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A Homozygous *RAB3GAP1:c.743delC* Mutation in Rottweilers with Neuronal Vacuolation and Spinocerebellar Degeneration

T. Mhlanga-Mutangadura, G.S. Johnson, A. Ashwini, G.D. Shelton, S.A. Wennogle, G.C. Johnson, K. Kuroki, and D.P. O'Brien

Background: A variety of presumed hereditary, neurologic diseases have been reported in young Rottweilers. Overlapping ages of onset and clinical signs have made antemortem diagnosis difficult. One of these diseases, neuronal vacuolation and spinocerebellar degeneration (NVSD) shares clinical and histological features with polyneuropathy with ocular abnormalities and neuronal vacuolation (POANV), a recently described hereditary disease in Black Russian Terriers (BRTs). Dogs with POANV harbor mutations in *RAB3GAP1* which codes for a protein involved in membrane trafficking.

Hypothesis: Rottweilers with NVSD will be homozygous for the *RAB3GAP1:c.743delC* allele associated with POANV in BRTs.

Animals: Eight Rottweilers with NVSD confirmed at necropsy, 128 Rottweilers without early onset neurologic signs, and 468 randomly selected dogs from 169 other breeds.

Methods: Retrospective case–control study. Dogs were genotyped for the *RAB3GAP1:c.743delC* allele with an allelic discrimination assay.

Results: All 8 NVSD-affected dogs were homozygous for the *RAB3GAP1:c.743delC* allele while the 128 NVSD-free Rottweilers were either homozygous for the reference allele (n = 105) or heterozygous (n = 23) and the 468 genotyped dogs from other breeds were all homozygous for the reference allele.

Conclusions and Clinical Importance: The *RAB3GAP1:c.743delC* mutation is associated with a similar phenotype in Rottweilers and BRTs. Identification of the mutation permits a DNA test that can aid in the diagnosis of NVSD and identify carriers of the trait so that breeders can avoid producing affected dogs. Disruption of membrane trafficking could explain the neuronal vacuolation seen in NVSD and other spongiform encephalopathies.

Key words: Canine; Molecular genetics; Peripheral nervous system disorders; Rab GTPase; Spongiform encephalopathies; Warburg micro syndrome.

A number of presumed hereditary neurologic diseases occur in young Rottweiler dogs.[1–9] As discussed in reviews of these diseases,[10,11] there is considerable overlap in clinical signs and ages of onset which can make antemortem differentiation of the conditions difficult. The development of DNA tests for the mutations associated with specific diseases can help diagnose dogs with those diseases.[12] Recently a deletion in *RAB3GAP1* was identified in Black Russian Terriers (BRTs) with a juvenile onset laryngeal paralysis and polyneuropathy.

Abbreviations:

BRT	Black Russian Terrier
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
GAP	GTPase activator protein
GEF	guanine exchange factor
NVSD	neuronal vacuolation and spinocerebellar degeneration
POANV	polyneuropathy with ocular abnormalities and neuronal vacuolation
WARBM	Warburg micro syndrome

Further investigation disclosed additional features of the disease which is now called polyneuropathy with ocular abnormalities and neuronal vacuolation (POANV).[13] A SINE insertion in *RAB3GAP1* was also reported in Alaskan Huskies with POANV.[14] Similarities between the phenotypes of POANV and one of the previously reported diseases in young Rottweilers, neuronal vacuolation, and spinocerebellar degeneration (NVSD),[7] prompted us to determine if the same mutation is found in dogs with NVSD.

Materials and Methods

Medical records of 8 Rottweilers diagnosed with NVSD were reviewed. Necropsies were performed on 4 dogs. Brain and other tissues collected at the necropsy were fixed in 10% formalin and histopathology examination was performed after routine histologic processing using H&E, LFB/PAS and Bielschowsky stains. In one of these dogs, fresh and fixed biopsies collected from the quadriceps, cranial tibial, and the cricoarytenoideus dorsalis muscles as well as the recurrent laryngeal and common peroneal nerves were

From the Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO (Mhlanga-Mutangadura, G.S. Johnson, Ashwini, G.C. Johnson, Kuroki); the Department of Pathology, University of California San Diego, La Jolla, CA (Shelton); the Department of Clinical Sciences, Colorado State University, Fort Collins, CO (Wennogle); and the Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO (O'Brien).

The work was performed at the University of Missouri, University of California San Diego, and Colorado State University

Corresponding author: D.P. O'Brien, Comparative Neurology Program, College of Veterinary Medicine, University of Missouri, 900 East Campus Drive, Columbia, MO 65211; e-mail: obriend@missouri.edu.

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sent by courier service to the Comparative Neuromuscular Lab at University of California San Diego. Upon receipt, the unfixed muscles were flash frozen in isopentane precooled in liquid nitrogen and processed with a standard panel of histochemical stains and reactions.[15] The fixed muscle was processed into paraffin by standard procedures and the fixed nerves resin embedded as previously described.[16]

EDTA-anticoagulated blood samples or buccal swab samples[a] were collected from these dogs and from 128 other Rottweilers that had no recorded neurologic signs at 1 year of age. Four of the sampled NVSD-free Rottweilers were diagnosed with laryngeal paralysis, polyneuropathy, or both between 3 and 10 years of age. DNA was prepared from these samples by previously described methods.[17,18] An additional 468 DNA samples from 169 other dog breeds were randomly selected from the University of Missouri Animal DNA Depository. The DNA samples from individual dogs were genotyped at *RAB3GAP1:c.743* with a TaqMan allelic discrimination assay[19] as previously described.[13] All studies were approved by the Animal Care and Use Committee of the University of Missouri and conducted with informed consent of the owners.

Results

Phenotype

Eight Rottweilers presented for respiratory distress at 3–4 months of age and 7 were diagnosed with laryngeal paralysis. Other reported signs were pelvic limb sensory ataxia and weakness (n = 8), cerebellar ataxia (n = 2), megaesophagus (n = 2), cataracts (n = 3), microphthalmia (n = 2), and persistent pupillary membranes (n = 1). Histopathology of the brain in the 4 dogs examined revealed frequent intracytoplasmic vacuolation within neurons confirming a diagnosis of NVSD (Fig 1). Vacuoles were found in the following neuroanatomic locations in the 4 dogs that were necropsied: cerebellar cortex (4/4), cerebellar roof nuceli (3/4), cuneate nuclei (4/4), gracilis nuclei (2/4), hypoglossal nuclei (2/4) inferior olives (2/4), vestibular nuclei (2/4), striate nuclei (1/4), substantia nigra (1/4), retina (1/4), and spinal cord (1/4). Irregular loss of cerebellar Purkinje cells with empty basket cell processes were prominent in all dogs, and occasional Purkinje cells had axonal torpedoes (Fig 2). Histopathology of peripheral nerve and muscle was performed on 1 dog (Fig 3). The overall density of myelinated fibers was subjectively appropriate in the common peroneal nerve (Fig 3A) with a decrease in the expected population of large caliber nerve fibers and an increase in the population of small caliber fibers consistent with mild axonal degeneration and presumptive regeneration. Myofiber size in the limb muscles was generally small (Fig 3C) with retention of a normal polygonal shape. In contrast, marked nerve fiber loss was evident in the recurrent laryngeal nerve (Fig 3B) with myofiber loss evident in the cricoarytenoideus dorsalis muscle (Fig 3D) consistent with more marked axonal degeneration.

Genotype

All 8 Rottweilers with NVSD were homozygous for the *RAB3GAP1:c.743delC* variant allele; whereas the 128 NVDS-free Rottweilers were either heterozygotes (n = 23) or homozygous for the reference allele (n = 105). The 4 Rottweilers who presented with laryn-

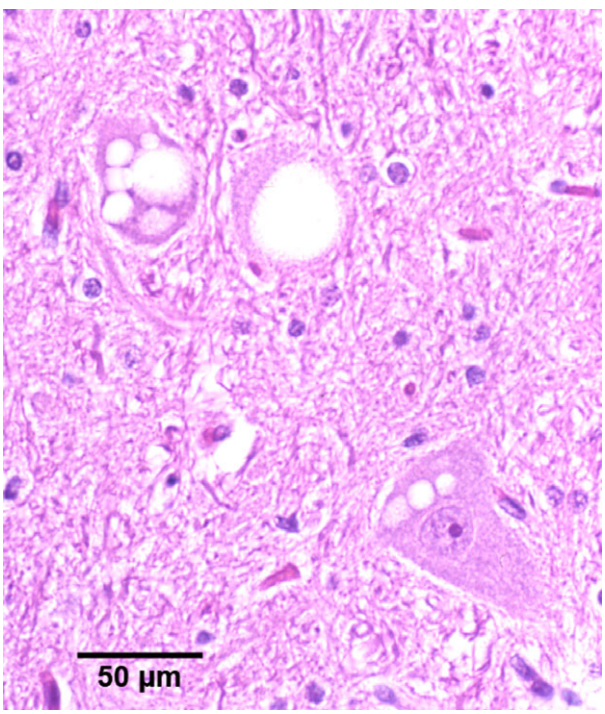

Fig 1. Neurons showed single or multiple large vacuoles characteristic of NVSD (H&E stain, bar = 50 µm).

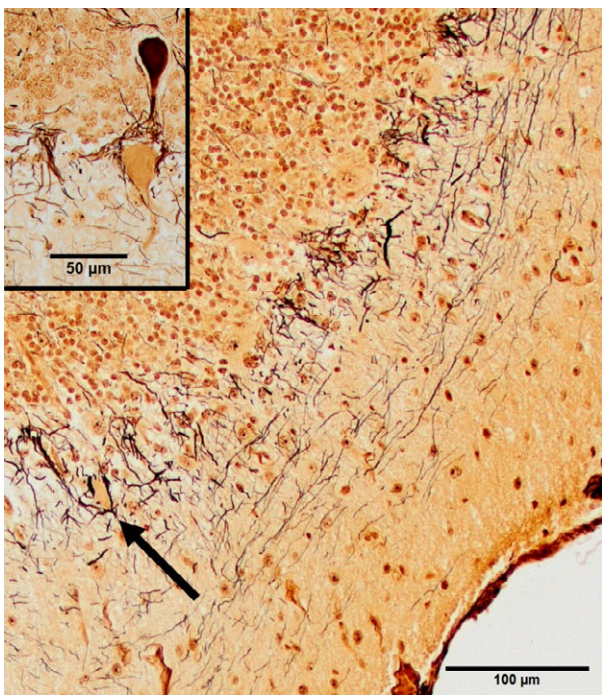

Fig 2. The cerebellar cortex contained segments where only empty Basket cell processes are apparent. The black arrow shows a residual Purkinje cell body (bar = 100 µm). Inset: A few Purkinje cells had swollen axons. This neuron is next to an empty basket. (Bielschowsky stain, bar = 50 µm).

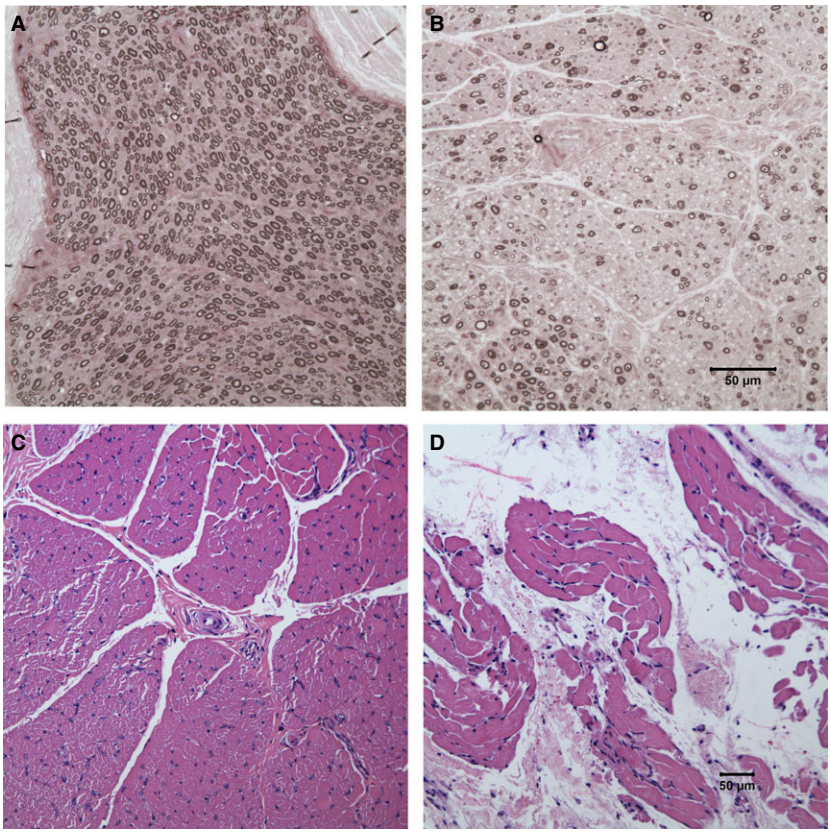

Fig 3. Muscle and nerve biopsies from a 3-month-old male Rottweiler puppy with a confirmed homozygous *RAB3GAP1:c.743delC* mutation. (**A**) Resin embedded 1-µm section from the common peroneal nerve showing a subjectively normal overall population of nerve fibers with a decreased population of large caliber fibers and an increased population of small caliber fibers. (**B**) In comparison, similar sections from the recurrent laryngeal nerve show marked loss of all calibers of myelinated nerve fibers consistent with chronic axonal degeneration. (Paraphenelenediamine stain, bar = 50 µm for both A and B). (**C**) Paraffin-embedded section from the vastus lateralis muscle showing generalized myofiber atrophy or hypotrophy with retention of the normal polygonal myofiber shape. (**D**) For comparison, myofiber loss was evident in the cricoarytenoideus dorsalis muscle with wide separation of remaining muscle fascicles. (H&E stain, bar = 50 µm for both C and D).

geal paralysis, polyneuropathy, or both at >1 year of age were all homozygous for the reference allele. All 468 dogs representing 169 breeds other than Rottweiler or BRT were homozygous for the reference allele.

Discussion

The *RAB3GAP1:c.743delC* variant previously identified in the homozygous state in BRTs with POANV[13] also occurred as a homozygous variant in all 8 of the NVSD-affected Rottweilers in our study. None of the normal Rottweilers or the Rottweilers with signs beginning at >1 year of age were homozygous for the variant. As previously reported[13] the variant was not detected in randomly selected samples from purebred dogs of other breeds besides the BRT. It is likely that the same founder mutation event was the source of the *RAB3GAP1:c.743delC* allele in both Rottweilers and BRTs. According to the BRT Club of America website (http://www.brtca.org/brt-information.html accessed November 16, 2015), the BRT breed was developed by interbreeding Rottweilers with other selected breeds during the 1930s in a military kennel near Moscow. The goal was to create a new Russian breed for the national security force that was large and rugged enough to endure the harsh Siberian winters. The mutation predicts a premature stop codon and a truncated gene product *RAB3GAP1:p.P248Lfs4** missing 730 C-terminal amino acids, including the catalytic domain. Thus, it is doubtful that the truncated gene product retains biological activity.

RAB3GAP1 codes for the catalytic subunit that combines with a noncatalytic subunit encoded by *RAB3GAP2* to form Rab3GAP. Rab3GAP was first recognized as a GTPase activator protein (GAP) that greatly enhances the inherent GTPase activity of Rab3.[20] Rab proteins function as molecular switches that regulate the formation, transport, tethering and fusion of a variety of membrane structures by cycling between inactive GDP-bound and active GTP-bound states.[21–23] GTP binding to Rab proteins is mediated by a guanine exchange factor (GEF), and subsequently Rab3GAP was shown to also function as a GEF for another Rab protein, Rab18.[24]

Homozygous and compound heterozygous mutations in human *RAB3GAP1, RAB3GAP2, and RAB18* cause a severe developmental disorder known as Warburg micro syndrome (WARBM1, WARBM2, and WARBM3,

respectively) and the somewhat milder disease phenotype called Martsolf syndrome.[25–29] Other patients (WARBM4) have had mutations in *TBC1D20,* which encodes a protein that functions as a GAP for Rab1 and Rab2.[29] Children with WARBM have microcephaly with severe developmental delays and seizures, ocular abnormalities including congenital cataracts and microphthalmia, and a predominantly axonal peripheral neuropathy.[28,30,31] There are no reports describing histopathology in WARBM, but MRI of affected children have shown predominantly frontal polymicrogyria and cerebellar atrophy.[28,32] Both Rottweilers with NVSD and BRT with POANV show microphthalmia, congenital cataracts and axonal peripheral neuropathy with laryngeal paralysis,[7,13,33–36] The spinocerebellar ataxia reported in some Rottweilers has not been observed in BRTs though both breeds show cerebellar pathology.[7,13,36] Cerebral cortical dysplasias have not been reported in either breed,[7,13,33–36] which would explain the absence in dogs of the cognitive changes and seizures reported in children with WARBM.[28]

A variety of progressive neurologic diseases occur in young Rottweilers and are presumed to be hereditary (Table 1).[1–6,8,9] The clinical histories of the *RAB3GAP1: c.743delC* homozygotes in the current study most closely resemble those previously reported for dogs with NVSD. In all reports of NVSD, the onset of clinical signs was around 3 months of age.[7,33,36–38] The initial report described respiratory difficulties in one case but laryngeal paralysis was not documented. Ataxia and weakness were the most prominent clinical signs observed.[7] Another report emphasized cerebellar ataxia and inspiratory stridor as the major clinical signs.[36] Another study described signs of laryngeal paralysis and weakness with electrodiagnostic and histopathology suggesting an axonopathy in Rottweilers with a sensorimotor neuropathy beginning around 3 months of age, but did not report any lesions in the brain or spinal cord[33] Later studies emphasized the laryngeal paralysis and polyneuropathy seen in NVSD.[35,37,39] Cataracts and microphthalmia have also been reported in some cases of NVSD or polyneuropathy in Rottweilers.[33–35] Compared to the NVSD cases, dogs with spinal muscular atrophy and distal myopathy had earlier ages at onset; whereas most dogs with neuroaxonal dystrophy, sensory neuropathy, and leukoencephalomyelopathy had later ages at onset. One dog that was diagnosed at necropsy with neuroaxonal dystrophy presented at 15 weeks of age with laryngeal paralysis[40] and might have, in fact, had NVSD. Now such cases could be genotyped for the *RAB3GAP1: c.743delC* allele to clarify whether or not they should be reclassified as cases of NVSD.

The cause of an NVSD-like disease phenotype in Boxers[41] is currently unknown. A recent report describes POANV in Huskies with a SINE insert in *RAB3GAP1*.[14] Affected dogs had microphthalmia, miosis, cataracts, and persistent pupillary membranes. Voice changes and megaesophagus were reported, but as in Rottweilers, the severe laryngeal paralysis seen in BRT[7] was not observed. The affected Huskies survived longer than the BRT and developed a severe sensory ataxia. Histopathology revealed neuronal vacuolation and axonal neuropathy.[14] The occurrence of similar disease phenotypes associated with 2 different *RAB3GAP1* mutations in 3 different breeds supports the existence of a causal relationship between the mutations and the disease.

The first cases of NVSD were seen soon after the recognition of variant Creutzfeldt–Jakob disease (CJD) in humans following the bovine spongiform encephalopathy (BSE) epidemic. Variant CJD was a novel form of CJD and transmission of the BSE prion to humans was suspected.[42] The recognition of a spongiform encephalopathy in dogs raised concerns that it could also be a prion disease. Further studies at the time, however, did not demonstrate protease-resistant prion protein on immunohistochemistry or Western immunoblot assay[7,36,37] and no mutations were identified in *PRNP,* the gene coding for prion protein, in an affected dog (Johnson GS and O'Brien, unpublished observation). The cause of the vacuolar change in prion diseases and its relationship with the pathogenesis of the disease are not known.[43] Studies of sporadic CJD have shown decreased expression of a Rab recycling protein and increased activated Rab3a in Purkinje cells which may contribute to the pathogenesis of the disease.[44,45] Further investigation of membrane trafficking in dogs with

Table 1. Comparison of some presumed hereditary neurologic diseases reported in Rottweilers

Disease	Age of Onset	Major Signs	Pathology
Spinal muscular atrophy[4,5]	4 weeks	Progressive paralysis and hypotonia	Motor neuron degeneration
Distal myopathy[8]	3–8 weeks	Planitgrade/palmigrade stance and weakness	Myofiber atrophy and endomysial fibrosis
Myotubular myopathy[9]	7–13 weeks	Weakness and hypotonia in males (x-linked)	Small muscle fibers with central nuclei and necklace fibers
Neuronal vacuolation and spinocerebellar degeneration[7,36]	12 weeks	Ataxia, weakness and laryngeal paralysis	Neuronal vacuolation
Neuroaxonal dystrophy[1,3]	1.5–4 years	Progressive sensory ataxia and nystagmus	Spheroids in sensory tracks
Distal sensorimotor neuropathy[6]	1.5–4 years	Progressive paralysis, hypotonia, muscle atrophy and slow NCV	Loss and thinning of myelin, axonal necrosis and neurogenic muscle atrophy
Leukoencephalomyelopathy[2]	3–4 years	Progressive cerebellar ataxia and weakness	Demyelination of spinal cord, brainstem, and cerebellum

RAB3GAP1:c.743delC could shed light on the pathogenesis of transmissible spongiform encephalopathies.

In conclusion, our data support a causal relationship between homozygosity for the *RAB3GAP1:c.743delC* allele and NVSD in Rottweilers and POANV in BRT as well as the *RAB3GAP1* SINE insertion and POANV in Huskies.[13,14] We would recommend using POANV to describe all 3 diseases since it encompasses more of the signs associated with the mutations. The availability of DNA tests for the deletion allele should aid in the diagnosis of the disease and clarify the relationship between the various young onset neurologic diseases in Rottweilers. It should also permit breeders to identify carriers of the mutant allele. They can then use wise breeding strategies to avoid producing affected dogs while maintaining desirable traits and genetic diversity in their line. Finally, affected dogs could serve as a model for investigating how altered membrane trafficking leads to neurodegenerative disease.

Footnote

[a] Whatman FTA® Elute cards, GE Healthcare Life Sciences, Marlborough, MA

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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