Neuronal ceroid lipofuscinosis associated with an *MFSD8* mutation in Chihuahuas

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ABSTRACT

The neuronal ceroid lipofuscinoses (NCLs) are hereditary neurodegenerative disorders characterized by progressive declines in neurological functions, seizures, and premature death. NCLs result from mutations in at least 13 different genes. Canine versions of the NCLs can serve as important models in developing effective therapeutic interventions for these diseases. NCLs have been described in a number of dog breeds, including Chihuahuas. Studies were undertaken to further characterize the pathology of Chihuahua NCL and to verify its molecular genetic basis. Four unrelated client owned Chihuahuas from Japan, Italy and England that exhibited progressive neurological signs consistent with a diagnosis of NCL underwent neurological examinations. Brain and in some cases also retinal and heart tissues were examined postmortem for the presence of lysosomal storage bodies characteristic of NCL. The affected dogs exhibited massive accumulation of autofluorescent lysosomal storage bodies in the brain, retina and heart accompanied by brain atrophy and retinal degeneration. The dogs were screened for known canine NCL mutations previously reported in a variety of dog breeds. All 4 dogs were homozygous for the *MFSD8* single base pair deletion (*MFSD8:c.843delT*) previously associated with NCL in a Chinese Crested dog and in 2 affected littermate Chihuahuas from Scotland. The dogs were all homozygous for the normal alleles at the other genetic loci known to cause different forms of canine NCL. The *MFSD8:c.843delT* mutation was not present in 57 Chihuahuas that were either clinically normal or suffered from unrelated diseases or in 1761 unaffected dogs representing 186 other breeds. Based on these data it is almost certain that the *MFSD8:c.843delT* mutation is the cause of NCL in Chihuahuas. Because the disorder occurred in widely separated geographic locations or in unrelated dogs from the same country, it is likely that the mutant allele is widespread among Chihuahuas. Genetic testing for this mutation in other Chihuahuas is therefore likely to identify intact dogs with the mutant allele that could be used to establish a research colony that could be used to test potential therapeutic interventions for the corresponding human disease.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are inherited progressive neurodegenerative diseases characterized by progressive declines in motor and cognitive functions, seizures, and vision loss [1]. Most forms of human NCL culminate in premature death. In affected patients, normal early development is followed by onset of clinical signs at ages ranging from infancy to adulthood. Human NCLs result from mutations in one of 13 genes, designated CLN1 through CLN14 (no mutation associated with CLN9 has been identified). Almost all cases exhibit an autosomal recessive pattern of inheritance [1]. The NCLs are a class of lysosomal storage diseases (LSDs), distinguished from the many other LSDs by the accumulation of storage material with distinct autofluorescence and ultrastructural properties and the predominance of proteins among the material that accumulates in the storage bodies [1-13].

Canine NCLs have been reported to result from mutations in the canine orthologs of 8 of the 13 known human NCL genes [14]. Phenotypic signs of NCL have also been reported in additional breeds for which the molecular genetic bases are not yet known [15-22]. Of the canine NCL mutations, 2 distinct *CLN8* mutations have been reported in English Setters and Australian Shepherds [23, 24], whereas identical *CLN5* mutations have been found in Border Collies and Australian Cattle Dogs [25, 26]. Recently a mutation in *MFSD8* (the gene associated with CLN7 disease) initially identified in a Chinese Crested dog with NCL [27] was reported in 2 NCL-affected littermate Chihuahuas [28]. Some other previously described canine NCL mutations have been identified only in either a single dog or an isolated line [23, 24, 29], whereas for some canine NCLs, the mutant allele has been found to be quite common in the affected breed [30, 31]. Making a distinction between these 2 possibilities is important in providing breeders advice on whether to screen their dogs for the mutant allele and in evaluating the potential for identifying dogs with the mutant alleles that could be used for breeding to develop research models. Therefore, a study was undertaken to better characterize the NCL phenotype in the Chihuahua disease, to confirm the association between the *MFSD8*

mutation and the disease, and determine whether this mutation is restricted to a single line of

dogs or is more widespread in the breed.

2. Materials and Methods

2.1 Subject dogs

Four Chihuahuas that exhibited similar progressive neurological signs were evaluated for this study. Two of these were unrelated longhaired dogs from Japan that were previously reported to have suffered from NCL [35]. Also evaluated were short-haired Chihuahuas from Italy and England (Figure 1) that exhibited clinical signs with a progression similar to those of the dogs from Japan. Descriptions of the clinical disease signs are included in the Results section of this paper. There were no known relationships between any of the affected Chihuahuas evaluated in this study. For molecular genetic analyses, in addition to the DNA from the 4 clinically affected Chihuahuas, we utilized archived DNA samples from 1818 dogs that were either clinically normal or suffered from an unrelated disease (see below for details). All procedures involving animals were reviewed and approved by the University of Missouri Animal Care and Use Committee and were performed in compliance with the EU Directive 2010/63/EU for animal experiments and were approved by the University of Missouri Animal Care and Use Committee. All samples utilized in this study were obtained with the owners' consent. Euthanasias were performed using standard protocols for clinical practice in the countries in which the dogs were euthanized.
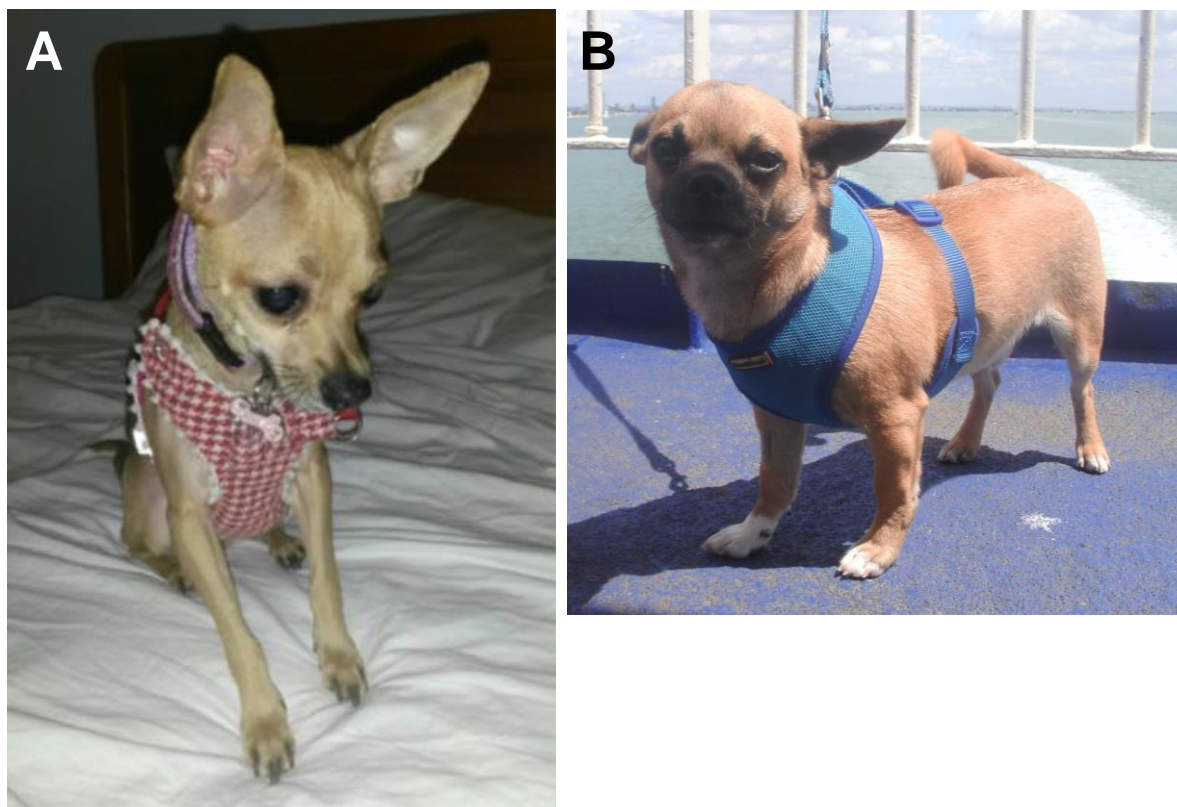
Figure 1. Photographs of the subject dogs from Italy (A) and England (B). The dogs exhibited the typical conformations of short-haired Chihuahuas.

2.2 Histopathology and electron microscopy

Brain histopathology in the Japanese cases was described previously [35]. Brain, eye and heart tissues were collected from the Italian Chihuahua at necropsy performed shortly after euthanasia. The eyes were enucleated and the corneas removed immediately. One eye from each dog was placed in a fixative consisting of 3.5% formaldehyde, 0.05% glutaraldehyde, 120 mM sodium cacodylate, 1mM $CaCl_2$, pH 7.4 (immuno fix), and the other eye was placed in 2.5% glutaraldehyde, 100 mM sodium cacodylate, pH 7.4 (EM fix). Likewise, slices of the cerebral cortex, cerebellum and heart ventricle wall were each placed in the same fixatives shortly after euthanasia. Samples were incubated in these fixatives at room temperature until being further processed for microscopic examination. Prior to further processing, the eyecups were dissected

to obtain regions from the posterior poles adjacent to the optic nerve heads, and these regions were used for examination.

Slices of each of these tissues were processed, embedded and frozen for cryostat sectioning. The tissues were incubated with gentle agitation successively in 170mM sodium cacodlylate buffer, pH 7.4, 10% sucrose in the cacodylate buffer, 25% sucrose in the cacodylate buffer, and a 1:1 mixture of the 25% sucrose solution and Tissue-Tek (Sakura Fintek, Torrance, CA) for a minimum of 30 minutes each.   The samples were then transferred to cryomolds filled with Tissue-Tek, incubated at 4^0C for 40 minutes then frozen on a block of dry ice. Cryostat sections of each of these tissues were cut at a thickness of 8 μm, mounted on Superfrost Plus slides (Fisher Scientific, Fairlawn, NJ) in 170mM sodium cacodylate. The sections were examined and photographed using fluorescence microscopy as previously described [32] except that images were acquired using an Olympus DP72 color digital camera.

Pieces of the tissue samples that had been fixed in 2.5% glutaraldehyde were post-fixed with osmium tetroxide and embedded in epoxy resin. Sections of the embedded tissues were cut on an ultramicrotome at thicknesses of 0.5 to 0.8 μm, mounted on glass slides and stained with toluidine blue. Areas of interest were identified by microscopic examination of these sections, and the blocks were trimmed to remove tissue from outside the areas of interest. Sections were then obtained from the trimmed blocks at thicknesses of 70 to 90 nm. The latter sections were mounted on 200 mesh copper thin-barred grids, stained with uranyl acetate and lead citrate, and were then examined and photographed using a JEOL 1400 transmission electron microscope.

Slices of immuno--fixed cerebellum from a normal 3 year old Beagle and from the Italian Chihuahua were embedded in paraffin. Four µm thick sections of these tissues were mounted on positively charged glass slides, deparffinized, and immunostained with an antibody directed against glial fibrillary acid protein (GFAP) as described previously [27]. Slides were

counterstained in hematoxylin (Biocare Medical) at a 1:10 dilution for 5 min followed by a Tris-buffered rinse, dehydrated, coverslipped, and then imaged with light microscopy.

2.3 Molecular genetic analyses

Genomic DNA was isolated from EDTA-anticoagulated whole blood or buccal swabs of each of the dogs as described previously [23, 33]. The NCL-affected Chihuahua from Italy was genotyped for previously reported NCL-causing mutations in *TPP1*, *PPT1*, *CLN5*, *CLN8*, *CTSD*, and *MFSD8* with assays described in earlier publications [14, 23, 27, 29, 31, 34]. The previously described TaqMan allelic discrimination for an *MFSD8* 1 bp deletion and frameshift [27] was also used to genotype the two NCL-affected Chihuahuas from Japan, the NCL-affected Chihuahua from England, and previously archived DNA samples from 57 Chihuahuas that were either clinically normal or suffered from an unrelated disease, and 1761 additional unaffected dogs representing 186 other breeds. The latter included 1478 Chinese Crested dogs [27].

3. Results

3.1 Clinical signs of neurological disease

The clinical signs and other aspects of the disease phenotype of the two affected dogs from Japan were described as cases 1 and 2 in a previous report in which multigenerational pedigree information was included [35]. The neurological signs in these dogs had an onset of 16 and 18 months respectively and included progressive vision loss, anxiety, ataxia, cognitive impairment, and seizures. Magnetic resonance imaging of both dogs demonstrated diffuse brain atrophy and ventricular enlargement [35]. The dogs died at 23 and 24 months of age respectively. These 2 dogs had no ancestors in common for at least three generations. Unrelated affected short-haired Chihuahuas from Italy and England (Figure 1) exhibited a clinical progression similar to the dogs from Japan. Starting at 16 to 18 months of age these signs included loss of house training, severe anxiety, especially when handled or on a lead, personality changes including altered interactions with other dogs and occasional aggressiveness toward people, profound loss of learned behaviors including responsiveness to commands and to being called by name, excessive sensitivity to loud noises, development of compulsive behaviors including circling, bouts of persistent vocalization, impaired ability to climb up or down stairs, loss of coordination, severe vision loss in both bright and dim light conditions, and bouts of trance-like behavior. Although pupillary light reflexes were intact, neither eye in these dogs exhibited a dazzle or menace response or visual tracking in either eye. Generalized brain atrophy and ventricular enlargement similar to that of the Japanese cases were observed with magnetic resonance imaging both dogs. The Italian dog was euthanized at approximately 25 months of age after suffering from severe seizures and the Chihuahua from England was euthanized at approximately 22 months of age due to the severity of the clinical signs. A DNA sample but no tissues were obtained from the English dog. Brain, eye and heart tissues were obtained from the Italian dog shortly after death and preserved as described previously. There were no known relationships between any of the affected Chihuahuas evaluated in this study.

3.2. Histopathology and electron microscopy

No postmortem tissues were available for examination from the English dog. Histopathological examination of tissues from the 2 Japanese cases was restricted to examination of stained paraffin sections of the cerebellum, cerebral cortex, medulla and meninges. Neurons throughout the brain exhibited massive perinuclear accumulations of storage bodies that stained with Sudan black [35], consistent with a lysosomal storage disease.

We were able to collect the brain, eyes and heart from the affected Italian Chihuahua shortly after euthanasia. These tissues were examined for the presence of the autofluorescent lysosomal storage material that is characteristic of the NCLs. The affected Chihuahua exhibited massive accumulations of autofluorescent storage material in the brain, retina, and heart (Figures 2 and 3). In the cerebral cortex storage material accumulation occurred throughout the tissue. In the cerebellum autofluorescent storage material was most abundant in the Purkinje cell layer. Within this layer storage material was present not only in what could be recognized as Purkinje cells, but also in masses much larger than the Purkinje cell bodies (Figure 2A). Substantial amounts of autofluorescent storage material were also present in the molecular and granule cell layers (Figures 2A and 2B). The retina was severely thinned, lacked clearly recognizable normal retinal layers (Figure 2E) and massive amounts of storage material were present throughout what remained of the retina (Figure 2D). Autofluorescent storage material was present throughout the ventricular wall muscles (Figure 3). At the ultrastructural level the contents of the storage bodies from the cerebellar, cerebral cortical and retinal neurons all were composed of numerous aggregates of compacted multilaminar membrane-like material (Figure 4).
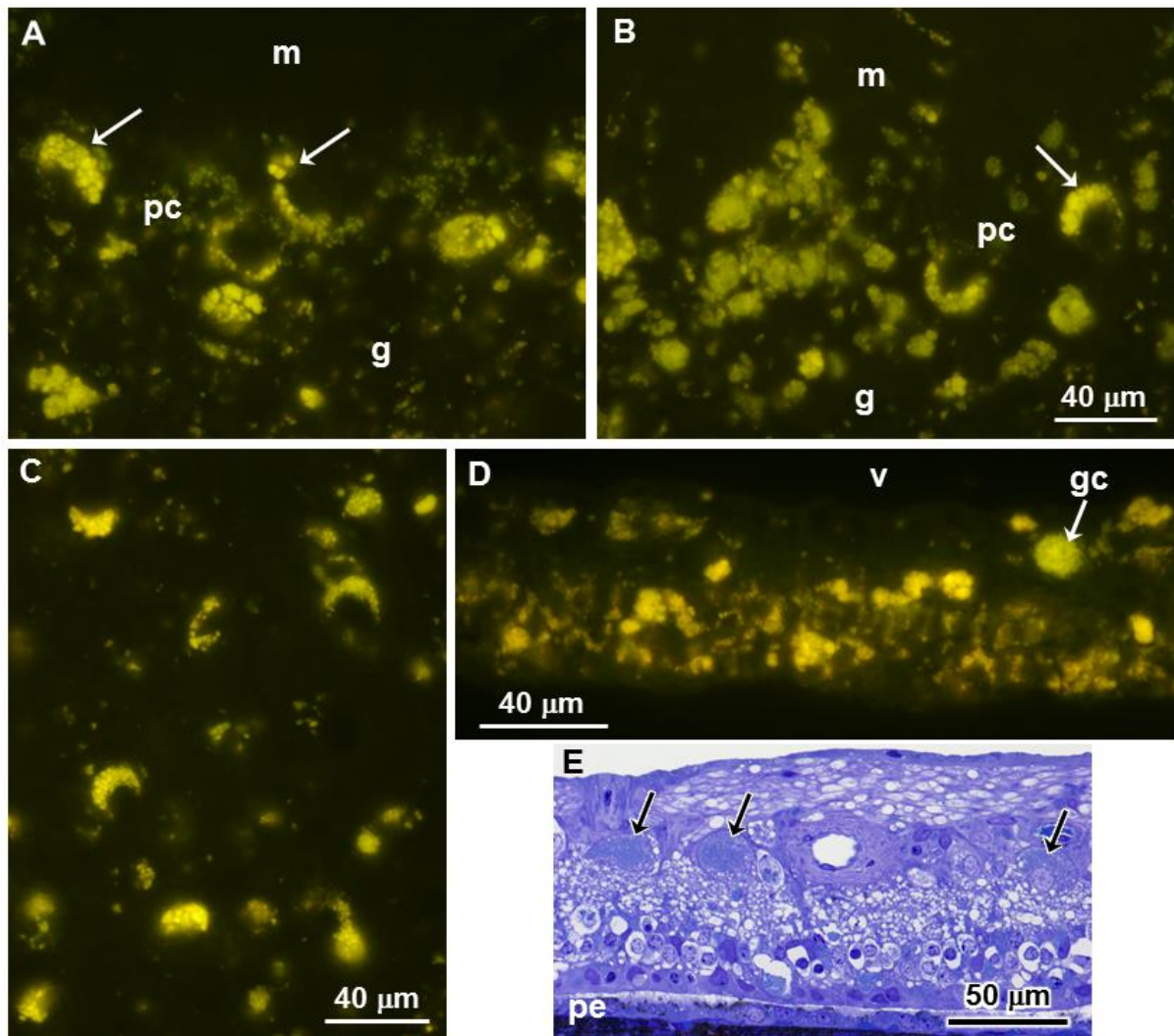
Figure 2. Fluorescence micrographs of cryostat sections of the cerebellum (A and B), cerebral cortex (C) and retina (D) and a light micrograph of a Toluidine blue-stained section of the retina from the affected Italian Chihuahua. Abbreviations: m: molecular layer; pc: Purkinje cell layer; g: granule cell layer; v: vitreous body of eye; gc: retinal ganglion cell; pe: retinal pigment epithelium. Arrows in (A) and (B) indicate Purkinje cells. Arrows in (E) indicate ganglion cells. Bar in (B) indicates the magnification for both panels (A) and (B).
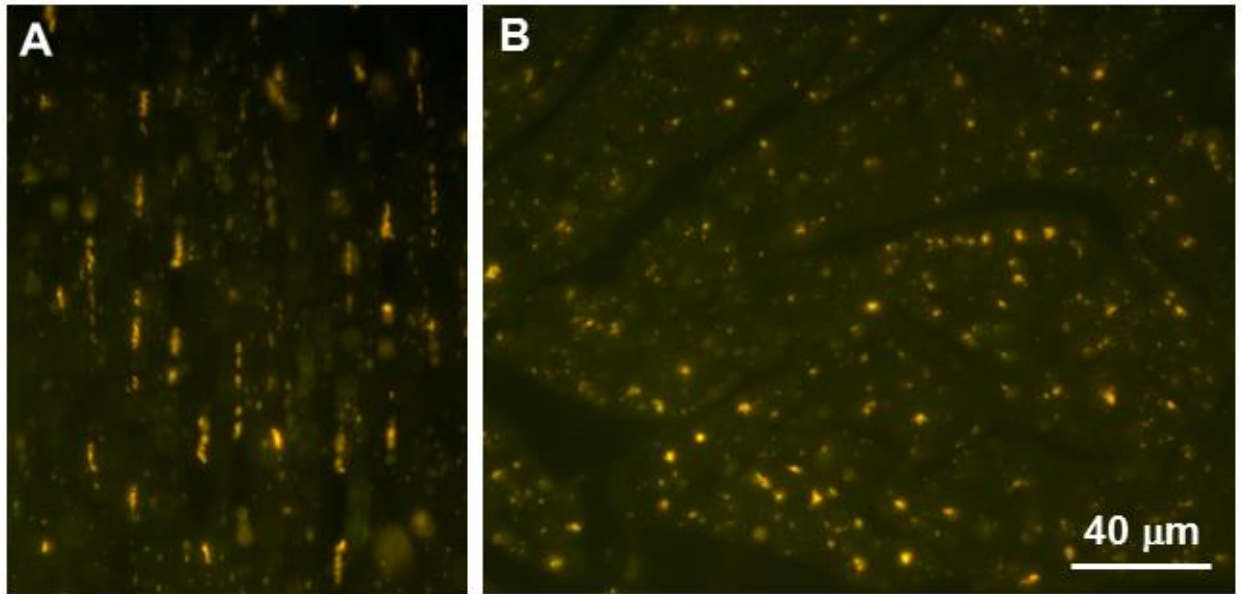
Figure 3. Fluorescence micrographs of the heart ventricular wall muscle with the muscle fibers oriented in longitudinal (A) and cross-section (B) orientations. Bar in (B) indicates magnification for both micrographs.
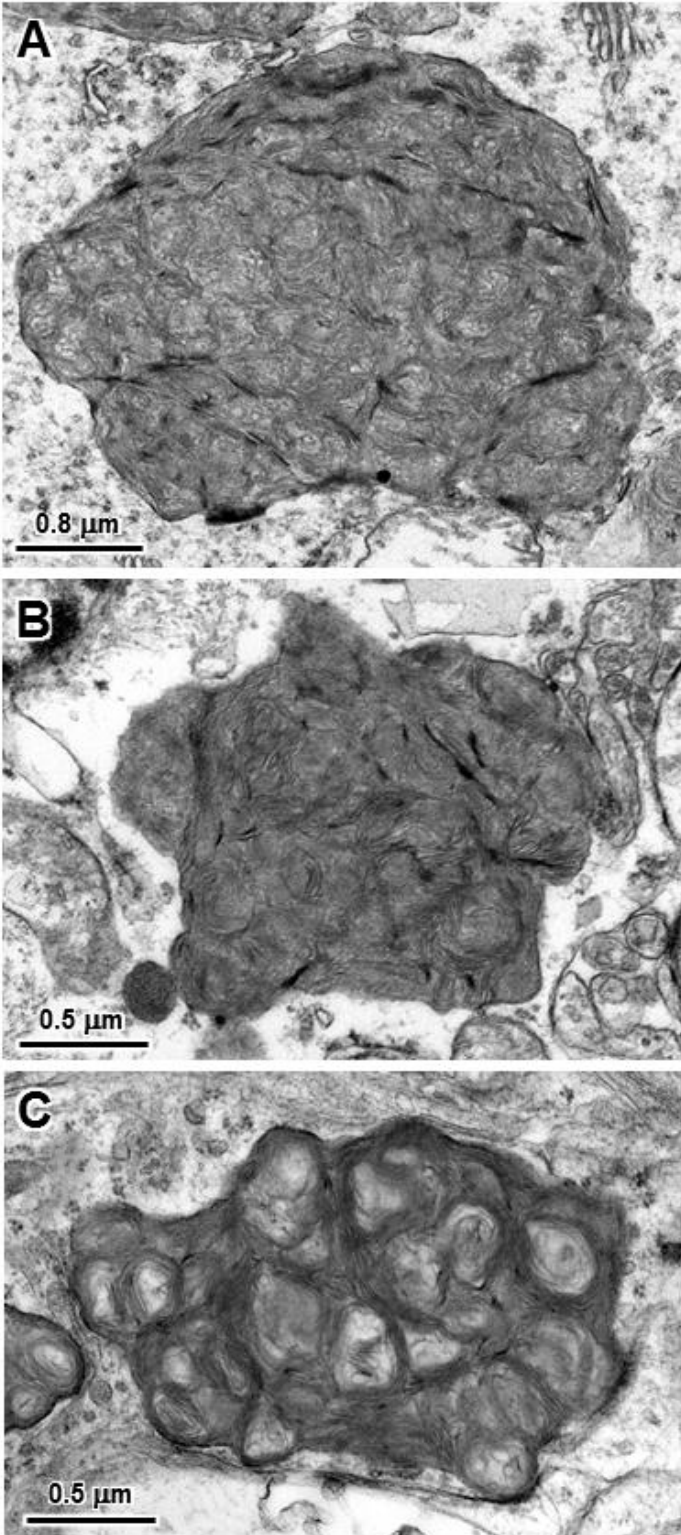
Figure 4. Electron micrographs of disease-specific storage bodies from a cerebellar Purkinje cell (A), a cerebral cortical neuron (B), and a retinal ganglion cell (C).

The NCLs, like many progressive neurodegenerative diseases, are characterized by astrogliosis, as demonstrated by increased amounts of glial fibrillary acid protein (GFAP) in astrocytes [24, 27, 35-37]. The cerebellum of the Italian Chihuahua was evaluated for GFAP expression using immunohistochemistry. Very high numbers of cells that stained strongly with an antibody directed against GFAP were present in both the cerebellar medulla (Figure 5A) and the granule cell layer (Figure 5C). By comparison, little GFAP immunostaining was observed in either of these cerebellar areas in a normal healthy dog (Figures 5B and 5D).
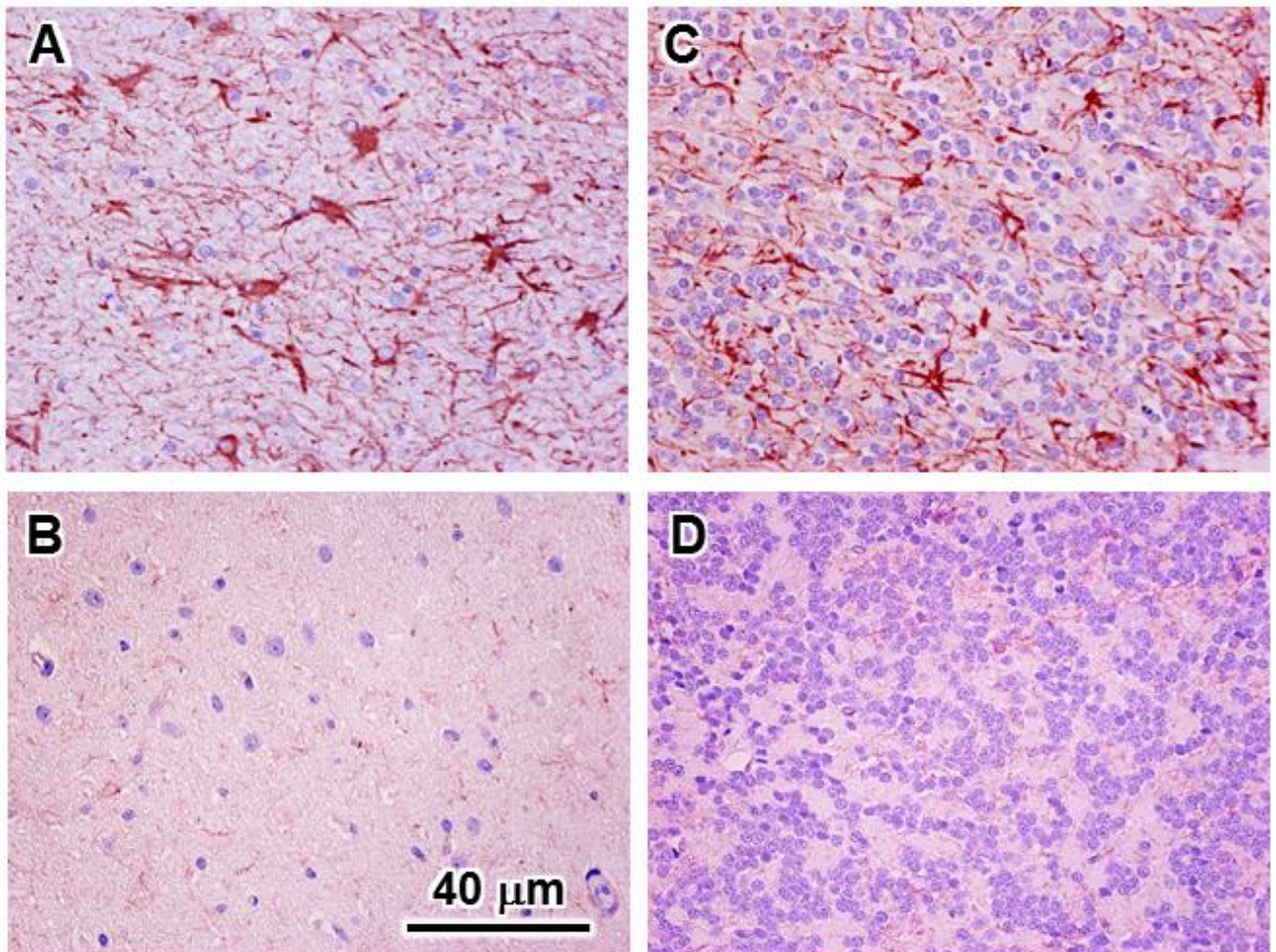

Figure 5. Light micrographs of sections of the cerebellar medulla (A and B) and granule cell layer (C and D). All sections were immunostained for GFAP. GFAP staining is a reddish brown color. Micrographs (A) and (C) are from sections from an affected Chihuahua and micrographs (B) and (D) are from a normal healthy Beagle of similar age. Bar in (B) indicates magnification of all 4 micrographs.

3.3. Molecular genetic analyses

      The NCL-affected Chihuahua from Italy was homozygous for the normal alleles at the loci in *TTP1*, *PPT1*, *CLN5*, *CLN8*, and *CTSD* previously associated with NCL in dogs. By contrast, this dog, along with the affected Chihuahuas from Japan and England was homozygous for the *MFSD8* single base pair deletion (*MFSD8:c.843delT*) previously associated with NCL in a Chinese Crested dog from Los Angeles, CA [27] and in 2 littermate Chihuahuas from Scotland [28]. Archived DNA samples from 57 Chihuahuas that were either clinically normal or suffered from an unrelated disease, and 1761 unaffected dogs representing 186 other breeds were tested for the mutant *MFSD8* allele. Of these dogs, all were homozygous for the normal allele except for one previously reported Chinese Crested dog that was heterozygous for the one base deletion [27].

4. Discussion

A homozygous one base pair deletion and frameshift in *MFSD8* is associated with the clinical and histopathological signs of NCL from 4 unrelated Chihuahuas from around the world. This same mutation was previously associated with NCL in 2 littermate Chihuahuas with no apparent close relationship with the dogs described in this study [28]. The widespread geographic distribution of the disease among Chihuahuas and the fact that it occurs in both long-haired and short-haired varieties suggests that the mutant allele may be relatively common among Chihuahuas, and warrants at least a broad random screening of unrelated Chihuahuas to obtain an estimate of the mutant allele frequency in the breed. Although screening of DNA samples from 57 archived DNA samples from Chihuahuas in our repository did not identify any dogs with the mutant allele, our archive does not represent a random sampling of the general Chihuahua population, since samples collected for this archive are usually targeted to specific diseases, which in the case of the Chihuahua samples did not include NCL. Until the *MFSD8* mutant allele frequency can be better estimated in the Chihuahua population, it would be prudent to screen Chihuahuas for this mutation prior to breeding, particularly for males that may be used widely for breeding based on certain perceived desirable characteristics. Such screening could identify intact dogs that could be used to establish a research colony or provide semen that could be preserved for use in establishing such a colony in the future. We already have preserved semen from dogs with the CLN2 and CLN5 forms of NCL.

The same *MFSD8* mutation associated with NCL in Chihuahuas was previously associated with NCL in a single Chinese Crested dog. It is likely that the mutation originated in one of these breeds and was transferred to the other breed by either intentional or accidental interbreeding. Although only one affected Chinese Crested dog has been reported to date, the finding of a heterozygote among the archived DNA samples from an apparently unrelated dog of this breed indicates that the mutant allele is not restricted to this single case and further screening for the mutant allele in Chinese Crested dogs may be warranted.

Although all of the dogs that were homozygous for the *MFSD8* mutation exhibited a similar pattern of clinical signs, the retina of the affected dog from Italy that was euthanized at 25 months of age was much more severely degenerated than that of the dog from Scotland that was euthanized at 23 months of age [28]. This suggests that retinal degeneration may accelerate late in the disease process, although this difference may simply represent variation between dogs. Unfortunately retinal tissue from the NCL-affected Chinese Crested dog that was homozygous for the *MFSD8* mutation was not available for examination [27]. Although visual deficits occur in most of the canine NCLs and in their human counterparts, the degree of retinal degeneration observed in the affected Chihuahua from Italy was much more profound than in most other canine NCLs [22, 27, 32, 38-43], and all 4 dogs included in this report exhibited profound visual deficits. Similar visual deficits have been reported in human subjects with *MFSD8* mutations associated with NCL [44, 45]. In most types of canine NCL the autofluorescent storage body accumulation in the retina is concentrated primarily in the ganglion cells and along the outer limiting membrane. However, in the Italian Chihuahua the storage material was present throughout the severely degenerated retina (Figure 2).

Although the NCLs are generally considered primarily neurological disorders, expression of the genes that harbor the disease-causing mutations are not restricted to the nervous system. It is likely that the NCLs considered primarily neurological diseases because the central nervous system and retina are more sensitive to the effects of the mutations than are other tissues. However, as we have shown in the Chihuahua and another canine NCL [14], the disease is accompanied by substantial accumulation of autofluorescent storage material in the heart muscle. Storage body accumulation has been demonstrated in other tissues in various other types of human NCL as well [1, 11, 46]. Although these storage body accumulations in non-neuronal tissues do not appear to be associated with substantial functional impairment in untreated individuals, such impairment may become evident if patients receive treatments targeted to the central nervous system. Indeed, we have found evidence for heart pathology in

dogs with the CLN2 form of NCL that have achieved extended lifespans as a result of CNS-targeted enzyme replacement therapy (unpublished finding).

Dogs with the various forms of NCL are potentially excellent models for the evaluation of potential therapeutic interventions that may then be translated to human application. Indeed, evaluation of enzyme replacement therapy in treating the CLN2 form of NCL in a Dachshund model [47] formed the basis of a human clinical trial of this treatment that has just been successfully completed (https://clinicaltrials.gov/ct2/show/NCT02678689?term=CLN2&rank=3), for the first time making an effective treatment available for children with any form of NCL. Unfortunately, for some of the canine NCLs in which the causative mutation has been identified, the mutations were discovered in isolated cases or specific lines and it was not possible to obtain and preserve semen from affected or carrier dogs so that development of a canine model has not yet been possible. Including the previously reported cases in NCL in 2 littermates [28], we have now shown that the NCL-causing mutation in *MFSD8* is widespread among Chihuahuas. Thus by screening relatives of the affected dogs or by genotyping of a large Chihuahua population, it should be possible to identify intact affected male carriers and preserve semen to use in generating dogs for future therapeutic studies. We have already initiated this effort which will expand on our current endeavors to obtain and preserve semen from other forms of canine NCL. Our goal is to make these semen samples widely available so that once promising treatment approaches have been developed the canine models will be available to validate their efficacy and optimize their application to the corresponding human disorders.

Acknowledgments

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