Achromatopsia caused by novel mutations in both CNGA3 and CNGB3

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C omplete achromatopsia or rod monochromatism is a stationary cone dystrophy, with an incidence of −1 in 30,000, in which functional cones are absent from the retina. A ffected individuals usually present in infancy with nystagmus, poor visual acuity (6/60–6/36), photophobia, and complete colour blindness. Fundal examination is normal, but electroretinography reveals absent photopic (cone) responses and normal scotopic (rod) responses. Individuals with incomplete achromatopsia retain some colour vision. Achromatopsia is recessively inherited and genetically heterogeneous. To date, three achromatopsia genes have been characterised, the first two described being CNGA3 and CNGB3, located at chromosome positions 2q11 and 8q21 respectively. CNGA3 and CNGB3 respectively code for the α and β subunits of the cGMP gated cation channel in cone cells. Recently, mutations within a third gene, GNAT2, which encodes the α subunit of cone transducin, have also been shown to cause achromatopsia. A high proportion (18/22 kindreds) was found to have a disease associated mutation in either CNGA3 or CNGB3. Five CNGA3 and two CNGB3 mutations were novel. The previously described CNGB3 1148delC was detected in seven of the nine families with CNGB3 mutations.

Studies of patients with achromatopsia have revealed more than 40 disease causing mutations in CNGA3, the majority being missense mutations. In the patient groups studied, four CNGA3 mutations (Arg277Cys, Arg283Trp, Arg436Trp, and Phe547Leu) accounted for 40% of all mutant CNGA3 alleles. In contrast, only eight different mutations of CNGB3 have been identified to date, with six of these being nonsense mutations. The most common mutation is a 1 bp frameshift deletion, 1148delC, which accounts for 84% (199/237) of CNGB3 mutations.

Current estimates suggest that mutations in CNGB3 account for 40–50% of achromatopsia, with mutations in CNGA3 contributing a further 20%. There is therefore a significant proportion of patients for whom neither CNGA3 nor CNGB3 mutations can be found (≈30%). GNAT2 is the third gene to be implicated in achromatopsia, but mutations in this gene are thought to be responsible for <2% of patients affected with this disorder. This suggests the presence of further genetic heterogeneity that may extend to a locus on chromosome 14, which was identified as a result of maternal isodisomy.

Here we report novel mutations in both CNGA3 and CNGB3 in a panel of patients with achromatopsia, which further addresses the question of mutation frequency in the two genes.

PATIENTS AND METHODS
A panel of patients with achromatopsia from 22 different families was ascertained from two British clinical centres. Some of the families were of non-European origin. After informed consent was obtained, blood samples were taken for DNA extraction.

Key points

- Achromatopsia (ACHM) is an autosomal recessive stationary cone dystrophy that occurs in complete and incomplete forms. Individuals with complete ACHM have nystagmus, photophobia, 6/60 vision, and total colour blindness. ACHM is genetically heterogeneous.
- Mutations in the CNGA3 and CNGB3 genes, which encode the alpha and beta subunits of the cone cyclic cGMP channel, a crucial component of phototransduction, cause achromatopsia. In addition, mutations in GNAT2, which encodes cone specific alpha transducin, are a rare cause of achromatopsia.
- We investigated 22 UK achromatopsia families in order to define the CNGA3 and CNGB3 mutation spectrum in this heterogeneous population. The diagnosis was confirmed by ophthalmological examination, electroretinography, and psychophysical assessment.
- A high proportion (18/22 kindreds) was found to have a disease associated mutation in either CNGA3 or CNGB3. Five CNGA3 and two CNGB3 mutations were novel. The previously described CNGB3 1148delC was detected in seven of the nine families with CNGB3 mutations.
- Overall, we have demonstrated in our cohort of complete achromatopsia patients that mutations in CNGA3 and CNGB3 show a similar prevalence, and a high mutation detection rate can be achieved by analysing both genes.

Clinical assessment
A full medical and ophthalmic history was obtained and an ophthalmological examination performed. Patients with clinical findings consistent with a diagnosis of achromatopsia underwent electrophysiological assessment that included a flash electroretinogram (ERG) and pattern ERG, according to the protocols recommended by the International Society for Clinical Electrophysiology of Vision. Psychophysical testing included the use of the American Optical Company, Hardy, Rand, and Rittle plates, Sloan achromatopsia plates, Farnsworth D-15, the Mollon-Reffin (MR) minimal test, a computerised colour vision test, and an anomaloscope. The Sloan achromatopsia plates, Farnsworth D-15, and the

Abbreviations: ERG, electroretinogram; MR, Mollon-Reffin minimal test; SNPs, single nucleotide polymorphisms
MR test were all performed under CIE Standard Illuminant C from a Sol-Source easel lamp (Gretag Macbeth).

Individuals were diagnosed as affected based on the presence of characteristic clinical and psychophysical findings, and electrophysiological evidence of absent or severely reduced photopic ERG, with normal scotopic responses.

**Molecular genetic analysis**

Total genomic DNA was extracted from blood samples using a Nucleon® Biosciences kit. The genomic structure of both CNGA3 and CNGB3 was established using published information and by screening the NCBI high throughput genomic sequences database with coding sequence. The coding sequences of CNGA3 and CNGB3 were amplified by PCR in each individual using primer sequences and conditions as published previously. PCR reactions (50 μl) were performed as follows: 1×NH₄ buffer, 1 mmol/l MgCl₂, 200 μmol/l each dNTP, 10 pmol/l each of sense and antisense primers, 200 ng–1 μg DNA, 1 U BioTaq. After resolution on a 1% LMT agarose gel, products were excised and eluted. Direct sequencing of PCR products was carried out on an ABI 3100 Genetic Analyser using the original PCR primers in the sequencing reactions. The sequence was examined for alterations utilising Sequencing Analysis (ABI Prism™) and GeneWorks™ software.

GenBank sequences were used to construct an alignment of channel protein sequences and to analyse the evolutionary conservation of the corresponding amino acid positions. The sequences used were the cGMP gated (CNG) channel cone α subunits of human (accession number AF065314), mouse (AJ243933), and chicken (X89598) photoreceptors, the rod α subunits of human (S42457) and mouse (XM132106), the cone β subunits of human (AF272900), mouse (NM_013927), and dog (AF490511), and the rod β subunits of human (AF042498) and rat (NM_031809). Sequences were aligned using Clustal W.

**RESULTS**

**Clinical assessment**

At least one affected individual was assessed from each family, and the clinical data obtained are summarised in table 1. All examined patients had complete achromatopsia.
with photophobia, variable degrees of nystagmus, decreased visual acuity (6/60–6/36), and absent photopic ERG with normal rod responses. Affected individuals in whom psychophysical testing was performed displayed no residual colour vision. The unaffected family members who were assessed were found to have normal ophthalmological examination.

Molecular genetics
The eight coding exons of CNGA3 and the 18 coding exons of CNGB3 were screened for mutations in the panel of 22 small families/probands with achromatopsia. All mutations and genetic variants were assigned a nucleotide/amino acid number starting at the first translated base/codon of the CNGA3 and the CNGB3 genes according to GenBank entries NM_001298 and NM_019098, respectively.

CNGA3 mutation analysis
For consistency with previous studies, the numbering of the CNGA3 sequence excludes the 55 amino acids (165 nucleotides) encoded by exon 2b, reported recently.1 Nine different mutations of CNGA3 were found that would appear to be disease associated. Of these, five are new mutations that have not previously been reported: a homozygous Arg23Stop mutation in family 7, a homozygous Gln196Stop mutation in family 8, a homozygous Arg221Stop mutation in family 10, a 1443insC (Ile482fs) frameshift mutation with the previously reported arg569His substitution in family 9, and a homozygous Gly548Arg substitution in family 6 (table 2). For the novel missense mutation (Gly548Arg), 100 control chromosomes were screened for mutations but none was found. Affected individuals in six of the families are homozygous and one family is a compound heterozygote. In families 3 and 12, only one mutation in each was identified. However, these two mutations, Arg223Trp and Arg436Trp, have both been reported previously as disease causing,56 so it is probable that the second mutation was missed in these families by our current mutation screening strategy.

The three novel nonsense mutations that introduce stop codons at Arg23, Gln196, and Arg221 would all generate (if translated and not subjected to nonsense mediated mRNA decay) significantly truncated proteins that would be unlikely to retain any normal function. The frameshift mutation (Ile482fs) would truncate the protein after adding five aberrant amino acids. The novel Gly548Arg is in the cGMP binding domain and is highly conserved across avian cone and mammalian rod and cone CNG channel α subunit proteins (table 3). The locations of these mutations in the α channel protein are shown in fig 1.

A homozygous Thr245Met substitution was found in the affected individual in family 4. Unlike the above mutations however, Thr245 is not conserved across other CNG sequences. Its role in disease pathogenesis is therefore less certain and so it has not been included in the list of novel disease associated mutations.

In addition to the above, three single nucleotide polymorphisms (SNPs) were found, a silent T→C transversion at nucleotide 72 that has been reported previously,3 a silent C→T change at nucleotide 1278, and a G→A change at nucleotide 171 that causes a Met57Ile substitution. This latter change was found as a polymorphism in eight of the families.

CNGB3 mutation analysis
The Thr383 frameshift (1148delC) mutation has been previously identified as the most common disease causing mutation.81 In the present study, this 1 bp deletion was found to segregate with disease status in seven of the nine families with CNGB3 mutations.

Two novel sequence variants in the coding region of CNGB3 were also identified (table 4). A 595delG frameshift mutation (Glu198fs) that results in a truncated polypeptide of 200 amino acids was found as a compound heterozygote in family 1 and a Phe525Asn substitution as a homozygous change in family 13. Phe525 is highly conserved across other CNG receptor subunits (table 5) and the mutation was not found in a screen of 100 control chromosomes. The locations of the novel Glu198fs and Phe525Asn mutations in the β channel protein are shown in fig 1.

An Ile307Val substitution was detected in affected and unaffected individuals in family 19. However, this is likely to represent a disease causing mutation, as it is a very conservative substitution at a site that is not conserved across species (table 5). In fact, Val307 is encoded by the normal canine gene.

In addition, five SNPs were identified. The common 892A→C (Thr298Pro) and 2264A→G (Glu755Gly) polymorphisms have been previously reported.4 New silent polymorphisms are 487T→G, 1752G→A, and 2214A→G.

In family 12 with a heterozygous CNGA3 mutation (Arg436Trp), a single T→G nucleotide change was also found.

Table 3 Amino acid conservation in cone and rod α subunits of CNG channel proteins at sites of missense mutations in CNGA3

<table>
<thead>
<tr>
<th>Residue</th>
<th>Human cone α</th>
<th>Mouse cone α</th>
<th>Human rod α</th>
<th>Mouse rod α</th>
<th>Chicken cone α</th>
</tr>
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<tbody>
<tr>
<td>245</td>
<td>Thr</td>
<td>Lys</td>
<td>Ser</td>
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<td>Gly</td>
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<tr>
<td>448</td>
<td>Gly</td>
<td>Gly</td>
<td>Gly</td>
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<td>Gly</td>
</tr>
</tbody>
</table>

Table 2 CNGA3 disease causing mutations in families with achromatopsia

<table>
<thead>
<tr>
<th>Family</th>
<th>Type</th>
<th>Nucleic acid change</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Hm</td>
<td>67C→T</td>
<td>Arg23Stop</td>
</tr>
<tr>
<td>8</td>
<td>Hm</td>
<td>586C→T</td>
<td>Gln196Stop</td>
</tr>
<tr>
<td>10</td>
<td>Hm</td>
<td>661C→T</td>
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<tr>
<td>3</td>
<td>Ht</td>
<td>607C→T</td>
<td>Arg2315Stop</td>
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<tr>
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<td>Ht</td>
<td>1306C→T</td>
<td>Arg436Trp</td>
</tr>
<tr>
<td>9</td>
<td>Ht</td>
<td>1443insC, 1706G→A</td>
<td>Ile482fs, Arg569His</td>
</tr>
<tr>
<td>15</td>
<td>Hm</td>
<td>1641C→A</td>
<td>Phe547Ileu</td>
</tr>
<tr>
<td>16</td>
<td>Hm</td>
<td>1642G→A</td>
<td>Gly548Arg</td>
</tr>
</tbody>
</table>

Ht, heterozygous; Hm, homozygous. Mutations in bold represent novel changes.

Figure 1 Model of CNG subunit showing the approximate position of the novel CNGA3 and CNGB3 (italic) mutations.
in the 5'UTR of the CNGB3 gene, 36 bp upstream of the ATG start codon. This is present in the heterozygous state in the affected male subject RM24, and was inherited from his mother RM26 (table 1). He also has a second β subunit variant that encodes an Asn27Ser substitution inherited from his father, RM25. As a heterozygous CNGA3 mutation had been already detected in this family, it is probable that both the 5'UTR and the Asn27Ser substitution are polymorphisms. However, in the absence of a second CNGA3 mutation in this family, the possibility remains that the achromatopsia may originate from an interaction between the CNGA3 and CNGB3 mutations.

Following the exclusion of a mutation in both CNGA3 and CNGB3 in family 22, a novel homozygous frameshift mutation in GNAT2 (c842_843insTCAG; M280fsX291) was identified.10

**DISCUSSION**

Mutations in the genes CNGA3 and CNGB3 encoding the α and β subunits of cone photoreceptor CNG channels have been described in subjects with achromatopsia.5,8,11 These proteins are functionally important, therefore, in all three classes of human cones. Moreover, analysis of the homologous CNGA3 knockout mouse model shows complete absence of physiologically measurable cone function, a decrease in the number of cones in the retina, and morphological abnormalities of the remaining cones.22

Unlike previous studies, which indicated that approximately 25% of patients with achromatopsia have alterations in CNGA3 and 40–50% in CNGB3,12,14 we have found a more even distribution of mutations across the two genes; 41% of families with mutations in CNGA3 and 36% with mutations in CNGB3. We failed to find CNG mutations in five families, although two of these had heterozygous substitutions (Thr245Met in CNGA3 and Ile307Val in CNGB3) that are most probably not disease associated, as neither residue is conserved in other CNG receptors. However, final confirmation of the lack of disease association will require a functional assay of the altered protein.

If the family that was subsequently shown to have a homozygous mutation in GNAT214 is included, 23% (5/22) of families did not appear to have a disease causing mutation in either the CNGA3 or CNGB3 gene. This is somewhat lower therefore than the ~30% of patients who did not appear to have either a CNGA3 or CNGB3 mutation in previous investigations,11 and may reflect sampling bias arising from the smaller number of families examined in this study. Alternatively, as some of our families are not of European origin, the difference may reflect a different prevalence of CNGA3 and CNGB3 disease mutations in different populations.

This study has identified five previously unreported CNGA3 mutations and two previously unreported CNGB3 mutations. Of these, one of the CNGA3 mutations and one of the CNGB3 mutations are missense mutations resulting in the replacement of a highly conserved amino acid. The remaining novel mutations result in premature termination of translation and therefore severely truncated polypeptides lacking important functional elements such as the pore or cGMP binding domain.

Homozygous mutations were present in 12 families (which may reflect consanguinity within the families, although data relevant to this were not collected), while compound heterozygous mutations were found in two families. Only single heterozygous mutations could be found in CNGA3 in three families and in CNGB3 in one family; the most likely explanation for this is that the second mutation is in either an intron or promoter region that is outside the regions sequenced, or an as yet unidentified exon. It is also possible that a larger deletion may be present that would not have been detected by our screening strategy.

The majority of CNGA3 mutations identified to date are missense mutations, indicating that there is little tolerance for substitutions with respect to functional and structural maintenance of the channel polypeptide. This notion is supported by the high degree of evolutionary conservation among CNG channel α subunits. By contrast, only one of the previously identified mutations of CNGB3 was a missense mutation, with the others being either stop or frameshift mutations. In the present study, however, one of the two novel mutations is a missense mutation, indicating that functional constraints are as important for the β as for the α subunit, and this is reinforced by the evolutionary conservation of residues at these sites in the β subunit genes of rods and cones. The novel Gly548Arg missense mutation in CNGB3 adds to the large cluster of 15 different mutations in the cGMP binding domain, indicating that an altered binding affinity for cGMP may be responsible for lack of function of the channel.

The Arg436Trp mutation in CNGB3 that is present in the heterozygous state in patient RM24 has been previously found in one case of complete and three cases of incomplete achromatopsia.7 In all instances, the affected individuals were compound heterozygotes, although as for RM24, the second mutation for one of the incomplete achromatopsia was not identified. From this, it is evident that the severity of the disorder may vary depending on the particular combination of mutant alleles.

There is to date a far greater allelic heterogeneity of CNGA3 mutations (over 50 described) compared with CNGB3 mutations (only 10 described). As it is known that CNGA3 subunits can form functional homomeric channels when expressed alone whereas CNGB3 subunits cannot,24 it is possible that mutant β subunits are only deleterious and therefore detected if they interact in an inhibitory fashion.
with wild type α subunits. However, this interpretation is not supported by recent work in which the channel proteins were expressed in Xenopus oocytes. In this system, mutant Thr383fs β subunits do not prevent homomeric α channel formation and function. Additionally, it is also unlikely that homomeric α channels are sufficient for normal channel function in intact cone photoreceptors.

An autosomal recessive canine form of cone degeneration of that occurs naturally in the Alaskan Malamute and German Shorthaired Pointer breeds is phenotypically similar to human achromatopsia. Canine CNGB3 mutations have recently been identified in both of these breeds, thereby establishing these affected dogs as the only naturally occurring large animal model of human achromatopsia. Such models provide a valuable system for exploring disease mechanisms and evaluating the potential for gene therapy.

The three achromatopsia disease genes described to date, CNGA3, CNGB3, and GNAT2, all encode proteins that are restricted to the cone phototransduction cascade. This finding confirms that all three classes of cone photoreceptor utilise a common cone specific cGMP gated channel and a common α transducin subunit. As other components of phototransduction such as the β and γ transducin subunits and the phosphodiesterase subunits are cone specific, it follows that these are also candidates for achromatopsia disease genes.

ACKNOWLEDGEMENTS

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