

Accumulation of glial fibrillary acidic protein and histone H4 in brain storage bodies of Tibetan terriers with hereditary neuronal ceroid lipofuscinosis

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Summary The neuronal ceroid lipofuscinoses (NCLs) are inherited neurodegenerative diseases characterized by massive accumulation of autofluorescent storage bodies in neurons and other cells. A late-onset form of NCL occurs in Tibetan terrier dogs. Gel electrophoretic analyses of isolated storage body proteins from brains of affected dogs indicated that a protein of approximately 50 kDa was consistently prominent and a 16 kDa component was present in some brain storage body preparations. Mass spectral analysis identified the 50 kDa protein as glial fibrillary acidic protein (GFAP), isoform 2. GFAP identification was supported by immunoblot and immunohistochemical analyses. Histone H4 was the major protein in the 16 kDa component. Specific accumulation of GFAP and histone H4 in storage bodies has not been previously reported for any of the NCLs. Tibetan terrier NCL may be the canine correlate of one of the human adult-onset NCLs for which the genetic bases and storage body compositions have not yet been determined.

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Abbreviations

GFAP	glial fibrillary acidic protein
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCL	neuronal ceroid lipofuscinosis

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are inherited neurodegenerative disorders characterized by massive accumulations of autofluorescent storage material in neurons and other cell types (Haltia 2006; Jolly 1995; Katz et al 1997; Sehafer and Pearce 2006; Wisniewski et al 2001). A distinctive feature of the NCLs is that the stored material contains predominantly a single protein or relatively few specific proteins (Haltia 2006; Palmer et al 1986, 1989, 1990, 1997; Tyynela et al 1993, 1997a). There are a number of different forms of human NCL that each result from mutations in one of at least eight different genes (*PPT1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, *CLN8*, *CTSD* and *MFSD8*) (Siintola et al 2006, 2007; Steinfeld et al 2006; Wisniewski et al 2001). In the majority of these diseases, the major stored proteins are F_0 subunit c of mitochondrial ATP synthase, a 7.6 kDa hydrophobic protein (Buzy et al 1996; Hall et al 1991; Kominami et al 1992; Tyynela et al 1997b) and/or the 16 kDa V_0 subunit c of vacuolar ATPase (Palmer et al 1997). In other forms of the disease, saposins A and D are prominent proteins in the storage bodies (Tyynela et al 1993; Tyynela et al 1997a). Naturally occurring NCLs have been reported in a number of nonhuman species, including mice, sheep, cattle and numerous

dog breeds (Awano et al 2006a, b; Jolly and Palmer 1995; Jolly et al 1992, 1994; Katz et al 2001). In cases where the underlying mutation occurs in a gene orthologous to one of the human NCL genes, the major stored proteins have been found to be the same as in the corresponding human disorders. For example, NCL in English setters results from a mutation in the canine *CLN8* gene, and the storage bodies contain large amounts of mitochondrial F₀ ATP synthase subunit c (Katz et al 1994, 2005a) as they do in the corresponding human disorder (Herva et al 2000; Ranta et al 2001).

Despite extensive research, there remain some cases of human NCL for which the genetic basis is unknown and in which the composition of the storage material has not been characterized. Among these are late-onset forms of NCL, including most cases that have been designated as Kufs disease (Martin 1991). An autosomal recessively inherited late-onset NCL has also been reported in Tibetan terrier dogs (Katz et al 2005b; Riis et al 1992). The genetic basis of the Tibetan terrier disease remains to be identified. To determine whether the NCL Tibetan terrier storage material is similar in protein composition to that of other known NCLs, storage material was isolated from brains of affected dogs and analysed.

Materials and methods

Animals and tissues

NCL in Tibetan terriers has been extensively characterized in our laboratories by analysis of behavioural signs and examination of brain and retinal tissues for accumulation of disease-specific storage bodies (Katz et al 2002, 2005b). Behavioural changes typically first become apparent at 5–7 years of age and progress until the dogs are euthanized, usually by age 10 years. Among the behavioural signs of affected dogs are the development of a nervous demeanour, increased aggressiveness, loss of training, hypersensitivity to various stimuli, loss of coordination, apparent visual impairment, tremors and seizures. Dogs with neurological signs were identified with the assistance of the Tibetan Terrier Club of America. We obtained health-survey information, including the evaluation of 29 potential behavioural signs of NCL, the age of onset for these signs, and other health and pedigree information on the dogs. Dogs identified by these surveys as exhibiting at least five of the primary behavioural signs of the disease with an age of onset greater than 5 years were designated as potentially affected. The

owners of these dogs were notified and asked to donate tissues from the dogs at the time of death. Using this approach, brain and retinal tissues were obtained from the Tibetan terriers used in this study.

The dogs were euthanized owing to the advanced stage of the disease. As soon as possible after death, the eyes and brain were collected from each dog, usually by a local veterinarian. One eye was placed in fixative (3.5% formaldehyde, 0.1% glutaraldehyde, 0.13 mol/L Na-cacodylate, 0.13 mmol/L CaCl₂, pH 7.4) and most of the cornea was removed. Small slices of cerebral cortex and cerebellum were placed in the same fixative, and the remainder of the brain was frozen. The other eye and portions of the same brain tissues were fixed for electron-microscopic examination (Katz et al 2005b). The preserved samples were shipped for next-day delivery to the laboratory. The fixed tissues were processed and examined using fluorescence microscopy and electron microscopy to determine whether there was storage body accumulation typical of this disease (Katz et al 2005b). Dogs that showed the typical pattern of behavioural signs with an age of onset greater than 5 years and that exhibited the characteristic pattern of storage body accumulation in retina, cerebral cortex and cerebellum were designated as being affected with NCL.

Storage body isolation

In the brain, the storage material accumulation was pronounced throughout the cerebral cortex (Fig. 1). Cortical grey matter was therefore used for storage body isolation. Approximately 1 g portions of cortical grey matter were dissected from the frozen brains of three affected Tibetan terriers that were not closely related. The samples were each weighed and homogenized in 1 mmol/L HEPES, 5 mmol/L NaCl, 0.1 mmol/L Na₂EDTA, pH 7.2 at a concentration of approximately 1 g of tissue per 10 ml homogenization buffer. Each homogenate was then subjected to centrifugation at 1000 rpm in a Sorvall HB-4 rotor at 4°C for 3 min. The supernatant was collected and diluted to approximately 13 ml and then centrifuged at 10 000 rpm with the Sorvall HB-4 rotor at 4°C for 10 min. The resultant pellet was suspended with sonication in 12 ml of homogenization buffer and the sample was centrifuged at 5000 rpm with the Sorvall HB-4 rotor at 4°C for 10 min. This produced a 2-part pellet consisting of a top white portion and a bottom yellow portion. The yellow portion of the pellet was suspended in 4 ml of homogenization buffer and the suspension was layered on top of 10 ml of 1.35 mol/L CsCl in homogenization buffer in a centrifuge tube. The sample was then centrifuged at 9500 rpm in the Sorvall

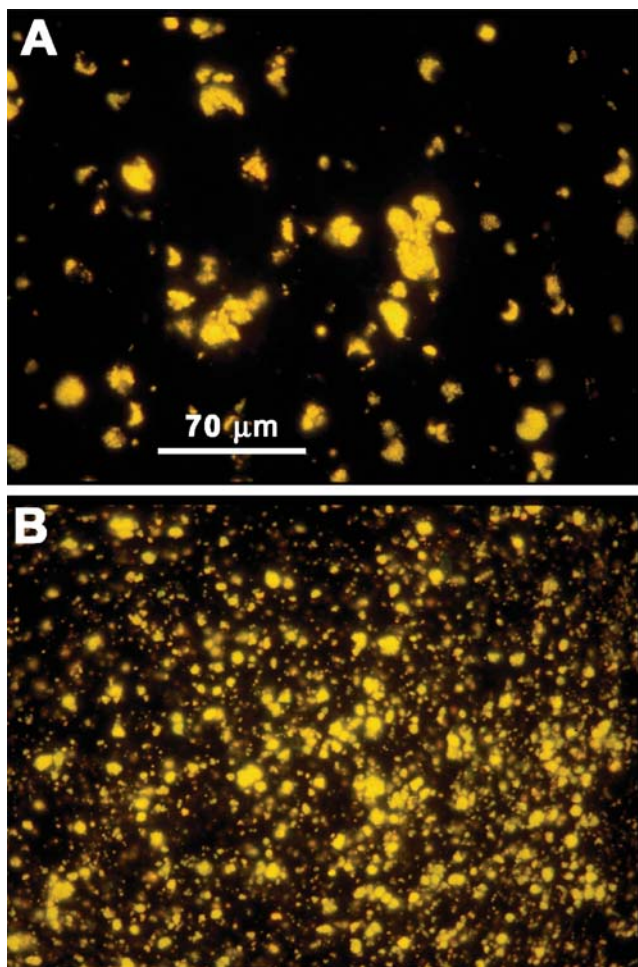


Fig. 1 Fluorescence micrographs of (A) storage bodies in cerebral cortex *in situ* and (B) isolated storage bodies. Bar in (A) indicates magnification for both micrographs

HB-4 rotor for 40 min. The resultant pellet was washed with homogenization buffer and portions of the pellet were then examined with fluorescence and electron microscopy and were used for protein characterizations.

A small fraction of each storage body preparation (less than 5 μ l) was applied to a glass microscope slide, covered with a coverslip, and examined with fluorescence microscopy under conditions optimized for visualization of storage body autofluorescence (Katz et al 2005b). Another portion of the isolated storage material was processed for electron-microscopic examination (Katz et al 1995). The remainder of the sample was used for protein characterization.

Protein characterization

Isolated storage bodies were solubilized in 2% SDS (Katz et al 1994) and subjected to SDS-PAGE using a Criterion Precast Gel System with Tris-HCl 10–20% gradient and 18% gels (Bio-Rad, Hercules, CA, USA).

In addition to the storage bodies from Tibetan terrier brains, storage bodies from the brain and pancreas of sheep with NCL were also analysed with SDS-PAGE. The NCL sheep storage bodies were a gift from David Palmer of Lincoln University in New Zealand (Palmer et al 1986). Protein bands were visualized with Coomassie blue or silver stain. Previous studies indicated that some protein components of NCL storage bodies do not stain with Coomassie blue, and special silver staining techniques are required to reveal these proteins (Fearnley et al 1990). Initial silver staining of SDS-PAGE gels of the storage bodies using established protocols failed to reveal the presence of a band corresponding to vacuolar ATPase subunit c that was previously reported to be associated with liver storage bodies from Tibetan terriers with NCL and pancreas storage bodies from sheep with NCL (Palmer et al 1997). However, after experimenting with a number of silver staining techniques an approach was developed that did reveal additional proteins in some samples, including a band with the same apparent molecular weight (16 kDa) as vacuolar ATPase subunit c. After electrophoresis, gels to be silver-stained were fixed in 50% acetone, 1% acetic acid, 0.02% paraformaldehyde for 10 min. The gels were then washed with water followed by incubation in 50% acetone for 15 min. This was followed by washing in water and then incubation in 1 mmol/L sodium thiosulfate for 10 min. The gels were again washed with water and then incubated in 5 mmol/L silver nitrate, 1% paraformaldehyde for 10 min in the dark. After washing the gels again in water, they were incubated in developer (500 mmol/L sodium carbonate, 1 mmol/L sodium thiosulfate, 0.05% paraformaldehyde) until protein bands appeared. Acetic acid was added to stop the development and the gels were then washed in 5% acetic acid followed by water washes. After these procedures most protein bands were visible but there was usually relatively high background staining of the gel. To specifically reduce the background, the gels were again incubated in 1 mmol/L sodium thiosulfate for 5 min., washed with water, incubated in the silver nitrate solution for 10 min., washed with water, incubated in 5% acetic acid, and washed again. The background reduction procedure was repeated until proper contrast between the protein bands and the background was achieved. As positive controls, extracts from NCL sheep brain and pancreas storage bodies were analysed on the same gel.

A major band of approximately 50 kDa that was consistently seen in the Tibetan terrier storage body extracts was excised from a Coomassie blue-stained gel. In addition, a prominent band of approximately

16 kDa that was present in some Tibetan terrier storage body extracts was excised from a silver-stained gel. The samples were destained, reduced with dithiothreitol, alkylated with iodoacetamide and digested overnight with trypsin. The digests were lyophilized to dryness, reconstituted in 0.9% formic acid, and desalted on a micro C18 Ziptip (Millipore Corp., Bedford, MA, USA). The digests were then analysed in alpha-cyano-4-hydroxycinnamic acid matrix with MALDI TOF/TOF mass spectrometry using an Applied Biosystems (Foster City, CA, USA) model 4700 instrument with a 355 nm Nd:YAG laser (200 Hz) in the positive-ion mode. For the 50 kDa protein, the eight most intense precursor ions were automatically selected for tandem MS/MS analysis. Spectra were processed and batch-analysed in the 'Combined MS plus tandem MS' mode with Applied Biosystems GPS Explorer software (version 3.6). The mass spectral data of ions with a signal/noise ratio >20 in the 700–4000 Da mass range (excluding trypsin autolysis peptides) and tandem MS ions with a signal/noise ratio >10 in the mass range from 60 Da up to the precursor mass were matched against the NCBI mammalian protein database (June 18, 2006 update) using the MASCOT search engine from Matrix Science (www.matrixscience.com). Similar analyses were performed on the 16 kDa bands from the storage body preparations from NCL Tibetan terrier and sheep.

For the 50 kDa band, the results of the MS analysis were confirmed by western blotting in which a portion of the isolated storage bodies was fractionated by SDS-PAGE as described above and transferred to a Millipore Immobilon-P PVDF membrane (Millipore Corp.). The membrane was probed with a rabbit anti-human glial fibrillary acidic protein (GFAP) antibody (AB5804, Chemicon, Temecula, CA, USA) at a 1:1000 dilution, and the bound antibody was visualized using Chemicon BCIP/TNBT substrate (cat. no. E5007) in conjunction with an alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. AP307A, Chemicon) at a 1:1000 dilution.

Immunohistochemical analysis was performed to confirm the localization of GFAP to the storage bodies. Small slices of cerebral cortex were collected from affected dogs immediately after euthanasia and placed in 3.5% formaldehyde, 0.1% glutaraldehyde, 0.13 mol/L Na-cacodylate, 0.13 mmol/L CaCl₂, pH 7.4. After incubation in the latter fixative for 1 h at room temperature, the samples were washed with 0.17 mol/L sodium cacodylate, pH 7.4 and embedded in Tissue-Tek freezing medium. The embedded samples were frozen on dry ice and sections of the frozen tissue were cut at a thickness of 5 µm with a cryostat. Sections were mounted on Superfrost/Plus slides (Fisher Scientific,

Waltham, MA, USA) and allowed to dry overnight. The sections were then incubated in phosphate-buffered saline (PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1 mmol/L KH₂PO₄, pH 7.4) for 15 min, followed by incubation in blocking solution (0.5% bovine serum albumin, 0.2% Tween 20, 0.1% sodium azide, 5% normal goat serum in PBS) for 1.5 h. The sections were then incubated at 4°C overnight with the same anti-GFAP antibody used for the immunoblot analysis diluted 1:800 in the blocking solution. Following incubation with the primary antibody, the sections were washed with PBS and then incubated for 3 h at room temperature in AlexaFluor 568-conjugated goat anti-rabbit IgG (Invitrogen A11036) diluted 1:200 in blocking solution. The sections were then washed with PBS and dried, and coverslips were mounted over the sections with Gel Mount (Biomedica, Foster City, CA, USA). Control sections of the same samples were treated identically except that the primary antibody was left out of the overnight incubation, which was done with blocking solution alone. Sections were examined and photographed using a Zeiss Axiophot fluorescence microscope using filter sets optimized for storage body and immunofluorescence as described previously (Narfström et al 2003). Autofluorescence and immunofluorescence images of the same areas of tissue were merged using the DP Manager program (Olympus Optical Co., Tokyo, Japan).

Results

Fluorescence and electron microscopy revealed massive accumulations of storage material throughout the cerebral cortexes of Tibetan terriers that were euthanized after exhibiting symptoms of NCL (Figs. 1A and 2A). The ultrastructure of the storage body contents was somewhat heterogeneous, but most of the storage bodies contained structures that had the appearance of stacks of membranes in whorls or parallel arrays (Figs. 2 and 3). Also present within the storage bodies were coarsely granular and lipid-like components (Fig. 3). Isolation procedures similar to those used for other NCLs generated almost pure preparations of these storage bodies from cerebral cortex grey matter homogenates (Figs. 1B and 2B).

SDS-PAGE analysis of solubilized storage bodies with gels stained with Coomassie blue revealed a single major protein band of slightly over 50 kDa that was present in all preparations from NCL Tibetan terrier brain (Fig. 4A). In addition to the 50 kDa band, in some samples silver staining revealed a strongly-stained band at approximately 16 kDa as well as

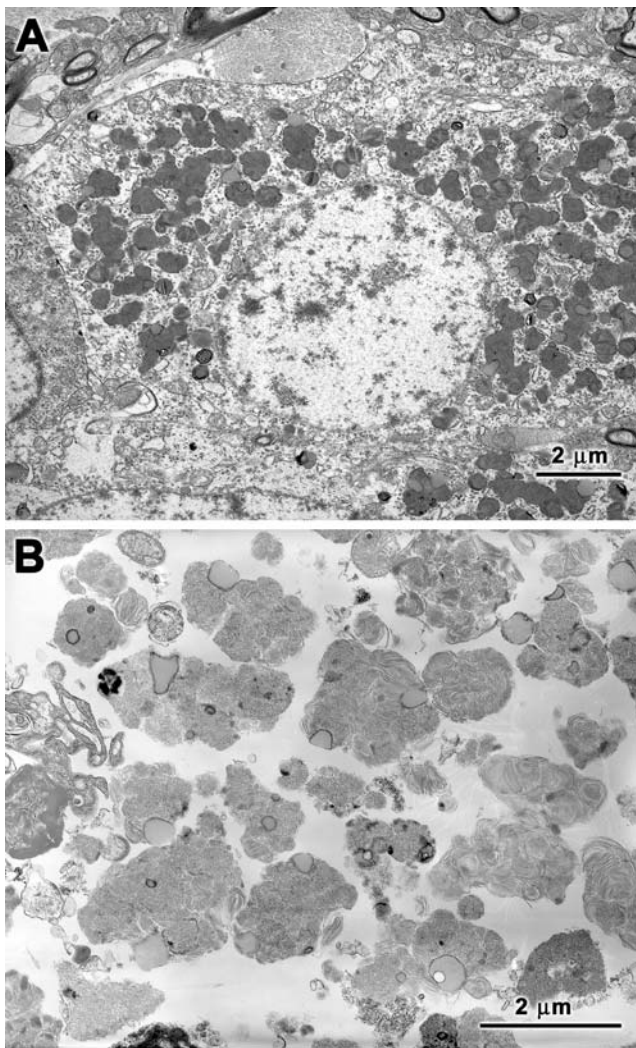


Fig. 2 Electron micrographs of (A) storage bodies in cerebral cortex *in situ* and (B) isolated storage bodies. (A) A cerebral cortical neuron, the perinuclear region of which is filled with electron-dense storage bodies. (B) Storage body material isolated from the cerebral cortex of a Tibetan terrier with NCL

several bands of over 200 kDa (Fig. 4B). Only the 50 kDa band was detected in all storage body preparations from Tibetan terrier brains. Analysis of purified storage bodies from NCL sheep brain and pancreas on the same gel revealed four bands with approximate apparent molecular weights of 8, 16, 18 and 22 kDa (Fig. 4B). The 50 kDa and high-molecular-weight bands were not observed in the extracts from NCL sheep brain or pancreas storage bodies.

Because the 16 kDa component in some of the Tibetan terrier storage body preparations co-migrated with a component from the sheep storage bodies, mass spectral analyses were performed to compare these 16 kDa bands. The mass spectral analyses of peptide fragments from the sheep pancreas 16 kDa gel band identified the most abundant component of this band as

histone H4 (gi | 57098917). Seven peptides in the tryptic digest of the sheep pancreas band had masses that matched those predicted for histone H4; moreover, tandem MS analysis of two of these peptides indicated amino acid sequences of VFLENVIR (ion score of 45; score >43 indicated 95% confidence) and ISGLIYEETR (ion score of 71) which matched peptides predicted for histone H4. Mass spectral analysis indicated that subunit c of mitochondrial F₀ ATP synthase was also present in this band as a minor component. Three peptides had masses that matched those predicted for mitochondrial F₀ ATP synthase subunit c; furthermore, tandem MS identified one of these peptides as FIGAGAATVGVAGSGAGIGTVFGSLIIGYAR (with an ion score of 117). Western blot analysis of the sheep pancreas storage bodies by Palmer and colleagues had previously shown that a gel band that they reported as having an apparent molecular weight of 12 kDa bound antibodies from a

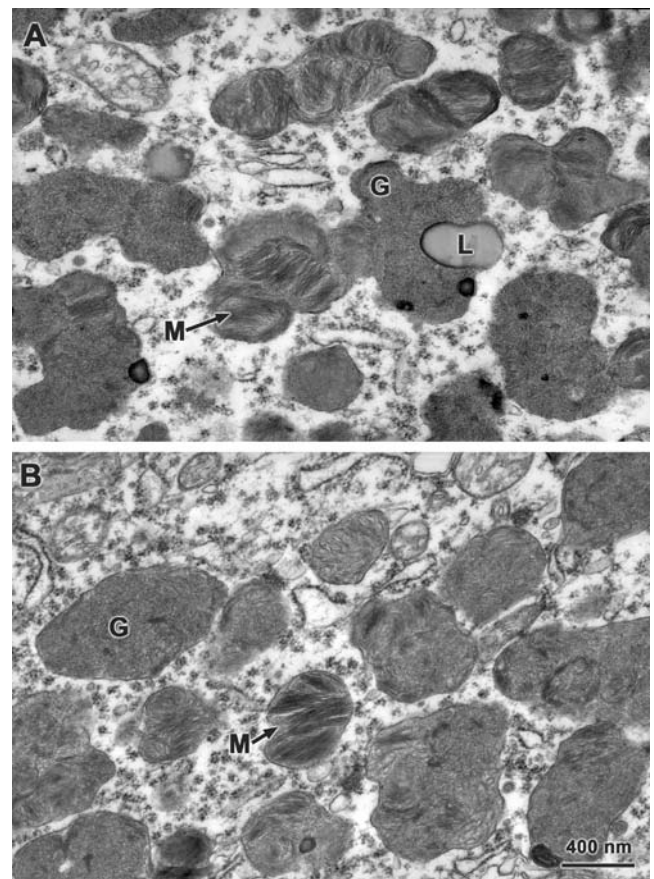


Fig. 3 High-magnification electron micrographs of storage bodies in the cerebral cortex of a Tibetan terrier with NCL. The storage body contents were heterogeneous in appearance, consisting of membrane-like (M), course granular (G), and lipid-like (L) components. Often all three types of material were present within the same storage body. Bar in (B) indicates the magnification of both micrographs

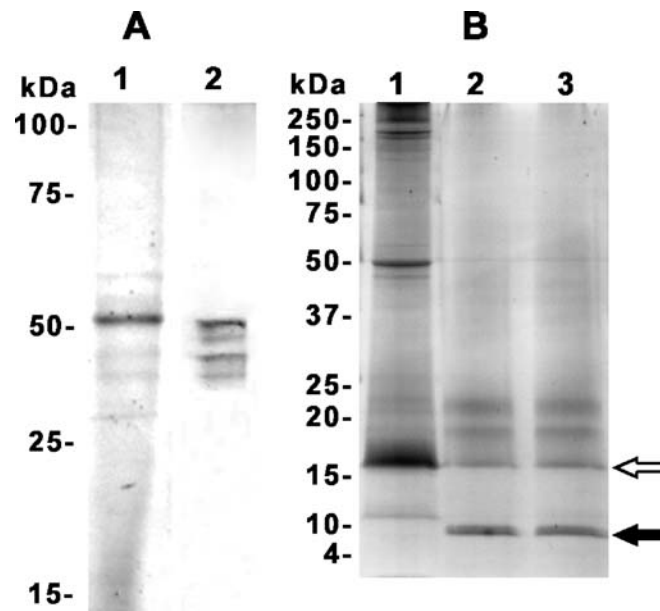


Fig. 4 In Coomassie blue-stained gels, a single 50 kDa protein is the predominant component of storage bodies isolated from the cerebral cortex of a Tibetan terrier with NCL (A, lane 1). Immunoblot analysis of the storage body extracts using rabbit anti-human GFAP antibody identified the 50 kDa band as GFAP (A, lane 2). Silver staining of gels using a special protocol revealed

an additional major band at approximately 16 kDa in storage body extracts (B, lane 1). Extracts of storage bodies from NCL sheep brain (B, lane 2) and pancreas (B, lane 3) revealed previously identified bands at approximately 8 kDa (mitochondrial ATP synthase subunit c) and 16 kDa (vacuolar ATPase subunit c), as well as additional bands at approximately 18 and 21 kDa

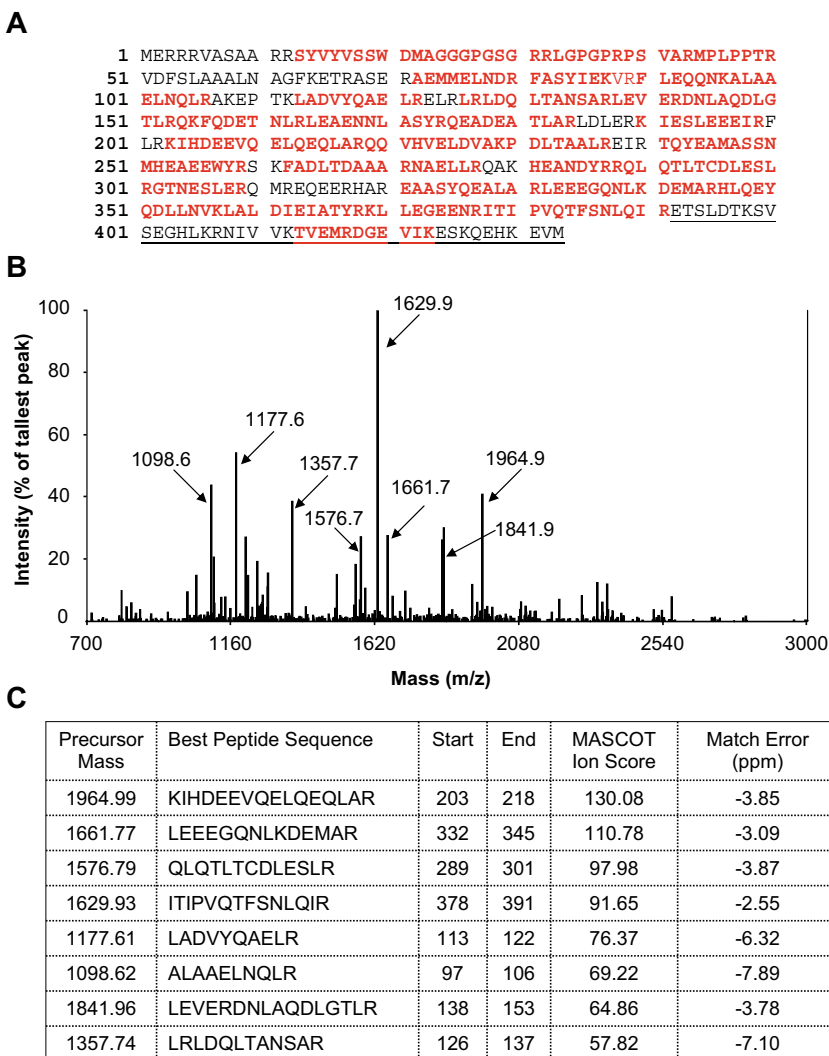
vacuolar V_0 ATPase subunit c antiserum (Palmer et al 1997). Since no 12 kDa band was observed in our silver-stained gels of the same storage body preparations, and since vacuolar V_0 ATPase subunit c has a molecular weight of 16 kDa, it is likely that the band that ran at 16 kDa in our gel electrophoretic analyses corresponds to the band reported as 12 kDa by Palmer and colleagues. Although mass spectral analysis of this band produced no confident identification of vacuolar V_0 ATPase subunit c in the sheep pancreas storage bodies, one lower-abundance peptide mass did match this protein. However, the identity of this peptide could not be confirmed by tandem MS owing to its low abundance.

Likewise, mass spectral analyses of the 16 kDa bands from the sheep and Tibetan terrier brain storage bodies failed to identify vacuolar V_0 ATPase subunit c. Mass spectral analysis of the 16 kDa band from the Tibetan terrier brain preparations identified histone H4 (gi|57098917) as the major component; the masses of four peptides from this band matched those predicted for histone H4 with 48% coverage of the protein. The peptides ISGLIYEETR (ion score of 56) and VFLENVIR (ion score of 41) were identified by tandem MS analysis of the tryptic digest from the Tibetan terrier brain storage body sample. Haemoglobin beta was also detected by MS analysis of the tryptic digest of the 16 kDa band from the Tibetan terrier brain storage bodies; nine peptides in the digest had

masses predicted for peptides from haemoglobin beta (gi|57113367) covering 68% of the protein and of these three were identified as EFTPQVQAAYQK (ion score of 52), VNVDEVGGEALGR (ion score of 61), and FFDSFGDLSTPDAVMSNAK (ion score of 104) by tandem MS analysis. The sheep brain storage bodies also had a band with an apparent molecular weight of 16 kDa (Fig. 4B). Mass spectral analysis of the tryptic digest of the 16 kDa band from the sheep brain storage bodies indicated that the most abundant protein in this band was ubiquitin (gi|51701909). Five peptides had masses that matched those predicted for ubiquitin and one of these peptides was identified as IQDKEGIPPDQQR (ion score of 77) by tandem MS analysis. MS analysis also indicated the presence of prosaposin as a minor component of the sheep brain 16 kDa band. Tandem MS analysis indicated the presence of the peptide GCSFLPDQYR (ion score of 47) in this band. No peptides with masses corresponding to those predicted for vacuolar V_0 ATPase subunit c were detected in the storage body preparations from NCL sheep or Tibetan terrier brains.

Mass spectral analysis of the 50 kDa gel band identified it as canine predicted protein GFAP isoform 2 (gi|73965502) with a MASCOT protein score of 997 (a MASCOT protein score >78 indicated a match at the 95% confidence level). A total of 39 peptides were matched to GFAP and these matched peptides

Fig. 5 Mass spectral analysis of the 50 kDa protein isolated from the storage bodies. (A) The amino acid sequence of the protein identified by mass spectrometry. The top hit from a search of NCBI nr limited to mammals was a predicted canine protein corresponding to glial fibrillary acidic protein, isoform 2 (gi|73965502). The red type indicates the sequence coverage by the 39 peptides observed in the MS spectrum of the trypsin digest. Peptide sequence was determined by mass matches (peptide mass fingerprinting) and/or by peptide fragmentation matches (amino acid sequencing). The underlined portion of the sequence indicates where isoforms 1 and 2 of canine GFAP differ. (B) Mass spectrum of peptides from the trypsin-digested protein band. For clarity, only those peptide masses selected for MS/MS fragmentation are indicated. The eight most abundant peptide ions were automatically selected for MS/MS fragmentation based on their relative intensities in the MS spectrum. (C) Table of MS/MS fragmentation matches showing mass of the precursor ion (peptide selected for MS/MS), the peptide sequence, the position of the matched peptide in the protein sequence, the MASCOT ion score, and the mass error associated with each peptide sequence. The low parts per million ($\text{ppm} = \frac{|\text{observed mass} - \text{theoretical mass}|}{\text{theoretical mass}} \times 10^6$) mass error indicates an almost exact match with the theoretical sequence



covered 76% of the protein (Fig. 5A, red type). The eight most abundant peptides in the MS spectrum (Fig. 5B) were subjected to tandem MS fragmentation. All of these peptides were matched to GFAP with MASCOT ion scores ranging from 58 to 130 (a MASCOT ion score >42 indicated a match at the 95% confidence level). The amino acid sequences of the fragmented and matched peptides are shown in Fig. 5C and the pertinent peaks are indicated on the MS spectrum. These tandem MS peptides alone covered 24% of the protein. Although peptide masses corresponding to the conserved regions of both isoforms 1 and 2 of canine GFAP were matched, the presence of the TVEMRDGEVIK peptide suggests that the protein present in the gel band is isoform 2. This peptide is from the variable C-terminal region of GFAP and it is only present in isoform 2. The identity of the storage body protein was confirmed by immunoblot analysis (Fig. 4B) and by immunohistochemistry (Fig. 6). Very few of the aggregates of storage

material in the tissue sections were associated with cells exhibiting typical astrocyte morphology (Fig. 6). When examined with the microscope filters used for immunofluorescence, sections of cerebral cortex from affected dogs that were either not immunostained or were carried through the immunostaining procedure with the primary antibody omitted were barely detectable. The storage body autofluorescence may have made a minor contribution to the intensity of the storage body-associated fluorescence observed when using the microscope filter set optimized for visualizing red-emitting fluorophores, but it was not sufficient to interfere with GFAP immunolocalization.

Discussion

Although human and animal NCLs result from mutations in more than eight different genes, they are all characterized by massive accumulations of autofluor-

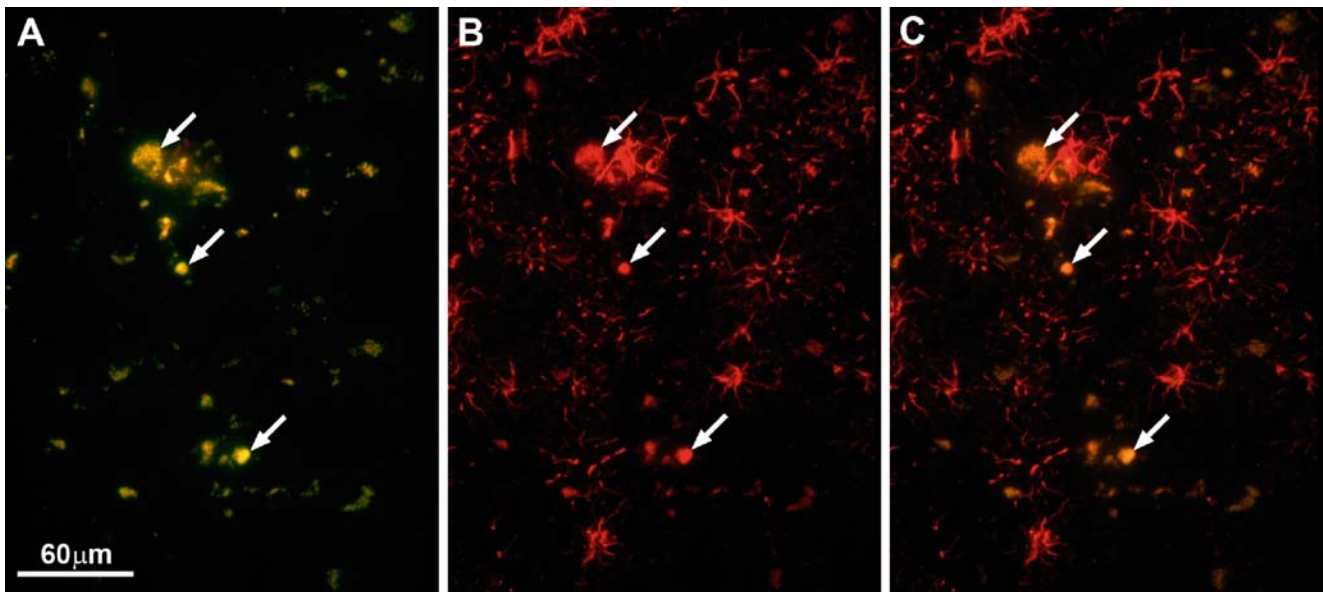


Fig. 6 Immunohistochemical analysis of cerebral cortex from a Tibetan terrier with NCL. Cryostat sections of fixed tissue were probed with an anti-GFAP antibody that was localized by a red-emitting fluorophore bound to a secondary antibody. (A) Fluorescence micrograph of the tissue using light filters optimized for demonstration of storage body autofluorescence (yellow). (B) Fluorescence micrograph of the same area of

tissue using light filters optimized for visualization of the GFAP immunofluorescence. (C) An image generated by merging micrographs (A) and (B) (overlapping autofluorescence and immunofluorescence shown in orange). The autofluorescent storage material (arrows) was almost completely restricted to cells that did not have the stellate GFAP-positive processes typical of astrocytes

escent lysosomal storage bodies throughout the nervous system (Jolly 1995; Katz et al 1997; Wisniewski et al 2001). In all types of NCL that have been characterized to date, the storage bodies have been reported to contain predominantly a single protein or a few major proteins: F₀ subunit c of mitochondrial ATP synthase, vacuolar V₀ ATPase subunit c, or saposins A and D (Palmer et al 1986, 1989, 1990, 1997; Tynnela et al 1993, 1997a). Additional proteins are variably associated with the isolated storage bodies, but these have not been well characterized. For example, Hall and colleagues analysed the protein compositions of storage bodies isolated from brains of human subjects with various forms of NCL and the silver-stained gels from their study contain a number of proteins with apparent molecular weights greater than 25 kDa that were not identified (Hall et al 1991).

The ultrastructural features of the storage bodies are somewhat distinct between the various forms of NCL. However, there is ultrastructural heterogeneity in the storage bodies within most particular forms of the disease and even between different tissues and cell types of the same individual (Koppang 1973; Neville et al 1980). Ultrastructural differences between the different forms of NCL have not been correlated with storage body protein composition (Goebel 1996, 2000; Goebel and Wisniewski 2004; Goebel et al 1979, 1982; Haltia 2003; Katz et al 1994; Nijssen et al 2003; Palmer

et al 1997; Wisniewski et al 2001). The storage bodies in the Tibetan terrier cerebral cortex have components that are somewhat similar in ultrastructure to the ‘fingerprint’ inclusions that accumulate in some of the NCLs. However, the predominant storage body proteins seen in other forms of NCL were not detected in the Tibetan terrier storage body preparations. On the other hand, the specific association of GFAP and histone H4 with the Tibetan terrier storage bodies has not been reported previously in other forms of NCL. Based on SDS-PAGE analyses, a number of unidentified proteins, in addition to those that have been well characterized, are present in storage bodies isolated from tissues of animals and humans with NCL (Fearnley et al 1990; Hall et al 1991; Palmer et al 1986, 1997). These proteins vary in size depending on the form of the disease with which the storage bodies were associated and the tissues from which they were isolated. These differences in protein composition could account, at least in part, for the ultrastructural heterogeneity of the storage material in the NCLs. Differences in protein composition between different Tibetan terrier storage body preparations may reflect differences in the disease stage at which the dogs were euthanized. The dogs were all privately owned and different owners had their dogs euthanized at different stages of the disease. The absence of the 16 kDa band in some of the Tibetan terrier brain storage body

preparations may indicate that a cell type in which the 16 kDa proteins accumulate are selectively lost as the disease progresses.

Vacuolar V_0 and mitochondrial F_0 ATPase subunit c have been previously reported to be associated with NCL sheep pancreas storage bodies (Fearnley et al 1990; Palmer et al 1986, 1997). Bands of approximately the same molecular weight as mitochondrial F_0 ATPase subunit c were detected in silver-stained gels of the extracts of storage bodies from NCL sheep brain and pancreas (Fig. 4B). However, neither vacuolar V_0 nor mitochondrial F_0 ATPase subunit c was detected in the storage body preparations from Tibetan terrier cerebral cortex. This is in contrast to a previous report that vacuolar V_0 ATPase subunit c could be demonstrated by western blotting in extracts from livers of Tibetan terriers with NCL (Palmer et al 1997). This suggests that V_0 ATPase subunit c may be present in the storage bodies from some tissues but not others in the same animal. However, in the study by Palmer and colleagues (Palmer et al 1997) it was also reported that western blotting demonstrated the presence of vacuolar V_0 ATPase subunit c in storage bodies from the pancreas of sheep with NCL. We analysed storage bodies from sheep pancreas provided by the same laboratory and did not detect vacuolar V_0 ATPase subunit c in this preparation. Extracts of the sheep pancreas storage bodies did contain a component with the same apparent molecular weight as vacuolar V_0 ATPase subunit c (16 kDa), but mass spectral analysis of this band only definitively identified histone H4 and mitochondrial F_0 ATP synthase subunit c. The V_0 ATPase may also have been present in this band, as suggested by the findings of Palmer and colleagues, but if so, its relative abundance was too low for us to confidently identify it by tandem MS analysis. The presence of mitochondrial F_0 ATP synthase subunit c in the 16 kDa band is not surprising as this 7.6 kDa protein tends to form oligomers (Fearnley et al 1990).

Of the NCLs where storage body protein compositions have been analysed, the specific accumulation of GFAP in brain storage bodies may be unique to the Tibetan terrier disorder, although the presence of this protein in storage bodies from other NCLs cannot be ruled out on the evidence currently available. In previous studies on storage bodies isolated from animals and humans with NCL, bands were present on gels that could correspond to GFAP, but attempts were not made to identify the proteins in these bands (Fearnley et al 1990; Hall et al 1991). The fact that the Tibetan terrier storage bodies are enriched in predominantly a single or a few proteins suggests that the mechanism underlying storage body accumulation in

these dogs has some features in common with the other NCLs. On the other hand, the distinct identity of the major accumulating proteins suggests that the genetic defect in the Tibetan terriers differs from those in the other forms of NCL. Indeed, candidate gene analyses and homozygosity mapping have excluded mutations in most of the known NCL genes as the basis for the Tibetan terrier disease, including some recently identified late-onset disorders in animals (Drögemüller et al 2005; Wöhlke et al 2005, 2006; Johnson et al unpublished data).

The specific accumulation of GFAP in Tibetan terrier NCL suggests that normal turnover of this protein may require a specific enzyme or interacting protein whose absence cannot be completely compensated for by alternative pathways for protein degradation. The pathways via which GFAP is normally degraded in cells have not been completely elucidated. GFAP undergoes a number of posttranslational modifications, including glycosylation, citrullination and phosphorylation, that could affect its turnover (Ishigami et al 2005; Korolainen et al 2005). It has been demonstrated that elimination of phosphorylation sites in GFAP enhances degradation of this protein (Takemura et al 2002). It is therefore possible that the accumulation of GFAP in the Tibetan terrier disease results from a defect in posttranslational modification of the protein or in recognition of a specific modified form of GFAP by the protein degradative machinery of the cell. It has been hypothesized that oxidative damage is involved in the pathology of at least some forms of NCL (Siakotos et al 1995), and there is evidence that GFAP is specifically subject to autoxidative modification in Pick disease, another neurodegenerative disorder (Muntane et al 2006). Similar oxidative damage could be involved in the accumulation of GFAP in Tibetan terrier NCL. However, the proteins that accumulate in most other NCLs do not appear to have abnormal posttranslational modifications such as those characterized by oxidative damage. GFAP accumulation may also result from alterations in its interactions with other cytoskeletal proteins (Tian et al 2006).

Abnormal intracellular accumulation of GFAP occurs in another neurodegenerative disorder called Alexander disease (Hsiao et al 2005; Li et al 2005). This disease results from mutations in *GFAP*, with variations in the age of onset and severity depending on the specific underlying mutation (Asahina et al 2006; Hsiao et al 2005; Johnson 2004; Li et al 2005). Alexander disease differs in significant respects from Tibetan terrier NCL. In Alexander disease, GFAP accumulation occurs in cytoplasmic aggregates called

Rosenthal fibres that differ morphologically from NCL storage bodies (Hsiao et al 2005; Li et al 2005). The accumulation is restricted to astrocytes in Alexander disease (Hsiao et al 2005), unlike in NCL where storage body accumulation occurs in both neurons and glia (Katz et al 2005b). In the Tibetan terrier disease, little if any of the storage body accumulation occurs in cells that have morphologies typical of astrocytes (Fig. 6). In addition, Alexander disease is inherited in a dominant fashion (Li et al 2005), whereas Tibetan terrier NCL is clearly a recessively inherited disorder (Katz et al 2005b; Riis et al 1992). We have recently identified affected Tibetan terriers that are heterozygous for single nucleotide polymorphism (SNP) markers immediately adjacent to *GFAP* (unpublished data); this confirms that mutations in or near *GFAP* are not responsible for this recessive disorder.

GFAP accumulation outside of astrocytes has also been reported in brains of Alzheimer disease and Down syndrome patients (Hol et al 2003). In affected subjects with no apparent GFAP gene mutations, GFAP expression, including several novel splice variants, was found in hippocampal neurons. This observation, along with the current findings, suggest that aberrant GFAP expression and accumulation may be secondary to neurodegenerative changes in general rather than being linked closely to the primary defect underlying a specific neurodegenerative disorder.

Two splice variants of GFAP have been reported in dogs: isoforms 1 and 2 (GenBank accession numbers XP 537614 and XP 848378 respectively). These two forms differ only from amino acid residue 392 to the carboxyl terminus (residue 432 in isoform 1 and 433 in isoform 2). Based on the mass spectral analysis, it appears that only isoform 2 is present in the storage bodies. A number of GFAP splice variants have also been reported in humans and other species (Condorelli et al 1999; Nielsen et al 2002; Singh et al 2003). Canine isoform 2 corresponds to the isoform that is normally expressed predominantly in humans (Fuchs and Weber 1994; Reeves et al 1989). Therefore, it is unlikely that Tibetan terrier NCL results from an abnormality in GFAP mRNA splicing or excessive production of a minor isoform.

Whatever the mechanism underlying specific accumulation of GFAP in lysosomal storage bodies, discovery of the association of this protein with the storage bodies in the canine disorder may lead to better characterization of some forms of human and animal NCLs for which the genetic basis has not yet been determined. In the Tibetan terrier disease, the onset of clinical symptoms occurs relatively late in life. A number of cases of adult-onset NCL have been

reported in humans (Martin 1991). These disorders are usually referred to as Kufs disease but, based on the heterogeneity in clinical signs, they probably represent a group of genetically diverse disorders. The storage material composition in most Kufs disease patients and in other late-onset NCLs has not been characterized, nor have the mutations responsible for these disorders been identified. Because of similarities in disease phenotypes between some of the human adult-onset NCLs and the Tibetan terrier disease, it would be of interest to determine whether any of the human cases are also characterized by specific GFAP accumulation. Accumulation of the same protein in the storage bodies would suggest that the human and canine diseases may result from mutations in orthologous genes. If this proves to be the case, the Tibetan terrier could serve as a useful model for some forms of adult-onset NCL in humans.

Identification of histone H4 in storage bodies from both Tibetan terrier brain and sheep pancreas suggests that storage of this protein in at least some cell types may be a common feature of the NCLs. However, this protein was not detected in the sheep brain storage body preparation. On the other hand, ubiquitin and prosaposin were detected in sheep brain storage bodies but not in the storage bodies from sheep pancreas or Tibetan terrier brain. Differences in storage protein composition between different tissues of the same animal and between animals with different forms of NCL mirror differences in ultrastructural appearances of the storage bodies. All of the NCLs appear to be characterized by selective accumulation of small numbers of specific proteins. Differences in which proteins accumulate in the different forms of NCL and in different tissues may provide important clues to the mechanisms underlying disease pathology and help to explain why certain cell types degenerate in the NCLs whereas other cell types that accumulate storage material appear to survive unimpaired. Therefore, it will be important to further study differences in storage body protein composition in different tissues, in different forms of NCL, and at different disease stages for each form of NCL.

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