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OPA1-related auditory neuropathy: site of lesion and outcome of cochlear implantation

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Hearing impairment is the second most prevalent clinical feature after optic atrophy in dominant optic atrophy associated with mutations in the *OPA1* gene. In this study we characterized the hearing dysfunction in *OPA1*-linked disorders and provided effective rehabilitative options to improve speech perception. We studied two groups of *OPA1* subjects, one comprising 11 patients (seven males; age range 13–79 years) carrying *OPA1* mutations inducing haploinsufficiency, the other, 10 subjects (three males; age range 5–58 years) carrying *OPA1* missense mutations. Both groups underwent audiometric assessment with pure tone and speech perception evaluation, and otoacoustic emissions and auditory brainstem response recording. Cochlear potentials were recorded through transtympanic electrocochleography from the group of patients harbouring *OPA1* missense mutations and were compared to recordings obtained from 20 control subjects with normal hearing and from 19 subjects with cochlear hearing loss. Eight patients carrying *OPA1* missense mutations underwent cochlear implantation. Speech perception measures and electrically-evoked auditory nerve and brainstem responses were obtained after 1 year of cochlear implant use. Nine of 11 patients carrying *OPA1* mutations inducing haploinsufficiency had normal hearing function. In contrast, all but one subject harbouring *OPA1* missense mutations displayed impaired speech perception, abnormal brainstem responses and presence of otoacoustic emissions consistent with auditory neuropathy. In electrocochleography recordings, cochlear microphonic had enhanced amplitudes while summating potential showed normal latency and peak amplitude consistent with preservation of both outer and inner hair cell activities. After cancelling the cochlear microphonic, the synchronized neural response seen in both normally-hearing controls and subjects with cochlear hearing loss was replaced by a prolonged, low-amplitude negative potential that decreased in both amplitude and duration during rapid stimulation consistent with neural generation. The use of cochlear implant improved speech perception in all but one patient. Brainstem potentials were recorded in response to electrical stimulation in five of six subjects, whereas no compound action potential was evoked from the auditory nerve through the cochlear implant. These findings indicate that underlying the hearing impairment in patients carrying *OPA1* missense mutations is a disordered synchrony in auditory nerve fibre activity resulting from neural degeneration affecting the terminal dendrites. Cochlear implantation improves speech perception and synchronous activation of auditory pathways by bypassing the site of lesion.

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Abbreviations: ABR = auditory brainstem response; DOA = dominant optic atrophy; ECochG = electrocochleography; PTA = pure tone average

Introduction

Dominant optic atrophy (DOA) is among the most common inherited optic neuropathies and is characterized by progressive bilateral visual loss beginning in childhood (Kjer, 1959). Retinal ganglion cell degeneration, affecting primarily the small fibres of the papillo-macular bundle (Carelli *et al.*, 2004), is the pathological hallmark of DOA. About 60–70% of DOA cases are associated with pathogenic mutations in the nuclear gene (*OPA1*) encoding for the OPA1 protein (Alexander *et al.*, 2000; Delettre *et al.*, 2000), a mitochondria-targeted dynamin-related GTPase that localizes to the inner mitochondrial membrane (Delettre *et al.*, 2000; Olichon *et al.*, 2006). OPA1 promotes fusion of the inner mitochondrial membrane (Olichon *et al.*, 2006), maintains the integrity and structure of mitochondrial cristae (Frezza *et al.*, 2006), and is also implicated in maintenance of membrane potential and oxidative phosphorylation (Lodi *et al.*, 2004).

More than 200 mutations have been identified so far (www.mitodyn.org), and most of them are predicted to generate protein truncation, possibly inducing haploinsufficiency. These mutations are responsible for the classic form of 'non-syndromic' optic neuropathy characterized by variable degrees of central vision impairment (Ferré *et al.*, 2009). Some patients may present with a syndromic form of DOA associated with sensorineural hearing loss, ataxia, sensorimotor neuropathy, progressive external ophthalmoplegia and mitochondrial myopathy (DOA+ phenotype) (Meire *et al.*, 1985; Amati-Bonneau *et al.*, 2008; Hudson *et al.*, 2008). Multiple deletions of mitochondrial DNA (mtDNA) accumulate in the skeletal muscle of these patients, thus pointing to a further function of *OPA1* in maintaining mtDNA stability (Amati-Bonneau *et al.*, 2008). The DOA+ phenotype has been associated mainly with missense mutations affecting the GTPase domain (Amati-Bonneau *et al.*, 2005; Yu-Wai-Man *et al.*, 2010), and a dominant negative mechanism has been proposed, which would result from abnormal protein structure.

The most common extra-ocular manifestation in DOA+ is sensorineural hearing loss, found in ~60% of such patients, most frequently associated with the R445H missense mutation (Yu-Wai-Man *et al.*, 2010; Leruez *et al.*, 2013; Yu-Wai-Man and Chinnery, 2013). Hearing loss starts in childhood or adolescence, and usually follows the onset of visual symptoms (Yu-Wai-Man *et al.*, 2010; Leruez *et al.*, 2013; Yu-Wai-Man and Chinnery, 2013). Although the majority of studies broadly qualify the hearing disorder as 'sensorineural hearing loss', some authors have proposed auditory neuropathy as the pathophysiological mechanism underlying the hearing impairment in DOA+ (Amati-Bonneau *et al.*, 2005; Huang *et al.*, 2009; Santarelli 2010).

Auditory neuropathy is a hearing disorder characterized by a disrupted temporal coding of acoustic signals in the auditory nerve fibres resulting in impairment of auditory perceptions relying on temporal cues (Starr *et al.*, 1996, 2008; Zeng *et al.*, 2005). The disruption of auditory nerve discharge underlies either the absence or profound alteration of auditory brainstem responses (ABRs) and severe impairment of speech perception. In contrast, cochlear receptor outer hair cell activities are preserved (otoacoustic emissions, cochlear microphonic) (Santarelli *et al.*, 2008; Starr *et al.*, 2008). The suggested mechanisms for hearing dysfunction include both pre synaptic and post synaptic disorders affecting inner hair cell depolarization, neurotransmitter release from ribbon synapses, spike initiation in auditory nerve terminals, loss of nerve fibres and impaired conduction, all occurring in the presence of normal physiological measures of outer hair cell activities (otoacoustic emissions, cochlear microphonic). The hearing impairment has peculiar features reflecting alteration in temporal coding of acoustic information in auditory nerve fibres, which is typically unaffected in cochlear hearing impairment resulting from hair cell loss and disruption of the cochlear amplifier (for a review see Starr *et al.*, 2008).

In the last decade, the use of electrocochleography (ECochG) recording has been proposed in the diagnosis of auditory neuropathy for defining the details of both receptor and neural responses in the various forms of the disorder (Santarelli and Arslan, 2002; McMahon *et al.*, 2008; Santarelli *et al.*, 2008). Recordings of cochlear potentials by transtympanic ECochG in two *OPA1* patients harbouring the R445H mutation have shown prolonged low-amplitude negative potentials replacing the auditory nerve compound action potential found in subjects with normal hearing (Huang *et al.*, 2009; Santarelli, 2010). These prolonged responses have been considered as originating from the activation of degenerated terminal portions of auditory nerve fibres (Huang *et al.*, 2009).

In this study we investigated the site of the lesion and the pathophysiological mechanisms behind the hearing impairment in patients with DOA carrying different mutations in the *OPA1* gene. To this end, we recorded the receptor and neural cochlear potentials using transtympanic ECochG, and compared them to the electrically-evoked neural and brainstem responses obtained after cochlear implantation.

Materials and methods

Subjects

We evaluated hearing function in two groups of subjects with *OPA1*-related DOA. One group included patients carrying

OPA1 mutations predicted to induce haploinsufficiency or rearranged protein (indicated as *OPA1*-H) while the second group included subjects harbouring *OPA1* missense mutations (indicated as *OPA1*-M). Details of neurological and genetic findings from all subjects are summarized in Supplementary Table 1, whereas clinical and audiological results are reported in Supplementary Table 2 and Table 1, for the *OPA1*-H and *OPA1*-M groups.

The *OPA1*-H group comprised 11 subjects (seven males; age range 13–79 years), all affected with variable degrees of impaired vision. Only two subjects complained of hearing loss.

The *OPA1*-M group included 10 subjects (three males; age range 5–58 years), three of whom (Subjects 1, 2 and 6) have been partially reported in previous studies (Santarelli and Arslan, 2002; Huang *et al.*, 2009). In all patients, visual loss started in childhood or adolescence. The onset of vision impairment preceded that of hearing loss in seven subjects, whereas in two patients the disease started with congenital deafness (Subject 7) or impaired speech perception (Subject 4). At the time of evaluation all but one patient (Subject 10) complained of difficulty in understanding speech. Two subjects reported tinnitus and vertigo. Subject 9 had been using a cochlear implant for 2 years at the time of our first evaluation.

All subjects underwent audiological assessment including pure tone and speech audiometry, speech perception measures and otoacoustic emissions and ABRs recording, all performed in the same session. The patients included in the *OPA1*-M group were also submitted to ECochG recording except for Subjects 9 and 10.

CT and MRI scans of the head and ear (including the internal acoustic canal) were performed in all patients of the *OPA1*-M group except for Subject 10. The results were normal; in particular, no cochlear malformations were found.

Seven *OPA1*-M patients underwent unilateral cochlear implantation at our department. Surgery was performed within 1 year of first evaluation except for Subject 2, who received a cochlear implant 2 years after the first audiological assessment. All patients underwent a further audiometric evaluation in the week preceding cochlear implantation. None showed worsening of the hearing threshold with respect to the first assessment.

Given poor performance with the cochlear implant, Subject 9 underwent the device manufacturer's integrity testing and a further CT scan of the ear was carried out to confirm correct positioning of the electrode array. This patient also showed no cochlear malformation on radiological imaging.

Audiological studies

Audiometry

We tested hearing thresholds at frequencies from 250 to 8000 Hz (Grason-Stadler GSI 61 audiometer) in a sound-attenuating room. The degree of hearing impairment was defined by the pure tone average (PTA) threshold levels at

Table 1 Clinical and audiological data from the *OPA1*-M group

Subjects	1	2	3	4	5	6	7	8	9	10
Clinical										
Gender	F	F	F	F	M	F	F	F	M	M
Age tested	21	48	41	18	46	5	31	11	48	58
Age onset	9	9	5	13	6	4	Congenital	5	6	6
Sign at onset	Vision	Vision	Vision	Hearing	Vision	Vision	Hearing	Vision	Vision	Vision
Deafness	Quiet	Quiet	Noise	Quiet	Quiet	Quiet	Quiet	Quiet	Quiet	–
Deaf onset	9	28	25	13	13	5	Congenital	5	15	–
Tinnitus	–	–	Yes	–	–	–	–	Yes	–	–
Vertigo	–	–	Yes	–	–	–	–	Yes	–	–
Audiology (right/left)										
Hearing	Mild/Mod	Mild/Mod	Mild/Mild	Mild/N	Sev/Mod	Mod/Mild	Prof/Prof	Mod/Mod	Prof/Prof	N/N
PTA (dB)	35/43	40/50	41/39	28/15	73/68	50/38	116/116	71/58	100/120	13/11
LF (dB)	45/54	30/40	48/33	33/23	71/68	56/45	105/105	71/69	103/115	11/10
HF (dB)	40/25	70/43	28/25	25/23	70/73	50/43	130/130	80/35	100/130	15/15
OAEs	+/+	+/+	+/+	+/+	+/+	+/+	ABS/ABS	+/+	ABS/ABS	+/+
ABR-V (ms)	ABS/8.0	ABS/7.6	7.8/ABS	ABS/ABS	ABS/ABS	ABS/ABS	ABS/ABS	ABS/ABS	ABS/ABS	5.8/5.9
Cochlear implantation										
CI	CI24RE	CI24RE	CI24RE	CI24RE	CI24RE	–	CI24RE	CI24RE	HiRes90K	–
Ear	R	L	R	R	R		R	R	L	
Aided Threshold (dB)	32	32	26	20	26		21	32	36	
ABRs in implanted patients										
Acoustic ABRs	No	–	Yes	No	No		No	No	–	
eABR-V electr. 20	4.6	NT	4.1	3.9	ABS	–	4.3	4.5	NT	
eABR-V electr. 13	4.2	NT	ABS	4.1	ABS	–	4.2	4.9	NT	
eABR-V electr. 6	5.0	NT	ABS	4.2	ABS	–	4.5	4.8	NT	

R/L = right ear/left ear; N = normal; Mod = moderate; Sev = severe; Prof = profound; PTA = pure tone average (average thresholds at 0.5, 1, 2, 4 kHz); LF = low frequencies (average thresholds at 0.5, 1, 2 kHz); HF = high frequencies (average thresholds at 4, 8 kHz); ABS = absent; OAEs = otoacoustic emissions; CI = cochlear implant; eABR = electrically evoked ABR; electr. = stimulating electrode number; NT = not tested. Aided threshold = average thresholds at 0.25, 0.5, 1, 2, 4 kHz as measured in the free-field with subjects wearing their sound processor; Acoustic ABRs refers to the presence of acoustically-evoked ABRs in the implanted ear before cochlear implantation.

0.5, 1, 2 and 4 kHz, and was classified as mild (PTA 21–40 dB HL), moderate (PTA 41–70 dB HL), severe (PTA 71–95 dB HL) and profound (PTA >95 dB HL) (Study group on terminology, definition and hearing assessment, 1996; Martini *et al.*, 1997).

Acoustic reflex thresholds were measured ipsilaterally and contralaterally to the stimulated ear (Grason-Stadler GSI TympStar impedance audiometer). They were considered absent when no response was found at intensities >110 dB HL.

In implanted patients, aided thresholds were measured (Interacoustic AC30 Audiometer connected to a Pioneer A 103 amplifier, JBL TLX130 loudspeakers) with subjects wearing their sound processor on user settings. Warble tone stimuli were presented in the free-field at octave frequencies from 250 to 4000 Hz. To avoid contralateral acoustic stimulation, the ear canal was occluded with an earplug.

Speech audiometry

Articulation gain curves were obtained using disyllabic, phonetically-balanced words from an Italian word list for adults (Bocca and Pellegrini, 1950). Ten words were presented for each stimulus intensity. At each level, scoring was based on the percentage of words correctly repeated by the subject.

Speech perception tests

Speech perception tests were performed in a sound-attenuated room. Speech stimuli were presented in the free-field through one loudspeaker placed 1 m away from the front of the subject's head. Tests were administered at 70 dB(A) in quiet, and in the presence of competing noise presented at two signal-to-noise ratios (+10, +5). Competing speech noise was presented through two additional loudspeakers placed laterally at an angle of 90° on either side of the subject's head at a distance of 1 m.

Speech material consisted of disyllabic words and comprised digital anechoic recordings of a native Italian female speaker. Subjects were presented with one of four randomly chosen lists, each list consisting of 25 items. The speech material was obtained from the protocol of patient candidacy for cochlear implantation for the Italian language (Quaranta *et al.*, 1996). Subjects were requested to respond by repeating the words they heard.

Distortion product otoacoustic emissions

Distortion product otoacoustic emissions were obtained using the ILO-92 OAE system. Primary tones were presented at 70 dB SPL and the f2/f1 ratio was kept at 1.21. The frequency was increased in 1/4 octave steps from 708 to 6299 Hz. Four spectral averages were summed for each stimulus condition.

Electrophysiological studies

Auditory brainstem responses

Potentials were recorded from scalp electrodes (vertex to mastoid ipsilateral to the stimulated ear) in response to 2000 trials of alternating polarity clicks presented monaurally (TDH-50 transducer earphone) at a maximum intensity of 125 dB SPL (corresponding to 90 dB nHL, referred to the psychoacoustical threshold of subjects with normal hearing).

Electrocochleography

Eight of 10 *OPA1*-M patients were administered this procedure as part of our standard cochlear implantation assessment protocol, which includes a signed patient consent form. ECochG was not performed in two subjects, one who showed normal hearing (Subject 10) and the second who was using a cochlear implant at the time of our assessment (Subject 9).

ECochG protocol was assessed by the regional body for quality control of clinical and therapeutic procedures (CCHSA, Veneto Region 2007–2010).

Adults were tested under local anaesthesia and children under general anaesthesia. A sterile stainless steel needle electrode was passed through the tympanic membrane and placed on the promontory wall with the aid of an operating microscope. Stimuli consisted of 0.1 ms rarefaction and condensation clicks, delivered separately in the free-field by means of two high frequency drivers (Electro-Voice DH1A/2MT 16 Ω) mounted on a single polyurethane horn (Electro-Voice HP420) with a maximum intensity of 120 dB SPL (corresponding to 90 dB nHL relative to the psychoacoustic threshold of subjects with normal hearing). The stimulus was calibrated in the free-field by means of a microphone (Brüel and Kjaer 4165) placed at 1 m from the base of the polyurethane horn, which corresponded to the distance of the patient's ear from the horn.

The stimulus paradigm consisted of an initial click, followed 15 ms later by 10 clicks with an interstimulus interval of 2.9 ms, and the sequence was repeated every 191 ms (Santarelli *et al.*, 2008). This stimulus paradigm was used to distinguish between neural and receptor potentials by taking advantage of the different effects of adaptation induced by high stimulation rates (Eggermont and Odenthal, 1974; Santarelli and Arslan, 2013).

The potentials were differentially amplified (50 000 times), filtered (5–8000 Hz) and digitized (25 μs) for averaging (500 trials). The procedure of averaging the responses evoked separately by condensation and rarefaction clicks was applied to cancel the cochlear microphonic and extract the compound action potential with the superimposed summating potential. The resulting curve was subtracted from the potential evoked by condensation clicks to obtain the cochlear microphonic. As cochlear microphonic attenuation was often incomplete at high stimulus intensity and cochlear microphonic spectral energy was at a maximum between 1500 and 3000 Hz, a low-pass digital filter (12 dB/octave, cut-off frequency 2000 Hz) was used to attenuate the residual cochlear microphonic, where needed (Santarelli *et al.*, 2008).

After cancelling the cochlear microphonic, the ECochG waveform begins with the receptor summating potential, which appears as an initial negative deflection arising from baseline and preceding the neural compound action potential (Eggermont, 1976; Schoohnoven, 2007; Santarelli and Arslan, 2013). Latency was defined relative to cochlear microphonic onset in milliseconds. Amplitude was computed relative to the period 1 ms before cochlear microphonic onset in microvolts (μV). We defined latency and amplitude of summating potential at the initial negative deflection arising from baseline while compound action potential peak amplitude was measured at maximum negative potential (with respect to baseline).

Cochlear potentials recorded from *OPA1*-M patients were compared to the ECochG data previously collected from two groups of children tested for presumed cochlear deafness. The first group included 20 children with normal hearing with normal

thresholds when evoking neural and receptor potentials (age range 3.5–6.5 years), whereas the second group comprised 19 children with cochlear hearing loss mainly related to genetic aetiology (mutations in the *GJB2* gene) with compound action potential thresholds between 80 and 100 dB SPL (age range 1–4 years) (Santarelli *et al.*, 2008; Santarelli and Arslan, 2013).

Electrically-evoked compound action potentials and auditory brainstem responses

Electrically-evoked compound action potentials were recorded with Cochlear Corporation Custom-Sound EP software. Stimulation consisted of trains of biphasic, 25 μs width per phase pulses presented at 80 Hz. The evoked electrical activity was recorded two electrodes apart.

Electrically-evoked ABRs were obtained by using biphasic pulses, 50 ms width per phase, presented at 20 Hz. Potentials were recorded from scalp electrodes (vertex to mastoid contralateral to the stimulated ear). Three electrodes (No.20 apical, No.13 intermediate and No.6 basal) were tested at decreasing stimulus levels starting from the upper limit of behavioral dynamic range.

Statistical analysis

ANOVA for repeated measures was carried out to analyse ECochG measures. Separate two-factor ANOVAs with factors of group and stimulus intensity were used to evaluate latency, amplitude and duration measures. *Post hoc* tests for multiple comparisons were conducted with the Tukey-Kramer procedure. The level of significance was $P < 0.05$.

Values contained in both text and figures indicate mean ± standard error.

Results

Hearing thresholds and middle ear muscle acoustic reflexes

The *OPA1*-H group showed normal hearing thresholds except for Subjects 1 and 7, who had mild and moderate hearing loss, respectively (Supplementary Table 2). High resolution CT scanning performed in Subject 7 revealed a thickened stapes footplate, suggestive of grade 1 otosclerosis (Marshall *et al.*, 2005). Moreover, Subject 1 had a positive history of exposure to occupational noise and showed a typical audiometric profile of noise-induced hearing loss.

All but one patient from the *OPA1*-M group had elevated hearing thresholds, and the severity of hearing loss ranged from mild to profound (Table 1). Differently from all the others, Subject 10 showed normal hearing thresholds.

Acoustic reflexes were detected in all *OPA1*-H patients, whereas they were absent in all but one (Subject 10) of the *OPA1*-M group.

Speech audiometry

Articulation-gain curves were obtained from all subjects. Ears were pooled into different classes of PTA. These were defined by minimum and maximum PTA levels in the *OPA1*-H group, whereas the *OPA1*-M subjects were pooled into three classes characterized by increasing PTA values (Fig. 1). Subjects with profound hearing loss were not included. Because of the high variability of scores, in the case of the *OPA1*-M patients, the articulation curves obtained from individual ears were considered.

Articulation-gain curves from *OPA1* patients were compared to the mean functions with 95% confidence limits, calculated for each class of PTA for a large sample of subjects, including normally-hearing individuals (394 ears, range 18–50 years) and patients with cochlear hearing loss (583 ears, range 18–50 years), submitted to audiometric evaluation at our department over the past 8 years.

The mean articulation-gain function calculated for the *OPA1*-H group closely followed the corresponding curve obtained from subjects with normal hearing. In contrast, *OPA1*-M patients showed lower scores compared to the

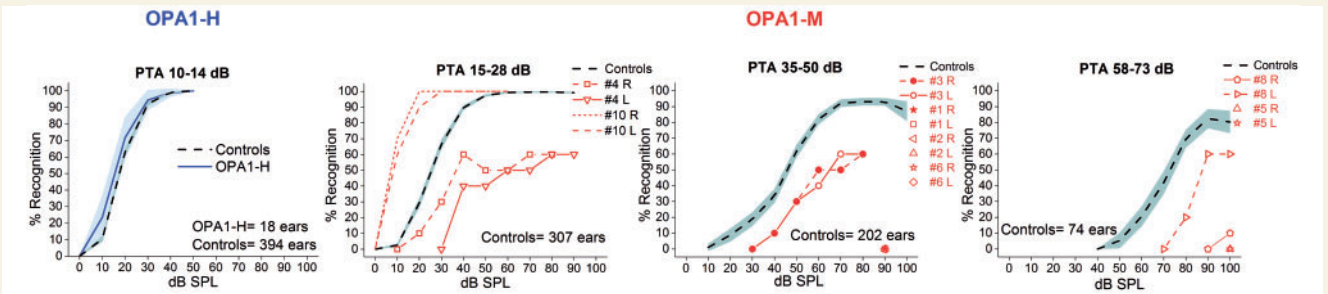

Figure 1 Articulation gain curves from patients with DOA. Articulation curves obtained from *OPA1*-H and *OPA1*-M groups have been superimposed on the mean articulation functions (dashed lines) with 95% confidence limits (shadowed areas) calculated for controls at corresponding PTA values. These were defined by minimum and maximum (10–14 dB) PTA values in the *OPA1*-H group, whereas the *OPA1*-M subjects were pooled into three classes characterized by increasing PTA values (15–28 dB, 35–50 dB, 58–73 dB). Mean function is displayed for the *OPA1*-H group, whereas articulation curves from individual ears have been considered for the *OPA1*-M patients due to the high variability of scores. Speech intelligibility was within normal limits in *OPA1*-H patients, whereas a remarkable decrease in reception scores compared to controls was found for all but one of the *OPA1*-M subjects. R = right, L = left.

hearing-impaired controls for all PTA classes, except for Subject 10, who displayed normal scores. These findings indicate that the decrease in speech intelligibility in the *OPA1*-M group cannot solely be attributed to the increase of hearing threshold, as is the case for hearing-impaired subjects with cochlear hearing loss.

Distortion product otoacoustic emissions

Distortion product otoacoustic emissions were recorded from all but one of the *OPA1*-H patients, and from the *OPA1*-M group except for the two subjects showing profound hearing loss (Table 1 and Supplementary Table 2).

Auditory brainstem responses

ABRs were recorded from all *OPA1*-H subjects with normal latencies and morphology (Fig. 2 and Supplementary Table 2). In contrast, ABRs were absent in 6 of 10 subjects of the *OPA1*-M group (Table 1). Of the remaining patients, one had normal responses (Subject 10), whereas in three subjects (Subjects 1–3) only wave V was recorded from one ear with prolonged latency (Fig. 2 and Table 1).

Electrocochleography

Cochlear microphonic potentials were recorded from all the tested *OPA1*-M patients (Fig. 3). The responses proved to be significantly larger compared to both controls and subjects with cochlear hearing loss (Fig. 3). An enhancement of cochlear microphonic amplitude in patients with auditory neuropathy might result from decreased activity of the efferent system secondary to abnormal auditory nerve fibre activation (Santarelli and Arslan, 2002).

ECochG responses obtained after cochlear microphonic cancellation showed remarkable differences in comparison with subjects with normal hearing and patients with cochlear hearing loss. The waveforms recorded from two representative *OPA1*-M subjects are superimposed on the corresponding potentials obtained from one normally-hearing control and from one hearing-impaired child at stimulus intensities from 120 to 60 dB SPL in Fig. 4. In the normal control, the response begins with the receptor summating potential, which is believed to derive from inner hair cell activation (Durrant *et al.*, 1998). This is followed by the neural compound action potential, originating from the synchronous activation of auditory nerve fibres innervating the basal portion of the cochlea (Eggermont, 1976). Decreasing the stimulus level results in a gradual latency increase and amplitude reduction of both summating potential and compound action potential peaks. The duration of the summating potential–compound action potential

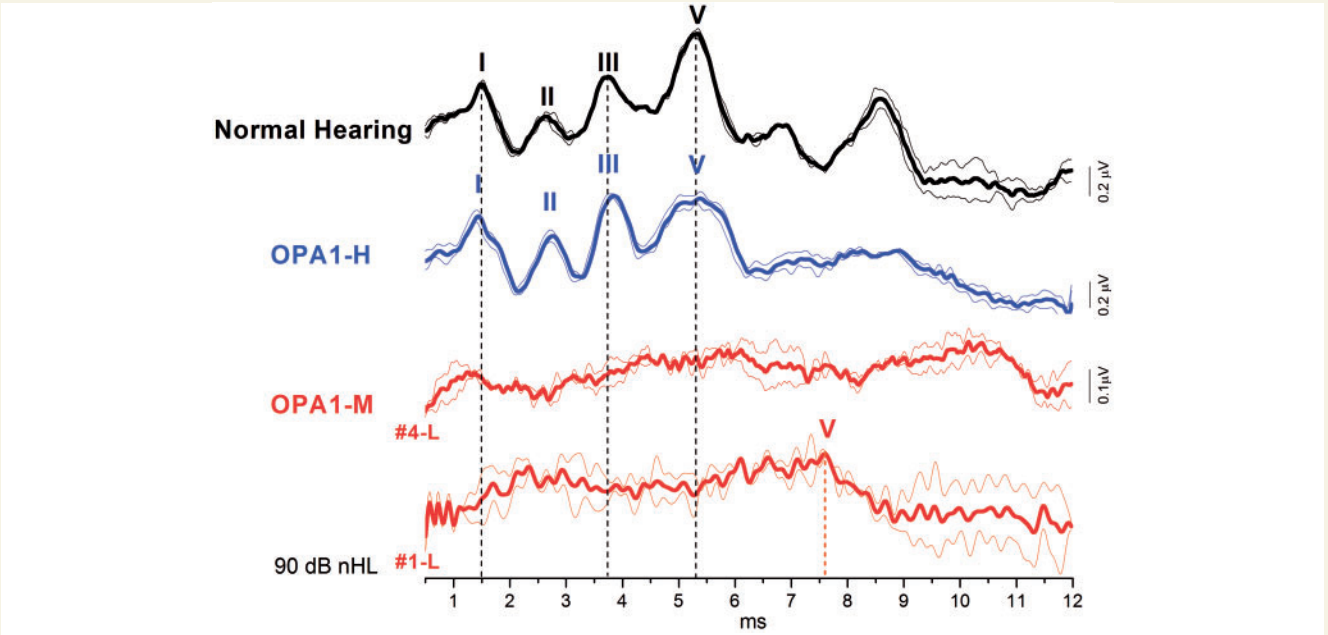

Figure 2 ABRs recorded at 90 dB nHL from one control with normal hearing and three subjects with DOA, one included in the *OPA1*-H and two in the *OPA1*-M group. ABRs were normal in the *OPA1*-H subject, whereas *OPA1*-M patients had no brainstem responses (#4-L) or showed a delayed wave V (#1-L). For each subject the thick line results from the average of individual recordings (thin lines). Vertical dashed black lines indicate wave I, III and V peaks in the control, while the vertical red line refers to wave V peak in *OPA1*-M Subject 1. L = left.

complex, as measured from initial negative deflection to return to baseline, is relatively constant at suprathreshold intensities but broadens at low stimulus level. The ECochG waveforms obtained from the subject with cochlear deafness showed comparable peak latencies and duration with respect to the control with normal hearing; however, the amplitude of both summating and compound action potentials was remarkably lower.

Two patterns of ECochG potentials were observed in the *OPA1*-M patients. In the most common pattern (10 of 14 ears, red line in Fig. 4), the response recorded at high intensity (120–100 dB SPL) began with a fast negative deflection, peaking at the same summating potential peak latency as in the normal control and showing a comparable amplitude. This was followed by a low-amplitude prolonged negative potential, which returned to baseline at ~8–9 ms from response onset. In the second pattern, which was found in a smaller sample (both ears in Subject 5, right ear in Subjects 2 and 3), at high intensity (120–100 dB SPL) only the prolonged potential was identified without the preceding summating potential component (blue line in Fig. 4).

At intensities lower than 100 dB, the prolonged potential was recorded for both ECochG patterns with increased peak latency and reduced amplitude compared to the compound action potential recorded from the normal control.

The means and standard errors of amplitude, latency and duration of ECochG potentials are plotted as a function of signal intensity in Fig. 4 for control subjects with normal hearing, hearing-impaired subjects and *OPA1*-M patients. The ANOVA results for these comparisons are summarized in Supplementary Table 3. Both amplitude and peak latency of the summating potential component calculated for the *OPA1*-M group was comparable with the corresponding values measured for control subjects with normal hearing. Compared with subjects with cochlear hearing loss, the ECochG responses from *OPA1*-M patients showed similar summating potential peak latencies but significantly larger summating potential amplitudes. The duration of the whole ECochG waveform, as measured from summating potential onset to return to baseline, was significantly prolonged in *OPA1*-M patients compared to the other two groups. Also the peak latency of the prolonged negative potential was significantly delayed in *OPA1*-M patients compared to the compound action potential latency calculated for both normally-hearing and hearing-impaired groups.

Differently from all other *OPA1-M* patients, Subject 7 showed only the cochlear microphonic potential without a superimposed negative activity at each stimulation intensity.

To clarify whether the prolonged potentials originate from neural or from receptor activation, we used an adaptation procedure that preferentially attenuates neural responses with minor changes in summating potential amplitude (Eggermont and Odenthal, 1974; Santarelli *et al.*, 2008). Figure 5 shows the recordings obtained at 100 dB SPL from one control subject with normal hearing and two representative *OPA1*-M patients in response to the click stimulation sequence reported at the bottom of the graph. Mean values of normalized amplitudes are reported in the right panel as a function of click position in the

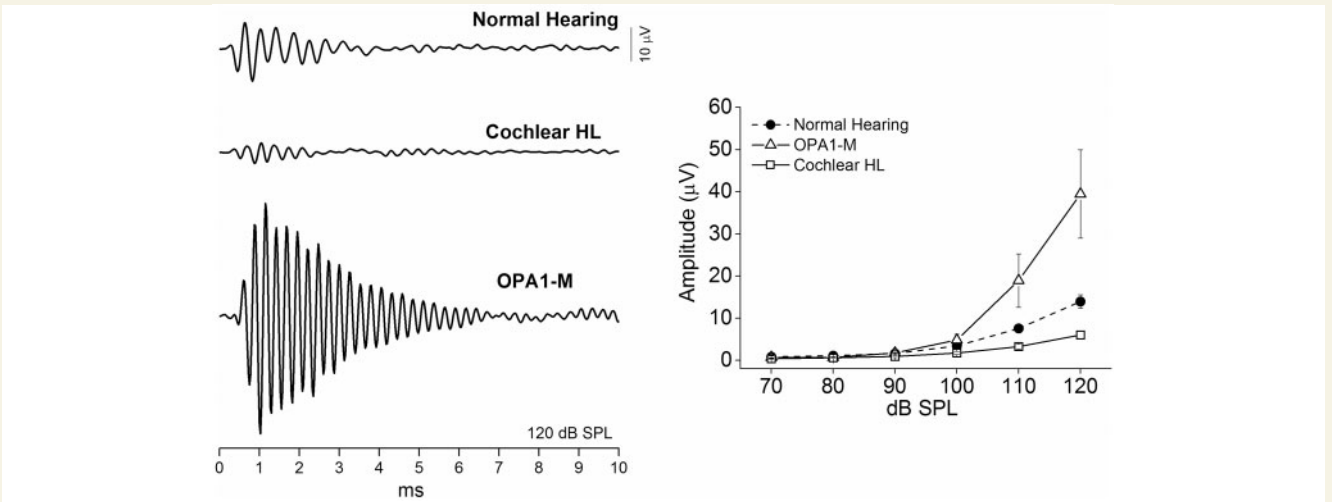

Figure 3 Cochlear microphonic potentials. *Left*: Cochlear microphonic potentials recorded at 120 dB SPL from one control with normal hearing, one hearing-impaired subject with cochlear hearing loss and one representative *OPA1*-M patient (Subject 7, left ear). *Right*: Mean cochlear microphonic amplitudes are reported as a function of stimulus intensity for the *OPA1*-M patients and for both normally-hearing and hearing-impaired subjects. Cochlear microphonic potentials recorded from *OPA1*-M patients are significantly larger compared to controls with normal hearing and hearing-impaired subjects with cochlear hearing loss (Cochlear HL).

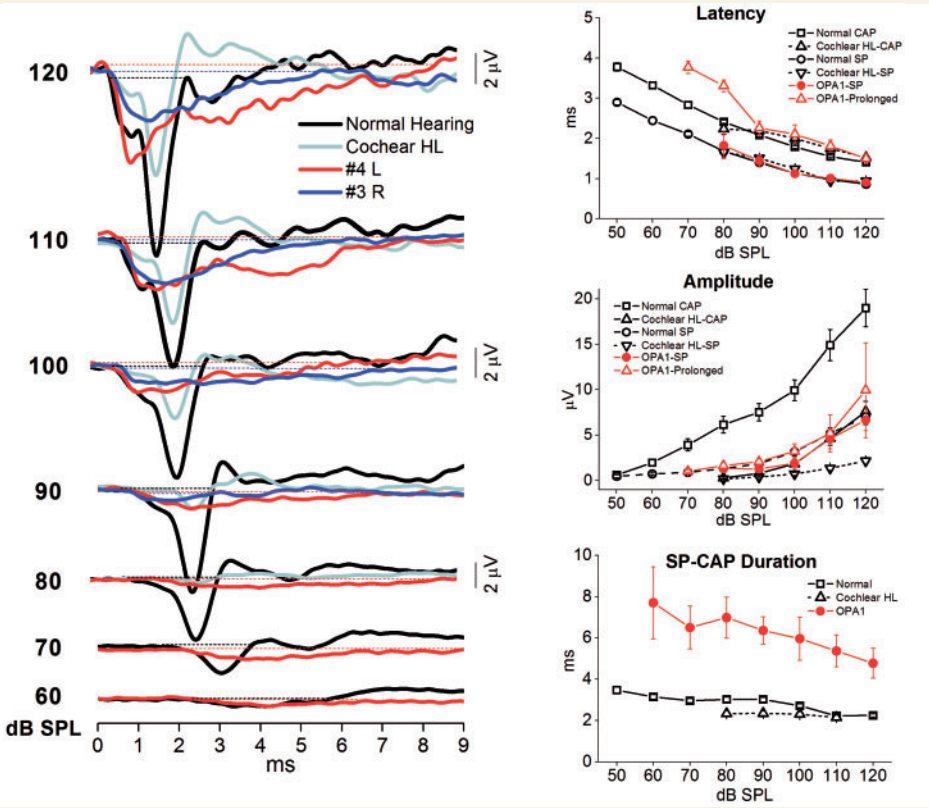

Figure 4 Cochlear potentials recorded from *OPA1*-M patients. *Left*: ECochG waveforms obtained after cochlear microphonic cancellation from two representative *OPA1*-M patients are superimposed on the corresponding responses recorded from one control with normal hearing and from one hearing-impaired child with cochlear hearing loss (Cochlear HL) at decreasing stimulus intensity. *Right*: Means and standard errors of summating potential (SP) peak latency and amplitude obtained from *OPA1*-M patients are superimposed on mean compound action potential (CAP) and summating potential amplitude and peak latency calculated for controls with normal hearing and hearing-impaired subjects with cochlear hearing loss. Mean amplitude and latency of the prolonged potentials (*OPA1*-Prolonged) are also reported. Mean response duration as calculated from onset to return to baseline (SP-CAP) is shown for *OPA1*-M subjects and for both normally-hearing and hearing impaired groups in the lower right corner. Time '0' refers to cochlear microphonic onset. In each recording the horizontal dashed line refers to baseline.

stimulus sequence for both controls with normal hearing and *OPA1*-M patients, superimposed on mean normalized summating potential amplitudes calculated for controls. In the normal controls, compound action potential amplitude was markedly attenuated after adaptation (61%), whereas summating potential attenuation was much lower (17%). Moreover, response duration as measured from summating potential onset to return to baseline was almost unchanged after adaptation. In *OPA1*-M patients, the prolonged response was markedly attenuated after adaptation, and the amount of peak amplitude attenuation was comparable with that of the normal compound action potential (52%). Moreover, a high stimulation rate reduced the duration of the response evoked by the last click in the stimulus sequence (range 2.1–3.5 ms) to the values seen in controls (range 1.9–3.4 ms) (Santarelli *et al.*, 2008). These findings point to a neural rather than a receptor origin for the generation of the prolonged negative potentials recorded from *OPA1*-M patients.

Aided thresholds and speech perception in implanted subjects

Aided thresholds were obtained from all implanted patients in the free-field at frequencies from 0.25 to 4 kHz. Hearing sensitivity was restored within 1 month of cochlear implant connection in all subjects (Table 1).

Open-set disyllable recognition scores were evaluated before cochlear implantation and after 1 year of cochlear implant use. Although there was considerable variation between subjects, scores significantly improved for all cochlear implant recipients in a quiet environment and in the presence of background noise, except for Subject 9 (paired *t*-test, $P < 0.01$). Speech recognition scores as evaluated in a quiet environment (Fig. 6) increased from 0% in the pre-implant condition to 50–90% after 1 year of cochlear implant use in four patients with mild-to-moderate hearing loss (Subjects 1, 2, 5 and 8) and in one subject (Subject 7) with profound deafness. In the remaining subjects

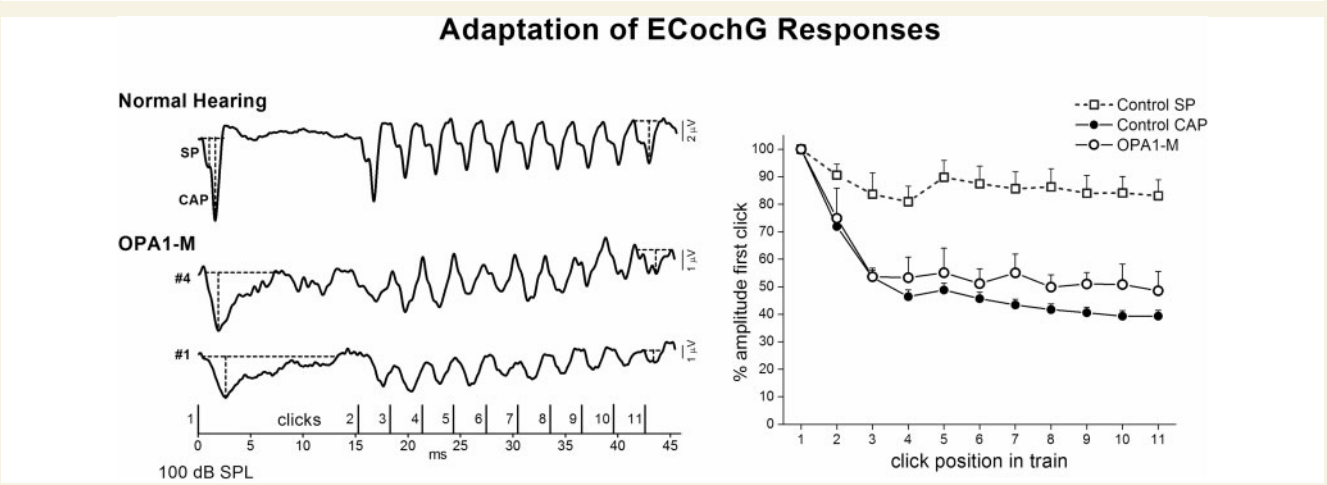

Figure 5 Adaptation of ECochG potentials in *OPA1*-M patients. ECochG recordings obtained at 100 dB SPL from one control with normal hearing and two *OPA1*-M patients in response to the stimulus sequence reported at the bottom are illustrated in the *left* panel. In the *right* panel the means and standard errors of normalized summating potential–compound action potential (SP-CAP) amplitudes are reported as a function of click position in the stimulus sequence for normally-hearing controls and *OPA1*-M patients superimposed on mean summating potential amplitudes calculated for controls. In *OPA1*-M patients the size of attenuation of cochlear potentials during adaptation was within the range of compound action potential attenuation calculated for controls. The vertical dashed lines indicate the summating potential and compound action potential peak in the control and the peak of the prolonged response in *OPA1*-M patients. Time '0' refers to cochlear microphonic onset. In each recording the horizontal dashed line refers to baseline.

(Subjects 3 and 4) the recognition scores increased from pre-implant values of 40–64% to 75–88% as evaluated after cochlear implantation. Overall, mean disyllable recognition scores measured in quiet increased from 16% in the pre-implant condition to 72% as evaluated after 1-year's experience with the cochlear implant. Differently from all others, Subject 9 had no improvement of speech perception with cochlear implant use (not shown).

In six patients speech perception was also evaluated in the presence of background noise at two different signal-to-noise ratios (+10, +5) (Fig. 6). For each level of noise, open-set recognition scores significantly increased after 1 year of cochlear implant use compared to the pre-implant condition ($P < 0.01$). Considering individual scores, all the *OPA1*-M patients improved performances when using the cochlear implant.

Electrically-evoked compound action potentials and auditory brainstem responses

Electrically-evoked compound action potentials were absent in all the implanted patients except for Subject 7, who showed the electrically-evoked neural response at each electrode location (Fig. 7).

Electrically-evoked ABRs were tested in six implanted patients (Table 1). The waveforms recorded from two subjects (Subjects 4 and 7) at decreasing current levels are shown for apical (n.20), intermediate (n.9, 13) and basal (n.6) electrodes in Fig. 7. In the most common pattern (Subject 4) electrically-evoked ABR recordings showed wave V, which was recorded with increasing latency from apical to basal electrodes (Fig. 7 and Table 1). For a given electrode location, decreasing current levels resulted in increased latencies and attenuated wave V amplitudes (Fig. 7). This response pattern was observed in three of six patients (Subjects 1, 4 and 8). In Subject 3, wave V was recorded in response to electrical stimulation only at the apical electrode location, whereas no brainstem responses were evoked in Subject 5 through the cochlear implant.

In *OPA1*-M implanted patients no acoustically-evoked ABRs had been obtained before cochlear implantation in the ear using the cochlear implant, except for Subject 3, who showed a markedly delayed wave V in response to acoustic stimulation.

In the patient with profound deafness (Subject 7), neural and brainstem potentials were recorded in response to electrical stimulation through the cochlear implant (Fig. 7). In this subject wave II was also identified in addition to wave V in the ABR recordings obtained at each electrode location (Firszt *et al.*, 2002).

Discussion

Our study demonstrates that the hearing dysfunction in *OPA1* patients is underlain by auditory neuropathy due to degeneration of auditory nerve fibres, and that electrical stimulation through the cochlear implant is able to improve hearing thresholds, speech perception, and synchronous activity in auditory brainstem pathways.

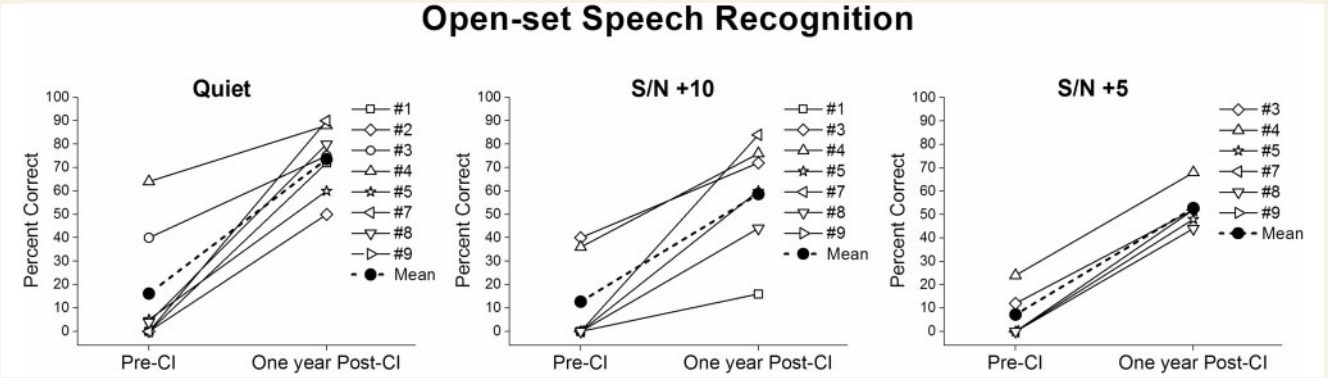

Figure 6 Speech perception scores obtained from *OPA1*-M patients using a cochlear implant. Individual and mean scores on open-set disyllable recognition test measured in quiet and in the presence of competing noise at two signal-to-noise (S/N) ratios (+10, +5) are reported for the pre-implant condition and within 1 year of cochlear implant (CI) experience. Speech perception improved in all *OPA1*-M patients after cochlear implantation except for Subject 9 (not shown).

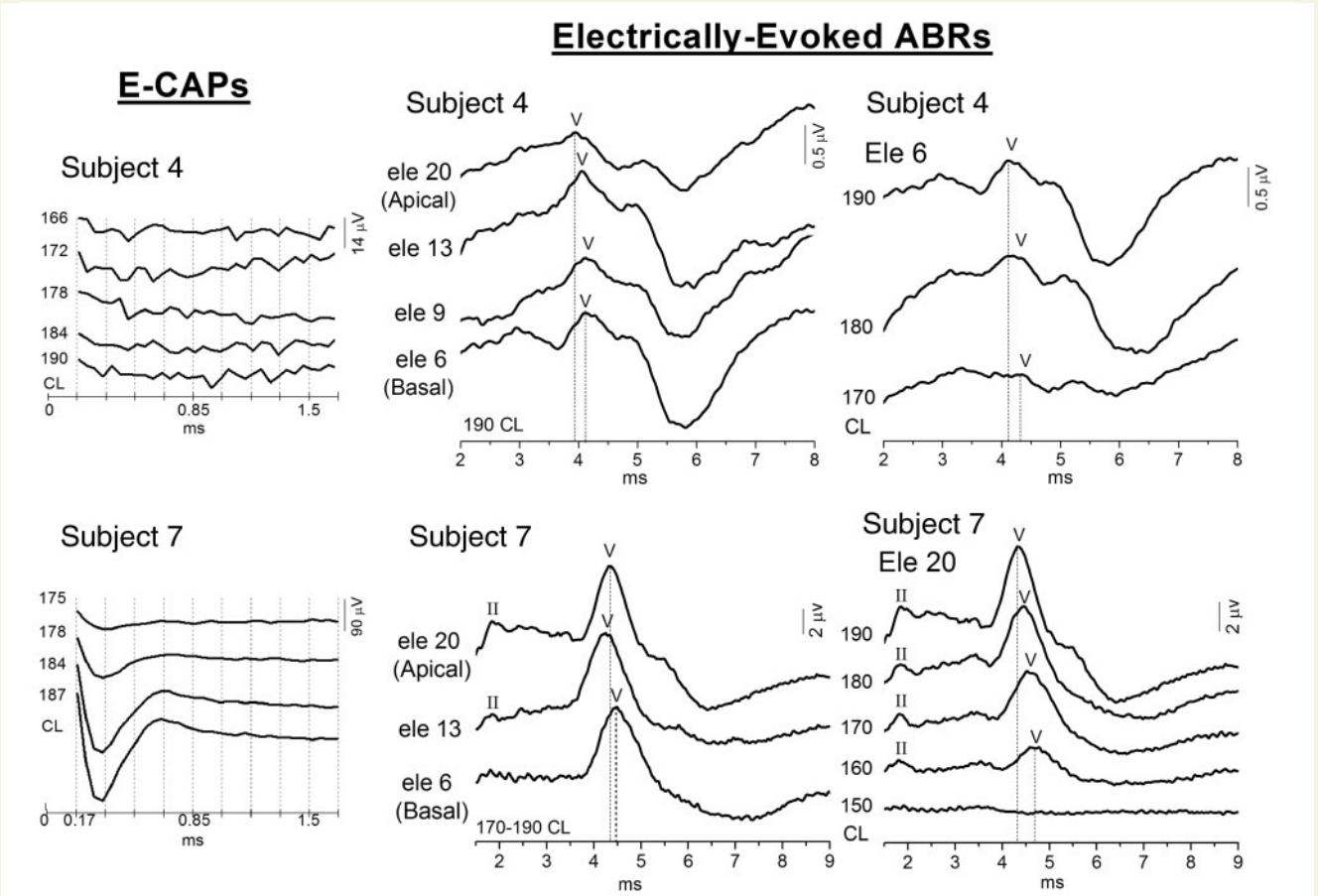

Figure 7 Electrically-evoked compound action potentials and ABRs from *OPA1*-M implanted patients. Electrically-evoked potentials from two representative subjects are displayed. In Subject 7 (*bottom*) both electrically-evoked compound action potentials (*left*) and electrically-evoked ABRs (*middle* and *right*) were recorded at all electrode locations; wave II was also identified in electrically-evoked ABR recordings in addition to wave V. No electrically-evoked compound action potentials were obtained from Subject 4, whereas electrically-evoked ABR wave V was recorded at all electrode locations. In both patients wave V was recorded with increasing latency from apical to basal electrodes (vertical dashed lines, *middle*). For a given electrode location, decreasing current levels resulted in increased latencies and attenuated wave V amplitudes (vertical dashed lines, *right*).

Previous studies have shown that mutations leading to haploinsufficiency are associated with a lower risk of developing the DOA + phenotype and hearing loss compared to missense mutations involving the GTPase domain (Yu-Wai-Man *et al.*, 2010; Leruez *et al.*, 2013). Overall, our results support this earlier observation as all but two *OPA1*-H patients showed normal hearing function. Of the two hearing-impaired subjects, one had a positive history of occupational noise exposure, whereas the second patient had otosclerosis. Thus, although our study included a limited series of patients, we suggest caution in causally relating any hearing impairment to the pathogenic *OPA1*-H mutations due to the possible coexistence of unrelated aetiologies.

All but one of the *OPA1*-M patients had hearing impairment. Of these, four subjects carried the R445H mutation. These findings confirm previous studies reporting that missense mutations occur in about two-thirds of patients with DOA +, and that R445H is by far the most frequent mutation in hearing-impaired patients (Yu-Wai-Man *et al.*, 2010; Leruez *et al.*, 2013). In this study, three novel missense mutations, p.S298N, p.A115V and p.R290Q, are reported for the first time in association with hearing impairment, and two of these, p.S298N and p.R290Q, affect the GTPase domain.

Only a few studies have reported the clinical profile of auditory neuropathy in patients harbouring mutations in the *OPA1* gene (Amati-Bonneau *et al.*, 2005; Huang *et al.*, 2009; Leruez *et al.*, 2013). Mild-to-moderate hearing loss with disproportionate impairment of speech perception, absent ABRs and presence of otoacoustic emissions have been reported by Amati-Bonneau *et al.* (2005) in three adult patients carrying the R445H mutation consistent with auditory neuropathy. In accordance with these data, the findings reported in our study point to auditory neuropathy as the pathophysiological mechanism underlying the hearing disorder in *OPA1*-M patients. First, an impairment of speech perception was observed in all but one subject of the *OPA1*-M group. This impairment was not related to the increase in hearing thresholds, as performance on speech audiometry was remarkably poorer compared to control subjects showing cochlear hearing loss and a comparable amount of hearing threshold elevation. Moreover, acoustically-evoked brainstem responses were absent or showed profound alteration in all *OPA1*-M patients irrespective of the severity of the hearing impairment. Third, otoacoustic emissions were detected bilaterally while cochlear microphonic was recorded through electrocochleography with normal or enhanced amplitude. All these findings point to disruption of auditory nerve discharge with preservation of outer hair cell function consistent with the occurrence of auditory neuropathy (Starr *et al.*, 1996, 2008).

In our previous study, transtympanic ECochG recordings performed in two related subjects with auditory neuropathy carrying R445H mutation showed that the compound action potential found in subjects with normal hearing was replaced by low-amplitude prolonged negative potentials, which have been interpreted as arising from abnormal activation of the terminal dendrites of auditory nerve fibres (Huang *et al.*, 2009; Santarelli, 2010). The current results extend these observations. First, in addition to the R445H mutation other *OPA1* mutations have been associated with the prolonged negative potentials found in ECochG recordings. More importantly, a rather uniform ECochG pattern emerged across subjects. In the majority of *OPA1* patients the ECochG response begins with an abrupt deflection with peak latency and amplitude falling within the range of summating potential latencies and amplitudes in controls with normal hearing, a picture consistent with preservation of inner hair cells.

The summating potential component is followed by prolonged low-amplitude negative potentials, replacing the synchronized compound action potential found in ears with normal hearing. The sensitivity of these potentials to rapid stimulation is consistent with their neural generation, thus indicating that they result from abnormal activation of degenerated auditory nerve fibres. Based on these findings, the hearing dysfunction found in *OPA1* patients could be considered essentially as a neural rather than a receptor disorder and thus qualified as 'post-synaptic auditory neuropathy' (Starr *et al.*, 2008). Specifically, the unmyelinated portion of auditory nerve terminals could be primarily involved by the degenerative process due to the high metabolic demand for spike conduction, as occurs for unmyelinated pre-laminar axons of the papillo-macular bundle in the optic nerve (Carelli *et al.*, 2004).

The ECochG pattern observed in *OPA1* patients appears profoundly different with respect to hearing-impaired subjects with cochlear hearing loss. In the latter, the morphology of ECochG waveforms is preserved as both summating potential and compound action potential had peak latencies and durations comparable to controls with normal hearing, whereas the amplitudes were remarkably smaller. This pattern is consistent with the lack of cochlear amplifier with consequent transition from the compressive behaviour found in normal hearing to the passive dynamics of a damaged cochlea. Differently from cochlear hearing loss, the distinctive feature of the ECochG waveforms recorded from *OPA1*-M patients is their prolonged duration reflecting dis-synchrony of auditory nerve firing. This might result from a limited probability of summation of the potentials arising from single auditory nerve fibres due to disturbances in spike initiation and conduction. These findings support the hypothesis that the hearing impairment found in *OPA1*-related auditory neuropathy reflects the alteration in temporal coding of acoustic information rather than the decrease of acoustic input due to the reduction of hair cell number.

In some ears, however, the cochlear potentials showed no clear separation between summating potential and the prolonged negative component. In these cases the coexistence of a lesion localized to inner hair cells cannot be ruled out. In addition, Subject 7 had profound deafness with absence

of both otoacoustic emissions and summating potential response in the ECochG waveform consistent with cochlear damage. The coexistence of neural and hair cell involvement in *OPA1* patients is in accordance with the expression of the *OPA1* protein in both outer and inner hair cells (Bette *et al.*, 2007). Nevertheless, as high density of mitochondria has been found only in spiral ganglion cells, it is plausible that auditory nerve fibres represent the most vulnerable site to *OPA1*-related lesions in auditory periphery.

Cochlear potentials recorded from *OPA1*-M patients were compared to ECochG data previously collected from children tested for presumed cochlear deafness. Bilateral transtympanic recording methods are part of our audiological evaluation of hearing disorders in children when the reliability of ABRs in hearing threshold estimation could be significantly reduced, possibly resulting from dysfunction or immaturity of brainstem generators of ABRs (Kraus *et al.*, 1984; Jiang *et al.*, 2008, 2011). Approximately 350 children have been tested by transtympanic ECochG at our department over the past 15 years. In some, the transtympanic results did not show objective evidence of a peripheral auditory disorder. Electrocochleography data from these subjects served, therefore, as normal hearing control data for comparison with the *OPA1*-M patients. In addition to normally-hearing controls, we extracted from the large sample a group of hearing-impaired subjects meeting specific inclusion criteria (genetic aetiology, compound action potential threshold between 80 and 100 dB SPL).

Although the controls were considerably younger than *OPA1*-M subjects, the age difference cannot be considered a major limitation (Santarelli *et al.*, 2008). Both amplitude and latency of the compound action potential peak recorded from children in response to click stimulation at several intensities were comparable to the corresponding values reported in other studies for normally-hearing and hearing-impaired adults (Eggermont, 1976; Noguchi *et al.*, 1999; Schoonhoven, 2007). This is in line with our knowledge of the timing of developmental maturation processes in the cochlea and auditory nerve. Indeed, the latency of ABR wave I, which reflects the synchronous activation of auditory nerve fibres, is comparable to adult values by 1–2 years of life (Eggermont *et al.*, 1991). Moreover, the rate-induced latency shifts of ABR wave I recorded from newborns show no, or only very slight, differences compared to adult values (Salamy *et al.*, 1978; Weber and Roush, 1993).

Two patients of the *OPA1*-M group had vertigo, which was reported as a disturbing symptom in Subject 3 and as occurring only occasionally in Subject 8. Caloric tests of vestibular function performed before cochlear implantation in Subject 3 revealed abnormally decreased velocity of the slow phase of nystagmus for the left ear consistent with decreased peripheral vestibular sensitivity. Bilateral vestibular hyporeflectivity was found in Subject 2, who has never complained of vertigo. Similar findings have been obtained in one Japanese patient harbouring the R445H mutation in the *OPA1* gene, who showed no response bilaterally on caloric testing in the absence of vestibular symptoms (Mizutari *et al.*, 2010). Impaired vestibular function has previously been reported in other patients with auditory neuropathy, particularly in those with a concomitant peripheral neuropathy, and attributed to degeneration of vestibular nerves (Starr *et al.*, 1996; Fujikawa and Starr, 2000; Sinha *et al.*, 2013). The lack of vestibular symptoms in these patients might reflect the slow rate of the degenerative process (Fujikawa and Starr, 2000).

The benefits of cochlear implantation in two *OPA1* patients were reported for the first time in our previous paper (Huang *et al.*, 2009). This study extends these observations, showing that cochlear implants constitute a viable therapeutic option in improving speech perception in patients with *OPA1*-related hearing impairment. First of all, hearing sensitivity was restored regardless of the degree of hearing loss. More importantly, speech recognition scores improved remarkably in quiet as well as in the presence of competing noise in all but one patient (Subject 9). In particular, the improvement of speech perception scores in the presence of background noise was striking, as one of the hallmarks of auditory neuropathy is the difficulty in understanding speech in noisy environments due to the impairment of temporal processing of acoustic signals in auditory nerve fibres (Zeng *et al.*, 2005; Starr *et al.*, 2008). Indeed, mean disyllable recognition scores as measured in the presence of competing noise at signal-to-noise ratio +5, increased from 7% in the pre-implant condition to 53% as evaluated after 1-year's experience with the cochlear implant.

Electrically-evoked responses in auditory nerve fibres (electrically-evoked compound action potentials) were absent in all but one (Subject 7) implanted *OPA1*-M patient. This finding, together with the detection of prolonged neural potentials in ECochG recording in the presence of normal cochlear receptor activities, points to degeneration of auditory nerve fibres as the primary damage underlying hearing dysfunction in patients with *OPA1* disease.

Differently from auditory nerve potentials, brainstem potentials were recorded in response to electrical stimulation in five of six implanted patients. Of these, four subjects showed ABR wave V in response to electrical stimulation at at least one electrode location. As the neural responses recorded at intracochlear electrodes are believed to be dominated by the activation of the terminal portion of auditory axons (Miller *et al.*, 2008), the detection of wave V in electrically-evoked ABR recordings in the absence of electrically-evoked neural responses supports the hypothesis that the hearing dysfunction in *OPA1*-M patients is underlain by degeneration of the distal portion of auditory nerve fibres, and that electrical stimulation through the cochlear implant evokes brainstem responses by bypassing the site of the lesion localized to the terminal dendrites. This hypothesis also fits in with the findings reported for a mouse model of *DOA* showing dendritic pruning of the optic nerve fibres at the very early stage of the disorder (Williams *et al.*, 2010).

Of the five *OPA1* patients who had electrically-evoked ABRs, the subject with profound deafness (Subject 7) also showed neural potentials (electrically-evoked compound action potentials) and both waves II and V in ABR recordings in response to electrical stimulation consistent with cochlear deafness. Nevertheless, it should be pointed out that this subject also showed a remarkable enhancement of cochlear microphonic amplitude in ECochG recordings as found in the other *OPA1* patients (Fig. 3).

Brainstem potentials were absent in response to electrical stimulation in Subject 5, while they were recorded only from the apical electrode location in Subject 3. As these patients were older compared to the other *OPA1* implanted subjects, we think that the duration of the disease could be a crucial prognostic factor in predicting the effectiveness of electrical stimulation in activating the auditory nerve fibres in *OPA1*-M subjects. Indeed, both demyelination and axonal loss affecting the entire auditory nerve have been described at an advanced stage of the *OPA1* disease (Kjer *et al*., 1983). Thus, it is reasonable to hypothesize that a possible involvement of more proximal portions of auditory nerve fibres in the progression of the disease results in decreased stimulating efficiency of the cochlear implant.

Although the improvement of speech perception with cochlear implant use may be related to the stimulation of preserved proximal portions of auditory fibres, there does not seem to be a straightforward correlation between improvement of speech perception scores with cochlear implant use and wave V detection in electrically-evoked ABRs. Indeed, in the *OPA1* patient (Subject 5) showing absence of electrically-evoked ABRs, speech perception scores considerably improved in both quiet and noise after 1-year's experience with the cochlear implant.

Overall, the results of cochlear implantation provide evidence of the effectiveness of cochlear implant use in improving speech perception in *OPA1*-M patients, and contribute to shedding light on the mechanisms and site of lesion of the primary degeneration affecting the auditory periphery.

In conclusion, we document that the hearing dysfunction affecting patients with mutations in the *OPA1* gene is underlain by degeneration of terminal dendrites at an early stage of the disease, whereas demyelination and axonal loss may become prevalent at an advanced stage. Cochlear implantation is a successful therapeutic option to improve speech perception. Further studies are needed to ascertain whether electrical stimulation through the cochlear implant can prevent further degeneration of auditory nerve fibres at an early stage of the disease.

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Supplementary material

Supplementary material is available at *Brain* online.

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