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ARTICLE

CNGB3 mutations account for 50% of all cases with autosomal recessive achromatopsia

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Achromatopsia is a congenital, autosomal recessively inherited disorder characterized by a lack of color discrimination, low visual acuity (<0.2), photophobia, and nystagmus. Mutations in the genes for *CNGA3*, *CNGB3*, and *GNAT2* have been associated with this disorder. Here, we analyzed the spectrum and prevalence of *CNGB3* gene mutations in a cohort of 341 independent patients with achromatopsia. In 163 patients, *CNGB3* mutations could be identified. A total of 105 achromats carried apparent homozygous mutations, 44 were compound (double) heterozygotes, and 14 patients had only a single mutant allele. The derived *CNGB3* mutation spectrum comprises 28 different mutations including 12 nonsense mutations, eight insertions and/or deletions, five putative splice site mutations, and three missense mutations. Thus, the majority of mutations in the *CNGB3* gene result in significantly altered and/or truncated polypeptides. Several mutations were found recurrently, in particular a 1 bp deletion, c.1148delC, which accounts for over 70% of all *CNGB3* mutant alleles. In conclusion, mutations in the *CNGB3* gene are responsible for approximately 50% of all patients with achromatopsia. This indicates that the *CNGB3*/*ACHM3* locus on chromosome 8q21 is the major locus for achromatopsia in patients of European origin or descent.

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Introduction

Achromatopsia (rod monochromacy/total colorblindness; OMIM 262300, 216900) is a congenital, autosomal recessively inherited disorder characterized by reduced visual acuity, pendular nystagmus, increased sensitivity to light (photophobia), a small central scotoma (which is often difficult to demonstrate), eccentric fixation, and reduced or complete loss of color discrimination. Hyperopia is common. Nystagmus develops during the first few weeks after birth followed by increased sensitivity to bright light. Best visual acuity varies with severity of the disease and is 20/200 or less in complete achromatopsia and may be as high as 20/80 in incomplete achromatopsia. Visual acuity is usually stable over time, but both nystagmus and sensitivity to bright light may improve slightly. Although the fundus is usually normal, macular changes and vessel narrowing may be present in some patients. In electroretinographic recordings, cone function cannot be detected, while rod function has been reported as essentially normal.[1]

Mutations in the *CNGA3* and *CNGB3* genes, which encode the α- and the β-subunits of the cyclic nucleotide-gated cation channel (CNG) in cone photoreceptors, and also in the *GNAT2* gene, encoding the α-subunit of the cone photoreceptor transducin, have been shown to cause this disorder.[2–6] All three gene products are involved in the cone phototransduction cascade (photopigment→G-protein→phosphodiesterase/cGMP→CNG channel). The CNG channels are located in the cell membrane of the outer segments of the photoreceptor. In the dark, they are kept open by high intracellular levels of cGMP (dark current). Illumination triggers a signal transduction cascade – involving transducin – that results in the decrease of the intracellular cGMP level. This decrease in ligand concentration leads to the closure of the cGMP-gated channels and thus generates a membrane hyperpolarization signal that subsequently modulates glutamate release at the photoreceptor synapse.[7]

While we recently reported the results of a comprehensive screen for mutations in *CNGA3*,[8] there are as yet only limited data for *CNGB3*.[3,4,9,10] The aim of this study was to determine the prevalence of *CNGB3* gene mutations and to establish a comprehensive *CNGB3* gene mutation spectrum in a large collection of patients affected by achromatopsia.

Materials and methods

Patients and clinical examination

Patients were informed about the objectives of the studies and consented to participate. The research program followed the tenets of the Declaration of Helsinki. The recruitment and clinical examinations were performed in several ophthalmic centers notably in Germany, the Netherlands, Denmark, Italy, the United States, Sweden, Hungary, France, Switzerland, and Israel. The clinical diagnosis was established by standard clinical examinations (visual acuity, color vision, ophthalmoscopy) and, if applicable, by further electroretinographic and psychophysical testings.

Venous blood was collected from all patients and available family members after informed consent and total genomic DNA was extracted according to standard procedures.

The patient sample comprised of 341 independent subjects with a diagnosis of congenital achromatopsia including patients diagnosed with complete (rod monochromacy) and incomplete achromatopsia.

Patients with blue cone monochromacy, caused by a defect in the red-/green opsin gene cluster, were excluded through prior genetic testing (data not shown).

In all, 80 cases of this patient sample were shown to carry homozygous or compound heterozygous *CNGA3* gene mutations[2,9] (unpublished data) and were excluded from screening of the *CNGB3* gene.

Clinical details of some of these patients with *CNGB3* mutations have recently been reported.[11–13]

Mutation screening in achromatopsia

In a first screening series, 70 patients, in which *CNGA3* mutations had been excluded, were analyzed by PCR amplification of all 18 exons and flanking intron sequences of the *CNGB3* gene from total genomic DNA and subsequent direct DNA sequencing. PCR products were purified using Gibco PCR Purification Columns (GibcoBRL, Life Technologies, Rockville, MD, USA) or treated with ExoSAP-IT (USB Corp., Cleveland, OH, USA) and sequenced employing Big Dye Terminator Chemistry (Applied Biosystems, Weiterstadt, Germany). Sequencing reactions were separated on ABI 377 and ABI 3100 DNA sequencers (PE Biosystems, Weiterstadt, Germany), analyzed

by manual inspection and comparative assembly with the SeqMan program (DNASTAR, Madison, WI, USA).

In a second screening series, DNA samples of 67 achromatopsia patients were subjected to whole *CNGB3* gene SSCP analysis. For exons 1, 5, 9, and 11–16, PCR products were separated on 10% polyacrylamide gels (37.5:1) without glycerol at 4°C, exons 3, 8, 10, and 18 were run on 10% polyacrylamide gels (37.5:1) without glycerol at room temperature, and exons 4, 6, 7, and 17 were analyzed on 10% polyacrylamide gels (37.5:1) with 10% glycerol at 4°C. All gels were run in 1 × TBE over night at constant voltage (100–300 V) depending on fragment size. Polyacrylamide gels were then silver-stained for visualization of single- and double-stranded DNA fragment bands.

In addition, to account for the most frequent mutations, DNA samples of patients were analyzed for common mutations as follows: c.819–826del8 – PCR with mismatch primers for exon 6 and RFLP with *Hin*fI; c.886–896del11insT – PCR with mismatch primers for exon 7 and RFLP with *Apo*I; c.991-3T>G – PCR with mismatch primers for intron 8/exon 9 and RFLP with *Hin*fI; c.1006G>T (p.E336X) – standard PCR for exon 9 and RFLP with *Apo*I; and c.1148delC – standard PCR for exon 10 and RFLP with *Bfa*I. Digested PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining in comparison to a healthy control subject.

Our most recent screening strategy combines these PCR/RFLP assays and subsequent direct sequencing of all coding exons (remaining number of patients).

Control experiments

To evaluate the different identified sequence variations, we analyzed 100 healthy control DNA samples for the following mutations (in parts reported previously):[3] c.467C>T (p.S156F), c.819–826del8, c.886–896del11insT, c.926C>T (p.P309L), c.991-3T>G, c.1006G>T (p.E336X), c.1148delC, c.1304C>T (p.S435F), and c.1578+1G>A. None of the mutations were observed in the control panel.

Segregation analysis

Segregation analysis for the presence and independent inheritance of two mutant alleles were performed in all cases (68/163), for whom samples from additional family members were available. Analysis was carried out either by PCR/RFLP analysis, SSCP analysis, or direct sequencing.

Results

Prevalence of *CNGB3* mutations in achromatopsia patients

Our original patient sample comprised 341 independent patients with a clinical diagnosis of achromatopsia. Of these patients, 80 were shown to carry mutations in the *CNGA3* gene (unpublished data)[2,8] and five patients carried *GNAT2* mutations.[5] In 163 patients, we were able to identify mutations in the *CNGB3* gene. The spectrum of *CNGB3* mutations comprised 28 different mutations including three missense mutations, 12 nonsense mutations, five small deletions of 1, 2, 8, 10, or 21 bp, respectively, three 1 bp insertions, and five putative splice site mutations (Table 1). The mutations observed in *CNGB3* were spread almost evenly over all coding exons (Figure 1). This is in contrast to the distribution of mutations in *CNGA3*, which are almost exclusively confined to the three terminal exons encoding the structural and functional domains of the channel α-subunit.[8]

Among the 163 patients with *CNGB3* mutations, both mutant alleles were identified in 149 subjects and only a single heterozygous mutation was detected in 14 subjects (Table 2).

Of the 149 achromatopsia patients with two mutations in the *CNGB3* gene, heterozygous mutations were observed in 44 cases and apparent homozygosity was present in 105 cases including 95 patients with homozygosity for the c.1148delC mutation allele. However, for most of the subjects with apparent homozygous mutations, there was no evidence for parental consanguinity. For 68 of the 163

Table 1 Mutations in the *CNGB3* gene

Location	*Alteration nucleotide sequence*[a]	*Alteration polypeptide*	*Total number of chromosomes*
Exon 1	c.29_30insA	p.K10fsX	1
Exon 1	c.112C>T	p.Q38X	1
Exon 4	c.391C>T	p.Q131X	1
Exon 4	c.467C>T	p.S156F[4]	3
Exon 5	c.607C>T	p.R203X[3]	1
Intron 5	c.644-1G>C	Splice site	1
Exon 6	c.646C>T	p.R216X	1
Exon 6	c.682_683insG	p.A228fsX	1
Exon 6	c.702G>A	p.W234X	2
Exon 6	c.706delAinsTT	p.I236fsX	1
Exon 6	c.819–826del8	p.P273fsX[3,4]	16
Exon 7	c.886–896del11insT	p.R296fsX	7
Exon 8	c.926C>T	p.P309L	1
Intron 8	c.991-3T>G	Splice defect	13
Exon 9	c.1006G>T	p.E336X[3]	6
Exon 10	c.1063C>T	p.R355X	2
Exon 10	c.1119G>A	p.W373X	2
Exon 10	c.1148delC	p.T383fsX[3,4]	233
Exon 11	c.1255G>T	p.E419X	1
Exon 11	c.1298_1299delTG	p.V433fsX	1
Exon 11	c.1304C>T	p.S435F[3,4]	4
Exon 12	c.1432C>T	p.R478X	1
Exon 12	c.1460G>A	p.W487X	1
Intron 13	c.1578+1G>A	Splice defect[3]	6
Exon 14	c.1635A>T	p.Y545X	1
Intron 15	c.1781+1G>C	Splice defect	2
Intron 15	c.1781+1G>A	Splice defect	1
Exon 18	c.2160−2180del21	p.Q720_K726del	1

[a]Numbering of nucleotide sequence as designated by Genbank Accession no. AF272900, with the adenosine of the start codon ATG denoting nucleotide position 1. fs = frame shift.

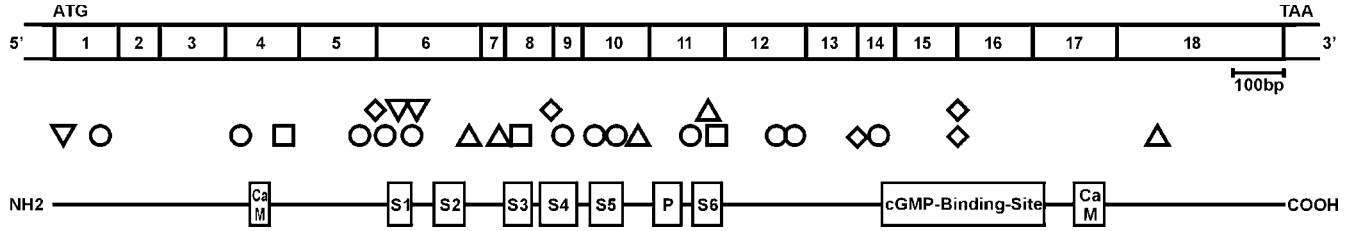

Figure 1 Distribution of the mutations found in the *CNGB3* gene and the CNGB3 polypeptide. The structure of the exon coverage of the *CNGB3* gene is displayed compared with the structural and functional CNGB3 channel domains. The mutations are shown in relative position to this arrangement. The different mutation types are displayed as follows: △ deletion, ▽ insertion, □ missense mutation, ○ nonsense mutation, ◇ splice site mutation. S1–S6: transmembrane domains; CaM: Ca^{2+}/calmodulin domains.

Table 2 Allele compositions of *CNGB3* gene mutations and their frequency in our patient sample affected by achromatopsia

Allele 1	*Allele 2*	*Number of observed cases*
c.1148delC	c.1148delC	95
c.1148delC	c.819–826del8	9
c.1148delC	c.886–896del11insT	6
c.1148delC	c.991-3T>G	4
c.991-3T>G	c.991-3T>G	3
p.S435F	p.S435F	2
c.1148delC	c.1578+1G>A	2
c.1148delC	p.E336X	2
c.1148delC	p.S156F	2
c.819–826del8	c.991-3T>G	2
c.1148delC	p.R203X	1
c.1148delC	p.E419X	1
c.1148delC	c.28insA	1
c.1148delC	p.W487X	1
c.1148delC	c.1781+1G>C	1
c.1148delC	c.1781+1G>A	1
c.1148delC	p.R216X	1
c.1148delC	p.R478X	1
c.1148delC	p.P309L	1
c.1148delC	c.1298_1299delTG	1
c.1148delC	c.706delAins2T	1
p.E336X	p.E336X	1
c.1578+1G>A	c.1578+1G>A	1
p.W373X	p.W373X	1
p.R355X	p.R355X	1
p.W234X	p.W234X	1
p.E336X	p.Q131X	1
p.E336X	c.1578+1G>A	1
c.819–826del8	c.1578+1G>A	1
c.819–826del8	c.682_683insG	1
c.819–826del8	p.Q38X	1
p.S156F	c.644-1G>C	1
c.1148delC	Unknown	7
c.819–826del8	Unknown	2
c.1781+1G>C	Unknown	1
c.991-3T>G	Unknown	1
c.886–896del11insT	Unknown	1
p.Y545X	Unknown	1
c.2160–2180del21	Unknown	1

CNGB3 mutation carrying patients, family members were available for segregation analysis, which provided no evidence for in-phase double mutations (ie heterozygous mutations in *cis*) or hemizygous mutations that mimic homozygosity. In fact, the presence and segregation of two independent mutation alleles could be unequivocally confirmed in 51 cases in which both parents were genotyped (data not shown). A total of 19 patients had affected family members. All affected family members were shown to carry the same genotype as the index patient.

In 14 achromatopsia patients, only a single heterozygous mutation was detected, although all 18 coding exons had been sequenced. In addition, the presence of larger rearrangements and deletions was excluded by means of semi-quantitative multiplex PCR and long-distance PCR covering all coding exons in overlapping segments (data not shown).

Discussion

Nature and impact of *CNGB3* mutations

A major finding of this study is that the majority of mutations in the *CNGB3* gene (25/28) – if expressed – give rise to truncated CNGB3 polypeptides and most probably represent null alleles. Four of the 12 nonsense mutations result in premature translation termination before transmembrane domain S1, and the remaining mutations well before or right at the beginning of the cGMP binding site (Figure 1). Similarly, all but one of the deletion/insertion mutations result in frameshifts that lead to largely truncated polypeptides. It is very likely that these mutations do not contribute to the formation of functional heteromeric cone CNG channels, since all of these mutant CNGB3 polypetides lack the cGMP binding site. The high proportion of protein truncation mutations in *CNGB3* contrasts with the mutation spectrum in the *CNGA3* gene, where missense mutations predominate.[8] The reason for this obvious discrepancy in the prevalence of different types of mutations is unclear. One might speculate that the CNGB3 polypeptide, which is less stringently conserved in evolution than CNGA3, tolerates to a larger extent substitution mutations. However, the presence of only few sequence variants/polymorphisms argues against this.

Five of the detected mutations most probably affect splicing of the *CNGB3* pre-mRNA. Three of them (c.1578 + 1G > A; c.1781 + 1G > C; c.1781 + 1G > A) alter the almost perfectly conserved guanosine of the splice donor 5′-terminal -GT- dinucleotide. Such mutations typically lead to a skipping of the preceding exon during transcript processing. The other two splice site mutations c.644-1G > C and c.991-3T > G affect the splice acceptor consensus sequence. The mutation c.644-1G > C alters the last nucleotide of the introns' almost perfectly conserved terminal -AG- dinucleotide. The mutation c.991-3T > G is located three nucleotides upstream of the intron 8/exon 9 boundary. Statistically, this nucleotide position is most frequently occupied by a cytidine or by a thymidine nucleotide. In most cases, mutations at the splice acceptor site lead to a skipping of the downstream exon. Arguments supporting the pathogenicity of the putative splice site mutations are their occurrence in association with a second clearly pathogenic mutation in compound heterozygotes (see Table 2: allele compositions) and the recurrency of the c.991-3T > G mutation in Dutch patients.

The association of the missense mutations with the achromatopsia phenotype is also worthy of discussion. Firstly, all three mutations have been excluded in over 100 healthy controls and shown to segregate with the achromatopsia phenotype in the respective pedigrees.

The missense mutation p.S156F was observed in two independent achromatopsia families in our study and has also been reported by Sundin and co-workers.[4] Although it affects an amino residue in the less stringently conserved amino-terminus, it was found as the sole alteration in *trans* with clear pathogenic mutations (Table 2). Most recent research has shown that p.S156F is located in the vicinity of an amino-terminal Ca^{2+}/calmodulin binding site (aa 132–147).[14]

The mutation p.P309L affects an amino-acid residue in transmembrane domain S3 that is evolutionarily highly conserved among human and murine CNGB3 and human CNGB1. It was observed in *trans* with the most frequent mutant allele c.1148delC.

The p.S435F mutation has been reported in prior studies to segregate with 'Pingelapese Blindness' (OMIM262300).[3,4] This kind of achromatopsia is very common in the Pingelap islander population of the Eastern Caroline Islands of Micronesia (incidence 5–10%).[4,15] Recent work achieved by heterologous coexpression of p.S435F mutant CNGB3- and wild-type CNGA3-subunits in *Xenopus* oocytes suggests that the mutation does not disturb subunit assembly or plasma membrane targeting, but rather seems to result in an increase of the affinity for cAMP and cGMP, and changes the pore properties of the channel including decreased single channel conductance and sensitivity to block by L-*cis*-diltiazem.[16]

Recurrent mutations

Nine of the 28 different mutations were found recurrently (Table 1). Some of these were confined to specific geographic locations suggesting local founder effects. The mutations c.819–826del8 and c.886–896del11insT that account for 16 and eight disease alleles, respectively, in our sample were found only in patients originating from Germany or the Netherlands. Even more restricted in its geographic distribution is the putative splice site mutation c.991-3T > G in intron 8. Except for one patient, this mutation was exclusively found in patients originating from the Netherlands. Of the 40 patients of Dutch origin, five patients were compound heterozygous and three homozygous for this mutation (11/13 alleles).

Most strikingly, we identified as many as 233 c.1148delC mutant alleles in our total of 163 independent patients with *CNGB3* mutations. In all, 95 patients were homozygous (or at least apparently homozygous) for this mutation (190 alleles) and 43 were heterozygous. In seven of the latter patients, the c.1148delC mutation was the sole *CNGB3* mutation detected (see below). In comparison, another study reports eight families with *CNGB3* mutations, of these five patients were apparently homozygous, one compound heterozygous, and one carried a single c.1148delC allele.[10] This means that the c.1148delC mutation accounts for over 70% of all *CNGB3* mutant alleles in our achromat sample. This predominance of a single mutation is one of very few examples among retinal diseases such as the p.P23H *RHO* gene mutation in the US patients with autosomal dominant retinitis pigmentosa,[17] the p.R345W *EFEMP1* gene mutation in Malattia Leventinese and Doyne honeycomb retinal dystrophy,[18] or the c.2588G > C *ABCA4* gene mutation in middle and northern European Stargardt disease patients.[19] In comparable samples, screening for the c.1148delC mutation will enable the molecular diagnosis of almost 40% of all achromatopsia cases with a single assay.

Singular mutations

In 14 index patients, we could only detect a single heterozygous mutation. The lack of a second mutation in these patients might be explained by several arguments. A general problem in PCR-based screening protocols – as used in this study – is the failure to identify large genomic deletions and rearrangements. We tried to address this problem by performing semiquantitative multiplex and long-distance PCR experiments for the *CNGB3* gene in these subjects. Yet, these investigations did not provide evidence for the presence of such deletions/rearrangements in our patients with single heterozygous mutations. Another possibility is that the missing mutations are located within introns ('deep intron mutations') or in yet unidentified upstream untranslated sequences critical for *CNGB3* splicing or expression.

Finally, given the large number of subjects screened in this study, we have to consider that one or the other of these single heterozygotes might be a carrier for a mutant *CNGB3* allele, while the disease is caused by mutations in another gene. Current estimates for the prevalence of achromatopsia are between 1:30 000 and 1:50 000, with about half of them caused by *CNGB3* mutations as shown in this study. The expected carrier frequency for *CNGB3* mutations therefore ranges between 0.006 and 0.008.

Prevalence of *CNGB3* mutations in achromatopsia

Our study demonstrates that mutations in *CNGB3* are the most common cause of achromatopsia in patients of European origin or descent. Of 341 patients in our sample, 163 (47%) carry mutations in *CNGB3*. In contrast, mutations in *CNGA3* account for only 23% of cases in this sample (unpublished results).[8] Mutations in the third gene known to be associated with achromatopsia, *GNAT2*, are even less prevalent and are found in less than 2% of achromatopsia patients.[5,6]

Although there has been considerable progress in the genetic basis of achromatopsia, and the genetic defect can now be defined in almost three of every four patients within our study sample, there still remains a fair proportion of achromats that tested negative for all three known achromatopsia genes. Discordant marker segregation patterns for all three loci in a few families with multiple affecteds (unpublished data) additionally support the existence of at least one further achromatopsia locus in the human genome.

Phenotypic presentation in human

All patients with *CNGB3* mutations initially presented with typical complete achromatopsia characterized by severely reduced visual acuity, pendular nystagmus, photophobia, and loss of color vision. Electrophysiological examination, when possible, regularly showed the absence of cone function. Although typical complete achromatopsia is usually a nonprogressive disease, a preliminary analysis suggests that the symptoms of a few of our patients are consistent with a progressive disease manifesting with decreasing visual acuity and central visual field defects.[13] Another case of progressive cone dystrophy associated with mutations in *CNGB3* has been described by Michaelides and co-workers.[20] However, while complete achromatopsia and progressive cone dystrophies have also been associated with mutations in *CNGA3*, so far incomplete achromatopsia has only been associated with mutations in *CNGA3*.[8,11,21]

Pathophysiology and comparison to animal models

Native cone photoreceptor CNG channels most likely represent heterotetramers of two α- and two β-subunits encoded by *CNGA3* and *CNGB3*, respectively.[22] In heterologous expression systems, the α-subunit is *per se* able to form functional cation channels.[23] In contrast, the β-subunit alone cannot conduct measurable ion currents.

However, it has been demonstrated for the mouse ortholog *cng6* that in coexpression experiments, the *cng6* gene product modulates the biophysical and electrophysiological behavior of the functional channel-forming α-subunit. It induces flickering channel gating, a decrease in outward rectification, and sensitivity to the blocking agent L-*cis*-diltiazem similar to native cone CNG channels.[24] Since mutations in *CNGB3* cause complete achromatopsia, it must be reasoned that the presence of the β-subunit is indispensable for appropriate channel function *in vivo*.

Very recent research has shown that autosomal recessive canine cone degeneration (cd) in the Alaskan malamute and the German shorthaired pointer breeds is caused by mutations in the canine *CNGB3* gene.[25] In the naturally occurring Alaskan malamut, cone-degenerate pups develop dayblindness and photophobia between 8 and 12 weeks postnatal, the age when retinal development is normally completed in dogs. Symptoms are present only in bright light, vision in dim light is normal. Affected dogs remain ophthalmoscopically normal throughout life. Cone function can be detected electroretinographically in very young cd-affected pups (3–6 weeks old), but begins to fail at 6–12 weeks of age and is nonrecordable in mature cd-affected dogs.[26] Adult cd-affected retinas lack all cones. Cones degenerate by extrusion of the nucleus into the inner segment and later displacement of the cone nuclei in the interphotoreceptor space.[27,28] The genetic analysis has shown that in the Alaskan malamute, the complete gene is deleted, while in the German shorthaired pointers, the disease is caused by a missense mutation c.784G>A, resulting in the amino-acid substitution p.D262N (Genbank Accession no. AF490511). This amino-acid residue is located in the second transmembrane domain and is known to be conserved between human and dog CNGB3.[27]

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