

Alzheimer's disease markers in the aged sheep (*Ovis aries*)

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Abstract

This study reports the identification and characterisation of markers of Alzheimer's disease (AD) in aged sheep (*Ovis aries*) as a preliminary step towards making a genetically modified large animal model of AD. Importantly, the sequences of key proteins involved in AD pathogenesis are highly conserved between sheep and human. The processing of the amyloid- β ($A\beta$) protein is conserved between sheep and human, and sheep $A\beta_{1-42}/A\beta_{1-40}$ ratios in cerebrospinal fluid (CSF) are also very similar to human. In addition, total-tau and neurofilament light levels in CSF are comparable to those found in human. The presence of neurofibrillary tangles in aged sheep brain has previously been established; here we report for the first time that plaques, the other pathological hallmark of AD, are also present in the aged sheep brain. In summary, the biological machinery to generate the key neuropathological features of AD is conserved between the human and sheep, making the sheep a good candidate for future genetic manipulation to accelerate the condition for use in pathophysiological discovery and therapeutic testing.

Keywords: Alzheimer's Disease, Amyloid- β , Sheep *Ovis aries*, Plaques, Tangles, Animal model.

1. Introduction

Dementia is the most common neurological condition among older adults, with the majority of cases being attributable to Alzheimer's disease (AD). AD is a devastating neurodegenerative disease that causes progressive memory loss, cognitive decline and finally dementia, leading to premature death and causing considerable stress to families. The prevalence of AD is expected to triple by 2050 due to an aging population (Alzheimers Ass. 2016, Alzheimers 2012) and therefore effective treatments for the disease are desperately needed. Mutations in three genes; amyloid precursor protein (*APP*), and presenilin 1 and 2 (*PSEN1* and *PSEN2*) (Levy-Lahad et al., 1995, Levy et al., 1990, Sherrington et al., 1995) are known to cause relatively rare (<1%) familial AD. Each of these mutations result in the enhanced production of, or imbalances favouring, the amyloidogenic 42 amino acid long amyloid- β peptide ($A\beta_{42}$) form of the APP protein. The risk of developing sporadic or late onset AD (LOAD) has been associated with variations in several genes including apolipoprotein E (*APOE*) (Harold et al., 2009, Saunders et al., 1993, Schellenberg and Montine 2012). These genes are also functionally linked to $A\beta$ peptide homeostasis, supporting the 'amyloid cascade hypothesis' as an initiating mechanism for AD pathogenesis (Hardy and Higgins 1992).

Due to the difficulty of making a diagnosis of AD in the earlier phases of the disease, patients recruited for clinical trials have typically been in the mild to moderate dementia stages of the disease (Blennow 2010). However it is generally agreed that the most effective treatment window would be early and ideally pre-symptomatic (Mangialasche et al., 2010). Cerebrospinal fluid (CSF) biomarkers are increasingly being used in the diagnosis of AD and also in the mild cognitive impairment (MCI) phase of AD (Blennow et al., 2010). These

biomarkers are also central in the recent research criteria for AD (Dubois et al., 2014) and preclinical AD (Dubois et al., 2016). Late-onset neurodegenerative diseases such as AD are difficult to model accurately in rodents because of their short lifespans. The commonly used rodent models of AD have been engineered to exhibit rapid and unnatural disease progression (Sabbagh et al., 2013), limiting their applications for early-stage disease research. Indeed, while several compounds have been beneficial in mouse models of AD, translation to humans has been very disappointing (Blennow K 2006, Dragunow 2008, McGonigle 2014). Successfully translated compounds have been those providing symptomatic relief rather than halting disease progression (McGonigle 2014).

To enable safer, more effective clinical trials, and to discover the early pathogenic mechanisms of AD we believe there is a need for a large animal model of AD, with a complex brain structure (including a more developed cortex with gyri and sulci) and longevity, which will accurately capture the disease as it progresses, including its pre-symptomatic phase. Dogs and non-human primates have been used as models of aging and show relevant AD pathology, as recently reviewed (Youssef et al., 2016); however these models are expensive and fraught with ethical issues. A transgenic AD minipig has been produced by random integration of mutant human APP into the minipig genome, driven by the PDGF β promoter to give high levels of expression (Kragh et al., 2009, Sondergaard et al., 2012). Minipigs are housed individually or in small groups, making long-term pre-clinical trials relatively expensive.

We see value in modelling AD in sheep (*Ovis aries*) due to the similarity of its brain structure and size relative to human. Sheep can live for at least 10 years, making them ideal for the study of later-onset diseases such as AD. Importantly, studies have shown AD-associated

neurofibrillary accumulation (tau pathology) in normal aged sheep (Braak et al., 1994, Nelson and Saper 1995); a feature which is absent in wild type rodents and has made AD modelling challenging in rats and mice (Hardy and Selkoe 2002). While the rate of naturally occurring dementia in sheep is unknown (as most farmed sheep are culled before reaching old age), sheep with cognitive deficits are studied due to natural mutations in genes causing Batters Disease in humans (Cook et al., 2002, Jolly et al., 1980, Weber and Pearce 2013) Sheep are readily trainable for use in tests of cognitive function (Morton and Avanzo 2011) and sheep suffering from a progressive neurological disease can be quantified longitudinally using modern methods, such as EEG (Perentos et al., 2015) and MRI (Sawiak et al., 2015). Sheep have face recognition systems for remembering specific individuals long term comparable to human (Kendrick et al., 2001). Furthermore, sheep can be kept in large numbers in a social environment on a farm, which is ethically more acceptable and cheaper than caged large laboratory animals. Genetically modified flocks can also be expanded relatively quickly from a few founder animals due to the JIVET reproductive technology that has been developed specifically in sheep (Kelly et al., 2005). A transgenic sheep model of the neurodegenerative disorder, Huntington's Disease, has been successfully established by our laboratory in this manner (Jacobsen et al., 2010) and is proving to be a valuable tool in HD research (Handley et al., 2016, Morton et al., 2014, Reid et al., 2013). The sheep genome has now been published and annotated (Jiang et al., 2014), and thus the genome of the sheep can now be precisely manipulated for human disease research.

In this report, we present data on the suitability of sheep as a model for AD. We compare the human and sheep peptide sequences for relevant AD proteins and peptides, and compare types and levels of common AD biomarkers in CSF that will be relevant for tracking

disease progression. We also looked for evidence of plaques and tangles, the hallmarks of human AD, in the aged sheep brain.

2. Methods

2.1. Human and sheep DNA sequence alignments

Key human AD-associated reference protein sequences (as at 26th July 2016) were used in BLAST analysis (utilising the blastp algorithm) against all *Ovis aries* protein sequences.

Where proteins have multiple isoforms, the longest recorded isoform for human was used.

For the APP protein, cleavage sites were compared, as well as the amino acid sequence of the A β ₁₋₄₂ fragment. The sheep protein with the highest homology against each human sequence is presented in Table 1.

2.2. Collection of tissue and CSF samples

Samples were obtained from four eight-year-old sheep, and a single fourteen-year-old sheep that were being euthanized for normal animal management reasons. Animals were humanely euthanized followed by immediate exsanguination. CSF samples were immediately collected from cisterna magna of each of the eight year old sheep, aliquoted and frozen. After extraction from the skull, the brains from all five sheep were blocked into five coronal blocks, split into two hemispheres and immersion fixed for 48 hours in 10–20 volumes of freshly prepared 10% buffered (0.1M phosphate buffer pH 7.4) formalin (Scharlau, Spain) at 4°C and then transferred to 0.1M phosphate buffer plus 0.1% sodium azide (Sigma, Australia). Fixed blocks were then cryopreserved for immunohistochemical analysis using sequential sucrose immersion

followed by freezing in dry ice powder prior to storage at -80°C as previously described (Waldvogel et al., 2007).

2.3. Fluid biomarkers in sheep CSF

2.3.1. Hybrid immunoaffinity-mass spectrometry for A β

To identify A β fragments, we used CSF taken from each of the eight year old sheep.

Immunoaffinity capture of A β was combined with mass spectrometry (MS) for analysis and study of the A β peptide pattern in sheep CSF as previously described (Portelius et al., 2007).

In brief, the anti-A β antibodies 6E10 and 4G8 were separately coupled to magnetic beads.

After washing of the beads, the 4G8 and 6E10 coated beads were used in combination for immunoprecipitation. After elution, the immune-purified A β peptides were identified using an UltraFLEXtreme matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS instrument (Bruker Daltonics, Bremen, Germany).

2.3.2. Total tau and phospo-tau181 concentrations

Total tau concentration in CSF was measured using using a sandwich enzyme-linked immunosorbent assay (ELISA) (INNOTEST hTAU-Ag; Fujirebio, Ghent, Belgium) that uses antibodies against the mid-domain of tau, thus measuring all tau isoforms irrespective of phosphorylation state. Phospo-tau181 concentration in CSF was measured using a sandwich ELISA (INNOTEST Phospo-tau[181P]; Fujirebio, Ghent, Belgium) that includes one antibody specific for the phosho-epitope at threonine 181 in combination with a mid-

domain anti-tau antibody. Intra-assay coefficients of variation were below 10% and all samples measured within the linear range of the standard curve.

2.3.3. Neurofilament light concentration

Neurofilament light concentration in CSF was measured using the NF-light ELISA as described by the manufacturer (UmanDiagnostics, Umeå, Sweden). The assay uses two monoclonal antibodies directed against the rod domain of the protein. The lower limit of quantification is 50 ng/L with intra-assay coefficients of variation <10%.

2.4 Tangles and plaques in the sheep brain

Coronal brain sections at the level of mid temporal gyrus and hippocampus were cut on a freezing sledge microtome and stored in phosphate buffered saline (PBS) plus 0.1% (w/v) azide (Sigma, Australia) at 4°C until use. For immunohistochemistry, free floating 50µm-thick sections were initially washed in a solution of 50% methanol (Scharlau, Spain) and 1% H₂O₂ to expose binding sites and block endogenous peroxidase activity. Sections were washed in PBST (PBS with 0.2% triton X-100 (BDH, NZ)) prior to incubation in primary antiserum diluted in immunobuffer, (1% Normal Goat Serum in PBS, 0.2% Triton-X100 and 0.4 g Methiolate, (Waldvogel et al., 2007) for 48 hours at 4°C. To visualise tangles, we used a polyclonal rabbit anti-Tau antibody (DAKO #A0024 diluted 1:15,000) generated against the C-terminus of human Tau (amino acids 243-445). Plaques were visualised using a monoclonal mouse anti-Aβ antibody (clone 4G8, Chemicon #MAB1561, diluted 1:1200) raised to amino acids 17-24 of Aβ, with the epitope between aa18-22. Sections were subsequently washed in PBST at room temperature and incubated for 24 hours at room

temperature in the appropriate biotinylated goat anti-mouse or goat anti-rabbit secondary antibody (Sigma, Australia), diluted to 1:1000 in immunobuffer. Subsequently, sections were washed with PBST and incubated for four hours at room temperature in extravidin peroxidase complex (Sigma, E2886, Australia), diluted to 1:1000 in immunobuffer. Sections had a further three 10 minute washes in PBST prior to visualisation. For visualisation, each series of sections was incubated in 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma, Australia) in phosphate buffer with the addition of 0.01% H₂O₂ (BDH, NZ) to visualize the tertiary complex. Subsequently, sections were mounted onto slides with gelatine, dried overnight and then dehydrated in a graded ethanol and xylene series. Sections were cover-slipped with DPX Mountant solution (Merck, Australia) prior to visualization. Negative control sections were processed to determine nonspecific staining using the same immunohistochemical procedures as detailed above, except that the primary or secondary antibodies were omitted from the procedure. Positive human controls were used for each antibody for comparison. Sections were Nissl stained with cresyl violet (Sigma, Australia) according to standard techniques (0.5% w/v), to assist in identifying the plane of cells relative to plaques and tangles, and to assist in identification of cell layers.

Tangles and plaques were visualised on a Leica DC 500 light microscope with Analysis LS Research 2.3 software, and the total number of tangles and plaques in the entire cortical region and hippocampus per slide were counted. The number per location (gyrus) was noted for each sheep, and nissl-stained nuclei enabled assessment with respect to cortical layers. As sample sizes were small and because of some inter-animal variability of the specific hippocampal region sections examined, a relative rather than absolute count was

generated for each animal (marked with a + sign; Table 3). Tangle densities, representative of all 5 sheep, were mapped on a MSU atlas image of the sheep brain (section 1240 from the Michigan State University Sheep Brain Atlas(Johnson et al.,). The hippocampal level seen in this MSU atlas image in Figure 3 differs between the left and right hemispheres, representing the range of sections examined in this study and also reflecting the difficulty in collecting comparative coronal sections from the hippocampal formation.

Thioflavin T staining (Sigma) was undertaken on 20 μ m sections which were mounted, dehydrated and then stained for eight minutes in 0.05% thioflavin solution followed by three washes in PBS solution. After adding coverslips the slides were viewed via fluorescence microscopy under a Leica DC 500 microscope with Analysis LS Research 2.3 software. Human sections were processed in parallel as positive control comparisons.

3. Results

3.1. Homology of Key AD proteins between human and sheep

The amino acid sequences of the key AD-related proteins show high homology between human and sheep, several close to 100% (Table 1). As seen in humans, sheep have multiple isoforms of APP. There were 6 predicted sheep isoforms for APP, ranging from 677 - 770 amino acid residues. There are 23 amino acid differences within the full length APP protein compared, however none of these are close to the C-terminal cleavage sites. The sheep $A\beta_{1-42}$ mRNA region has 6 nucleotide differences when compared to human, but results in a peptide sequence which is 100% homologous. BACE1, the β -cleavage enzyme which contributes to formation of the disease-associated $A\beta_{1-42}$ fragment, is 98.4% homologous between human and sheep. Presenilin 1 and Presenilin 2; components of the gamma-cleavage complex, also show high homology. Ovine NF-L homology to human is 95.3%. A 10 amino acid insertion in the sheep sequence interrupts an otherwise 97.1% homology. The sheep ApoE protein had the lowest homology to humans overall (70%), however it does possess the same amino acids in the key positions (112 and 158) that define the human *APOE* $\epsilon 4$ allele.

3.2. $A\beta$ and Tau levels in sheep CSF

3.2.1. $A\beta$ levels in sheep CSF

All of the main fragments of APP that are found in humans were detectable in sheep CSF, including $A\beta_{1-42}$ (Figure 1). As seen in humans (Figure 1E), the $A\beta_{1-17}$ and $A\beta_{1-40}$ peptides were the most abundant cleavage products. The ratio of $A\beta_{1-42}$ over $A\beta_{1-40}$ averaged at 0.113

for the sheep, which is very similar to the human ratio of $A\beta_{1-42}$ over $A\beta_{1-40}$ (Hansson et al., 2007), indicating the utility of sheep CSF as a tool for following AD progression.

3.2.2. Tau and phospho-tau181 processing in sheep CSF

Levels of total tau were variable within the three sheep CSF samples assessed (Table 2), but were comparable with human samples, and average 319.3 ng/L, which is within the normal range for humans (Sjögren et al., 2001). Levels of phospho-tau181 in sheep CSF were very low, the mean level in normal humans is around 45 ng/L (Vanderstichele et al., 2006), and all three sheep samples were within 15.1 - 15.4 ng/L, which was close to the lower limit of quantification for the assay.

3.2.3. Neurofilament light in sheep CSF

Levels of neurofilament light were between 600 and 1200 ng/L in all sheep samples (Table 2) which is within the normal range seen in humans (Zetterberg et al., 2016).

3.3. Plaques in the sheep brain

$A\beta$ immunopositive plaques were identified in all animals and were visualised as large dense structures many times larger than a single neuron as shown in Figure 2A-C, as well as abundant smaller structures about the size of a single neuronal nucleus (Figure 2A, arrow). Quantification of plaques was limited to large dense structures and excluded the smaller structures which were very numerous, particularly in supragranular cortical layers. Plaques were found in both supragranular and infragranular layers within each of the sheep and in all cortical regions, including the hippocampus. The plaques observed within each sheep

were present at similar densities in all cortical areas, and the average number of plaques per slide in each sheep ranged between 60 and 109, equating to an average of 24-44 plaques per cm² of cortical tissue. Most of the plaques were diffuse, although a few resembled the dense-core plaques seen in the positive human control. Thioflavin-T stained structures were seen in all of the sheep (Figure 2D), although at considerably lower abundance than of those seen with immunostaining. The human positive control sections contained large numbers (>500) immuno-stained plaques, with adjacent sections revealing many (>100) thioflavin positive structures similar to those seen in the sheep.

3.4. Tangles in the sheep brain

Tangles were identified in fixed brain tissue from the five aged sheep using Tau antibodies. A range of tau immunopositive structures were detected in all of the sheep assessed, and included small neuropil threads to the larger classical tangles shown in Figure 3A and B. There was some evidence for dystrophic neurites, based on thickened neurites seen within tau labelled sections (Figure 3C), although as we did not double stain the sections we cannot confirm their association with plaques. The tangle structures were almost exclusively within the upper middle layers (layer II – IV) of the sheep cortex and most abundant in the temporal lobe and entorhinal cortex of all sheep than elsewhere in the section (Figure 3D). Tangle-like structures were not identified in the hippocampus of the fourteen-year-old sheep, but were detected in 3 of the 4 eight-year old sheep. All sheep had tangles within entorhinal cortex. Quantification of tangles was limited to the larger neuronal tangles, excluding neuropil threads, which were very numerous. The average number of tangles per slide for each sheep ranged between 3 and 58.

4. Discussion

The aim of this study was to assess the suitability of sheep for future genetic manipulation to produce a large animal model of AD. We examined the similarities between key human and sheep proteins known to be involved in AD, and measured CSF levels of proteins and peptides that are known to be associated with the disease. Additionally, we report evidence of plaques and tangles; the neuropathological hallmarks of the disease, in the aged sheep brain.

Aged sheep naturally develop the PHF-tau positive tangles associated with AD, as seen in our and previous studies (Braak et al., 1994, Nelson and Saper 1995). Our identification of tangle structures in entorhinal cortex from all animals and within hippocampus of three of the five animals is consistent with the progression of tangles in human brain described in Serono Pozo et al (2011) and Braak and Braak (2006). Levels of CSF total tau in normal sheep are shown here to be comparable to that found in humans. In contrast, phospho-tau181 concentrations were below the limit of quantification in sheep CSF. The amino acid threonine that is phosphorylated at this position is present in the sheep peptide, but the flanking region in the sheep tau amino acid sequence differs from human tau. This sequence divergence may mean that phosphorylation at tau181 cannot take place, or alternatively, that the AT270 antibody used, with a minimal epitope of P₁₇₆PAPKT(p)P₁₈₂ (Vanmechelen et al., 2000), does not react with sheep tau phosphorylated at this position. Either way, the ratio of total tau to phospho-tau181 cannot be used as a biomarker or indicator of disease progression in sheep. However, the human tau protein can potentially be phosphorylated at 19 sites or more (Augustinack et al., 2002), and because tau tangles do form in the sheep

brain, phosphorylation of other sites may be more suitably used to track disease progression, such as phospho-tau231 (Hampel et al., 2010). Neurofilament light levels in human CSF can also potentially be used as a biomarker of AD progression (Zetterberg et al., 2016), and our results show that neurofilament light can be detected and measured in sheep, and is found at levels comparable to that of humans.

The A β fragments detected in CSF from the four eight-year-old sheep show that sheep utilise the same mechanisms for degrading APP and A β peptide as humans, which is a major part of the amyloid cascade hypothesis. Importantly, we have identified that sheep produce the disease associated A β ₁₋₄₂ fragment, suggesting they will be a good model for testing the amyloid cascade hypothesis of AD. The A β ₁₋₄₂ peptide sequence is completely conserved between humans and sheep. Mice and rats show a three amino acid difference in the A β ₁₋₄₂ peptide sequence (Johnstone et al., 1991), suggesting that sheep may make a more comparable model to humans than rodent models. Our results show that the α -, β - and γ -secretase cleavage sites, and processing pathways, in the APP protein are most likely identical between sheep and humans (Portelius et al., 2011). This evidence, taken together, suggests that sheep should be able to form the amyloid plaques that define AD, indeed, here we report for the first time the presence of plaques in sheep brain tissue, detected by A β immunohistochemistry. Although the sample size in this study was limited, the fourteen-year-old sheep showed more plaques than any of the four eight-year-old sheep (Table 3), suggesting that plaques are likely to accumulate in an age-dependant manner in sheep as they do in humans. The relatively low thioflavin staining in sheep compared to human may be due to most of the plaques in sheep being diffuse rather than dense-cored plaques, as it

was the dense centres that showed the strongest thioflavin staining in human positive control sections.

All of the key proteins implicated in human AD are found in sheep and show high homology. Interestingly, the ApoE amino acid sequence in sheep contains two amino acids at the 112 and 158 positions, which correspond with the *APOE* ϵ 4 allele in humans that is known to increase the chances of developing AD.

The evidence presented here suggests that the amyloid pathway has been highly conserved between humans and sheep, and that the pathological mechanisms for human AD are likely also found in sheep. Sheep are not known to develop AD naturally, although this is likely due to their shorter lifespan relative to humans. Most sheep in typical farming conditions are culled once past their useful lifespan for the farmer, and so do not die of age related disorders.

Our findings demonstrate that sheep are a clinically translatable model, displaying the pathological hallmarks of AD, and are thus a good species for consideration of genetic manipulation to generate a large animal model of AD. Because of the conservation of the key constituents of the amyloid generating pathway an approach would be to introduce a mutation through gene editing in APP, PSEN1 or 2 that results in early onset human disease. As the typical AD pathogenic markers are present in aged wild-type sheep our expectation is that a model made in this way without overexpression of transgenes or using multiple mutations will develop early AD changes. The indication that prodromal disease status could be monitored via CSF testing dramatically improves the potential utility of a sheep AD model. Moreover, the ability to keep sheep in large cohorts in standard paddocking

arrangements will enable research and drug testing at lower cost, hopefully bringing the field of AD research closer to finding an effective cure.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Tables

Table 1: Percentage identity between key human AD-related proteins and the sheep reference sequence or closest sheep sequence hit on BLAST, showing that high homology exists between human and sheep.

	Sheep vs Human %	Human Sequence		Coverage
	Identity	(Length)	Sheep Sequence (Length)	%
APP770	97%	NP_000475.1 (770)	XP_004002849.1 (770)	100
A β 1-40	100%	NP_000475.1 (770)	XP_004002849.1 (770)	100
A β 1-42	100%	NP_000475.1 (770)	XP_004002849.1 (770)	100
α -cleavage site	100%	NP_000475.1 (770)	XP_004002849.1 (770)	100
β -cleavage site	100%	NP_000475.1 (770)	XP_004002849.1 (770)	100
γ -cleavage sites	100%	NP_000475.1 (770)	XP_004002849.1 (770)	100
BACE1	99%	NP_036236.1 (501)	XP_004016104.1 (501)	100
PSN1	91%	NP_000012 (467)	XP_012037385.1 (468)	100
PSN2	96%	NP_000438.2 (448)	XP_014954666.1 (445)	100
APOE	69%	NP_000032.1 (317)	XP_011950465.1 (342)	100
APOE4 (112 position)	100%	NP_000032.1 (317)	XP_011950465.1 (342)	100
APOE4 (158 position)	100%	NP_000032.1 (317)	XP_011950465.1 (342)	100
Tau isoform 1 (758aa)	87%	NP_058519.3 (758)	XP_012004971.1 (601)	80
NF-L	98%	NP_006149.2 (543)	XP_014948576.1 (536)	75

Table 2: CSF levels of three protein fragments associated with AD. Total tau and neurofilament light levels were measurable in sheep and comparable to human levels, while levels of Phospho-tau 181 were not much higher than the lower level quantification limits of the test.

	Total Tau (ng/L)	Phospho-Tau181 (ng/L)	Neurofilament Light (ng/L)
Sheep 1	594	15.1	800
Sheep 2	73	15.4	615
Sheep 3	291	15.1	1185

Figure Legends (images as separate TIFF files)

Figure 1: Amyloid-beta CSF profiles, showing that all of the cleaved fragments of the APP protein present in humans appear in similar levels in sheep, including the $A\beta_{1-42}$ fragment. **A-D** represent the four eight year old sheep samples, with **E** as a human control for comparison.

Figure 2: Plaques are present in aged sheep brain. **A.** Large amyloid immuno-positive structures are observed in the entorhinal cortex of the 14 year old sheep along with smaller structures indicated by the arrow. **B.** High magnification image of the largest plaque seen in **A.** **C.** Two plaques adjacent to granule cells of dentate gyrus within the hippocampus from 8 year old sheep 3. DAB is the chromogen used in **A, B** and **C.** **D.** A thioflavin-positive structure from the 14 year old sheep cortex is shown. Scale bars are 50 μ m in length.

Figure 3: Tangles are present in the aged sheep brain. **A.** A neuronal tangle found in the entorhinal cortex of the 14 year old sheep. **B.** A tangle within the temporal lobe of 8 year old Sheep 2. **C.** Possible dystrophic neurites in the 14 year old sheep observed as a cluster of thickened neurites within entorhinal cortex. DAB is the chromogen used in **A, B** and **C.** **D.** Section 1240 from the Michigan State University Sheep Brain Atlas is representative of the coronal level of hippocampus examined in this study showing relative densities of tangles in different regions of the cortex. Red circles represent high density; yellow represents moderate density, and green is low density. Scale bars are 50 μ m in length.

Table 3: Relative numbers of tangles and plaques observed in the cortex and hippocampus of the 5 sheep in this study, showing that the fourteen year old sheep had more tangles and plaques than the eight year old sheep. One + equates to approximately 5 plaques or tangles.

	Average No. of Tangles per 50µm	
	Section	Average No. of Plaques per 50µm Section
8yo Sheep 1	+	+++++
8yo Sheep 2	++++	+++++
8yo Sheep 3	+	+++++
8yo Sheep 4	+	+++++
14yo Sheep	+++++	+++++

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