TITLE PAGE

The novel homozygous KCNJ10 c.986T>C (p.(Leu329Pro)) variant is pathogenic for the SeSAME/EAST homologue in Malinois dogs.

running title: KCNJ10 variant causes SeSAME/EAST in Malinois dogs

Mario Van Poucke[1,*], Kimberley Stee[2,*], Sofie F.M. Bhatti[2], An Vanhaesebrouck[3], Leslie Bosseler[4], Luc J. Peelman[1,*] and Luc Van Ham[2,*]

[1]Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

[2]Department of Small Animal Medicine and Clinical Biology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

[3]Department of Clinical Neurosciences, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom

[4]Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

*These authors contributed equally to this work

The authors declare no conflict of interest

Correspondence: Dr Mario Van Poucke, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium; Tel +32 9 2647806; Fax +32 9 2647849; E-mail: Mario.VanPoucke@UGent.be

ABSTRACT

SeSAME/EAST syndrome is a multisystemic disorder in man, characterised by seizures, sensorineural deafness, ataxia, developmental delay and electrolyte imbalance. It is exclusively caused by homozygous or compound heterozygous variations in the KCNJ10 gene. Here we describe a similar syndrome in 2 families belonging to the Malinois dog breed, based on clinical, neurological, electrodiagnostic and histopathological examination. Genetic analysis detected a novel pathogenic KCNJ10 c.986T>C (p.(Leu329Pro)) variant, which is inherited in an autosomal recessive way. This variant has an allele frequency of 2.9% in the Belgian Malinois population, but is not found in closely related dog breeds nor in dog breeds where similar symptoms were already described. The canine phenotype is remarkably similar to humans, including ataxia and seizures. In addition, in half of the dogs clinical and electrophysiological signs of neuromyotonia were observed. Because there is currently no cure and treatment is non-specific and unsatisfactory, this canine translational model could be used for further elucidating the genotype/phenotype correlation of this monogenic multisystem disorder and as an excellent intermediate step for drug safety testing and efficacy evaluations, before initiating human studies.

Keywords: KCNJ10, spinocerebellar ataxia, epilepsy, myokymia, neuromyotonia, animal model

INTRODUCTION

SeSAME (Seizures, Sensorineural deafness, Ataxia, Mental retardation and Electrolyte imbalance) or EAST (Epilepsy, Ataxia, Sensorineural deafness, Tubulopathy) syndrome (Phenotype MIM number 612780) is a rare, autosomal recessive multisystemic disorder.[1,2] It is caused by homozygous or compound heterozygous variations in the voltage-gated potassium channel (VGKC), inwardly rectifying, subfamily J, member 10 gene (KCNJ10, alias KIR4.1; Gene/Locus MIM number 602208). Functional KCNJ10 subunits form homotetrameric or heterotetrameric (in coassembly with KCNJ16, alias KIR5.1) channels with 2 putative transmembrane regions. The KCNJ10 channel is mainly expressed in the brain, spinal cord, inner ear and kidneys. Depending on their tissue localisation, these channels have distinct physiologic properties. Variations described so far have all been loss-of-function variations.[3]

The human KCNJ10 gene is very well characterised. It is annotated in all genome browsers, has a reviewed RefSeq status (NCBI Gene ID 3766) and one transcript (CCDS1193.1; annotated by both Ensembl and HAVANA). It is located on chromosome 1q23.2 and consists of a first non-coding exon and a second exon containing the complete CDS of 1140 bp encoding a protein of 379 amino acids. Until now, 14 variants have been described causing SeSAME/EAST, all in the KCNJ10 coding sequence (Figure 1).[4]

A similar disease (OMIA 001820-9615) has also been described in a number of dog breeds (Border collie, Dachshund and Terrier breeds; reviewed in Vanhaesebrouck *et al*[5]). Until now only the KCNJ10 c.627C>G (p.(Ile209Met)) variant has been reported to be associated with spinocerebellar ataxia and myokymia, seizures or both (SAMS) in

certain Terrier breeds.[6,7] In addition, Forman *et al*[8] reported that the CAPN1 c.344G>A (p.(Cys115Tyr)) variant is strongly associated with late onset ataxia in Parson Russell Terriers, suggesting that CAPN1 may represent a novel candidate gene for ataxia in humans. According to OMIA there are no spontaneous disease causing variants in KCNJ10 described in other species.

Here, we describe a syndrome in 2 Malinois dog families with a phenotype strikingly similar to SeSAME/EAST syndrome in humans and the identification of a new pathogenic missense variant in KCNJ10.

MATERIALS AND METHODS

Clinical examination

Three 3-month-old intact (2 males and 1 female) Malinois dogs (dogs 1-3) were presented at the Department of Small Animal Medicine and Clinical Biology, Faculty of Veterinary Medicine, Ghent University for an uncoordinated gait since the age of 6 to 8 weeks. A clinical and neurological examination was performed in all 3 dogs, and a complete bloodwork in dog 1 and 3. Urinalysis was performed only in dog 3. Electromyography (EMG) and brainstem auditory evoked response (BAER) tests were performed in all 3 dogs. EMG recordings were made from facial, truncal and appendicular muscles of front and hind limbs. Motor nerve conduction velocity studies and repetitive nerve stimulation of the radial and sciatic nerves were performed in dog 1 and 2. A commercially available electrophysiological unit (Medelec/TECA, Sapphire 2M) was used for electrodiagnostic recordings. EMG and BAER tests were performed under sedation and motor nerve conduction velocity studies were performed under general anesthesia. Cerebrospinal fluid analysis and MRI (0,2 Tesla magnet) of the

brain and cervical spinal cord was performed in dog 1. For affected dog 4, only video footage was available.

Pathological examination

Post-mortem examination was performed in dog 2, immediately following euthanasia. Central and peripheral nervous tissue samples were collected, formalin-fixed, paraffin-embedded and stained with hematoxylin-eosin or luxol fast blue. An immuno-histochemical staining with neurofilament (monoclonal mouse anti-human neurofilament protein clone 2F11; Dako, Cat. no. M0762, Glostrup, Denmark) was performed.

Genetic analysis

EDTA blood samples from the 4 affected dogs and 9 healthy family members were collected for genetic analysis (pedigree analysis in Figure 2). Genomic DNA was isolated from the blood by performing a proteinase K digestion as described in Van Poucke *et al.*[9] cDNA transcribed from canine brain RNA in Van Poucke *et al*[10] was used in this study to experimentally investigate the existence of the 3 predicted KCNJ10 transcript variants (Acc. no.: X1: XM_005640901.2, X2: XM_014111261.1, X3: XM_545752.5).

Primer pairs were designed with Primer-BLAST[11] based on the canine KCNJ10 reference sequence (Acc. No. NC_006620.3), taking into account all described transcript variants, sequence variants and repeat sequences. Primers were chosen in regions that were free of secondary structures (Mfold).[12] PCR amplicons were analysed via agarose gel electrophoresis. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the individual PCR primers as sequencing primers and run at Eurofins Genomics

(Ebersberg, Germany). Sequence analysis was performed with Unipro UGENE v1.16.1.[13] An assay with dual labelled probes (Eurofins) was designed as described by Van Poucke *et al*[9] to genotype the KCNJ10 c.986T>C variant in additional animals. Experimental details on the genetic analysis are given in Supplementary Information File S1A.

Bioinformatics tools for sequence variant interpretation

Variant information was gathered from population databases (Exome Aggregation Consortium[14], Exome Variant server[15], 1000 Genomes[16], dbSNP[17] and dbVAR[18]), disease databases (humsavar.txt release 2016_05[19], ClinVar[20], HGMD-public[21], OMIM[22] and OMIA[23]) and sequence databases (RefSeqGene[24], NCBI Genome[25], UCSC Genome[26] and Ensembl Genome[27]). I-Mutant2.0[28] was used as a predictor of protein stability changes upon variations. Computational prediction of disease related variants was performed with the consensus tools CONDEL[29] (combines Logre, MAPP, Massessor, Pph2 and SIFT), Meta-SNP[30] (combines PANTHER, PhD-SNP, SIFT and SNAP), PredictSNP[31] (combines MAPP, PhD-SNP, Pph1, Pph2, SIFT and SNAP) and PON-P2[32].

RESULTS

Clinical features

General clinical examination was unremarkable in all 3 presented dogs. Neurological examination revealed a severe generalized hypermetric ataxia and absent patellar reflexes in all dogs (Supplementary Information File S2A). Generalized involuntary vermicular muscle contractions (myokymia) triggered by excitement were present in dogs 1 and 2 (Supplementary Information File S2B). Dog 1 had demonstrated a

neuromyotonic episode of extreme generalized muscle stiffness with normal consciousness, triggered by stress. The dog also developed generalized seizures a few weeks later.

Complete blood count and serum biochemistry, including electrolytes (Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}), was normal in dog 1 and 3. Urinalysis (including electrolyte clearance for Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and PO^{4-}), was unremarkable in dog 3. EMG showed neuromyotonic discharges[5,33] in those muscles also visibly showing myokymia in dogs 1 and 2 (Figure 3), and was normal in dog 3. BAER recordings showed disappearance of wave components III/IV and V, and mildly prolonged latencies of waves I and II in dogs 1 to 3 (Figure 4). Results of motor nerve conduction velocity studies and repetitive nerve stimulation in dogs 1 and 2 did not differ from two age-matched Malinois control dogs. MRI of the brain/cervical spine and cerebrospinal fluid analysis, performed in dog 1, did not reveal any abnormalities.

The neurological condition of dogs 1, 2 and 4 deteriorated further (Supplementary Information File S2C) and all were euthanized because of the severity of the ataxia before the age of 6 months. Despite severely debilitating non-ambulatory ataxia, dog 3 is still alive (9 months after presentation).

Pathological features

A full necropsy was performed on dog 2 (ataxia and myokymia). No macroscopic abnormalities were found. On histopathology, bilateral myelopathy with predominant axonopathy and myelin vacuolization was found. Changes were most obvious in the corticospinal tracts of the brainstem and the ventral funiculi of the cervical, thoracic and lumbar spinal cord. Similar lesions were found in the cerebellum (white matter) and medulla oblongata (predominantly at the pyramid bodies). In the peripheral nervous

system, subtle changes were seen in a few axons (swollen with vacuolated myelin) of the brachial plexus, the femoral nerve and the sciatic nerve. Luxol fast blue and neurofilament stainings confirmed these findings (Supplementary Information File 3).

Screening for known spinocerebellar ataxia-related/causal variants

All 13 members of the 2 Malinois families (dogs 1-13, Figure 2) were first screened for the KCNJ10 c.627C>G (p.(Ile209Met)) variant described by Gilliam *et al*[6] and the CAPN1 c.344G>A (p.(Cys115Tyr)) variant described by Forman *et al*[8], but none of the variants (so far only described in certain Terrier breeds) were detected. In addition, none of the variants were found in 28 healthy Malinois from different families.

Canine KCNJ10 gene characterisation

Contrary to the human KCNJ10, its canine ortholog is not yet annotated in any of the genome browsers (although assigned to chromosome 38), only has a model RefSeq status (NCBI Gene ID 488635) and 3 predicted transcript variants (X1-X3, with X3 being orthologous with the human transcript variant). Therefore, we first validated the predicted canine KCNJ10 transcripts experimentally. Via RT-PCR we could only confirm the presence of transcript variant X3 in the canine brain (primers 1F/1R could only amplify the 360-bp X3 fragment and not the 535-bp X1 fragment, while primers 2F/R2 could not amplify the 140-bp X2 fragment although they could amplify a 231-bp fragment with genomic DNA as a template; see Supplementary Information File S1A for experimental details). The other 2 canine transcript variants were not or very lowly (below detection limit) expressed in the canine brain, or wrongly predicted. Sequencing the complete X3 transcript showed that the canine KCNJ10 CDS was correctly predicted and has the same length compared to its human transcript ortholog, showing 90% nucleotide sequence identity and 98% amino acid sequence identity

(Supplementary Information File S1B-C).

KCNJ10 variant detection

The complete confirmed canine KCNJ10 CDS was resequenced in both parent dogs of family 1, their 5 offspring (including 2 affected dogs) and one healthy Malinois from another family. Only one variant was discovered, a c.986T>C (p.(Leu329Pro)) variant (NCBI_ss#1966650805), homozygous in the 2 affected offspring (dogs 1-2), heterozygous in both healthy parents (dogs 10-11) and 2 healthy offspring (dogs 6-7), and not present in 1 healthy offspring (dog 8) and 1 non-related healthy Malinois (Figure 2, Supplementary Information File S1B-D).

KCNJ10 variant screening

A genotyping assay with dual labelled probes was used to screen this variant in another healthy offspring of the father in family 1 (dog 5, heterozygous) and in family 2 (healthy heterozygous parents (dogs 12-13), 2 affected homozygous mutant offspring (dogs 3-4) and 1 healthy heterozygous offspring (dog 9; Figure 2). The assay was also used to estimate the frequency of this variant in 103 additional healthy Malinois from different families: 97 were homozygous wild type, 6 heterozygous and none were homozygous mutant. We also investigated if the variant was present in the other closely related Belgian Shepherd breeds (Groenendaeler (N = 52), Laekenois (N = 28) and Tervueren (N = 56)), other Sheepdogs (Australian shepherd, German shepherd and Shetland sheepdog; all N = 25) and breeds where similar symptoms were already described (Border collie, Dachshund, Jack Russell terrier, Maltese and Yorkshire terrier; all N = 25). However, the variant was not detected in any of these breeds.

DISCUSSION

Based on the Standards and Guidelines for the Interpretation of Sequence Variants, we conclude that the c.986T>C (p.(Leu329Pro)) variant is pathogenic for the SeSAME/EAST-like syndrome in Malinois dogs in an autosomal recessive way because of 1 strong (PS), 1-2 moderate (PM) and 3-4 (PP) supporting criteria for pathogenicity.[34]

All 4 affected Malinois dogs were homozygous mutant, while none of the 112 healthy Malinois dogs were. Because there were no false negatives/positives, odds ratios were infinite (PS4). Apart from the 13 members of the affected families (Figure 2), only 6 carriers were observed in the other 103 tested healthy Malinois dogs. The variant was not found in 136 dogs from 3 closely related Belgian breeds, 75 dogs from 3 other sheepdog breeds and 125 dogs from 5 breeds where similar symptoms were already described (all dogs came from different families). In addition, a Leu was present at this position in the intracellular C-terminus in all known eutherian mammal KCNJ10 reference sequences and a variant in this codon was not found in popular population databases. A variation in this highly conserved residue is assumed to be pathogenic (PM2).

In humans, SeSAME/EAST is exclusively caused by variants in KCNJ10. Until now, 14 different pathogenic variants have been described with functional confirmation (Figure 1). A KCNJ10 missense variant was also found to segregate with a similar disease in certain Terrier breeds. Here we describe a novel KCNJ10 missense variant that segregates with the disease in 2 Malinois families (with 2 affected family members in each family; PM/PP1). Since the same variation segregates in both families, they probably share a common ancestor carrying the founder variation. But because of the

lack of pedigree information we could not identify the founder, nor the relatedness between both families. The allele frequency of the variant in the Belgian Malinois population is estimated at 2.9%. Breeders are advised to screen for the variant in order to breed with variant-free animals and get variant-free offspring.

Missense variants are the most common mechanism in this disease (11/14 in human and 2/2 in dog) and missense variants in KCNJ10 have a low rate of benignity (z-score for missense variants in KCNJ10 is 1.78 according to the ExAC Browser, indicating intolerance to variation; PP2)

There are multiple lines of computational evidence that support the deleterious effect of this Leu → Pro variant. First, the introduction of a proline is known to affect the protein stability because of its exceptional structure. It is conformational more rigid than any other amino acid because of its pyrrolidine ring and unlike all other amino acids it contains a secondary amide (instead of a primary amide) being unable to form a hydrogen bond.[35] According to I-Mutant2.0, the p.(Leu329Pro) variation indeed decreases stability (RI = 8). Second, according to humsavar.txt (release 2016_05) 65% of all Leu → Pro variants are disease associated, while the average for all variants is only 38%. Leu → Pro variants are together with Gly → Arg and Arg → Cys variants the most predominant disease associated variants (all more than 1000 hits). In addition, all nine classified Leu → Pro variants known to exist in the KCN gene family are disease associated. Third, frequently used consensus tools for computational prediction of disease related variants all confirm this assumption (CONDEL: 0.55, deleterious; Meta-SNP: 0.68, disease; PredictSNP: 0.55, deleterious; PON-P2: 0.91, pathogenic). Last, the patients' phenotype and family history is highly specific for a disease with a single genetic etiology (PP4).

The clinical features of the SeSAME/EAST syndrome are the consequence of a KCNJ10 potassium channel dysfunction.[1,36] In our 4 affected dogs, ataxia was always present, with or without myokymia, neuromyotonia and/or seizures. It is believed that, as in human, the novel KCNJ10 variant affects the function of the potassium channel in the brain and spinal cord, resulting in seizures and ataxia in the affected Malinois dogs. Interestingly, clinical signs of myokymia and neuromyotonia (nor cramps) have not been described in humans, neither in knock-out KCNJ10 mice models. Myokymia and neuromyotonia were clinically and electrodiagnostically detected in respectively 2 and 1 of the affected Malinois dogs. The authors believe that the association between myokymia/neuromyotonia and KCNJ10 variation is not coincidental, as another KCNJ10 variation also caused myokymia and neuromyotonia in a large number of dogs.[6,7] In humans, myokymia and neuromyotonia have been associated with genetic and immune-mediated defects of voltage-gated potassium channels of the peripheral nerve.[37-42] However, it has not yet been associated with a potassium channel in the central nervous system. One hypothesis would be that impairment of the "potassium sink" role by a KCNJ10 variation not only can result in increased neuronal firing within the brain and resulting in seizures, but also in the lower motor neurons of the spinal cord, resulting in hyperexcitability of the peripheral nerve and consequently resulting myokymia and/or neuromyotonia. Another hypothesis would be that the KCNJ10 channel is also present in the peripheral nerve itself, as suggested in literature, but this has not been investigated yet. Moreover, next to direct malfunctioning of potassium channels, demyelination of the peripheral nerve has also been reported together with myokymia and neuromyotonia, which is another hypothesis in our animal model as demyelination of peripheral nerves was observed. The authors do not believe that

285 systemic electrolyte disturbances would have resulted in myokymia and neuromyotonia
286 in the affected Malinois dogs, as serum electrolytes and electrolyte clearance were
287 normal. The fact that all dogs had ataxia, but only some dogs had seizures, myokymia
288 or neuromyotonia is not surprising, as it is well known that potassium channel diseases
289 can be phenotypically heterogeneous (variability of clinical signs and severity) in dogs[33]
290 and humans[1,2,43].
291 The mildly delayed latencies of waves I and II, and the loss of waves III, IV and V was
292 repetitively found on the BAER tests of our affected dogs. This suggests a peripheral as
293 well as central localization for dysfunction within the auditory pathway.[44] This is a
294 major difference to humans and mice, in which a cochlear dysfunction is assumed to be
295 the origin of the hearing impairment.[1,2] The extent of hearing impairment in man with
296 KCNJ10 variation is variable and can sometimes be absent or only appreciated with
297 specific testing.[45] None of the affected dogs showed any signs of clinically relevant
298 hearing impairment.
299 Another major difference with the SeSAME/EAST syndrome in man is that the dogs in
300 this study did not appear to show any signs of developmental delay as, apart from gait
301 abnormalities, they behaved and were able to obey as well as their healthy siblings. As
302 intelligence and language testing as in man are not applicable to dogs, we cannot
303 exclude that a certain degree of developmental delay could have been missed in our
304 dogs.
305 The histopathological findings in our dogs are identical to the ones described in the
306 Russel Terrier group.[6,8] In knock-down mice, hypomyelination in the spinal cord with
307 severe spongiform vacuolation, axonal swelling and degeneration is also described.[46] To
308 the author's knowledge, there are currently no pathological reports available of humans

with SeSAME/EAST syndrome. A nerve biopsy report from a human patient showed axonal neuropathy with hypomyelination, which is consistent with the findings in dogs.[45]

In man, the ataxia seems to be non-progressive and the seizures are in most cases easily controlled with anti-epileptic drugs.[43] In mice, general knock-out of KIR4.1 leads to neonatal mortality.[47] In Jack Russel Terriers, the ataxia also seems to be non-progressive, but the myokymia and neuromyotonia worsen gradually over several months, leading to euthanasia of most dogs.[33] Due to the larger stature of Malinois dogs compared to Jack Russell Terriers, they seemed to cope less easily with the ataxia during growth of the animal. So, mid- to long-term prognosis in these dogs is reserved to poor.

In conclusion, we described a pathogenic c.986T>C (p.(Leu329Pro)) variant for a SeSAME/EAST-like syndrome in Malinois dogs. Because, currently, there is no cure and treatment is non-specific and unsatisfactory, the canine translational model could, in addition to the existing valuable model organisms such as Xenopus, mice and zebrafish, that are mainly used for basic research and drug screening,[1,48] be used for further elucidating the genotype/phenotype correlation of this monogenic multisystem disorder and as an excellent intermediate step for drug safety testing and efficacy evaluations, before initiating human studies.[49]

ACKNOWLEDGEMENTS

We wish to thank Dominique Vander Donckt, Linda Impe and Ruben Van Gansbeke for excellent technical assistance, as well as the breeders, owners and veterinarians who collaborated to this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest

Supplementary Information (SI) is available at European Journal of Human Genetics' website.

REFERENCES

1) Bockenhauer D, Feather S, Stanescu HC, *et al*: Epilepsy, ataxia, sensorineural deafness, tubulopathy, and KCNJ10 mutations. *New Eng J Med* 2009; **360**: 1960-1970.

2) Scholl UI, Choi M, Liu T, *et al*: Seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by mutations in KCNJ10. *Proc Natl Acad Sci* 2009; **106**: 5842-5847.

3) Sala-Rabanal M, Kucheryavykh LY, Skatchkov SN, Eaton MJ, Nichols CG: Molecular mechanisms of EAST/SeSAME syndrome mutations in Kir4.1 (KCNJ10). *J Biol Chem* 2010; **285**: 36040-36048.

4) Parrock S, Hussain S, Issler N, *et al*: KCNJ10 mutations display differential sensitivity to heteromerisation with KCNJ16. *Nephron Physiol* 2013; **123**: 7-14.

5) Vanhaesebrouck AE, Bhatti SF, Franklin RJ, Van Ham L: Myokymia and neuromyotonia in veterinary medicine: a comparison with peripheral nerve hyperexcitability syndrome in humans. *Vet J* 2013; **197**: 153-62.

6) Gilliam D, O'Brien DP, Coates JR, *et al*: A Homozygous KCNJ10 Mutation in Jack Russell Terriers and Related Breeds with Spinocerebellar Ataxia with Myokymia, Seizures, or Both. *J Vet Intern Med* 2014; **28**: 871–877.

7) Rohdin C, Gilliam D, O'Leary CA, *et al*: A KCNJ10 mutation previously identified in the Russell group of terriers also occurs in Smooth-Haired Fox Terriers with hereditary ataxia and in related breeds. *Acta Vet Scand* 2015; **57**: 26.

8) Forman OP, De Risio L, Mellersh CS: Missense Mutation in CAPN1 Is Associated with Spinocerebellar Ataxia in the Parson Russell Terrier Dog Breed. *PLoS ONE* 2013; **8**: e64627.

9) Van Poucke M, Vandesompele J, Mattheeuws M, Van Zeveren A, Peelman LJ: A dual fluorescent multiprobe assay for prion protein genotyping in sheep. *BMC Infect Dis* 2005; **5**: 13.

10) Van Poucke M, Martlé V, Van Brantegem L, *et al*: A canine orthologue of the human GFAP c.716G>A (p.Arg239His) variant causes Alexander disease in a Labrador retriever. *Eur J Hum Genet* 2016; **24**: 852-856.

11) Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T: Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012; **13**: 134.

12) Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003; **31**: 3406-3415.

13) Okonechnikov K, Golosova O, Fursov M, the UGENE team. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 2012; **28**: 1166-1167.

14) Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org/) [05, 2016 accessed].

15) Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [05, 2016 accessed].

16) The 1000 Genomes Project Consortium: A global reference for human genetic

variation. *Nature* 2015; **526**: 68-74. (URL: http://www.1000genomes.org/) [05, 2016 accessed].

17) Sherry ST, Ward MH, Kholodov M, *et al*: dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 2001; **29**: 308-311. (URL: http://www.ncbi.nlm.nih.gov/SNP/) [05, 2016 accessed].

18) Lappalainen I, Lopez J, Skipper L: dbVar and DGVa: public archives for genomic structural variation. *Nucleic Acids Res* 2013; **41**: D936-D941. (URL: http://www.ncbi.nlm.nih.gov/dbvar/) [05, 2016 accessed].

19) Wu CH, Apweiler R, Bairoch A, *et al*: The Universal Protein Resource (UniProt): an expanding universe of protein information. *Nucleic Acids Res* 2006; **34**: D187-D191. (URL: http://www.uniprot.org/) [05, 2016 accessed].

20) Landrum MJ, Lee JM, Benson M, *et al*: ClinVar: public archive of interpretations of clinically relevant variants. *Nucleic Acids Res* 2016; **44**: D862-D863. (URL: http://www.ncbi.nlm.nih.gov/clinvar/) [05, 2016 accessed].

21) Stenson PD, Mort M, Ball EV, Shaw K, Phillips AD, Cooper DN: The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Hum Genet* 2014; **133**: 1-9. (URL: http://www.hgmd.cf.ac.uk/ac/index.php/) [05, 2016 accessed].

22) Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD). (URL: http://omim.org/) [05, 2016 accessed].

23) Online Mendelian Inheritance in Animals, OMIA. Faculty of Veterinary Science, University of Sydney. (URL: http://omia.angis.org.au/) [05, 2016 accessed].

24) Pruitt KD, Brown GR, Hiatt SM, *et al*: RefSeq: an update on mammalian reference

405 sequences. *Nucleic Acids Res* 2014; **42**: D756-763. (URL:
406 http://www.ncbi.nlm.nih.gov/refseq/rsg/) [05, 2016 accessed].
407 25) NCBI Genome. (URL: http://www.ncbi.nlm.nih.gov/genome/) [05, 2016 accessed].
408 26) Kent WJ, Sugnet CW, Furey TS, *et al*: The human genome browser at UCSC.
409 *Genome Res* 2002; **12**: 996-1006. (URL: https://genome.ucsc.edu/) [05, 2016 accessed].
410 27) Yates A, Akanni W, Amode MR, *et al*: Ensembl 2016. *Nucleic Acids Res* 2016; **44**:
411 D710-716. (URL: http://www.ensembl.org/index.html/) [05, 2016 accessed].
412 28) Capriotti E, Fariselli P, Casadio R: I-Mutant2.0: predicting stability changes upon
413 mutation from the protein sequence or structure. *Nucleic Acids Res* 2005; **33**: W306-
414 310.
415 29) González-Pérez A, López-Bigas N: Improving the assessment of the outcome of
416 nonsynonymous SNVs with a consensus deleteriousness score, Condel. *Am J Hum*
417 *Genet* 2011; **88**: 440-449.
418 30) Capriotti E, Altman RB, Bromberg Y: Collective judgment predicts disease-
419 associated single nucleotide variants. *BMC Genomics* 2013; **14**: S2.
420 31) Bendl J, Stourac J, Salanda O, *et al*: PredictSNP: robust and accurate consensus
421 classifier for prediction of disease-related mutations. *PLoS Comput Biol* 2014; **10**:
422 e1003440.
423 32) Niroula A, Urolagin S, Vihinen M: PON-P2: prediction method for fast and reliable
424 identification of harmful variants. *PLoS One* 2015; **10**: e0117380.
425 33) Bhatti SF, Vanhaesebroeck AE, Van Soens I, *et al*: Myokymia and neuromyotoni
426 in 37 Jack Russel terriers. *Vet J* 2011; **189**: 284-288.
427 34) Richards S, Aziz N, Bale S, *et al*: Standards and guidelines for the interpretation of
428 sequence variants: a joint consensus recommendation of the American College of

429 Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet*
430 *Med* 2015; **17**: 405-424.
431 35) Bajaj K, Madhusudhan MS, Adkar BV, *et al*: Stereochemical criteria for prediction
432 of the effects of proline mutations on protein stability. *PLoS Comput Biol* 2007; **3**: e241.
433 36) Reichold M, Zdebik AA, Lieberer E, *et al*: KCNJ10 gene mutations causing EAST
434 syndrome (epilepsy, ataxia, sensorineural deafness, and tubulopathy) disrupt channel
435 function. *Proc Natl Acad Sci U S A* 2010; **107**: 14490-14495.
436 37) Hart IK, Newsome-Davis J (eds). *Generalized Peripheral nerve hyperexcitability*
437 *(Neuromyotonia)*. Mc-Graw-Hill: New York, USA, 2004.
438 38) Browne DL, Gancher ST, Nutt JG, *et al*: Episodic ataxia/myokymia syndrome
439 associated with point mutations in the human potassium channel gene, KCNA1. *Nat*
440 *Genet* 1994; **8**: 136-140.
441 39) Wuttke TV, Jurkat-Rott K, Paulus W, Garncarek M, Lehmann-Horn F, Lerche H:
442 Peripheral nerve hyperexcitability due to dominant-negative KCNQ2 mutations.
443 *Neurology* 2007; **69**: 2045-2053.
444 40) Irani SR, Alexander S, Waters P, *et al*: Antibodies to Kv1 potassium channel-
445 complex proteins leucine-rich, glioma inactivated 1 protein and contactin associated
446 protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia.
447 *Brain* 2010; **133**: 2734-2748.
448 41) Kleipa KA, Elman LB, Lang B, Vincent A, Scherer SS: Neuromyotonia and limbic
449 encephalitis sera target mature Shaker-type K^+ channels: subunit specificity correlates
450 with clinical manifestations. *Brain* 2006; **129**: 1570-1584.
451 42) Odabasi Z, Joy JL, Claussen GC, Herrera GA, Oh SJ: Isaac's syndrome associated
452 with chronic inflammatory demyelinating polyneuropathy. *Muscle Nerve* 1996; **19**: 210-

215.

43) Cross JH, Arora C, Heckemann RA, *et al*: Neurological features of epilepsy, ataxia, sensorineural deafness, tubulopathy syndrome. *Dev Med Child Neurol* 2013; **55**: 846-856.

44) Dewey CW, da Costa RC (eds). *Practical guide to canine and feline neurology*, 3rd edn. Wiley Blackwell: Iowa, USA, 2016.

45) Scholl UI, Dave HB, Lu M, *et al*: SeSAME/EAST syndrome – phenotypic variability and delayed activity of the distal convoluted tubule. *Pediatr Nephrol* 2012; **27**: 2081-2090.

46) Neusch C, Rozengurt N, Jacobs RE, Lester HA, Kofuji P: Kir4.1 potassium channel subunit is crucial for oligodendrocyte development and i*n vivo* myelination. *J Neurosci* 2001; **21**: 5429-5438.

47) Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD: Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. *J Neurosci* 2007; **27**: 11354-11365.

48) Mahmood F, Mozere M, Zdebik AA, *et al*: Generation and validation of a zebrafish model of EAST (epilepsy, ataxia, sensorineural deafness and tubulopathy) syndrome. *Dis Model Mech* 2013; **6**: 652-660.

49) Patterson EE. Canine epilepsy: an underutilized model. *ILAR J* 2014; **55**: 182-186.

50) Trager EH, Khanna R, Marrs A *et al*: Madeline 2.0 PDE: a new program for local and web-based pedigree drawing. *Bioinformatics* 2007; **23**: 1854-1856.

TITLES AND LEGENDS TO FIGURES

Figure 1. A predicted model of the KCNJ10 monomeric subunit (adapted from Reichold *et al*[36]), showing described SeSAME/EAST syndrome causing variants in human (in grey)[4] and dogs (in black)[6]. The KCNJ10 c.986T>C (p.(Leu329Pro)) variant described here is indicated with an asterisk.

Figure 2. Sample pedigree of the 2 families (family 1 at the left and family 2 at the right) drawn with Madeline 2.0 PDE[50]. Squares are males and circles females. The white shaded icon represents a not blood-sampled dog, grey were healthy dogs and black affected dogs. Strikethrough icons represent deceased animals. The KCNJ10 c.986T>C genotype is shown underneath the icon.

Figure 3. EMG: neuromyotonic discharges in a muscle clinically affected with myokymia. Multiple waveforms consisting of trains of 2 to 6 motor unit action potentials.

Figure 4. BAER potentials of an affected (A) and a control (C) dog (1 ms/division): loss of waves III/IV and V, prolonged latencies of all waves and prolonged interpeak latencies.

SUPPLEMENTARY INFORMATION

Supplementary Information file S1 (*.pdf)

A. Experimental details on the genetic analysis.

B. Coding and protein sequence of canine KCNJ10. The c.986T>C (p.(Leu329Pro)) variation is shown in red.

C. KCNJ10 protein BLAST alignment between human (Acc. No. NP_002232.2) and dog (Acc. No. XP_545752.3).

D. Chromatogram of the region containing the c.986T>C (p.(Leu329Pro)) variation (in red) in a (1) homozygous wild type, (2) heterozygous and (3) homozygous mutant animal.

Supplementary Information file S2 (*.mpg4)

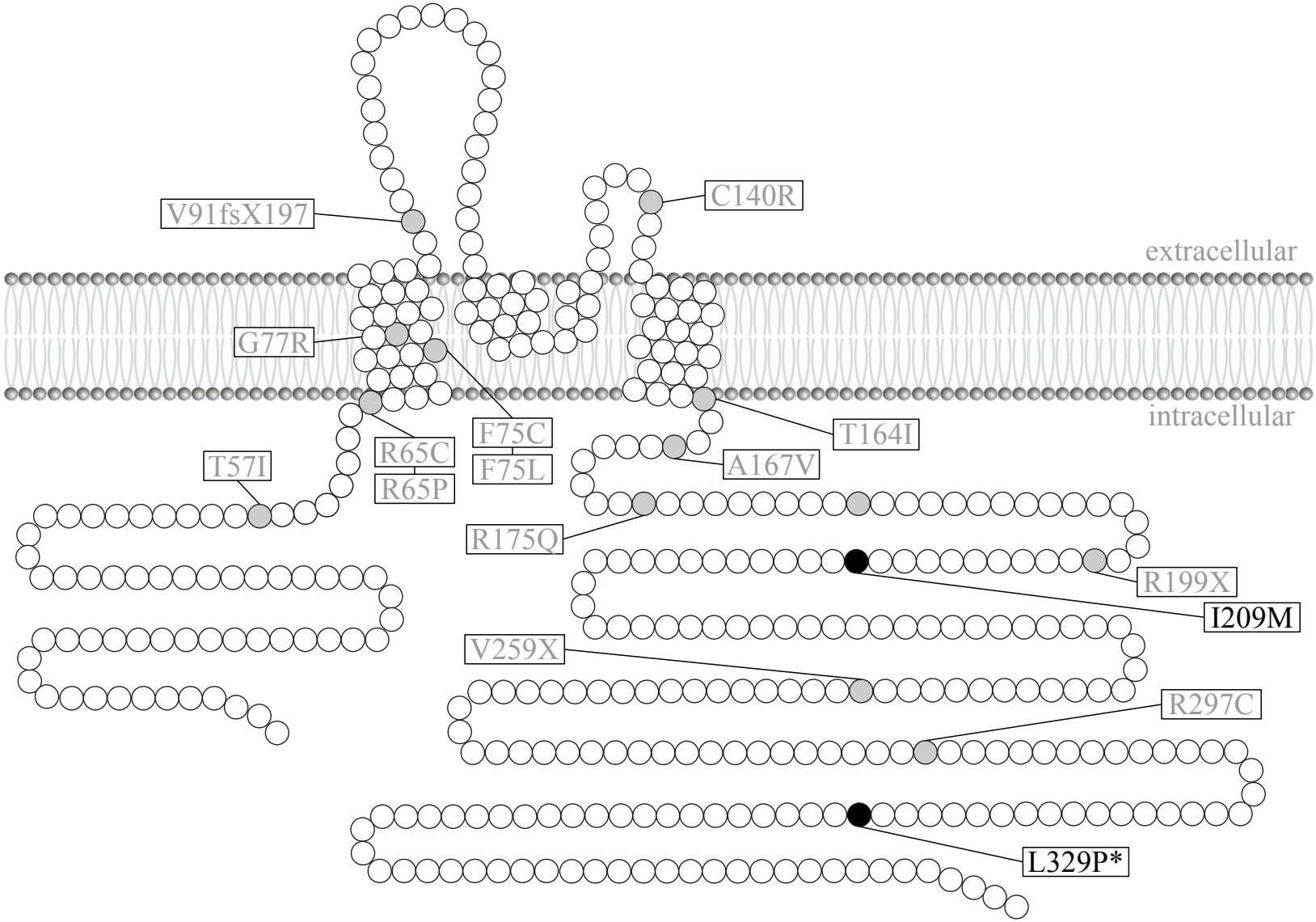
A. Video showing the severe generalized hypermetric ataxia on presentation in dog 2.

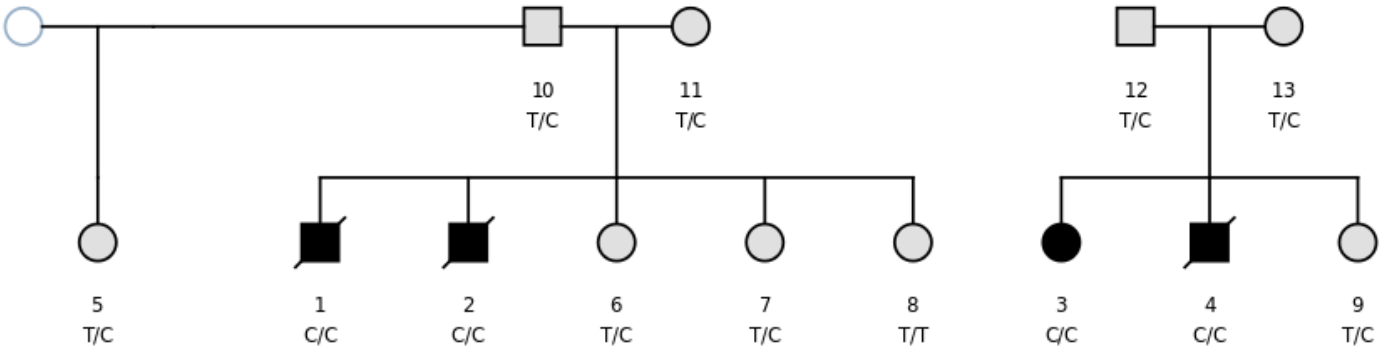
B. Video showing involuntary vermicular muscle contractions triggered by excitement in dog 1.

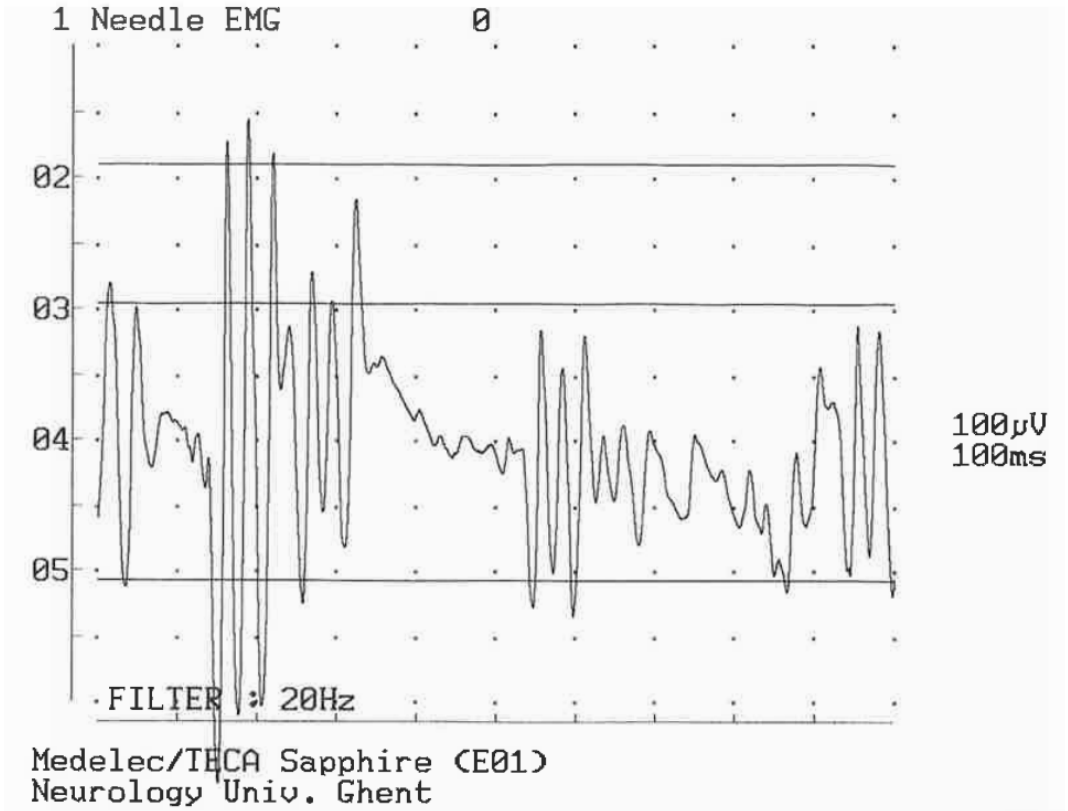
C. Video showing the evolution of the severe generalized hypermetric ataxia in dog 2, 2 months after presentation.

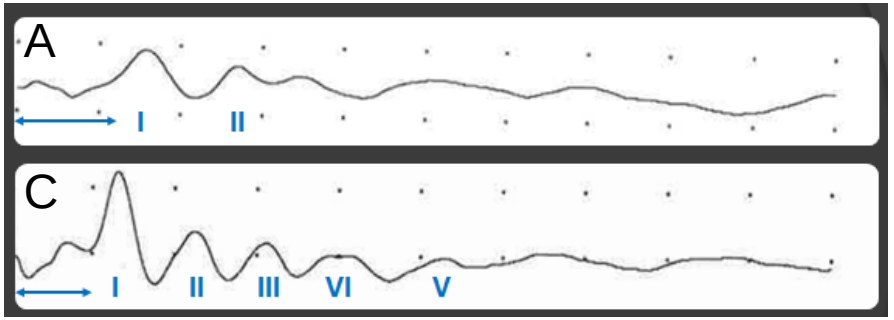
Supplementary Information file S3 (*.pdf)

Spinal cord at the level of C1, close-up of the ventral horn and ventral funiculus, hematoxylin-eosin staining. The myelin sheaths of the ventral funiculus are diffusely vacuolized.


V91fsX197
C140R
extracellular
G77R
intracellular
F75C
F75L
R65C
R65P
T164I
T57I
A167V
R175Q
R199X
I209M
V259X
R297C
L329P*


10
T/C
11
T/C
12
T/C
13
T/C
5
T/C
1
C/C
2
C/C
6
T/C
7
T/C
8
T/T
3
C/C
4
C/C
9
T/C


1 Needle EMG
0
02
03
04
05
100µV
100ms
FILTER : 20Hz
Medelec/TECA Sapphire (E01)
Neurology Univ. Ghent


A
I
II
C
I
II
III
VI
V