Opa1 is essential for retinal ganglion cell synaptic architecture and connectivity

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Retinal ganglion cell dendritic pruning has been reported in association with a 50% reduction in Opa1 transcript and protein in retinal and neural tissue, which manifests as visual dysfunction in the heterozygous mutant mouse, B6;C3-Opa1Q285STOP. Here we report a marked reduction in retinal ganglion cell synaptic connectivity in the absence of soma loss and explore the mechanism and relationship between mitochondrial integrity and synaptic connectivity. We observed decreased levels of postsynaptic density protein 95 in Opa1+/− mutant mice consistent with synaptic loss in the inner plexiform layer. Glutamatergic but not γ-aminobutyric acid-ergic synaptic sites were reduced in Opa1+/− mice. We observed increased synaptic vesicle number in bipolar cell terminal arbours assessed by immunohistochemistry, electron microscopy and western blot analysis. These changes occur without significant loss of mitochondrial membrane potential in retina and optic nerve. Analysis of biolistically transfected retinal ganglion cells shows the retraction of mitochondria towards the soma, and mitochondrial fragmentation, preceding dendritic loss. These processes cast light on the intimate relationship between normal mitochondrial fusion and fission balances, as influenced by the OPA1 protein, in neural cell connectivity in the mammalian retina.

Keywords: Opa1; retinal ganglion cell; synapse; mitochondria; connectivity

Abbreviations: GABA = γ-aminobutyric acid

Introduction

Neurons live on a metabolic knife-edge with their viability reliant on an energy supply limited by mitochondrial function (Li et al., 2004; Chen and Chan, 2006; Mattson and Magnus, 2006). Many factors can influence the mitochondrial efficiency, in particular, the integrity of critical mitochondrial shaping proteins: Mfn1, Mfn2 and Opa1 (mitochondrial fusion) (Olichon et al., 2002, 2006; Santel et al., 2003; Davies and Votruba, 2006); and Drp1 and Fis1 (mitochondrial fission) (Mozdy et al., 2000; Frank et al., 2001; Liesa et al., 2009). In humans, the OPA1 protein is coded for by the OPA1 gene, which is mutated in patients with the disease autosomal dominant optic atrophy (Olichon et al., 2006). Autosomal dominant optic atrophy is the most common inherited optic neuropathy, with an estimated prevalence of 1:12,000, although this may be as high as 1:10,000 in certain populations (Carelli et al., 2002; Delettre et al., 2002). Autosomal dominant optic atrophy typically presents in the first
decade of life as bilateral visual loss with pallor of the optic disc, centrocaecal visual field scotoma and strabismus (Votruba et al., 1998; Yu-Wai-Man et al., 2011). Histological assessment of eyes from a limited number of aged and severely affected subjects shows thinning of the retinal ganglion cell layer (Kjer et al., 1983). Demyelination has also been observed in the optic nerve, chiasm and tract in histological assessments of patient donor eyes (Kjer et al., 1983; Milea et al., 2010). OPA1 is ubiquitously expressed and is essential for life (homozygous Opa1−/− mice die at embryonic Day 8.5) (Davies et al., 2007; Williams et al., 2011), however, little is known about the mechanisms that precede or underlie optic atrophy in those with mutations in OPA1 (>200 mutations reported) (Ferré et al., 2005; Amati-Bonneau et al., 2009).

The putative role of OPA1 and disrupted mitochondrial fusion in the pathophysiology of autosomal dominant optic atrophy is not a unique association; mitochondrial dysfunction is increasingly recognized as a key contributor to neuronal dysfunction and loss in classic neurodegenerative diseases such as Alzheimer's (Wang et al., 2008), Huntington's (Kieper et al., 2010) and Parkinson's disease (Abou-Sleiman et al., 2006). In these disease states, the rate of neuronal loss is slow and accompanied by prolonged periods of neural dysfunction in the absence of demonstrable neuronal loss and atrophy. The critical dependence of neurons on mitochondria leaves them highly susceptible to any changes in energy stasis (Mattson and Magnus, 2006). It should be noted that autosomal dominant optic atrophy is not the only mitochondrial disease that specifically affects retinal ganglion cells. Leber's hereditary optic neuropathy, caused by point mutations in mitochondrial DNA affecting subunits of complex 1, also leads to a specific degeneration of retinal ganglion cells (Carelli et al., 2009).

But why do retinal ganglion cells show a specific vulnerability to mitochondrial dysfunction? The retinal ganglion cell population is uniquely challenged in the CNS as retinal ganglion cell axons only myelinate when they exit the eye and enter the optic nerve, with lengthy sections of unmyelinated axon residing within the retina (up to 2000 μm for retinal ganglion cells in the periphery of the mouse retina). This, as well as their large dendritic field, places a high metabolic demand on the retinal ganglion cell, which manifests as a large number of mitochondria within the dendrite and axon. Mitochondrial structure, number and their distribution within the axon, dendrite and synapse are thus integral to neuronal physiological function and health (Selkoe, 2002; Mattson et al., 2008; Cheng et al., 2010; Cho et al., 2010).

Since mitochondria underpin neuronal health we reasoned that OPA1 mutations might manifest, at least in the early stages of disease, as changes in mitochondrial structure and location within the dendrite and the architecture of synaptic sites leading to reduced connectivity of retinal ganglion cells.

Here we explore the subcellular changes underlying our previously documented retinal ganglion cell dendropathy associated with Opa1 deficit. We investigate the relationship between the dendritic distribution of synapses, associated mitochondrial architecture, number and distribution and loss of synaptic connectivity.

Materials and methods

Animals and genotyping

Mice were kept in a 12-h light (10lx)/dark cycle with food and water available ad libitum. Breeding and all experimental procedures were undertaken in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and approved by ethical and legal authorities. We have described the mutant strain in detail elsewhere (B6; C3-Opa1Q285STOP) (Davies et al., 2007). All animals were out-crossed to a C57Bl6 background (F1) and the experiments were performed on mice bred to generations F4–F5. Animals were genotyped by Opa1 allele-specific polymerase chain reaction (Davies et al., 2007).

RNA purification and complementary DNA synthesis

Tissues (retina, kidney, liver and spleen) were dissected from wild-type and Opa1+/− animals and RNA was extracted using the RNeasy® Mini Kit (Qiagen). Two retinas were pooled into one sample. RNA quantification was carried out using Spectrophotometer U-2800 equipped with UV solutions software.

For complementary DNA synthesis, 200 ng of total RNA was mixed with 1 μl (270 ng) oligo (d)T18 primers and 1 μl dNTP mix: dATP, dGTP, dCTP and dTTP, 10 mM each (Bioline) in a total volume of 13 μl. The mixture was heated to 65°C for 5 min and incubated on ice for 1 min. Afterwards 4 μl 5 × First Strand Buffer (Invitrogen), 1 μl 0.1 M dithiothreitol (Invitrogen), 1 μl RNase Inhibitor 100 U/μl (Bioline) and 1 μl SuperScript® III room temperature 200 U/μl (Invitrogen) were added to each tube followed by 1 h incubation at 50°C. The enzyme was inactivated by heating at 70°C for 15 min.

Reverse transcriptase–polymerase chain reaction of Opa1 transcript isoforms

Primers were designed using Primer3 software. Polymerase chain reaction amplification was performed on a TC-512 Thermo Thermal Cycler (Techne Inc.) in a 25-μl reaction mixture containing 2 μl complementary DNA, 1 μM forward and reverse primers and 12.5 μl 2× BioMixRed (Bioline). The polymerase chain reaction conditions were identical for all designed primers: initial denaturation for 5 min at 94°C; 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 54°C, elongation for 1 min at 72°C and final elongation for 10 min at 72°C. Product was run out on 1–2% agarose gels.

Isolation of mitochondria from mouse tissue

Mitochondria were isolated from freshly excised mouse tissue (120 mg brain, six whole retinas and optic nerves attached) using Mitochondria Isolation Kit (Sigma). All steps were performed on ice. Samples were centrifuged at 4°C. Mitochondria in the pellet were suspended in 80 μl (brain) or 10 μl (retina) of Storage Buffer (Sigma). Mitochondrial protein concentration was quantified using UV spectrophotometer (Picodrop Limited). Mitochondria were aliquoted and stored at −80°C. The mitochondrial fraction was probed by cytochrome c on a western blot, to confirm the presence of a single band at 15 kDa (data not shown).
Mitochondrial membrane potential measurement

Membrane potential was measured in isolated mitochondria by JC-1 staining and analysis of JC-1 dye fluorescence at 595 nm in an F-4500 fluorescence spectrophotometer (Hitachi High Technologies). The excitation wavelength was set at 490 nm. Assays were performed in a quartz cuvette with a 10-mm optical pathway. Fifty micrograms of mitochondrial proteins were suspended in a Storage Buffer (Sigma) and added to 1.9 ml JC-1 Assay Buffer (Sigma) to total volume of 2 ml. After addition of 2 μl JC-1 stain (Sigma), the sample was mixed by inversion and left at room temperature in the dark for 7 min to allow complete uptake of JC-1 dye into the mitochondria. The blank fluorescence measured in a cuvette with Assay Buffer lacking mitochondrial fraction was subtracted from the fluorescence of each sample.

Electron microscopy

Wild-type \((n = 6)\) and \(\text{Opa1}^{+/−}\) \((n = 6)\) mice were killed by cervical dislocation and their whole retinas fixed in 1% glutaraldehyde in Sorensen’s phosphate buffer. Retinas were post-fixed in 1% osmium tetroxide for 2 h, washed, dehydrated through graded ethanol (50, 70, 90 and 100% for 15 min each) followed by three exchanges of propylene oxide for 10 min each. Retinas were then infiltrated for 45 min in 50% TAAB embedding resin (TAAB Laboratories Equipment Ltd) in propylene oxide, followed by 3 x 1 h in 100% TAAB embedding resin before being included in 100% TAAB embedding resin at 60°C for 48 h. Eighty nanometre thick sections were collected onto 300 mesh copper grids and stained for 30 min in saturated uranyl acetate, washed and stained again using Reynolds lead citrate (Reynolds, 1963) for 15 min before being air dried. Samples were examined in a Philips CM12 TEM (FEI UK Ltd) at 80 kV. Images captured with a Megaview III camera and AnalySIS software (Soft Imaging System GmbH).

For mitochondrial analysis, total mitochondrial and cristae areas were measured using the freehand selection tool in ImageJ and to calculate average mitochondrial length the longest length of single mitochondria was measured (the morphometrics of 1323 mitochondria were analysed in this way). Synapses were counted using ImageJ’s Cell Counter Plugin with a region of interest with fixed area of 85 μm² selected from sublaminas a and b of the inner plexiform layer. For vesicle densities, bipolar cell terminal areas were measured and all synaptic vesicles counted with and results expressed as a density (synaptic vesicles/μm²).

Western blot analysis

Retina and brain protein samples from \(\text{Opa1}^{+/−}\) \((n = 6)\) and wild-type \((n = 6)\) mice aged 12, 14 and 15 months were boiled for 5 min at 95°C in a sample loading buffer. Twenty micrograms of protein extracts were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis in Tris/Glycine/SDS running buffer (Bio-Rad).

Precision Plus ProteinTM Standard (Bio-Rad) was loaded in a volume of 10 μl/ lane to show the location of proteins of distinct sizes in the gel. Electrophoresis was run for 1 h at 15 min at 100 V followed by transfer to 0.2 μm nitrocellulose membrane (Bio-Rad,) for 75 min at 250 mA in a cold Tris/glycine transfer buffer (Bio-Rad) with 20% methanol (v/v). After the transfer, membranes were blocked with 5% bovine serum albumin (Sigma) in phosphate-buffered saline/0.2% Tween for 1 h at room temperature.

The membranes were incubated with either mouse anti-cytochrome c (monoclonal; 1:1000; BD Pharmingen), rabbit anti-PSD-95 (monoclonal; 1:500), rabbit anti-synaptophysin (polyclonal; 1:500), rabbit anti-γ-aminobutyric (GABA)A receptor α51 (polyclonal; 1:500) or rabbit anti-mGluR2 and 3 (polyclonal; 1:500) (Abcam) or 1% bovine serum albumin in phosphate-buffered saline/0.2% Tween; β-actin (monoclonal; 1:400) was used as a loading control. The membranes were washed three times in phosphate-buffered saline/0.2% Tween and incubated for a further 1 h with a secondary goat anti-rabbit antibody (1:10000) or goat anti-mouse (1:10000) in 1% bovine serum albumin in phosphate-buffered saline/0.2% Tween. After three washings in phosphate-buffered saline/0.2% Tween, blots were subjected to chemiluminescent detection with Pierce ECL Western Blotting Substrate (Thermo-Fisher Scientific) for 5 min and exposed to blue sensitive radiographic film (Kodak).

Immunohistochemical labelling of retina sections and retinal flat-mounts

Adult \(\text{Opa1}^{+/−}\) experimental mice \((n = 12)\) and their littermate controls (wild-type) \((n = 12)\) were analysed in three age groups: 12, 14 and 15 months. Mice were killed by cervical dislocation and the eyes quickly enucleated, punctured at the limbus and then submerged in 4% paraformaldehyde at 4°C for 1 h. Eyes were washed (1% phosphate-buffered saline) and placed in a 25% sucrose solution in phosphate-buffered saline at 4°C for 24 h. Eye cups were then frozen in Optimal Cutting Temperature compound (Sakura Finetek) and cut at 20 μm thickness using a cryostat at −23°C. For immunohistochemical staining, the techniques outlined in the literature (Chetkovich et al., 2002) were followed with minor alterations. Briefly, slide mounted sections were warmed to room temperature for 30 min and then permeabilized with 0.2% Tween (phosphate-buffered saline/0.2% Tween). Sections were blocked with 5% chick serum in phosphate-buffered saline at room temperature for 1 h and then incubated with rabbit anti-PSD-95 (monoclonal; 1:400), rabbit anti-PKC-α (monoclonal; 1:200), rabbit anti-synaptophysin (polyclonal; 1:250), rabbit anti-GABA A receptor α51 (polyclonal; 1:250), rabbit anti-mGluR2 and 3 (polyclonal; 1:250) (Abcam) or rabbit anti-γ-synuclein (monoclonal; 1:200) in 5% chick serum in phosphate-buffered saline at 4°C overnight. Sections were washed three times for 2 min in phosphate-buffered saline/0.2% Tween and incubated with donkey anti-rabbit AF488-conjugated antibody (1:500) and TO-PRO® iodide (1:500) (Invitrogen) at room temperature for 2 h. They were washed again three times for 2 min in phosphate-buffered saline/0.2% Tween, mounted in ProLong® Gold AntiFade Reagent, cover-slipped and sealed with nail polish. For intrinsically photosensitive retinal ganglion cell labelling, retinal flat-mounts were warmed to room temperature for 30 min and permeabilized with 0.2% Tween (phosphate-buffered saline/0.2% Tween), blocked with 5% chick serum in phosphate-buffered saline at room temperature for 1 h and then incubated with rabbit anti-melanopsin (monoclonal; 1:2500) in 5% chick serum in phosphate-buffered saline at 4°C for 72 h. Following this, sections were washed three times for 2 min in phosphate-buffered saline/0.2% Tween and incubated with chick anti-rabbit AF488-conjugated antibody (1:500) and TO-PRO® iodide (1:500) at 4°C for 72 h. Retinas were washed again three times for 2 min in phosphate-buffered saline/0.2% Tween, mounted in ProLong® Gold AntiFade Reagent, cover-slipped and sealed with nail polish.
Image acquisition and analysis

Z-stack images were taken 24 h after mounting using a Zeiss LSM510 laser scanning confocal microscope at ×20. All images were processed using ImageJ. For bipolar cell counts the ‘cell counter’ plugin was used and cell populations expressed as a percentage change from wild-type. For quantification of synaptic and neurotransmitter markers image stacks were z-projected, the colour channel with the secondary fluorophore cropped out and the pixel density measured. For quantification of electron microscope images ImageJ’s ‘freehand’ selection tool and ‘cell counter’ plugin were used.

Retinal thickness

Cross sections of 24-month-old wild-type and Opa1+/− mice (n = 6) were mounted and stained with haematoxylin and eosin. Images of the whole retina cross-sections were obtained using a Leica DMRA2 microscope equipped with a DC500 camera (Leica) and QWinV3 software allowing the retinal thickness to be measured using ImageJ.

Biologic transfection

For biologic transfection, retinas from six 12-month-old mice were used (wild-type n = 3, Opa1+/− n = 3). The preparation and delivery of plasmid DNA biologically has been described in detail elsewhere (O’Brien and Lummis, 2006). In brief, gold particles (1 μg per bullet; 1 μm in diameter; Bio-Rad) were coated with either PSD-95-GFP and pCMV-E2-Crimson (Clontech) for synaptic analysis or pDsRed2-Mito (MitoDsRed) (Clontech) and pEGFP-N1 (GFP) (Clontech) (1 μg DNA per bullet) for mitochondrial analysis and delivered to retinal ganglion cells in a flat-mount preparation using the Helios Gene Gun (Bio-Rad) at ~150 psi. Transfected retinas were incubated on cell culture inserts in a custom media containing NeuroBasal Dendrites and mitochondria were measured using ImageJ’s ‘freehand’ selection tool and ‘cell counter’ plugin were used.

Quantitative reverse transcriptase–polymerase chain reaction

Retinas from 12-month wild-type (n = 3) and Opa1+/− (n = 3) mice were harvested and placed in RNA later® (Ambion). Total RNA was isolated using TRIzol® and purified using the RNeasy® Clean Up kit (Qiagen). One microgram of total RNA from each sample was reverse-transcribed using the high capacity complementary DNA reverse transcription kit (Applied Biosystems). Quantitative polymerase chain reaction reactions were prepared using TaqMan® Universal PC Master Mix, No AmpErase® UNG (Applied Biosystems) and mixed with complementary DNA, TaqMan® primers and probe gene specific assay mix. TaqMan® gene expression assays (Applied Biosystems) were used for Mus musculus Opa1 (Assay ID: Mm00453879_m1), Syt (Mm00436850_m1) and Ptd95 (Mm00492193_m1). Quantitative polymerase chain reaction was performed using an ABI Prism 7900HT (Applied Biosystems). Assays were carried out in triplicate with the mean Ct values used to calculate the relative gene expression levels after normalizing to 18S RNA levels (endogenous control: VIC/MGB Probe, Primer Limited). Analysis of relative gene expression data was performed using the ΔΔCt method. Statistical analysis was carried out by Tukey’s post hoc test and expressed as ANOVA P-values. All analyses of wild-type and Opa1+/− data were undertaken in a blinded fashion.

Results

Opa1 transcript isoforms found in neurological tissues are the same in wild-type and Opa1+/−

Reverse transcriptase–polymerase chain reaction analysis of Opa1 transcripts showed the presence of three messenger RNA isoforms: isoform 1 (exons 4 and 5), isoform 7 (exons 4 and 5b) and isoform 8 (exons 4, 4b and 5b) present in mouse retina; isoform 1 was most abundant. The same expression profile was seen in both wild-type and heterozygous mutants. Reverse transcriptase–polymerase chain reaction analysis revealed the presence of exon 4 in all mouse Opa1 isoforms indicating that alternative splicing occurred only between exons 4b and 5b.

Opa1 protein is reduced in neurological tissues and mitochondria but Opa1 processing in retina of heterozygous mice is normal

Mouse Opa1 protein exists in two long isoforms and three short isoforms. The level of both long and short Opa1 isoforms was reduced by ~50% in 5-month-old Opa1+/− mice compared with the control wild-type group. In the Opa1+/− mouse model the relative mean expression of long to short isoforms calculated from western blot was comparable to the control [wild-type: 0.83 ± 0.21; Opa1+/−: 0.87 ± 0.04 (mean ± SD)], which indicated that pathological proteolysis of Opa1 was not induced.

Mitochondrial membrane potential is unchanged in Opa1+/− retina

To investigate differences in mitochondrial membrane potential (ΔΨ) in Opa1+/− mice, mitochondria were isolated from brain and retina and assessed by spectrofluorimetry for JC-1 red, which is sensitive to changes in mitochondrial membrane potential. The mean Opa1+/− mitochondrial membrane potential did not differ significantly from wild-type counterparts (P > 0.05). In Opa1+/− retina the mean mitochondrial membrane potential was 87.6 ± 5.6% of that measured in wild-type mice. In brain, mitochondrial membrane potential was 91.7 ± 11.2% (mean ± SEM) of the values in wild-type mice. As a positive control mitochondrial membrane potential was measured in four samples (two wild-type and two Opa1+/− samples) 2 min after the addition of CCCP (carbonyl cyanide m-chlorophenyl hydrazone),
Figure 1 Aberrant mitochondrial morphology in Opa1+/- mice. In order to explore mitochondrial morphology in wild-type and Opa1+/- retinal ganglion cell dendrites, mitochondria were imaged using electron microscopy or ex vivo in retinal whole mounts using Biolistics and their morphometrics [average length, cristae to matrix ratio and mitochondria to dendrite ratio (Den. Mito. Index)] measured using ImageJ. (A) Total cristae area as a function of mitochondrial area is reduced in Opa1+/- mice compared with wild-type controls. (B) Representative panel of mitochondria from the three age groups observed (12, 14 and 15 months). (C–H) Samples of biolistically labelled on-centre retinal ganglion cells from wild-type (C–E) and Opa1+/- mice (F–H). Square panels show the en face view (compressed z-stack, max intensity) while the underlying rectangular panels shows a single dendrite typical of the selection used for quantification. Inset plots show the change in mitochondria number (J) (mitochondria/µm dendrite) and a decreased dendritic mitochondrial index (I) (total mitochondrial length per total dendrite length) as a function of genotype. Scale bar = 500 nm (B), 50 µm (C–H; en face view panels), 10 µm (C–H; single dendrite view panel). Error bars = standard error of the mean. *P < 0.05, **P < 0.001, ***P < 0.0001 (Mann–Whitney U).
Figure 2  Retinal cell populations are preserved in Opa1\(^{+/-}\) mice. Immunohistochemical labelling against \(\gamma\)-synuclein (green) as a marker of retinal ganglion cells reveals no loss of retinal ganglion cell populations (B) in the ganglion cell layer in Opa1\(^{+/-}\) mice (A) \((P > 0.05)\). Additionally, labelling against melanopsin (green) as a marker of intrinsically photosensitive retinal ganglion cells on retinal flat mounts (C, wild-type; D, Opa1\(^{+/-}\)) reveals stable intrinsically photosensitive retinal ganglion cell populations (E). Labelling against PKC-\(\alpha\) (green) as a marker of bipolar cells reveals no loss of bipolar cell populations (F) and no gross anatomical differences in bipolar cell morphology in Opa1\(^{+/-}\) mice (H) compared with their wild-type controls (G) \((P > 0.05)\). GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; Meln = melanopsin; ONL = outer nuclear layer; OPL = outer plexiform layer; RGC = retinal ganglion cell. Error bars = standard error of the mean. Scale bars = 50 \(\mu\)m.
which uncouples the mitochondrial proton gradient (1 nM/mg proteins). In all samples the mitochondrial membrane potential dropped to ~30% of original values (33.88 ± 4.66).

Average mitochondrial length, cristae to matrix ratio and dendritic mitochondrial index is decreased in Opa1+/- retinal ganglion cells

Mitochondrial morphology was assessed quantitatively by electron microscope. Mitochondrial average length was significantly reduced in Opa1+/- in all age groups [15 months, average length (nm) ± SEM: wild-type, 730 ± 20; Opa1+/-, 602 ± 17; P < 0.0001], as well as cristae to matrix ratio [15 months, cristae to matrix ratio (%) ± SEM: wild-type, 31 ± 1; Opa1+/-, 15 ± 1; P < 0.0001]. The mitochondrial average length and cristae to matrix ratio were similar in sublamina a and b within animal groups (Fig. 1A and B). Results show aberrant, fragmented mitochondria compared with wild-type controls. On centre retinal ganglion cells of 12-month-old mice were biologically labelled with pDsRed2-Mito and pEGFP-N1 to label neuronal processes and mitochondria (Fig. 1C–H). We observed a decrease in the dendritic mitochondrial index (Fig. 1I) in Opa1+/- mice (i.e. fewer mitochondria/μm of dendrite) as well as an increase in mitochondrial number in those dendrites (Fig. 1J) (i.e. many small, fragmented mitochondria).

Cell populations are unchanged in Opa1 deficient retinas

Immunohistochemical labelling of γ-synuclein as a specific retinal ganglion cell marker (Surgucheva et al., 2008) indicated preservation of retinal ganglion cell populations (P > 0.05) (Fig. 2) confirming the absence of significant retinal ganglion cell loss in Opa1+/- retinas (Davies et al., 2007; Williams et al., 2010). To account for intrinsically photosensitive retinal ganglion cells flat-mounted retinas were labelled using a melanopsin antibody. Intrinsically photosensitive retinal ganglion cell populations were unchanged at 24 months (wild-type; 2.15% retinal ganglion cells = intrinsically photosensitive retinal ganglion cells, Opa1+/-; 2.21%, P > 0.05) (Fig. 2C–E). Neuron counts in the inner and outer nuclear layers were based on cell labelling with anti-PKC-α antibodies (inner nuclear layer; bipolar cells) or TO-PRO® iodide (outer nuclear layer; photoreceptors; no change; P > 0.05; data not shown). There was no change from wild-type bipolar cell count in either the 12 (+3.6%; P > 0.05), 14 (−8.3%; P > 0.05) or 15 (+2.6%; P > 0.05) month age groups, suggesting dysfunction of post- but not presynaptic sites. The retinal thickness was similar at all ages (P > 0.05), and a summary of individual nuclear layer thicknesses is shown in Table 1. There was no change in nuclear layer thicknesses between Opa1+/- and wild-type mice (P > 0.05 in all instances).

<table>
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<tr>
<th>Layer</th>
<th>Age (months)</th>
<th>Wild-type (μm ± SEM)</th>
<th>Opa1+/- (μm ± SEM)</th>
<th>t-test (P)</th>
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<tr>
<td>Outer nuclear layer</td>
<td>12</td>
<td>51.9 ± 1.6</td>
<td>53.7 ± 1.9</td>
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<tr>
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<td>35.9 ± 1.8</td>
<td>37.2 ± 1.9</td>
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<td>9.2 ± 0.4</td>
<td>10.5 ± 0.5</td>
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<tr>
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<td>35.9 ± 1.3</td>
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<tr>
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<td>10.6 ± 0.4</td>
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<td>9.8 ± 0.8</td>
<td>10.1 ± 0.7</td>
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Nuclear layer thickness was calculated from retinal sections of wild-type and Opa1+/- mice (wild-type n = 12; Opa1+/- n = 12) labelled with TO-PRO® as a nuclear counter stain. There is no change observed in individual nuclear layer thicknesses between wild-type and Opa1+/- mouse retinas in all ages. There is no change in retinal cell populations across the retina (Fig. 2). SEM = standard error of the mean, P = Student’s t-test P-value.

Opa1 deficiency leads to decreased synaptic density

Twenty micrometre-thick sections of fixed retinal tissue were labelled with antibodies against PSD-95 to quantify synaptic integrity within the inner plexiform layer in Opa1+/- mice (Fig. 3A). Secondary fluorophore luminescence was reduced in Opa1+/- mice at 12 (~21.51%; P = 0.0626), 14 (~29.090%; P = 0.0246) and 15 (~44.22%; P = 0.0018) month age groups. Electron microscopy analysis of postsynaptic sites in Opa1+/- and wild-type inner plexiform layer revealed a steady decrease in postsynaptic density with age and was significant for sublamina b (15 months; %Δ sublamina a, −19.1%, P > 0.05; %Δ sublamina b, −33.3%, P < 0.01) (Fig. 3). These observations were supported by western blot analysis (Fig. 7). Interestingly, quantitative polymerase chain reaction analysis of 12-month-old whole retinas for PSD-95 showed an increase in PSD-95 transcript levels in Opa1 deficiency leads to decreased synaptic density (Barnard et al., 2011).

To determine the change at a cellular level in the dendritic distribution of synapses and associated mitochondria, retinal ganglion cells from 12-month-old mice were biologically labelled with pE2-Crimson and PSD-95:GFP to label neuronal processes and mitochondria (Fig. 4A-F). The left shift in the Sholl analysis of postsynaptic sites (Fig. 4I) and mitochondria (Fig. 4J) indicate a synaptic and mitochondrial deficit towards the most distal portion. The area under the Sholl curve (Fig. 4H) showed a 79% (P < 0.001) reduction for postsynaptic sites and an 88% reduction (P < 0.01) for mitochondria.
Figure 3 Reduced retinal connectivity in inner plexiform layer of Opa1+/- mice. To explore synaptic density within the inner plexiform layer retinal sections (A) were labelled with antibodies against PSD-95 and the relative intensity of the secondary fluorophore measured. There was a general decrease in synaptic density within the inner plexiform layer (B) observed in Opa1+/- inner plexiform layer (A, right) over wild-type controls (A, left). The number of synaptic sites on and off-centre retinal ganglion cell dendrites were calculated from electron microscope images from sublamina a (off-centre) and sublamina b (on-centre) of the inner plexiform layer (C). On (C) at 12, 14, and 15 months sublamina a there is no significant differences between groups. In (C) sublamina b there is significance between the groups. The reduction in synaptic density is greater in sublamina b and significant by 12 months. GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; ONL = outer nuclear layer; OPL = outer plexiform layer; wt = wild-type. Error bars = standard error of the mean. *P < 0.05, ***P < 0.001 (Student’s t-test).
The selective vulnerability of glutamatergic synapses increases with age

Sections of the same retinas were labelled with antibodies against mGluR2 and 3 (the most common glutamate receptor on on-centre retinal ganglion cell dendrites) and against GABA<sub>A</sub> receptor α51 (the most common GABA<sub>A</sub>ergic receptor expressed on off-centre retinal ganglion cell dendrites), for which the relative luminance of the secondary fluorophores were quantified. mGluR2 and 3 labelling was reduced at all ages but this was only significant from 14 months (Fig. 5; 12 months, −5.96%, P = 0.7535; 14 months, −22.96%, P = 0.0347; 15 months, −23.76%, P = 0.0113) consistent with preferential loss of on-centre retinal ganglion cell dendrites. By contrast, GABA<sub>A</sub>ergic relative luminance increased in all age groups (12 months; +21.96%; P = 0.4350. 14 months; +37.12%; P = 0.0457. 15 months; +58.81%; P < 0.0001) (Fig. 5). These results are mirrored in the western blot analysis (Fig. 7).

To determine the subcellular changes underlying the loss of synaptic connectivity we explored synaptic vesicle levels within the synaptic bouton. Retinal sections were labelled with synaptophysin as a marker of synaptic vesicles. An increase in the relative luminance of the secondary fluorophore in the 12 ( +122.51%; P = 0.0026), 14 ( +129.88%; P = 0.0001) and 15 ( +48.85%; P = 0.0124) month age groups was observed (Fig. 6).

Electron microscopy analysis of Opa1<sup>+/−</sup> bipolar cell terminals confirmed the increase in the number of synaptic vesicles in aged Opa1<sup>+/−</sup> compared to wild-type (at 15 months, density synaptic vesicles/μm<sup>2</sup>± SEM: wild-type sublamina a (93.9 ± 15), b (100.0 ± 11); Opa1<sup>+/−</sup> sublamina a (194.6 ± 16.7; P = 0.0001), b (240.8 ± 30.3; P = 0.0003) (Mann–Whitney U)). Western blot analysis of 12-, 14- and 15-month-old retinas (Fig. 7) and quantitative polymerase chain reaction of 12-month retinas (Fig. 8) for synaptophysin further confirmed the increase in synaptophysin levels.

Discussion

Here we explore the retinal synaptic events occurring between 12 and 15 months of age in the Opa1<sup>+/−</sup> mouse, a mouse model of dominant optic atrophy, which has previously been shown to have active retinal ganglion cell dendritic atrophy starting at 10–15 months. During this period in the pathophysiology of Opa1<sup>+/−</sup> there is active dendritic pruning accompanied by significant changes in synaptic density, synaptic structure and processes and postsynaptic sites showing a decrease in synaptic density. Percentage comparisons of the area under the curve (AUC) (J) show a 79% reduction in the area under the curve for postsynaptic sites and an 88% reduction for mitochondria. Filled circle/triangle = wild-type; open circle/triangle = Opa1<sup>+/−</sup>; error bars = standard error of the mean; scale bars = 50 μm, **P < 0.01, ***P < 0.0001 (Mann–Whitney U).
mitochondrial morphology within the synaptic bouton. These data imply that synaptic atrophy coincides or precedes dendritic atrophy, suggesting that dendritic atrophy (and consequently visual dysfunction) may be driven at the level of the synapse.

An important finding of the study is that the neuropharmacology of the retinal ganglion cell has a strong influence on neuronal viability in the absence of normal functioning mitochondria. We found a selective loss of glutamatergic, but not GABAergic, synaptic sites with age in Opa1+/−/C0 mice, as indicated by changes observed by immunohistochemistry and western blot analysis as well as a reduction in the number of postsynaptic density sites determined by electron microscopy and biolistic transfection of PSD-95:GFP. In addition, there is a corresponding increase in synaptic vesicle count and a change in distribution within the presynaptic bouton (bipolar cell presynaptic site), with a failure to recruit synaptic vesicles to synaptic sites. Electron microscopy reveals a marked change in mitochondrial morphology in all retinal cells types observed, as expected in Opa1-deficient mice. These results are consistent with our previous work indicating selective atrophy of on but not off-centre retinal ganglion cells.

One explanation for this selective loss is that on-centre retinal ganglion cells need to support high energy glutamatergic synapses with bipolar cells. By contrast, off-centre retinal ganglion cells connect to bipolar cells using relatively lower energy GABAergic synapses. This higher energy requirement may be the cells’ weakness as the OPA1-deficient mitochondria may not be able to support the cells’ metabolic needs. The retinal ganglion cell may go through a phase of low bioavailability of ATP leading to a lower rate of synaptic firing. This leads to a period of low activity whereby synaptic sites are depleted, synaptic connections fail and the dendrites atrophy, leading to the loss of visual function we see in this mouse phenotype. It has been previously shown that the maintenance of retinal ganglion cell dendritic architecture depends on glutamatergic signalling in both the mature and immature retina, supporting our findings that an absence of glutamatergic synapses leads to dendritic degeneration (Bodnarenko et al., 1995; Wong et al., 2000; Sernagor et al., 2001).

The retinal ganglion cell soma and dendrite is unique in the CNS in that it is frequently exposed to high levels of light. This has lead to theories (Wataha et al., 2004; Osborne et al., 2006) that ambient light may exacerbate mitochondrial dysfunction by mechanisms including higher generated levels of reactive oxygen species from mitochondrial photosensitizers and suppressed levels of oxidative phosphorylation. Light exposure studies on rat retinal cultures show enhanced levels of apoptosis (increased number of terminal deoxynucleotidyl transferase dUTP nick end labelling-positive cells) caused by cleavage of caspase-3 by light into the caspase-3 active form (Lascaratos et al., 2007).
This selective loss provides a useful insight into the selective vulnerability of neurons in mitochondrial disease associated with chronic neurodegeneration, and may help elucidate similar mechanisms of neurodegeneration in other diseases where mitochondrial dynamics have shown to be altered (Wang et al., 2008; Cho et al., 2010).

We detected three Opa1 isoforms in wild-type retinas and mutant mice. Isoform 1 (exons 4 and 5), which does not contain the alternatively spliced exons 4b and 5b, is predominant, similar to findings in human retina. The protein encoded by this variant is the most highly expressed isoform in murine neural tissue, including retina. The two other isoforms found in mouse retina of wild-type and mutant Opa1+/- mice were isoform 7 (exons 4 and 5b) and isoform 8 (exons 4, 4b and 5b). The presence of an additional isoform 5 (exons 4 and 4b) was detected in other mouse tissues (liver, kidney and spleen). The expression of different Opa1 isoforms in mitochondria has been shown to be important for Opa1 pro-fusion activity. Dissipation of the mitochondrial membrane potential can disturb this process by stimulation of activity of specific proteases that target long Opa1 isoforms.
culminating in the accumulation of short Opa1 isoforms. Opa1 processing has been reported in some mitochondrial diseases and in mouse models with impaired mitochondrial biogenesis. This may be of relevance in relation to autosomal dominant optic atrophy and the specific vulnerability of retinal ganglion cells in this disease. Furthermore it is known that OPA1 alternative exons 4b and 5b are involved in the apoptotic process. The predominance of the exons 4 and 5 isoform in retina may help to explain the absence of apoptosis we see in our mutant mouse (M. Piechota and M. Votruba, unpublished results).

The pathological changes in retinal ganglion cell synaptic density and structure and mitochondrial architecture, number and distribution within the synaptic bouton together with demonstrable retinal ganglion cell dendropathy in the absence of widespread cellular loss, represents a tangible marker for early disease. This constellation of cellular changes also has the potential to serve as a sensitive biomarker for rescue and recovery therapeutic strategies. This study highlights the importance of OPA1-mediated mitochondrial fusion in neuronal health.

**Figure 7** Western blot densitometry data from retinas of 12 mice (wild-type, n = 6; Opa1+/−, n = 6) validates findings found by immunohistochemistry and electron microscopy. There is a general decrease with age in PSD-95 (postsynaptic densities) and mGluR2 and 3 (glutamatergic synapses), which supports previous findings of on-centre retinal ganglion cell specific degeneration. There is also a general increase in synaptophysin (presynaptic vesicles) and GABA_A receptor αS1 (GABA_Aergic synapses). β-actin was used as a loading control.

**Figure 8** Quantitative polymerase chain reaction analysis of Opa1 (A), PSD-95 (B) and synaptophysin (C) transcripts from 12-month-old wild-type and Opa1+/− retina show an expected decrease in Opa1 transcript and increase in synaptophysin transcript as mirrored in the data collected from electron microscopy, immunohistochemistry and western blot analysis. Interestingly, PSD-95 transcription levels are higher in Opa1+/− mice suggesting upregulation of PSD-95 in response to the destruction of functional synapses on the retinal ganglion cells. RQ = .

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