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The effects of Nembutal on the intracerebellar EEG activity revealed by spectral and fractal analysis

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Abstract: A detailed analysis of the anesthetic-induced modulation of intracerebellar electrical activity is an important step to understand the functional brain responses to anesthesia. We examined the electrical activity recorded from different cortical layers: molecular layer (ML), Purkinje cell layer (PCL), granular layer (GL) and the white matter (WM) in the vermian part of rat cerebellar lobule V during Nembutal anesthesia using spectral and fractal analysis. Spectral analysis revealed a difference in the mean relative power of delta (0.1-4.0 Hz) and theta (4.1-8.0 Hz) frequencies through the cerebellar layers. Compared to the ML, delta activity increased significantly in the GL, while theta activity decreased in the GL and the WM. Fractal analysis revealed that the mean value of Higuchi's fractal dimension (HFD) increased, starting from the ML to the WM. Theta activity exhibited a negative correlation with the HFD value in the ML. In contrast, the gamma activity showed a positive correlation with the HFD value in the ML and the GL. The combined use of spectral and fractal analyses revealed that Nembutal displays different effects on rat cerebellar electrical activity, which largely depends on the neurochemical and electrophysiological organization of the cerebellar layers.

Keywords: Nembutal; intracerebellar EEG; spectral analysis; fractal analysis

Abbreviations and acronyms: bispectral index (BIS); depth of anesthesia (DOA); electroencephalogram (EEG); granular layer (GL); Higuchi's fractal dimension (HFD); molecular layer (ML); Purkinje cell layer (PCL); white matter (WM)

INTRODUCTION

Research on the impact of anesthetics on brain neuronal circuits based on electroencephalography (EEG) is still a topical issue in fundamental and clinical neuroscience. One reason for this prolonged interest is that monitoring of brain's oscillatory electrical activity is crucial for determining the depth of anesthesia (DOA) in patients undergoing surgery [1,2]. In this respect, efforts have been made in recent years to understand the effects of different inhalation and non-inhalation anesthetics such as ketamine, Nembutal, propofol, sevoflurane, isoflurane, and desflurane on the cerebral, pontine and hippocampal electroencephalographic (EEG)/electrocorticographic (ECoG) activities of experimental animals [3-7]. The application of nonlinear measurements and linear methods such as Higuchi's fractal dimension (HFD), Lempel-Ziv complexity and spectral analysis allows for a reasonable approach to understanding the mechanism of anesthetic-induced alterations of EEG/ECoG activity as confirmed by many studies [2-8].

The cerebellum is a central brain structure deeply integrated into major loops with the cerebral cortex, brainstem and spinal cord, and it is involved in motor, cognition and executive control, with an impact on pathologies like dyslexia and autism [9]. It is made up of grey matter located on the surface of the cerebellum,

forming the cerebellar cortex and white matter (WM) located underneath the cerebellar cortex. The cerebellar cortex consists of three functionally and anatomically distinct layers: the molecular layer (ML), the Purkinje cell layer (PCL) and the granular layer (GL) [10,11]. The systematic effects of anesthetics such as ketamine or Nembutal on cerebellar EEG activity are initially examined indirectly mainly as part of research efforts to understand the cerebellar cortex/cerebral cortex interactions in epilepsy, sleep and traumatic brain injury [12-14]. In recent years there has been an increase in research into the direct effects of anesthesia on the electrical activity of deep brain structures, including the cerebellum [5-7]. These studies addressed surface cerebellar EEG activity [5,13], while intracerebellar EEG dynamics were scarcely explored.

Nembutal (pentobarbital) is a short-acting barbiturate, an isomer of amobarbital with $GABA_A$ agonistic effects that has been widely used over decades in research, human and veterinary medicine [15-21]. Despite this fact, we still do not fully understand how Nembutal affects cerebellar neural networks. Namely, the formation and maintenance of oscillatory electrical activity of the cerebellum are essential given the anatomical and functional interplay between the cerebellum and cerebrum in both health and disease. The activation of GABA receptors suppresses neuronal excitability, maintaining a balance between excitation and inhibition [22]. In the cerebellum, there is specificity in the distribution of GABAergic neurons and GABA receptors, including $GABA_A$, which makes this brain structure a useful system for examining the functional significance of the diversity of inhibitory circuits [23-27]. Therefore, in this study, we aimed to test whether there are layer-dependent changes in EEG activity of the vermian part of rat cerebellar lobule V under Nembutal anesthesia by a combined use of linear spectral analysis and nonlinear HFD. The vermian part of lobule V functionally belongs to the spinocerebellum and is responsible for the static and dynamic balance of the body [28]. Spectral analysis is one of the principal methods used in the field of neuroscience for the quantification of EEG signals associated with different physiological and pathological states [29]. HFD is a nonlinear measure of signal complexity in the time domain, and it has been used in neurophysiology and anesthesiology for some time now [8]. The value of HFD increases when a complex system expresses more chaotic behavior, while the value of decreases when the system is less chaotic, as in the brain during epileptic discharges [30-33]. Thus far, many studies have successfully used HFD alone or in combination with other nonlinear and linear methods to estimate the impact of different anesthetics on human and animal brain electrical activity [2-8,34]. Given the lack of knowledge about anesthesia-induced alteration of cerebellar EEG activity, it is crucial to understand not only the spectral and fractal behaviors of cerebellar layers but also the nature of the anesthetic-induced EEG regimen across these layers. In this pilot study, our primary goal was to examine how Nembutal modulates EEG rhythmic activity across cerebellar layers by employing spectral and fractal analyses.

MATERIALS AND METHODS

Experimental animals and surgical procedures

The experiments were performed on 6 adult (2-2.5-month-old) male Wistar rats. Experimental animals were reared in a 12-hour light-dark cycle at an optimal temperature of 21-24°C with unrestricted access to food and water. All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia (No. 12/06).

The rats were anesthetized by Nembutal (Pentobarbitalnatrium, Serva, Germany) that was administered intraperitoneally (i.p.) at a dose of 40 mg/kg. During the experiment, anesthesia was applied at a dose of 8 mg/kg every 50-60 min as needed. The anesthetized animal was fixed in a stereotaxic apparatus. The operative procedure for electrode placement included drilling a hole 2 mm in diameter on the occipital bones, 10.5 mm posterior to the bregma and 1.5 mm left of the sagittal suture [35].

Electrophysiological recordings

The electrical activity of the cerebellum was recorded with one tungsten microelectrode (diameter 0.25 mm, impedance 2.0 MΩ). Recording electrodes were placed

on the left side of the vermian part of lobule V, starting from the cerebellar surface, using the Microdriving Terminal apparatus (Alpha Omega Engineering, Israel) for fine electronic navigation. With a step increment of 0.05 mm, a recording electrode was scrolled down through the cerebellar cortex and the WM (Supplementary Fig. S1). The recording electrode was placed in the ML (at a depth of 0.45-0.55 mm), the PCL (at a depth of 0.60 mm), the GL (at a depth of 0.70-0.85 mm) and the WM (at a depth of 0.90-1.00 mm). A reference electrode (ground) was placed on the frontal bone muscle.

Electrical signals from the cerebellum were observed on a 2-channel memory oscilloscope (Tektronix, USA). A Multi-Channel Processor Plus (Alpha Omega Engineering, Israel) was used for signal amplification and filtering. The filter parameters were direct current (DC) for the high pass filter, 150 Hz for the low pass filter, and 50 Hz for the notch. Analog to digital (A/D) conversion of EEG signals was performed at a sampling frequency of 256 Hz. Every signal recording lasted 121 s (Burr-Brown, Multifunctional board PCI-20428W-1) with a SIGVIEW program [36].

Spectral analysis

Spectral analysis of registered cerebellar EEG signals was performed using programs written in Matlab 8.5. All recorded signals were filtered to avoid artifacts at 50, 60, 100, 106 and 120 Hz. Each signal (121 s) was divided into 15 epochs with a duration of 8 s. As a result of Fourier spectral analysis, we obtained the absolute and relative power spectra, with a total frequency range between 0.1-128 Hz divided into 5 frequency ranges: delta (0.1-4.0 Hz), theta (4.1-8.0 Hz), alpha (8.1-15.0 Hz), beta (15.1-32.0 Hz) and gamma (32.1-128.0 Hz).

Fractal analysis

Fractal analysis of recorded signals was performed using programs written in Matlab 8.5. The HFD value was calculated according to Higuchi's algorithm [8,30,37,38], with a non-overlapping window size of 200 points, which at a sampling frequency of 256 Hz corresponds to an epoch duration of 0.781 s [39]. An optimal value of k_{max}=8 was chosen [40]; the HFD values ranged from 1.000 to 2.000, as expected.

Statistical analysis

Statistical analysis included a total of 24 EEG signals recorded in the cerebellum of 6 experimental animals at the following depths: 0.45-0.55 mm (ML), 0.60 mm (PCL), 0.70-0.85 mm (GL), and 0.90-1.00 mm (WM). The null hypothesis was postulated as the mean relative power spectrum in the delta, theta, alpha, beta and gamma frequency ranges of cerebellar electrical activity which did not change, as observed by scrolling down the recording electrode through the cerebellar layers. Because of the limited amount of data, testing the null hypothesis was performed using nonparametric statistics. Kruskal-Wallis ANOVA for five dependent variables (5 frequency ranges) and one factor with four levels (ML, PCL, GL and WM) were obtained. For paired comparison, a Mann-Whitney U test was used. Spearman's correlation was used to assess the relationship between the relative power of each frequency range and the HFD values in different layers of the cerebellum.

RESULTS

In general, delta rhythmic activity dominated the cerebellar mean relative power spectra in Nembutal anesthesia (Fig. 1A). However, there were two distinct shapes of the mean relative power spectra obtained from a vermian part of rat cerebellar lobule V. One was obtained from the ML and others were obtained from the PCL, the GL and the WM. The spectral profile of the EEG signals suggested an increase in the mean relative power in the delta frequency range (Fig. 1A). Thus far, the mean relative power in the theta, alpha and beta frequency ranges decreased compared to the EEG recorded from the superficial ML (Fig. 1B, C and D, respectively). These findings indicate that EEG activity in different cerebellar layers has a distinctive spectral characteristic.

Comparing all EEG rhythms through the cerebellar layers, the Kruskal-Wallis test showed a significant difference only in the mean relative power of theta (4.1-8.0 Hz) ($H_{(3,24)}$=7.87, p<0.05). This value was the highest in the ML (29.05%). By scrolling down the recording electrode through the PCL, the GL and the WM, the mean relative power of theta transiently increased from 22.94% to 23.92% and then decreased

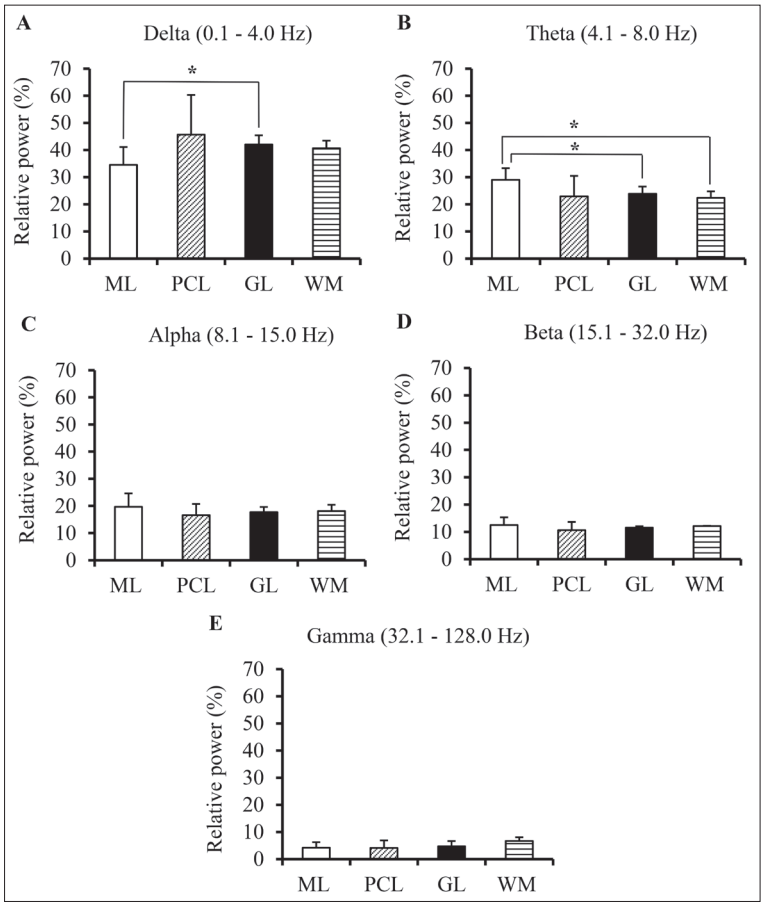

Fig. 1. The relative power of **A** – delta (0.1-4.0 Hz); **B** – theta (4.1-8.0 Hz); **C** – alpha (8.1-15.0 Hz); **D** – beta (15.1-32.0 Hz); **E** – gamma (32.1-128.0 Hz) frequency ranges in the molecular layer (ML), the Purkinje cell layer (PCL), the granular layer (GL) and the white matter (WM) of the cerebellum. Each bar represents the mean±SD; **P*<0.05 (Mann-Whitney U test).

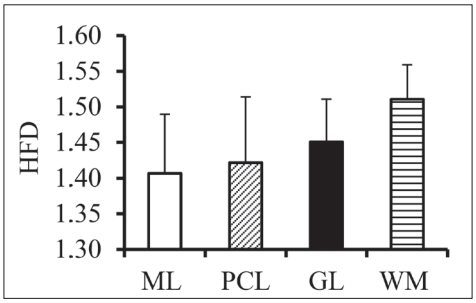

Fig. 2. The value of the fractal dimension (HFD) in the molecular layer (ML), the Purkinje cell layer (PCL), the granular layer (GL), and the white matter (WM) of the cerebellum. Each bar represents the mean HFD for each layer±SD.

to 22.38%, respectively (Fig. 1B). Compared to the ML, the mean relative power of theta in the GL and the WM was significantly reduced (U=10, P<0.05 and U=3, P<0.05, respectively). In the delta frequency range (Fig. 1A), the increase in the mean relative power with increasing depth of the recording electrode in the cerebellum was barely below the level of significance ($H_{(3.24)}$=7.39, P=0.06). Further paired comparisons revealed that this increase in the delta frequency range was significant in the GL when compared to the ML (U=10, P<0.05). The mean relative power of alpha, beta and gamma (Fig. 1C, D and E, respectively) did not change significantly in the cerebellum ($H_{(3.24)}$=1.58, P>0.05, $H_{(3.24)}$=1.69, P>0.05, and $H_{(3.24)}$=3.35, P>0.05, respectively). However, there seemed to be differences in gamma rhythmic activities between the three cortical layers on one side and the WM on the other.

The HFD value of the cerebellum in Nembutal anesthesia was consistently increased through the layers of this structure, according to the depth of the recording electrode, starting from the ML (1.4067) to the PCL (1.4217), the GL (1.4507) and the WM (1.5107) (Fig. 2). Although the observed change was not significant ($H_{(3.24)}$=4.17, P>0.05), it seems that Nembutal induced an increase in the HFD through the cerebellar layers. Post-hoc comparisons revealed that the difference between the ML and the WM approached the level of significance (U=4.5, P=0.08).

To further verify that changes in spectral and fractal parameters across the cerebellar layers are interrelated, we performed statistical correlation analysis between the relative power of each of the five frequency bands and the HFD values across different layers. Regarding the relationship between the relative power and HFD value, theta activity showed a strong negative correlation with the HFD value in the ML (r_s=-0.92, p<0.05). In contrast, gamma activity showed a strong positive correlation with the HFD value in the ML (r_s=0.96, P<0.05) and the GL (r_s=0.86, p<0.05).

DISCUSSION

The potential benefits of an improved understanding of anesthetic-induced modulation of EEG activity are an accurate assessment of the DOA and neural

connections underlying cognitive processes and consciousness. The results of this pilot study suggest that Nembutal increases slow delta rhythm and decreases theta rhythm starting from the ML to the WM, and that it has no effect on alpha, beta and gamma rhythms in the cerebellum. Also, the HFD value increases steadily through the cerebellar layers, starting from the ML to the WM. We also found that the value of the HFD negatively correlated with the theta activity in the ML, and that it positively correlated with the gamma activities in the ML and the GL.

As noted, Nembutal potentiates GABAergic inhibition throughout the central nervous system, including the cerebellum [5-7,17]. We can hypothesize that the specific and localized distribution of GABAergic neurons in the cerebellum [26,27] might be responsible for layer-dependent differences in EEG activity induced by Nembutal anesthesia. First, GABAergic neurons in the cerebellum are Purkinje cells, basket cells, stellate cells, Golgi neurons, and certain neurons in the cerebellar nuclei. Several types of GABAergic neurons can be found in the cerebellar layers and deep nuclei where each of them seems to play specific roles [26,27]. While basket and stellate cells in the ML inhibit Purkinje cells owing to GABAergic inhibition, thus far, GABAergic receptors on Golgi cells in the GL form an inhibitory feedback loop by receiving parallel fiber inputs and suppressing granule cell activity [26]. Information from the cerebral cortex is first sent to the deep cerebellar nuclei through the axons of Purkinje cells, and then to the inferior olive nuclei via GABAergic neurons where the regulation of synchrony of neuronal activities takes place [26].

This GABAergic transmission of information is also supported by the cytochemical distribution of GABA receptors in the cerebellum. An earlier study by Palacios et al. [23] revealed that in slide-mounted tissue sections of rat cerebellum, there is a very high density of GABA receptors in the GL, a low level in the ML and a negligible density in the WM. This layer-related distribution of GABA receptors could explain a significantly higher presence of theta rhythm in the GL as compared to the ML. Indeed, earlier studies identified granule cells as a critical for theta-frequency bursting and resonance, which may play an essential role in determining synchronization, rhythmicity and learning in the cerebellum [41]. Similarly, Hartmann and Bower [42] described 7-8 Hz oscillatory activity (upper theta activity) in the GL of the cerebellar folium Crus IIa in immobile rats. Studies confirm a high localization of GABA transporter in the nerve plexus around the bases of rat cerebellar Purkinje cells [43,44]. Purkinje cells receive GABAergic, axodendritic synapses from stellate cells and axosomatic synapses from basket cells, both of which contain $GABA_A$ receptors [25]. In general, theta rhythmic activity is highly sensitive to changes in GABAergic transmission [45,46]. Besides, it appears that gamma rhythm requires the activation of a heterogeneous GABAergic neuronal population and very likely $GABA_A$ receptor-mediated inhibition; both Purkinje cell and local interneuronal involvement were necessary for maintaining local field gamma activity [47]. These findings could be used to explain the tendency of Nembutal to increase delta activity in the PCL and GL. It is very likely that the increase in GABAergic transmission by Nembutal slows EEG activity at the expense of reduced theta and increased delta rhythms, with a statistically unproven incidental increase in the mean relative power of gamma rhythm. These results are in agreement with our previous study, indicating an increase in the spectral entropy of delta rhythm and a decrease in the spectral values of the theta frequency range recorded at the surface of both the cerebrum and cerebellum under Nembutal anesthesia [5].

Our pilot study shows that there is an increase in the HFD value through the cerebellar layers, starting from the ML to the WM, under Nembutal anesthesia. Other studies, in contrast to our findings, have reported that brain activity, measured by the complexity of the EEG signal, becomes less chaotic when consciousness is "switched off" during anesthesia, ischemia or traumatic brain injury [48]. However, we hypothesize that oscillatory brain dynamics are too complex and heterogeneous to be described only by a uniform increase or decrease in the value of HFD. It seems that the cerebellum has its own unique infrastructural complex sub-dynamics in health and disease, which probably depend on its specific molecular, functional and anatomical organization. Based on previous reports, it is to be expected that a decrease in higher frequency bands such as beta and gamma contributes to a reduction of the HFD value [6]. In general, the reduction of spectral power at higher frequency ranges, as in many clinical conditions, is associated with the loss of

complexity that reflects impaired regional structural and functional unity of the brain [49]. In our study, an increase in functional complexity (expressed as an increased HFD value) that was accompanied by a simultaneous decrease in theta rhythm through the cerebellar layers, could be explained by increasing the participation of gamma rhythm in the total EEG spectrum. Indirect confirmation of this is the strong positive correlation between the gamma activity and the HFD value in the ML and GL. This observation could be explained by the fact that the activation of cerebellar GABAergic receptors plays a significant role in modulating gamma rhythm [47]. From these pieces of evidence, one could hypothesize that Nembutal activation of $GABA_A$ receptors in the PCL and the GL leads to increased gamma activity and, consequently, HFD value.

On the other hand, the different lobes of the cerebellum exhibit different functional properties. It appears that the cognitive and limbic regions of the cerebellum are located in the posterior lobe, with cognitive areas situated laterally. In contrast, autonomic/affective/limbic functions are represented in the vermis [50,51]. By considering this functional topography, we might expect different spectral and fractal characterization across layers if the EEG signals are collected from these functionally distinct cerebellar regions. Only future studies can confirm or disprove such expectations.

Having in mind that the cerebellum and cerebrum are connected via several afferent projections originating from the primary motor cortex and prefrontal cortical areas [51, 52], it can be expected that the cerebellum is involved in establishing and maintaining levels of generalized anesthesia. Probably, the functional connections of the cerebellum with other regions of the brain also help to create the state of general anesthesia (i.e. cerebello-hippocampal interactions). In this respect, spectral and fractal analyses and measurements based on these two approaches to EEG signals are of particular importance for monitoring the DOA. For example, one of the earliest methods used to assess DOA via EEG monitoring involved a form of bispectral analysis called the bispectral index (BIS) [2], which integrates several different EEG parameters into a single variable, or better yet, the statistical BIS algorithm combines the contribution of each of the vital EEG features to generate the BIS index [2].

Due to its reliability and potential for automated computational application, HFD has found its place in intensive care units for monitoring the depth of propofol- and isoflurane-induced anesthesia, where only one EEG channel was enough for its estimation [53]. This suggests that both analyses can be used in a complementary manner to assess the DOA and to estimate the functional interplay between the cerebrum and cerebellum as well as other brain regions at various stages of general anesthesia, including Nembutal anesthesia.

CONCLUSION

This study shows that spectral analysis and HFD can be used complementarily for measuring anesthesia-induced intracerebellar EEG dynamics. Given the results presented here, it is indicative that cerebellar EEG activity in Nembutal anesthesia is layer-dependent. Understanding global neural dynamics is not possible without an understanding of the operations of locally functional neural networks such as those that build different layers of the cerebellum. Further studies with higher statistical power and larger sample sizes are necessary to provide improved insight into the Nembutal-induced modulation of intracerebellar EEG activity.

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Author contributions: G.S., Lj.M. and S.K. designed and carried out the experiments. G.S. and S.K. analyzed the EEG signals and interpreted the results. S.S. carried out the fractal analysis. G.S. and S.K. wrote the manuscript. Lj.M., J.P., B.P. and S.S. critically revised the manuscript.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Stojadinovic%20et%20al_5405_Supplementary%20Material.pdf