Title
A Missense Mutation in the Vacuolar Protein Sorting 11 (VPS11) Gene Is Associated with Neuroaxonal Dystrophy in Rottweiler Dogs.

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**ABSTRACT** Canine neuroaxonal dystrophy (NAD) is a recessive, degenerative neurological disease of young adult Rottweiler dogs (*Canis lupus familiaris*) characterized pathologically by axonal spheroids primarily targeting sensory axon terminals. A genome-wide association study of seven Rottweilers affected with NAD and 42 controls revealed a significantly associated region on canine chromosome 5 (CFA 5). Homozygosity within the associated region narrowed the critical interval to a 4.46 Mb haplotype (CFA5:11.28 Mb – 15.75 Mb; CanFam3.1) that associated with the phenotype. Whole-genome sequencing of two histopathologically confirmed canine NAD cases and 98 dogs unaffected with NAD revealed a homozygous missense mutation within the Vacuolar Protein Sorting 11 (VPS11) gene (g.14777774T>C; p.H835R) that was associated with the phenotype. These findings present the opportunity for an antemortem test for confirming NAD in Rottweilers where the allele frequency was estimated at 2.3%. VPS11 mutations have been associated with a degenerative leukoencephalopathy in humans, and VPS11 should additionally be included as a candidate gene for unexplained cases of human NAD.

**KEYWORDS** autophagy canine lysosome neurodegenerative inherited genetic

Neuroaxonal dystrophy (NAD) is a relatively non-specific histopathological diagnosis for a group of neurodegenerative disorders characterized by dystrophic changes of the neuron followed by development of axonal swellings or spheroids (Revesz et al. 2015). First described by Cajal, (Cajal 1928) axonal swellings may occur in the central or peripheral nervous system and the underlying pathogenesis of the variable structured material found in these swellings is often poorly defined. The dystrophic phenotype may vary depending on age of onset, clinical manifestations, and specific disorder.

NAD can be divided into three major etiological groups: physiological, secondary, and primary. Physiological NAD can be seen commonly in humans and domesticated species as a component of aging (Suzuki et al. 1979; Saito 1980; Summers et al. 1995; Borràs et al. 1999; Gavier-Widen et al. 2001; Revesz et al. 2015), and secondary NAD may be seen focally, or more widely throughout the nervous system in response to a wide variety of conditions, including trauma, infection, toxin exposure and metabolic disease such as vitamin E deficiency or organophosphate exposure (Yagishita 1978; Summers et al. 1995; Revesz et al. 2015). Axonal spheroids have also been described in human patients with amyotrophic lateral sclerosis, Alzheimer’s, Parkinson’s disease and hereditary spastic paraparesis. Primary NAD is generally associated with a group of genetically heterogeneous, inherited neurodegenerative diseases where the presence of neuroaxonal dystrophy is a major pathological component of the...
disease. In human patients, neuroaxonal dystrophic pathology has been associated to varying degrees with several genetically defined disease syndromes, most prominently in Infantile Neuroaxonal Dystrophy (INAD) and Pantotetanate Kinase-associated Neurodegeneration associated with alterations in the PLA2G6, and PANK2 genes respectively. Many of the human NAD syndromes are also associated with brain iron accumulation, including alterations in the PLA2G6, PANK2, FTL, C19orf12, FA2H, and WDR45 genes (Revesz et al. 2015; Arber et al. 2016). Neuroaxonal dystrophy in humans without iron accumulation is seen in Wilson’s disease and Nasu-Hakola disease involving the ATP7B and DAP12/TREM2 genes respectively, and in “neuroaxonal leukoencephalopathy with axonal spheroids” which has no defined genetic cause to date (Revesz et al. 2015). Primary NAD has been reported in most domesticated species including dogs (Clark et al. 1982; Chrisman et al. 1984; Blakemore and Palmer 1985; Evans et al. 1988; Sacre et al. 1993; Franklin et al. 1995; Bennett and Clarke 1997; Siso et al. 2001; Nibe et al. 2007; Fyfe et al. 2010; Fyfe et al. 2011; Hahn et al. 2015; Pintsut et al. 2016; Degl’Innocenti et al. 2017; Tsuoi et al. 2017), cats (Woodard et al. 1974; Carmichael et al. 1993; Rodriguez et al. 1996; Rébibois and Poncelet 2004), cattle (Hanshaw et al. 2015), sheep (Cordy et al. 1986; Nuttall 1988; Harper and Morton 1991; Kessell et al. 2012; Finnie et al. 2014), pigeons (Barrows 2010; Blythe et al. 2016), and horses (Beech 1984; Blythe et al. 1991; Aleman et al. 2011; Finno et al. 2013; Finno et al. 2016), where an association with vitamin E deficiency, along with a genetic susceptibility, has been reported.

In dogs, breed related NAD has been reported as fetal onset in Giant Schnauzer-Beagle mix breed dogs (Fyfe et al. 2010; Fyfe et al. 2011), juvenile onset in Dachshund mix breed dogs (Pintsut et al. 2016), Border collies (Clark et al. 1982), Chihuahuas (Blakemore and Palmer 1985; Degl’Innocenti et al. 2017), Jack Russell Terriers (Sacre et al. 1993), Papillons (Franklin et al. 1995; Nibe et al. 2007; Tsuoi et al. 2017), Spanish Water Dogs (Hahn et al. 2015), and young adult or adult onset in Rottweilers (Cork et al. 1983; Chrisman et al. 1984; Evans et al. 1988; Bennett and Clarke 1997; Siso et al. 2001) and English Cocker Spaniels (McLellan et al. 2003). NAD in Cocker Spaniels is accompanied by retinal degeneration and is associated with vitamin E deficiency (McLellan et al. 2003). Specific genetic mutations associated with the PLA2G6, TECPR2 and MFN2 genes have been identified in the Papillon, Spanish Water Dog and Schnauzer-Beagle cross dogs respectively (Fyfe et al. 2011; Hahn et al. 2015; Tsuoi et al. 2017).

Rottweiler NAD was first reported in the early 1980s and is characterized by a young adult age of onset with mild progression of clinical signs, including ataxia, hypermetria, intention tremor and nystagmus (Cork et al. 1983; Chrisman et al. 1984). Clinical signs reflect the predominantly sensory topographical distribution of pathology within the central nervous system (CNS) consisting of mild cerebellar atrophy, large numbers of axonal spheroids, and demyelination of axons in the vestibular nucleus, lateral and medial geniculate nuclei, sensory nuclei of the trigeminal nerve, gracilis and cuneate nuclei, and in the spinal cord dorsal horn (Cork et al. 1983; Chrisman et al. 1984). Based on a small pedigree, it was hypothesized to be an autosomal recessive disorder (Cork et al. 1983).

Defining underlying genetic mechanisms for breed related neuroaxonal dystrophies in dogs has the potential to provide biological insight and potential translational models for the heterogeneous disease phenotypes seen in human patients (Shearin and Ostrander 2010; Hytönen and Lohi 2016). A genome-wide association was therefore performed with samples from the original four reported Rottweiler cases (Cork et al. 1983; Chrisman et al. 1984) and three additional cases, together with whole-genome sequencing of selected cases to identify candidate genes for NAD in Rottweilers.

MATERIALS AND METHODS

Canine Samples

Buccal swabs or blood samples were collected from privately owned dogs through the Willam R. Pritchard Veterinary Medical Teaching Hospital at UC Davis. Collection of canine blood samples was approved by the University of California, Davis Animal Care and Use Committee (protocol #16892). Additional Rottweiler DNA samples were provided by the University of Minnesota and the University of Bern, Switzerland. These studies were approved according to the national guidelines for animal welfare by the Institutional Animal Care and Use Committees (IACUC) of the University of Minnesota, and by the Cantonal Committee for Animal Experiments (Canton of Bern; permits 23/10, 48/13 and 75/16) for the University of Bern. Owners specified the breed of each dog. Genomic DNA was extracted using the Qiagen kit (QIAGEN, Valencia, CA). Neurological phenotypes were determined by a veterinarian and confirmed postmortem via necropsy when available.

Genome-wide SNP Genotyping

Genome-wide SNP genotyping was performed on seven cases and 42 controls, using the Illumina CanineHD 220k BeadChip (Illumina, San Diego, CA, USA). All samples had a genotyping rate of ≥ 90%. 62,193 SNPs were excluded due to a minor allele frequency ≤ 5% and 7,421 SNPs were excluded due to a high genotype failure rate (≥ 10%), leaving 151,799 SNPs after quality control. A Chi-square analysis and a genomic inflation factor (λ) was calculated with PLINK (Purcell et al. 2007). Homozygosity throughout the associated interval was analyzed by visual inspection assisted by color-coding homozygous genotypes in Excel. Homozygosity in the affected dogs, that passed the Bonferroni threshold (P ≤ 3.29×10^-7), was used to narrow down the regions of association and was visualized using Haploview (Barrett et al. 2005; Barrett 2009; Clarke et al. 2011). Figures were made in R using the ggplot2 package (Wickham 2009).

Whole-Genome Sequencing

Whole-genome sequencing was performed as described on 100 canine genomes, (Brown et al. 2017) with two histopathologically confirmed Rottweiler cases and 98 dogs unaffected with NAD, across 25 different breeds, including two Rottweilers. Sequencing was performed on the Illumina HiSeq 2000 using 100bp paired-end reads with approximately 8.7x coverage per sample. The reads were aligned to the canine reference genome (CanFam3.1) (Lindblad-Toh et al. 2005). Local realignment and variant calls were performed using the Genome Analysis Tool Kit (GATK version 2.5-2g57256b) pipeline (McKenna et al. 2010). Biological consequences of variants were predicted using Ensembl’s Variant Effect Predictor (VEP), PolyPhen-2 (v2.2.2r398), and SIFT (Adzhubei et al. 2010). SNPs with low coverage were filtered out of analysis.

Genotyping by Sanger Sequencing

Primers were designed using Primer3 (Rozen and Skaltsky 2000) to validate the putative functional mutation uncovered in VPS11 (F: CTGCCAGGTCCCTGTCTCTAAAG; R: TGTAAGACGTCTTGCGCTCT). PCR products were sequenced using the Big Dye termination kit on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were evaluated using Chromas (Technelysium, South Brisbane, QLD, Australia). Sequences were aligned to CanFam3.1 using BLAT (UCSC Genome Browser). Allele frequency was calculated excluding the seven affected cases.
RNA Extraction and cDNA Sequencing

RNA was isolated from liver using Qiagen QIAGEN Blood Mini Kit tissue protocols (QIAGEN, Valencia, CA). RNA was reverse transcribed into cDNA using Qiagen QuantiTect Reverse Transcription Kit. Ubiquitously expressed VPS11 (F: TGGTCCAAAAACTGCAGAAA; R: CTCTAAACGATTGGTGGGA) and the housekeeping gene RPS5 (Brinkhof et al. 2006) cDNA were PCR amplified from liver tissue from two affected Rottweilers, one Gordon Setter, and one mixed breed dog. RPS5 was amplified in liver to ensure equivalent amounts of cDNA were produced. The PCR products were sequenced on an ABI 3500 Genetic Analyzer and analyzed using Chromas (Technelysium, South Brisbane, QLD, Australia). The sequences were aligned to Can Fam3.1 using BLAT (UCSC Genome Browser) to confirm the missense mutation in the cDNA of VPS11.

Data Availability

The SNP genotyping data can be found in files Supplemental 1 (File S1), and Supplemental 2 (File S2). Whole-genome sequencing files reported in this paper can be found in the NCBI Sequence Read Archive (SRA Bioproject no. PRJNA377155). Sequences from four Pugs were made available in 2012 by TGEN (https://www.tgen.org/patients/canine/). Supplemental material available at Figshare: https://doi.org/10.25387/g3.6214010.

RESULTS

Case Definition

DNA samples were available from the four original NAD affected Rottweilers from Cork et al. (Cork et al. 1983) and three additional cases. One case was evaluated at the Veterinary Medical Teaching Hospital VMTH (University of California, Davis) with neurologic deficits and histopathological findings at necropsy consistent with previously reported cases. Blood samples from two additional dogs were submitted by their respective owners. Both dogs were presented to veterinarians with a history and clinical signs consistent with NAD and were noted to be “clumsy” as puppies. One dog was presented at approximately 1 year of age with generalized ataxia and hypermetria and absent menace responses. The second dog was presented for progressive ataxia and hypermetria that had been present for several years. Both dogs were alive and ambulatory at 2 and 3 years of age respectively. The second dog had been tested previously for mutations associated with two other neurodegenerative diseases reported in Rottweilers (leukoencephalomyelopathy (Minor et al. 2018), laryngeal paralysis-polyneuropathy (Mhlanga-Mutangadura et al. 2016)) and was negative for both mutations.

Genome-Wide Association Study

To identify loci associated with the NAD phenotype in the Rottweiler dog, a genome-wide association study was performed, followed by homozygosity analysis using seven cases affected with NAD and 42 healthy controls. Four of the seven cases were directly related resulting in a genomic inflation (λ) value of 1.52. A chi-square analysis of the 151,799 SNPs, identified preliminary associations on canine chromosomes (CFA) 4, 5, 12, 14, 16, 19, and 37 (Figure 1A). The lowest P value was on CFA 5 (P = 1x10^-14) and 26 SNPs in this region were more associated than on the next highest chromosomal location. Since this disease is uncommon and pedigree analysis was consistent with a recessive mode of inheritance, a homozygosity analysis was performed in the cases. To identify regions of homozygosity in the cases, P values from markers with an allele frequency of 1 in the cases were plotted. There were 45 markers that met the Bonferroni correction (P ≤ 1x10^-5), and all but one (CFA 34; P = 3.71x10^-3) were on CFA 5, making it the only statistically significant region of association that met the allele frequency criteria (Figure 1A-C). Homozygosity throughout the associated interval was used to narrow down the region of interest to 4.46 Mb (Chr5:11,292,754-15,754,443; CanFam3.1).
Whole-Genome Sequencing

Variants within the critical interval, identified in the GWAS, were analyzed for association using 98 dogs unaffected with NAD and two Rottweilers histopathologically confirmed to have NAD. Within the critical interval, there were 73 genes, and 31,749 SNPs and 17,421 indels were identified. 164 SNP variants and 15 indels segregated with the phenotype were within the region identified on CFA 5. Only a single SNP was found to be protein coding (CFA 5:14,777,774) among the segregating variants. The remaining variants were 3’UTR (n = 4), downstream (n = 23), intergenic (n = 74), intronic (n = 56), a non-coding transcript (n = 5), or upstream variants (n = 16).

VPS11 Non-Synonymous Variant

A non-synonymous variant was identified on CFA 5 (g.14777774T > C; CanFam3.1) in the Vacuolar Protein Sorting 11 (VPS11) gene. The variant leads to an amino acid change (p.H835R) in the Zinc RING finger domain of the protein (Figure 2B), which is ultimately predicted to be deleterious (VEP: moderate; PolyPhen-2: 0.999; SIFT: 0). The cDNA of VPS11 was sequenced from liver from two NAD affected Rottweilers, one Gordon Setter, and one mixed breed dog, to confirm the presence of the mutation in the mRNA (VPS11c:2504A > G). VPS11 is highly conserved across species (Table S1), with humans and dogs having 98.2% conservation at the amino acid level (Figure 2B).

VPS11 Variant Genotyping

440 dogs, consisting of 288 Rottweilers, and 152 dogs from 19 other breeds, were genotyped for the VPS11 mutation (Table 1). Of the 288 Rottweilers, 13 were identified as heterozygous for the mutation, seven were homozygous for the mutation (cases as described above), and the remaining 268 Rottweilers, along with the 152 other dogs, were all homozygous for the reference allele. Of the Rottweilers genotyped, 211 were from the United States of America (204 wild type, three heterozygous, and seven homozygous mutants); 75 were from Europe (65 wild type and 10 heterozygous). The frequency of the mutant allele in this population is estimated to be 2.3%.

DISCUSSION

Seven Rottweilers that presented with clinical signs consistent with the NAD phenotype were homozygous for a non-synonymous mutation within the RING-finger domain of the Vacuolar Protein Sorting 11 (VPS11) gene (Figure 2). In order to overcome significant population stratification based on relatedness of the affected cases, a genome-wide association followed by homozygosity mapping was used. This type of approach has been used successfully in the past to identify breed specific Mendelian recessive diseases in dogs (Drögemüller et al. 2009; Kropatsch et al. 2010; Forman et al. 2016).

Sorting and degradation of internalized cell surface proteins and lipids in eukaryotic cells is controlled through the “endocytic network” (Balderhaar and Ungermann 2013; Spang 2016), such that surface proteins may progress through early and late endosomes before they are degraded in lysosomes, or may be sorted and recycled. Disposal and recycling of cytoplasmic components is similarly achieved through the autophagosome-lysosome pathway during autophagy (Levine and Klionsky 2004; Nixon 2013) (Figure 3). Two VPS class C complexes, CORVET and HOPS, each composed of multiple different VPS proteins are essential for control of the membrane fusion machinery and trafficking of material through these endosome-lysosome organelles. CORVET and HOPS act as tethers, in coordination with other key proteins such as RAB5 and RAB7, and bring
appropriately targeted vesicles into close proximity with the target membrane (Richardson et al. 2004; Balderhaar and Ungermann 2013; Perini et al. 2014; van der Kant et al. 2015; Spang 2016). Both CORVET (class C core vacuole/endosome tethering complex) and HOPS (homotypic fusion and protein transport) contain a four-subunit core consisting of VPS11 (PEP5), VPS16, VPS18 (PEP3), and VPS33, which are conserved across yeast, insects, plants, and mammals (Nickerson et al. 2009). VPS11 has been shown to have a key role in determining selective binding to either early or late endosomes, and as an integrator of the complex assembly (Plemel et al. 2011; van der Kant et al. 2015). The RING domain of VPS11 that harbors the non-synonymous mutation in Rottweiler NAD has been shown to be important specifically in fusion at the vacuole (lysosome) in yeast (Plemel et al. 2011).

Mutation of the VPS11 gene in humans is associated with an infantile onset neurological syndrome characterized by hypomyelination and variable neurological deficits including motor and cognitive impairment, dystonia, ataxia, visual deficits, and seizures (Figure 2B) (Edvardson et al. 2015; Hörtnagel et al. 2016; Zhang et al. 2016). Histopathological characterization has not been done. However, the syndrome is classified as a leukoencephalopathy based on MRI (magnetic resonance imaging) findings, and skin and bone marrow biopsies were suggestive of a lysosomal storage type disease (Hörtnagel et al. 2016). Consistent with the known function of VPS11 (Plemel et al. 2011), in vitro studies of the mutant human protein resulted in disruption of late endosome/lysosome fusion and the autophagic pathway (Edvardson et al. 2015; Zhang et al. 2016). Although the Rottweiler VPS11 mutation is in a similar location to one of the documented human mutations within the VPS11 RING finger domain (Figure 2B) (Edvardson et al. 2015; Zhang et al. 2016), the clinical phenotypes appear to have distinct differences, most notably the apparent white matter vs. gray matter distribution of lesions in humans vs. dogs. The human VPS11 syndrome also appears to be characterized by lysosomal accumulations compared to axonal spheroids (Hörtnagel et al. 2016), although detailed histopathological characterization of human CNS lesions is not available. However, this spectrum of intracellular accumulations of varying types is within the rational consequences of disruption of the endosome-autophagosome-lysosome system predicted following VPS11 (CORVET/HOPS) dysfunction. Additionally, species and site specific differences in pathological phenotype for mutations within the same gene are well documented across a broad range of genetic diseases. For example, a variety of clinical and pathological phenotypes have been reported for alterations within the same genes that cause some of the neuroaxonal dystrophy syndromes in humans (Revesz et al. 2015; Arber et al. 2016); PLA2G6 gene mutations can give a spectrum of disease phenotypes as well as classical INAD including dystonia-parkinsonism syndromes and spastic paraplegia (Gregory et al. 2008; Ozes et al. 2017). Similarly, alterations in the MFN2 gene give rise to fetal onset NAD in dogs (Fyfe et al. 2010; Fyfe et al. 2011); however, human alterations result in peripheral nervous system syndromes (Charcot-Marie-Tooth disease type 2A2 and hereditary motor and sensory neuropathy type 6A (Del Bo et al. 2008)), while mouse knockouts and transgenic overexpression and cattle with altered MFN2 have CNS and PNS neurodegeneration but do not have NAD (Drögemüller et al. 2011). Protein site-specific effects, species differences in pathological responses (such as lack of iron accumulation in canine NAD), and differences in gain or loss of function mutations may all contribute to the phenotypic heterogeneity.

Despite this heterogeneity within the NAD disease phenotype, common pathological pathways are implicated in many NAD syndromes, including the primary and secondary diseases. Vitamin E deficiency has been associated to varying degrees with axonal dystrophy in both experimental and clinical settings, in several species including

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Table 1 VPS11 (g.14777774T > C) MUTATION GENOTYPING RESULTS

| BREED                | TOTAL | VPS11/ VPS11 | VPS11/ vps11 | vps11/ vps11 |
|----------------------|-------|--------------|--------------|--------------|
| Boston Terrier       | 2     | 2            | 0            | 0            |
| Boxer                | 33    | 33           | 0            | 0            |
| Brittany             | 3     | 3            | 0            | 0            |
| Bulldog              | 5     | 5            | 0            | 0            |
| Dachshund            | 1     | 1            | 0            | 0            |
| French Bulldog       | 3     | 3            | 0            | 0            |
| German Shorthaired Pointer | 4     | 4            | 0            | 0            |
| Golden Retriever     | 39    | 39           | 0            | 0            |
| Great Dane           | 2     | 2            | 0            | 0            |
| Irish Setter         | 1     | 1            | 0            | 0            |
| Labrador Retriever   | 17    | 17           | 0            | 0            |
| Mixed Breeds         | 6     | 6            | 0            | 0            |
| Newfoundland         | 4     | 4            | 0            | 0            |
| Nova Scotia Duck      | 13    | 13           | 0            | 0            |
| Tolling Retriever    |       |              |              |              |
| Pug                  | 5     | 5            | 0            | 0            |
| Rottweiler           | 288   | 268          | 13           | 7            |
| Saluki               | 2     | 2            | 0            | 0            |
| Weimaraner           | 5     | 5            | 0            | 0            |
| West Highland White  | 2     | 2            | 0            | 0            |
| Terrier              |       |              |              |              |
| Whippet              | 5     | 5            | 0            | 0            |

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Figure 3 Schematic representation of the endosome-autophagosome-lysosome pathway. VPS11 is a key constituent of the VPS class C complexes CORVET (red) and HOPS (blue). Disruption of the CORVET/HOPS tethering complexes, and subsequently the membrane fusion processes required for appropriate trafficking, would be consistent with both the lysosomal storage and NAD phenotypes seen in human and dog disease, secondary to accumulation of membrane and cytosolic constituents. Blue arrows represent fusion events mediated by HOPS, red arrows represent fusion events mediated by CORVET, dashed gray arrows represent pathways of exocytosis and plain gray lines represent pathways of endocytosis.
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