotype variation and linkage disequilibrium in 313 human genes. Science 293, 489-493.

Tsakiridou,E., Bertollini,L., de Curtis,M., Avanzini,G., Pape,H.C., 1995. Selective increase in T-type calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J Neurosci 15, 3110-3117.

Vitko,I., Chen,Y., Arias,J.M., Shen,Y., Wu,X.R., Perez-Reyes,E., 2005. Functional characterization and neuronal modeling of the effects of childhood absence epilepsy variants of CACNA1H, a T-type calcium channel. J Neurosci 25, 4844-4855.

Weale,M.E., Depondt,C., Macdonald,S.J., Smith,A., Lai,P.S., Shorvon,S.D., Wood,N.W., Goldstein,D.B., 2003. Selection and evaluation of tagging SNPs in the

neuronal-sodium-channel gene SCN1A: implications for linkage-disequilibrium gene mapping. Am J Hum Genet 73, 551-565.

Zondervan,K.T., Cardon,L.R., 2004. The complex interplay among factors that influence allelic association. Nat Rev Genet 5, 89-100.