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Sleep disturbance in clinical and preclinical scrapie-infected sheep measured by polysomnography

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ABSTRACT

Neurodegenerative diseases are characterised by neuronal loss and abnormal deposition of pathological proteins in the nervous system. Among the most common neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease and transmissible spongiform encephalopathies (TSEs). Sleep and circadian rhythm disturbances are one of the most common symptoms in patients with neurodegenerative diseases. Currently, one of the main objectives in the study of TSEs is to try to establish an early diagnosis, as clinical signs do not appear until the damage to the central nervous system is very advanced, which prevents any therapeutic approach. In this paper, we provide the first description of sleep disturbance caused by classical scrapie in clinical and preclinical sheep using polysomnography compared to healthy controls. Fifteen sheep classified into three groups, clinical, preclinical and negative control, were analysed. The results show a decrease in total sleep time as the disease progresses, with significant changes between control, clinical and pre-clinical animals. The results also show an increase in sleep fragmentation in clinical animals compared to preclinical and control animals. In addition, sheep with clinical scrapie show a total loss of Rapid Eye Movement sleep (REM) and alterations in Non Rapid Eyes Movement sleep (NREM) compared to control sheep, demonstrating more shallow sleep. Although further research is needed, these results suggest that prion diseases also produce sleep disturbances in animals and that polysomnography could be a diagnostic tool of interest in clinical and preclinical cases of prion diseases.

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Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative diseases, with fatal outcomes, TSEs are caused by an unconventional agent called prion, an abnormal protein (PrP^{Sc}) that is generated by a conformational conversion of a cell surface glycoprotein (PrP^{c}) naturally present in the host. PrP^{Sc} is capable of inducing the transformation of the PrP^{c} to PrP^{Sc}, thus accumulating in the nerve tissue and other peripheral tissues, slowly and without remission, until it causes the death of the individual. Prion diseases affect both humans and domestic and wild animals, and a total of sixteen different diseases have been described, seven in animals and nine in humans, all of which are characterised by a fatal outcome leading to the death of the individual (Imran and Mahmood 2011). Classical scrapie in sheep is considered one of the most important TSEs (Pattison and Jones 1967) due to its wide distribution and large economic impact. Other prion diseases in animals, chronic wasting disease (CWD) of cervids which is acquiring great importance in Europe with the appearance of new strains (Benestad et al. 2016; Sola et al. 2023) and the most relevant of all, given its zoonotic potential, bovine spongiform encephalopathy (BSE) causing the variant of Creutzfeld-Jakob disease (vCJD) (Hill et al. 1997). The most prevalent prion disease in humans is sporadic Creutzfeldt-Jakob disease (sCJD), characterized by rapidly progressive dementia and ataxia, and develops in patients without known risk factors (Ironside et al. 2005; Puoti et al. 2012; Sato 2021). Prion genetic diseases, which have been described exclusively in humans, are caused by mutations in the gene that encodes PrP^{C} (*PRNP*) (Minikel et al. 2016). These include familial Creutzfeldt-Jakob disease (fCJD) (Gambetti et al. 2003), fatal familial insomnia (FFI) (Kretzschmar et al. 1995; Khan and Bollu 2018),

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and Gerstmann-Sträussler-Scheinker syndrome (GSS) (Takada et al. 2017).

Prion diseases share common neurodegenerative features with other neurodegenerative protein misfolding diseases like Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies and Huntington's disease (HD) among others (Dobson 2003). These neurodegenerative diseases are characterised by neuronal loss and abnormal deposition of proteins (phosphorylated α-synuclein, tau, β-amyloid, huntingtin or PrP^{Sc}) in the nervous system, mainly in the central nervous system (Scheckel and Aguzzi 2018; Soto and Pritzkow 2018). The origin of most neurodegenerative diseases is uncertain. Some of these diseases are idiopathic (Iranzo and Santamaria 2015) and others are hereditary (Tanner et al. 1999; Frisoni et al. 2022), and prion diseases deserve special mention as they can be sporadic, hereditary or acquired (Imran and Mahmood 2011).

A variety of neurological symptoms and signs, including dementia and parkinsonism (tremor, bradykinesia, postural rigidity and gait imbalance) (Braak et al. 2003) are typical of these diseases, which typically have a chronic and progressive course. In addition to all of them, sleep disturbances are among the most common clinical manifestations that have the greatest negative effects on the quality of life of those who experience them. In a retrospective study of 126 patients with sCJD, they found that the most commonly reported symptoms were sleep disturbances (Wall et al. 2005). As a result, because they appear before the beginning of the diseases' signature symptoms, sleep disturbances can be a crucial diagnostic sign that predicts the progression of the disease (Mattis and Sehgal 2016; Lauretti et al. 2017). The most commonly detected sleep disorders in neurodegenerative diseases are: insomnia, excessive daytime sleepiness (EDS), changes in circadian rhythm, rapid eye movement (REM) sleep behaviour disorder and nocturnal stridor (Landolt et al. 2006; Weissová et al. 2016; Dufort-Gervais et al. 2019).

PrP^{c} has multiple roles in the body through different physiological processes. It has been implicated in cell survival and adhesion (Cazaubon et al. 2007). It has an important role in protection against oxidative stress and apoptosis (Westergard et al. 2007; Kovacs and Budka 2008). In terms of its involvement in cell adhesion, PrP^{c} is involved in neuronal differentiation, epithelial and endothelial barrier integrity, transendothelial monocyte migration and T-lymphocyte activation (Steele et al. 2006; Cazaubon et al. 2007; Castle and Gill 2017). In addition to this, it has been found to play an important role in the regulation of circadian rhythms and physiological sleep processes (Martins and Brentani 2002; da Luz et al. 2015). As early as 1996, the first research directly linking the cellular prion protein to circadian rhythms and sleep showed that there were significant differences in sleep periods between PrP^{c} deprived mice and PrP^{c} mice. They suggested that loss of PrP^{c} leads to neurological dysfunction in at least one of the prion diseases such as fatal familial insomnia (FFI) (Tobler et al. 1996).

In this study, we have used sheep with classical scrapie as an animal model of neurodegenerative disease to study sleep patterns by polysomnography. Several authors described studies of sleep in the ovine species, both in healthy animals and in animals with some neurodegenerative disease (Perentos et al. 2016; Schneider et al. 2020, 2021; Vas et al. 2021), but none has focused on prion diseases. The monitoring of animal activity by means of polysomnography is a breakthrough in the way of understanding the sleep/wake cycle in sheep as it represents the introduction of an innovative and non-invasive method in animal research that will allow us to thoroughly investigate the sleep/wake cycle of healthy sheep and sheep with scrapie. This will be of great help for future treatments to improve the quality of life of individuals, as it could help us detect possible cases early and help them with specific treatments to extend life as much as possible.

Materials and methods

Animals

Fifteen female animals aged 4–5 years were selected from local herds in the autonomous community of Aragón, Zaragoza, Spain. This is the native breed called Rasa Aragonesa, widely distributed throughout the community. They were divided into three groups of five sheep each: five clinical sheep naturally infected with scrapie agent; five preclinical sheep naturally infected with scrapie; and five negative control sheep. The five negative control sheep came from a controlled and scrapie-monitored farm which has no problems with scrapie disease.

To carry out this selection, biopsies of the lymphoid tissue associated with the rectal mucosa were performed, using anti prion protein antibodies (González et al. 2005) to establish whether the sheep were preclinical. With the animal's hind limbs raised, a speculum was introduced through the anus to visualise the rectal columns. In this position, the target lymphoid tissue was removed with the aid of forceps and scissors without the need for local anaesthesia as the procedure was well tolerated. The removed tissue (approximately 8 mm in length) was fixed in formalin for subsequent detection of PrP^{Sc} by immunohistochemistry using the monoclonal primary antibody L42 (1:500, R-Biopharm, Darmstadt, Germany) (Monleón et al. 2005). As they were animals without clinical signs of scrapie and positive for PrP^{Sc} in lymphoid tissue, they were classified as preclinical. Animals in the clinical stage were identified by the observation of scrapie associated clinical signs, which included pruritus and scratching of the tail root, lumbar area, and limbs, neurological signs, such as ataxia and head tremors, teeth grinding, wool loss, and cachexia. The brain was removed after euthanasia. Clinical and preclinincal animals were confirmed to be scrapie-positive by the detection of

PrP^{Sc} in the brain by immunohistochemical analyses using the monoclonal primary antibody L42 (1:500, R-Biopharm, Darmstadt, Germany) (Monleón et al. 2005). Negative animals came from a scrapie-free herd, and their negativity was confirmed by ELISA tests IDEXX HerdChek BSEScrapie Antigen EIA (IDEXX Laboratories, Westbrook, USA).

Genotyping

All the animals displayed a *PRNP* gene genotype ARQ/ARQ.

For genotyping, blood samples were taken from the jugular vein using vacuum tubes with EDTA as anticoagulant. Subsequently, DNA was extracted using genomic DNA purification kits from blood (e.g. GFXTM Genomic from Amersham Pharmacia Biotech Inc.).

The PCR technique based on previous studies (Acutis et al. 2006) was used to carry out ORF amplification, with a number of modifications as detailed below.

The open reading frame (ORF) of *PRNP* gene (750 bp) of all animals was amplified from the genomic DNA with forward and reverse gene-specific primers. The reaction was performed using the reagents of the commercial kit of QIAGEN® Taq PCR Core Kit. The polymerase chain reaction (PCR) was performed in a final volume of 50 µL which contained 20 pmol of each primer, 5 µL of 10× PCR Buffer, 10 µL of 5× Q-Solution Buffer, 1 µL of dNTP mix (10 mM), and 2.5 U of Taq DNA polymerase. The following PCR conditions were used: denaturation at 94 °C for 2 min, 35 amplification cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min 30 s; followed by a final 5–10 min extension at 72 °C. PCR was performed using an S-1000 Thermal Cycler (Bio-Rad, Hercules, CA). Once the PCR was completed, all PCR products were analysed by electrophoresis on a 1.0% agarose gel stained to verify that the gene had been correct. Amplified PCR reactions were purified using the vacuum manifold from Millipore® at 24 Hg of pressure for 3 min. Once the amplified DNA was purified, it was sent to be sequenced together with the primers corresponding to the company Stab-Vida in Portugal. Chromatograms were analysed using Chromas 2.6.6. (Technelysium Pty Ltd, Australia).

Sleep/wake cycle monitoring

For monitoring the sleep/wake cycle in sheep, the Embletta MPR PG XS system was available together with the Ambulatory Polysomnography Module already used by several authors for sleep monitoring in humans. This device is designed to perform ambulatory studies by means of a PC-USB cable connection. By using this device, we simultaneously performed electroencephalography (EEG), electromyography (EMG) and electro-oculography (EOG) measurements. To apply the electrodes (Ambu Neuroline Cup), the cranial area of the animals was shaved. While the animal was held by two people, the researcher proceeded to attach the electrodes. The setup took around 30 min. Electrodes to measure eye movements were placed on top of each eye (O), and the two electrodes for measuring muscle activity on the submandibular muscles (M), two more electrodes were placed on the mastoid bone behind each ear (E) (Figure 1(A)).

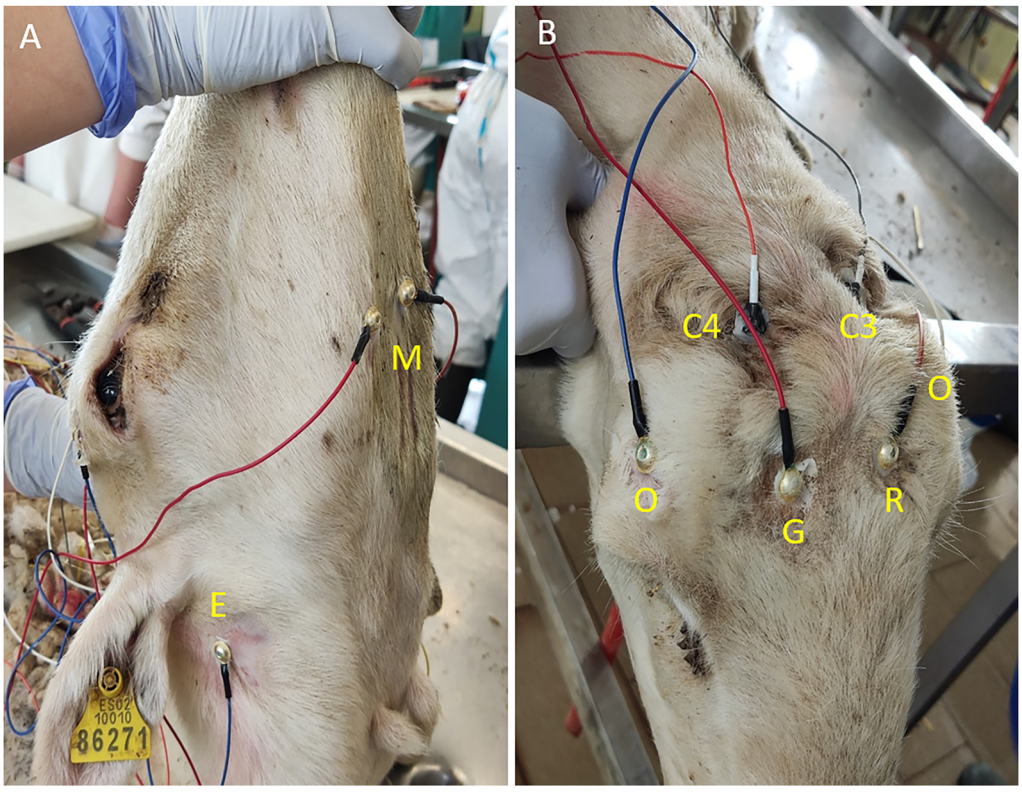

Figure 1. Electrode distribution on the sheep's head.

These electrodes were attached to the skin of the sheep by means of cyanoacrylate. For electroencephalographic measurement (surface EEG), two electrodes were placed on the front of the sheep's skull, corresponding to Reference (R) and Ground (G). On the medial side of the head, C3 and C4 were placed and just below Occipital 1 (O1) and 2 (O2) were placed (Figure 1(B)). The recording device was fixed with a bag on the sheep's back, thus avoiding any discomfort and snagging. The sleep recordings were conducted once for 12 h, from 8 pm to 8 am. They were performed in the facilities where they are normally housed in which they were fully adapted. During the recording there was no light and they were not disturbed until 8:00. The sun set at 19:00 and rose at 7:00. During the recording there was no researcher, which allowed the animals to be calm in a natural environment. These studies are technically challenging as sleep measurements in animals are expensive and difficult to carry out, repeating measurements in several animals on different nights.

Wake activity was characterized by fast, low amplitude EEG, and large amplitude variable EMG and eye movements as measured by the EOG. Non Rapid Eyes Movement (NREM) sleep was characterized by EEG with large amplitude slow-wave oscillations in the delta frequency band (0.5–4 Hz), a reduction of EMG tone and a flat EOG. REM sleep was characterized by a further reduction in muscle tone, a wake-like EEG and occasional eye movements. EEG channels were bandpass-filtered between 0.5–50 Hz, EOG between 0.3–15 Hz and EMG between 10–100 Hz (Supplementary file 1). All the studies were reviewed manually and studies were divided in epochs of 30 s. Two independent clinical neurophysiologists performed a visual analysis.

Data analysis and statistics

GraphPad Prism 8.0 (San Diego, CA) software was used for the statistical analyses. Graphs were generated with GraphPad Prism 8.0 (San Diego, CA). All quantitative data were tested for normality with the Shapiro–Wilk test. Between-group differences in electroencephalography, electrooculography and electromyography scores were determined by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Differences between groups were considered significant at $p < 0.05$.

Results

Sleep-wake patterns of clinical and preclinical scrapie sheep differ from those of healthy control sheep

Polysomnography showed that sheep spend most of their resting time awake, since control sheep, i.e. healthy sheep, spent 73% of their resting time awake (525 min). Clinically scrapie-affected animals spent the most time awake at 85% of the total (606 min), closely followed by pre-clinical sheep at 81% (590 min) (Figure 2(A,B)). Total recording time was 720 min. Statistically significant differences were detected between control and clinical animals in the percentage of total awake time ($p < 0.01$). Statistically significant differences were also detected between control and preclinical animals ($p < 0.05$). No differences were found between preclinical and clinical animals (Figure 2(A)).

Clinical scrapie sheep have increased sleep fragmentation

When analysing the number of sleep-wake cycles, i.e. the number of sleep fragmentations in each group of sheep, it was observed that control sheep had an average of eight sleep fragmentations during the night, preclinical sheep had eleven and clinical sheep had fourteen sleep fragmentations (Figure 3(A,B)). Statistically significant differences were detected between control and clinical animals in the number of sleep fragmentations that occurred in a night ($p < 0.01$). Significant differences were also detected between preclinical and clinical animals ($p < 0.05$). No

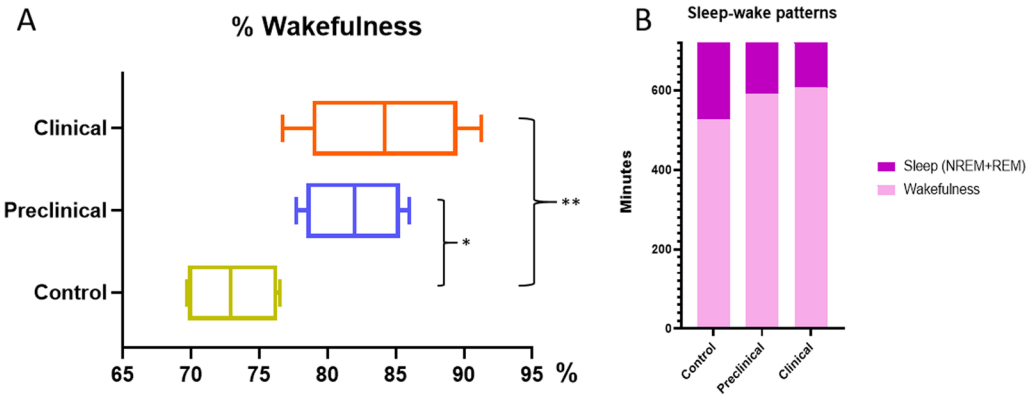

Figure 2. Sleep-wake patterns. (A) Percentage of wakefulness. The graph shows the total percentage of wakefulness of the different groups of animals over the course of a night. The Central line is the median and the whiskers are the maximum and minimum values. Evaluation of differences between groups was performed using the one-way ANOVA test followed by the Bonferroni post hoc test (*$p < 0.05$ and **$p < 0.01$). (B) Sleep-wake patterns. The graph shows the mean of the total minutes of wakefulness and sleep (NREM + REM) of the different groups of animals over the course of a night.

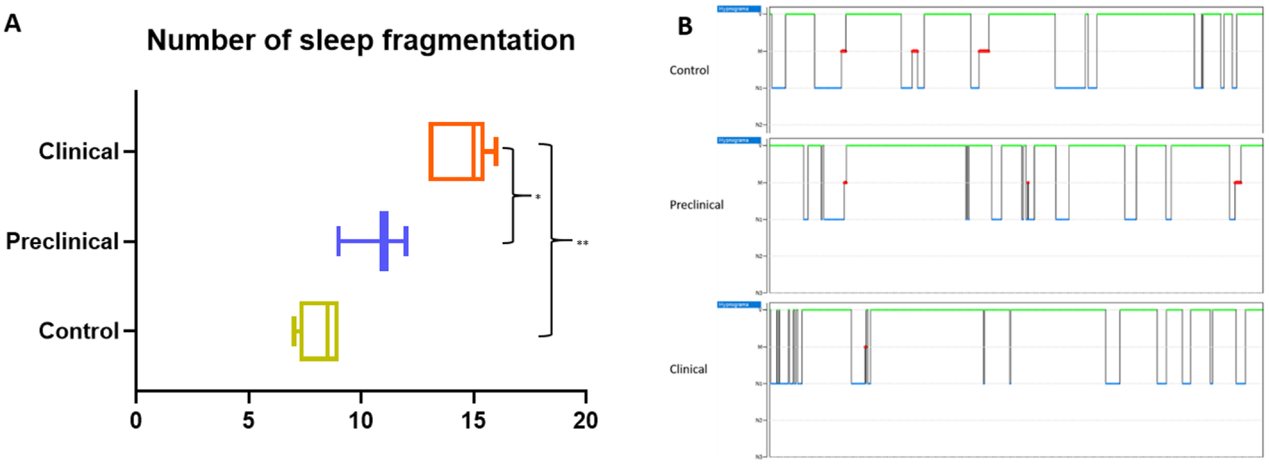

Figure 3. Sleep fragmentations. (A) Number of sleep fragmentations. The graph shows the sleep fragmentations of the different groups of animals over the course of a night. The Central line is the median and the whiskers are the maximum and minimum values. Evaluation of differences between groups was performed using the one-way ANOVA test followed by the Bonferroni post hoc test (*$p < 0.05$ and **$p < 0.01$). (B) Hypnogram. Shows the different phases of sleep in the three different groups of sheep. The wakefulness is shown in green, the NREM sleep phase in blue and the REM sleep phase in red.

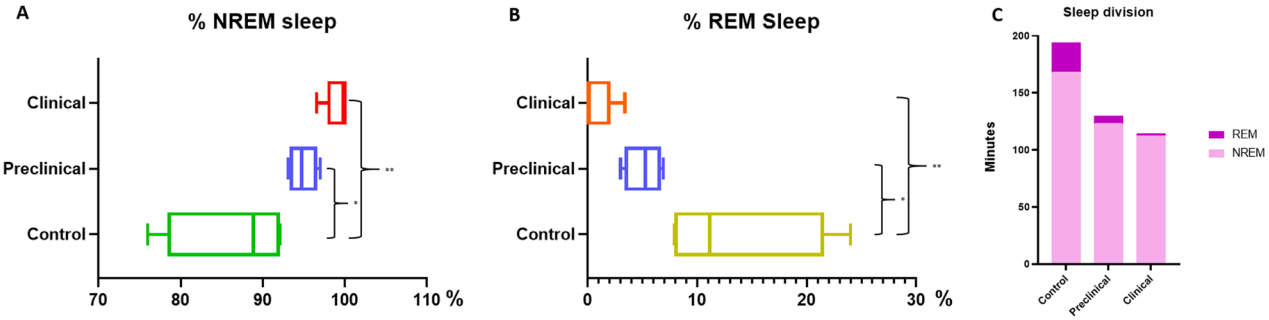

Figure 4. Alterations in NREM and REM sleep. (A) Percentage of NREM sleep. The graph shows the total percentage of NREM sleep within the total sleep of the different groups of animals over the course of a night. The Central line is the median and the whiskers are the maximum and minimum values. (B) Percentage of REM sleep. The graph shows the total percentage of REM sleep within the total sleep of the different groups of animals over the course of a night. The Central line is the median and the whiskers are the maximum and minimum values. Evaluation of differences between groups was performed using the one-way ANOVA test followed by the Bonferroni post hoc test (*$p < 0.05$ and **$p < 0.01$). (C) Sleep division. The graph shows the division of sleep in minutes.

differences were found between control and preclinical animals (Figure 3(A)).

Clinical and preclinical scrapie sheep show alterations in NREM and REM sleep compared to control sheep

In sheep, sleep was divided into two clearly differentiated types of sleep in sheep, NREM sleep, and REM sleep. The % of NREM sleep obtained by polysomnography shows that sheep with clinical scrapie have about 100% of their sleep in the NREM phase. Preclinical ewes have 95% compared to control sheep with 86% (Figure 4(A)). Statistically significant differences were detected between control and clinical animals in the percentage of total NREM sleep time ($p < 0.01$). Statistically significant differences were also found between control and preclinical animals ($p < 0.05$). No differences were found between preclinical and clinical animals (Figure 4(A)).

The % of REM sleep obtained by polysomnography shows that clinical scrapie sheep have virtually no REM sleep (1 min). Preclinical sheep have around 5% (7 min) REM sleep followed by control sheep with 13% (24 min) (Figure 4(B,C)). Statistically significant differences were detected between control and clinical animals in terms of percentage of total REM sleep time ($p < 0.01$). Statistically significant differences were also found between control and preclinical animals ($p < 0.05$). No differences were found between preclinical and clinical animals (Figure 4(B)).

EEG Delta frequency distributions during NREM sleep are altered in clinical scrapie sheep

The EEG frequency distribution was fully examined using classical frequency bands and the Delta frequency was established as a reference. Delta frequencies are analysed to determine whether NREM sleep is lighter or deeper.

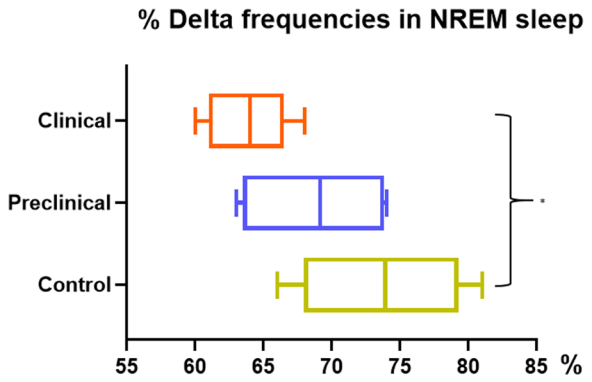

Figure 5. Percentage of Delta frequencies in NREM sleep. The graph shows the total percentage of Delta frequencies within NREM sleep for different groups of animals over the course of a night. The Central line is the median and the whiskers are the maximum and minimum values. Evaluation of differences between groups was performed using the one-way ANOVA test followed by the Bonferroni *post hoc* test (*$p < 0.05$).

Delta frequencies between control and preclinical sheep were very similar, around 70% of NREM sleep frequencies. Clinical sheep had an average Delta frequency of 64% in NREM sleep. Statistically significant differences were detected between control and clinical animals in terms of the total percentage of Delta frequencies ($p < 0.01$). No differences were found between pre-clinical and clinical animals or between control and pre-clinical animals (Figure 5).

Discussion

Sleep-wake disorders are a major source of morbidity and negatively affect the quality of life of neurodegenerative disease patients. The disorders may occur during the prodromal stage of the disease, years before the onset of the cardinal symptoms that define the diagnosis of the disease (Kondratova and Kondratov 2012; Hastings and Goedert 2013; Abbott and Videnovic 2016; Mattis and Sehgal 2016).

PrP^c plays a key role in a biochemical pathway that affects several physiological processes. There are several animal models of PrP^c dysfunction or PrP^C knockout, in which marked alterations in circadian rhythms occur (Tobler et al. 1996), suggesting that PrP^c plays an important role in one of the most essential processes for life: sleep/wakefulness.

This is the first study to provide a detailed comparison of sleep-wake patterns and the distribution of EEG spectra in healthy and scrapie-infected sheep. Sheep have a number of advantages over other species for studying the neurobiology of sleep. They have a human-like brain anatomy with convoluted cortexes, they are diurnal and their sleep structure is more similar to that of humans than rodents (Toth and Bhargava 2013). In addition, extended life expectancy is particularly useful when studying late onset diseases such as prion diseases. Their husbandry and welfare are easily managed (Morton and Howland 2013). Another important factor for using sheep in our study is that the sleep-wake pattern and EEG signatures are similar to those observed in humans and non-human primates (Hsieh et al. 2008). In addition, scrapie is the prototypical prion disease in animals, as it has been the subject of the largest number of studies in TSEs (Zabel and Reid 2015).

Polysomnography results have shown that sheep do not excel at sleeping in quantity. Rumination artefacted the EEG making it impossible to analyse, so these epochs were considered wakefulness. Video recording was not available so artefacts in the EEG could not be attribute to any specific movement artefact. Control sheep spent 73% of the night awake. Clinical sheep spent more time awake during the night than preclinical sheep and much more time awake than healthy sheep, suggesting that their response to sleep pressure is abnormal. It is becoming increasingly evident that chronic neurodegenerative diseases are commonly accompanied by sleep dysfunction and may appear even several years before the main symptoms (Ju et al. 2014; Schrempf et al. 2014). In a study carried out in 2016, all patients with fCJD showed sleep abnormalities, including increased periods of night-time wakefulness (Givaty et al. 2016).

When assessing the number of sleep fragmentations, it was observed that clinical sheep had a significantly higher number of sleep fragmentations during the night compared to control and preclinical sheep. It has been shown that rhesus monkeys infected with kuru show sleep fragmentation during the night (Bert et al. 1978). It has also been observed that mice inoculated with murine prions show sleep fragmentation very early in the incubation period (Steele et al. 2007).

Clinical and preclinical scrapie sheep showed alterations in NREM and REM sleep compared to control sheep whose results may be somewhat different from those published in articles such as the one by Perentos et al. in 2016 (Perentos et al. 2016) as they use a completely different invasive methodology than the one employed. In the clinical sheep, a total loss of REM sleep was observed. In the case of the preclinical sheep, a significant decrease in REM sleep was also demonstrated. In the case of Parkinson's disease, REM sleep disturbance is described even decades before the development of synucleinopathy (Postuma et al. 2013). In prion diseases, a total loss of REM sleep is described in rhesus monkeys infected with kuru (Bert et al. 1978) All patients with fCJD present with loss and decrease of REM sleep (Givaty et al. 2016). In addition, recently in 2022, a Creutzfeld-Jakob disease (CJD) patient was observed to have severe REM sleep disturbance 5 years before the disease onset (El Sammak et al. 2022).

Analysis of normalised EEG showed less content in delta frequencies in clinical scrapie sheep than in control sheep, this result suggests that the NREM sleep of clinical sheep is much shallower than that of control sheep. In previous experiments in which monkeys were infected with Kuru, they also observed that NREM sleep became increasingly lighter (Bert

et al. 1978). Delta-wave disruption is a key early event in HD mouse models (Burgold et al. 2019). Shallow sleep without solid periods of slow-wave sleep has also been observed in patients with fCJD, leading to abnormal sleep (Givaty et al. 2016).

In GSS disease, no sleep disturbances have been reported in patients suffering from the disease (Provini et al. 2009). This suggests that sleep disturbances are specific to some prion diseases, maybe related to the different strains causing them, which is a field that still needs much research to clarify the role of the pathological prion protein in normal sleep function.

One of the main objectives of TSEs studies is to try to perform an early diagnosis of these diseases, as clinical signs do not appear until the nervous damage is very advanced, which prevents any therapeutic approach. This is why preclinical biomarker studies in TSEs are of great relevance. Our main objective was to try to determine the possible alterations that prion diseases can produce in sleep and to evaluate the possible use of polysomnography as a diagnostic tool, as it provides a non-invasive and objective measure of cortical brain activity. It is true that it is a tool that by itself does not imply any type of clear diagnosis, since, as has been mentioned, there are prion strains that do not produce sleep alterations, in addition, there are also other neurodegenerative diseases that produce these alterations. For all these reasons, the combination of different diagnostic tools is of great importance. This study is the first approach to the alterations produced by scrapie in the sleep/wake cycle in sheep. We have observed that clinical sheep have a poorer quality of sleep, as the total percentage of sleep is lower, with a high fragmentation of sleep, less deep NREM sleep and a complete disappearance of REM sleep. In the case of preclinical sheep, they also have milder sleep disturbances. These results are a good starting point for future research on sleep disturbances in prion diseases. Studies with a more extensive evaluation of animals, with different genotypes and different prion strains are needed to give us a better understanding of sleep disturbances in prion diseases. All of this will be fundamental in the search for treatments to improve these sleep disorders and try to prolong the quality of life of the individual.

Author contributions

The conception and design of the study were carried out by CA. data acquisition was carried out by DS and BM. Data analysis was carried out by JF, ES and DS. the writing of the manuscript was carried out by DS as well as the figures. All authors contributed with the writing of various parts of the paper and editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

All animal experiments were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (permit number PI17/21).

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Data availability statement

The data presented in this study are available within the article text and figures.

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