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ORIGINAL INVESTIGATION

A glycine transporter SLC6A5 frameshift mutation causes startle disease in Spanish greyhounds

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Abstract

Startle disease, or hyperekplexia, is a glycinergic disorder characterized by hypertonia and apnea that is triggered by noise and/or touch. Mutations in five genes have been associated with startle disease in humans, dogs, cattle, and mice. We identified a novel recessive startle disease in a family of Spanish greyhounds. Whole genome resequencing of an affected dog revealed a homozygous two base pair deletion in the ninth exon of *SLC6A5*, encoding the presynaptic glycine transporter. The deletion is predicted to cause a frameshift, p.S460FfsX47, leading to a premature stop codon that truncates over a third of the protein. Family members were genotyped for the deletion, and findings were consistent with an autosomal recessive inheritance pattern. The pathogenic variant was absent from 34 unrelated greyhounds, 659 domestic dogs of pure and mixed breeds, and 54 wild canids, suggesting it occurred recently and may be private to the family. The findings of this study can be used to inform future breeding decisions and prevent dissemination of the deleterious allele in greyhounds.

Introduction

Startle disease, or hyperekplexia, is a rare disorder of newborn children caused by defective neurotransmission of inhibitory glycinergic signals (Harvey et al. 2008). When exposed to acoustic or tactile stimuli, affected individuals display an exaggerated startle reflex characterized by hypertonia and apnea (Harvey et al. 2008). The cessation of breathing that occurs during a startle episode is highly dangerous, as it can lead to brain damage and sudden infant death (Bakker et al. 2006; Thomas et al. 2010). Familial startle disease may be inherited in an autosomal dominant, autosomal recessive, or X-linked fashion (Harvey et al. 2008). Approximately 80% of cases are attributed to deleterious alleles of *GLRA1*, which encodes the alpha one subunit of inhibitory glycine receptors (Shiang et al. 1993; Tijssen and Rees 2007). Pathogenic variants have also been identified in genes encoding glycine receptor GLRB (Rees et al. 2002); receptor clustering proteins GPHN (Rees et al. 2003) and ARHGEF9 (Harvey et al. 2004); and presynaptic glycine transporter SLC6A5 (Eulenberg et al. 2006; Rees et al. 2006).

Naturally occurring forms of startle disorders have also been reported in three domesticated species: dogs (Gill et al. 2011), horses (Gundlach et al. 1993), and cattle (Charlier et al. 2008). In dogs, a startle disease identified in the Irish wolfhound breed was attributed to a *SLC6A5* microdeletion (Gill et al. 2011). Herein, we describe a novel startle disease in two Spanish greyhounds that presented at 2 months of age with limb rigidity that was exacerbated by handling. We generated whole genome resequencing data from a single affected dog to establish the genetic basis for the disease.

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Materials and methods

Animals

Two 2-month-old male Spanish greyhound puppies with muscle stiffness and five apparently normal siblings were obtained by a greyhound rescue group in Spain. From the start of ambulation, the affected puppies showed an inability to stand and rigidity of all limbs that was exacerbated by movement or handling. A genetic disease was suspected. The sire and dam of the affected dogs did not display clinical signs of the disease, indicating that the inheritance pattern is likely recessive.

Muscle or whole blood samples were obtained from greyhound family members (sire, dam, two affected littermates, and one unaffected littermate) and genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen). Buccal cell samples were obtained from 25 healthy, unrelated Spanish greyhounds. Genomic DNA was isolated using the Gentra Puregene DNA Isolation kit (Qiagen). DNA concentration was quantitated by a NanoDrop 1000 spectrophotometer (Thermo Scientific), and samples were diluted to 50 ng/µL.

Whole genome resequencing

Whole genome resequencing (WGS) data were generated at approximately 26 × coverage for one affected dog. After preparation of a 350 base pair (bp) insert fragment library, a HiSeq X Ten instrument (Illumina) was used to generate paired-end reads (2 × 150 bp). High-quality reads were aligned against the reference dog genome assembly CanFam3.1 using Burrows–Wheeler Aligner (BWA; Li and Durbin 2009) software. Reads were visualized using integrated genomics viewer (IGV; Thorvaldsdóttir et al. 2013). Exons of *SLC6A5, GLRA1, GLRB, GPHN*, and *ARHGEF9* were manually scanned for variants. WGS data are publically available from NCBI under BioProject PRJNA509599.

Genotyping of c.1379_1380delCT

Primers for PCR were designed to amplify a 372 bp region containing c.1379_1380delCT (forward 5′-GCCCCCTCC AGTGACCCT-3′, reverse 5′-CATCCCGTGAAGAGCCCC -3′). PCR was carried out following manufacturer's instructions for 2X ReddyMix (Thermo Scientific). The following amplification specifications were used: initial denaturation at 95 °C for 5 min; 5 cycles of 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 10 s; and 30 cycles of 95 °C for 20 s, 56 °C for 15 s, and 72 °C for 10 s; with a final extension of 72 °C for 10 min. Sanger sequencing was performed on an ABI 3730xl Genetic Analyzer (Applied Biosystems) to validate c.1379_1380delCT. The greyhound family and 25 additional Spanish greyhounds were genotyped for c.1379_1380delCT. We further investigated the presence of c.1379_1380delCT in a publically available VCF file containing SNPs and small insertions and deletions from 668 domestic dogs of various pure and mixed breeds, including 9 greyhounds, and 54 wild canids (Plassais et al. 2019).

Results

Animals

Apart from muscle stiffness, the general physical examination of the affected dogs was normal. On neurological examination, affected dogs were in lateral recumbency and displayed a rigid posture of all limbs and trunk with normal mental status (Fig. 1). The dogs were not able to stand or walk unassisted. With assistance, they developed extensor rigidity in all limbs after a few steps. Cranial nerve examination and postural reactions were normal. Spinal reflexes could not be accurately evaluated due to stiffness. Rigidity lessened during sleep and was absent during general anesthesia. A neuromuscular disease was suspected, but a central nervous system disorder causing defective inhibitory neurotransmission was also considered.

For evaluation of a neuromuscular disorder, electromyography was performed under general anesthesia and no evidence of abnormal spontaneous activity was detected. Consequently, a form of myotonia and peripheral nerve hyperexcitability syndrome were ruled out. Sciatic and ulnar motor nerve conduction velocities were also normal, eliminating peripheral nerve disease from consideration. Skeletal muscle biopsies from the biceps femoris and triceps brachii muscles ruled out primary myopathic disease. A form of startle disease or hyperekplexia was considered most likely.

Fig. 1 Clinical presentation of startle disease in an affected Spanish greyhound. An affected puppy displays lateral recumbency and rigid posture

Both affected dogs were treated with a combined therapy of diazepam (0.5 mg/kg BID to TID) and mexiletine (8.3 mg/kg TID) PO that resulted in increased physical activity. Despite treatment, one dog developed apneic episodes during handling and ultimately died of respiratory arrest. The second dog is still alive 18 months after diagnosis.

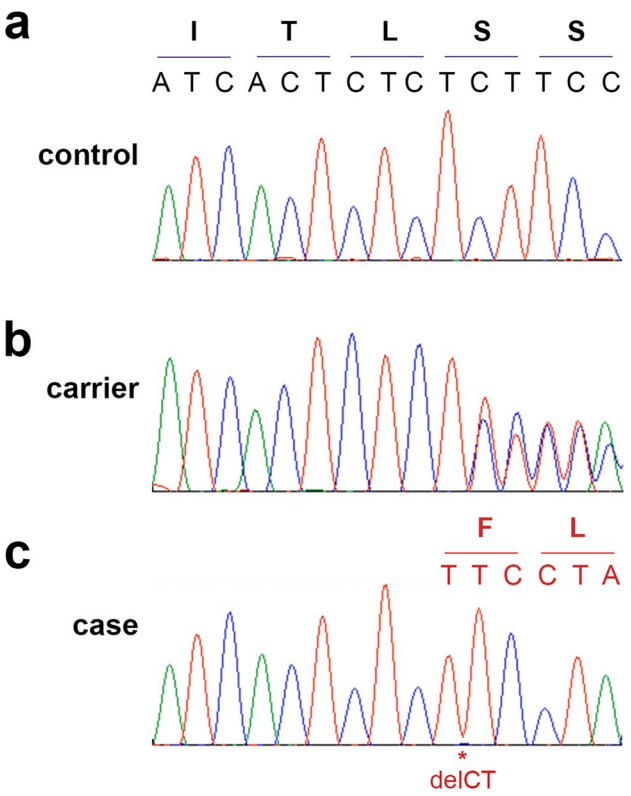

Fig. 2 Validation of the *SLC6A5* exon nine deletion detected by whole genome resequencing. Sanger sequencing chromatograms are shown for an unrelated, healthy greyhound (**a**), the heterozygous sire (**b**), and an affected dog homozygous for the mutant allele (**c**). Sequences from the affected dog display homozygosity for a cytosine and thymine deletion, causing a frameshift in SLC6A5 (p.S460FfsX47)

Whole genome resequencing

We used whole genome resequencing data from one affected dog to manually scan the coding regions of five functional candidate genes (*SLC6A5, GLRA1, GLRB, GPHN*, and *ARHGEF9*) for homozygous variants. We hypothesized that the affected dogs are homozygous for a deleterious allele inherited identical by descent from heterozygous parents. In the case of the X-linked gene, *ARHGEF9*, we considered that a hemizygous variant inherited maternally would appear homozygous in the affected dogs, both males. We identified a single homozygous coding variant: a two base pair deletion (c.1379_1380delCT) in exon nine of *SLC6A5* (Fig. 2). The deletion causes a frameshift (p.S460FfsX47) that leads to a premature stop codon at amino acid residue 506 of the 768 residue full-length protein (Fig. 3). No homozygous or compound heterozygous variants were found in the coding regions of the other four candidate genes.

Genotyping of c.1379_1380delCT

We developed a PCR assay and used Sanger sequencing to validate the 2-bp *SLC6A5* deletion and genotype the remaining family members. The second affected dog was homozygous for the pathogenic variant, while the dam, sire, and unaffected littermate were heterozygous, a pattern consistent with simple autosomal recessive inheritance. Because startle disease has not been reported in greyhounds outside of this family, we posited that the mutation is recent and would not be found in unrelated greyhounds or dogs of other breeds. The mutation was absent in a control population of 34 unrelated greyhounds, 659 domestic dogs of various pure and mixed breeds, and 54 wild canids.

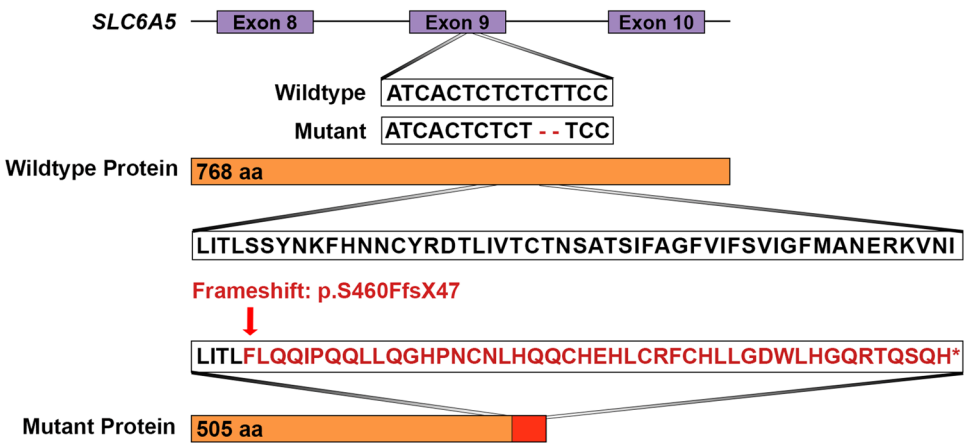

Fig. 3 Impact of the frameshift mutation in *SLC6A5*. A schematic representation depicting *SLC6A5* c.1379_1380delCT, which predicts a frameshift at amino acid 460 and premature stop codon (*) at position 506

Discussion

The early clinical presentation of touch-induced rigid posture and lateral recumbency with rigidity at 2 months of age in the Spanish greyhounds is consistent with hyperekplexia or startle disease (Harvey et al. 2008). The normal electromyographic evaluation ruled out a form of congenital myotonia (Vite 2002) and peripheral nerve hyperexcitability syndrome (Vanhaesebrouck et al. 2013), and normal muscle biopsies eliminated primary myopathic disease. Based on these clinical findings, we deemed a form of startle disease most likely.

Due to the phenotypic similarity of the affected dogs with previously described cases of startle disease, we prioritized genes involved in glycinergic neurotransmission that are associated with startle disorders. These parameters left us with five functional candidate genes that we interrogated for homozygous coding variants using whole genome resequencing data from a single affected dog. The homozygous 2-bp deletion present in exon nine of *SLC6A5* leads to a frameshift and premature stop codon that predicts truncation of over one-third of the open reading frame for the wildtype SLC6A5 protein. Thus, it is likely that the mutant transcript is subject to nonsense-mediated decay resulting in an absence of SLC6A5 and startle disease in homozygous individuals.

SLC6A5 is a glycine transporter critical for maintaining a plentiful presynaptic pool of neurotransmitter at glycinergic synapses (Rees et al. 2006). Recessive *SLC6A5* variants are considered the second most common cause of startle disease (López-Corcuera et al. 2018). Loss of SLC6A5 function in knockout mouse models is lethal in the second postnatal week and produces phenotypes similar to human startle disease including muscle rigidity and spasticity (Gomeza et al. 2003). A spontaneous microdeletion encompassing exons two and three of canine *SLC6A5* is associated with startle disease in Irish wolfhounds (Gill et al. 2011). Affected puppies displayed muscle rigidity and tremor in response to handling similar to the exaggerated startle response observed in the greyhounds described herein. In both the Irish wolfhounds and greyhounds, the clinical phenotype is likely attributed to insufficient synaptic glycine due to a loss of function of glycine transporter SLC6A5. A disorder having clinical signs similar to startle disease was previously described in Labrador retrievers (Fox et al. 1984; March et al. 1993); however, a causative mutation has not been identified.

This study illustrates the ease with which simple Mendelian disorders of dogs can be dissected at the genetic level when the phenotype is clearly analogous to a human disorder for which causal genes have been identified. Genetic testing can prevent dissemination of recent deleterious alleles and facilitate the preservation of genetic variation within purebred dog populations. This report also identifies another large animal model for the study of new therapeutic possibilities for the treatment of startle disease.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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