A sheep Genetic Investigation with human medical benefits

- B Strugnell, SBRT 13.11.22

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Introduction

- **13th June 2017**: Swaledale lambs gathered for the first time since turnout for ovivac P, tagging, worming etc.
- Had been on some allotments with their mothers adjacent to the fell
- Some lambs noticed with mild to moderate hindlimb ataxia and mild tremor.
- Submitted for further work up

Flock Background

- This was a 350 Ewe Swaledale Flock in Co. Durham, over 300 acres of fell and grassland. Small flock of BF Leicester ewes to breed own tups. No cattle.
- The flock was closed except for tups (Swaledale & BFL).
- The main business was producing **North Country mules** for sale as breeding females.
- 60 or so Swaledale ewes were bred pure every year to maintain the pure flock.
- Different tups go in on the same day. The sheep stay in-bye after tupping until they are scanned in late December. Then they all go onto the fell until the last week in March when they come back into the in-bye fields. They are fed hard feed until they lamb in late April. They are not given any treatments before turnout onto the fell (No copper bolus). They are on the Heptavac system. They lamb outside. Lambing % for the pure Swaledale shearling this year (2017) was 188% and 206% for the ones bred to the Leicester.

What to do?

- All lambs blood sampled
- One (wether) lamb euthanased and post mortemed. No gross findings (including brain and spinal cord)
- The rest of them sent home for close monitoring

Progression of Disease

- Of the 10 or so lambs which had initially presented with moderate ataxia, all progressed downhill pretty rapidly.
- One died on 19th June 2017 and was necropsied the next day
- One was euthanased showing severe tremor and paddling on 27th June 2017
- The others were lost, presumed dead, wandered into a ditch, rushes etc.
- Three more were euthansed and necropsied on 28th June 2017
- So: In all , samples were collected from **5** affected lambs, from a total of 15 lambs (from a crop of 100)

Lambs progressed quite rapidly

Differential Diagnoses

- Delayed Swayback
- Border Disease
- Lead Toxicity
- White Muscle Disease
- Ryegrass Staggers

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What to do?

- Bloods from all lambs and fixed brain, spinal cord and visceral organs from necropsied lamb submitted to SAC Edinburgh

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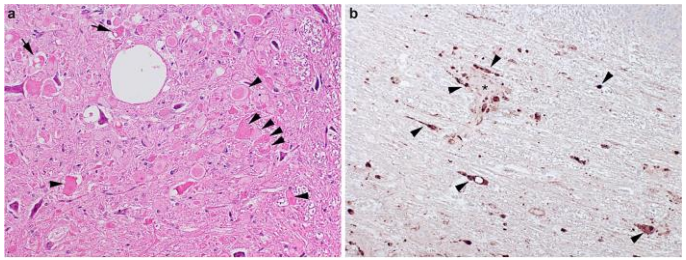
Results



- Brain (cerebrum, striatum, hippocampus, thalamus, midbrain, cerebellum, medulla).
  Widespread spheroid formation is widespread in brainstem nuclei, involving particularly relay nuclei, including accessory cuneate, olives, rostral colliculus, lateral geniculate body, caudal colliculus, medial geniculate body, and oculomotor nuclei, variably associated with gliosis. Purkinje proximal axonal spheroid (torpedo) formation is widespread together with variable smaller spheroid formation in internal granule cell layer, spheroid formation in dendritic tree and occasional Purkinje neuronal degeneration/loss with gliosis in the Bergmann layer.
  Spinal cord (C3, C7, T7, lumbosacral intumescence/L5).
  Spheroid formation and associated change are most prominent in intermediate grey matter at segment T7.
- Comment.
  This lamb has severe **neuroaxonal dystrophy (NAD)** which is consistent with an intrinsic (genetic) defect. Antioxidant deficiency has been implicated in some presentations of NAD mainly in horses on dirt paddocks, however a genetic component is still suspected. These lesions are different from delayed swayback and copper deficiency is not a differential. Further to follow.

1 Swaledale Tup

Leicester Tups

Replacement Swaledales

50 Shearlings

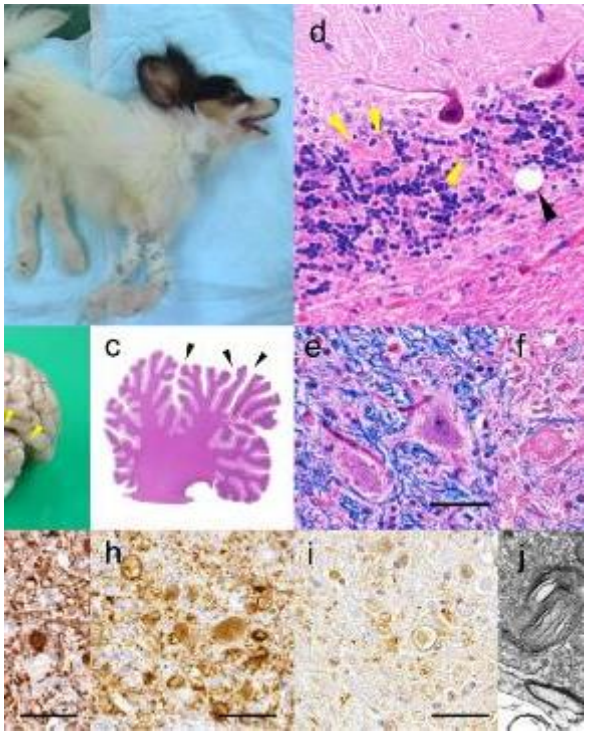
300 2+ crop Ewes

Replacement Swaledales

Mules for Sale

Neuroaxonal Dystrophy

- **Histopathology:**
- Widespread spheroid formation is widespread in brainstem nuclei, involving particularly relay nuclei, including accessory cuneate, olives, rostral colliculus, lateral geniculate body, caudal colliculus, medial geniculate body, and oculomotor nuclei, variably associated with gliosis. Purkinje proximal axonal spheroid (torpedo) formation is widespread together with variable smaller spheroid formation in internal granule cell layer, spheroid formation in dendritic tree and occasional Purkinje neuronal degeneration/loss with gliosis in the Bergmann layer.
- Diagnosis: **Neuroaxonal Dystrophy**
- Pathogenesis: Usually a mutation in PLKA2G6 gene (codes for a phospholipase involved in membrane phospholipids).
- Heterologous but usually autosomal recessive defect. Reported in humans, Merinos, Suffolks, Zwartbles, and various dog breeds (Paillon, Chihuahua)
- Clinical presentation is variable (depends on mutation) but always progressive and fatal



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NAD in Sheep

- Kessell, AE, Finnnie, JW, Blumbergs, PC, Manavis, J, Jerrett, IV (2012) Neuroaxonal Dystrophy in Australian Merino lambs. Journal of Comparative Pathology 147 (1) 62-72

- Cordy, DR, Richards, WP, Bradford, GE (1967) Systemic neuroaxonal dystrophy in Suffolk Sheep. Acta Neuropathologica 8(2) 133-40

- Nuttall, W.O. (1987) Ovine neuroaxonal dystrophy in New Zealand. New Zealand Veterinary Journal 36 (1) 5-7 (Coopworth Sheep)
- APHA Emerging Threats Quarterly Report Q3 2015: NAD in Zwartbles. Older report / memory of NAD in Swaledale sheep difficult to trace

Next Steps

- On 28th June 2017, we went out to the farm and blood samples ALL 58 shearling Swaledales which had gone to the Swaledale tup.
- We also blood sampled 13 unaffected Swaledale lambs and the tup.
- It was not possible to identify unaffected siblings to affected lambs, so some normal lambs were sampled; their parentage and relationship to affected lambs would be established later.
- Bloods were sent to Cord Droegmuller at Berne University via SAC Edinburgh for genetic analysis

Possible Genetic Explanation 1: Mosaic?

- Pure germline mosaicism refers to mosaicism found exclusively in the gametes and not in any somatic cells. Germline mosaicism can be caused either by **a mutation that occurs after conception**, or by epigenetic regulation, alterations to DNA such as methylation that do not involve changes in the DNA coding sequence.

Agerholm *et al. BMC Veterinary Research* (2016) 12:100
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BMC Veterinary Research

RESEARCH ARTICLE Open Access

Lethal chondrodysplasia in a family of Holstein cattle is associated with a *de novo* splice site variant of *COL2A1*

Jørgen S. Agerholm[1*], Fiona Menzi[2], Fintan J. McEvoy[3], Vidhya Jagannathan[2] and Cord Drögemüller[2]

Abstract

Background: Lethal chondrodysplasia (bulldog syndrome) is a well-known congenital syndrome in cattle and occurs sporadically in many breeds. In 2015, it was noticed that about 12 % of the offspring of the phenotypically normal Danish Holstein sire VH Cadiz Captivo showed chondrodysplasia resembling previously reported bulldog calves. Pedigree analysis of affected calves did not display obvious inbreeding to a common ancestor, suggesting the causative allele was not a rare recess
with incomplete penetrance or a mosaic

Results: Three malformed calves were ex
scanning. These calves were morphologic
body weight. The syndrome was characte
the spine and the long bones of the limb
small irregular diaphyses and enlarged ep
The sire and a total of four affected half-si
map the defect in the genome. Significan
including chromosome 5 where whole ge
32473300 G > A). This private sequence va
sequence GT at the 5'-end of *COL2A1* intr
allele in heterozygous state and all five da
a gonadal and somatic mosaic as assesse
blood and 15 % in semen.

Conclusions: The phenotypic and gene
mutation underlying lethal chondrodysp
identified independent spontaneous sp
have occurred during the early foetal de
COL2A1 splice site variant as candidate
mosaicism is a relatively frequent mechan
fraction of affected offspring. Paternal dor
ratio of defective offspring may be very h

Keywords: Congenital, Malformation, Rar

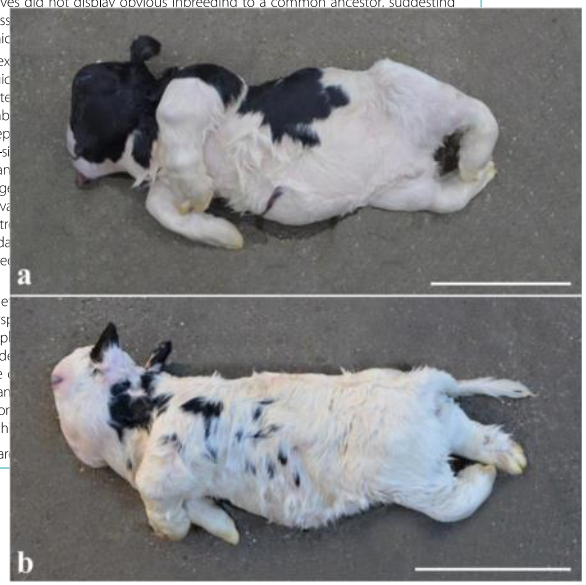
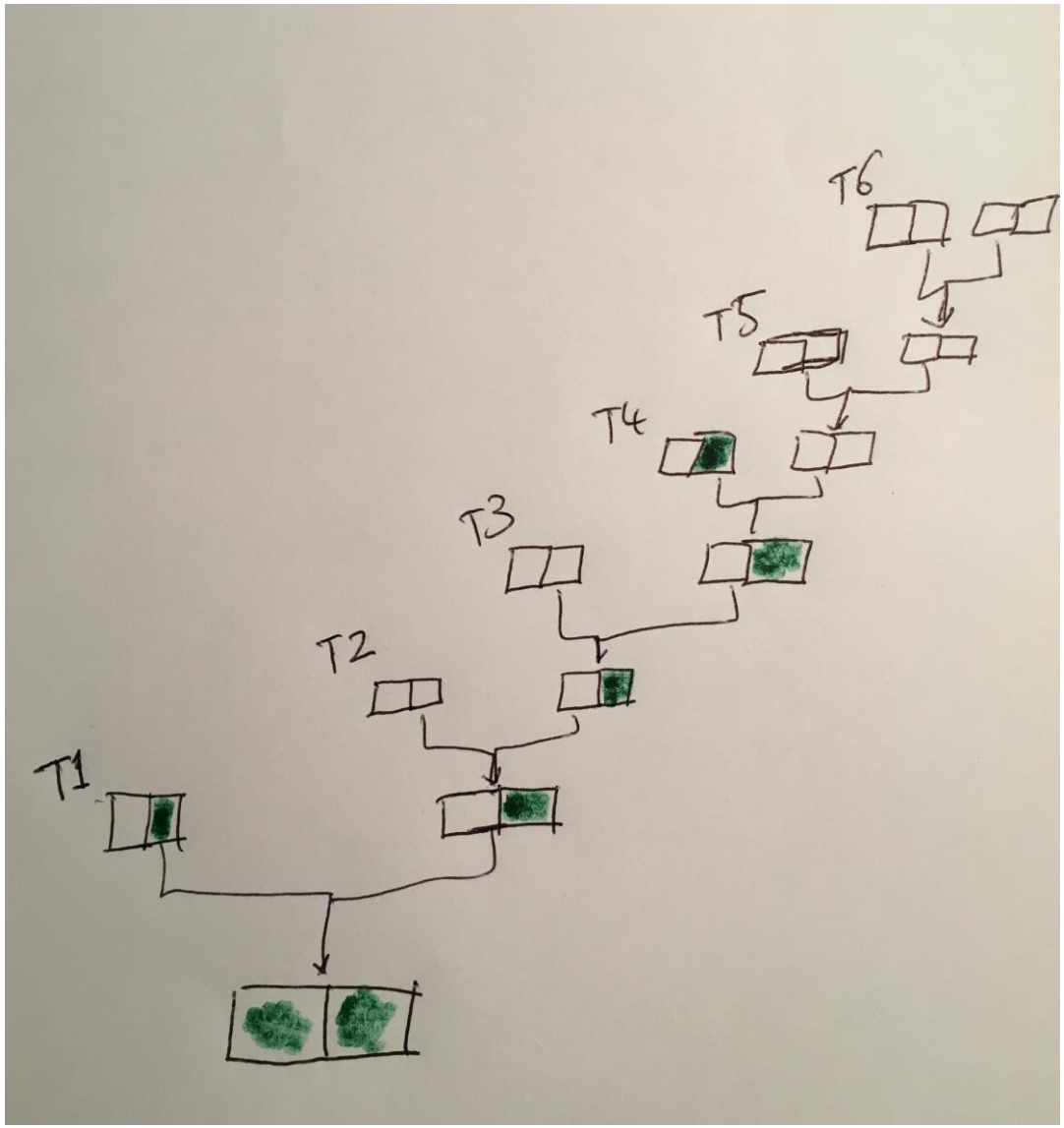


Fig. 1 Gross morphology of chondrodysplastic calves. Notice the severe disproportionate dwarfism with short and compressed body and limbs and severe dysplasia of the facial bones. **a** case 1, **b** case 2. Bar = 30 cm

Possible Genetic Explanation 2: Autosomal Recessive?



Results of Berne Testing: SNP Analysis (2019)

- **1. SNP (Single Nucleotide Polymorphism) Analysis**
- The ram was confirmed as the sire of all affected lambs. Maternity status was established for the 5 affected lambs. This enabled construction of 2 complete parent-offspring trios for Whole Genome Sequencing (WGS).
- SNP analysis also confirmed that all sampled Swaledale dams had a common sire (the one before this one).
- They tried autozygosity mapping, assuming an autosomal recessive mode of inheritance. This is a screen for homozygous alleles. However, there were no intervals of extended homogygous regions with alleles shared by all five NAD-affected lambs

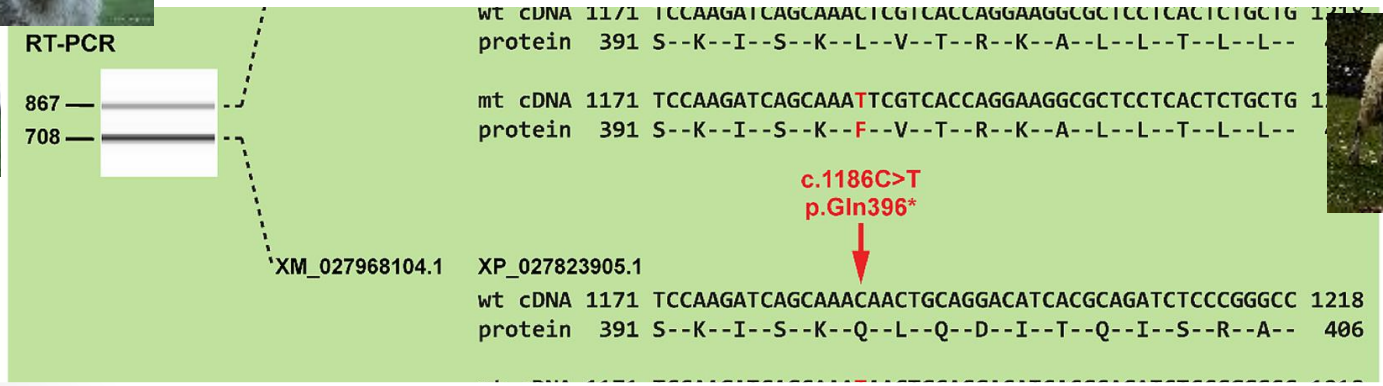
Results of Berne Testing: Whole-Genome Sequence Analysis (WGS)

- WGS were obtained from the two parent-sibling trios (5 animals in all)
- These WGSs were compared to archived WGSs from a total of 453 genomes from 54 breeds.
- This identified 4 possible candidate gene mutations:

1. Chromosome 15: Homogygous in affected lambs but heterozygous in parents. But uncharacterized gene
2. HERC1 gene (chromosome 7). Associated with macrocephaly & psychomotor retardation in humans. But not on PLA2G6 gene
3. Chromosome 3: Two mutations noted in the PLA2G6 gene: **intron 2 (c.336-2A.G)** and **exon 8 (c.1312C>T)**. All affected lambs had one of each mutated copy.

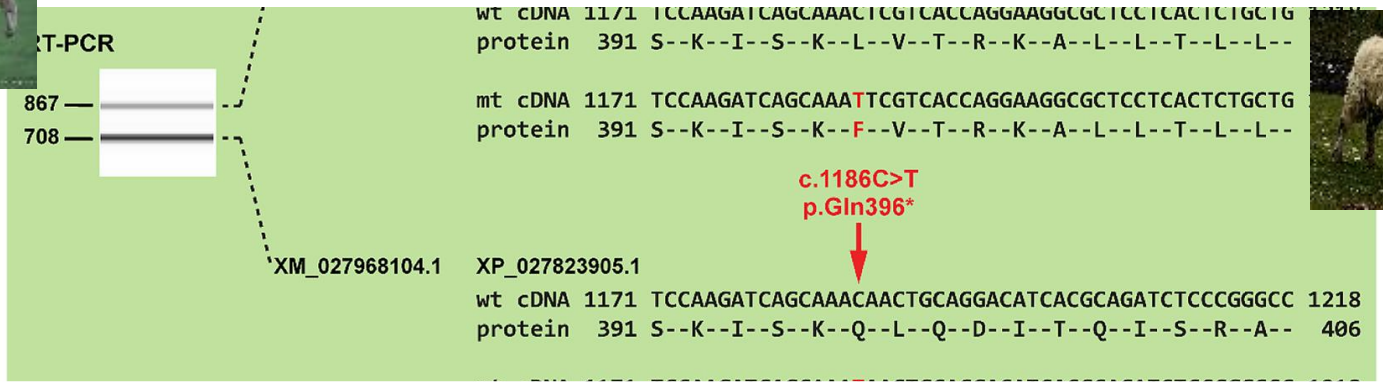
Results of Berne Testing: Mode of Inheritance

- Genotyping of the parents revealed that the **SIRE** was a heterozygous carrier of the **exon 8 (c.1312C>T).** mutation
- The **dams** of all affected lambs were heterozygous carriers of the **intron 2 (c.336-2A.G)** mutation
- Of the 13 clinically normal lambs, 3 were homozygous wild type, 7 were heterozygous for the **exon 8 (c.1312C>T)** and 3 were heterozygous for the **intron 2 (c.336-2A.G)** mutation.
- Of the 52 ewes sampled, 25 were homogygous wild type and 27 were heterozygous carriers of the **intron 2 (c.336-2A.G)** mutation
- The sire of the dams (the previous Swaledale tup) was assumed to be a heterozygous carrier of the **intron 2 (c.336-2A.G)** mutation, but was not available for sampling



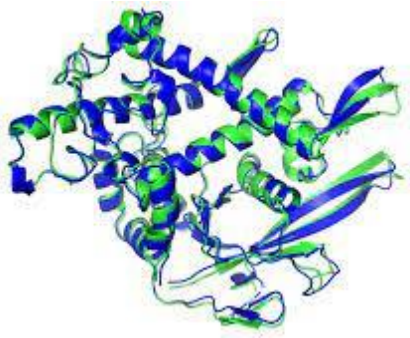
Compound Heterozygous Inheritance

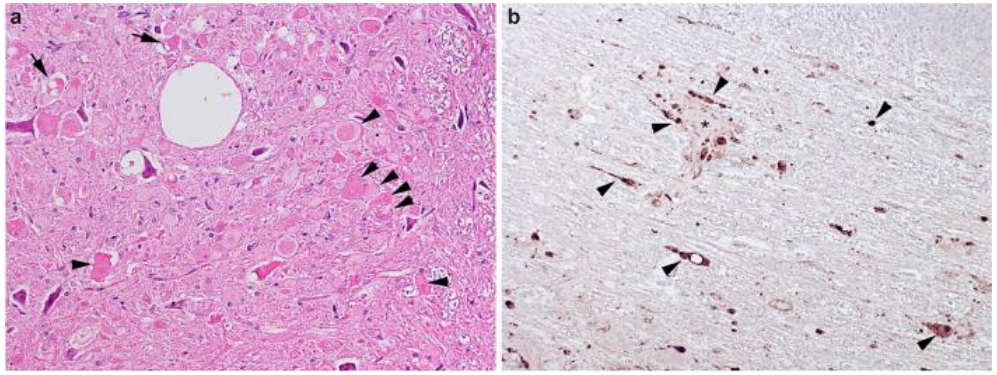
- **The presence of two different mutated alleles at a particular gene locus.**



Functional confirmation of PLA2G6 variants

- At post mortem examination, samples of spinal cord, sciatic nerve, and cerebellum from 3 lambs had been collected and put into RNeasy reagent
- mRNA was extracted from these tissues and reverse transcriptase used to create cDNA using primers based on predicted sequences around mutations.
- Intron 2 Variant (splice-site mutation): The PLAG26 protein was predicted to be significantly truncated (72 amino acids) – lacking more than 90% of the original protein
- Exon 8 variant (stop codon): exchange of a leucine for a phenylalanine and a 50% protein truncation





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ORIGINAL ARTICLE

Compound heterozygous *PLA2G6* loss-of-function variants in Swaledale sheep with neuroaxonal dystrophy

Anna Letko[1] · Ben Strugnell[2] · Irene M. Häfliger[1] · Julia M. Paris[1] · Katie Waine[3] · Cord Drögemüller[1] · Sandra Scholes[4]

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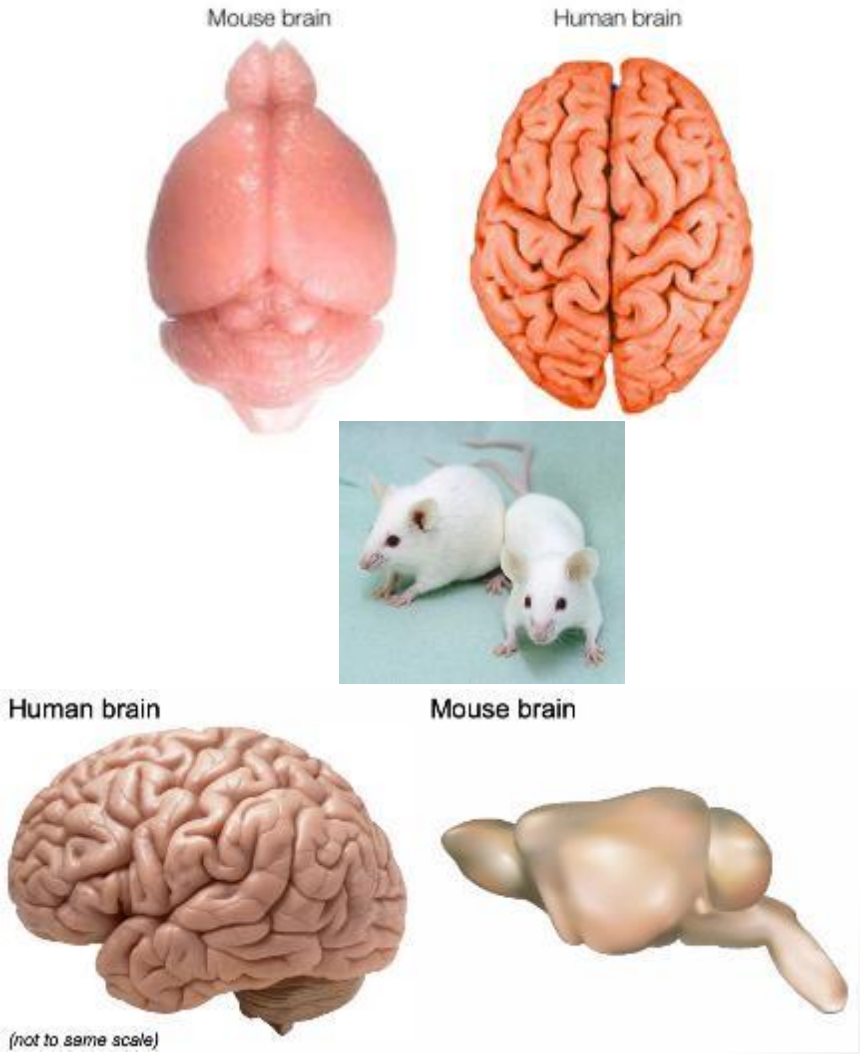
Abstract

Sporadic occurrences of neurodegenerative disorders including neuroaxonal dystrophy (NAD) have been previously reported in sheep. However, so far no causative genetic variant has been found for ovine NAD. The aim of this study was to characterize the phenotype and the genetic aetiology of an early-onset neurodegenerative disorder observed in several lambs of purebred Swaledale sheep, a native English breed. Affected lambs showed progressive ataxia and stiff gait and subsequent histopathological analysis revealed the widespread presence of axonal spheroid indicating neuronal degeneration. Thus, the observed clinical phenotype could be explained by a novel form of NAD. After SNP genotyping and subsequent linkage mapping within a paternal half-sib pedigree with a total of five NAD-affected lambs, we identi
by whole-genome sequencing in the ovine PLA2G6 gene situated in a NAD-linked geno
cases were carriers of a compound heterozygous splice site variant in intron 2 and a nons
present evidence for the occurrence of a familial novel form of recessively inherited NAD
neity at *PLA2G6*. This study reports two pathogenic variants in *PLA2G6* causing a novel
which enables selection against this fatal disorder.

Then in January 2021..

- We were contacted by Prof. Ahad Rahim, Professor of Translational Neuroscience at University College London (UCL).
- He was working on iNAD in children with colleagues at Great Ormond St and elsewhere. They had developed an experimental mouse model but were having problems because of the lissencephalic nature of mouse brains
- He wanted to develop a gyrencephalic animal experimental model, to trial gene therapy, metabolome, imaging
- He wondered if the flock was still there, genes still present and would they sell him some heterogygous carriers.



Were there any heterozygous Females left?

- Flock had been partly dispersed in 2019 but some original females left
- Farmer was needing to screen flock for carriers as didn't want to sell carrier males or females (so this work was for *diagnostic* purposes)
- Feb 2021: Blood sampled 116 sheep: **27** female carriers of the **intron 2 (c.336-2A.G** and **7** female carriers of the **exon 8 (c.1312C>T)** mutation
- The new Swaledale tup was homozygous recessive for the wild type gene.

Did they produce any heterozygous males?

- We blood sampled the male offspring of the carrier ewes of both genes (35 ewes in total).
- From 30 male lambs sampled, **8** were carriers of the **intron 2 (c.336-2A.G** and **3** were carriers of the **exon 8 (c.1312C>T**) mutation
- Ahad's team has bought these tups and semen will be collected and stored later in the year
- They may also buy carrier cull ewes for some embryo transfer and storage work.
- **BUT……**

Veterinary Surgeons Act/ ASPA?

- ..We have now reached the limits of the Veterinary Surgeon's Act as all future work will not be categorisable as 'for diagnostic purposes.'
- It will therefore have to be under Home Office Licence (person, place, project) and a specific project licence is being applied for by Roslin and AB Europe.

Future Plans

- Semen will be collected in November/ December 2021 at AB Europe, under HO licence.
- Hopefully it will also be possible to buy/ borrow some carrier females, to superovulate, and thus to store embryos
- These could be homozygous wild type, homogygous for either mutant (if so, likely clinically affected) or heterozygous for both mutants.
- These can be stored long-term and implanted into recipients if and when funding secured
- If not enough semen (tups too immature), can keep them until November 2022.
- Considered epididymal harvest (no HO licence needed) but:
  1. A bit late if not enough semen
  2. Embryos better under Refine Reduce Replace lab animal guidelines

Conclusions

- NAD was confirmed histologically in 6-10 week old Swaledale lambs.
- Disease progression was rapid and we needed to act fast before they all died off
- A genetic cause was strongly suspected from the circumstantial evidence and a composite heterozygous mode of inheritance identified.
- Sheep from this flock could provide a useful animal model for human disease for neurodegenerative diseases
- Material collected from FSCCs, with appropriate escalation up the chain, can contribute to endemic disease surveillance for New and Emerging Disease, and One Health principles to progress human health.

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- The Farmer
- Ahad Rahim and his team at UCL

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Discussion