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ORIGINAL ARTICLE

Presynaptic and postsynaptic mechanisms underlying auditory neuropathy in patients with mutations in the OTOF or OPA1 gene

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Abstract
Objective: Our objective was to compare acoustically- and electrically-evoked potentials of the auditory nerve in patients with postsynaptic or presynaptic auditory neuropathy with underlying mutations in the OPA1 or OTOF gene. Study design: Transtympanic electrocochleography (ECochG) was recorded from two adult patients carrying the R445H OPA1 mutation, and from five children with mutations in the OTOF gene. Cochlear potentials to clicks or tone-bursts were compared to recordings obtained from 16 normally hearing subjects. Electrically-evoked neural responses recorded through the cochlear implant were also obtained. Results: The cochlear microphonic (CM) was recorded from all subjects, with normal amplitudes. After cancelling the CM, cochlear potentials were of negative polarity with reduced amplitude and prolonged duration compared to controls in both groups of patients. Prolonged negative responses were recorded as low as 50–90dB below behavioural threshold in subjects with OTOF mutations whereas in the OPA1 disorder the prolonged potentials were correlated with hearing threshold. A compound action potential (CAP) was superimposed on the prolonged activity at high stimulation intensity in two children with mutations in the OTOF gene while CAPs were absent in the OPA1 disorder. Electrically-evoked compound action potentials (e-CAPs) were only recorded from subjects with OTOF mutations following cochlear implantation. Conclusions: The findings are consistent with abnormal function of distal portions of auditory nerve fibres in patients carrying the OPA1 mutation whereas the low-threshold prolonged potentials recorded from children with mutations in the OTOF gene are consistent with abnormal neurotransmitter release resulting in reduced dendritic activation and impairment of spike initiation.

Key words: electrocochleography, auditory brainstem responses, cochlear implant, electrically-evoked neural response

Introduction
Auditory neuropathy (AN) is a disorder characterized by disruption of auditory nerve activity with preservation of outer hair cell (OHC) function (otoacoustic emissions, OAE, and/or cochlear microphonic, CM) (1). The disruption of auditory nerve discharge underlies both the absence of or profound alterations in auditory brainstem responses (ABRs) and severe impairment of speech perception. The impairment of auditory nerve function results from demyelination and axonal loss (postsynaptic AN) or lesions involving inner hair cells (IHCs) and/or the synapses with auditory nerve fibres (presynaptic AN) (1). The disorder has a wide range of aetiologies (e.g. genetic, infectious, toxic-metabolic, immunological); however, in half the patients no aetiological factors can be identified (1,2). It occurs in all age groups (1) and may be present in isolation or associated with multisystem involvement (1,3,4).

Among non-isolated AN disorders, mutations in the OPA1 gene are believed to cause disruption of auditory nerve discharge by affecting the unmyelinated portions of auditory nerve fibres (5). The OPA1 gene encodes a mitochondrial GTPase involved in the regulation of oxidative phosphorylation (6), mitochondrial fusion (7), and apoptosis (8). Patients carrying OPA1 mutations present with slowly progressive loss of visual acuity, with underlying atrophy of optic nerve fibres; two-thirds also show hearing
impairment possibly associated with the clinical picture of AN (5,9). Studies performed on an OPA1-deficient mouse model have shown that early lesions involve the terminal unmyelinated portions of retinal ganglion cells (10). Moreover, the restoration of speech perception after cochlear implantation in two patients with the R445H mutation has led to the hypothesis that the lesion must be localized to the distal portion of auditory nerve fibers (postsynaptic AN) (5).

Mutations of the OTOF gene result in a very homogeneous phenotype of prelingual, profound hearing loss associated with the absence or marked threshold elevation of ABRs and the presence of OAEs, which have been found in over 50% of subjects (11). The OTOF gene encodes otoferlin, a transmembrane protein belonging to the ferlin protein family, which contains several repeating C2 domains involved in calcium binding (12). Studies performed in mice indicate that otoferlin plays a crucial role in vesicle release at the synapse between IHCs and auditory nerve fibers by interacting with syntaxin1 and SNAP25 (13). In addition, otoferlin involvement in vesicle replenishment at the presynaptic membrane has been suggested (14). On the basis of these findings it can be hypothesized that a presynaptic disorder underlies AN in patients carrying OTOF mutations.

Abnormalities of auditory nerve fiber discharge underlain by presynaptic or postsynaptic AN cannot be evaluated by far-field recording techniques such as of ABR due to the low signal-to-noise ratio (15). Both receptor (cochlear microphonic, CM; summating potential, SP) and auditory nerve (compound action potential, CAP) activities can be recorded using a near-field recording technique such as transtympanic electrocochleography (ECoG) (15). Our previous studies have shown that ECoG recordings to click stimulation obtained from patients with OPA1 (5) or OTOF (16) mutations consist of low-amplitude prolonged responses originating from abnormal activation of auditory dendrites.

In this study we compared the cochlear potentials to click or tone-burst stimulations in patients with postsynaptic or presynaptic AN, with underlying mutations of the OPA1 or OTOF genes, respectively. In addition, electrically-evoked neural response recordings obtained through the cochlear implant were obtained.

**Material and methods**

**Subjects**

Two subjects carrying the R445H mutation in the OPA1 gene, a mother (M) (47 years of age) and daughter (D) (21 years of age), were affected by reduction in visual acuity associated with optic atrophy since the age of nine years. Speech comprehension difficulties were first experienced at about the same age by the daughter, but by the mother in her early twenties. At the time of evaluation, speech perception was severely impaired and relied on visual cues. The details of clinical, neurological and audiological evaluation are reported in a previous paper (5). Both patients underwent cochlear implantation (right side in the daughter, left side in the mother) resulting in restoration of speech perception (5).

Five subjects carrying the OTOF mutations were children (three male and two female, ranging in age from 12 to 20 months), and showed congenital profound hearing loss with absent ABRs and presence of OAEs. Three of the children, two brothers and a sister, were compound heterozygous for mutations c.2732_2735dupAGCT and p.Ala964Glu; one subject was homozygous for mutation p.Phe1795Cys, and one was compound heterozygote for mutations c.1609delG in exon 16 and c.1966delC in exon 18 (16). Four of the children received a cochlear implant and showed almost normal language development within one year of cochlear implant use.

**Electrocochleography (ECoG)**

ECoG recordings were performed under general anaesthesia in children and local anaesthesia in adults. Recordings were obtained by means of a needle electrode placed on the promontory wall with the aid of an operating microscope.

Stimuli consisted of 0.1-ms rarefaction and condensation clicks, separately delivered in the sound field with a maximum intensity of 120dB p.e. SPL (corresponding to 90dB nHL, with reference to the psychoacoustical threshold of normally-hearing subjects). The stimulus was calibrated in the sound field by means of a Brüel and Kjaer 4165 microphone (mounted on an 800 B Larson-Davis sound level meter) placed at 1 m from the base of the polyurethane horn, corresponding to the distance of the patient’s ear from the horn. The procedure of comparing the peak-to-peak click amplitude to the peak-to-peak amplitude of a 2-kHz tone was utilized to calibrate the click level (p.e. SPL). The stimulus paradigm consisted of an initial click, followed 15 ms later by 10 clicks with an inter-stimulus interval of 2.9 ms. This sequence was repeated every 191 ms. This particular stimulus paradigm was used to help distinguish CAP and SP potentials by taking advantage of different effects of adaptation induced by high stimulation rates on these responses (15,17).

Tone-bursts (1 ms rise-decay time, 10 ms plateau, 151 ms inter-stimulus interval) with a maximum
intensity of 100dB SPL were used in three patients, two carrying the OPA1 mutation and one child with OTOF mutations.

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 CM and extract the CAP with the superimposed SP. The resulting curve was subtracted from the potential evoked by condensation clicks to obtain the CM (15).

Cochlear potentials recorded from all patients were compared to the ECochG data previously collected from 16 normal hearing children (mean 3.7 years, range 3.5–6.5 years). They underwent ECochG testing for presumed cochlear deafness but proved to have normal cochlear function with normal thresholds when evoking neural and receptor potentials.

Latency was defined relative to CM onset in milliseconds (ms). Amplitude was computed relative to the period 1 ms before CM onset in microvolts (μV). Values contained in the text indicate mean ± standard error.

Since in the majority of patients the SP was followed by a slow neural potential without an identifiable CAP, we indicated the waveform obtained after CM cancellation as the SP-CAP complex, and defined the SP-CAP onset at the initial negative deflection arising from baseline and the SP-CAP end at the return to baseline (15). Analogously, both amplitude and duration changes during adaptation were evaluated on the SP-CAP as a whole and were compared to the corresponding values obtained from controls. Since the SP and CAP could not be identified separately, the amplitude attenuation during adaptation was calculated at the latency of the CAP in controls.

Electrically-evoked compound action potential (e-CAP) and auditory brainstem (e-ABR) recordings

The electrically-evoked compound action potential (e-CAP) was obtained by utilizing the Cochlear Corporation Custom Sound EP software. Stimulation consisted of a train of alternate polarity, biphasic, 25-μs width per phase pulses presented at 80 Hz. Stimulation mode was MP1 + 2. The evoked electrical activity was recorded two electrodes apart.

e-ABRs were only tested on one patient with the OPA1 mutation (D) by using biphasic pulses, 50 ms width per phase, which were presented at 35 Hz. Stimulation mode was MP1. Potentials were recorded from scalp electrodes (vertex to mastoid contralateral to the stimulated ear). Signals were differentially amplified (50,000 times), filtered (10–4000 Hz) and digitized (25 μs) for averaging (1000 trials).

Results

Both adult patients carrying the OPA1 mutation had a moderate hearing loss (pure tone average PTA threshold levels at 0.5, 1, 2 and 4 kHz were in the 41–70dB HL range according to the European Concerted Action Project on Genetics of Hearing Impairment, 1996).

ABRs were absent in the right ear and showed a delayed wave V in the left ear (wave V latency M-Left (L), 7.5 ms; D–L, 6.9 ms). OAEs direction products were obtained from both ears while ECochG recordings showed normal amplitudes of the CM (mean control amplitude at 120dB p.e. SPL 13.40 ± 1.92 μV, mean values from OPA1 patients 10.55 ± 1.30 μV).

Cochlear potentials obtained at 120dB p.e. SPL after CM cancellation from the left ear of both OPA1 patients are shown in Figure 1, together with the grand average of corresponding control recordings with 95% confidence limits (shadowed area). In controls, cochlear potentials show an initial fast SP deflection followed by the CAP which returns to baseline by 2.4 ms. The waveforms obtained in the OPA1 disorder consisted of the SP appearing at the same latency as in controls, which was followed by a sustained negative potential returning to baseline by 12 ms. This response was identified at intensities close to the audiometric threshold (Figure 3)

In order to assess whether the prolonged responses were neural or receptor potentials, we used a neural adaptation paradigm aimed at distinguishing between neural and receptor potentials by taking advantage of the different effects of adaptation induced by high stimulation rates (15,17). In controls, the CAP amplitude as measured at 110dB from the first to the eleventh click, was attenuated by 70% (68.3 ± 2.3%) during adaptation whereas SP attenuation was much lower, averaging 27% (27.2 ± 3.6%) (compare the response to the first and eleventh click in the waveforms from controls reported in the left and middle upper panels in Figure 1). No change in response duration was found in controls (mean change from the first to the eleventh click at 110dB p.e. SPL −0.08 ± 0.10). In contrast, the potentials recorded from patients with the mutation in the OPA1 gene were reduced in both amplitude (amount of attenuation at 110dB p.e. SPL for the right ear M, 8%; D 57%; amount of attenuation at 110dB p.e. SPL for the left ear M 59%; D 50%) and duration (change in duration at 110dB p.e. SPL for the right ear M 0.1 ms; D 2.3 ms; change in duration at 110dB p.e. SPL for the left ear M 1.9 ms; D 13 ms) after adaptation. The amount of response attenuation was within the range of the mean attenuation calculated for the whole SP-CAP complex in controls (56.7 ± 2.2%). However, recordings obtained from one ear (M-R) failed to show changes in either amplitude or duration after adaptation.
Figure 1. Cochlear potentials recorded from patients carrying R445H mutation in the OPA1 gene in response to a high intensity click stimulus (120dB SPL). Recordings obtained from the left ear of mother (M-L) and daughter (D-L) are superimposed on the mean potentials obtained by averaging all control responses to the first (non-adapted responses, left graphs) and the last three (adapted responses, middle graphs) clicks of the stimulus sequence. The shadowed area refers to 95% confidence limits in controls. The ECochG responses recorded from the OPA1 subjects show no CAPs and consist of the SP followed by a low-amplitude negative potential with increased duration compared to controls. On the right side the responses evoked by the first (black line) and eleventh (grey line) click of the stimulus sequence at 110dB SPL are superimposed. Changes in amplitude were measured at the latency of the CAP in controls (circles). The SP-CAP complex was decreased in both amplitude and duration after adaptation. The amount of response attenuation was comparable to that found in controls. The horizontal dashed lines extend from the SP-CAP onset to return to baseline; time ‘0’ refers to CM onset in this and in the subsequent figures.

Cochlear potentials to tone-burst stimuli at frequencies ranging from 1 to 8 kHz were recorded from only one patient (mother, right ear). The waveforms obtained at 100dB p.e. SPL are superimposed on the corresponding traces recorded from one control in Figure 4 (left panel). CAPs were clearly identified in the control whereas in the patient with the OPA1 mutation the recorded traces mainly consisted of a DC-coupled component, e.g. the SP.

CM potentials recorded from patients with OTOF mutations were of normal amplitude (mean control amplitude at 120dB p.e. SPL 13.40 ± 1.92 μV, mean values from OTOF patients 14.10 ± 3.84 μV).

ECochG waveforms obtained after CM cancellation followed three main patterns (Figure 2, upper panel). In the first pattern the SP (subjects 4, 5 right ear) was followed by a small CAP, and both responses were superimposed on a low-amplitude prolonged negative potential returning to baseline by 10–12 ms. The second pattern (subjects 2, 5 left ear) showed a normal SP that was followed by the sustained low-amplitude activity without a superimposed CAP. Only a prolonged negative response peaking approximately at the same latency as the CAP in controls was identified in the third pattern (subject 3). Moreover, whatever the response pattern, the prolonged responses were identified as low as 60dB SPL, which is 90dB below behavioural threshold (Figure 3).

When comparing the response to the eleventh click with that of the first click of the sequence (Figure 2, lower panel), both amplitude (mean attenuation in amplitude 57.17 ± 2.68%) and duration (mean change in duration 5.69 ± 1.10 ms) of the adapted responses appeared reduced in all but one subject (subject 1). The amount of response attenuation was within the range of the mean attenuation calculated for the whole SP-CAP complex in controls (56.7 ± 2.2%).

Cochlear potentials evoked by tone-burst stimulation were recorded from both ears of one patient with OTOF mutations (subject 3). Waveforms obtained from the right ear at frequencies from 1 to 8 kHz are superimposed in Figure 4 (right panel) on the corresponding traces recorded from one control, the latter
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Figure 2. Cochlear potentials from patients with mutations in the OTOF gene. In the upper panel the responses recorded from three patients (black line) at 120 dB p.e. SPL are superimposed on the mean potentials obtained by averaging all control responses (grey line). Three patterns of ECochG waveforms can be identified. In the lower panel the responses evoked by the first (black line) and eleventh (grey line) click of the stimulus sequence at 110 dB SPL have been superimposed. Changes in amplitude were measured at the latency of the CAP in controls (circles). Both amplitude and duration of the SP-CAP responses were markedly decreased after adaptation.

displayed on a different time scale. The potentials from the OTOF patient were of low amplitude, peaked about 1 ms later than the CAP in control, and were identified as low as 70–80 dB p.e. SPL (motgh over).

Electrically-evoked compound action potentials (e-CAPs) were tested in all the implanted subjects through their cochlear implant (Figure 5). One example is reported in Figure 5 for OPA1 (D-R) and OTOF

Figure 3. Cochlear potentials from patients with mutations in OPA1 or OTOF gene at decreasing stimulation intensities. In both subjects the SP was followed by a slow negative potential returning to baseline by 12 ms. However, the threshold of this prolonged response was close to the hearing threshold in the patient with OPA1 disorder whereas in subjects with OTOF mutations the sustained potential was identified 60 dB below the audiometric threshold.
Electrically-evoked compound action potentials (e-CAPs)

Figure 5. Electrically-evoked compound action potentials (e-CAPs) from two implanted patients (Nucleus C124RE with Freedom processor) with OPA1 (D-R) or OTOF (subject 2, right ear) mutations. Individual waveforms recorded at increasing stimulation intensities (from 180 to 220 CL) by stimulating three different electrodes are displayed (basal 3, intermediate 10, apical 21). No responses were recorded from the OPA1 subject whereas e-CAPs with typical morphology were obtained from the child carrying OTOF mutations.

Discussion

We recorded acoustic- and electrically-evoked cochlear potentials from patients with auditory neuropathy with underlying mutations in the OPA1 or OTOF gene.

Detection of OAEs and CMs with normal amplitudes in all patients points to a preserved function of OHCs in both disorders. The ECochG waveforms obtained after CM cancellation from both groups of patients appeared as negative deflections markedly prolonged in duration and reduced in amplitude compared to controls. The prolonged negative activity was sensitive to a neural adaptation paradigm consistent with a neural origin of the prolonged responses (15). Nevertheless, we found several differences between the two disorders, which possibly indicates that different mechanisms and sites of lesion underlie abnormal activation of auditory nerve fibres. In previous studies we hypothesized that the prolonged negative potentials recorded from patients with OPA1 (5) or OTOF (16) mutations resulted from abnormal activation of auditory nerve terminals. Specifically, in patients with OPA1 mutations disturbances of spike initiation and slowing of conduction velocity were believed to result from a lesion involving the auditory nerve fibres themselves (5). This hypothesis is consistent with studies on animal models showing that the early lesion in OPA1 involves the terminal unmyelinated portions of optic nerve fibres (10). Moreover, the restoration of speech
perception after cochlear implantation suggests that
the lesion should be localized to the auditory nerve
dendrites since it could be by-passed by electrical
stimulation through the cochlear implant (5). Fur-
ther support to the hypothesis that the lesion should
be confined to the distal portion of auditory nerve
fibres is given by the findings reported in this study
showing that no CAPs were recorded in response to
electrical stimulation while ABR was restored after
cochlear implantation.

The cochlear responses obtained from the right
ear of one patient with OPA1 mutation did not
change either in amplitude or duration during adap-
tation. This result is consistent with the prolonged
responses originating from receptor rather than neu-
ral sources (15). Accordingly, cochlear potentials to
tone-burst stimulation recorded from the same ear
consisted mainly of a DC-coupled response showing
the same amplitude as the SPs in controls.

Mutations in the OTOF gene are likely to result
in a presynaptic disorder since the product of this
gene, otoferlin, is known to be involved in neurotrans-
mitter release (13) and vesicle replenishment at
the presynaptic site in IHCs (14). Analogous to
patients with the OPA1 mutation, prolonged negative
responses were recorded from children with muta-
tions in the OTOF gene.

However, Differently from patients with the OPA1
mutation, the prolonged negative responses were
recorded from children with mutations in the OTOF
gene at intensities that were well below the hearing
threshold (Figure 3). These potentials seem analo-
gous to the dendritic responses recorded by Sellick
et al. (18) from the scala tympani of guinea pigs after
administration of tetrodotoxin to block neural spik-
ing in terminal dendrites of auditory nerve fibres.
Moreover, a CAP was superimposed on the pro-
longed negative activity at high stimulation intensity
in two patients. On the basis of these findings it can
be hypothesized that the prolonged responses
recorded at intensities below the hearing threshold
may reflect local dendritic activation. This may be
followed by spike initiation for some ears at high
stimulation intensity leading to CAP recording
whereas at low intensity, dendritic activation fails to
evoke a synchronized neural activation. Abnormal
dendritic activation, in turn, may result from reduc-
tion and alteration in timing of the neurotransmitter
release. Therefore, unlike those patients with the
OPA1 mutation, auditory nerve terminals should
retain their function in children with mutations in the
OTOF gene. This hypothesis is further supported
by the detection of electrically-evoked neural
responses (e-CAPs), which were recorded through
the cochlear implant from all patients with OTOF
mutations.

Cochlear potentials evoked by tone-burst stimula-
tion from the child with OTOF mutations showed the
prolonged negative potential without a superimposed
SP or CAP component. These prolonged potentials
were of low amplitude, peaked 1 ms later than the CAP
in controls, and were identified at intensities as low as
50–90dB below behavioural threshold. These responses
may also result from the sum of local dendritic poten-
tials, which fail to trigger spike initiation or synchro-
nized activation of auditory fibres.

We conclude that in subjects with the OPA1
mutation, lesions localized to the distal portion of
auditory nerve fibres underlie AN, whereas abnormal
synaptic release results in abnormal dendritic activa-
tion and disruption of auditory nerve discharge in
children with mutations in the OTOF gene.

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