Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines

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Summary

Leber hereditary optic neuropathy (LHON) is a maternally inherited form of retinal ganglion cell degeneration leading to optic atrophy which is caused by point mutations in the mitochondrial genome (mtDNA). Three pathogenic mutations (positions 11778/ND4, 3460/ND1 and 14484/ND6) account for the majority of LHON cases and they affect genes that encode for different subunits of mitochondrial complex I. Excitotoxic injury to retinal ganglion cells and the optic nerve has been previously hypothesized, especially given the high susceptibility of this neural cell type to glutamate toxicity. Osteosarcoma-derived cytoplasmic hybrids (cybrids) generated from six unrelated LHON patients, two cell lines for each pathogenic mutation, were compared with cybrids obtained from three healthy controls. Molecular and biochemical analyses showed that excitatory amino acid transporter 1 (EAAT1)/GLAST is the most active glutamate transporter in this cellular model. The glutamate uptake maximal velocity was significantly reduced in all LHON cybrids compared with control cybrids. This reduction was correlated in a mutation-specific fashion with the degree of mitochondrial production of reactive oxygen species, which is enhanced in LHON cybrids. Our findings support the hypothesis that the genetically determined mitochondrial dysfunction in LHON patients leads to impaired activity of the EAAT1 glutamate transporter. This observation is particularly relevant since EAAT1 is the major means of glutamate removal in the inner retina and this prevents retinal ganglion cells being damaged as a result of excitotoxicity.

Keywords: Leber hereditary optic neuropathy; glutamate transport; EAAT1; cybrids; reactive oxygen species

Abbreviations: cybrid = osteosarcoma-derived cytoplasmic hybrid; DHK = dihydrokainic acid; EAAT = excitatory amino acid transporter; LABH = L-aspartate-β-hydroxamate; LHON = Leber hereditary optic neuropathy; Km = glutamate uptake affinity; mtDNA = mitochondrial DNA; ROS = reactive oxygen species; Vmax = glutamate uptake maximal velocity; SOS = L-serine-O-sulphate; THA = L(-)-threo-3-hydroxyaspartic acid.


Introduction

Leber hereditary optic neuropathy (LHON), the first disease to be associated with point mutations in the mitochondrial DNA (mtDNA), has been recently estimated as the most frequent mitochondrial disease (Wallace et al., 1988; Man et al., 2003). LHON is characterized by sub-acute, bilateral loss of central vision due to degeneration of retinal ganglion cells and their axons (Newman, 1998; Carelli, 2002). Three mtDNA pathogenic mutations at positions 11778, 3460 and 14484 are
found in the vast majority of LHON cases. A few differences in clinical expression are related to the specific mutation—the 14484 being unequivocally associated with the most favourable visual outcome. The 11778, 3460 and 14484 mutations affect ND4, ND1 and ND6 subunit genes, respectively, of complex I (NADH-ubiquinone oxidoreductase), the first site of the mitochondrial respiratory chain (Chalmers and Schapira, 1999).

The biochemical effect of these mutations has been extensively studied in different patient-derived tissues and cellular experimental models. However, the results of biochemical investigations in LHON remain controversial (Brown, 1999; Howell, 2003). While the 3460/ND1 mutation induces a severe defect of complex I activity, the 11778/ND4 and 14484/ND6 mutations are apparently less severe (Larsson et al., 1991; Majander et al., 1991; Carelli et al., 1997; Cock et al., 1999). Moreover, a consistent decrease of respiration with NAD-linked substrates was shown in isolated mitochondria, with 11778/ND4 and 3460/ND1 being the most severely affected (Majander et al., 1991). These results were reproduced in cytoplasmic hybrid (cybrid), where only the mutant mitochondria from the patient are transferred into an immortalized host cell line, previously devoid of its own mtDNA (Vergani et al., 1995; Hofhaus et al., 1996; Brown et al., 2000). Another set of experiments, exploring complex I function with different inhibitors (Degli Esposti et al., 1994; Carelli et al., 1999), suggested an altered interaction of complex I with its quinone substrate, coenzyme Q, as a common feature of LHON pathogenic mutations (Carelli et al., 2002). Thus, besides the variably deficient electron transfer through complex I, an increased production of reactive oxygen species (ROS) may play a relevant role (Wong et al., 2002). Notably, mitochondria are the major intracellular source of ROS and a recent study has shown that most of the superoxide radicals are produced at complex I in brain mitochondria (Kudin et al., 2004).

Despite the effort to dissect the molecular basis of complex I defects in LHON, the pathophysiology of retinal ganglion cell degeneration remains poorly understood. Excitotoxicity refers to neuronal death caused by over-activation of excitatory amino acid receptors. Glutamate acts as the major excitatory neurotransmitter and, concurrently, represents a potential neurotoxin for the CNS. The maintenance of physiological levels of extracellular glutamate, which is necessary to prevent excitotoxicity, is prominently mediated by a family of high-affinity excitatory amino acid transporters (EAATs) (reviewed by Beretta et al., 2003). Administration of glutamate to neonatal mice resulted in degeneration of the inner retinal layers (Lucas et al., 1957; Olney, 1969), including the same cell type affected in LHON. Although excitotoxic injury has been postulated as a putative concurrent pathogenic mechanism in LHON (Howell, 1998), no experimental evidence has been provided so far to support this hypothesis.

In the present study, we investigated the specific effect of the three LHON pathogenic mutations on glutamate transport—in relation to the bioenergetic and oxidative status—using cybrid cell lines. The osteosarcoma acceptor cell line was chosen because it may represent a suitable model for studying the role of excitotoxicity in the pathophysiology of LHON, since preliminary data indicated that such cells functionally express EAAT1 as their major EAAT—as occurs in the neuroretina.

**Methods**

**Cell lines and culture conditions**

Cybrid cell lines were constructed using enucleated fibroblasts from three controls and six LHON probands as mitochondria donors, and the osteosarcoma (143B.TK⁻)–derived 206 cells as acceptor rho⁰ cell line (143B.TK⁻ and rho⁰ 206 cell lines were kindly provided by Giuseppe Attardi and Michael King). The choice of the parental cell line was determined by our preliminary results showing that it expresses EAAT1 as the dominant, functional glutamate transporter (see Results and Fig. 1). This finding is of particular relevance for LHON, in light of the report of EAAT1 as being the major glutamate transporter.
transporter in Müller astrocytes (Rauen et al., 1996). To avoid possible confounding effects due to different functional profiles of different batches of 143B.TK-, the parental cell line used for these experiments belonged to the same batch from which the rho0/206 cell line was developed, and was kept in cycling culture for a comparable length of time. It is therefore unlikely that these cells present significant divergence in the nuclear genome from the cybrids considered in this study. Both definition of the mtDNA haplogroup and identification of the LHON pathogenic mutations were performed by the PCR/restriction fragment length polymorphism method carried out as described previously (Danielson et al., 2002; Torroni et al., 1997). Parental (143B.TK-) and cybrid cell lines were grown in Dulbecco’s modified eagle’s medium (DMEM) medium supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, 1% non-essential amino acid solution, 1% minimal essential medium (MEM) vitamins solution and 0.1 mg/ml bromodeoxyuridine at 37°C in an incubator with a humidified atmosphere of 5% CO2. The medium was changed 2–3 times per week and always on the day of the experiment.

Glutamate transporter expression
EAAT1, EAAT2 and EAAT3 mRNA levels were assayed by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated by using RNeasy kits (Qiagen, Milano, Italy). The following amplification primers were used: EAAT1 (forward, bp 649–667; reverse, bp 1,112–1,133; GenBank accession no. U03504); EAAT2 (forward, bp 638–656; reverse, bp 1,002–1,023; GenBank accession no. U03505); EAAT3 (forward, bp 651–672; reverse, bp 1,088–1,107; GenBank accession no. U03506). β-actin was used as the internal standard. Imaging densitometry was employed to evaluate cDNA signals.

Glutamate transporter expression was also assayed by western blot analysis. Cells were sonicated in ice-cold phosphate-buffered saline (PBS) containing 50% (w/v) sucrose, 1 mM EDTA, 5 mM EGTA, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mM antipain and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). Cell extracts (~40 μg of proteins for each cybrid line) were separated by 7.5% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked, incubated with affinity-purified anti-EAAT1, anti-EAAT2 or anti-EAAT3 antibodies, and then with horseradish peroxidase-linked secondary antibodies. Signals were revealed by chemiluminescence, visualized on X-ray film, and protein expression was evaluated using an imaging densitometer.

High-affinity sodium-dependent glutamate uptake
Cell monolayers were harvested with 5% trypsin and cultured in a non-transportable unselective EAAT5 inhibitor; (ii) l-serine-O-sulphate (SOS), a transportable EAAT1/EAAT3 inhibitor; (iii) l-aspartate-b-hydroxamate (LABH), a transportable, EAAT3-partially selective inhibitor; (iv) dihydrokainic acid (DHK), a non-transportable EAAT2-selective, non-transportable inhibitor. Details of the pharmacological properties of the glutamate uptake inhibitors used in this study are discussed by Bridges et al. (1999).

Measurement of intracellular ATP levels
ATP was determined luminometrically (Victor2 1420 multilabel counter, Wallac, Boston, USA) using an ATP Bioluminescence Assay Kit (Sigma, St.Louis, MO, USA) according to the provided protocol. Briefly, cells cultured in flasks were trypsinized, collected and incubated with lysis buffer. The aliquots of cellular extracts were assayed for ATP content using the ATP dependency of the light-emitting luciferase-catalysed oxidation of luciferin. ATP nanomoles were calculated from a freshly prepared standard curve and related to total protein content, assessed using Bradford’s method.

Mitochondrial ROS levels
The dye dihydrorhodamine-123 (RH-123) was used to quantify levels of mitochondrial ROS. RH-123 is an oxidation-sensitive lipophilic dye that enters mitochondria and fluoresces when oxidized by ROS—in particular superoxide anions and peroxynitrite—to the positively charged rhodamine-123 derivative (Lievre et al., 2001). Cells were cultured in flasks and incubated for 40 min in Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 2.3 mM CaCl2, 5.6 mM glucose, 5 mM HEPES, 1.2 mM MgCl2, pH 7.4) containing 10 mM RH-123 and washed twice in dye-free Locke’s buffer. Cells were trypsinized, collected and centrifuged. Fluorescence was quantified using a Cary Eclipse fluorimeter (Varian, Palo Alto, CA, USA), with excitation at 488 nm and emission at 525 nm, after resuspension of the cells in 3 ml of Locke’s buffer containing 0.5% Triton X-100. The values of rhodamine-123 fluorescence were related to total protein content and represented on a graph. For some experiments, cells were cultured on coverslips and observed using a Radiance 2100 confocal microscope (Biorad, Hercules, CA, USA) (excitation 488 nm, emission 515–540 nm).

Statistical analysis
All results are expressed as mean ± SD. One-way ANOVA (analysis of variance), followed by Tukey’s multiple comparison test, was used to assess the significance of differences between values for cybrid cell lines. Correlation was computed with Pearson’s r test.
**Results**

**Glutamatergic properties of human 143B.TK⁻ osteosarcoma cells**

Preliminary studies were carried out with the parental cell line, 143B.TK⁻, to assess whether the major glutamate transporters and receptors typical of brain plasma membranes were expressed and functionally active in this experimental model. RT-PCR analyses (Fig. 1A) showed that this human osteosarcoma cell line naturally expresses mRNAs for the three glutamate transporters tested, with a relative abundance of sarcoma cell line expressing mRNAs for the three glutamate transporters and receptors typical of brain plasma membranes were expressed and functionally active in this experimental model.

When exposed to L-glutamate (0.1–1 mM) or the glutamate receptor agonists N-methyl-D-aspartate (NMDA, 100–250 μM) and kainate (50–200 μM), no cell death was detected in osteosarcoma cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or lactate dehydrogenase assays after 24 h, even at high concentrations. This suggested that this cell line is fully insensitive to excitotoxicity (data not shown).

**Glutamate transport is defective in LHON cybrids**

Glutamate uptake was investigated in three control and six LHON cybrid cell lines as used in previous studies (Ghelli et al., 2003). Two different cell lines for each LHON pathogenic mutation were tested (see Table 2). All cybrid cell lines displayed a high-affinity, sodium-dependent glutamate uptake that fitted a hyperbolic kinetic curve (see Fig. 2A–D and Table 2). Control cybrids showed a higher glutamate uptake rate (mean Vmax = 5793 ± 729 pmol/mg protein/30 min) compared with parental cells. All LHON cybrid cell lines showed a reduction of Vmax compared with both control cybrids and parental cell line, clustering in pairs according to the carried mutations (ANOVA P < 0.0001). Cybrids bearing the 14484/ND6 mutation showed a reduction of 60% compared with control cybrids (mean Vmax = 2327 ± 326 pmol/mg protein/30 min; P < 0.01 versus control group), while the 11778/ND4 determined a

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**Table 1** Effect of non-selective and selective glutamate transporter inhibitors on glutamate uptake in osteosarcoma 143B.TK⁻ cells

<table>
<thead>
<tr>
<th>Glutamate transporter inhibitors</th>
<th>Glutamate uptake rate (60 μM), % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>THA (500 μM)</td>
<td>1</td>
</tr>
<tr>
<td>SOS (200 μM)</td>
<td>6</td>
</tr>
<tr>
<td>LABH (130 μM)</td>
<td>80</td>
</tr>
<tr>
<td>DHK (250 μM)</td>
<td>97</td>
</tr>
</tbody>
</table>

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**Table 2** LHON mutations, mtDNA haplogroups and glutamate uptake values of the cybrid cell lines utilized in the present study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LHON mutation</th>
<th>mtDNA haplogroup</th>
<th>Km</th>
<th>Vmax</th>
<th>Vmax (mean ± SD)</th>
<th>Vmax (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>None</td>
<td>H</td>
<td>60.80</td>
<td>6822</td>
<td>5793 ± 729</td>
<td>100</td>
</tr>
<tr>
<td>HPS</td>
<td>None</td>
<td>T</td>
<td>59.81</td>
<td>4514</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGA</td>
<td>None</td>
<td>J</td>
<td>58.98</td>
<td>6044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFF</td>
<td>11778</td>
<td>U</td>
<td>43.74</td>
<td>1952</td>
<td>1903 ± 158</td>
<td>32.8***</td>
</tr>
<tr>
<td>HPE</td>
<td>11778</td>
<td>J</td>
<td>57.54</td>
<td>1854</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL180</td>
<td>14484</td>
<td>J</td>
<td>67.01</td>
<td>2050</td>
<td>2327 ± 326</td>
<td>40.1***</td>
</tr>
<tr>
<td>HBA</td>
<td>14484</td>
<td>J</td>
<td>53.15</td>
<td>2604</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMM</td>
<td>3460</td>
<td>H</td>
<td>59.13</td>
<td>1646</td>
<td>1324 ± 269</td>
<td>22.8***</td>
</tr>
<tr>
<td>RJ206</td>
<td>3460</td>
<td>T</td>
<td>58.39</td>
<td>1003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mean Vmax values for both the control group and the LHON mutation subgroups (11778, 14484 and 3460) are shown as absolute value and percentage of control cells. ANOVA P < 0.0001, followed by Tukey’s multiple comparison test. **P < 0.01 versus control group. ***P < 0.001 versus control group.
decrease of nearly 70% (mean Vmax = 1903 ± 158 pmoles/mg protein/30 min; P < 0.01 versus control group). The effect of the 3460/ND1 mutation was the most severe, causing a glutamate uptake impairment of nearly 80% compared with control cybrids (Vmax = 1324 ± 269 pmoles/mg protein/30 min; P < 0.001 versus control group). No gross differences in affinity (Km) were detected among the various cell lines. RT-PCR and western blot analyses were carried out to assess whether the decreased glutamate uptake rate in LHON cybrids was due to altered expression of EAAT1. No difference in EAAT1 mRNA content (data not shown) or immunoreactivity (Fig. 3), following normalization to the β-actin signal, was detected among the various cell lines. Similar results were obtained for EAAT3 mRNA and immunoreactivity (data not shown). The difference in mtDNA haplogroup, in particular haplogroup J, did not show any clear influence on glutamate transport activity.

### Glutamate uptake and whole-cell bioenergetic status

Total intracellular ATP content was assessed in control and LHON cybrids under basal conditions (see Fig. 4). A significant decrease of ATP levels was observed for cybrids bearing the 3460/ND1 mutation (48% reduction, compared with control cybrids, P < 0.01). On the other hand, the 11778/ND4 and 14484/ND6 mutant cybrids did not show any significant energetic failure compared with the control cybrid group. These results were similar to those obtained from independent sets of experiments performed by others (A. Baracca and C. Zanna, personal communication). When tested for linear regression, ATP levels for each single cybrid line did not correlate with the glutamate uptake Vmax (P = 0.11, r² = 0.31, data not shown).

**Fig. 2** Glutamate uptake studies in cybrid cell lines. Glutamate uptake kinetics (extracellular glutamate concentration from 1 to 120 μM) of single cybrid cell lines from (A) control subjects and LHON patients bearing (B) 11778, (C) 14484 and (D) 3460 mutations. For each point of the cumulative curve, the data from three separate experiments were averaged.

**Fig. 3** Representative EAAT1 western immunoblot in cybrid cell lines. No difference in EAAT1-like immunoreactive protein content was shown among control and LHON cybrid cell lines: lane 1 = HPC (control); lane 2 = HPS (control); lane 3 = HFF (11778 mutation); lane 4 = HL180 (14484 mutation); lane 5 = HMM (3460 mutation); lanes 6–7 = positive control: rat cerebellum homogenate (~5 and ~2.5 μg of proteins, respectively).
Glutamate uptake strongly correlates with mitochondrial free radical generation

Mitochondrial-derived ROS production was evaluated with the fluorochrome RH-123 (see Fig. 5A, C and D). A significant increase in RH-123 fluorescence was observed for all the LHON cybrid cell lines. In particular, cybrids bearing the 3460/ND1 mutation showed the highest ROS levels (264% compared with control cybrids), followed by the 11778/ND4 (212%) and 14484/ND6 mutations (190%). A strong negative correlation was established between mitochondrial ROS production and glutamate uptake Vmax (P = 0.001, r² = 0.77) for each cybrid cell line, as represented in Fig. 5B.

To strengthen the hypothesis for a causative role of ROS in the observed impairment of EAAT1-mediated glutamate uptake, the parental cell line was challenged for 4 h with increasing concentrations of hydrogen peroxide (Fig. 6A) or the selective complex I inhibitor rotenone (Fig. 6B). After the incubation, glutamate uptake was measured as previously described. Both hydrogen peroxide and rotenone caused a dose-dependent decrease in the glutamate uptake rate compared with untreated cells.

Discussion

Despite the well-established genetic aetiology of LHON, relatively little is known about the molecular mechanisms that link complex I mutations to retinal ganglion cell degeneration (Carelli et al., 2004). Excitotoxicity has been hypothesized to be a concurring factor, based on the well-known sensitivity of retinal ganglion cells to injury mediated by over-activation of NMDA-type glutamate receptors (Luo et al., 2001). Nevertheless, no experimental evidence has so far been provided to document this hypothesis. The present study reports a novel finding in LHON research, demonstrating for the first time that LHON-associated mtDNA mutations negatively affect the activity of EAATs, in particular EAAT1 [glutamate-aspartate transporter (GLAST) in mice]. Our results suggest that the reduced activity of EAAT1, which varies among the different mtDNA mutations, might be the biochemical consequence of an oxidative modification of the transporter, caused by mitochondria-derived ROS. Thus, it is tempting to speculate that retinal Müller astroglial cells, which mostly rely on EAAT1

Fig. 4 Total intracellular ATP content expressed as nmoles/mg protein, of control and LHON cybrid cell lines under basal conditions (see Results). Data were obtained from nine separate experiments. **P < 0.01 compared with control cybrids.

Fig. 5 Mitochondrial production of ROS and correlation with glutamate uptake rate. (A) Mitochondrial ROS production, expressed as RH-123 fluorescence units/µg cell protein of control and LHON cybrid cell lines under basal conditions (see Results). Data were obtained from three separate experiments. **P < 0.01 compared with control cybrids. ***P < 0.001 compared with control cybrids. (B) A strong (P = 0.001, r² = 0.77) negative correlation was observed between mean mitochondrial ROS levels (RH-123 fluorescence/µg cell protein) and the mean glutamate uptake Vmax (pmoles/mg protein/30 min) in single cybrid cell lines, after linear regression analysis. RH-123 fluorescence was also imaged in cybrid lines using a laser scanning microscope (600 × ) to further confirm the mitochondrial source of ROS production as is shown for (C) control (HPC) and (D) 3460 mutant (RJ206) cells.
for glutamate clearance from the synaptic cleft, would remove glutamate less efficiently in LHON patients. This would imply a toxic rise of extracellular glutamate in the inner retina, which may contribute to the degenerative process affecting retinal ganglion cells.

We have investigated expression and function of glutamate transporters in cybrids obtained from six LHON patients and three healthy subjects. This cellular model is designed to study the cellular changes induced by mutant mtDNA dissected from the original nuclear background, given that only mitochondria from patients or controls are used to repopulate by cell fusion the perennial osteosarcoma-derived rho0 cell line (206), previously devoid from its own mtDNA (King and Attardi, 1989). The resulting fusion cybrids express the exogenous mtDNA using the ‘neutral’ nuclear background of the host parental osteosarcoma (143B.TK−) cell line, which was found to express naturally all three of the major EAATs. In particular, our data indicate that glutamate transport in 143B.TK− cells is mostly mediated by EAAT1. This finding is not surprising, since it has already been reported that EAAT1/GLAST is expressed and exerts a metabolic role in bone tissue whose osteosarcoma cell line represents a malignant variant (Mason et al., 1997; Itzstein et al., 2000). Interestingly, EAAT1/GLAST is the same transporter that accounts for most glutamate removal in the neuroretina by Müller astroglial cells (Rauen et al., 1996). In addition, the observed glutamate transport Km and Vmax values in osteosarcoma cells are of the same order of magnitude of those described in Müller glia in culture (Gonzalez et al., 1999). A direct link between EAAT1 function and excitotoxicity in retinal tissue was reported by Harada et al. (1998), who showed that degenerative changes in the inner retinal layers after ischemic injury and glutamate toxicity were more severe in EAAT1-deficient mice compared with wild-type mice.

The cybrid cell system represents a convenient experimental model to study the effect of mtDNA point mutations, which affect complex I function, on EAAT1-mediated glutamate transport. The 3460/ND1 mutation, which consistently lowers complex I activity (Brown, 1999) and is associated with a severe neuropathology (Sadun et al., 2000), also resulted in a drastic decrease in glutamate transport. Furthermore, the 14484/ND6 mutation, which is associated with a benign visual prognosis and normal complex I activity (Carelli et al., 1999), had the mildest effect. The 11778/ND4 mutation, which is the most common worldwide and is associated with the poorest visual outcome, showed an intermediate impairment in glutamate transport. Despite the degree that glutamate uptake impairment mirrors the known effect of single LHON mutations on complex I activity, it does not correlate exactly with the clinical severity of the disease, suggesting that other independent pathways might operate synergistically in the development of the overall clinical picture. The presence of the J haplogroup in the mtDNA background, which has been associated with the 11778/ND4 and 14484/ND6 mutations (Torroni et al., 1997), did not seem to influence the glutamate uptake rate. We refer in particular to the direct comparison of HPE/HFF cybrid lines, carrying both the 11778/ND4 mutation but differing in their haplogroup, J and U, respectively.

EAAT1 expression did not show any change in LHON cybrids with respect to controls, suggesting that glutamate transporter molecules are synthesized correctly. Several recent studies showed that EAATs possess specific redox-sensing elements, consisting of cysteine residues, which regulate the transport rate by thiol-disulphide redox interconversion (Trotti et al., 1998). The rate of glutamate uptake is maximal when the reactive cysteine residues are in the reduced state and minimal when they are in the oxidized state, without any change in the affinity for glutamate itself (Km). This regulatory mechanism probably reflects variation in transporter conformation. Hydrogen peroxide and xanthine oxidase activation generate ROS, which rapidly and irreversibly inhibit glutamate uptake in astrocyte cultures (Volterra et al., 1994). Peroxynitrite, formed by the combination of superoxide and nitric oxide, potently inhibits glutamate

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Fig. 6 Glutamate uptake sensitivity to hydrogen peroxide and rotenone. Glutamate uptake Vmax values for the parental cell line 143B.TK− in untreated cells and in cells exposed to (A) hydrogen peroxide (100–500 μM, 4 h) or to the (B) the complex I specific inhibitor rotenone (20–500 nM, 4 h). Data were obtained from three separate experiments. ** P < 0.01 compared with untreated cultures. *** P < 0.001 compared with untreated cultures.
uptake by purified or recombinant high affinity glutamate transporters reconstituted in liposomes (Trotti et al., 1996). Lipids are especially vulnerable to oxidative stress and products of lipoperoxidation, such as acrolein and 4-hydroxy-2-nonenal, are able to reduce glutamate uptake (Lovell et al., 2000). Thus, increased ROS generation from LHON mitochondria seems to represent a likely culprit for the observed inactivation of glutamate transporters. Preliminary studies on optic nerve and retinal histological specimens obtained from LHON patients indicate an increased staining for nitrotyrosine, possibly affecting the astroglial cellular component at the optic nerve head, and thus providing indirect evidence that the proposed pathway might be active in LHON (Carelli et al., 2000).

In line with this hypothesis, we have shown that the impairment of glutamate uptake correlates strongly with mitochondrial ROS production in the single cybrid cell lines. Remarkably, an increase in hydroperoxide and mitochondrial-derived superoxide production with no changes in mitochondrial membrane potential were reported recently in neuronal-like LHON cybrids carrying the 11778/ND4 and 3460/ND1 mutations that were obtained using the neuronal precursor cell line Ntera-2/D1 (Wong et al., 2002). On the contrary, no correlation was observed between the glutamate uptake rate and total ATP content. Energy production was assessed as total cellular ATP content, which is known to be almost unchanged in cybrids containing non-LHON mtDNA mutations when cultured in a complete medium (Gajewski et al., 2003). On the other hand, when assaying ATP synthesis specifically driven by complex I substrates, a drastic decrease was shown in LHON cybrids (A. Ghelli, personal communication). As far as glutamate transporters are concerned, it is interesting to note that, even in mtDNA mutants, the subcellular concentration of ATP is preserved in the cytosolic and subplasma membrane compartments—those compartments that fuel the activity of glutamate transporters—while being depleted in the mitochondrial matrix (Gajewski et al., 2003).

Lactate production has not yet been investigated in our cybrid cell lines. Although it is reasonable to speculate that LHON mutations might lead to lactic acid overproduction due to altered oxidative phosphorylation, this is unlikely to have biased glutamate uptake measures in this study, as the culture medium was changed frequently and the uptake experiments were conducted in a pH-controlled buffer.

Rotenone, a selective inhibitor of complex I, was used as a pharmacological tool to investigate the effect of altered complex I activity on glutamate transport. Rotenone shares with LHON mutations the toxic properties of both inhibiting complex I activity and promoting the production of mitochondrial-derived ROS (Genova et al., 2003; Kudin et al., 2004). In particular, one study showed that rotenone both reduces cell respiration and induces a dose-dependent oxidative damage in 143B.TK- cells within the same concentration range tested in the present study (Barrientos et al., 1999). The detrimental effect of both hydrogen peroxide and rotenone on EAAT1-mediated transport further confirms the sensitivity of glutamate transporters to increased ROS production and this occurs either as a result of external stimuli or from mitochondrial sources.

Although LHON mutations are usually homoplasmic and occur systemically, most patients report visual failure as the only symptom, raising the question of the specific vulnerability of the optic nerve. Conversely, it is noteworthy to recall that a number of patients with LHON have been described to have associated neurological abnormalities such as movement disorders, multiple sclerosis-like illness, polyneuropathy, epilepsy and dementia (Nikoskelainen et al., 1995), indicating a potentially widespread neurodegenerative process, which might be the manifestation of an excitotoxic damage enhanced by unknown factors.

Multiple observations support the view that retinal ganglion cell death occurs preferentially via apoptosis under various physiological and pathological conditions, and this may apply to LHON. For example, surplus retinal ganglion cells die during development through an apoptotic pathway (Martinou et al., 1994) and optic nerve axotomy induces retinal ganglion cell apoptosis in animal models (Rabacchi et al., 1994). In the acute phase of LHON, the absence of overt signs of inflammation at the examination of the fundus oculi has been suggested to argue against a necrotic type of cell death (Howell et al., 1997). Moreover, two recent studies showed respectively an increased sensitivity of LHON cybrids bearing the 11778/ND4 and 3460/ND6 mutations to Fas-induced apoptosis (Danielson et al., 2002), and the rapid induction of a mitochondrial-dependent apoptotic death in LHON cybrids carrying primary LHON mutations when cells were forced to use oxidative metabolism for ATP synthesis by replacing glucose with galactose in the medium (Ghelli et al., 2003).

There is increasing evidence that mitochondrial dysfunction and activation of NMDA channels may both contribute to neuronal apoptosis (Schinder et al., 1996). Our findings indicate that interaction between mitochondrial dysfunction and excitotoxicity may also occur in LHON. The impaired glutamate uptake points to the possible relevant role of Müller astrogial cells, which express EAAT1 as their major glutamate transporter, in promoting excitotoxic injury to retinal ganglion cells. Although these findings need more in-depth study, this working hypothesis might open new opportunities for the prevention or early treatment of LHON subjects and families with currently available anti-glutamatergic drugs.

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