A missense mutation in the murine Opa3 gene models human Costeff syndrome

Vanessa J. Davies,1 Kate A. Powell,1 Kathryn E. White,2 Wanfen Yip,1 Vanessa Hogan,2 Andrew J. Hollins,1 Jennifer R. Davies,1 Malgorzata Piechota,1 David G. Brownstein,3 Stuart J. Moat,4 Philip P. Nichols,2 Michael A. Wride,1,5 Michael E. Boulton1,6 and Marcela Votruba7

1School of Optometry and Vision Sciences, Cardiff University, Cardiff, 2Neurology, Medical School, Newcastle upon Tyne, 3Research Animal Pathology Core Facility, University of Edinburgh, UK, 4Department of Medical Biochemistry and Immunology, University Hospital of Wales, Cardiff, UK, 5Zoology Department, School of Natural Sciences, University of Dublin Trinity College, Dublin 2, Ireland, 6Department of Ophthalmology and Vision Sciences, University of Texas Medical Branch, Galveston, TX, USA and 7Cardiff Eye Unit, University Hospital Wales, Cardiff, UK

Correspondence to: Marcela Votruba, School of Optometry and Vision Sciences, Cardiff University, Maindy Road, Cathays, Cardiff CF24 4LU, UK
E-mail: votrubam@cardiff.ac.uk

Opa3 mRNA is expressed in all tissues examined to date, but currently the function of the OPA3 protein is unknown. Intriguingly, various mutations in the OPA3 gene lead to two similar diseases in humans: autosomal dominant inherited optic atrophy and cataract (ADOAC) and a metabolic condition; type 3-methylglutaconic aciduria (MGA). Early onset bilateral optic atrophy is a common characteristic of both disorders; retinal ganglion cells are lost and visual acuity is impaired from an early age. In order to investigate the function of the OPA3 protein, we have generated a novel ENU-induced mutant mouse carrying a missense mutation in the OPA3 gene. The heterozygous mutation in exon 2, causes an amino acid change p.L122P (c.365T>C), which is predicted to alter tertiary protein structure. In the heterozygous state, the mice appear uncompromised however; in the homozygous state mice display some of the features of MGA. Visual function is severely reduced, consistent with significant loss of retinal ganglion cells and degeneration of axons in the optic nerve. In the homozygous optic nerve, there was evidence of increased mitochondrial activity, as demonstrated by the increased presence of mitochondrial marker Cytochrome C Oxidase (COX) histochemistry. Mice homozygous for the opa3L122P mutation also display a severe multi-systemic disease characterized by reduced lifespan (majority dying before 4 months), decreased weight, dilated cardiomyopathy, extrapyramidal dysfunction and gross neuro-muscular defects. All of these defects are synonymous with the phenotypic characteristics of Type III MGA found in humans. This model will be of major importance for future studies of the specific function of the OPA3 gene.

Keywords: OPA3; inherited optic atrophy; 3-methylglutaconic aciduria; mouse model

Abbreviations: ADOA = autosomal dominant optic atrophy; ADOAC = autosomal dominant optic atrophy and cataract; CMT4A = Charcot–Marie-Tooth type 4A; CMT2A = Charcot–Marie-Tooth type 2A; COX = cytochrome C oxidase; DAB = 3,3’-diaminobenzidine; ENU = N-ethyl-N-nitrosourea; FRDA = Friedreich’s ataxia; H&E = haematoxylin and eosin; HMSN VI = hereditary motor and sensory neuropathy type VI; INL = inner nuclear layer; IPL = inner plexiform layer; mfn = mitofusin; MGA = type 3-methylglutaric aciduria; MRC = medical research council; MTP = mitochondrial trifunctional protein; NBF = neutral buffered formalin; NF = neurofilament; NFL = nerve fibre layer; MRI = magnetic resonance imaging; OKN = optokinetic drum; ONL = outer nuclear layer; PINK1 = PTEN-induced kinase 1; RGCs = retinal ganglion cells; SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary’s; Royal London Hospital, St Bartholomew’s and the Royal London School of Medicine; Phenotype A ssessment (SHIRPA)

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**Introduction**

Hereditary optic atrophy encompasses a group of disorders with varying modes of inheritance in which optic atrophy is a primary characteristic. Autosomal dominant optic atrophy (ADOA; OMIM 165500; (Votruba et al., 1998a, b)) is the most common of these disorders, and is characterized by progressive loss of visual acuity, central scotoma, colour vision defects, bilateral loss of retinal ganglion cells (RGCs) and ascending atrophy of the optic disc (Kjer, 1959). To date, over 100 mutations in the OPA1 gene, which localizes to chromosome 3q28-q29, have been identified as responsible for ADOA (http://ibbma.univ-angers.fr/eOPA1). More rarely, ADOA has been linked to mutations in the OPA3 gene. In this instance, a blue-dot cerulean cataract accompanies dominantly inherited optic neuropathy [ADOAC; OMIM 165300; (Garcin et al., 1961; Reynier et al., 2004)].

Two dominant missense mutations have been identified in the OPA3 gene: one causes a heterozygous 277G-A transition in exon 2 (c.277G>A) change and the other a heterozygous 313C-G transversion in exon 2 (c.313C>G) (Reynier et al., 2004).

Mutations in the OPA3 gene are also responsible for another syndrome with bilateral optic atrophy as a primary characteristic, that of type III 3-methylglutaconic aciduria (MGA; OMIM 25801; (Anikster et al., 2001)). Two homozygous mutations are reported: G→C change at the -1 position of intron 1 in the 3’ (acceptor) splice site (c.143-1G>C) (Anikster et al., 2001) and the in frame deletion c.320_337del (AGCAGCGCCACAGGAGG) (Kleta et al., 2004). Type III MGA (Costeff syndrome) is one of four disorders that comprise MGA, a rare neuro-ophthalmological syndrome characterized by increased urinary excretion of 3-methylglutaconic acid and 3-methylglutaric acid, early-onset bilateral optic atrophy, pallor of the optic disc, reduced visual acuity, late onset spasticity, extra pyramidal dysfunction and cognitive deficit. Type I MGA is a mild neurological disease that results from a deficiency in 3-methylglutaryl-CoA hydratase in the leucine-oxidation pathway. Type II MGA (Barth syndrome) is an X-linked disorder that consists of dilated cardiomyopathy, short stature and neutropenia. Finally, Type IV MGA is a moderate to severe neurological disease that is associated with cardiac, ophthalmic, hepatic and renal symptoms.

The unique existence of type III MGA in a subset of Iraqi–Jews and its distinct phenotype led to the mapping of the gene responsible for this disorder, OPA3, to chromosome 19q13.2–q13.3 (Nystuen et al., 1997), and the identification of the founder mutation (Anikster et al., 2001). The OPA3 gene contains two exons and encodes for a mitochondrion-related protein of unknown function (Da Cruz et al., 2003). mRNA is ubiquitously expressed throughout the body and in many regions of the brain (Anikster et al., 2001).

The distinct, yet common characteristics shared by MGA type III and ADOAC, and the variable mode of inheritance of these disorders has shed little light on the possible function of the OPA3 protein. Proteomics suggest that OPA3 localizes to the inner membrane of the mitochondria (Da Cruz et al., 2003), while fibroblasts from a patient with ADOAC are particularly susceptible to apoptosis (Reynier et al., 2004). However, little more than this is known about Opa3 protein function, since no abnormalities in the respiratory chain, mitochondrial membrane potential or morphology of the mitochondrial network were identified in these fibroblasts [in contrast to what is known of the consequences of OPA1 haploinsufficiency; (Olichon et al., 2003; Cipolat et al., 2004; Griparić et al., 2004)].

In order to understand the function of the OPA3 protein and its role in disease, we generated a novel mouse model by ENU mutagenesis, carrying a point mutation in the OPA3 gene. Our model contains a missense mutation that is predicted to alter the tertiary structure of the OPA3 protein by creating an amino acid change from leucine to proline at position 122. In the heterozygous state, these mice are indistinguishable from wild-type littermate controls. However, homozygous mutant animals display a severe multi-systemic disease, characterized by retinal abnormality and optic atrophy, reduced lifespan, decreased weight, dilated cardiomyopathy, extra-pyramidal dysfunction and gross neuro-muscular defects. The loss of vision displayed by these mice was consistent with degeneration of axons in the optic nerve. Thus, we have generated a mutant Opa3 murine model that displays a clinical phenotype comparable to Type III MGA.

**Material and Methods**

**Genotype analysis**

Opa3 genotyping was performed using specific primers from mOpa3 exon2 (Forward 5’-GCACGAGATCGCAAATGG-3’ and reverse 5’-GTACAGTGAGCGATTGCAGACC-3’; product size 343 bp).

Cycling conditions were 35 cycles of 1 min denature at 92°C, 1 min anneal at 63°C and 1 min extension at 72°C with a final extension at 72°C for 5 min.

The 343-bp product was digested using Pst1 enzyme (sequence CGAGCA; 10 U/ml) giving a 205-bp product from the wild-type (T) allele and a 247-bp product from the recessive (C) allele (Fig. 1).

**Rd1** genotyping was carried out as an multiplex reaction, with the following three primers in equal combination: RD3: 5’-TG ACAATTACCTCTTTTTTTTCTAGTCTGA-3’, RD4: 5’-GAAAACT GCAAGGCTTATGAGGAACT-3’, and RD6: 5’-TACCCCACTCT TCTTATTTTCAGC-3’ (Gimenez and Montoliu, 2001; Qiao et al., 2003). The wild-type Rd1 allele was 300 bp and the mutant allele was 450 bp (Davies et al., 2007).

**Breeding strategy and embryo analysis**

pa3 and rd1 allele-specific PCR genotyping was used to direct breeding with wild-type C57Bl/6JCr1 mice and the removal of the rd1 allele of pdeB (carried by the CSH paternal line) was thus ensured. Opa31+/− ‘founder’ mice were crossed with C57Bl/6JCr1
using the RNeasy Mini kit (Qiagen, UK). First-strand cDNA synthesis was performed using a cDNA synthesis kit (BioLine) with oligo (dT)$_{18}$ primers. PCR was performed using 20 pmol mOpa3 primers designed to span an intron/exon boundary (forward 5'-GGCGAAGCTTGCTACTGG; reverse 5'-CTGCTGGCTCTCTAGCTGAG) and a Taq polymerase mastermix (BioMix Red; BioLine, UK). Cycling conditions as above, giving a final product of 202-bp.

**Functional visual testing**

**Animals**

Opa3$^{+/+}$ ($n=7–9$), Opa3$^{+/−}$ ($n=12–16$) and Opa3$^{−/−}$ ($n=10–15$) mice were tested at 3–4 months on the SHIRPA primary neurological screen and the optokinetic drum (OKN) task. Founder Opa3$^{+/−}$ ($n=6$) and littermate controls ($n=6$) were also tested at 8 and 12 months of age.

**SHIRPA primary screen and clinical examination of the eye**

Mice were assessed on 36 separate screening tests that form part of the SHIRPA primary protocol (Rogers et al., 1997). This included tests of muscle tone, power and coordination, and hearing, using a click box (90D and 18–20Hz). Behaviours were ranked according to a standard behavioural score (http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_1.html).

Statistical analysis to assess any differences between groups was performed using parametric t-test or non-parametric Mann–Whitney U-test, where appropriate.

Slit lamp biomicroscopy (Haag Streit) was performed on restrained, but non-sedated mice with pupillary dilation (1% atropine MINIMS) in order to examine the anterior segment and lens. Indirect ophthalmoscopy, employing a Volk Super field and 66 D lens, was used to visualize the fundus.

**OKN**

Mice were tested on the optokinetic response test with the use of a rotating OKN. A digital video camera (JVC, GR-D250) linked to a monitor and DVD recorder was used to record movements (http://www.eumorphia.org/) (Thaung et al., 2002; Hart et al., 2005). Briefly, mice were placed on the platform and allowed to settle for 2 mins. The drum was rotated for 1 min and the mice were observed for a head-tracking response. After a 30-s break, the drum was rotated in the opposite direction for 1 min. The mice were assessed on 36 separate screening tests that form part of the SHIRPA primary protocol (Rogers et al., 1997). This included tests of muscle tone, power and coordination, and hearing, using a click box (90D and 18–20Hz). Behaviours were ranked according to a standard behavioural score (http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_1.html).

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**Biochemical Investigations**

Urine samples were obtained from 12-month-old Opa3$^{+/−}$ founders ($n=6$) and littermate controls ($n=6$). Blood samples extracted from 3–4-month-old Opa3$^{+/−}$ ($n=3$), Opa3$^{−/−}$ ($n=3$), Opa3$^{+/−}$ ($n=3$) mice.
Histology

Eyes were enucleated from 3-month-old Opa3+/− (n = 3), Opa3−/− (n = 3) and Opa3+/+ (n = 3) littermate control mice. The eyes were fixed at 4°C in 10% neutral buffered formaldehyde (NBF), dehydrated through a graded ethanol series and embedded in paraffin wax (RA Lamb, UK) for serial sectioning at 7 μm. Coronal sections were taken at 1 in 20 intervals and haematoxylin & cosin (H&E) stained in order to identify the optic nerve. Five retinal sections either side of the optic nerve were then mounted and alternately stained with H&E. Images were taken of each section using a Leica DMIR microscope and a Leica DCS500 camera with Qwin software for manual RGC counts. Individual retinal layer thickness was measured using the Qwin software.

Brain, heart, skeletal muscle, liver, kidney and spleen from 4–5-month-old Opa3+/− (n = 4), Opa3−/− (n = 4) and Opa3+/+ (n = 5) littermate controls were fixed in 10% NBF for wax histology and stained with picric red to demonstrate collagen, or with H&E or Cresyl violet.

Magnetic resonance imaging (MRI)

To examine the fat distribution within the 3-month-old mice, ‘healthy’ Opa3+/− (n = 1), Opa3−/− (n = 1) and Opa3+/− (n = 1) mice with a ‘sick’ Opa3−/− mouse (n = 1) were imaged using a Bruker Biospin Advance 9.4T (400 MHz) MRI system (Ettlingen, Germany). The mice were culled and positioned inside a 35-mm quadrature whole-body mouse coil. For each animal a series of RARE scans with a RARE factor of 1 (55 slices, 0.5-mm thickness) were run in coronal orientation. The weighting of the images was designed to be of proton-density: repetition time: 500 ms; echo time: 12.6 ms, and were imaged with a field of view 7.0 × 3.5 cm² and a matrix size 512 × 256. The images were acquired with and without fat suppression, at the same receiver gain, and reconstituted with the same scaling. The fat distribution images were then created by subtracting the fat suppressed image from the unsuppressed image.

Electron microscopy

Optic nerves, brain and spinal cord from 3-month-old Opa3+/− (n = 3) Opa3−/− (n = 3) and Opa3+/+ (n = 3) mice were fixed in a mixture of 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in epoxy resin. Ultrathin sections from optic nerve, spinal cord and brain corpus callosum were stained with uranyl acetate and lead citrate and examined using a Philips CM100 transmission electron microscope (EM Research Services, Newcastle University). Images (×19 000) were taken of 15 random areas per optic nerve from three Opa3+/− and three Opa3−/− mice. The appearance of the mitochondrial matrix was described as either ‘clear’ or ‘opaque’ depending on intensity of staining (Fig. 7G and H). The number density (Nₜ) of mitochondria in the optic nerve was estimated from the high magnification images (Andrews et al., 2006).

Neurofilament and cytochrome C oxidase (COX) histochemistry

For neurofilament (NF) staining, tissues were fixed in 4% PFA in 0.1M phosphate buffer pH 7.4 for 15 min at 4°C. Endogenous biotin was blocked on sections using a commercially available kit (Vector laboratories, UK). A mouse monoclonal primary antibody to NF-H (Novocastra, UK) was used in conjunction with an animal research kit (DAKO ARKTM kit) to minimize reactivity of anti-mouse secondary antibody with endogenous immunoglobulin. Sections were first incubated with peroxidase block from the DAKO ARKT kit for 5 min, followed by biotinylated primary NF-H antibody in a humidified chamber for 30 min, then with streptavidin-peroxidase reagent from the kit for 30 min in a humidified chamber. Reaction product was visualized using 3,3′-diaminobenzidine (DAB) from the DAKO ARKT kit for 5 min, washed, dehydrated, cleared in Histoclear™ and mounted in DPX™. Images were taken on a digital camera using ImagePro Plus.

For COX histochemistry 10 μm longitudinal serial sections were cut from optic nerve and brain samples taken from 3-month-old Opa3+/− (n = 3) Opa3+/− (n = 3) and Opa3+/+ mice (n = 3). Sections were processed for COX histochemistry to assess cellular enzyme activity (Old and Johnson, 1989) by incubating in a medium containing 4 mM DAB, 100 μM cytochrome c in 0.1M phosphate and catalase for 35 min at 37°C. Images were obtained using a Zeiss Axioiplan microscope with an Axiohc HRc digital camera and axiovision image-capture software. Densitometric measurements were made using Zeiss KS-300 densitometry software in the whole optic nerve from each section stained for COX histochemistry. The densitometry scale is an inverse linear scale ranging from 0 (black) to 255 (white). Higher COX activity is reflected by an increasingly dense, insoluble indamine polymer.

Results

Generation of Opa3 mutant mice: the p.L122P mutation

An ENU mutagenized DNA archive from 10000 C3H male mice was screened for point mutations in Opa3 exons 1 and 2, using heteroduplex analysis by temperature gradient capillary electrophoresis, run at two temperatures: 55–60°C and 60–70°C (Ingenium, SA). Positive fragments were sequenced and three SNPs were found, one of which was a heterozygous missense mutation in exon 2 coding for T to C transition at position 365 in the open reading frame (c.365T>C) (Fig. 1C). This mutation is predicted to cause an amino acid change within the peptide (Leucine 122 to Proline: L122P) in exon 2, which is expected to alter the tertiary protein structure. Sperm were used (IVF with C57BL/6 females) to generate heterozygous hybrid Opa3+/− ‘founders’ (the B6; C3-Opa3p.L122P mouse line). All procedures complied with local ethical, national and international regulatory bodies.

Effects of Opa3+/− mutation on transcription

Opa3 mRNA was present in all tissues examined and its transcription was unaffected by the presence of the T/C mutation (Fig. 1B).

Progeny

Opa3+/− mutant animals are robust and indistinguishable from littermate control Opa3+/+ mice, surviving well into adulthood. They are produced with the expected ratio from
intercross (60%) and outcross (54%) matings (Table 1). In this generation (G1), pregnancies of carrier Opa3+/C0 dams from outcross or inter-cross matings are phenotypically normal and without any complications. However, the majority of Opa3/C0 progeny are compromised from birth. Seventeen per cent die before weaning (3 weeks), with a possible gestational loss; 15% are born against an expected birth rate of 25% (Table 1). Those that survive post-weaning display a very variable phenotype. The majority die within 12–16 weeks, although two animals have survived to 7 months of age. The phenotype of these mice varies, but characteristics include: a ‘runted’ appearance at birth, stunted growth, frail appearance, cranial facial abnormalities (a snubbed snout), splayed gait and reduced activity (Fig. 2). Those Opa3/C0 mice that appear ‘normal’ at birth have a shorter life span than littermate Opa3+/+ and Opa3+/+ mice. Once Opa3/C0 mice become compromised they display piloerection, pronounced tremor, hunched physique, reduced body fat and have limited movement due to abnormal gait (Fig. 2). Animals that initially appear to be healthy deteriorate rapidly and often die within 7 days. The cause of death is not obvious, occurring suddenly in the absence of any preceding illness, undue stress or fasting. In the majority of instances, we were unable to investigate the progeny of Opa3/C0 inter-cross or outcross matings due to the frail state of the Opa3/C0 females. Two Opa3/C0 inter-crosses were set up, but pregnancy was never established, despite an extensive period allowed for mating.

Neurological defects and functional visual anomalies
A basic neurological examination of the Opa3+/+, Opa3+/− and Opa3−/− mice was performed at 3–4 months of age to assess the extent of the behavioural phenotype before testing the mice for visual function. Founder Opa3+/+ and littermate Opa3+/+ mice were tested at 8 months of age, to assess whether ageing compromises the Opa3−/− mice further. Due to deteriorating health and declining numbers, we were unable to test the Opa3−/− mice beyond 4 months of age.

Slit lamp biomicroscopy of the lens and anterior segment and dilated fundal examination by indirect microscopy in restrained, non-sedated mice was performed on all animals in the study. No evidence of cataract was seen by 8–12 months. No gross retinal anomalies were detected, within the limits of resolution possible with these techniques. The optic nerve appeared to be normal; however, its shape and size were not quantified and are particularly difficult to evaluate in mice, due to their small size.

Primary SHIRPA screen
The primary SHIRPA screen allows observational assessment of 36 different general health and neurological measures (Rogers et al., 1997; http://www.eumorphia.org). In the 3–4-month-old Opa3−/− mice, there was no significant effect of genotype on 35 of the separate behavioural measures. However, there was a significant

| genotype | mean litter size | survival at weaning
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Opa3+/− × Opa3+/−</td>
<td>8.26</td>
<td>84</td>
</tr>
<tr>
<td>Opa3+/− × Opa3+/−</td>
<td>8.0</td>
<td>93</td>
</tr>
<tr>
<td>Opa3+/− progeny, n (%)</td>
<td>25 (25)</td>
<td>46 (50)</td>
</tr>
<tr>
<td>Opa3+/− progeny, n (%)</td>
<td>60 (50)</td>
<td>54 (50)</td>
</tr>
<tr>
<td>Opa3−/− progeny, n (%)</td>
<td>15 (25)</td>
<td>—</td>
</tr>
</tbody>
</table>

Obtained and predicted (bold, in parentheses) percentages of genotypes from outcross (n = 40) and inter-cross (n = 256) mouse matings.
Table 2 Primary SHIRPA screen of wild-type and homozygous mutant mice

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Opa3+/+</th>
<th>Opa3+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body position</td>
<td>Rearing on hind legs</td>
<td>Sitting or standing</td>
</tr>
<tr>
<td>Spontaneous activity</td>
<td>Vigorous scratch/groom, moderate movement</td>
<td>Casual scratch/groom, slow movement</td>
</tr>
<tr>
<td>Tremor</td>
<td>None</td>
<td>Mild</td>
</tr>
<tr>
<td>Transfer arousal</td>
<td>Momentary freeze, then swift movement</td>
<td>Prolonged freeze, then slight movement</td>
</tr>
<tr>
<td>Piloerection</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Startle response</td>
<td>Preyer reflex</td>
<td>Jump ~1 cm</td>
</tr>
<tr>
<td>Gait</td>
<td>Normal</td>
<td>Fluid but abnormal</td>
</tr>
<tr>
<td>Tail elevation</td>
<td>Horizontally extended</td>
<td>Dragging</td>
</tr>
<tr>
<td>Visual placing</td>
<td>Before vibrasse contact</td>
<td>Upon vibrasse contact</td>
</tr>
<tr>
<td>Toe pinch</td>
<td>Brisk, rapid withdrawal</td>
<td>None</td>
</tr>
<tr>
<td>Provoked biting</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Aggression</td>
<td>Provoked biting or attack</td>
<td>None</td>
</tr>
</tbody>
</table>

Deficits recorded in SHIRPA primary observation assessment for 3- to 4-month-old Opa3+/+ controls (n = 10), and Opa3+/- mutants (n = 9). The median ranked behavioural score is given, together with its description in the SHIRPA primary screen.

The effect of genotype on transfer arousal from the viewing jar to the open field arena $U(12,10) = 31.00, P < 0.05$. The Opa3+/+ mice moved more actively in the open field arena (median 4.5, interquartile range 4, 5) compared with Opa3+/- mice (median 3.5, interquartile range 3–4).

In the 3–4-month-old Opa3+/- mice, there was a significant effect of genotype on 14 of the 36 behavioural measures compared with Opa3+/+ littermates (Table 2, Fig. 3). Opa3+/- mice weighed less ($t = -4.336, P < 0.001$; Fig. 3A) and were less active ($t = -3.636, P < 0.005$; Fig. 3B) than Opa3+/+ mice. In the viewing jar, Opa3+/- mice were significantly different to Opa3+/+ mice with respect to their body position: $U(9, 10) = 13, P < 0.005$, spontaneous activity: $U(9, 10) = 14.00, P < 0.01$ and tremor: $U(9, 10) = 10.00, P < 0.05$ (Table 2 for description). In the arena, the Opa3+/- mice were significantly different from Opa3+/+ mice with respect to five measures: transfer arousal from the viewing jar to the arena: $U(9, 10) = 8.5, P < 0.005$; startle response tested by the MRC standard click box: $U(9, 10) = 18.00, P < 0.05$; gait: $U(9, 10) = 5.0, P < 0.001$; tail elevation: $U(9, 10) = 25.0, P < 0.05$ and piloerection: $U(9, 10) = 25, P < 0.05$. For behaviours measured outside of the arena, there were significant differences between the Opa3+/- and Opa3+/+ mice with respect to four measures: visual placing: $U(9, 10) = 18.00, P < 0.05$; toe response: $U(9, 10) = 17.5, P < 0.05$; provoked biting: $U(9, 10) = 22.5, P < 0.03$ and aggression: $U(9, 10) = 23.0, P < 0.05$. Results from this basic neurological screen suggest that Opa3+/- mice might have compromised aspects of muscle and lower motoneuron function, spinocerebellar function, sensory function, autonomic function and neuropsychiatric function (Rogers et al., 1997). Further testing is required to define the specific nature of these deficits. Importantly, locomotion and visual function appear to be compromised in the Opa3+/- mice.

It was impossible to examine the Opa3+/- mice beyond 4 months of age using the primary SHIRPA screen. However Opa3+/- founder mice were tested at 8 months of age and were found to be significantly different to the Opa3+/+
littermates only with respect to the pinna reflex: \( U(6, 6) = 3.0, P < 0.005 \). Therefore, at 8 months of age, the \( \text{Opa3}^{+/−} \) mice remain relatively indistinguishable from littermate controls.

**OKN test**

One of the key clinical characteristics of ADOAC and Type III MGA is reduced visual acuity. For this reason, and given the impaired visual placing phenotype identified in the \( \text{Opa3}^{+/−} \) mice on the primary SHIRPA screen, we examined the visual acuity of 3–4-month-old \( \text{Opa3}^{+/−} \) mice. The \( \text{Opa3}^{+/−} \) founder mice were tested at 8 and 12 months of age to assess any age-related deterioration in visual function of carrier mice. Sighted C57Bl/6JCrI mice were capable of tracking the moving acuity square wave grating at all frequencies (defined as at least one head tracking movement in the same direction and speed of the drum (Thaung et al., 2002)). Non-sighted C3H mice failed to track any of the gratings, confirming the validity and sensitivity of our test data [data not shown; (Hart et al., 2005)].

\( \text{Opa3}^{+/−} \) and \( \text{Opa3}^{+/+} \) mice at 3–4 months of age displayed normal visual function by tracking the 2° grating, consistent with the published literature [Fig. 3; (Thaung et al., 2002)]. However, only 1 out of 7 (14%) of the \( \text{Opa3}^{+/−} \) mice tested tracked the 2° grating (Fig. 3C). If a mouse failed to track any grating, it was tested on the same grating on a separate occasion (http://www.eumorphia.org/). Non-responding \( \text{Opa3}^{+/−} \) mice were tested on the 4° grating; two out of the remaining six (33%) mice tracked this grating, indicating reduced visual acuity. Finally, one of the four (25%) remaining non-responding \( \text{Opa3}^{+/−} \) mice tracked the larger 8° grating. Therefore, three of the seven \( \text{Opa3}^{+/−} \) mice failed to track the 2°, 4° and 8° grating, indicating that the \( \text{Opa3}^{+/−} \) mice have no or limited visual function.

Older \( \text{Opa3}^{+/−} \) founder mice were tested at 8 and 12 months of age using the 2° grating (Fig. 3D and E). No difference was recorded in terms of the ability to track, or time spent tracking the grating over a 2-min period between the \( \text{Opa3}^{+/−} \) and \( \text{Opa3}^{+/+} \) mice at either 8 months \( t = 1.378 \), \( P > 0.05 \) or 12 months of age \( t = 10.570, P > 0.05 \). Therefore, the \( \text{Opa3}^{+/−} \) mice are not functionally blind.

**Biochemistry**

Increased urinary excretion of 3-methylglutaconic acid and 3-methylglutaric acid is diagnostic of type III MGA. We were unable to obtain urine from \( \text{Opa3}^{+/−} \) mice owing to their poor health. \( \text{Opa3}^{+/−} \) mice became extremely dehydrated despite being observed to drink normally, and we were unable to devise any strategy that would enable us to collect urine. As an alternative, blood was collected for analysis of acylcarnitines by tandem mass spectrometry, to determine if organic acids could be detected; but using this method we were unable to detect any differences between the three phenotypes. However, we were able to test the urine of 12-month-old founder \( \text{Opa3}^{+/−} \), but no differences were observed between these and littermate \( \text{Opa3}^{+/+} \) mice.

**General histology**

In \( \text{Opa3}^{+/−} \) mice, the liver was lipid/glycogen depleted. A marked cardiomyopathy was also observed and the left and right ventricular chambers were dilated (Fig. 4H). Both the interventricular septum and left ventricular free wall were thinned (Fig. 4H). The left and right ventricular myocardium had diffuse changes consisting of matrix expansion, inducing interstitial fibrosis, cardiac muscle fibre deposits, atrophy and hyperatrophy of residual muscle fibres, as well as vacuolation of muscle fibres (Fig. 4K). Such severity of cardiomyopathy would explain the observed dramatic deterioration of health and is a likely cause of death.

No gross structural abnormalities were observed between the \( \text{Opa3}^{+/+} \), \( \text{Opa3}^{+/−} \) or \( \text{Opa3}^{−/−} \) mutant mice in the skeletal muscle, spleen or brain. Kidneys also appeared to be normal by H&E histology despite the dehydrated state of \( \text{Opa3}^{−/−} \) mice; however, this analysis will only detect gross deficits.

**MRI**

Compromised \( \text{Opa3}^{−/−} \) mice were shorter and contained less fat in the body cavity than other mice (Fig. 5). Changes in length and body fat were observed between compromised \( \text{Opa3}^{−/−} \), \( \text{Opa3}^{+/−} \) and \( \text{Opa3}^{+/+} \) mice; however no further differences were identified. In a healthy state, the \( \text{Opa3}^{−/−} \) mice appear indistinguishable from their \( \text{Opa3}^{+/+} \) and \( \text{Opa3}^{+/−} \) littermates (Fig. 5).

**Retinal histology**

A marked reduction in retinal ganglion cells (RGCs) was apparent in the RGC layer of \( \text{Opa3}^{−/−} \) retina (Fig. 6A and D). At 3–4 months of age RGC counts from H&E stained retinal sections were statistically different between \( \text{Opa3}^{−/−} \) and \( \text{Opa3}^{+/+} \) mice \( t = −4.806, P < 0.01 \); Fig. 6A). Furthermore, there was a considerable difference between the thicknesses of the \( \text{Opa3}^{−/−} \) compared to \( \text{Opa3}^{+/−} \) retinal layer (Fig. 6E–H). The whole retina, measured from retinal pigment epithelium to nerve fibre layer (NFL), was significantly thinner, with most marked differences in the inner plexiform layer (IPL), outer nuclear layer (ONL), combined RGC and NFL (Fig. 6E–H).

Retinal morphology appeared normal in the 3- to 4-month-old \( \text{Opa3}^{+/−} \) mice (Fig. 6C) when compared to \( \text{Opa3}^{+/+} \) littermates.

**Electron microscopy and neurofilament immunostaining**

Degenerating axons were observed in spinal cord, brain and, most abundantly, in optic nerve of \( \text{Opa3}^{−/−} \)
mice (Fig. 7). This type of degenerating axon is referred to as ‘dark’ degeneration and may be a consequence of neurofilament aggregation. To address this, neurofilament staining, using NF-H, a standard neurofilament marker, was performed on homozygous optic nerve and an increase in staining was observed (Fig. 8).

Mitochondria with either clear or opaquely stained matrix were found in both wild-type and homozygous optic nerves (Fig. 7G and H). The proportion of opaque mitochondria in the Opa3<sup>−/−</sup> mice was significantly higher.

Fig. 4 Histopathology of 3- to 4-month homozygote Opa3<sup>−/−</sup> compared with Opa3<sup>+/+</sup> controls. (A) and (B) show the anterior surface of Opa3<sup>+/+</sup> hearts, (C–E) of Opa3<sup>−/−</sup> hearts. Sections from Opa3<sup>−/−</sup> hearts display reduced ventricular mass and atrial enlargement. Panels (F–I) are transverse sections through the base of the ventricles. (F) and (H) show H&E stained sections from Opa3<sup>+/+</sup> (F) and Opa3<sup>−/−</sup> (H) mice. Panels (G) and (I) show sections stained with picro-sirius red for collagen from Opa3<sup>+/+</sup> (G) and Opa3<sup>−/−</sup> (I) mice respectively. The ventricular cavities of Opa3<sup>−/−</sup> mice are dilated and there is an increase in picro-sirius red staining in the myocardium of both chambers. Panels (J) and (K) are H&E stained sections showing septal myocardium from (J) Opa3<sup>+/+</sup> and (K) Opa3<sup>−/−</sup> animals. Panel (K) also demonstrates matrix expansion, cardiac muscle fibre dropout and vacuolation and hypertrophy of residual cardiac muscle fibres. Insets in (J) and (K) are of sections stained using picro-sirius red to demonstrate collagen in the expanded matrix.

Fig. 5 Magnetic resonance images displaying the variation in abdominal body fat distribution in (A) Opa3<sup>+/+</sup>, (B) ‘healthy’ Opa3<sup>−/−</sup>, (C) Opa3<sup>+/−</sup> and (D) ‘sick’ Opa3<sup>−/−</sup> mice. The ‘sick’ Opa3<sup>−/−</sup> mouse has considerably less fat compared to ‘healthy’ Opa3<sup>−/−</sup>, Opa3<sup>+/−</sup> or Opa3<sup>+/+</sup> animals. Arrows show the position of abdominal fat in panels (A–C). Arrow in (D) shows the absence of an abdominal fat pad in compromised Opa3<sup>−/−</sup> animals.
than in Opa3+/+ mice (mean ± SD: 44 ± 4 versus 24 ± 8, \(P = 0.021\)); however, there was no significant difference in mitochondrial number density (0.99 ± 0.4 versus 0.97 ± 0.3 \(\mu\)m\(^{-3}\)).

No significant abnormalities were observed by electron microscopy in the optic nerve, brain and spinal cord from 3-month-old Opa3+/+ and Opa3+/+ mice.

**COX histochemistry**

Mitochondrial activity was increased in the optic nerve of Opa3−/− mice compared with Opa3+/+ mice (mean density 191.97 ± 5.74 versus 225.46 ± 2.4, \(P < 0.001\); Fig. 8C and D) assessed by COX histochemistry (Old and Johnson, 1989). Opa3−/− brain, which displayed evidence of degenerating axons by EM, showed no difference in COX activity in the corpus callosum compared to Opa3+/+ mice.

**Discussion**

In this study, we describe for the first time the generation and characterization of a novel ENU-induced mutant mouse with a clinical phenotype reminiscent of MGA Type III and carrying a mutation in the opa3 gene. In the heterozygote state, the mutant mice (Opa3+/−), are clinically and pathologically indistinguishable from...
littermate controls. However, in the homozygote state (Opa3+/−) the mutant mice are severely compromised. These mice are runted, display a marked tremor and piloerection, and have less core body fat. They present with a splayed gait and limited movement. Their reduced visual acuity is consistent with the thinning of various retinal layers and a significant reduction in RGCs, extending to a distinct degeneration of axons within the optic nerve. The majority of these mutant mice fail to live beyond 4 months of age, possibly as a direct consequence of cardiomyopathy. The phenotype of the homozygous mice is variable and, in a few instances, these mice are identical in appearance to their littermates at weaning; however, their health status declines rapidly thereafter.

Mutations in the OPA3 gene cause two distinct diseases in humans, which share some common characteristics. Similar to the clinical phenotype of ADOAC (5) and Type III MGA (Anikster et al., 2001), our Opa3+/− mice lose retinal ganglion cells leading to optic atrophy and reduced visual acuity. However, the detrimental mouse phenotype is only observed in the recessive state, and thus is not a
consequence of a dominantly inherited missense mutation, as is the case for ADOAC. Furthermore, there is no evidence of any type of cataract, a key characteristic of ADOAC (Reynier et al., 2004) in the homozygote mice up to 4 months of age or in the heterozygote mutant mice up to 12 months of age; although we cannot exclude the possibility that cataract would develop with age thereafter. Moreover, our Opa3$^{+/−}$ mutant mice do develop many of the other clinical features of MGA Type III (Anikster et al., 2001; Kleta et al., 2002). Through the SHIRPA neurological screen we observed symptoms that could be interpreted as spasticity (due to their abnormal gait and locomotion), extrapyramidal dysfunction (due to their reduced and inability to initiate movement) and ataxia (due to their splayed gait and unsteady motion of hind limbs). In addition, this phenotype is apparent in the recessive progeny, since their heterozygous parents are unaffected. These results indicate that the mode of inheritance is analogous to MGA Type III.

The missense mutation we have modelled is located in exon 2. The two mutations reported in ADOAC c.277G$⇒$A and c.313C$⇒$G are also located in exon 2 and may have a dominant negative effect causing a phenotype in the heterozygous state (Reynier et al., 2004). The homozygous mutations that are causal in Type III MGA either abolish OPA3 mRNA expression through a splice site mutation (Anikster et al., 2001), or cause a homozygous 18-bp deletion in exon 2 of the OPA3 gene, resulting in deletion of six amino acids between codons 108 and 113 (Kleta et al., 2002), both of which result in loss of function. The effect of the p.L122P missense mutation on OPA3 protein, introducing a proline residue, would be to alter the protein’s tertiary structure causing disruption to its α-helix and β-sheet conformation (Silvestri et al., 2005). This could have far reaching consequences for the function of the protein.

OPA3 mRNA is widely expressed throughout the body; most abundantly in the skeletal muscle, kidney and brain. The multi-systemic phenotype displayed by our Opa3$^{+/−}$ mice is consistent with this expression. Results from this and other studies may implicate a function related to mitochondrial activity. Mitochondrial disorders affect high-energy consuming structures such as the basal ganglia, cerebellum, neurons and primary retinal ganglion cells, causing ataxia and optic atrophy (Kaplan, 2002; Chen and Chan, 2006), consistent with the phenotype of our Opa3 mice. Many of their phenotypic characteristics and mode of inheritance are shared by other diseases caused by mutations that code for mitochondrial-related proteins; for example: ADOA (Alexander et al., 2000; Delettre et al., 2000), Charcot–Marie–Tooth disease type 2A (CMT2A; Zuchner et al., 2006), type 4A (Pedrola et al., 2005) Friedreich’s ataxia (FRDA; Puccio and Koenig, 2000) and hereditary motor and sensory neuropathy type VI (HMSN V1; Zuchner et al., 2004). Furthermore, it is known that OPA3 contains a mitochondrial targeting sequence (Anikster et al., 2001) and localizes to the inner mitochondrial membrane (Da Cruz et al., 2003). This allows us to speculate that the OPA3 protein either belongs to the family of proteins involved in mitochondrial homeostasis, or it is involved in one of the many mitochondrial pathways. Future studies into the subcellular localization of the OPA3 protein will provide us with a greater insight into the function of the protein.

The major characteristic of this mouse model is the loss of cells in all retinal layers, in particular the significant loss in the RGC layer, leading to optic atrophy and resulting in reduced visual acuity. From this, we are able to speculate that in the mouse, OPA3 is expressed in the optic nerve, and the various layers of the retina, in particular the RGC layer, and that it is important in either retinal development or maintenance. Further work will be needed to distinguish between these two mechanisms and to characterize the developmental expression profile of Opa3 in the retina.

Mitochondria are concentrated in the non-myelinated parts of optic nerve axon, optic nerve head and just past the laminar cribrosa, as well as at the nodes of Ranvier. Axonal transport is an ATP-dependent process, mitochondria being the major source of this ATP (Carelli et al., 2002), thereby providing an explanation for increased mitochondrial activity in the unmyelinated axons (Barron et al., 2004). In our Opa3$^{+/−}$ mice we observed increased mitochondrial activity in the post-laminar myelinated region of the optic nerve, demonstrated by increased COX histochemistry. This was not because of demyelination or increased numbers of mitochondria, as neither was observed, but might be a direct consequence of inefficient mitochondria increasing their activity to meet energy demand following the degeneration of optic nerve axons. Adaptive changes involving the mitochondria take place in the optic nerve axon; in the Shiverer mouse, dysmyelination results in a higher number of mitochondria within the axon in response to the lack of myelin (Andrews et al., 2006). This increased energy requirement also promotes the production of reactive oxygen species in mitochondria, which damage the axons further (Andrews et al., 2006). There is extensive evidence of degenerating axons in our Opa3$^{+/−}$ mice, consistent with the consequences of increased mitochondrial activity. However, consistent with the published literature (Gallyas et al., 2006) the myelin sheaths surrounding these degenerating axons remained unchanged. This type of degeneration has been classified as ‘dark’ degeneration; the axons contain a dense axoplasm (Marques et al., 2003). In our Opa3$^{+/−}$ model, the dark degenerating axons coincided with increased NF staining, in accord with NF proteins participating in the process of axonal dark degeneration (Marques et al., 2003).

The cause of early death in our Opa3$^{+/−}$ mice is not immediately apparent; they die suddenly in the absence of preceding illness, undue stress or fasting. If the OPA3 protein is linked with mitochondrial function or processes, mitochondrial dysfunction may be the cause of their
lethality. Cardiomyopathy could be the primary cause of their death, or it could be a secondary consequence of either mitochondrial dysfunction or multiple organ failure. At the time of illness and death, our Opa3<sup>-/-</sup> mice are severely dehydrated (making urine collection impossible) and have a near complete absence of internal body fat, despite the kidneys appearing normal by histological analysis and being witnessed drinking and eating normally.

In summary, we describe a novel mutant mouse B6; C3–Opa3<sup>L122P</sup> which displays bilateral optic atrophy, reduced visual acuity, extrapyramidal dysfunction, spasticity, ataxia and a shortened life span. This novel mouse will be a valuable tool, providing a means to investigate directly the functional role of Opa3 in vivo and the pathophysiology of both Type III MGA.

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