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Original article

Electroencephalographic recordings of physiological activity of the sheep cerebral cortex

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Abstract

This paper presents the physiological activity of the cerebral cortex in sheep in electroencephalographic findings. The study was performed to evaluate and improve understanding of brain monitoring methods in freely moving animals without the use of any anaesthetic methods during the acquisition stage. The aim of the study was to determine the physiological activity of the cerebral cortex in animals in a sheep model (using clinically healthy Polish Merino rams, aged 1 yr.) to determine its clinical EEG protocol. The EEG was registered using an in-lab EEG device as well as ambulatory systems (Holter EEG). The bioelectrical activity of the sheep cerebral cortex was recorded using gold disc and needle electrodes placed on experimentally determined locations on the scalp. The main finding of this study was the rhythmic EEG activity of the sheep brain in wakeful and conscious states (eyes open). The rhythm was bilaterally synchronous and determined a basic pattern in the registration of physiological activity of the cerebral cortex. The acquired rhythm may correspond to the theta rhythm in humans, which is a normal activity controlled by the hippocampus. This background activity comprised intermittent episodic slow waves and synchronous beta waves.

Key words: brain, electroencephalography, sheep

Introduction

Electroencephalography (EEG) is a method that allows summarised electrical activity generated by neurons in the cerebral cortex to be recorded. This phenomenon is a specific tool used for registration of the central nervous system (CNS) bioelectrical activity from the scalp surface, as well as for analysing its basic functions. The procedure is characterised by high precision and high usability, especially in brain function

studies in humans for the diagnosis of physiological, functional disturbances of the CNS and as a diagnostic tool for epilepsy (Wrzosek et al. 2009). The EEG studies used for scientific purposes in animals contain an equally broad and comprehensive range of analytical methods, which justified the analysis of encephalitis, the suitability of anaesthetics (evaluations of general anaesthesia), epileptic seizures and the detection of BSE (bovine spongiform encephalopathy) (Cwynar and Zawadzka 2006). However, for a proper evalu-

ation and interpretation of the pathological findings, it is important to determine the physiological state of the brain in healthy individuals. Unfortunately, EEG patterns in the physiological state in animals without anaesthesia are practically unavailable (Brauer et al. 2011). Therefore, the aim of this study was to investigate the physiological activity of the cerebral cortex in animals in the sheep model to determine its EEG patterns.

Materials and Methods

Experimental animals

Fifteen clinically healthy Polish Merino rams (body weight 40.1 ± 1.4 kg; age 1 year ± 2 weeks) from a national breeding farm certified by the Polish Sheep Association were used in this study. Analyses of the animals' physiological state, as well as laboratory blood analyses, were performed. The sheep were kept in climatic chambers with thermal and acoustic isolation. Feeding during the whole experiment was maintained at a constant level with grain oats (0.2 kg per head; 100% of intake), grass hay and water (*ad libitum*). Microclimatic conditions were under constant monitoring by electronic sensors using Visual System Scada Pro (Microbe, Poland). In order to ensure full control of the conditions in the chambers as well as the observations of animal behaviour, a video monitoring system was also installed. All experimental procedures, including animal handling were reviewed and accepted by the 2nd Local Ethical Committee of Experimental Procedures on Animals in Wrocław, Poland (decision No. 82/ 2009). The study was carried out on the Experimental Farm of the Wrocław University of Environmental and Life Sciences in Wrocław.

Electroencephalography

The neurophysiological experiments in this study were carried out with both in-lab and ambulatory EEG equipment. In-lab registration of bioelectrical brain activity was performed using the COMET EEG system (Grass Technologies, West Warwick, RI, USA), which was connected by Ethernet with the PC located in the monitoring room. The bioelectrical activity was registered from the surface of the sheep's scalps using gold disc electrodes (Grass Technologies, USA) (Fig. 1). The hair was shaved only at the electrode placement points and the skin was defatted and disinfected with alcohol (ethyl alcohol). Each electrode was glued to the skin of sheep with EC2 Elec-

trode Cream (Grass Technologies, USA). Biopotentials from 10 electrodes (8 recording electrodes and 2 for reference and ground) were recorded. Electrodes AL (left frontal electrode) and AR (right frontal electrode) were both located over the rhinencephalon. Electrodes LL (left temporal electrode) and LR (right temporal electrode) were located over the temporal lobe. The C (central) electrode was located over the bone suture between the frontal bones. The P (central parietal) electrode was located over the parietal lobe. The PL (left occipital) and PR (right occipital) electrodes were located over the occipital lobe. All EEG records were analysed using the referential and bipolar derivations. The referential system was based on 8 channels (AL, AR, C, LL, LR, P, PL, PR) with the reference electrode (Ref) and ground (Gnd) located in the region of the anatomical structures of the nasal bone. Carrying out the study of bipolar montage, a 14 channel system was used as follows: AL – AR, AL – LL, AL – PL, AR – LR, AR – PR, LL – LR, LL – C, LL – PL, LR – PR, C – LR, C – P, PL – P, PR – P and PL – PR. The EEG data were recorded on a PC running under Windows XP and supported by Twin software (Grass Technologies, USA). The long-term (24 h) EEG recordings were done with AURA24™ ambulatory amplifiers (EEG Holter device; Grass Technologies, USA). Five AURA24 units allowed the EEG data from five sheep to be recorded simultaneously. During the study needle electrodes (Grass Technologies, USA) were implanted subcutaneously (Fig. 2). The needle electrodes' application (at 10 points) on the skulls was performed in accordance with the same system as their deployment in the stationary experiment. The electrode placement in the two previous methods was based on patent application (Cwynar and Kołacz 2011), as illustrated in Fig. 3. The invention of a new method in our study is based mainly on the electrode placement system where the electrodes are lying along the three parallel lines, where the position of the centre line was determined in the area of nasal bone connection and the frontal bone. Four electrodes were located along the centre line. The first two electrodes, ground (Gnd) and reference (Ref), were located above the nasal bones, due to the high electric detachment of this region of the animal's head. The experimental placement of the electrodes was made on the basis of the brain anatomy in sheep and numerous impedance and bioelectrical conduction tests in these regions, which was similarly confirmed by Strain et al. (1988). When using a larger number of electrodes, the minimum distance between the electrodes has also to be used. In our study, it was found in an experimental way that the optimum distance between the recording electrodes (disc as well

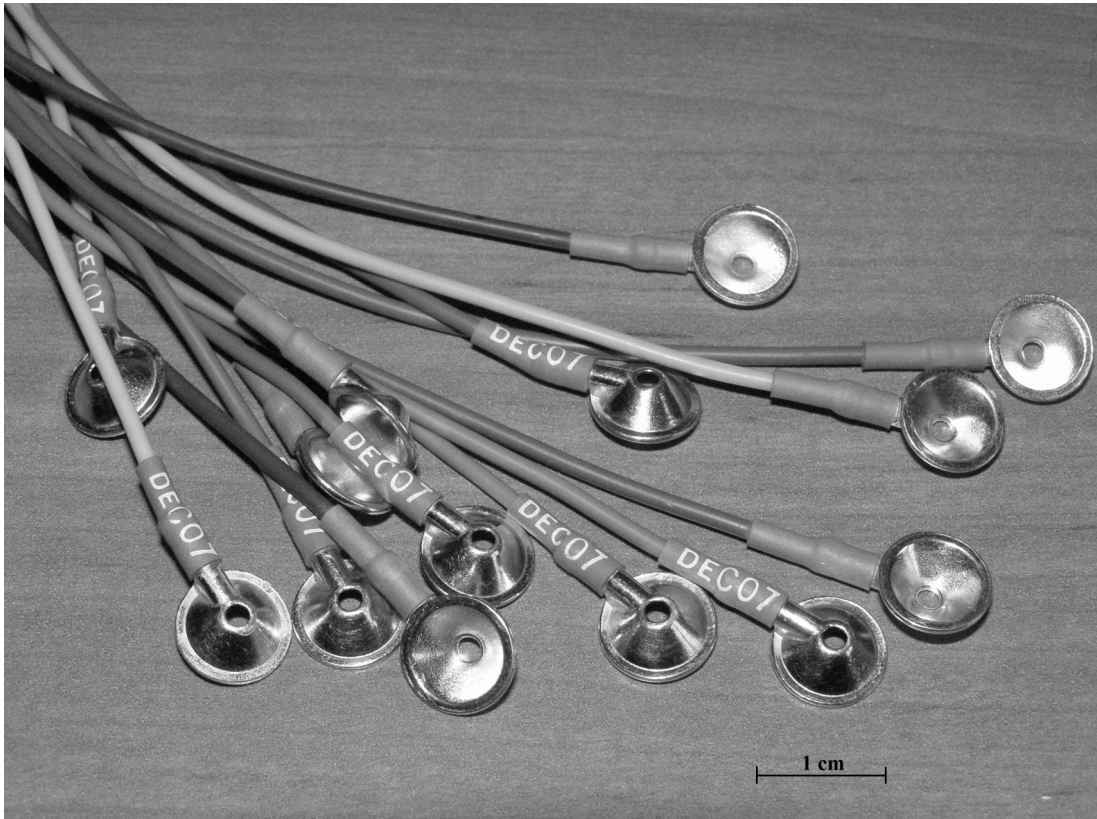


Fig. 1. Surface gold disc electrodes (Grass Technologies, USA) used during in-lab EEG registration in sheep.

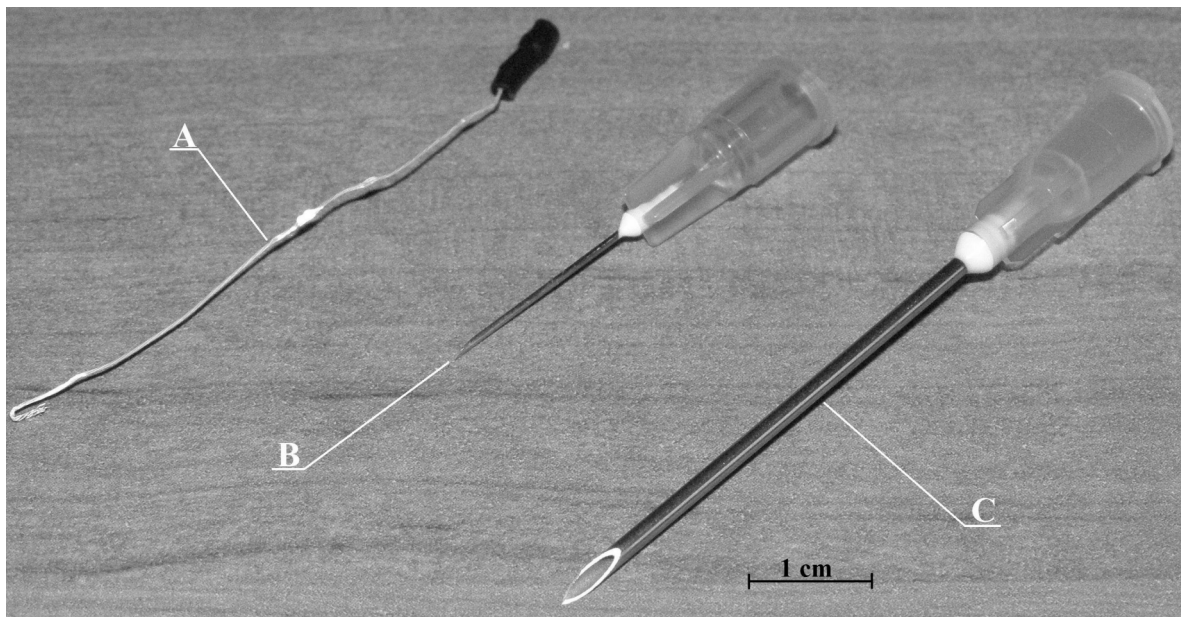


Fig. 2. Implantable needle electrodes (Grass Technologies, USA) used during long-term (24 h) EEG monitoring in sheep. A – main electrode; B – implant needle; C – standard needle \varnothing 1.2 mm.

as needle electrodes) should be at least 15% of the transverse length of the sheep skull, measured from the centre of the electrode. Moreover, the distance between the recording electrodes (AL, AR, C, LL, LR, P, PL, PR) and the ground (Gnd) or reference

(Ref) should be at least double the distance previously mentioned. The implantation of the subcutaneous electrodes was a single procedure performed 3 days before the experiment started. The implantation was made under general anaesthesia (to avoid unnecess-

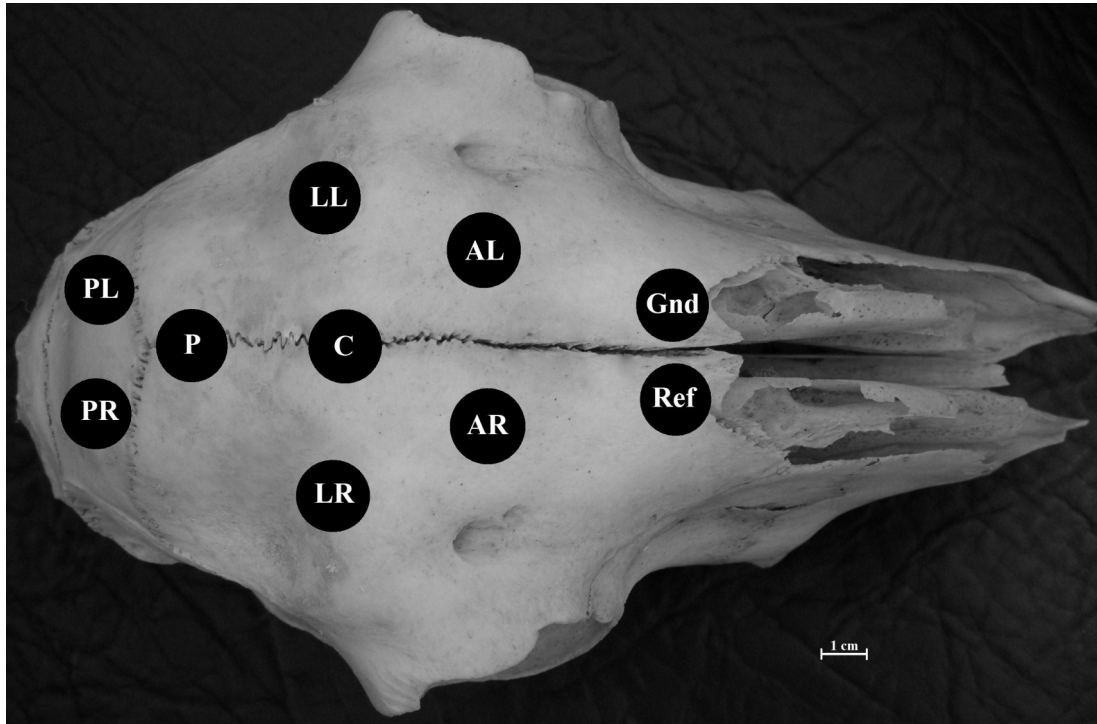


Fig. 3. The electrode placement on a sheep skull. The Ref (reference electrode) as well as the Gnd (ground electrode) are located on the nasal bone. There are two frontal electrodes (AL, AR), two temporal electrodes (LL, LR) and two occipital electrodes (PL, PR). The central electrode (C) was located between the frontal and temporal region on a bone suture, similarly to the posterior electrode (P), located between the temporal and occipital region.



Fig. 4. The ambulatory system (Holter EEG) and its installation possibility on the back of the experimental animals.

ary pain) using xylazine (Sedazin®, Biowet Pulawy, Poland), given intravenously via the jugular vein at a dose of 0.25 mg/kg, and a solution of ketamine (Ketamine 10%, Biowet Pulawy, Poland), also given

intravenously via the jugular vein at a dose of 10 mg/kg. No other anaesthetics or antibiotics were used in this study. The subcutaneous electrodes (Fig. 2) with a length of 20 mm were placed in the same loca-

tion system as the disc electrodes. The implantation procedure was based on the needle (subcutaneous) electrodes being positioned under the skin for the whole experimental period. The ambulatory system (Holter EEG) was fixed on the back of the experimental animals using linen belts (Fig. 4) All electroencephalograms were stored digitally on the PC's hard drives. The electroencephalographic apparatus setup was carried out according to the recommendations of the American Electroencephalographic Society (1994 ab), with a chart speed of 30 mm/s and equipment sensitivity of 750 $\mu\text{V}/\text{mm}$. The high-pass filter (low frequency) was set at a constant time of 1 second, while the low-pass filter (high frequency) was set at 70 Hz to reduce electromyographic artefacts. In order to prevent EEG distortion, notch filters were used occasionally to avoid brain wave distortion. During all experiments the electrode resistance was kept below 5 k Ω . It should be noted that the results derived from in-lab EEG equipment and ambulatory records did not differ among themselves. The EEG devices (electroencephalographs) are based on similar installations as well as the same software; therefore the results from neurophysiological studies may be compared. The EEG analysis included only the physiological states of the animals, in resting conditions. The remainder of the behavioural reactions of the laboratory animals as well as the electroencephalographical studies during these states were not under consideration in this study.

Experimental procedures

Sheep were placed in air-conditioned chambers for 14 days for acclimatization purposes. All of the environmental parameters met the requirements for sheep handling. Feeding and watering during the whole experiment were maintained at the same level. During the experimental period the microclimatic conditions were monitored daily. The EEG studies were performed on each subject using the COMET EEG system (Grass Technologies, USA). The examination was used to record the bioelectric activity of the cerebral cortex of the animals and to diagnose any event which consisted of a central nervous system dysfunction. The in-lab test procedure was necessary in order to standardise the animals in the experimental groups, which allowed collation of the obtained results. The experiment enabled the determination of EEG clinical records containing a physiological model of bioelectrical activity of the cerebral cortex in sheep at rest.

Data analysis

The results derived from the stationary and ambulatory EEG equipment were compiled in order to develop clinical records, taking into account the reactions and behaviour of the experimental animals. All data from the EEG studies were saved in digital records. The evaluation of the electroencephalograms was based on results obtained by online and offline methods using Twin software (Grass Technologies, USA). A comparative analysis was made taking into account artefact free samples of the EEG. All data were analysed using a standard visual method as well as the quantitative evaluations (quantitative EEG, q-EEG), taking into account the absolute values of amplitude and frequency of the EEG. Frequency (Hz) and amplitude ($\mu\text{V}/\text{mm}$), wave shapes, symmetry and time sequence of records were analysed using Neurotrac III neuromonitoring software (Grass Technologies, USA). The relationship between the amplitude and frequency of traces was carefully examined. The quantitative-qualitative analysis enabled the frequency spectrum distribution in the individual bands to be determined (Fig. 8). Additionally, the Fast Fourier Transformation was carried out according to Otto's (2007) methodology, where the EEG variable median power frequency was processed and the percent distribution of total power was as follows: alpha (8-13 Hz), beta (13-30 Hz), theta (4-8 Hz) and delta (0.5 – 4 Hz).

Results

The main finding of the electroencephalographic records in sheep registered in wakeful and conscious states (eyes open) was a rhythmic activity within the range of 5-6 Hz and 8 Hz. The amplitude of the EEG did not exceed 50 μV (mean 21 μV). The higher values do not constitute the existence of pathological features. The rhythms were bilaterally synchronous and determined a basic activity in the registration of physiological activity of the cerebral cortex with a clear prevalence in the frontal (AL, AR), central (C, P) and occipital region (PL, PR) channels. The recorded rhythms may be generated by two different generators – cortical and subcortical. A difference between sleep and wakefulness was also clearly observed; however, analysis of sleep is not included in this paper. The basic activity was intermittent, with episodic slow waves, especially in the AL, AR, LL and LR channels, which were recognized as artefacts arising from horizontal eye movements. The records also showed the episodic occurrence of synchronous beta waves of frequency 18-20 Hz, which were found to be

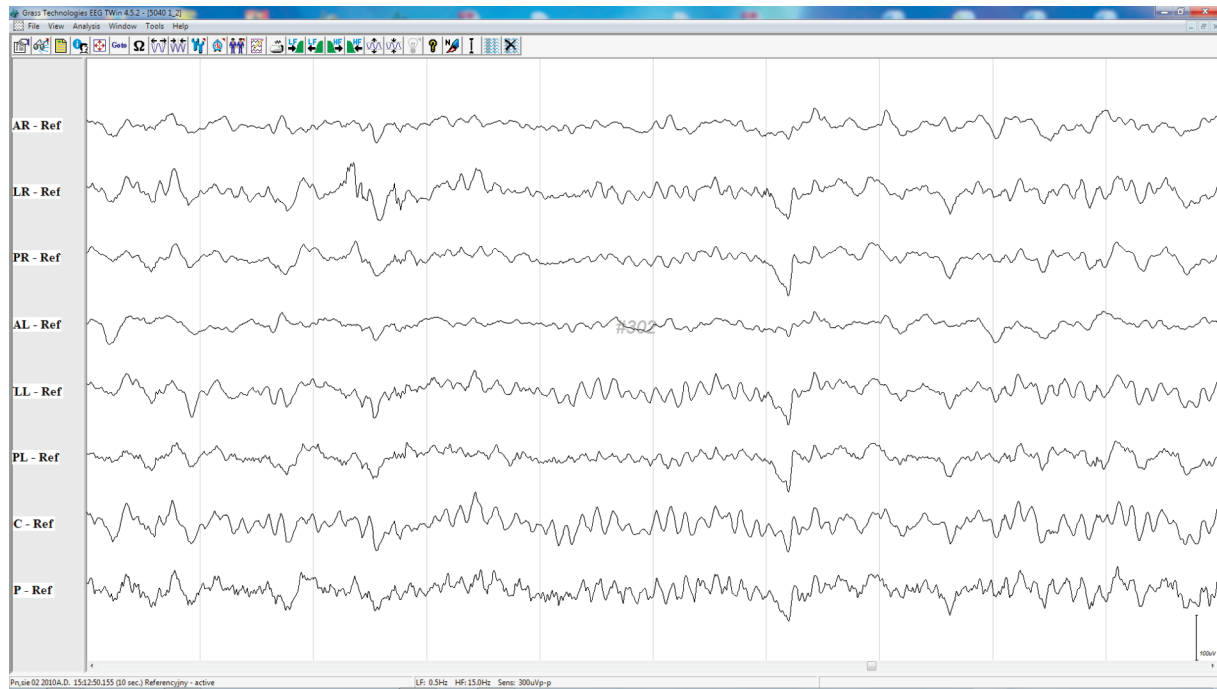


Fig. 5. Physiological activity of the sheep cerebral cortex in electroencephalographic record in monopolar (referential) montage during the wakeful state (standing, slowly walking) using the in-lab EEG system. Part I: page size 10 sec/page; sensitivity: 300 uVp-p; filters: LF 0.5 Hz, HF 15 Hz.

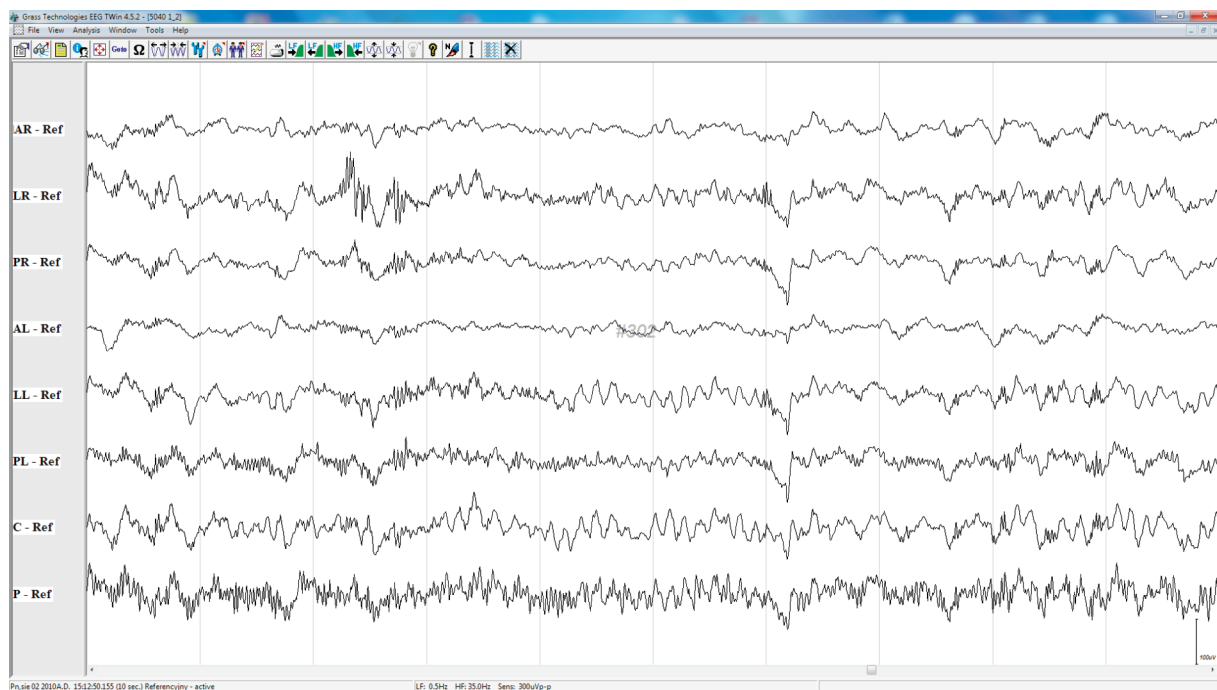


Fig. 6. Physiological activity of the sheep cerebral cortex in electroencephalographic record in monopolar (referential) montage during the wakeful state (standing, slowly walking) using the in-lab EEG system. Part II: page size 10 sec/page; sensitivity: 300 uVp-p; filters: LF 0.5 Hz, HF 35 Hz.

superimposed over the background rhythm. Nevertheless, especially in the frontal and temporal channels, such waves were the effect of the diffuse beta activity associated with the muscular artefacts caused by rumination. The electrophysiological studies showed that

in the frontal left channel (AL) the average frequency was 10.96 Hz, although the divergence in the study ranged from 3.6 up to 47 Hz. The average cerebral cortex voltage was 18.37 μ V, but the lowest value in the frontal biopotentials was 6.1 μ V, and the highest

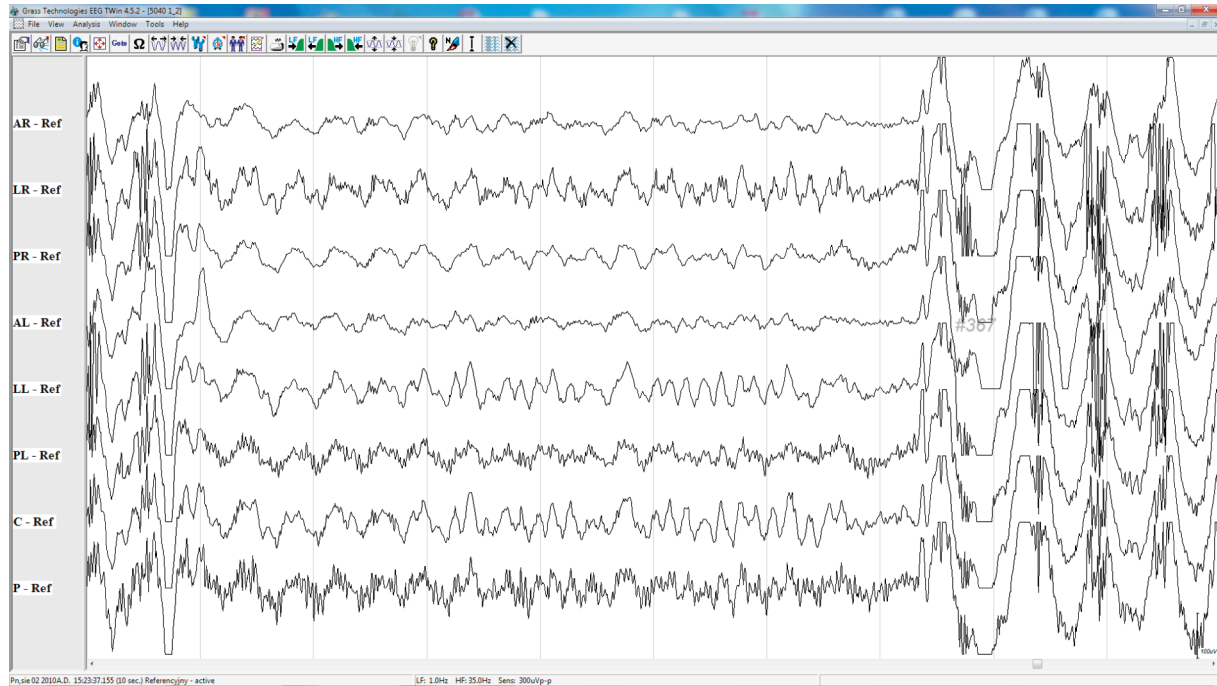


Fig. 7. Physiological activity of the sheep cerebral cortex in electroencephalographic record in monopolar (referential) montage during the wakeful state (standing, slowly walking) using the in-lab EEG system. Part III: page size 10 sec/page; sensitivity: 300 uV-p; filters: LF 1 Hz, HF 35 Hz.

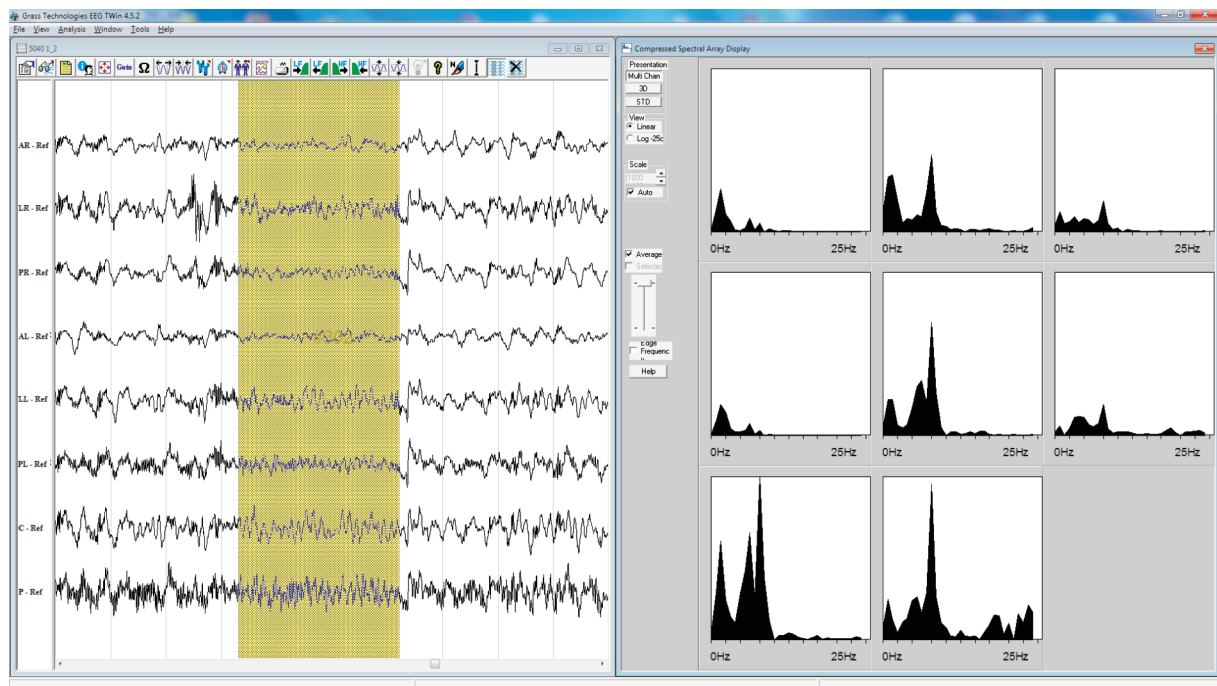


Fig. 8. Physiological activity of sheep cerebral cortex in the Compressed Spectral Array Display.

46 μ V. It was found that in the frontal right channel (AR) the brain waves were 3.9-44 Hz with a mean of 9.8 Hz. The amplitude range was between 7.8 and 49 μ V, and the mean voltage was 18.54 μ V. The mean wave frequency of the LL channel (left temporal lobe)

was 10.1 Hz, often reaching 21.1 Hz. It is not relevant that the LL and LR channels, located near the temporal line (linea temporalis), also detected an activity of low frequency (1.6 Hz) and occasionally occurring slow waves. The presence of this rhythm in the state

of full awareness and consciousness of the animals suggested delta wave activity (δ). Despite this, the mean amplitude of both mentioned lobes was 16.5 μV and its basic level fluctuated in the range of 8.3-25.5 μV . The LR channel (right temporal region) was characterised by a very similar amplitude and frequency compared to the left hemisphere, assuming a mean value of 10.2 Hz (range 3.9-21.1 Hz). The bioelectric voltage exhibited values of 8.3-34.3 μV , although the mean was estimated at 16.23 μV . The central channel (C) recorded a frequency range from 3.9 Hz to 21.9 Hz, while the estimated mean of this range was 10.57 Hz. Biopotentials occurring in the central region had a mean value of 20.3 μV , with a range from 11.5 to 32.8 μV . The P channel, located at the central-parietal region, presented higher values than the preceding C, showing a mean frequency of 11.94 Hz, which falls in the range 4.7-22.7 Hz. Despite higher levels of voltage from 9.7 up to 42.4 μV , the mean amplitude of waves was 18.01 μV . The location of PL and PR electrodes determined the bioelectrical activity of the cerebral cortex of the occipital region. The CNS activity of PL (left occipital lobe) was characterised by a mean frequency of 20.68 Hz and a range of 4.7-39.1 Hz, which may determine the typical beta (β) rhythm in humans. Despite the fact that the amplitude fluctuated from 7.8 μV up to 37.41 μV , the mean voltage of the electroencephalograms in sheep was 14.82 μV . Similar results were also obtained in the PR channel (right occipital lobe), where the mean frequency was 18.91 Hz with a range of 5.3-34.2 Hz, while the mean amplitude was 15.22 μV with a range of 7.9-36.9 μV . The EEG visual analysis showed generalised low-voltage activity, beta rhythm and theta waves. The frontal and central lobes were characterised by wave activity occurring at 10-12 Hz with an upward trend in the central region, where it reached the highest voltage of 20 μV . Electroencephalographic studies in sheep also had the episodic presence of morphological parameters of sleepiness, as well as short periods of low frequency delta wave activity, which did not exceed 3.5 Hz. Suggested clinical recordings containing physiological EEG patterns in sheep are presented in Fig. 5 and 6. It should be noted that the analysis of the present study excludes sleep states and behavioural circumstances, especially the interactions between the experimental subjects, since the bioelectrical activity during sleeplessness is not well known, especially in farm animals. Similarly, the brain waves representing interactions between the animals may significantly disturb the EEG registration with the numerous muscle artefacts. Moreover, the EEG analyses in sheep were taken only for conscious states or typical behaviour of the species, with slow motions, but watering and feeding time was not taken

into account. A great number of these reactions induced many artefacts, especially related to tongue muscular activity or rumination, which were eliminated to ensure that the registered electroencephalograms included only physiological activity of the cerebral cortex.

Discussion

Methodology

The main aim of this study was to evaluate the physiological state of the bioelectrical activity of the cerebral cortex in sheep. The study was performed using a sophisticated EEG method, which enabled detailed analysis of the central nervous system reactions. The original procedures in this study, which were also presented in the patent application by Cwynar and Kolacz (2011), may reveal certain advantages in animal EEG research, especially by avoiding most of the artefacts during neurophysiological tests, as well as during off-line analysis of the electroencephalograms. These problematic circumstances in EEG registration were also pointed out by Strain et al. (1986) during electroencephalographic studies of scrapie in sheep. Particular difficulties are encountered at the stage of the selection of appropriate species of animals for the planned experiments, taking into account the anatomical structure and intra-individual factors, as well as model behaviours. Cwynar and Zawadzka (2006) found that sheep are easy to use in neurophysiological experiments and are characterised by relevant anatomical and behavioural conditions, which justified their participation in this study. The most common experimental animals used in neurophysiology are rodents, such as mice or guinea pigs (Weiergräber et al. 2005). Experiments in rats are also important when analyzing CNS reactions (Ichinose et al. 1999). It can be concluded that laboratory animals will have small genetic differences, and therefore they enable the standardisation of results, which is also confirmed by other authors (Ashequr and Kamei 2005). There is also a need for electrophysiological studies in dogs, which were pointed in EEG studies in animals (Pellegrino and Sica 1997, Ives et al. 2006). Wrzosek et al. (2009) argued that a great advantage in conducting EEG research in animals is the use of the feline model. It should be noted that all experimental procedures conducted by the mentioned researchers were conducted in conscious sedation, which greatly restricts the use of this method in veterinary practice.

The monitoring of CNS functions, including the response to various stimuli, can play a fundamental role in research concerning the evaluation of the cer-

erebral cortex in animals. Despite the unification of many aspects of the use of EEG procedures, the number of electrodes is still changing in animal studies and is not standardised as in humans. Pellegrino and Sica (1997) used 12 electrodes in dogs. Bioelectrical registration of sheep cerebral cortex activity in the present study reduced this number to 10 electrodes for each experimental animal. Chang et al. (2002) propose a uniform number of 32 electrodes, based on the generally accepted international system of 10-20 (10-20 International System of Electrode Placement), which was also recommended by Rowan and Tolunsky (2004). Razoumnikova (2003) expressed a different view, that analysis of 16 electrodes is sufficient. Taking into account the ratio of the surface of the human skull to the sheep skull in our study, we decided to use a total of 10 electrodes to provide a multi-channel data acquisition system, by making 8 reference channels and 14 bipolar derivations.

EEG interpretation

There are multiple ways to interpret the data, especially because of the variety of parameters used in the experimental process, as well as their subsequent evaluation. Therefore, it is important to unify both methods, i.e. both the reading of the results and their explanation. The visual assessment method is the simplest way of interpreting the EEG records, since it consists of visual verification and evaluation of the graphic results and the possibility of comparing them. Unfortunately, this method is not objective, and one determinant of its proper application is the accuracy and precision of the analytical researcher. Moreover, it is hard to present the obtained graphical results in numerical form, so it is not possible to verify them using standard statistical analysis. As visual analysis is only partly an objective act and depends highly on the person performing the assessment; it is vulnerable to the subjective feelings of the investigator. EEG spectral analysis is a highly objective study, based solely on the values obtained by the calculations of the computer software. The results of the present study were obtained using methods suitable for human EEG processing, which are also used occasionally in animals. It should be noted that EEG standardisation in humans, suggested by Salansky et al. (1998), Rowan and Tolunsky (2004) and Marrosu et al. (2005), should involve the proper and multifaceted interpretation and analysis of incoming neural potentials and brain waves, as also mentioned by other authors (Aurlien et al. 1999, Razoumnikova 2003, Velis 2005). Guided by the above research, as well as analogous studies performed on animals (Ichinoze et al 1999, Pellegrino

and Sica 2004), we have established a unique, hitherto unused method for recording neural electrical signals from the brain, which was presented in our patent application as a method for measuring and monitoring the bioelectrical activity of the cerebral cortex in animals, especially in sheep (Cwynar and Kołacz 2011). In our study, using an experimental method, the electroencephalographic patterns in sheep were established together with a standard physiological registration in these animals. Yoshida et al. (2004) discussed these kinds of inventions and recommended that great attention should be paid towards the interpretation of animal experimental methods. Even so, most authors try to maintain the current convention in wave classification in frequency bands regardless of the EEG wave recording methods (Cwynar and Zawadzki 2010). It was also noted that during the EEG recording in dogs the interpretation and classification of rhythms were made according to the criteria of human medicine, as in the study by Pellegrino and Sica (2004) and Brauer et al. (2011). Therefore, the presented results of our experiments were based on the same practice. It was found that there are both beta waves, confirmed earlier by Cwynar and Zawadzki (2010), and also intermittent theta and delta rhythms. Pellegrino and Sica (2004) believe that dogs under sedation, without access to visual stimuli, may be characterised by the appearance of alpha waves, thus having lower values of neural voltage potentials, as well as lower frequencies. Pellegrino and Sica (2004) also noted that delta waves are associated with epileptic seizures. This observation is also supported by Chang et al. (2002) and Marrosu et al. (2005), who tend towards the same conclusions. However, in experiments conducted in sheep, there were no epileptic symptoms either in behaviour, or in the characteristics of the electroencephalographic records. Unfortunately, among the available literature there is a lack of electrophysiological experimental procedure data in sheep. Mahla (1997) argues that the size of the cortical origin rhythms, with particular emphasis on the amplitude, and their cyclical nature, are essential for the proper determination of brain bioelectrical activity and the consequent precise definition of the physiological state of the CNS. Different opinions were presented by Ives et al. (2006), Yoshida et al. (2004) and Weiergräber et al. (2005), who performed electroencephalographic measurements in animals, without taking into account these kinds of relationships, and did not connect the delta wave pairing with the pathological phenomena. The explanation of the presented theory is due to the fact that the mentioned authors are only interested in the physiological recording of bioelectrical activity of the cerebral cortex and specify the range of normal values

(Cwynar and Zawadzka 2006). It is safe to say that the aspects of the analytical approach to electroencephalographic studies discussed here have many supporters (Wolpaw et al. 2002, Ashequr and Kamei 2005), who may contribute to the development of this field in veterinary medicine. Brain monitoring using EEG methods may throw some new light on previously unexplored physiological activity of the cerebral cortex with all the surrounding environment and potentially stressful conditions (Riniolo et al. 2006), which was the main aim of this project.

Conclusions

This study indicates that it is possible to record the bioelectrical activity of the cerebral cortex in animals using simple methods and systems used as standard in human medicine. The observations reported in this study suggest that the typical physiological activity of the sheep cerebral cortex is bilateral synchronous rhythms of 5-6 Hz and 8 Hz in healthy sheep. It was found that the best locations for EEG registration are in the frontal (AL, AR), central (C, P) and occipital regions (PL, PR). Moreover, recorded EEG data correspond with normal CNS activity, mainly with the theta rhythm classified in humans. Despite many methodological problems that may arise during EEG procedures (e.g. artefacts caused by rumination), the analysis and interpretation of EEG seems to be very successful in applied veterinary practice as a basic, non-invasive method of cerebral cortex diagnosis.

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