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Cathepsin D deficiency underlies congenital human neuronal ceroid-lipofuscinosis

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Congenital neuronal ceroid-lipofuscinosis (NCL) is a devastating inherited neurodegenerative disorder of unknown metabolic basis. Eight patients with this rare disorder, all with similar clinical and neuropathological findings, have been reported, and here we describe two further patients. Previously, we showed that a mutation in the cathepsin D gene causes congenital NCL in sheep. On the basis of the neuropathological and ultrastructural similarities between the sheep and patients affected with congenital NCL, we screened the cathepsin D gene for mutations in a patient of Pakistani origin. We identified a nucleotide duplication, c.764dupA, in the cathepsin D gene in homozygous form in the patient, and in heterozygous form in his father. This duplication is likely to be disease-causing, as it creates a premature stop codon, predicting a truncation of the protein. When transiently expressed in cell cultures, the mutant protein was enzymatically inactive, but stable. In paraffin-embedded brain tissue samples of two affected siblings of the Pakistani patient, cathepsin D was absent, suggesting rapid degradation of the c.764dupA mutant cathepsin D at mRNA or protein level *in vivo*. Further, we were able to confirm lack of cathepsin D in the brain tissue of yet another, unrelated, patient of English origin with congenital NCL. On the basis of the present data, and the nearly identical clinical and/or pathological phenotype of the other reported cases of congenital NCL, it is reasonable to suggest that cathepsin D deficiency caused by mutations in the corresponding gene may underlie all cases of congenital NCL. The present observations also suggest that cathepsin D deficiency should be considered as a possible diagnosis in microcephalic neonates, who present with seizures at or before birth.

Keywords: lysosomal storage disorder; molecular biology; mutation; neuronal degeneration; newborn infant

Abbreviations: BHK = baby hamster kidney; CTSD = cathepsin D; GFAP = glial fibrillary acidic protein; NCL = neuronal ceroid-lipofuscinosis

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Introduction

Congenital neuronal ceroid-lipofuscinosis (congenital NCL, earlier called congenital amaurotic idiocy) is a devastating storage disorder of unknown metabolic basis. This disease represents the earliest-onset and the most aggressive form of the NCL, leading to extreme brain atrophy and death soon after birth (for reviews, *see* Barohn *et al.*, 1992; Kohlschütter and Lake, 1999). The NCLs are a genetically heterogeneous group of lysosomal storage disorders characterized by progressive neurodegeneration, and they collectively constitute the most common cause for children's progressive encephalopathies (for reviews, *see* Haltia, 2003; Tyynelä, 2005). Congenital NCL was first described in 1941 (Norman and Wood, 1941), and since then seven further cases of congenital NCL have been reported, including both male and female offspring of both consanguineous and non-consanguineous marriages (Brown *et al.*, 1954; Sandbank, 1968; Humphreys *et al.*, 1985; Garborg *et al.*, 1987; Barohn *et al.*, 1992). In two of these families, there

was more than one affected child with identical clinical history and/or neuropathological findings in a large family of otherwise healthy siblings and parents, and thus autosomal recessive inheritance has been considered likely (Brown *et al.*, 1954; Sandbank, 1968).

Congenital NCL presents clinically with post-natal respiratory insufficiency and status epilepticus, and death occurs within hours to weeks (Norman and Wood, 1941; Brown *et al.*, 1954; Sandbank, 1968; Humphreys *et al.*, 1985; Garborg *et al.*, 1987; Barohn *et al.*, 1992). The patients are microcephalic, with extremely small brains, weighing only 65–200 g instead of the normal 385 g after 40 weeks gestation. Typically, there is a subtotal loss of neurons in the cerebral cortex, as well as generalized activation of astrocytes and microglia. The white matter appears to lack myelin, and the cerebellum is extremely atrophic (Sandbank, 1968; Humphreys *et al.*, 1985; Garborg *et al.*, 1987; Barohn *et al.*, 1992). Most cells of the central nervous system are loaded with autofluorescent storage bodies, showing a granular ultrastructure, the so-called granular osmiophilic deposits (GRODs; Humphreys *et al.*, 1985; Garborg *et al.*, 1987; Barohn *et al.*, 1992).

Previously, an active site mutation (corresponding to human p.Asp295Asn) in the ovine cathepsin D (*CTSD*) gene was identified as a cause of congenital NCL in sheep (Tyynelä *et al.*, 2000). Newborn lambs with congenital NCL are severely affected at birth and die within a few days (Järplid and Haltia, 1993; Tyynelä *et al.*, 2000). Neuropathological findings in these sheep closely resemble those reported in patients with congenital NCL, including not only the extreme neuronal loss and glial activation but also the presence of storage deposits with granular ultrastructure (Humphreys *et al.* 1985; Garborg *et al.*, 1987; Barohn *et al.*, 1992; Tyynelä *et al.*, 2000). The storage deposits in affected sheep show accumulation of sphingolipid activator proteins, but not of mitochondrial ATP synthase subunit c (Tyynelä *et al.*, 2000). Recently, another naturally occurring CTSD deficiency was reported in American bulldogs with a late-onset, fatal NCL (Awano *et al.*, 2005). The underlying mutation (p.Met199Ile) results in relatively high residual enzyme activity (Awano *et al.*, 2005). In addition, targeted disruption of the *Ctsd* gene results in an early-onset NCL phenotype and progressive neurodegeneration in mice and *Drosophila* (Saftig *et al.*, 1995; Koike *et al.*, 2000; Myllykangas *et al.*, 2005).

In the present work, we provide the first molecular explanation for human congenital NCL, showing that a mutation in the *CTSD* gene underlies the neurodegeneration in an infant of Pakistani origin with healthy parents. Moreover, we characterize the disease-associated mutant CTSD protein by transiently expressing it in baby hamster kidney (BHK) cells, and show the absence of CTSD protein and other abnormal immunohistological changes in the paraffin-embedded brain specimens of patients with congenital NCL.

Material and methods

Clinical history and patient tissues

Family A: The parents of the affected children were healthy first cousins of Pakistani origin. Of their seven children, three boys born in 1985 (Patient 1), 1989 (Patient 2) and 1999 (Patient 3) exhibited clinical and neuropathological findings consistent with congenital NCL, as previously reported in Patient 1 (Garborg *et al.*, 1987). Clinical data concerning foetal and post-natal development were retrieved from the medical charts. EDTA-blood samples were retrieved from Patient 3 and his healthy father after informed consent, and genomic DNA was extracted using standard techniques. Paraffin-embedded brain tissue samples of Patients 1 and 2 were obtained at routine autopsies for diagnostic purposes.

Family B: The mother of Patient 4 (born in 1982) was British, but no information is available concerning the nationality of the father. The clinical and neuropathological findings were consistent with congenital NCL reported by Humphreys *et al.* (1985). Paraffin-embedded tissue material from this case and controls was obtained in a routine autopsy for diagnostic purposes. The controls for the identified *CTSD* mutation consisted of 225 Caucasian (Centre d'Etude du Polymorphisme Humain, CEPH, and Finnish) individuals. An institutional review board of the Helsinki University Central Hospital approved the study.

Mutation scanning in the *CTSD* gene

The nine exons, the exon–intron boundaries and at least 60 nucleotides of the exon-flanking non-coding regions in the *CTSD* gene (GenBank accession no. M11233) were screened for mutations by genomic sequencing. Polymerase chain reactions (PCRs) were performed using 50 ng of genomic DNA in a volume of 20 μl containing 1 × PCR buffer, 125 μM of dNTPs, 0.325 μM of each exon-flanking primer and 1 U of AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA, USA). In addition, the reaction for exon 1 contained 10% dimethyl sulphoxide (Sigma-Aldrich, Saint Louis, MO, USA) and the reaction for exons 7 and 8 contained 0.5 M betaine (Sigma-Aldrich). Primer sequences are available from the authors on request. PCR conditions were as follows: 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58–64°C for 45 s and synthesis at 72°C for 45 s, and an extension at 72°C for 5 min. PCR products were visualized on 1% agarose gels, purified using enzymatic Exo/SAP treatment (USB Corporation, Cleveland, OH, USA) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were analysed with Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI, USA). The identified mutation in the *CTSD* gene was screened in a panel of 550 control chromosomes by genomic sequencing.

Construction of the expression plasmid and site-directed mutagenesis

The coding region of the human *CTSD* cDNA was digested from the IMAGE clone 3506977 (RZPD clone IRAUp969A1048D, RZPD, Berlin, Germany) with restriction enzymes *Eco*RI and *Xho*I (New England Biolabs, Ipswich, MA, USA) and ligated into the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA, USA) cleaved with the same enzymes. The insert was sequenced as described above. Two different mutations, c.764dupA and c.883G→A, corresponding to genetic defects causing the human and ovine congenital NCL, respectively, were introduced into the human wild-type pcDNA3.1(+)-*CTSD* construct sequence by

site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primer sequences used in the mutagenesis are available from the authors on request. The mutation constructs were verified by sequencing as described above.

Cell culture and transfections

BHK cells were seeded on 35-mm plates at a density of 5×10^5 cells/well 24 h before transfection. Cells were cultured in EMEM (Sigma-Aldrich)/10% fetal calf serum without any antibiotics and transiently transfected with a mixture of 4 μg of each *CTSD* cDNA construct and 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 0.4 ml of OPTIMEM I (Invitrogen, Grand Island, NY, USA). As controls, the cells were transfected with pcDNA3.1(+) vector or left non-transfected. After transfection, the cells were incubated at 37°C for 24 h before the media and cells were collected.

Western blotting

After transfection, the proteins from the collected BHK cells were extracted by ultrasonication in phosphate-buffered saline (PBS)/0.5% sodium deoxycholate/0.05% Triton X-100/protease inhibitors (Complete, Roche, Mannheim, Germany). The samples were centrifuged at 20 000 g for 10 min and the supernatant's protein concentration was determined with a BCA protein quantification kit (Interchim, Montluçon, France). Aliquots of supernatants containing 5 μg of protein were separated in 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, USA). After blocking with 4% bovine serum albumin in 10 mM Tris–HCl (pH 7.5)/150 mM NaCl/0.1% Tween (TBST), the membrane was incubated with rabbit anti-human CTSD antibody (Dako, Glostrup, Denmark, 1 : 500) overnight at 4°C. Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad, Hercules, CA, USA).

CTSD activity

CTSD activity of transfected BHK cell extracts was measured using the recently developed CTSD activity assay using 6-[(7-amino-4-methylcoumarin-3-acetyl)amino]hexoid acid (AMCA)-haemoglobin as a substrate (Partanen *et al.*, 2003). Shortly, 20 μl of cell extracts were incubated with 20 μl of the substrate solution (0.1 μg/μl AMCA-Hb/0.4 μg/μl Hb/25 mM formic acid buffer, pH 3.7/50 μM leupeptin) for 24 h at 37°C. The reaction was terminated by addition of 10 μl of 50% trichloroacetic acid. After incubation for 30 min on ice the samples were centrifuged and the supernatants were neutralized with 1 M Tris–HCl (pH 9.0). The fluorescence was measured on a microtitre plate using 365 nm (5 nm slit) for excitation and 460 nm (10 nm slit) for emission with a Varian CARY Eclipse Fluorescence Spectrophotometer (Varian Australia, Mulgrave, Victoria, Australia).

Immunohistochemistry

The autopsy tissue samples from Patients 1, 2 and 4 were fixed in 4% neutral buffered formaldehyde and subsequently processed and embedded in paraffin. Four-micrometre paraffin sections were immunohistochemically stained for glial fibrillary acidic protein (GFAP) (rabbit anti-GFAP, Dako, Glostrup, Denmark; 1 : 10 000) and CD68 (mouse anti-CD68, clone PG-M1, Dako, Glostrup, Denmark; 1 : 800), sphingolipid activator protein D (SAP D; antiserum is a kind gift of Prof. Konrad Sandhoff, University of Bonn, Germany; 1 : 300), subunit c of mitochondrial ATP synthase (antiserum is a kind gift of Dr David N. Palmer, Lincoln University, New Zealand; 1 : 600), as well as for CTSD (rabbit anti-CTSD, Dako, Glostrup, Denmark; 1 : 400). Sections were dewaxed in xylene, pretreated either with 0.5% trypsin at 37°C for 40 min (GFAP antiserum) or with 4% (w/v) pepsin in 1% (v/v) HCl at 37°C for 20 min (all other antisera), and endogenous peroxidase activity was blocked by incubating the sections in methanol containing 1.6% hydrogen peroxide at RT for 30 min. Sections were blocked and stained for each antigen using an appropriate Vectastain Elite kit (Vector Laboratories, Peterborough, UK), and immunoreactivity was visualized using 3-amino-9-ethylcarbazole and hydrogen peroxide and counterstained with haematoxylin.

Results

Clinical findings

The clinical and neuropathological findings in Patients 1–3 in family A and Patient 4 in family B were compatible with the diagnosis of congenital NCL, and the findings in Patients 1 and 4 have been reported previously (Humphreys *et al.*, 1985; Garborg *et al.*, 1987). Within family A, ultrasound examinations of all three affected foetuses demonstrated deceleration of head growth in the last trimester. Delivery at term was followed by intractable seizures, spasticity and apnoea in all patients. In Patient 3, the jerky foetal movements reported by the mother before delivery were recorded by an ultrasound examination and interpreted as myoclonic seizures. All affected infants were microcephalic with overriding sutures and obliterated fontanels. Low-set ears were noted in Patients 1 and 2, while Patient 3 had a low-set ear on the left side and a rudimentary external ear on the right side. In addition, Patient 2 had a claw hand, and Patient 3 had a broad nasal bridge and a receding forehead. Patients 1–3 died at 10, 1 and 4 days of age, respectively. Brain weights of Patients 1 and 2 were 146 and 130 g. Family A also has four unaffected children, of whom two boys have a hearing deficit. In family B, Patient 4 was born after a normal pregnancy and showed post-natal apnoea with cyanosis progressing to respiratory failure and death within 29 hours after birth. Brain weight was 75 g. Interestingly, this patient also exhibited a broad nasal bridge and low-set ears (Humphreys *et al.*, 1985).

Identification of the mutation in the *CTSD* gene

Previously, we identified an active-site mutation in the ovine *CTSD* gene as a cause of congenital NCL in sheep (Tyynelä *et al.*, 2000). Owing to the close resemblance of the neuropathological findings between the human and ovine congenital NCL, we screened the *CTSD* gene for mutations in a patient with congenital NCL. In the genomic DNA isolated from Patient 3, we identified a nucleotide duplication, c.764dupA, in exon 6 of the *CTSD* gene in a homozygous

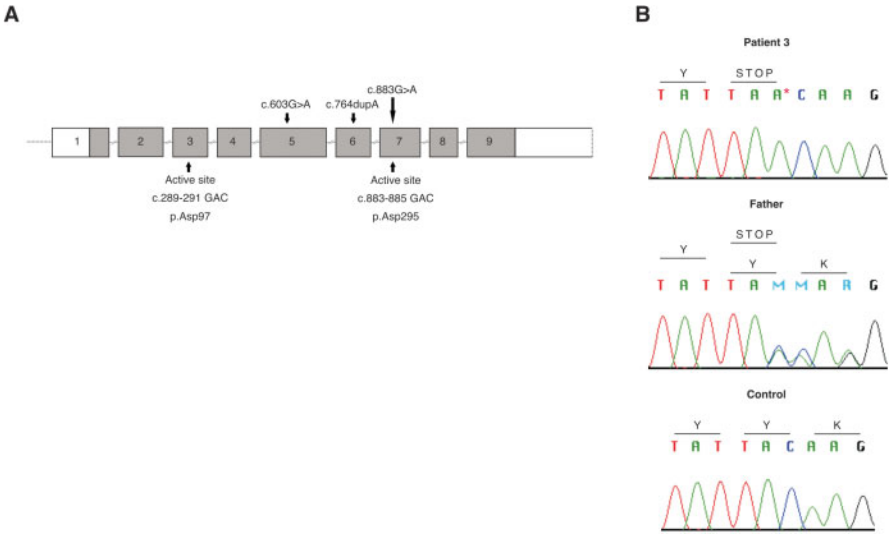

Fig. 1 Human *CTSD* gene and the c.764dupA mutation in congenital NCL. (**A**) *CTSD* genomic structure, positions of NCL-associated mutations and active site encoding nucleotides. CTSD is encoded by nine exons (boxes 1–9) of which the coding regions are shown in grey and untranslated regions in white. Nucleotide changes associated with diseases are indicated by arrows above the gene: c.764dupA is the novel human mutation; c.603 G→A and c.883G→A correspond to the canine and ovine mutations in the human cDNA sequence. Nucleotides (c.289-291 and c.883-885) encoding the active site aspartic acids (p.Asp97 and p.Asp295) of the CTSD enzyme are indicated by arrows below the gene. (**B**) Sequence chromatograms showing the c.764dupA mutation in homozygous form in Patient 3 and in heterozygous form in his father with a respective control sequence (all in forward orientation). The duplicated base in the patient chromatogram is indicated by an asterisk (*). In the chromatogram of the heterozygous father the sequences from both the mutant and normal alleles can be seen as an overlay of two sequences differing by one nucleotide distal to the duplication. M represents an overlay of adenine (A) and cytosine (C) and R an overlay of adenine and guanine (G).

form (Fig. 1A and B). The father of the patient was a heterozygous carrier of the mutation (Fig. 1B), but no DNA was available from the mother, who is his first cousin. The duplication creates a premature stop codon (TAC→TAAC) at position 255 (p.Tyr255X), predicting a truncation of the protein by 158 amino acids. The mutation was not found in any of the 550 control chromosomes. An additional nucleotide change, c.845G→A, in exon 7, distal to the stop codon created by the duplication, was also detected in Patient 3 in homozygous form and in his father in heterozygous form (data not shown). If translated the nucleotide change would result in an amino acid substitution of glycine to arginine at position 282 (p.Gly282Arg).

Transient expression of the c.764dupA mutant CTSD in BHK cells

In order to monitor the effect of the c.764dupA mutation in CTSD at protein level, we transiently expressed the mutant and the wild-type human CTSD in BHK cells. As a control, we used a human *CTSD* cDNA construct with c.883G → A mutation, corresponding to an enzymatically inactive mutant CTSD described earlier in congenital ovine NCL (Tyynelä *et al.*, 2000; Partanen *et al.*, 2003). When expressed in BHK cells, the c.764dupA mutant CTSD was enzymatically inactive in cell lysates and showed activity values similar to non-transfected controls and those with the inactive c.883G → A mutant (Fig. 2A). In contrast, expression of the wild-type human CTSD in BHK cells resulted in 4-fold activity compared with non-transfected cells (Fig. 2A). The production of the mutant and wild-type proteins in BHK cells was confirmed by western blotting, and their expression levels were found to be roughly equal (Fig. 2B).

As many other lysosomal enzymes, human CTSD is produced as a preproenzyme, which undergoes several proteolytic processing steps, first resulting in a single-chain active form, and finally in the mature enzyme composed of two polypeptide chains (Rawlings and Barrett, 1995). When transiently expressed in BHK cells, the wild-type human CTSD showed two protein bands on western blots corresponding to the single-chain active polypeptide band at 43 kDa and the mature form of the enzyme at 31 kDa (Fig. 2B). In contrast, the c.764dupA mutant CTSD appeared as a single band with a molecular weight of ~27 kDa, in agreement with the calculated size of the prematurely truncated protein (Fig. 2B). As a control, we followed the production and processing of the inactive c.883G→A mutant in BHK cells: the mutant c.883G→A protein was normally processed, producing two protein bands with slightly faster mobility than those in the wild-type (Fig. 2B), as reported before (Partanen *et al.*, 2003).

Neuropathological findings and absence of CTSD immunostaining in the brains of patients with congenital NCL

The neuropathological changes in the brains of Patients 1 and 2 (family A) and Patient 4 (family B) were extremely severe

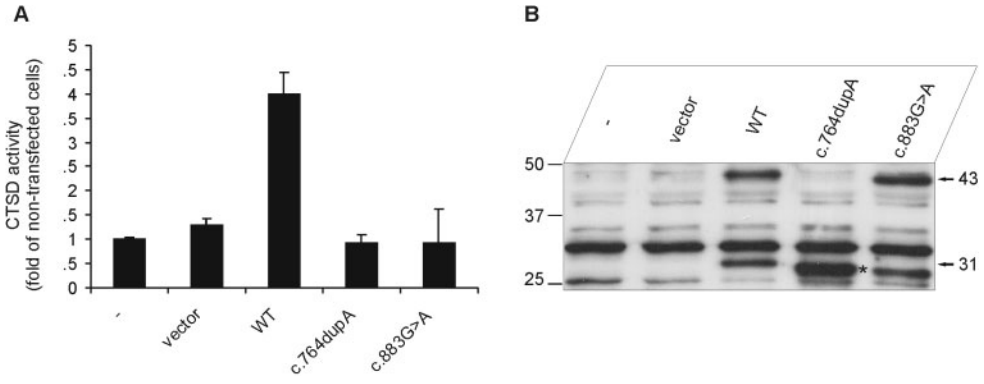

Fig. 2 Activity and processing of the c.764dupA mutant CTSD. (**A**) CTSD enzyme activity of BHK cells transfected with pcDNA 3.1(+)-vector, human wild-type and mutant CTSD constructs. The activity values are represented as a fold of non-transfected (–) BHK cells, which possess endogenous activity considered as a background. The c.764dupA mutant CTSD (c.764dupA) is enzymatically inactive, while the wild-type (WT) protein shows 4-fold activity compared with non-transfected (–) and vector-transfected (vector) cells. The c.883G→A mutant CTSD (c.883G→A) is known to be enzymatically inactive, and is used as a negative control in this experiment. The values are means ± SD of three independent experiments carried out in duplicate. (**B**) Western blot analysis of CTSD enzyme in BHK cells transiently expressing the human wild-type and mutant CTSD compared with non-transfected and pcDNA 3.1(+)-vector-transfected samples. In cells transfected with the wild-type (WT) CTSD, the single-chain (43 kDa) and mature (31 kDa) forms of CTSD are visualized (indicated by arrows on the right side). In marked contrast, the cells transfected with the c.764dupA mutant CTSD (c.764dupA) produce a truncated protein with an apparent molecular weight of 27 kDa (indicated by an asterisk, *). The previously characterized c.883G→A mutant CTSD (c.883G→A) is used as a control, and shows the single-chain and mature forms of CTSD, with slightly higher mobilities than the wild-type enzyme, as expected. The polyclonal CTSD antiserum also recognizes an additional protein band (~33 kDa) in all cell extracts, including the non-transfected (–) and vector-transfected (vector) cells, and this protein is likely to be endogenous for BHK cells. Molecular weights of markers are indicated on the left (kDa).

and similar. In all cases, the normal architecture of the cerebral and cerebellar cortex was abolished, showing extreme neuronal loss and glial activation, and the white matter was almost devoid of axons and myelin, as previously reported for Patients 1 (Garborg *et al.*, 1987) and 4 (Humphreys *et al.*, 1985). Hypertrophic astrocytes were abundant throughout the brain tissue, particularly in the white matter, and often contained a dense core strongly immunoreactive for GFAP (Fig. 3A–C). Microglial activation was most pronounced within the deeper layers of the cortical grey matter, as shown by CD68 staining (Fig. 3D–F). Storage deposits in all types of cells stained intensely positive for sphingolipid activator protein D (Fig. 4A), but not for subunit c of the mitochondrial ATP synthase (Fig. 4B), which is the main storage component in most forms of the NCLs, including Finnish variant late-infantile NCL (CLN5; Fig. 4C). These findings closely resemble the findings in ovine congenital NCL and human infantile NCL.

CTSD staining was practically absent in tissues of patients with congenital NCL, where neurons and microglial cells were devoid of any staining (Fig. 5A). Sometimes, however, the hypertrophic astrocytes showed a pale, diffuse and probably unspecific positive staining for CTSD in patient tissue (Fig. 5B). In marked contrast, CTSD antiserum produced a typical lysosomal punctate staining pattern in normal neurons (Fig. 5C). As an additional affected control, we stained brain tissue from a CLN5 patient with CTSD antiserum, and found strong immunoreactivity for CTSD within the storage neurons (Fig. 5D).

Discussion

A truncating *CTSD* gene mutation underlies human congenital NCL

In the present work, we provide the first molecular explanation for human congenital NCL by identification of a novel homozygous nucleotide change, c.764dupA, in the *CTSD* gene in a patient with congenital NCL. As expected, we also identified the same molecular change in heterozygous form in the healthy father. The mutation is likely to be disease-causing, as it creates a premature stop codon in the CTSD polypeptide. This mutation was observed in a homozygous form in Patient 3 and in a heterozygous form in his father, while it was absent in controls. Although there were four affected infants from two families, DNA was available for molecular studies in only one affected individual in family A. Despite multiple efforts, we were unable to extract good-quality DNA from the paraffin-embedded tissues from the other affected individuals in order to confirm the presence of the c.764dupA mutation in the siblings of Patient 3 and to determine the underlying mutation(s) in Patient 4. However, on the basis of the immunohistochemical findings it is reasonable to suggest that a deficiency in CTSD caused congenital NCL in all these patients.

CTSD (EC 3.4.23.5) is a lysosomal aspartic protease that belongs to the pepsin family (Press *et al.*, 1960; Rawlings and Barrett, 1995). The mature form of human CTSD is composed of two polypeptides encoded by the *CTSD* gene, interlinked by disulphide bridges (Metcalf and Fusek, 1993). Both of these polypeptide chains contain one aspartic acid

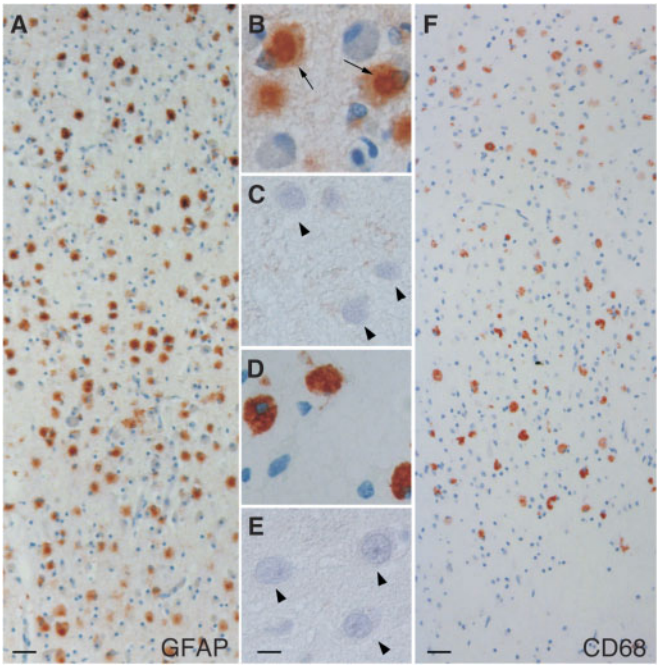

Fig. 3 Immunohistochemical staining of paraffin-embedded brain specimens from patients with congenital NCL indicates marked glial activation (**A**–**B**). Immunohistochemical staining of tissue samples using anti-GFAP antibodies reveals a number of GFAP-positive astrocytes throughout the cerebral cortex in samples from patients with congenital NCL. The hypertrophic astrocytes (indicated by arrows) often contain a dense core, which is intensely positive for GFAP. (**C**) In the deeper layers of the normal cerebral cortex, only slight punctate staining of the astrocytic processes between the neurons (indicated by arrowheads) was observed. (**D** and **F**) Immunohistochemical staining using anti-CD68 antiserum indicates that activated microglial cells were particularly abundant in the deep layers of the cerebral cortex. (**E**) In the normal cerebral cortex, healthy-looking neurons (indicated by arrowheads) were numerous but CD68-positive microglial cells were not observed. Scale bars, 40 μM in A and F, 10 μM in E.

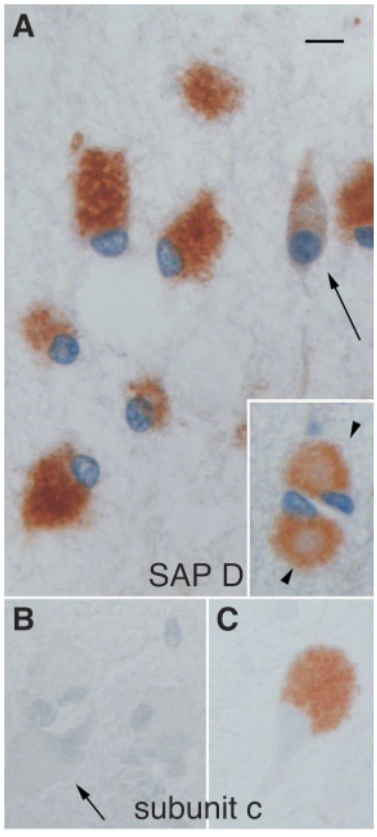

Fig. 4 Immunohistochemical staining of paraffin-embedded brain specimens from patients with congenital NCL reveals accumulation of sphingolipid activator protein D. (**A**) Immunohistochemical staining of tissue samples from patients with congenital NCL using sphingolipid activator protein D antiserum shows that the storage deposits within most cells, particularly in cells of glial origin, are strongly positive for sphingolipid activator protein D (SAP D). The few remaining neurons (indicated by an arrow) are variably positive for sphingolipid activator protein D, and the hypertrophic astrocytes (indicated by arrowheads) often show a core devoid of any staining (inset). (**B**) The storage material within the remaining neurons (indicated by an arrow) and the surrounding glial cells in samples from patients with congenital NCL is negative for subunit c of mitochondrial ATP synthase (subunit c), (**C**) while the storage material in Finnish variant late-infantile NCL (CLN5) is strongly positive for subunit c. All panels have the same magnification; scale bar, 10 μm, shown in **A**.

residue that is essential for the enzymatic activity of the protein (Faust *et al.*, 1985; Rawlings and Barrett, 1995). The novel congenital NCL mutation, c.764dupA, creates a premature stop codon at tyrosine 255 (p.Tyr255X) in CTSD, resulting in a truncation of the polypeptide by 158 amino acids, and in a deletion of the active site aspartic acid residue at position 295. In addition to the duplication, we found a nucleotide change c.845G→A (p.Gly282Arg) in homozygous form in the patient and in heterozygous form in his father. This nucleotide change is not likely to be disease-causing because it is located distal to the c.764dupA, which creates a premature stop codon in the *CTSD* gene. Moreover, the affected amino acid (Gly282) is not conserved among species, and, for example, sheep, mouse and man have different amino acids in that position.

The observed mutation resulting in a premature stop codon in the *CTSD* gene leads to production of an abnormally short mRNA, which may be degraded through the nonsense-mediated decay system (Hentze and Kulozik, 1999). Alternatively, post-translational quality control may result in an early degradation of the truncated protein. Early degradation of the mutant CTSD either at mRNA or protein level is likely to occur *in vivo*, because of the absence of CTSD staining in paraffin-embedded brain tissue specimens of Patients 1, 2 and 4. However, when the c.764dupA mutant CTSD was transiently expressed in BHK cells, a truncated form of CTSD was produced and appeared stable, but was enzymatically inactive, as expected owing to the absence of the active site aspartic acid residue. This may be due to the differences between the *in vivo* situation and the heterologous expression system, where the protein is produced in excess and the post-translational processing may occur differently.

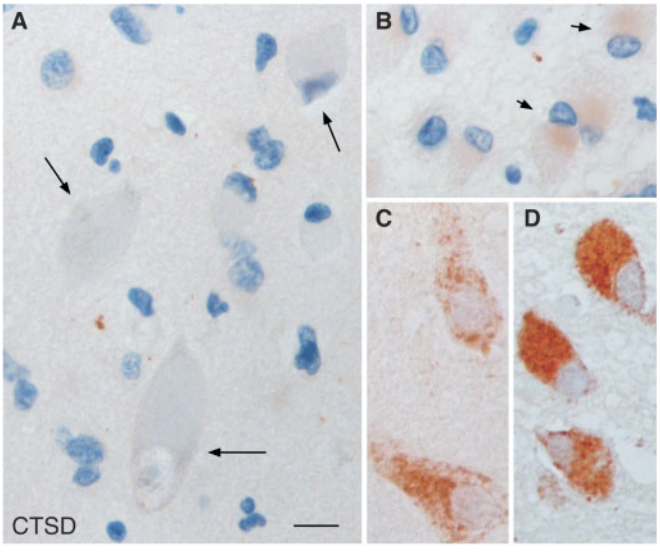

Fig. 5 CTSD staining is absent in paraffin-embedded brain specimens from patients with congenital NCL. (**A**) Immunohistological staining with a polyclonal CTSD antiserum reveals complete absence of CTSD in neurons (indicated by long arrows) and most other cells in samples from patients with congenital NCL. (**B**) Sometimes the hypertrophic astrocytes (indicated by short arrows), particularly within the white matter, stain diffusely for CTSD in the patient samples. (**C**) Normal neurons show a punctate staining for CTSD, (**D**) while the storage neurons in a patient with Finnish variant late-infantile NCL (CLN5) are strongly positive for CTSD (**D**). All panels have the same magnification; scale bar, 10 μm, shown in **A**.

CTSD deficiency in humans defines a distinct clinical NCL phenotype

The clinical findings in all four patients affected with congenital NCL were nearly identical. The extreme clinical phenotype, including severe post-natal central apnoea, seizures and death after birth is compatible with complete inactivation of the CTSD protein. The complete lack of the CTSD enzyme activity also leads to an early-onset and progressive neurodegenerative NCL disease in mice and sheep (Koike *et al.*, 2000; Tyynelä *et al.*, 2000), while the missense mutations observed in American bulldogs result in a partial inactivation of the CTSD, and, accordingly, to a late-onset form of NCL (Awano *et al.*, 2005). In addition, all patients with congenital NCL exhibited nearly identical neuropathological changes, characterized by complete disorganization of the neurons in the cerebral cortex, loss of Purkinje cells and inner granule cells in the cerebellum, as well as extreme glial activation (the present observations; Humphreys *et al.*, 1985; Garborg *et al.*, 1987). In all patients studied, the storage deposits were positive for sphingolipid activator protein D but not for subunit c of the mitochondrial ATP synthase, resembling the findings in human infantile NCL, caused by mutations in the palmitoyl protein-thioesterase 1 gene (Vesa *et al.*, 1995), and ovine congenital NCL, caused by a mutation in the *CTSD* gene (Tyynelä *et al.*, 2000). In addition, the storage material in all these patients showed granular ultrastructure (Humphreys *et al.*, 1985; Garborg *et al.*, 1987), as did yet another previously reported patient with congenital NCL (Barohn *et al.*, 1992), and animal models with CTSD deficiency, including sheep (Tyynelä *et al.*, 2000), American bulldogs (Awano *et al.*, 2005), mice (Koike *et al.*, 2000) and *Drosophila* (Myllykangas *et al.*, 2005).

Currently, there is no effective treatment for any form of NCLs. However, promising results from the adeno-associated virus-mediated gene therapy in mouse models of NCLs are emerging (Griffey *et al.*, 2005; Passini *et al.*, 2006). Unfortunately, prenatal treatment of congenital NCL seems very difficult, as it would imply strategies for appropriate substances or vectors to pass the placenta and reach the foetal brain early in pregnancy. However, the potential later-onset forms of CTSD deficiency may prove to be better targets for therapeutic trials.

In conclusion, we identified a disease-causing mutation in the *CTSD* gene of a patient with congenital NCL. On the basis of our present data as well as the nearly identical clinical and/or pathological phenotype of our patients and the other reported cases of congenital NCL, it is reasonable to suggest that CTSD deficiency caused by mutations in the corresponding gene may underlie all cases of congenital NCL. Thus, CTSD deficiency should be considered as a possible diagnosis in microcephalic neonates, who present with seizures at or before birth.

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