

ORIGINAL INVESTIGATION

Chiara M. Mazzanti · Andrew Bergen
Mary-Anne Enoch · Stefano Michelini
David Goldman

Identification of a Ser857-Asn857 substitution in DRK1 (KCNB1), population frequencies and lack of association to the low voltage alpha EEG trait

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Abstract A nonconservative amino acid substitution (Ser857Asn) in the human delayed-rectifier potassium channel DRK1 (KCNB1 locus), a candidate gene for the low voltage alpha electroencephalogram (EEG) trait locus (LVEEG1) at 20q13.2, and its frequency in ethnic population samples are described. The frequency of Asn857 in seven different ethnic population samples, totalling more than 1600 individuals, ranged from zero to greater than 3%. However, no association was found between Asn857 and the low voltage alpha EEG trait (LVA) in a population of 105 subjects assessed for the EEG, 24 of whom actually had LVA.

Introduction

The LVA trait, EEGV1 locus and KCNB1

The low voltage alpha trait (LVA) is a stable, naturally occurring electroencephalogram (EEG) trait difference, which is found in 4.2%–11.6% of the normal population (Vogel et al. 1970). In approximately one-third of families with LVA, a locus (EEGV1) was mapped to chromosome 20q13.2–13.3 with the variable number tandem repeat (VNTR) probe D20S19 (Steinlein et al. 1992). As the KCNB1 gene has been mapped to 20q13.2 (Albrecht et al. 1993; Melis et al. 1995), it is thus a candidate gene for the EEGV1 locus. The KCNB1 protein is the delayed-rectifier potassium channel (DRK1), which belongs to the voltage-gated potassium channel family and to the Shab

subfamily of potassium channels (Pongs 1992). DRK1 activates slowly after membrane depolarization and inactivates only after several seconds. The DRK1 channel is thought to consist of four transmembrane subunits, with the amino and carboxy termini of each subunit facing the cytoplasmic side of the membrane while the core domain of each subunit appears to be folded such that it spans the lipid bilayer six times (Yool and Schwarz 1991). The sequences of the transmembrane segments of mouse Shab, rat KCNB1 and human KCNB1 are identical, which suggests that this protein can tolerate few substitutions in these domains. However, clear pharmacological and conductance differences exist between the human and the rat KCNB1 channels. These functional differences obviously cannot be explained by sequence differences within the membrane-spanning domain (Albrecht et al. 1993).

To evaluate KCNB1 as a candidate gene for EEGV1, we used single-strand conformational polymorphism (SSCP) analysis (Orita et al. 1989) to detect KCNB1 variants in a population of 75 individuals whose EEG phenotype had been assessed quantitatively and qualitatively. An SSCP that corresponded to a nonconservative amino acid substitution was detected. Using mass allele detection (Bergen et al. 1996), the frequency of this variant allele was determined in large ethnic population samples and subjects evaluated for EEG phenotype.

Materials and methods

Polymerase chain reaction (PCR) amplification

Genomic human DNA was analyzed for polymorphisms in the KCNB1 gene by SSCP analysis (Orita et al. 1989) followed by direct sequencing of amplified DNA. The genomic DNA was isolated from Epstein-Barr virus immortalized lymphoblastoma cell lines or directly from lymphocytes. The amplification was accomplished with four sets of primers:

KCNB1PF1: 5'GGCGGCCGGATCAGCGATGCC3', nt 42–nt 61;
KCNB1PR1: 5'ATTCCTCTGTGGCTGCCAAG3', nt 607–nt 626;
KCNB1PF2: 5'AGCTACAGAGCCTCGATGAG3', nt 695–nt 715;
KCNB1PR2: 5'TGAGGATGGGTTGGGATTGG3', nt 1694–nt 1674;
KCNB1PF3: 5'CAGCACCTGAACGTTACGA3', nt 1620–nt 1640;

C. M. Mazzanti* · A. Bergen* · M.-A. Enoch · S. Michelini
D. Goldman (✉)
Laboratory of Neurogenetics,
National Institute on Alcohol Abuse
and Alcoholism, National Institutes of Health,
Rockville, MD, USA
Tel.: +1-301-443-0059; Fax: +1-301-443-8579;
E-mail: dgmneuro@pop.nih.gov

C. M. Mazzanti · S. Michelini
Institute of Psychiatry, University of Pisa, Pisa, Italy

* The first two authors contributed equally to this report.

Table 1 Populations and genotypes

Population	n	Heterozygote	Homozygote	Asn857 allele frequency
U.S. Caucasian ^a	255	3		0.006
Swedish	78	2		0.010
Finnish	724	46	2	0.033/0.035 ^b
Cheyenne Native American	143	0		0.000
Pima Native American	176	0		0.000
Yakut (Siberian)	196	3		0.008
Italian	99	1		0.005
Total	1671			

^a 105 were evaluated for EEG phenotype while 150 were not evaluated

^b Gene counting/maximum likelihood estimation

KCNB1PR3: 5'TAGAACGGGGAGGAGTGGAC3', nt 2122-nt 2102; KCNBI PF4: 5'AGCTCCGAGCACTTAAAGTC3', nt 2060-2080; KCNBI PR4: 5' AACAGCTCTCTCTTTGGAC3', nt 2690-nt 2670.

These primer pairs amplify four regions, which are 565, 999, 502 and 630 bp in length and which cover 97% of the KCNB1 channel-coding sequence (Albrecht 1993). The unamplified 3% of the coding sequence is nt 607 to nt 695 and corresponds to the first membrane-spanning segment, S1. Amplification was performed using 100 ng of DNA, 0.25 mM of each primer, 50 mM each of dATP, dCTP, dGTP, dTTP and 5 U/100 ml of AmpliTaq with the appropriate buffer (Perkin Elmer). For SSCP analysis, 1 µCi of [α -³²P]dCTP was included in the PCR reaction. Reactions were carried out in a volume of 25 µl. Samples were amplified for 30 cycles, each consisting of 20 s at 95°C, 15 s at 61°C, and 30 s at 72°C in a Perkin Elmer 9600 apparatus.

To increase the sensitivity of the SSCP analysis, the amplified fragments were digested with restriction enzymes to yield sub-fragments ranging from 100 to 257 bp in length. Fragments were digested with the following enzymes: *HinfI* for the KCNBI PR1 fragment, *EcoRI* and *MspI* for the KCNBI PR2 fragment, *BbvI* for the KCNBI PR3 fragment and *Eco109I* and *BsaI* for the KCNBI PR4 fragment (all enzymes were obtained from New England Biolabs, Beverly, Mass.). Mixtures consisted of 7.5 µl of PCR mixture, 5 units of enzyme and the recommended buffer. After incubation, the reaction mixture was diluted with 15 µl of 95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, incubated at 95°C for 3 min and then placed on wet ice. A 5-µl sample of the denatured DNA was loaded per lane in a 0.5 × mutation detection enhancement gel (AT Biochem, Malvern, Penn.). Electrophoresis was carried out at 4°C for 16 h at 6 W. The gel was dried and autoradiography was performed at -70°C.

Sequence determination

The polymorphic DNA fragment was gel purified and then extracted with glass beads (Geneclean, BIO 101, La Jolla, Calif.). After purification, the DNA was sequenced with [³²P] end-labeled primers using dideoxy cycle-sequencing reagents (Life Technologies). Samples were subjected to electrophoresis in a 6% polyacrylamide gel, dried and visualized using autoradiography.

Mass allele detection (MAD)

Two primers were synthesized, one specific the Asn857 allele (KCNBI Sp: 5' GGAAGCACACGAGATCAGTA 3' bases 2608-2627) and the other for complementary to the gene sequence (KCNBI NSp: 5' CTTCTCTCTGAGGGCTTACA3' bases 2741-2760). Specificity of amplification is conferred by the substitution AG→TA at the end of the allele-specific primer, KCNBI Sp. For the mass allele detection, allele-specific amplification of some pooled DNAs with biotin- and ruthenium-labeled primers was performed as described (Bergen et al. 1996). For some

pooled DNA samples, amplification was performed with unlabeled primers and the specific PCR products were detected on PAGE gels. Positive DNA pools were identified as those that yielded the specific 152-bp PCR product and the DNAs in these pools were subsequently screened individually.

All Asn857 heterozygotes and homozygotes were confirmed using restriction enzyme digestion with *SfaNI* (New England Biolabs) of a 320-bp PCR product amplified using primers (KCNBI For (2440-2459): 5'AACCACTTTGAAAGCTCCCC3' and KCNBI NSp). The KCNBI G2627A transition eliminates the *SfaNI* restriction site. Thus the normal homozygote (Ser857/Ser857) shows two bands upon digestion of the PCR product, the heterozygote (Ser857/Asn857) three bands, and the transition homozygote (Asn857/Asn857) only the original PCR product.

Subjects characterized for resting EEG

Genomic DNA samples were obtained from a total sample 105 U.S. individuals (99 Caucasians and 6 African-Americans) whose EEG phenotypes were determined by quantitative spectral analysis and by qualitative blind rating of the EEG tracing as described (Enoch et al. 1995). There were 24 subjects with the LVA phenotype in this group. The KCNB1 coding region of 75 of these individuals (14 with LVA) was evaluated by SSCP. Once the Asn857 variant was found, the remaining 30 individuals were screened using MAD.

Population samples and frequency calculations

The frequency of Asn857 was determined in population samples collected from various ethnic groups (Table 1), including three European populations, one Asian population, two Native American populations and U.S. Caucasians and African-Americans not evaluated for EEG phenotype for a total of 1671 genotypes determined by MAD. Significance figures were obtained by randomization and computing the likelihood of obtaining the experimentally derived figures.

Results

Amplification of genomic DNA with primers KCNBI PF4 and KCNBI PR4 followed by restriction digestion and SSCP analysis revealed a mobility shift in 3 of 75 U.S. Caucasians (Fig. 1a). Sequencing of the polymorphic DNA fragment revealed that the variant is caused by a G-to-A transition at nucleotide 2627 of the KCNB1 coding sequence (Fig. 1b). The effect of the transition is that the penultimate amino acid, serine 857 (AGC), is replaced by asparagine (AAC). The frequency of Asn857 was deter-

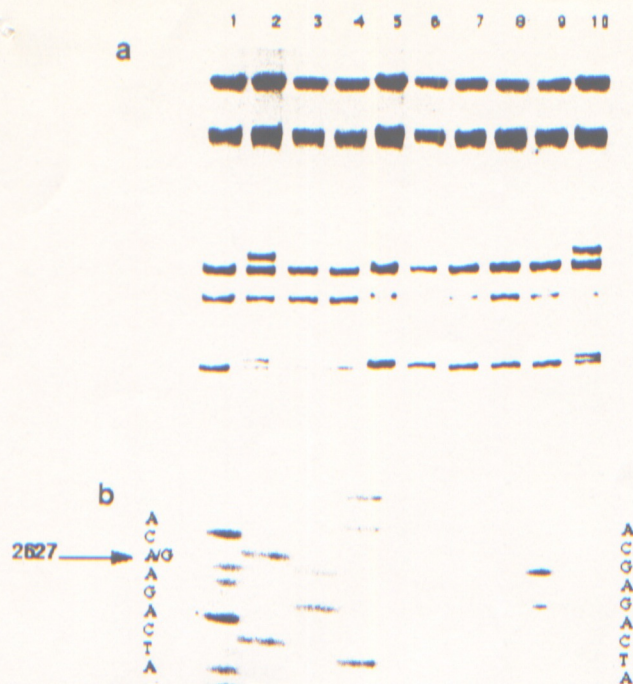


Fig. 1a Single-strand conformational polymorphism (SSCP) analysis of the region of the *KCNB1* gene corresponding to the carboxy terminus of DRK1. The PCR product of 630 bases was digested with *BsaI* and *Eco0109I*. Lanes 2 and 10 contain the variant SSCP phenotype. **b** Sequences of two individuals: on the left is a 2627A heterozygote, while on the right is a normal 2627G homozygote. This G→A transition causes a nonconservative substitution of serine 857 to asparagine 857.

mined in 105 individuals typed for trait differences in the resting EEG and in 1566 individuals from seven ethnic population samples.

Screening of a total of 1671 individual DNAs revealed statistically significant differences in the frequency of the *KCNB1* variant in different populations. In the U. S. population sample, Asn857 had an allele frequency of 0.6% (Table 1). Caucasians from Sweden and Finland showed Asn857 allele frequencies of 1% and 3.3%, respectively. The maximum likelihood estimate of the Asn857 allele frequency in Finns is 3.5%. Only two homozygotes were observed and both were Finnish. Italian Caucasians had an Asn857 allele frequency of 0.5%, similar to the US Caucasian sample. The Yakut population had an Asn857 allele frequency (0.8%), similar to the European populations. However, the three individuals identified with the Asn857 allele are all first-degree relatives and thus the true frequency in this sample is probably much lower. Two Native American populations did not manifest the Asn857 allele. The Asn857 allele frequency was significantly more common in Finns than in the U. S. population sample, Italian Caucasians and Native Americans ($P < 0.001$, $P = 0.01$ and $P < 0.001$, respectively).

Three of the 105 U.S. Caucasian individuals assessed for EEG phenotype were found to have the Asn857 allele, but none of these individuals had the LVA phenotype. The

Table 2 Effect of *KCNB1* Asn857 on alpha power

DRK1 genotype	Relative alpha power		Absolute alpha power	
	Mean	SD	Mean	SD
Total sample $n = 105$	0.66	0.15	177.25	98.63
Ser/Ser $n = 102$	0.65	0.15	175.66	96.6
Ser/Asn $n = 3$	0.79	0.065	266.5	123.04
Means of Ser/Asn vs. Ser/Ser	$t = 3.71$	$0.05 < P < 0.1$	$t = 1.29$	$P < 0.20$

average values for absolute and relative alpha power of the three individuals with the Asn857 allele did not differ significantly from the average values for the 105 individuals whose EEGs were measured (Table 2).

Discussion

KCNB1 Asn857 allele and population frequencies

The Asn857 allele is the first naturally occurring structural variant of the human *KCNB1* potassium channel to be identified. It is a moderately common and non-conservative substitution of the penultimate amino acid of the *KCNB1* protein, a phylogenetically conserved serine. Rare genetic variants of two other potassium channels are associated with pathology. The gene coding for an inwardly rectifying potassium channel, *KCNAl*, is associated with episodic ataxia/myokymia (Browne et al. 1994) and the mouse homolog of *KCNJ6* may be responsible for the weaver mutant (Patil et al. 1995).

The population frequencies observed in the different population samples suggest that the Asn857 allele is uncommon throughout Eurasian populations, is absent from Native American populations, but is more abundant in Finns, a population commonly regarded as having been affected by genetic drift and founder effects (Nevanlinna 1972). The lack of Asn857 alleles in the Native American population samples tested is probably not due to sample size because 638 chromosomes were tested. The possibility that a founder effect is responsible for the increased frequency of the Asn857 allele in the Finnish population relative to the other Caucasian populations tested could be investigated by performing linkage disequilibrium mapping (Hoglund et al. 1995).

The observation of two unrelated homozygotes in the Finnish population sample at the expected frequency might suggest that homozygosity for the Asn857 allele is not highly deleterious. However, the Finnish population sample is derived from a large collection of psychiatric patients and psychiatrically interviewed controls. One of the Asn857/Asn857 homozygotes is schizophrenic and the other has significant paranoia (M. Virkunen, D. Goldman, M. Eggert, M. Linnoila, unpublished data). There-

fore, the possible role of Asn857/Asn857 homozygosity in psychiatric disease is not completely resolved.

Possible functional alteration of KCNB1

We have not established whether the Asn857 substitution alters KCNB1 function, but there are several lines of evidence that indicate this is a reasonable possibility. The Shab subfamily has an RXXSI carboxy terminal amino acid consensus sequence (Strong et al. 1993) of which RXXS is a consensus phosphorylation sequence for Ca⁺⁺/calmodulin-dependent kinase (Kennelly and Krebs 1991). Delayed rectifying potassium channels are sensitive to phosphorylation state; the current is reduced by increased phosphorylation, increasing cell excitability (Levitan 1985). These observations suggest that the carboxy terminal serine is phosphorylated and may be involved in the regulation of the protein's function. Therefore the Asn substitution could obliterate a phosphorylation site resulting in increased excitability of the cell, or otherwise affect DRK1 function by altering the conformation of the carboxy terminus.

LVA and KCNB1

The low-voltage alpha trait is a stable, naturally occurring, and dominantly inherited EEG trait difference (Anokhin et al. 1992). In its inheritance, LVA appears to exhibit genetic heterogeneity with about one-third of LVA families showing significant evidence for a locus on the same region of chromosome of 20q where KCNB1 has also been localized. Of the 105 U.S. Caucasian individuals assessed for EEG phenotype, 3 were Ser857/Asn857 heterozygotes, but none of these individuals had LVA. A comparison of quantitative measures of alpha power was performed between the 3 Asn857 individuals and the other 102 individuals whose EEG phenotype had been evaluated. While not significant ($t = 3.61$, $0.05 < P < 0.1$), the small number of Asn857 heterozygotes could contribute to a lack of statistical power. Regions of the KCNB1 gene that could alter its expression have not been screened. Also, the sensitivity of the SSCP method used is probably about 85%, and, therefore, it is still possible that other KCNB1 variants exist that could be responsible for the LVA trait in this population.

As discussed, Asn857 is not only a naturally occurring, nonconservative substitution allele, but the serine that is replaced may be phosphorylated to regulate the function of the DRK1 channel. One way to explore this possibility would be through electrophysiological studies of both alleles. In addition, it may be useful to measure the frequency of Asn857/Asn857 homozygotes in schizophrenics and to perform additional screening of the coding and noncoding region of DRK1 for other substitutions that could alter function.

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