



Published in final edited form as:

Mol Genet Metab. 2019 May ; 127(1): 107–115. doi:10.1016/j.ymgme.2019.04.003.

A mixed breed dog with neuronal ceroid lipofuscinosis is homozygous for a *CLN5* nonsense mutation previously identified in Border Collies and Australian Cattle Dogs

Natalie A. Villani^a, Garrett Bullock^a, Jennifer R. Michaels^b, Osamu Yamato^c, Dennis P. O'Brien^d, Tendai Mhlanga-Mutangadura^a, Gary S. Johnson^a, and Martin L. Katz^{e,*}

^aDepartment of Veterinary Pathobiology, University of Missouri, Columbia, MO, U.S.A.

^bAngell Animal Medical Center, Boston, MA 02130, U.S.A.

^cLaboratory of Clinical Pathology, Kagoshima University, Kagoshima, Japan

^dDepartment of Veterinary Pathobiology, University of Missouri, Columbia, MO, U.S.A.

^eDepartment of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO, U.S.A.

Abstract

The neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative disorders characterized by progressive declines in neurological functions following normal development. The NCLs are distinguished from similar disorders by the accumulation of autofluorescent lysosomal storage bodies in neurons and many other cell types, and are classified as lysosomal storage diseases. At least 13 genes contain pathogenic sequence variants that underlie different forms of NCL. Naturally occurring canine NCLs can serve as models to develop better understanding of the disease pathologies and for preclinical evaluation of therapeutic interventions for these disorders. To date 14 sequence variants in 8 canine orthologs of human NCL genes have been found to cause progressive neurological disorders similar to human NCLs in 12 different dog breeds. A mixed breed dog with Labrador Retriever and Beagle parents developed progressive neurological signs consistent with NCL starting at approximately 11 to 12 months of age, and when evaluated with magnetic resonance imaging at 21 months of age exhibited diffuse brain atrophy. Due to the severity of neurological decline the dog was euthanized at 23 months of age. Cerebellar and cerebral cortical neurons contained massive accumulations of autofluorescent storage bodies the contents of which had the appearance of tightly packed membranes. A whole genome sequence, generated with DNA from the affected dog contained a homozygous C-to-T transition at position 30,574,637 on chromosome 22 which is reflected in the mature *CLN5* transcript (*CLN5:c.619C>T*) and converts a glutamine codon to a termination codon (p.Gln207Ter). The identical nonsense mutation has been previously associated with NCL in Border Collies, Australian Cattle Dogs, and a German Shepherd-Australian Cattle Dog mix. The

*Corresponding author at: Mason Eye Institute, Room EC-203, University of Missouri School of Medicine, Columbia, MO 65121, USA. katzm@health.missouri.edu (M.L. Katz).

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current whole genome sequence and a previously generated whole genome sequence for an Australian Cattle Dog with NCL share a rare homozygous haplotype that extends for 87 kb surrounding 22:30,574,637 and includes 21 polymorphic sites. When genotyped at 7 of these polymorphic sites, DNA samples from the German Shepherd-Australian Cattle Dog mix and from 5 Border Collies with NCL that were homozygous for the *CLN5:c.619 T* allele also shared this homozygous haplotype, suggesting that the NCL in all of these dogs stems from the same founding mutation event that may have predated the establishment of the modern dog breeds. If so, the *CLN5* nonsense allele is probably segregating in other, as yet unidentified, breeds. Thus, dogs exhibiting similar NCL-like signs should be screened for this *CLN5* nonsense allele regardless of breed.

Keywords

dog; neuronal ceroid lipofuscinosis; CLN5; lysosomal storage disease; neurodegeneration; whole genome sequencing

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are rare inherited progressive neurodegenerative diseases that in people are characterized by apparently normal development followed by progressive declines in cognitive and motor functions, loss of vision, seizures, respiratory impairment, and in most cases premature death [1]. The neurological signs are usually accompanied by generalized brain atrophy, retinal degeneration, and massive accumulation of autofluorescent lysosomal storage bodies in the central nervous system as well as other tissues and organs [1]. Prior to the advent of modern molecular genetics, NCL was classified into different types based primarily on the age of onset, pattern of clinical signs, and rate of disease progression. Currently, however, the mutations responsible for most cases of human NCL are known, and the different types are now classified as CLN1 through CLN14 based on the gene in which the disease-causing mutation resides [2]. In people, NCL-causing sequence variants occur in at least 13 genes [2]. The disease phenotype, including age of onset, spectrum of clinical signs, and rate of disease progression can vary not only according to the gene in which the causative mutation occurs, but also influenced by the specific mutation within that gene. The onset of disease signs can occur as early as infancy to as late as middle age, with a similar variability in the severity of disease signs.

Progress in developing treatments for the NCLs has been hampered by the lack of adequate animal models. Transgenic mouse models have been made for most of the NCLs, but many of these models fail to recapitulate the human disease phenotype and therapies tested in these models have not translated to human application [3-32]. Large animal models with nervous systems more similar to those of humans in size and complexity are likely to be more suitable for therapy development. Indeed, an enzyme replacement therapy for treating CLN2 disease that was successful in delaying disease onset and slowing the progression of clinical signs in a dog model is now being used successfully to treat children with this disorder [33-37]. The availability of dog models for the other NCLs would likely accelerate therapy development for these disorders as well.

Diseases with clinical signs similar to the human NCLs have been described in over 20 dog breeds and in several mixed breed dogs [38, 39]. NCL has been associated with sequence variants in 8 canine orthologs of human NCL genes [38, 39]. Among these canine disorders the onset of clinical signs ranges from a few months to 7 years of age [38].

With one exception, the parents of mixed breed dogs with NCL were purebred dogs of breeds in which the same causative mutation had been found to occur [38]. The exception was an offspring of a cross between an Australian Cattle Dog and a German Shepherd Dog. The affected dog was homozygous for a *CLN5:c.619C > T* variant previously associated with NCL in purebred Australian Cattle Dogs and Border Collies [38]. To our knowledge, neither an NCL-like disease phenotype nor the *CLN5* sequence variant have been identified in German Shepherd Dogs. A mixed breed dog exhibiting NCL-like signs reported to be the offspring of a Beagle and a Labrador Retriever was brought to our attention. A late-adult onset NCL has been reported in the latter breed, but no causative mutation was identified [40]. There have been no published reports of NCL-like disease in Beagles. Studies were therefore undertaken to determine whether the affected dog did indeed suffer from NCL, and if so, to identify the causative mutation(s).

2. Materials and methods

2.1. Subject dog

These studies were conducted with approval of the University of Missouri Institutional Animal Care and Use Committee and with the informed consent of the dog's owner. The affected dog (Fig. 1) was one of a litter of four produced by a mating between a female Beagle and a Labrador Retriever. The affected dog first began to exhibit obvious neurological signs at approximately 10 to 12 months of age, and these signs became progressively worse over time. However, abnormalities in the dog's stance were observed as early as 6 months of age. The neurological signs included anxiety, loss of housetraining, almost complete loss of responsiveness to previously learned commands and verbal prompting, compulsive circling, disruption of previously normal sleep patterns, loss of the ability to vocalize, impaired ability to prehend and swallow food, severe loss of coordination and ability to climb up or down stairs, development of tremors and seizures that were not responsive to phenobarbital, exhibition of trance-like behavior, and severe visual impairment in both dim and bright lighting conditions. Based on behavioral observations, visual impairment began with a loss of visual acuity, as the dog could visually track large but not small objects until at least 18 months of age. Due to the severity of the neurological signs, particularly the difficulty in eating, the owner elected to have the dog euthanized at approximately 23 months of age and the body was submitted for necropsy.

The affected dog's owner was informed by the owner of one of the littermates that the littermate had died after having exhibited similar signs. No DNA or tissues samples were collected from the littermate, and no disease diagnosis was made. Definitive health information on the two remaining littermates could not be obtained. We were unable to obtain information about the health histories of the parents or to obtain samples for DNA isolation from these dogs. Based on physical appearance, the mother of the affected dogs appeared to be of Beagle lineage and the father appeared to be of Labrador Retriever

lineage, but we were unable to obtain pedigree information on these dogs. The breeding that produced the affected puppies was apparently unplanned since all four littermates were donated to a dog rescue service.

2.2. Clinical evaluations and necropsy

The affected dog was examined by a veterinary neurologist (JRM) at approximately 21.5 months of age. The examination included full general physical examination and neurologic examination, as well as magnetic resonance imaging of the brain performed using a GE 1.5 Tesla Optima MR360 instrument. Cerebrospinal fluid cytology and fluid analyses as well as complete blood count and blood chemistry analyses were also performed at this time.

After euthanasia a complete necropsy was performed by a veterinary pathologist in the Pathology Department of Angell Animal Medical Center. Whole blood and parts of the formalin-fixed brain were shipped to the University of Missouri for further analyses. Fixed tissue samples were examined with fluorescence and electron microscopy [41]. For electron microscopy, small pieces of the cerebellar cortex, the deep cerebellar nuclei, and the parietal cerebral cortex were washed in 0.17 M sodium cacodylate buffer and then transferred to electron microscopy fixative (2% glutaraldehyde, 1.12% paraformaldehyde, 0.13 M sodium cacodylate, 1 mM CaCl₂, pH 7.4) and incubated in the latter fixative at room temperature with gentle agitation for at least 48 hours prior to being further processed for electron microscopic examination.

2.3. DNA analysis

DNA from the affected dog was purified from EDTA anti-coagulated blood as previously described [42] and submitted to the University of Missouri DNA Core Facility for PCR-free library preparation and sequencing on the Illumina NextSeq 500 platform. The resulting paired-end sequence data was analyzed using a modified best-practices GATK pipeline to align to the reference genome (CanFam3.1) and call variants. VCF files were generated from the affected dog's whole genome sequence and from the whole genome sequences of 141 other dogs with a variety of diseases that served as controls. Based on owner reports, 135 of the control dogs were purebred. The breeds represented in the control cohort are listed in the supplementary material. The 142 VCF files were loaded into commercial software (Golden Helix SVS) for sorting the variants by Alternative Allele Counts and by Gene Name. A previously described, a TaqMan allelic discrimination assay [43] was used to genotype archived DNA samples from randomly selected Beagles, Labrador Retrievers, German Shepherd Dogs for a C-to-T transition at position 30,574,637 on chromosome 22. The direct sequencing of PCR amplicons was used to determine the genotype at 7 polymorphic sites near 22:30,574,637, as detailed in the supplementary material. In an attempt to more definitively determine the breed ancestry of the affected dog we shipped 50 µg of purified DNA from the affected dog to each of four different commercial testing companies to perform DNA tests that are advertised to identify the breed backgrounds of mixed breed dogs. To avoid potential legal issues, we identify the companies here only by the code letters A,B, C and D. However companies offering this type of testing can easily be found by performing an internet search.

3. Results

3.1. Physical, clinical, and neurological findings

The affected dog was evaluated by a veterinary neurologist (JRM) when the dog was 22 months of age due to intermittent facial spasms and focal seizures of up to 45 minutes duration for each episode during which the dog remained alert. These episodes began when the dogs was approximately 21 months of age and occurred on an almost daily basis, usually multiple times each day, and were not prevented with phenobarbital. Some episodes included shaking of one hind limb. The dog was extremely anxious and would pace and bump into objects. Mild proprioceptive ataxia was observed and the dog would cross over her front legs when walking or standing. Crainial nerves, postural reactions, spinal reflexes and touch sensation were assessed as normal.

Magnetic resonance imaging of the brain at this time (Precontrast: T2WFSE, T2 FLAIR, MERGE (T2*GRE), T1WFSE; DWI; postcontrast (2.8 mL Omniscan IV): T1WFSE; 3D FSPGR) revealed diffuse brain atrophy characterized by widening of the cerebral and cerebellar sulci, generalized ventriculomegaly, and decreased size of the interthalamic adhesion (Fig. 2). In addition, there was diminished grey-white matter distinction. No abnormal contrast enhancement or intracranial mass effects detected. Cerebrospinal fluid evaluation and blood count and chemistries did not indicate any central nervous system inflammation.

3.2. Pathology findings

Gross examination of the affected dog at necropsy confirmed the diffuse brain atrophy observed with MR imaging. The brain was diffusely reduced in size, evidenced by increased space separating the brain from the cranial vault. There was mild dilation of the lateral ventricle. Edema of the lungs was the only other gross finding.

Multiple tissues were examined microscopically including cerebrum, thalamus, cerebellum, brainstem, pituitary gland, liver, spleen, kidney, adrenal gland, heart, lung, thyroid gland, parathyroid gland, stomach, small intestine, pancreas, and eye globe. Neuronal cell bodies in the brain and periphery (retina, autonomic ganglia, myenteric/submucosal plexuses) exhibited accumulation of eosinophilic large granules to globules, often with an eccentric or perinuclear distribution. The cytoplasmic inclusions are PAS-positive (Fig. 3). Scattered degenerate neurons were observed in the cerebral cortex. Numerous reactive glial cells identified by GFAP immunostaining were present in layer 6 of the cerebral cortex (Fig. 4) and the granular layer and deep cerebellar nuclei of the cerebellum (Fig. 5). Intense GFAP immunostaining was also present in the nerve fiber layer of the retina (Fig. 6). Purkinje cell numbers were significantly decreased in the cerebellar cortex, and the granular layer appeared thinned. The optic nerve head was vacuolated with increased glial cells. Hepatocytes and renal tubular epithelial cells had finely globular/granular eosinophilic cytoplasm, with no PAS reactivity. Lung sections are congested with mildly increased macrophages in alveoli.

The disease-related inclusion bodies that were present in brain and retina exhibited autofluorescence typical of lipofuscin and ceroid [44-48] (Fig. 7). In the cerebellar cortex,

the autofluorescent inclusions were present primarily in the Purkinje cell bodies, with few scattered autofluorescent bodies in the molecular or granule cell layers (Fig. 7A, arrows). In the deep cerebellar nuclei, large neurons contained abundant autofluorescent inclusions that were aggregated at one pole of the cell body (Fig. 7B, arrows). In the cerebral cortex, almost all neurons contained the disease-related autofluorescent inclusions (Fig. 7C, arrows). In the retina, these inclusions were present only in ganglion cells (Fig. 7D, arrow). The ganglion cells were much more sparse and smaller in size than ganglion cells from normal dog retinas.

Electron microscopic examination of the disease-specific storage bodies in cerebral cortical neurons and cerebellar Purkinje cells revealed that the stored material consisted primarily of whorls of densely-packed membrane-like structures (Figs. 8 and 9). The storage body contents were very similar in appearance in both the Purkinje cells and cortical neurons. In the cerebral cortex, however, a minority of the storage bodies contained scattered electron-dense amorphous inclusions (Fig. 8) that were not observed in the cerebellum. The material in the storage bodies was enclosed by surrounding membranes, but due to the initial fixation in formalin, these outer membranes were only partially preserved (Figs. 8 and 9).

3.3. Molecular genetic analyses

The affected dog's genome contained 8 variants that were predicted to alter the primary structure of an encoded polypeptide, absent from 141 control canine whole genome sequences, and homozygous in the affected dog. None of these variants were in genes predicted to cause lysosomal storage diseases. Therefore, the exclusion criteria were relaxed so that variants that occurred in two or fewer of the 282 reference alleles were retained for consideration. These relaxed exclusion criteria identified 7 additional rare variants that were homozygous in the affected dog, including a C-to-T transition at position 30,574,637 on chromosome 22 (*CLN5:c.619C>T*). As previously reported, the 22:30,574,637T (*CLN5:c.619T*) allele was homozygous in the whole genome of an Australian Cattle Dog with NCL [43]. The variant occurs in exon 4 of *CLN5* where it converts codon 207 from CAA (Gln) to TAA (Stop). DNA samples from 68 Beagles, 65 Labrador Retrievers, and 70 German Shepherd Dogs were randomly selected from those in the University of Missouri canine DNA Repository and genotyped at the chromosome 22:30,574,637 locus. All of the randomly selected samples were homozygous for the 22:30,574,637C allele.

We compared the genomic region surrounding chromosome 22:30,574,637 in the whole genome sequence of the affected dog and in the previously reported whole genome sequence of an Australian Cattle Dog with that was homozygous for the 22:30,574,637T allele [43]. Both dogs were homozygous for an identical 21-variant haplotype that extends for 87 kb from 22:30,523,216 to 22:30,610,464. Of the 141 whole genome sequences from control dogs, 140 were homozygous for at least one allele that was not part of the 21-variant haplotype. Thus, this full-length haplotype could have occurred in only one of the whole genome sequences, an epileptic Standard Schnauzer that was potentially heterozygous for the haplotype but homozygous for 22:30,574,637C allele (Table 1). In addition, we genotyped 6 individual DNA samples at 7 of the 21 polymorphic sites in the haplotype region. These 6 samples were all from dogs previously diagnosed with NCL due to

homozygosity for the 22:30,574,637T nonsense allele. Five of the genotyped DNA samples were from Border Collies from Japan and the other genotyped sample was from the previously described mixed breed dog of Australian Cattle dog and German Shepherd ancestry. As indicated in Table 1, all of these samples were homozygous for the same alleles that were found in the whole genome sequences from the affected dog and the Australian Cattle Dog with NCL.

3.4. Breed background analysis

Since breed background determination based on physical appearance can be unreliable, DNA samples from the affected dog were submitted to four different companies that analyze the breed backgrounds of mixed breed dogs based on patterns of polymorphic markers in the genome of the dog being evaluated. The results provided by the companies are summarized in Table 2. Although there were significant differences in the results provided by the different companies, analyses from 3 of 4 companies were in agreement on significant contributions from the following breeds: Treeing Walker Coonhound, Beagle, Shetland Sheepdog, and Siberian Husky. To our knowledge, NCL has not been reported in any of these breeds. On the other hand, only Company C indicated a contribution from a breed (Border Collie) in which NCL associated with the *CLN5:c.619T* variant has been reported [49].

4. Discussion and Conclusions

In 2005, Australian investigators reported that a young-adult onset NCL in Border Collies was caused by a homozygous nonsense sequence variant in *CLN5* [49]. Eleven years later, whole genome sequence analysis identified the identical homozygous *CLN5* variant in Australian Cattle Dogs with NCL [43]. More recently, the same homozygous *CLN5* variant was found in a dog with an Australian Cattle Dog dam and a German Shepherd Dog sire [38]. In the present study whole genome sequence analysis identified the same *CLN5* nonsense mutation in a mixed breed dog in which Broder Collie, Australian Cattle Dog, or German Shepherd Dog ancestry appears to be minimal or non-existent (Table 2).

A homozygous 2 bp deletion and frameshift variant in *CLN5* was identified in Golden Retriever siblings with NCL [50]. Although the precise cellular function of the *CLN5* protein remains to be determined, both the latter deletion and the *CLN5* 22:30,574,637C>T nonsense variant are predicted to encode truncated polypeptides with little or no residual biological activity [50]. As might be expected with homozygous nullifying mutations, similar disease phenotypes have been reported for the affected Golden Retrievers and the dogs of various breed backgrounds with the homozygous *CLN5* nonsense mutation. In all these cases, clinical signs have typically become apparent at 12 to 20 months of age. The affected dogs exhibited seizures and progressive declines in vision, cognition and coordination of movement which culminated in euthanasia due to reduced quality of life before the third birthday. Also similar to the affected dog, previous descriptions of dogs with *CLN5*-related NCL described MRIs indicative of diffuse brain atrophy, and autofluorescent storage bodies with varied ultrastructure that included aggregates of membrane-like structures [38, 41, 50].

Most cases of genetically defined canine NCL have been found only in the breed from which the variant was discovered [38]. One exception was a homozygous single base pair deletion and frame shift in *MFSD8* that was first identified as the likely cause of NCL in a Chinese Crested dog [51] but subsequently also identified in Chihuahuas with NCL [52-54]. Since the members of both breeds are very small and share other physical attributes, a founding event may have occurred in one breed and the variant may have been introduced into the other breed by an interbreed mating. We proposed that interbreed mating may also explain the occurrence of the same homozygous *CLN5* nonsense variant in both Border Collies and Australian Cattle Dogs with NCL [43]. When the *CLN5* nonsense variant was found to be homozygous in a cross between a German Shepherd Dog and an Australian Cattle Dog, we suggested that the German Shepherd Dog parent may have had an Australian Cattle Dog ancestor with the disease allele [38]. With the new finding of the identical NCL-causing *CLN5* sequence variant in a mixed breed dog with little or no apparent Australian Cattle Dog or Border Collie ancestry, recent transfer of the disease allele from the breed in which it originated to other breeds seems less plausible. An alternative explanation for the distribution of the *CLN5* nonsense variant among breeds is that the *CLN5* nonsense variant arose in a common ancestor of multiple breeds. The breed background data in Table 1 and our previous work [38] suggest that the *CLN5* 22:30,574,637T variant is particularly likely to be present in Treeing Walker Coonhounds, Beagles, Shetland Sheepdogs, Siberian Huskies and German Shepherd Dogs. Therefore dogs from these breeds in particular that are exhibiting NCL-like neurological signs should be genotyped for the presence of this variant. To our knowledge, NCL has not been reported in any of these breeds.

A rare, homozygous, 21-variant, 87 kb haplotype surrounds the *CLN5* nonsense variant in whole genome sequences from both the affected dog and a previously described Australian Cattle Dog with NCL [43], indicating that the mutation in both dogs stems from a common foundation event. Demonstration that 5 Border Collies and a previously reported mixed breed dog with Australian Cattle Dog dam and a German Shepherd Dog sire were homozygous for at least 7 of the haplotype alleles suggests that the same founding event was responsible for their NCL and supports the possibility that the founding event occurred before the establishment of the modern dog breeds. The *PRCD:c.5A* transition that causes progressive retinal atrophy [55] and the *ADAMTS17:c.1473+1 G>A* transition that causes lens luxation [56] are examples of other sequence variants that are responsible for recessive diseases in many breeds [57, 58] and probably have an ancient origins. If, in fact, the founding *CLN5* nonsense mutation event predated breed establishment, it is likely that *CLN5* nonsense allele is segregating in additional as yet unrecognized breeds. The fact that *CLN5*-associated NCL has not been reported to date in more breeds indicates that the nonsense allele is almost certainly rare in most breeds in which it occurs. However, the lack of reports of NCL in more breeds is also likely to be due in part of the failure to publicize most cases of NCL-like disorders in dogs. Indeed, after the first report that NCL in Border Collies is associated with the 22:30,574,637 T nonsense variant in *CLN5*, a survey of Border Collies from four kennels in Japan found the frequency T allele to be almost 35% [59], indicating that dogs from lines that certainly produced affected offspring continued to be used for breeding. In addition, by the time we described a form of NCL in American Bulldogs and subsequently reported the causative sequence variant in *CTSD* [60, 61], the

frequency of the mutant allele among American Bulldogs had become quite high [60]. Based on these two examples, it is quite possible that cases of NCL associated with homozygosity for the *CLN5* 22:30,574,637T variant have occurred in Treeing Walker Coonhounds, Beagles, Shetland Sheepdogs, Siberian Huskies, German Shepherd Dogs and other breeds but gone unreported. It is not unusual for owners of dogs with progressive neurological diseases to have their dogs euthanized without seeking to identify the cause of the disease and without the attending veterinarian publishing a description of the disease phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was funded in part by grant EY023968 from the U.S. National Institutes of Health and AKC Canine Health Foundation grant 02257. Our thanks to Cheryl Jensen for expert technical assistance.

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Fig. 1.
Photograph of the affected dog at approximately 20 months of age.

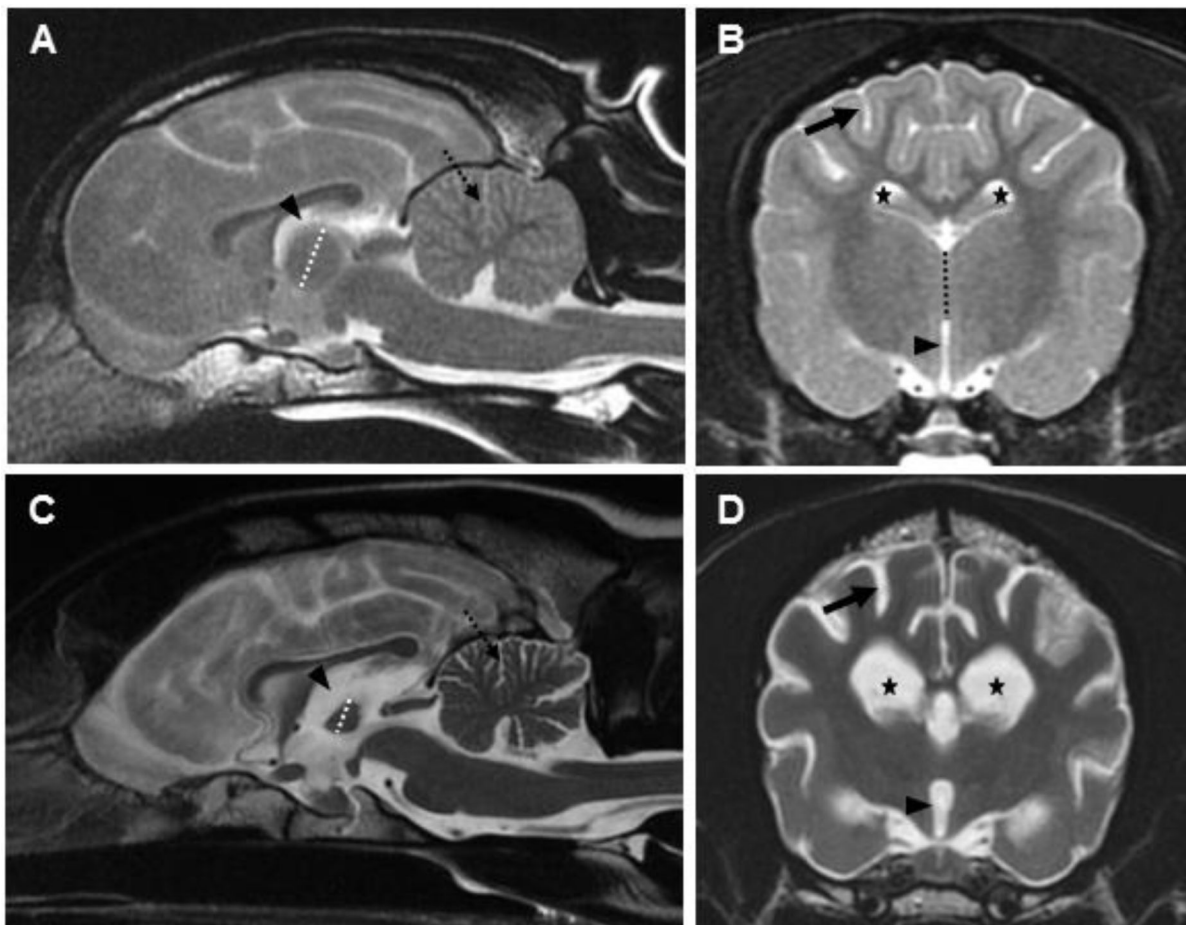


Fig 2.

T2 weighted magnetic resonance (MR) mid-sagittal (A) and axial (B) images at the level of the interthalamic adhesion from an approximately 2 year old normal dog. T2 weighted MR mid-sagittal (C) and axial (D) images at the level of the interthalamic adhesion from the affected dog. Diffuse brain atrophy is noted in the affected dog (C, D) as evidenced by enlargement of the lateral (5-pointed star) and third (arrowhead) ventricles, widening of the cerebral (solid arrow) and cerebellar (dotted arrow) sulci, and narrowing of the interthalamic adhesion (dotted line). Comparable structures in the age-matched, control dog (A, B) are marked with identical symbols to highlight normal structure.

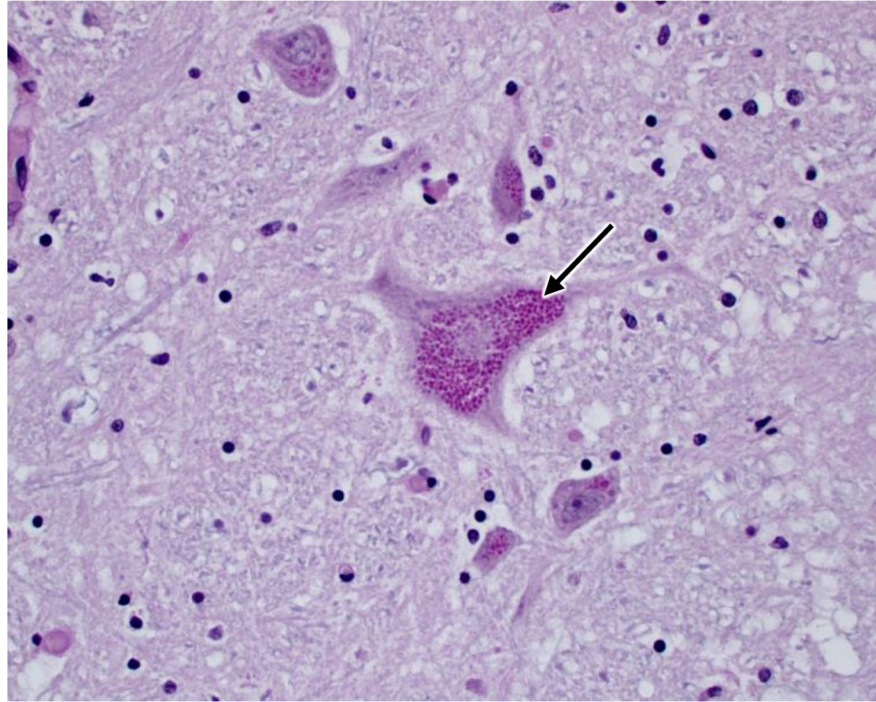


Fig. 3. Light micrograph of PAS-stained section of a brainstem nucleus from the affected dog depicting massive accumulation of PAS-stained cytoplasmic inclusions in large neurons (arrow).

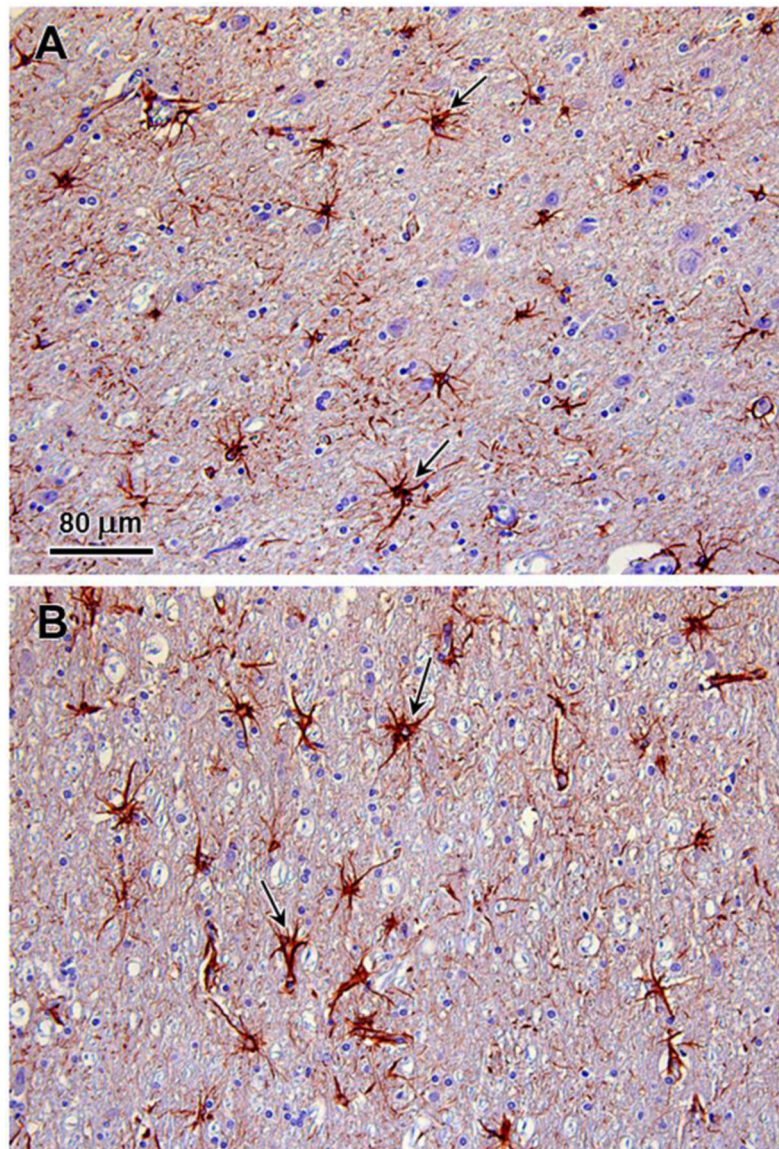


Fig. 4. Immunohistochemical demonstration of reactive astrocytes in the parietal cerebral cortex of the affected dog. GFAP immunostaining of sections of the cerebral cortex demonstrated numerous reactive astrocytes (arrows) in cortical layers 6A (polymorphic cell layer) (A) and 6B (B). Reactive astrocytes were sparse elsewhere in the cerebral cortex.

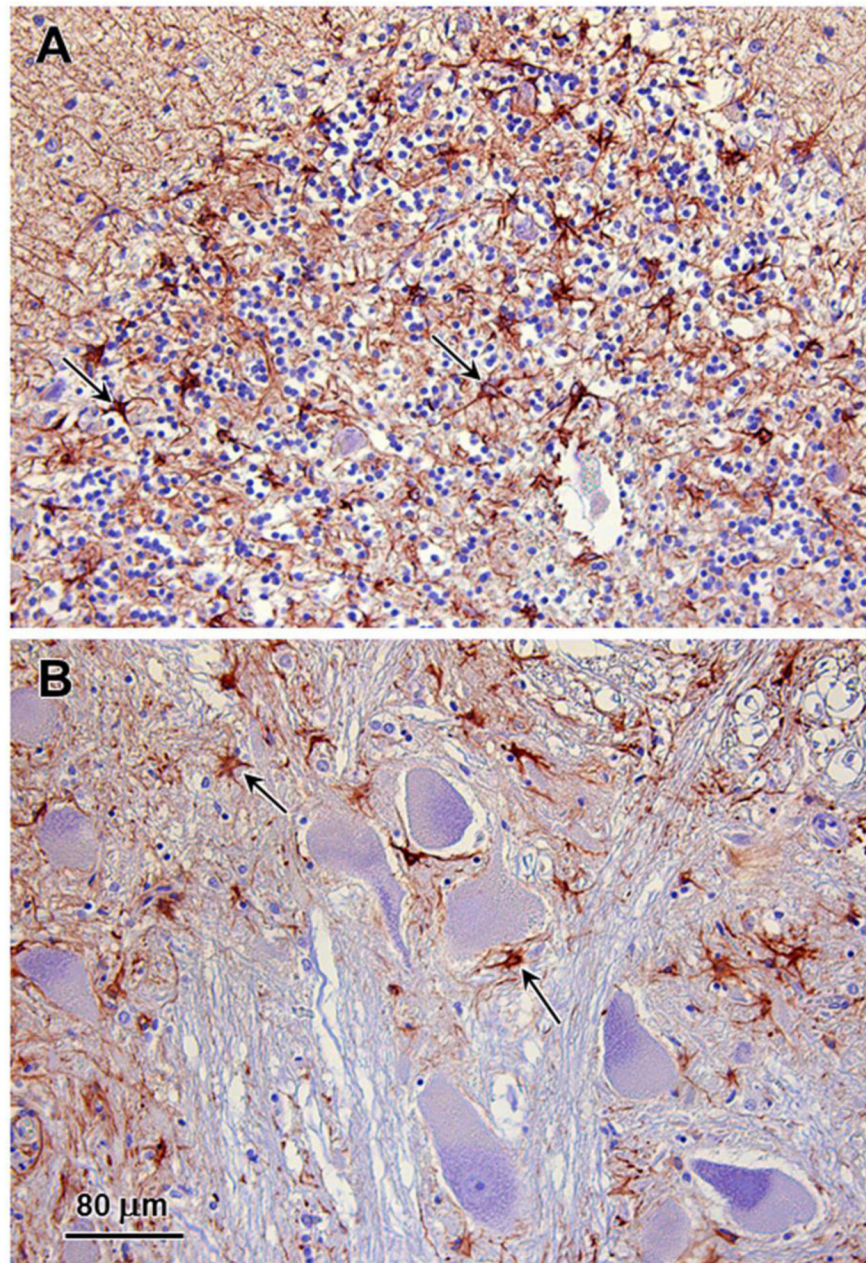


Fig. 5. Immunohistochemical demonstration of reactive astrocytes in the cerebellum of the affected dog. GFAP immunostained reactive astrocytes (arrows) were abundant in the granular layer of the cerebellar cortex (arrows in A) and in the deep cerebellar nuclei (arrows in B).

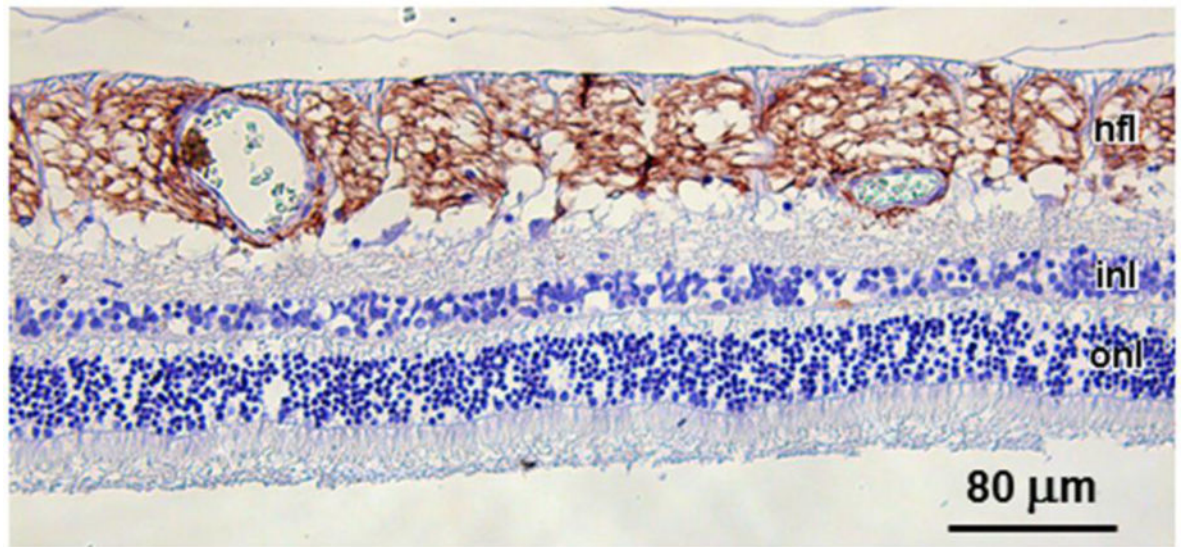


Fig. 6. GFAP immunostaining was intense in the nerve fiber layer (nfl) of the affected dog, but was not present in any other retinal cell layers, including the inner nuclear layer (inl) or outer nuclear layer (onl).

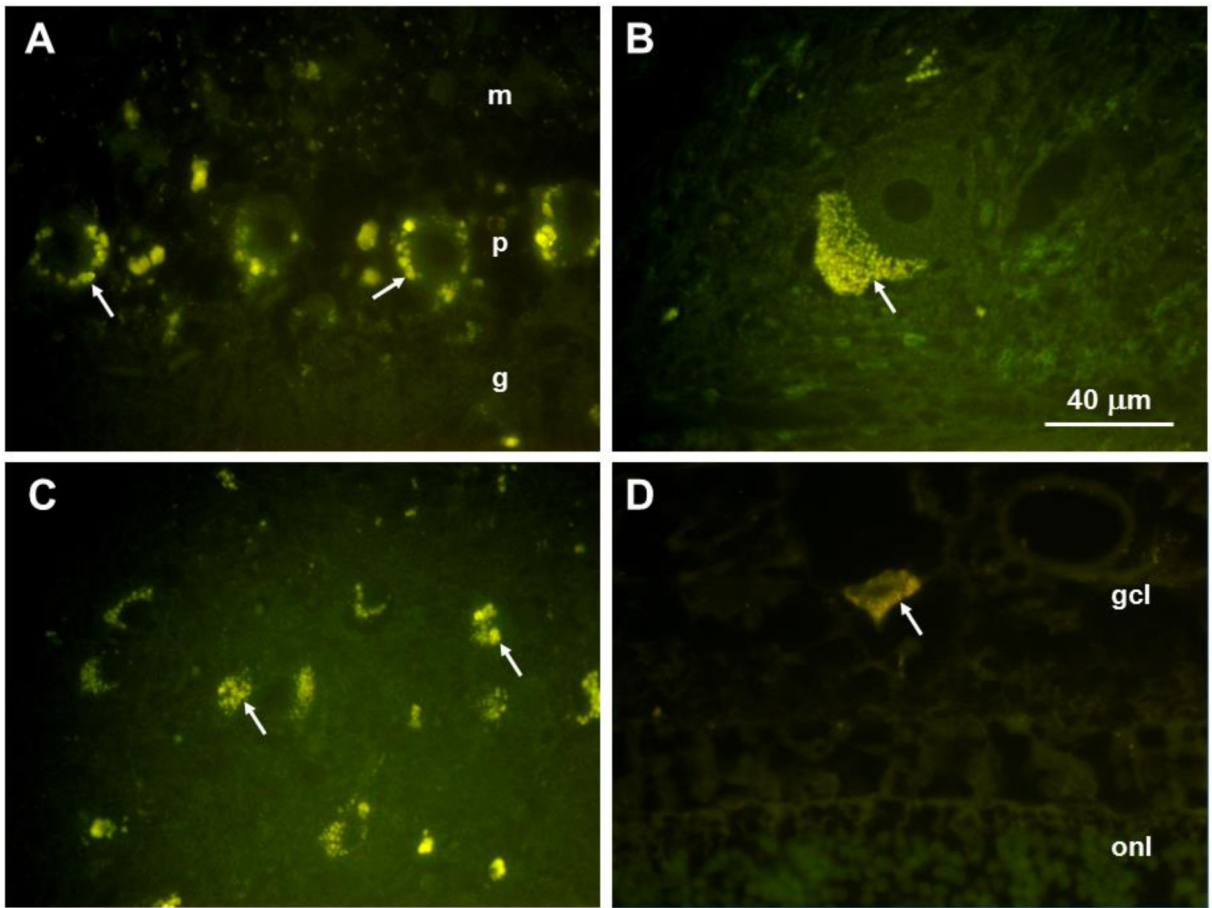


Fig. 7. Fluorescence micrographs of sections of the cerebellar cortex (A), a deep cerebellar nucleus (B), the parietal cerebral cortex (C), and the retina (D) from the affected dog. Disease-specific autofluorescent cytoplasmic inclusions are indicated by arrows. In (A) m:molecular layer, p:Purkinje layer, g:granule cell layer. In (D) gcl:ganglion cell layer; onl:outer nuclear layer. Bar in (B) indicates the magnification of all four micrographs.

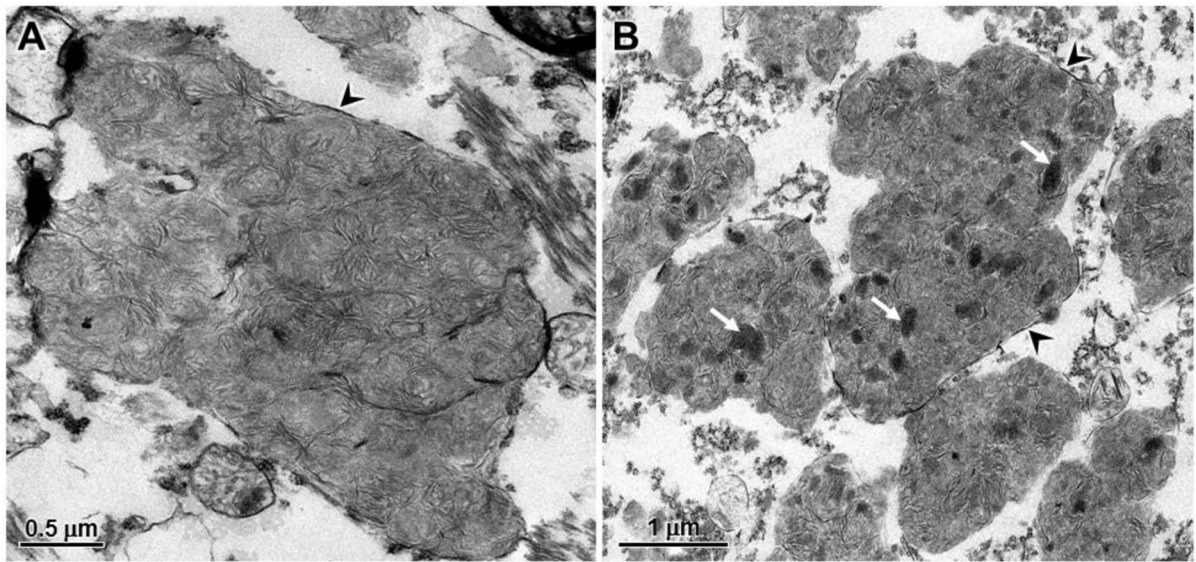


Fig. 8. Electron micrographs of storage bodies in cerebral cortical neurons of the affected dog. The contents of most storage bodies consisted of tightly packed clusters of membrane-like structures (A). In some storage bodies, electron dense amorphous patches of material were embedded within the membrane-like aggregates (arrows in B). Membranes enclosing the storage material (arrowheads) were only partially preserved due to the initial fixation in formalin.

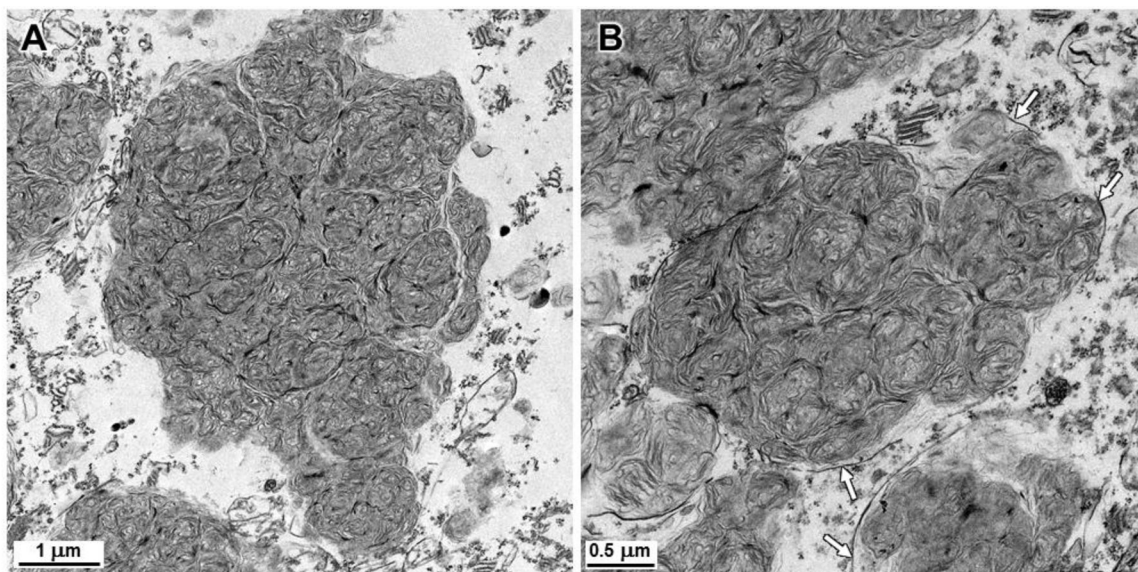


Fig. 9. Electron micrographs of storage bodies in cerebellar Purkinje cells of the affected dogaffected dog. As with the cerebral cortical neurons, the contents of most storage bodies consisted of tightly packed clusters of membrane-like structures, but none of the electron dense amorphous patches of material were observed in the Purkinje cell storage bodies. As with the cerebral cortex, membranes enclosing the storage material (arrows) were only partially preserved due to the initial fixation in formalin.

Table 1:Genotypes of dogs with NCL at polymorphic sites near the *CLN5* missense mutation at 22:30574637

Chromosome 22 Position	Reference Allele	Variant Allele	Variant Allele Frequencies	Genotypes		
				WGS for NCL Dogs [†]	WGS for Epileptic Std. Schnauzer [‡]	DNA from NCL Dogs [#]
30523216	G	T [€]	0.152	T [€] /T	T/T	T/T
30524512	AAAAAAAAA	-	0.192	-/-	-/-	N.D.*
30535524	T	TT	0.408	TT/TT	TT/TT	N.D.
30538412	-	CT	0.461	CT/CT	CT/CT	N.D.
30542756	C	A	0.755	A/A	A/A	N.D.
30546129	AC	-	0.069	-/-	AC/-	N.D.
30550783	-	G	0.755	G/G	G/G	N.D.
30553284	AAA	-	0.111	-/-	AAA/-	N.D.
30553293	AAATTA	-	0.367	-/-	AAATTA/-	-/-
30567376	C	T	0.193	T/T	T/T	T/T
30569433	TTT	-	0.536	-/-	-/-	N.D.
30569456	A	G	0.768	G/G	G/G	N.D.
30574637	C	T	0.013	T/T	C/C	T/T
30578990	A	c	0.178	C/C	C/C	C/C
30589554	A	-	0.565	-/-	A/-	N.D.
30594474	C	A	0.938	A/A	A/A	N.D.
30598322	G	C	0.383	C/C	C/C	C/C
30600625	-	ATA	0.925	ATA/ATA	ATA/ATA	N.D.
30601475	T	-	0.732	-/-	-/-	N.D.
30603161	T	C	0.925	C/C	C/C	N.D.
30608518	T	A	0.183	A/A	A/T	A/A
30610464	c	T	0.105	T/T	C/T	T/T

[†] Genotypes in whole genome sequences for the affected dog and an Australian Cattle Dog with NCL that was homozygous for the 22:30,574,637T allele

[‡] Genotypes in the whole genome sequence for an epileptic Standard Schnauzer that did not suffer from NCL

[#] Genotypes in genomic DNA samples from dogs with NCL including 6 Border Collies and a dog with a German Shepherd Dog sire and an Australian Cattle Dog dam (all of these dogs were homozygous for the 22:30,574,637T allele)

[€] Red alleles are in the NCL-associated haplotype

* N.D. = not determined

Table 2.

Breed contributions to the genome of the affected dog as determined by 4 companies.

Breed Contribution to Affected Dog (%)				
Breed	Company A	Company B	Company C	Company D
Treeing Walker Coonhound	38.2	25	--	25
Beagle	--	12.5	25	12.5
Harrier	--	--	25	--
Mountain Cur	19.4	--	--	--
Miniature Poodle	--	12.5	--	12.5
Shetland Sheepdog	12.5	12.5	--	12.5
Siberian Husky	9.4	--	12.5	12.5
Border Collie	Trace	--	12.5	--
Fox Terrier	--	--	12.5	--
Labrador Retriever	--	--	12.5	--
Rat Terrier	--	--	12.5	--
German Shepherd Dog	5.3	--	--	--
Chow Chow	trace	--	--	--
Dalmatian	trace	--	--	--
Herding breeds	--	trace	--	--
Guard breeds	--	trace	--	--
Sporting breeds	--	trace	--	--
Terrier breeds	--	trace	--	--
Unknown mix	--	--	--	25