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Canine neuronal ceroid lipofuscinoses: Promising models for preclinical testing of therapeutic interventions

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Abstract

The neuronal ceroid lipofuscinoses (NCLs) are devastating inherited progressive neurodegenerative diseases, with most forms having a childhood onset of clinical signs. The NCLs are characterized by progressive cognitive and motor decline, vision loss, seizures, respiratory and swallowing impairment, and ultimately premature death. Different forms of NCL result from mutations in at least 13 genes. The clinical signs of some forms overlap significantly, so genetic testing is the only way to definitively determine which form an individual patient suffers from. At present, an effective treatment is available for only one form of NCL. Evidence of NCL has been documented in over 20 canine breeds and in mixed-breed dogs. To date, 12 mutations in 8 different genes orthologous to the human NCL genes have been found to underlie NCL in a variety of dog breeds. A Dachshund model with a null mutation in one of these genes is being utilized to investigate potential therapeutic interventions, including enzyme replacement and gene therapies. Demonstration of the efficacy of enzyme replacement therapy in this model led to successful completion of human clinical trials of this treatment. Further research into the other canine NCLs, with in-depth characterization and understanding of the disease processes, will likely lead to the development of successful therapeutic interventions for additional forms of NCL, for both human patients and animals with these disorders.

1. Introduction: neuronal ceroid lipofuscinoses in humans and animals

The neuronal ceroid lipofuscinoses (NCLs) consist of a group of inherited neurodegenerative lysosomal storage disorders that result from mutations in at least 13 genes (Mole and Cotman, 2015). Most forms of NCL are autosomal recessive in inheritance. In addition to affecting people, NCL-like disorders have been reported to occur naturally in a number of

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other mammalian species including dogs, cats, cattle, horses, sheep, mice, and monkeys, as well as in some non-mammalian species (Bildfell et al., 1995; Bond et al., 2013; Broom et al., 1998; Cesta et al., 2006; Chalkley et al., 2014; Evans et al., 2012; Fiske and Storts, 1988; Frugier et al., 2008; Gao et al., 2002; Green and Little, 1974; Hafner et al., 2005; Houweling et al., 2006; Jasty et al., 1984; Jolly et al., 1994a; Jolly and Palmer, 1995; Kay Read and Bridges, 1969; Nakayama et al., 1993; Nibe et al., 2011; Ranta et al., 1999; Reece and MacWhirter, 1988; Tammen et al., 2006; Tyynela et al., 2000; Url et al., 2001; Weissenbock and Rossel, 1997; Wheeler et al., 2002; Woods et al., 1993). Mouse, pig, fish, and insect models of the NCLs have also been created through genetic engineering (Bond et al., 2013; Bouchelion et al., 2014; Faller et al., 2015; Miller et al., 2015; Schultheis et al., 2013). Although many naturally occurring progressive neurological disorders in dogs have been reported as NCLs, for the purposes of this review only those disorders in which the disease has been associated with mutations in the canine ortholog of one of the 13 known human NCL genes will be considered to be confirmed cases of NCL. Other canine disorders that have the neurological and histopathological features of the NCLs will be considered as "putative" NCLs until the gene causing mutation is discovered. When the latter occurs, the disease-causing mutation will confirm the classification of the disease as an NCL if the mutation occurs in one of the established NCL genes. If the mutation occurs in any other gene, a determination will need to be made as to whether the disease should be classified as a new form of NCL or should be placed with another established group of inherited lysosomal storage disorders. Both of these situations have occurred. In 2011 a mutation in *ATP13A2* was found to cause a late-onset NCL-like disease in Tibetan Terriers (Farias et al., 2011). As a result, a human disease known as Kufor-Rakeb syndrome was classified as a form of NCL (CLN12) (Bras et al., 2012; Mole and Cotman, 2015). On the other hand, an NCL-like disorder in American Staffordshire Terriers that results from a mutation in *ARSG* was originally designated as an NCL, even though no known human form of NCL had been shown to result from mutations in this gene (Abitbol et al., 2010). Subsequent analyses using a transgenic mouse model led to the reclassification of the canine disorder to the group of lysosomal storage diseases known as mucopolysaccharidoses (Kowalewski et al., 2015; Kowalewski et al., 2012; Kowalewski et al., 2014). In addition, we evaluated Shiba Inu dogs that exhibited a progressive neurological disease and massive accumulation of autofluorescent storage material in brain, retina and heart similar to confirmed canine NCLs (Figs. 1 and 2) (Kolicheski et al., 2017b). Although the ultrastructural appearances of the storage material in brain and retina were somewhat similar to those of other canine NCLs (Ashwini et al., 2016; Gilliam et al., 2015; Guo et al., 2014; Guo et al., 2015; Katz et al., 2011; Kolicheski et al., 2017a; Kolicheski et al., 2016; Kolicheski et al., 2017b; Sanders et al., 2010), the histological appearance of the storage material in the affected Shiba Inu heart was quite distinct from that of other NCLs in which heart histopathology had been examined (Figs. 3 and 4). This suggested that the Shiba Inu disease was either a previously undescribed form of canine NCL or a phenotypically similar disease. Indeed, whole genome sequence (WGS) analysis indicated that the disease did not result from a mutation in any of the known NCL genes but rather from a mutation in *HEXB* (Kolicheski et al., 2017b). Mutations in the human ortholog of this gene cause Sandhoff disease, a form of gangliosidosis, so on the basis of the molecular genetic analyses the Shiba Inu disease could be excluded as a form of NCL.

At present, no formal process has been established for determining whether diseases should be classified as NCLs if they are characterized by the clinical and pathological features typical of the NCLs but result from mutations in genes not already accepted as causes of human NCL. NCL classifications have generally been established on an ad hoc basis by experts on this group of disorders. Although there is not universal consensus on the criteria for classifying a disease as an NCL, there is general agreement that only those disorders associated with mutations in one of the known NCL genes should automatically be classified as an NCL (Mole and Cotman, 2015).

2. Identification of potential cases of NCL in dogs

For a canine disorder to be considered as a candidate NCL, the disease should be characterized by progressive neurological symptoms that include at least 4 of the following clinical signs: loss of vision, behavioural changes including changes in personality (e.g. development of aggressive behaviour) and loss of learned behaviors, tremors, cerebellar ataxia, cognitive and motor decline, sleep disturbance, and seizures. In most cases the progression in the severity of the neurologic signs ultimately lead to premature death or euthanasia. Since all of the NCLs are hereditary, it is important to determine if possible whether any close relatives or dogs of the same breed have exhibited clinical signs similar to those of a dog in which a diagnosis of NCL is being considered. A universal histopathologic feature of the NCLs is accumulation of autofluorescent lysosomal storage bodies in many neural and extraneural cell types, particularly in the neurons of the brain and retina (Fig. 1) (Haltia et al., 1973; Mole et al., 2011; Palmer et al., 1997a; Zeman and Donahue, 1963). The pigment resembles ceroid and lipofuscin storage material (Katz and Robison, 2002; Seehafer and Pearce, 2006), hence the name of the disease. The term ceroid refers to intracellular autofluorescent material that accumulates in cells of various tissues as a result of pathological processes or nutritional deficiencies (Davidson et al., 1998; Katz et al., 1986; Katz et al., 1984; Katz and Robison, 2002; Katz et al., 1978; Sulzer et al., 2008). Lipofuscin, on the other hand, refers to autofluorescent lysosomal storage material that accumulates primarily in postmitotic cells as part of the normal process of senescence (Katz and Robison, 2002; Seehafer and Pearce, 2006). Therefore, the storage material that accumulates in the NCLs is properly classified as ceroid. Because the clinical signs of canine NCL overlap with those of many other diseases, demonstration of the accumulation of autofluorescent ceroid storage material in neurological tissues is required to classify a disease as a potential NCL. Ultrastructurally and biochemically, there is heterogeneity in the lysosomal storage bodies between different forms of NCL and even between tissues within the same form. Despite this heterogeneity, detailed analyses of the ultrastructure or biochemical composition of the storage material can be useful in determining whether the disease should be considered a potential case of NCL. In some forms, specific proteins make up a large proportion of the storage body content (Haltia, 2006; Palmer, 2015; Palmer et al., 1992; Tyynelä et al., 1993), which differentiates the NCLs from most other lysosomal storage diseases. In addition, in some forms of NCL, in particular CLN2 disease, the ultrastructure of the storage material is quite distinctive compared to that of other NCLs and other lysosomal storage diseases (Fig. 5).

Minimum criteria for presumptive diagnosis of a canine NCL are evidence of heritability, progressive neurological dysfunction within the spectrum of clinical signs described above, and accumulations of autofluorescent storage material at least in neuronal tissues. The NCLs are also characterized by diffuse brain atrophy (Fig. 6). Magnetic resonance imaging is useful to detect brain atrophy based on increased volume in the ventricular system, and prominence of sulci in the cerebrum and folia in the cerebellum. Since there are some diseases that meet these criteria but clearly fall into one of the other established groups of lysosomal storage diseases, definitive classification as an NCL is dependent on demonstration that the disease results from a mutation in one of the known NCL genes (Mole and Cotman, 2015).

In this review, we will summarize the canine NCLs that result from mutations in orthologs of the genes containing mutations responsible for the established human NCLs. We will also review putative canine NCLs for which the mutations have not yet been identified. Finally, we will describe research aimed at developing effective treatments for human NCLs using naturally occurring canine models.

3. Canine NCLs with known causative mutations

The first putative cases of canine NCL were described in the 1950s by Nils Koppang, a Norwegian veterinarian who discovered a line of English Setters that included dogs that developed a progressive neurodegenerative disease that closely resembled what at the time was referred to as the classical juvenile form of NCL (Diezel et al., 1965; Hagen, 1953; Koppang, 1966; Koppang et al., 1988). Dr. Koppang obtained dogs from the affected pedigree and for many years maintained a breeding colony of these dogs on his private farm. Over the subsequent decades, numerous studies were performed on the affected dogs he generated in attempts to determine the underlying cause of the disease (Katz et al., 1994a; Katz et al., 1995a; Shibuya et al., 1998). With advances in molecular genetic technologies, the mutations responsible for various forms of human NCL began to be reported, starting with the discovery in 1995 of a mutation in the *PPT1* gene that was responsible for an infantile-onset form of NCL (Vesa et al., 1995). In the same and subsequent years, the genes harbouring the mutations for almost all reported cases of human NCL were identified (Mole and Cotman, 2015). With these discoveries, it became possible to screen dogs that were exhibiting NCL-like clinical signs for mutations in the orthologous canine genes using Sanger sequencing technologies. This candidate-gene approach led to the discovery of a mutation in *CLN8* as the cause of the English Setter NCL decades after the disease was first observed in this breed (Katz et al., 2005a).

Subsequently, the candidate-gene approach led to identification of the mutations responsible for various forms of NCL in Dachshunds, Border Collies, Australian Shepherds, and American Bulldogs (Awano et al., 2006a; Awano et al., 2006b; Katz et al., 2011; Melville et al., 2005; Sanders et al., 2010) (Table 1). The mutation responsible for NCL in Tibetan Terriers was discovered via homozygosity mapping (Farias et al., 2011). As a result of technological advances in DNA sequencing technology and data analysis, the entire canine genome sequence became available along with annotations that continue to be refined. Because of these advances, in order to find a causative mutation it is currently more efficient

to sequence the entire genome of a dog with putative NCL than it is to sequence individual candidate genes. This approach enables one to screen a dog for candidate mutations in all of the known NCL genes at once with the added advantage of enabling one to search for candidate disease causing mutations in other genes if an affected dog does not have a mutation in one of the known NCL genes. To date, the mutations responsible for NCL have been discovered in Australian Cattle Dogs, Golden Retrievers, Chinese Crested dogs, Chihuahuas, Cane Corsos, and Alpenlandische Dachsbrackes using the whole genome sequencing approach (Faller et al., 2016; Gilliam et al., 2015; Guo et al., 2015; Hirz et al., 2016; Kolicheski et al., 2017a; Kolicheski et al., 2016). In total to date, 12 mutations in eight genes have been identified as causative for NCL in dogs (Table 1).

NCLs occur not only in purebred dogs, but have also been found in mixed breed dogs. For example, in a mixed breed dog with Australian Shepherd ancestry suffering from NCL, a causative mutation was found in *CLN8* using whole genome sequencing (Guo et al., 2014). More recently a dog with a German Shepherd Dog dam and Australian Cattle Dog sire (Fig. 7) presented with clinical signs consistent with NCL, including seizures (See Supplemental Video 1). This dog exhibited accumulation of autofluorescent lysosomal storage bodies typical of the NCLs in the cerebellum, cerebral cortex, and retina (Fig. 8). These storage bodies exhibited ultrastructural features observed in other dogs with NCL (Fig. 9). An allelic discrimination assay revealed that this dog was homozygous for the *CLN5* nonsense mutation previously reported in Border Collies and purebred Australian Cattle dogs (Kolicheski et al., 2016; Melville et al., 2005). Since NCL has not been previously reported in German Shepherd Dogs, this suggests that the dam of the affected dog probably had either Border Collie or Australian Cattle Dog ancestry. These findings indicate that a diagnosis of NCL should be considered for any dog exhibiting progressive neurological signs typical of this disease regardless of putative ancestry, especially if good pedigree records and health histories of close relatives of the dog are not available.

4. Identifying the genetic basis for candidate canine NCLs

Prior to performing molecular genetic analyses to determine whether a dog exhibiting neurologic signs harbours a mutation in one of the known NCL genes, assessments are performed to determine whether the clinical signs and pathology are consistent with a tentative diagnosis of NCL. The age of onset, and rate and pattern of progression of neurological signs in the canine NCLs varies with the specific form of disease and the underlying mutation. In some forms of the disease that result from null mutations, signs can appear as early as several months of age (Awano et al., 2006a), whereas in others the first behavioural signs may not become apparent until the dogs are approximately 7 years old (Alroy et al., 1992; Farias et al., 2011; Katz et al., 2005b). The clinical disease signs for each of those forms of canine NCL for which the causative mutations have been identified have been described (see publications cited in Table 1). The most common behavioural signs include anxiety, personality changes including the development of aggressive and compulsive behaviours, cognitive decline including loss of responsiveness to previously learned commands, increased sensitivity to loud noises, and neurologic signs of ataxia, tremors, seizures, and impaired vision. In some cases affected dogs also exhibit bouts of trancelike behaviour. Not all of these signs may be present in all forms of canine NCL, and

some of the signs may be transient during the disease progression. For example, in some forms of NCL dogs may become aggressive in the early stages of the disease, but the aggressiveness subsides as the disease progresses. In all cases, the neurological signs become progressively worse over time and almost invariably lead the owners to eventually elect to have the dog humanely euthanized. For any dog that exhibits at least several of these signs that become progressively worse over time, a diagnosis of NCL should be considered.

Dogs suspected of suffering from NCL can easily be tested for any of the known canine NCL mutations using DNA obtained from a blood sample or cheek swab. All of the known canine NCL mutations are specific to one or two breeds (Table 1), so if the suspect dog comes from one of these breed backgrounds, genetic testing is likely to identify the cause of the disorder. Two different NCL-causing mutations have been identified in both Dachshunds and Australian Shepherds (Table 1), so for these breeds, dogs exhibiting NCL-like signs should be tested for both breed-specific mutations. It is possible that even within these breeds there are additional NCL-causing mutations. In mixed breed dogs of unknown ancestry, testing for all of the known canine NCL mutations may prove diagnostic, but negative results will not rule out NCL in these dogs. In cases of clinically affected dogs when the presence of the known canine NCL-causing mutations have been ruled out, a presumptive diagnosis can be supported by examining brain, retina, and other tissues, including heart muscle, for the accumulation of autofluorescent lysosomal storage bodies with the ultrastructural features characteristic of the NCLs (e.g. Figs. 8 and 9). However, as noted above, similar autofluorescent lysosomal storage bodies accumulate in some canine lysosomal storage diseases that are not classified as NCLs and that may not be ultrastructurally distinct from all of the storage body types found in the NCLs.

We have adopted a systematic approach to identifying NCL in dogs based on our past successes in this endeavour. As soon as a case of potential NCL is reported to us, we request a blood sample for DNA isolation, a complete clinical history on the affected dog, and completion of a survey form in which the presence and age of onset of a large panel of neurological signs is recorded. We also request the dog's pedigree if available, as well as health histories and blood samples from any close relatives (particularly parents and littermates). Under ideal conditions, we would obtain all of the information and samples, but often we are only able to obtain a blood sample from the affected dog and a description of that dog's history of clinical signs. Based on the information we obtain, we determine whether the dog is likely to be suffering from a form of NCL, and if appropriate based on the information received, we will screen the dog for the known canine NCL mutations using established mutation-specific assays. If all of these NCL-causing mutations are excluded, we will request that tissues including brain, eyes, and heart be collected at the time of euthanasia and preserved and shipped to us using prepared kits. Once we receive the tissues, we examine them for the presence of autofluorescent lysosomal storage bodies characteristic of the NCLs and evaluate the ultrastructural appearance of the storage material. We also use routine histology to evaluate the tissues for signs of neurodegeneration. If the results of these analyses are consistent with a diagnosis of NCL, we will perform a whole genome sequence analysis that includes alignment of the sequence with the CanFam3.1 reference assembly (http://useast.ensembl.org/Canis_familiaris/Info/Index). The WGS of the affected dog will then be examined for homozygous variants in the known NCL genes that do not occur in

unaffected dogs from an in-house WGS database and are likely to encode a change in amino acid sequence that would affect the function of the protein encoded. If a candidate mutation is found, we screen genomic DNA from a large number of dogs with no signs of NCL within the same and of different breeds to determine whether the candidate sequence variant is specific to the affected dog. If possible, we will also screen relatives of the affected dog and other dogs of the same breed exhibiting similar neurological and/or histopathological signs and dogs likely to be carriers. In most cases these analyses will lead to identification of the disease-causing mutation. In cases where no candidate mutations are found in any of the known NCL genes, the WGS will be evaluated for homozygous variants in other genes that could cause a similar disease. In some cases this will lead to identification of the disease-causing mutation in a non-NCL gene. For example, this is how we found a mutation in *HEXB* that results in NCL-like clinical signs and histopathology in Shiba Inus suffering from the canine equivalent of Sandhoff disease (Kolicheski et al., 2017b).

5. Putative canine NCLs for which the causative mutation has not yet been identified

Since the first report of an NCL-like disease in English Setters in the 1950s, numerous cases of putative NCL of currently unknown genetic basis have been reported in a large number of dog breeds (see Table 2). These reports range from very limited descriptions of clinical signs in one or a few isolated cases (Appleby et al., 1982; Bichsel and Vandevelde, 1982; Cantile et al., 1996; Cummings and de Lahunta, 1977; Hoover et al., 1984; Jolly et al., 1994b; Pickett et al., 1992; Rossmeisl et al., 2003; Vandevelde and Fatzer, 1980), to more thorough characterizations of the disease phenotypes, including histopathology, in multiple affected dogs of the same breed (Goebel, 1992; Goebel and Dahme, 1985; Goebel and Dahme, 1986; Jolly et al., 1994a; Jolly et al., 1997; Kirchhoff and Kobe, 1994; Minatel et al., 2000; Narfström et al., 2007; Nimmo Wilkie and Hudson, 1982; Palmer et al., 1997b; Smith et al., 1996). There is a wide range in the amount of evidence used to support putative diagnoses of NCL in these cases. Except for the disorders listed in Table 1, the genetic bases for these NCL-like disorders have not been determined, so at this point they must be considered putative NCLs, but could well be other diseases with clinical signs and pathology that are similar to the NCLs.

Among the dog breeds in which a disease was reported as an NCL with no molecular genetic confirmation are the Cocker Spaniel, Dalmatian, Labrador Retriever, Longhaired Dachshund, Wirehaired Dachshund, Australian Shepherd, Miniature Schnauzer, Polish Lowland Sheepdog (PON), Yugoslavian Shepherd Dog, Saluki, Corgi, Poodle, Spitz, and a mixed breed dog (see Table 2). For some of these breeds including Corgi, Spitz, and Yugoslavian Shepherd only limited clinical descriptions have been published (Bichsel and Vandevelde, 1982; Jolly et al., 1994b; Pickett et al., 1992). Histopathological confirmation of NCL-like storage body accumulation has been presented for Dalmatians (Goebel, 1992; Goebel and Dahme, 1985; Goebel and Dahme, 1986), a single Labrador Retriever (Rossmeisl et al., 2003), two related Miniature Schnauzers (Palmer et al., 1997b; Smith et al., 1996), a number of apparently unrelated Cocker Spaniels (Jolly et al., 1994a; Minatel et al., 2000; Nimmo Wilkie and Hudson, 1982), three Australian Shepherd littermates (Katz et

al., 2008), a Standard Poodle (Cantile et al., 1996), two related Saluki dogs (Appleby et al., 1982), two Longhaired Dachshunds (Vandevelde and Fatzer, 1980), a Wirehaired Dachshund (Cummings and de Lahunta, 1977), a mixed-breed dog with terrier ancestry (Hoover et al., 1984), and at least 9 PON dogs, many of which were not closely related (Narfstrom and Wrigstad, 1995; Narfström et al., 2007; Wrigstad et al., 1995).

Among the more thoroughly characterized of the putative canine NCLs of unknown genetic cause is the disease in the Polish Lowland Sheepdog breed, also known as Polish Owczarek Nizinny dogs (PON). The most extensive phenotypic characterization of the disease in this breed was performed by Narfström and colleagues (Narfström et al., 2007). In a series of nine affected PON dogs, the age of onset of clinical signs occurred at approximately 6 months in 6 of the dogs and not until 3 to 4 years of age in the remaining 3 dogs. This suggested that there may be two distinct diseases among the PON dogs tentatively classified as suffering from NCL. There was significant overlap in clinical signs between the two groups of dogs. These signs included behavioural abnormalities such as aggression, dementia, and confusion; neurological dysfunction causing ataxia; and retinal blindness (Narfström et al., 2007). Ophthalmic examinations revealed abnormal pupillary light reflexes (PLRs) in all affected dogs and generalized retinopathy that ranged from slight to severe.

The mutations responsible for NCL in PON dogs have not yet been identified, although a number of candidate genes have been evaluated for mutations in DNA samples from affected dogs. In the reports on candidate gene analysis, very little phenotypic information was provided on the single dog from which the DNA was obtained, so based on the possibility that there are 2 different forms of NCL in this breed, these studies cannot rule out mutations in the candidate genes as potential causes for NCL in all PON dogs. Sequencing the exons and flanking regions of the introns of *TPP1* and *CTSD* in a single 4.5 year old affected PON did not reveal any candidate mutations (Drogemuller et al., 2005b; Wohlke et al., 2005). No information was provided on the age of disease onset for this dog. Subsequently the same group evaluated a whole genome sequence from the same PON dog and did not find any candidate disease-causing mutations in *PPT1*, *CLN8* or *CLCN3* or in 3 of 4 exons of *CLN5* and 6 of 7 exons of *CLN6* (Drogemuller et al., 2005a; Wohlke et al., 2006). It may be possible to identify the disease-causing mutation in both the early-onset and late-onset forms of putative NCL in the PON using WGS dog if DNA samples can be obtained from dogs in which the disease phenotype has been thoroughly characterized.

Most of these cases of putative NCL were reported many years ago and we are not aware of live dogs from any of these breeds that are currently exhibiting signs of NCL. This may indicate that the disease is no longer present in any of the dogs of these breeds or that the disease incidence is very low. In addition, potential NCL probably goes unrecognized in many affected dogs that are euthanized with progressive neurological diseases and these cases are unreported to those who would be able to identify causative mutations. That this is likely the case is supported by the fact that we receive reports of new NCL-like disease in dogs on the average of once every six months, primarily from board-certified veterinary neurologists who are familiar with signs of neurodegenerative diseases and seek out expertise from researchers who are familiar the NCLs.

6. Investigation of therapeutic interventions for treating the NCLs using canine models

To date, systematic therapeutic interventions for canine NCLs have only been evaluated for the CLN8 disease in English Setters(Siakotos et al., 2001) and for the CLN2 disease in Miniature Longhaired Dachshunds (Katz et al., 2014; Katz et al., 2017; Katz et al., 2015; Tracy et al., 2016a; Vuillemenot et al., 2011; Vuillemenot et al., 2015; Whiting et al., 2016; Whiting et al., 2014). It has been reported that a major component of the storage material that accumulates in a number of the NCLs is subunit c protein, a subunit of mitochondrial ATP synthase (Palmer et al., 2013; Palmer et al., 1989) It was discovered that both the form of the subunit c protein stored in some of the NCLs and the form present in mitochondria from normal animals contains a trimethylated lysine (TML) residue (Katz et al., 1994b; Katz et al., 1995b; Katz and Gerhardt, 1992; Katz and Rodrigues, 1991; Katz et al., 1997). TML is a precursor in the normal biosynthesis of carnitine, which plays a critical role in lipid metabolism (Steiber et al., 2004; Strijbis et al., 2010). We therefore hypothesized that the apparent lack of adequate degradation of the subunit c protein in NCL would result in decreased tissue carnitine levels. Indeed, blood plasma carnitine and TML levels were found to be low in both human subjects with CLN3 NCL and in English Setters with CLN8 NCL (Katz, 1996; Katz and Siakotos, 1995). This led to the hypothesis that NCL-related pathology in at least some forms of the disease can result in part from inadequate tissue levels of carnitine. To test this hypothesis, English Setters with CLN8 NCL were provided with long-term dietary carnitine supplements. This resulted in a delay in the progression of some disease symptoms, including cognitive decline (Siakotos et al., 2001). However, most signs of the disease progression were not affected by the carnitine supplementation. Based on these studies, it was recommended that blood plasma carnitine levels be measured in children with any of the NCLs, and only if plasma levels are below the normal reference range should supplements be provided (Evangeliou and Vlassopoulos, 2003).

More extensive studies have been conducted on developing treatments for the CLN2 form of NCL using the Dachshund model for this disease. Miniature Dachshunds with a null mutation in *TPP1* have been bred and maintained at the University of Missouri since 2006. Enzyme replacement therapy using periodic infusion of recombinant human TPP1 protein into the cerebrospinal fluid (CSF) resulted in a dramatic delay in the onset and progression of the neurological signs of the disease, preservation of cognitive function, and significant prolongation of healthy lifespan (Katz et al., 2014). The treatment was so effective that, based on the efficacy of the treatment in the Dachshund model, a successful clinical trial of the same treatment was conducted in children with CLN2 disease (https://clinicaltrials.gov/ct2/show/NCT02963350?term=CLN2&rank=1), and the treatment is now available in multiple clinical centers. Subsequently, gene therapy was tested as an alternative approach to treatment in the Dachshund CLN2 model. An AAV2.TPP1 gene therapy vector was injected into the CSF of the lateral ventricles of the brain of affected dogs early in the disease. This treatment resulted in long-term high levels of TPP1 gene expression by the cells lining the ventricles and widespread distribution of functional TPP1 throughout the central nervous system (CNS) (Katz et al., 2015). The therapeutic efficacy of this treatment was similar to that obtained with the periodic infusions of recombinant TPP1. While both

treatments resulted in significant delays in onset of signs and slowed disease progression, the treated dogs did eventually progress to end-stage neurologic disease. However, in the enzyme infusion studies, it was found that the higher the dose of recombinant TPP1, the more prolonged was the therapeutic benefit. This suggests that at least with respect to many of the neurological signs, the disease could be “cured” if high enough sustained levels of TPP1 could be maintained.

Neither of these treatments had effects on the disease-related progressive decline in retinal function and retinal degeneration (Whiting et al., 2016; Whiting et al., 2014). To address this problem, affected dogs received single intravitreal injections of autologous bone marrow-derived mesenchymal stem cells that had been transduced to overexpress the normal human TPP1 protein. This treatment resulted in a dramatic long-term preservation of retinal function and structure with minimal apparent complications (Tracy et al., 2016b). Research is currently under way to determine whether this approach can be used to treat the neurologic signs of CLN2 disease in the Dachshund model.

Because the canine NCLs are relatively rare in occurrence and genetic testing is enabling breeders to deplete the gene pools of the mutations, it will probably be necessary to establish canine disease models and preserve semen. Research colonies are practical for the relatively early-onset forms of the disease, but it would be logistically very difficult to evaluate therapies in a laboratory setting in dogs with disease onsets of as late as 7 years, as in the Tibetan Terrier form of NCL. Our research team is currently attempting to collect and preserve semen from affected or carrier dogs with the earlier-onset forms of NCL so that the laboratory models can be developed should a promising therapy become available for testing.

7. Conclusions

Most of the canine NCLs appear to be rare disorders, and based solely on the clinical signs, can be difficult to distinguish definitively from other progressive neurological diseases. This makes the NCLs a challenging group of disorders to diagnose for the clinician. It is likely that the great majority of affected dogs are euthanized due to the progression of signs without a diagnosis having been made, in large part likely due to the poor prognosis regardless of pursuing a specific diagnosis. Presumptive diagnoses of NCL are more likely in cases where dogs are evaluated by board-certified veterinary neurologists and ophthalmologists based on recognition of the spectrum of clinical signs associated with these diseases. For those breeds in which the causative mutation has been identified, a definitive diagnosis can usually be made on the basis of available genetic testing (Table 1). Even if a dog exhibiting NCL-like signs is negative for the mutations that cause NCL in that dog’s breed, this will not completely rule out NCL as a cause of the dog’s disorder since, as we have seen, multiple different forms of NCL can occur within the same breed and an individual patient may be suffering from a form of NCL for which the causative mutation has not yet been identified.

Despite these obstacles, attempting to make a diagnosis of NCL based on genetic testing in dogs exhibiting signs consistent with a form of this group of disorders is worthwhile, even if

it will not directly benefit the affected patient. Identification of the causative mutation in an affected dog will provide the tools for testing related dogs that may be used for breeding so that owners can avoid breeding dogs that could produce affected offspring. In addition, identification of intact carriers of the NCL mutation could enable establishment of a laboratory model that can be used to test potential therapies that would benefit both dogs and children suffering from the corresponding form of NCL.

With current technologies, it is possible to screen dogs with NCL-like clinical signs for the causative mutations using either DNA tests for the known canine NCLs or whole genome sequencing for those dogs in which known canine NCL mutations have either been excluded or are unlikely. Commercial and academic research testing for the known canine NCL mutations is available at relatively moderate costs or at no charge from some research laboratories. To identify new canine NCL mutations, laboratories such as those of some of the authors will perform whole genome sequencing on a research basis at no cost to the dog owners if the affected dog to be tested has exhibited the neurological and pathological signs of NCL described earlier. In some cases whole genome sequencing will rule out NCL as the cause of a disorder, but because this technique allows the entire genome to be screened for potential disease-causing mutations, the same whole genome sequence analysis initially targeted to finding an NCL mutation can identify a mutation in any other gene that could underlie a dog's NCL-like disease. Because of the potential benefits of identifying the genetic bases of NCL-like neurological diseases in dogs, veterinarians are encouraged to contact NCL research laboratories if they have canine patients exhibiting signs consistent with a form of NCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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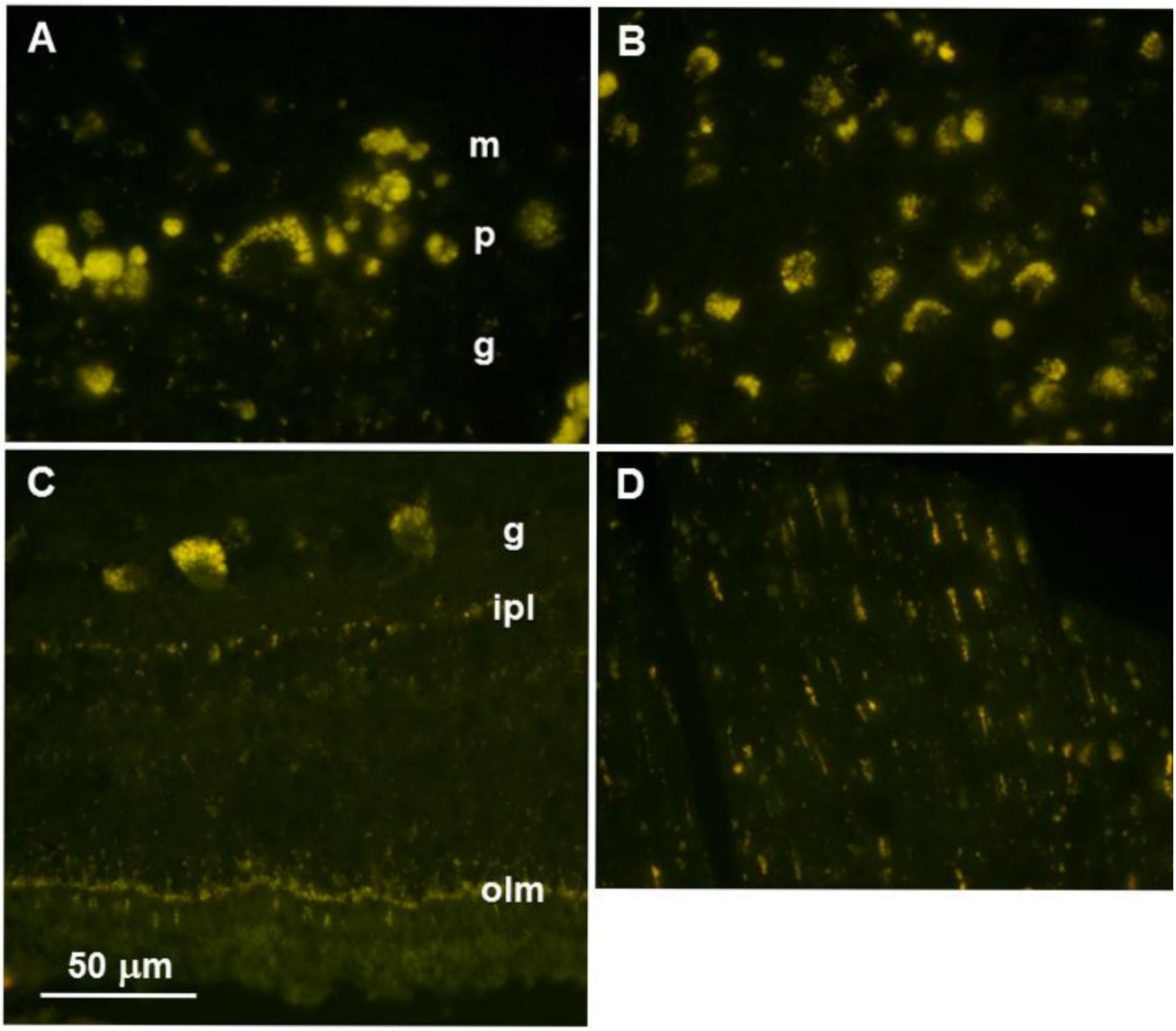

Figure 1.

Fluorescence micrographs of unstained sections of cerebellum (A), cerebral cortex (B), retina (C) and heart (D) from an Australian Cattle Dog that suffered from the CLN5 form of NCL. In the cerebellum autofluorescent inclusions were present in cells in the Purkinje cell (p), molecular (m), and granular (g) layers. Neurons throughout the cerebral cortex had abundant autofluorescent inclusions. In the retina the most pronounced accumulation of autofluorescent material was seen in ganglion cells (g), but some accumulation was also present in the inner plexiform layer (ipl) and along the outer limiting membrane (olm). In the heart strings of autofluorescent storage material were present in the muscle fibers sectioned in the longitudinal orientation. Bar in (C) indicates magnification of all 4 micrographs.

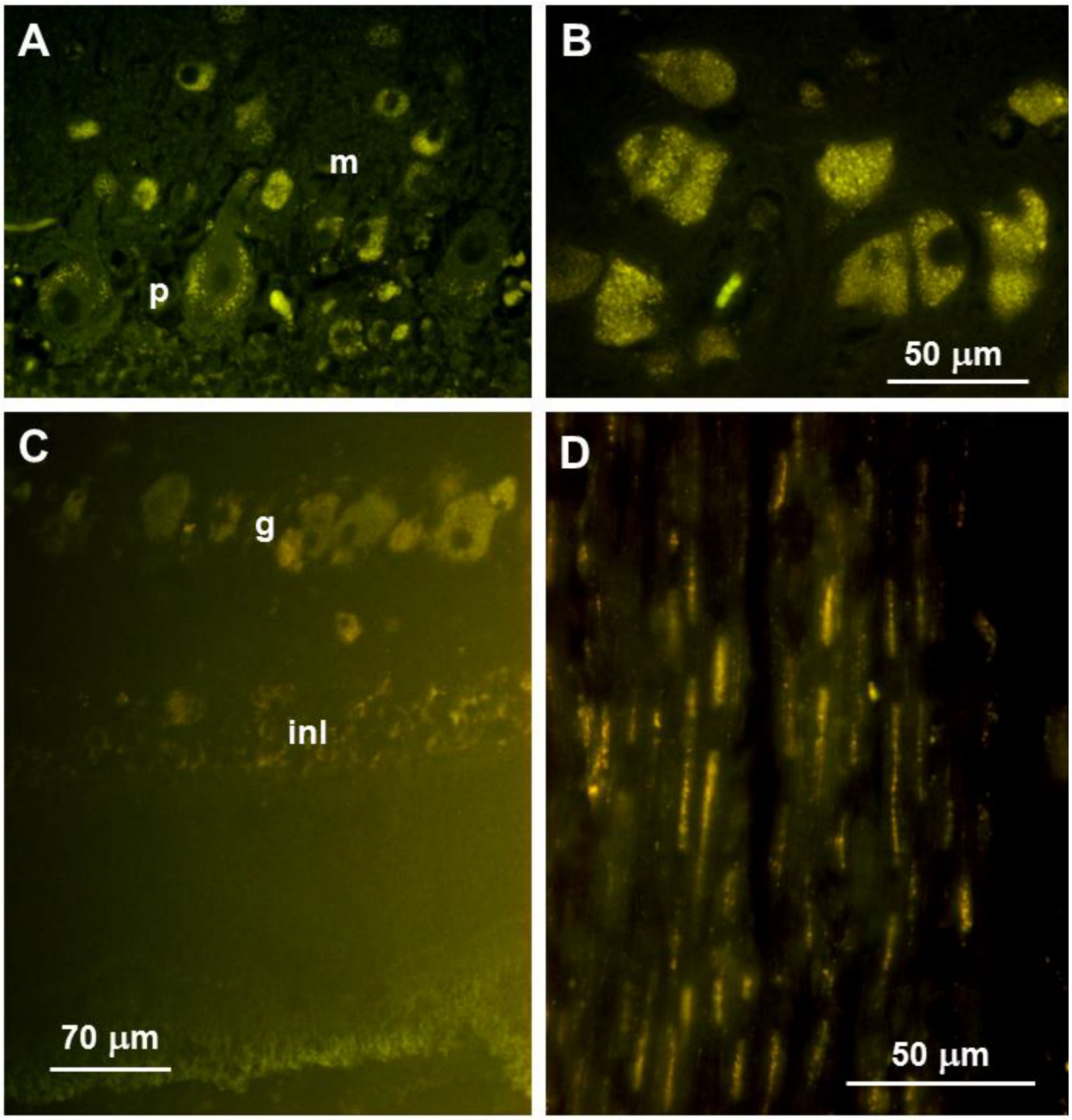

Figure 2.

Fluorescence micrographs of unstained sections of cerebellum (A), cerebral cortex (B), retina (C), and heart (D) from a Siba Inu with GM2 gangliosidosis resulting from a mutation in *HEXB*. In the cerebellum autofluorescent inclusions were present in both the Purkinje cell (p) and molecular (m) layers. Neurons throughout the cerebral cortex had abundant autofluorescent inclusions. In the retina the most pronounced accumulation of autofluorescent material was seen in the ganglion cell layer (g), but some accumulation was also present in the inner nuclear layer (inl). In the heart long strings of autofluorescent

storage material were present in the muscle fibers. Bar in (B) indicates magnification of micrographs (A) and (B).

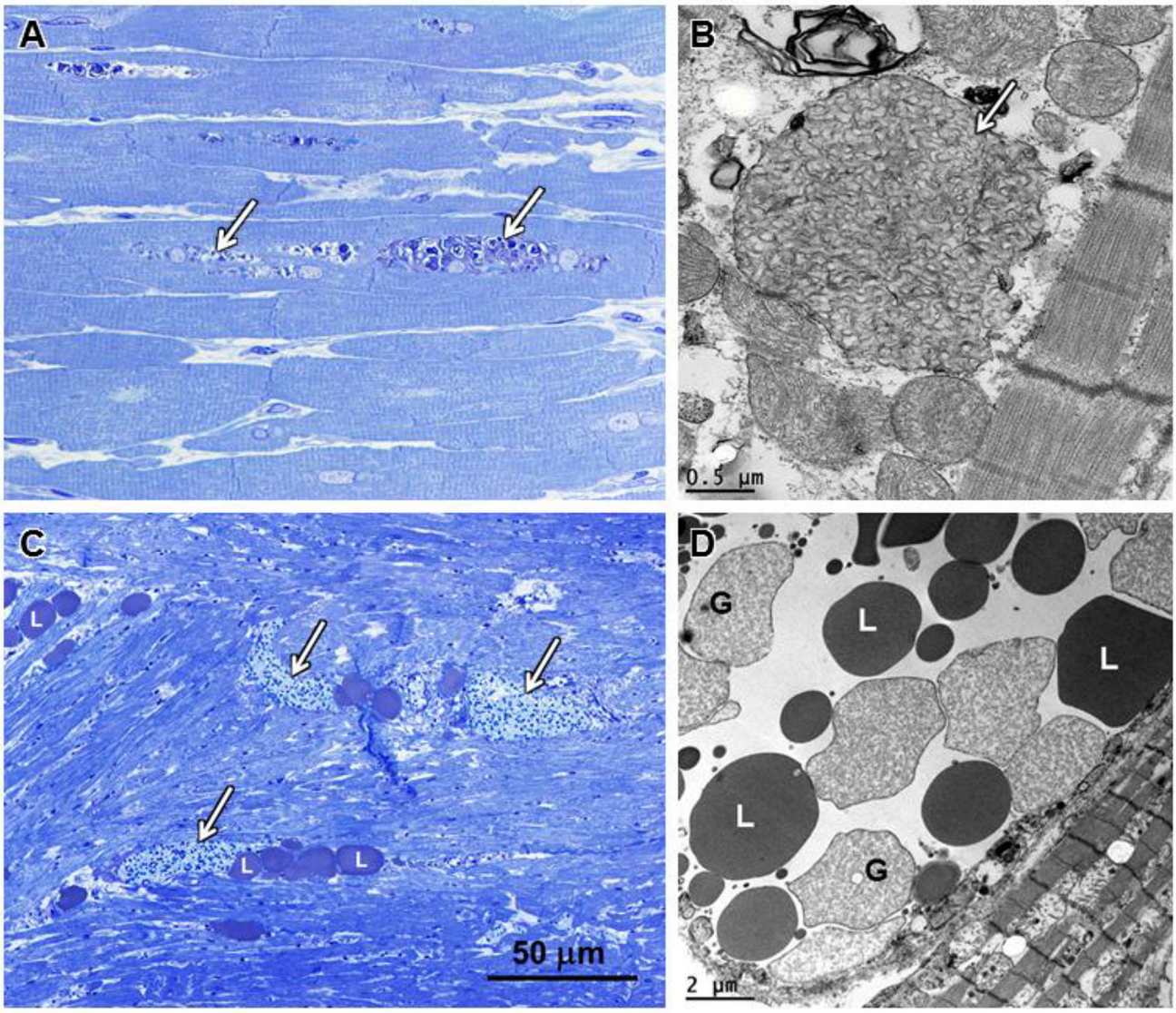

Figure 3.

Light and electron micrographs of storage material from cardiac muscle from a Dachshund with CLN2 NCL (A and B) and from a Shiba Inu with GM2 gangliosidosis due to a *HEXB* mutation (C and D). The storage material in the dog with CLN2 disease consisted of clusters of inclusion bodies embedded in the muscle fibers (arrow in A). At the ultrastructural level the material within these clusters contained membrane-bound inclusions indistinguishable from those found in neural tissues of dogs with CLN2 disease (stow in B). These abnormal clusters also contained loose whorls of membrane-like structures not present in neural tissues (Fig. 4). Although the storage material from the dog with GM2 gangliosidosis had similar fluorescence properties to that of dogs with CLN2 diseased (Fig. 1), the morphology of the material was quite different, consisting of large lipid droplets [L in (C)] and aggregates of small inclusions that consisted of lipid droplets [L in (D)] and membrane-bound structures with uniform granular contents [G in (D)].

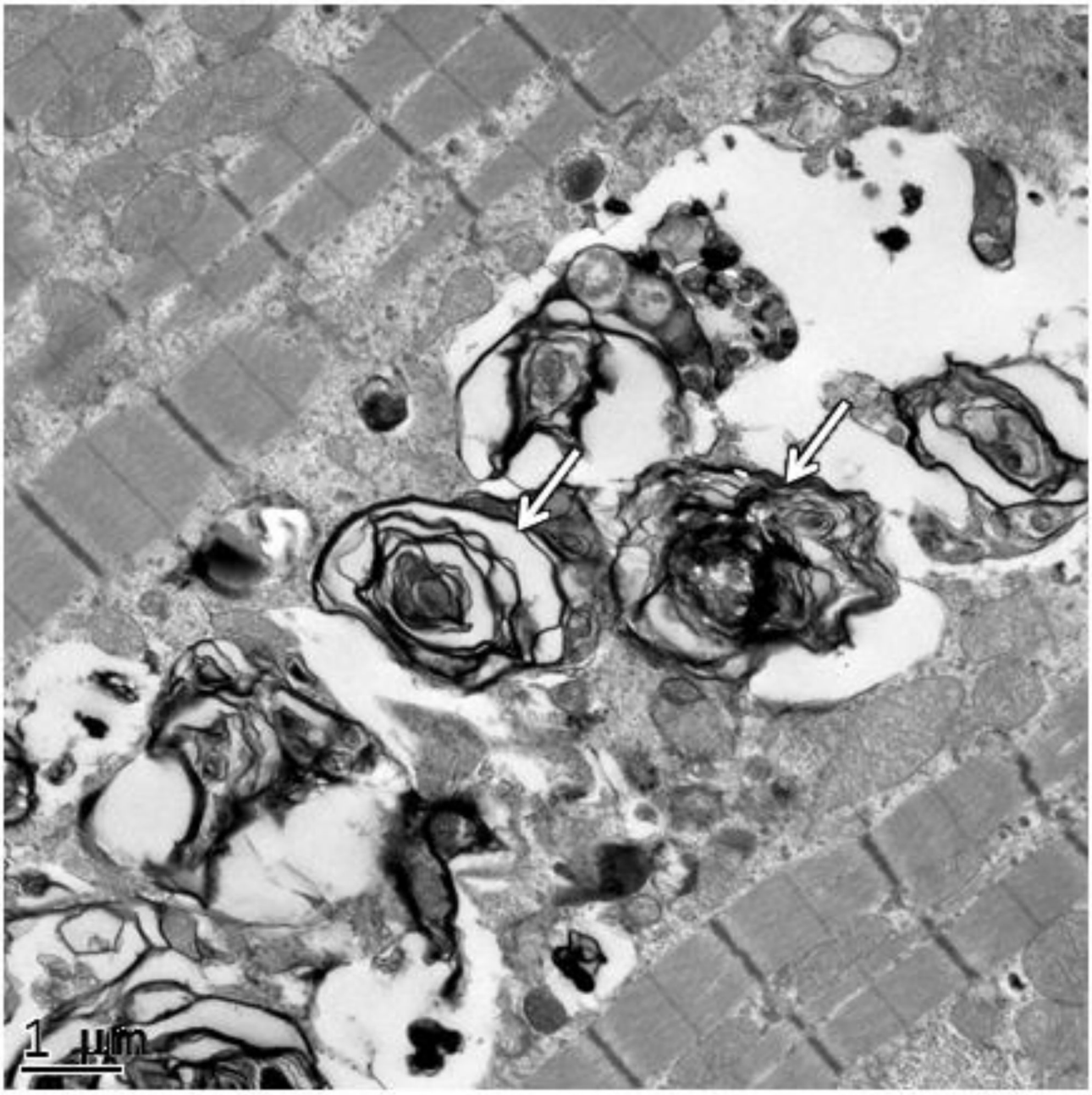

Figure 4.

Electron micrograph of showing structures consisting of whorls of membrane-like material (arrows) within the abnormal clusters of material present within the cardiac muscle of a Dachshund with CLN2 disease.

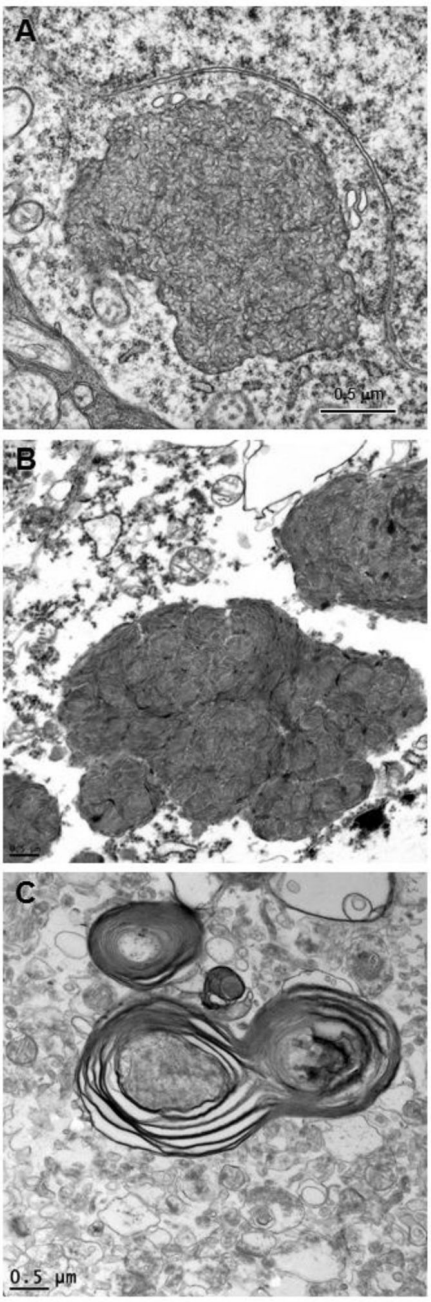

Figure 5.

Electron micrographs of disease-related storage bodies in the cerebral cortex from (A) a Dachshund homozygous for the *TPP1* mutation that causes the CLN2 form of NCL, (B) an Australian Cattle Dog homozygous for the *CLN5* mutation that causes NCL in this breed, and (C) a Shiba Inu dog homozygous for a *HEXB* mutation that causes GM2 gangliosidosis.

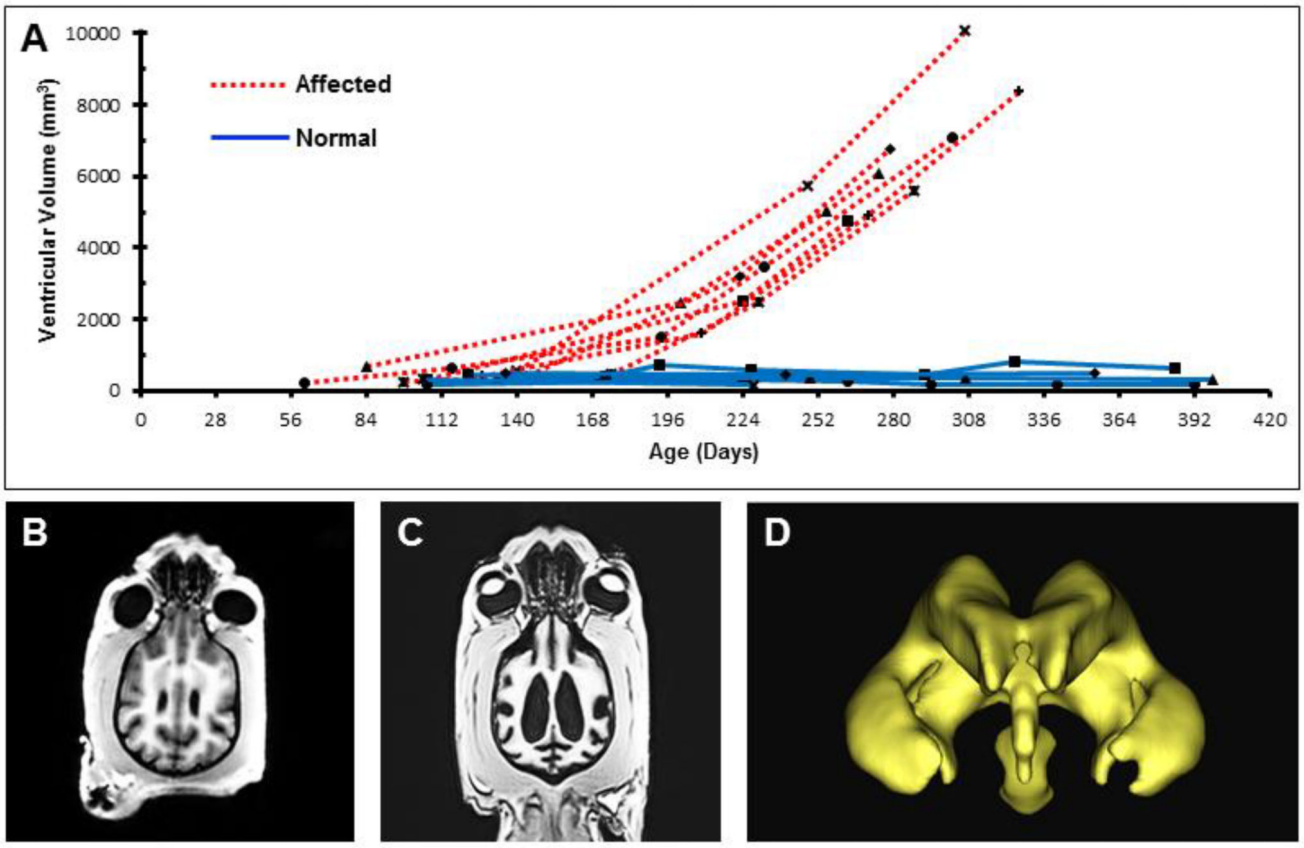

Figure 6.

(A) Brain ventricular volumes plotted as a function of age for normal Dachshunds (n=4) and Dachshunds affected with CLN2 disease (n=7). Each line trace represents a single dog. All of the dogs were from a research colony and were closely related. (B) Transverse MRI image of the brain from a 228 day old normal Dachshund. (C) Transverse MRI image of the brain from a 228 day old CLN2-affected Dachshund. (D) 3-dimensional reconstruction of the brain ventricular system from MR images from the 228 day old affected dog.

Figure 7.

German Shepherd Dog – Australian Cattle Dog mix that suffered from NCL and was homozygous for the *CLN5* mutation previously associated with NCL in purebred Border Collies and Australian Cattle Dogs. See video in supplemental materials illustrating disease-related seizure activity.

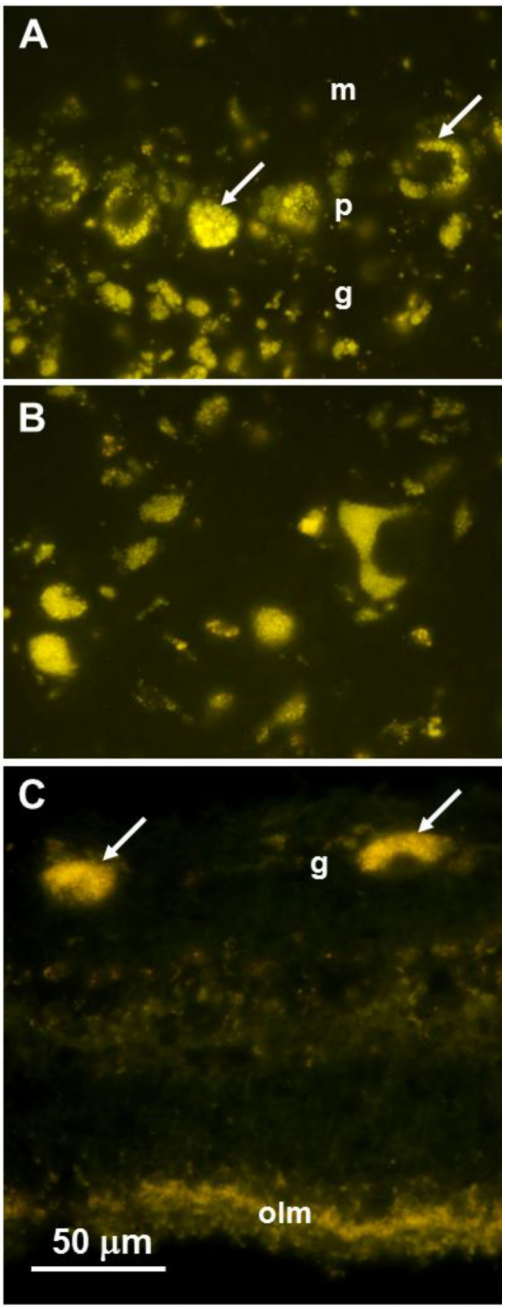

Figure 8.

Fluorescence micrographs of the cerebellum (A), cerebral cortex parietal lobe (B), and retina (C) from a German Shepherd Dog-Australian Cattle Dog mix that was homozygous for an NCL-causing mutation in *CLN5*. In the cerebellum the autofluorescent storage bodies were abundant in Purkinje neurons (arrows) in the Purkinje cell layer (p) as well as cells in the granular layer (g), but were sparse in the molecular layer (m). In the cerebral cortex almost all neurons contained abundant autofluorescent storage material. In the retina, the ganglion neuron cell bodies in the ganglion cell layer (g) were filled with this material (arrows in (C)).

Disease-specific autofluorescence was also abundant along the outer limiting membrane (olm). Bar in (C) indicates magnification of all 3 micrographs.

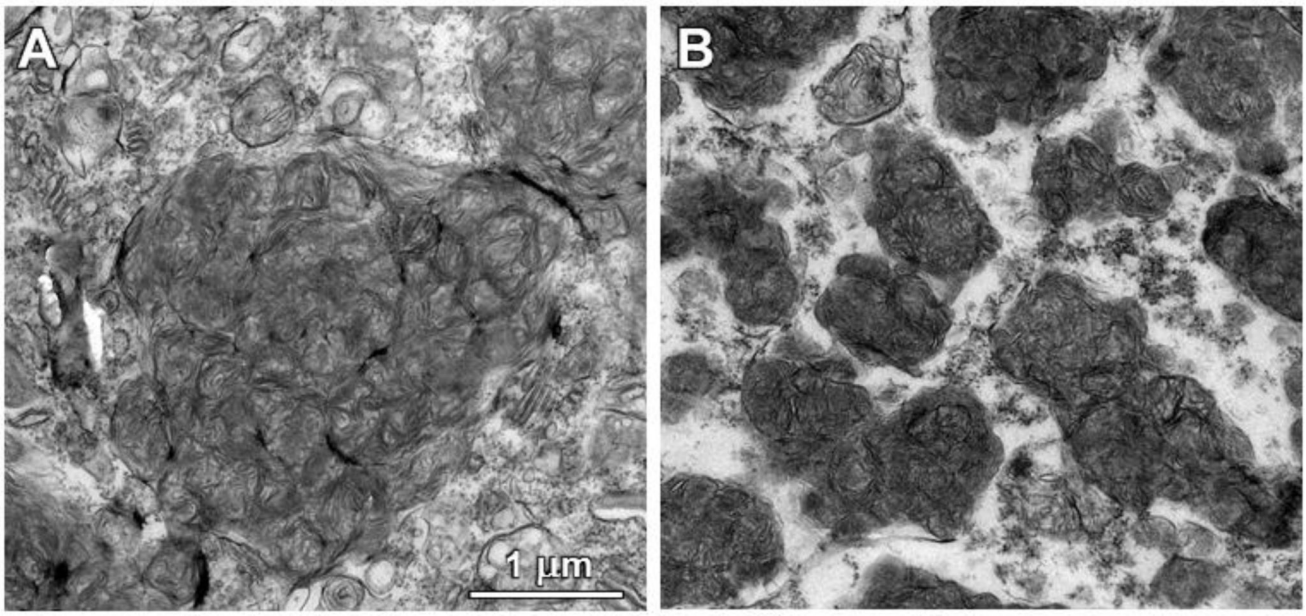

Figure 9.

Electron micrographs of the disease-specific storage bodies from a German Shepherd Dog-Australian Cattle Dog mix that was homozygous for an NCL-causing mutation in *CLN5* in a cerebellar Purkinje neuron (A) and in a cerebrocortical neuron (B). Bar in (A) indicates the magnification of both micrographs.

Table 1

Summary of canine NCL-associated mutations.

Disease	Gene	Mutation	Amino Acid Sequence	Affected Dog Breed
CLN1	*PPT1*	*PPT1:c.736_737insC*	p.F246Lfs*29	Dachshund (Katz et al., 2011)
CLN1	*PPT1*	*PPT1:c.124 + 1G>A*	Splice variant	Cane Corso(Kolicheski et al., 2017a)
CLN2	*TPP1*	*TPP1:c.325delC*	p.A108Pfs*6	Dachshund (Awano et al., 2006a)
CLN5	*CLN5*	*CLN5:c.619C>T*	p.Q207X	Border Collie(Melville et al., 2005), Australian Cattle Dog (Kolicheski et al., 2017a)
CLN5	*CLN5*	*CLN5:c.934_935delAG*	CLN5:p.E312Vfs*6	Golden Retriever (Gilliam et al., 2015)
CLN6	*CLN6*	*CLN6:c.829T>C*	p.W277R	Australian Shepherd (Farias et al., 2011)
CLN7	*MFSD8*	*MFSD8:c.843delT*	p.F282Lfs*13	Chinese Crested Dog(Guo et al., 2015), Chihuahua (Faller et al., 2016)
CLN8	*CLN8*	*CLN8:c.491T>C*	p.L164P	English Setter (Awano et al., 2006b)
CLN8	*CLN8*	*CLN8:c.585G>A*	p.W195X	Australian Shepherd (Guo et al., 2014)
CLN8	*CLN8*	*CLN8:g.30852988_30902901del*	Absence of CLN8	Alpenländische Dachsbracke (Hirz et al., 2016)
CLN10	*CTSD*	*CTSD:c.597G>A*	p.M199I	American Bulldog (Awano et al., 2006a)
CLN12	*ATP13A2*	*ATP13A2:c.1623delG*	p.P541 fs*56	Tibetan Terrier (Farias et al., 2011)

Table 2

Summary of canine breeds with suspected NCL of unknown genetic cause

				Storage material				
Breed	**Onset of clinical signs Retinal**	**Involvement?**	**Age of Death or Euthanasia***	**Autofluorescent?**	**Ultrastructure**	**Composition**	**Distribution**	**References**
Australian Shepherd	17m	nd	19–21m	Y	Lamellar	nd	Neuronal	(Katz et al., 2008)
Cocker Spaniel	18m-6y	Y	18m-6y	Y	Lamellar	nd	General	(Jolly et al., 1994a)
Dalmatian	6m	Y	6–8y	Y	Lamellar	nd	General	(Goebel et al., 1988; Goebel and Dahme, 1985)
Labrador Retriever	7y	N	8y	Y	Lamellar	nd	Neuronal	(Rossmeisl et al., 2003)
Longhaired Dachshund	5.5–6y	nd	5.5–7y	Y	Lamellar & granular	nd	Neuronal	(Vandevelde and Fatzer, 1980)
Miniature Schnauzer	2–3y	Y	3–4y	Y	GRODs	SAP	Neuronal	(Jolly et al.,1997; Smith et al., 1996)
Polish Owczarek Nizinny	6m-4y	Y	1.5–8y	Y	GRODs	SAP	General	(Narfstrom and Wrigstad, 1995 {Narfström, 2007 #102)
Standard Poodle	2y	Y	2.5y	Y	Lamellar	nd	General	(Cantile et al., 1996)
Saluki	1 y	N	2y	Y	Lamellar	nd	Neuronal	(Appleby et al., 1982)
Spitz	nd	Y	2y	nd	nd	nd	nd	(Pickett et al., 1992)
Terrier, mixed breed	4m	nd	9y	Y	Lamellar & granular	nd	Neuronal	(Hoover et al., 1984)
Welsh Corgi	6–8y	Y	6–9y	nd	nd	nd	nd	(Jolly et al., 1994a)
Wirehaired Dachshund	3y	nd	4.5y	Y	Lamellar & granular	nd	nd	(Cummings and de Lahunta, 1977)
Yugoslavian Shepherd	6m	nd	2y	Y	nd	nd	nd	(Bichsel and Vandevelde, 1982)

nd=not done, y=years, m=months, Y=yes, N=no, GRODs=granular osmophilic deposits, SC=subunit c of ATP synthase, SAP=sphingolipid activator protein

*Most dogs were euthanized due to the progressive nature of the symptoms