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CONTENTS

CUPRINS

COGNITIVE AND AFFECTIVE DISORDERS ASSOCIATED WITH DYSFUNCTIONAL CEREBELLO-LIMBIC-PREFRONTAL CIRCUITS

IOANA ANTOANETA GEORGESCU[#,*], ELENA LAURA GEORGESCU MĂRGĂRINT[#], CARMEN-DENISE-MIHAELA ZAHIU, ANA-MARIA ZĂGREAN, LEON ZĂGREAN

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ABSTRACT
The belief that the cerebellum is involved only in the motor function has been lately changed, as new research data supports its participation also in high cognitive functions and affection. A dysfunctional cerebellum has been proven to be associated with the existence of abnormal cerebral networks that communicate with the limbic system. Therefore, this organization can offer the cerebellum the ability to influence several higher functions of the cerebral cortex and the regulation of emotions. Functional neuroimaging, histological, and electrophysiological studies revealed surprising details regarding cerebellum-related pathologies and justified a careful reconsideration of the traditional cerebellar organization model. This paper has identified and summarized potential mechanisms facilitating the occurrence of neurological and behavioral abnormalities, providing new perspectives regarding cerebellum functions.
Keywords: emotion, cognition, cerebellum, schizophrenia, ADHD, psychiatric disorders

INTRODUCTION

Cerebellum has numerous anatomical and functional connections with different structures of the brain [1]. Its indirect connections with non-motor areas in the prefrontal cortex offer cerebellum the ability to participate in cognitive control [2], whereas its connections with the limbic system allow it to influence behavior [3][4]. Several authors advanced even the concept of limbic cerebellum and proposed the cerebellum as an extension of the Papez circuit [5][6]. Deep cerebellar nuclei have interconnections with several limbic structures, including the hippocampus [4], hypothalamus, septal nuclei, amygdala and accumbens [7], but probably the link between the nuclei and thalamus weights more than the others in terms of cognition and behavior. Fastigial nucleus, the one situated the closest to the midline of the cerebellum, projects directly on the midline thalamus, which has bidirectional interconnections with several areas in the prefrontal cortex, including the lateral orbitofrontal cortex, medial frontal, lateral prefrontal, and cingulate cortex. This anatomy offers medial thalamus the capacity to influence different cognitive functions, depending on the prefrontal activated region [8]. The cerebellar nuclei form cerebello-thalamo-cortical loops that are in close relationship with limbic areas, thus influencing affectivity and behavior. As stipulated before, the communication between the cerebellum and the cerebral cortex is fundamental for emotional regulation. The primary determinant of behavior is the fastigial nucleus. The exercise of this function is possible due to its anatomical connections with the amygdala, hippocampus, mammillary bodies, ventral tegmental area, hypothalamus, and septal nuclei. Fastigial glutamatergic projection neurons cross the midline and are the only type of fastigial neurons that can influence behavior [9][10].

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Recent evidence suggests that the cerebellum is necessary for habit formation and promotes a flexible behavioral control by exerting a modulatory response for the initiation and finalization of specific actions, in collaboration with the prefrontal cortex. Prediction and complex sequential learning (including Pavlovian conditioning) require a cerebellar contribution, which seems to be distorted in impulsive and compulsive disorders and other diseases with related symptomatology, such as attention-deficit hyperactive disorder (ADHD), autism or addictions [11]. High levels of dopamine and norepinephrine were detected in the fastigial nucleus, suggesting a cerebellar contribution in ADHD [12]. Cerebello-thalamo-cortical circuits appear to be distorted in several psychiatric disorders. Resting-state functional connectivity studies indicated a significant decrease in thalamic-cerebellar connectivity in schizophrenia and mildly reduced connectivity in bipolar disorder [13]. Moreover, numerous cognitive impairments can occur in cerebellar lesions, and linguistic, visual, and executive dysregulations are just a few of them [14][15]. Our research summarizes important information about the functional anatomy of the major systems in the brain and shows that the dynamics of the cerebello-limbic-prefrontal circuits are vital in regulating emotions and the use of cognitive functions.

CEREBELLUM AND SCHIZOPHRENIA

Cerebellum function is important in controlling the motor, emotional, and cognitive activities. The cerebellum agenesis causes not only abnormal movements, but also deficits in other regions of the brain [16,17]. An impaired cerebello-thalamo-prefrontal circuit is considered to be involved in the pathophysiology of schizophrenia [18][19]. Structural and functional imaging studies revealed that the most frequently affected regions in the schizophrenic brain were the lateral ventricles, dorsolateral prefrontal cortex, anterior and posterior cingulate, superior temporal sulcus, parahippocampal gyrus, and cerebellum. A better functional outcome in schizophrenic patients was associated with increased cerebellum gray matter volume. As a matter of fact, the onset of the disorder brings substantial alterations to the neuronal maturation, resulting in reduced gray matter, especially if the condition appears in adolescence or early adulthood. Also, a bigger posterior cingulate volume, together with higher activation of the prefrontal cortex, especially anterior cingulate, dorsolateral prefrontal cortex, and superior temporal sulcus, facilitated the social and global functioning of these patients. Moreover, higher activations of the amygdala and medial prefrontal regions were observed when performing social cognitive tasks. The increased connectivity between structures improved their functional disability (particularly social and cognitive), suggesting the importance of the fronto-limbic-cerebellar circuitry in maintaining sufficient communication and balance in the networks, and provide a good functional structure for high-demanding processes [20]. The lack of gray matter integrity might mirror an excessive synaptic pruning and unsuccessful tissue growth [21].

The sequencing of cDNA of post-mortem cerebellar cortices from schizophrenic patients revealed the existence of twenty-three genes with altered expression in the analyzed samples. These genes are involved in presynaptic vesicular transport, Golgi function, and gamma aminobutyric acid (GABA) neurotransmission [22]. Also, schizophrenic individuals presented postsynaptic functional deficits. Monoaminergic, GABAergic, and glutamatergic systems developed changes in the postsynaptic receptor expression and density proteins [23]. The right cerebellar regions of schizophrenic patients revealed a higher expression of N-methyl-D-aspartate (NMDA) receptor subunit 2D, which is thought to occur secondary to the dysfunctionality of the receptor, as an upregulation phenomenon. A low expression of NMDA receptor subunit 2C was associated with the neuregulin 1 risk variant, representing a schizophrenia susceptibility gene. Strikingly, an NMDA dysfunction can bring along a defect in the GABAergic system, a fact proven by the post-mortem studies from schizophrenic patients [18].

The deep cerebellar nuclei are interconnected with the prefrontal cortex through two different pathways: via thalamus and via ventral tegmental area (VTA) [2]. The circuits are known to exist in rodents, primates, and humans and several deviations from the normal structure and function of the cerebello-prefrontal circuits were found to be associated with schizophrenia. Using this connectivity, the cerebellum is able to modulate the activity in the prefrontal cortex. A deep cerebellar nucleus that is highly involved in the disease is the fastigial nucleus, as it sends projections to the VTA, a major supplier of dopamine to the cingulate cortex.

Dopamine has long been believed to have a role in the etiology of psychosis. Several genes encoding the DRD2 receptor and the ones taking part in the regulation of dopamine synthesis through glutamatergic and GABAergic pathways can make an individual more vulnerable to dopamine dysregulation [24].

The anterior cingulate cortex is invariably hypoactive during cognitive tasks in schizophrenia [25], and cerebellar functional abnormalities were correlated with the disease [26], suggesting that an abnormal cingulo-cerebellar circuit can trigger the cognitive impairments in schizophrenia.

The volume of the cerebellum can be decreased in schizophrenic patients, and the anterior lobe of the cerebellar vermis seems to be mostly affected. Fundamental cognitive deficits are often referred to as cognitive dysmetria. Long-lasting psychosis, persistent negative symptoms and substantial psychosocial impairments were correlated with decreased cerebellar volume [27]. Also, magnetic resonance imaging revealed low gray matter volume in Crus I and II of lobule VII [28] and a selective volume reduction of

the cerebellar vermis [29]. Moreover, damage to the cerebellum can cause cognitive-affective syndrome [30]. In addition, cerebellar repetitive transcranial direct current stimulation modulated verbal working memory [31], the perceptive aspects of temporal information processing [32]. Also, it influenced cognitive operations' execution, such as procedural memory and learning through observation [33].

Schizophrenia can also be regarded as a neural network disorder, where disturbances in the glutamatergic and dopaminergic systems can trigger a transformation in the fronto-temporal-thalamic-cerebellar networks that are interconnected with other limbic-related structures [18]. Deficits can occur at molecular and cellular levels. Low numbers of oligodendrocytes, deficient myelin, and significant reductions in the SNARE (SNARE stands for SNAP receptor, SNAP refers to soluble NSF attachment protein, and NSF stands for N-ethylmaleimide sensitive factor) protein complex, which support the neural connectivity, were noticed in schizophrenia [34]. All this evidence suggests that the disorder can be classified as both neurodevelopmental and neurodegenerative pathology.

The thalamus was shown to be involved in network synchronization, and to filter the sensory information, transferring it to the cortex. By projecting to the thalamus, the cerebellum can, therefore, modulate the neural activity in the prefrontal cortex, and regulate thalamic cognitive and emotional input to the prefrontal cortex. Schizophrenic patients suffer from dysmetria of thought (Figure 1), which involves a reduced capacity to receive and process information at high speed, to evoke different memories or associations, to exercise fine-tuned responses and realize smooth coordination of mental processes [35]. This suggests that the existence of a misconnection syndrome in the cortico-cerebellar-thalamic-cortical neural circuitry can cause symptomatology characteristic to schizophrenia.

CEREBELLUM AND ATTENTION-DEFICIT HYPERACTIVITY DISORDER

ADHD represents a disruptive mood deregulation disorder and is characterized by inattentiveness, impulsive behavior, and hyperactivity. The condition is often comorbid with depression, substance abuse or other disruptive behavior disorders [36]. Several pathophysiological mechanisms are believed to be involved in the development of ADHD symptomatology. Elevated dopamine and norepinephrine concentrations were detected in the fastigial nucleus, suggesting a cerebellar contribution to ADHD. This disorder also presents an impaired regulation of dopamine release in the medial prefrontal cortex, which is ultimately modulated by the glutamate [12].

Nonetheless, the dopaminergic release in medial prefrontal cortex can also be modulated through glutamatergic synapses via contralateral mediodorsal thalamus activation, initiated in the dentate nucleus [37]. Interestingly, deep brain stimulations (which simulate lesions) in the mediodorsal thalamus increased the impulsive behavior without altering the motor function, diminished the c-Fos expression in all deep cerebellar nuclei and increased the c-Fos expression in the prefrontal cortex, indicating a decreased selective attention induced by a disrupted cerebello-thalamo-cortical pathway [38].

Cerebellar injury during childhood is frequently linked with worse consequences than cerebellar injury in adulthood, indicating that the cerebellum is especially significant during development [39]. In ADHD, a condition described by inattentiveness, impulsive behavior, and hyperactivity, several studies found cerebellar disturbances such as reduced cerebellar volumes [40], cerebellar structural differences [41] and grey matter reductions bilaterally in lobule IX [40]. Also, the severity of the symptoms correlated to the degree of posterior vermis volume reduction and decrease in structural and functional cerebellar connectivity [42]. Nevertheless, ADHD is not a permanent disorder and can be treated. One pharmacologic treatment is methylphenidate that was shown to bring towards normal the alterations form the ADHD brain. Thus, treatment with methylphenidate induces changes in cerebellar activation [43], and children chronically treated for ADHD did not show the volume reduction, indicating a possible influence on the development of the vermis. Collected data suggests that cerebellum can modulate the activity in the forebrain and cause ADHD by changing the local and long-distance functional networks [44].

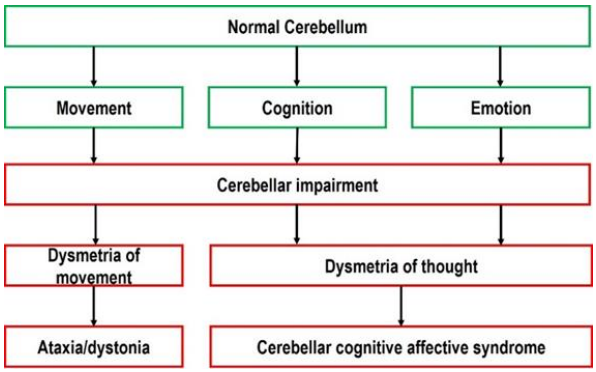

Fig. 1. Schematic of the dysmetria of thought theory (adapted after Guell et al., 2014 [45])

CONCLUSION

The functional connectivity studies presented in this work have shown different neuroanatomical substrates for the cognitive and affective disorders that are associated with cerebellar damage. Any deviations in the cerebello-limbic-prefrontal circuits can cause asynchronies in all related

networks and cause the development of cognitive affective syndromes and other behavioral abnormalities. The correction of these defects can ameliorate the severity of the symptoms, slow down the deterioration of the mind and improve the quality of life.

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TULBURĂRI COGNITIVE ȘI AFECTIVE ASOCIATE CU DISFUNCȚIA CIRCUITELOR CEREBELO-LIMBIC-PREFRONTALE

REZUMAT

Convingerea că cerebelul este implicat doar în funcția motorie s-a schimbat de curând, existând noi rezultate care susțin participarea sa si la procese cognitive superioare și afect. Un cerebel disfuncțional s-a dovedit a fi asociat cu existența rețelelor cerebrale anormale care comunică în permanență cu sistemul limbic. Această organizare poate așadar să ofere cerebelului abilitatea de a influența mai multe funcții înalte ale cortexului cerebral, precum și reglarea emoțiilor. Neuroimagistica funcțională, studiile histologice și electrofiziologice au dezvăluit detalii surprinzătoare în ceea ce privește patologiile legate de cerebel și au justificat o reconsiderare atentă a modelului tradițional de organizare a cerebelului. Lucrarea de fata prezinta potențialele mecanisme ce facilitează apariția anormalităților neurologice și comportamentale, oferind noi perspective în ceea ce privește funcțiile cerebelului.

Cuvinte cheie: emoție, cogniție, cerebel, schizofrenie, ADHD, tulburări psihiatrice

EXPERIMENTAL DEVICE FOR THE STUDY OF THE PHOTOSYNTHESIS PROCESS

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ABSTRACT
In this paper is presented an experimental electrophysiological device that allows the determination of the optimal light spectrum for the cultivation of plants under artificial conditions. The device contains a light source consisting of red and blue LEDs that are switched on in varying proportions, a biosignal amplifier and a digital data acquisition system. The electric potential collected at the level of the leaves from the stem of the plant in response to exposure to light of a certain spectrum is measured. These potentials generated by the leaves of the plant Crassula ovata illuminated with various associations of red and blue LEDs were compared. It was found that light radiation containing 60% red light and 40% blue light is the most favorable in terms of the efficiency of the photosynthesis process.
Key words: photosynthesis, biopotential, measurement amplifier, light spectrum, plant growth optimization

INTRODUCTION

The technology of cultivating plants in artificial conditions is more and more attractive because it allows obtaining crops of great interest throughout the year. Plants can be grown either by traditional methods, on the ground, in solariums, or in so-called hydroponic systems. These involve the existence of a liquid culture medium in which the roots of the plants are either immersed directly or an inert material is added, thus forming an artificial soil. This hydroponic agriculture is increasingly used both in developed countries (USA, Germany) and in arid areas of the planet [1,2,3].

The serious problems that this type of agriculture raises are: electricity consumption for pump operation and plant lighting that must be optimized both to reduce electricity consumption and to generate a light spectrum suitable for the normal and rapid development of crop plants. The study of the photosynthesis process is the central element for choosing the best light sources for growing plants under artificial conditions. Exploration of photosynthesis can be achieved through several techniques: for example, measuring carbon dioxide consumption and oxygen production in a sealed laboratory enclosure ensures accurate results but is difficult to achieve in field conditions. Another technique used in research is to measure the fluorescence of chlorophyll which also requires sophisticated equipment and well-trained staff. Determining the bioelectric potentials generated by leaves also provides good results and has the advantage of being easier to apply even in greenhouse conditions, using portable electronic equipment. It is known that plant biopotentials are the result of concentration gradients of intracalular and extracellular ions, respectively, and obviously reflect the integrity and magnitude of physiological processes of plants, and therefore photosynthesis. Current studies seek to explain the relationship between these bioelectric potentials and the wavelengths of light used [4,5,6].

In this paper we aim to establish the dependence between the size of these biopotentials and the quality of artificial light used to optimize the ratio of red light and blue light needed for plant growth in artificial environments [7,8,9].

EXPERIMENTAL DEVICE

The experimental device ensures the controlled illumination of the plant on which the determinations are performed, collects, amplifies and converts into digital signal the biopotentials generated by the plant during the

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photosynthesis process. Those signals are stored on a computer. It contains the following constructive structures: the support on which the plant sits, the light source made with 5 high intensity red LEDs and 5 high intensty blue LEDs, the light source control module powered by an adjustable laboratory source, the module for collecting and amplifying electrical biosignals generated by the plant, the power supply of the amplifier module and the interface with the computer - the acquisition board type Arduino UNO R3. Figure 1 shows the overall appearance of the experimental device.

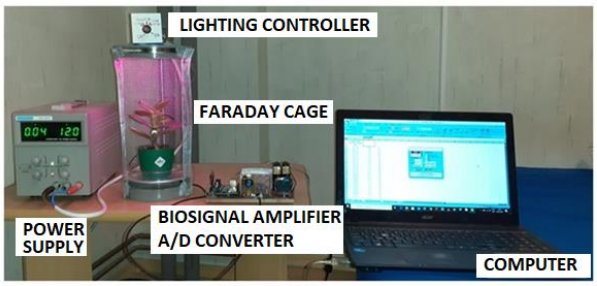

Fig. 1. The experimental device

a. Plant support

It is made in the form of a cylinder with a diameter of 110 mm and a height of 300 mm of wire mesh, and at the bottom is provided with an aluminum cap in electrical contact with the wire mesh, thus forming a real Faraday cage that shields the plant during measurements against disturbing external electromagnetic fields [10].

b. Light source controller

Ensures controlled lighting of the light source consisting of 5 high intensity red light emitting diodes (LEDs) and 5 high intensity blue LEDs. These modules are controlled by an electronic device so that they are switched on in turn in various combinations by manipulating a potentiometer. Its operation is based on the sequential opening of BD139 type transistors by means of 1N4001 diodes mounted in series [11,12].

c. Measuring amplifier

It is made using an operational amplifier type TL081, which is provided at the input with FET type transistors that provide the input impedance necessary to amplify this type of biopotential (approximately 10^{12} Ω). The amplifier is powered using a 2 x 9V double voltage from the batteries; which gives it excellent operating stability [13,14]. Two small stainless steel discs were used as electrodes for collecting biopotentials. The electrodes were fixed to the plant using a small amount of electroconductive gel for electrocardiography. The electrodes were mounted as follows: the positive electrode, which connects to the non-inverting terminal 3 of the operational amplifier, was fixed to the distal end of the tongue of a leaf, and the negative one, on the stem a few centimeters below the studied leaf [15]. Figure 2 shows how to fix the electrodes on the plant Crassula ovata.

Fig. 2. Electrodes fixed on the plant

The acquisition board type Arduino UNO R3 is used as an A / D converter and makes the connection with the computer. For the acquisition of the data obtained experimentally, the PLX-DAQ Excel application was used, which is free and compatible with Arduino. For the needs of the experiments performed in this paper, it was decided to record the value of the potential read from the plant every 3 seconds, because the electrophysiological processes in plants are extremely slow, consequently and the electric potential varies extremely slowly [16,17]. The block diagram, with the highlighting of all the modules, is presented in Figure 3.

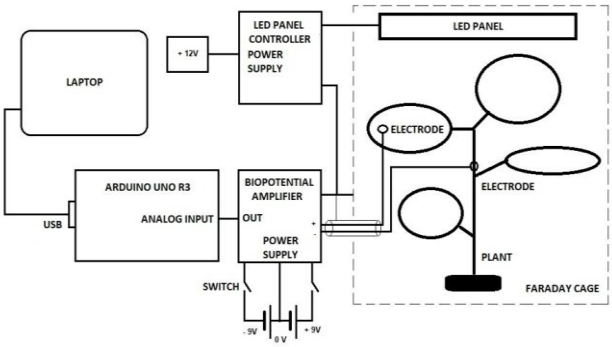

Fig. 3. Block diagram of the experimental device

EXPERIMENTAL PROTOCOL

From the literature data it is known that chlorophyll has 2 absorption maxima in the visible field of the light spectrum. The first maximum is in the blue region of the spectrum (wavelengths approximately 400-500 nm), and a second maximum in the red area of the spectrum (wavelengths in the range 700-750 nm). This is why the proposed experimental device is equipped with red and blue LEDs as light sources.

Depending on the lighting regime, 7 experiments were proposed, characterized by the following configurations (Figure 4):

All experiments were performed under reproducible conditions in a room with relatively constant humidity and temperature. The working protocol for all 7 experiments is identical.

The duration of each experiment is 3000 seconds. After each experiment the plant is left to rest in the Faraday cage in dark conditions for at least 1-1.5 hours, to restore basal physiological conditions

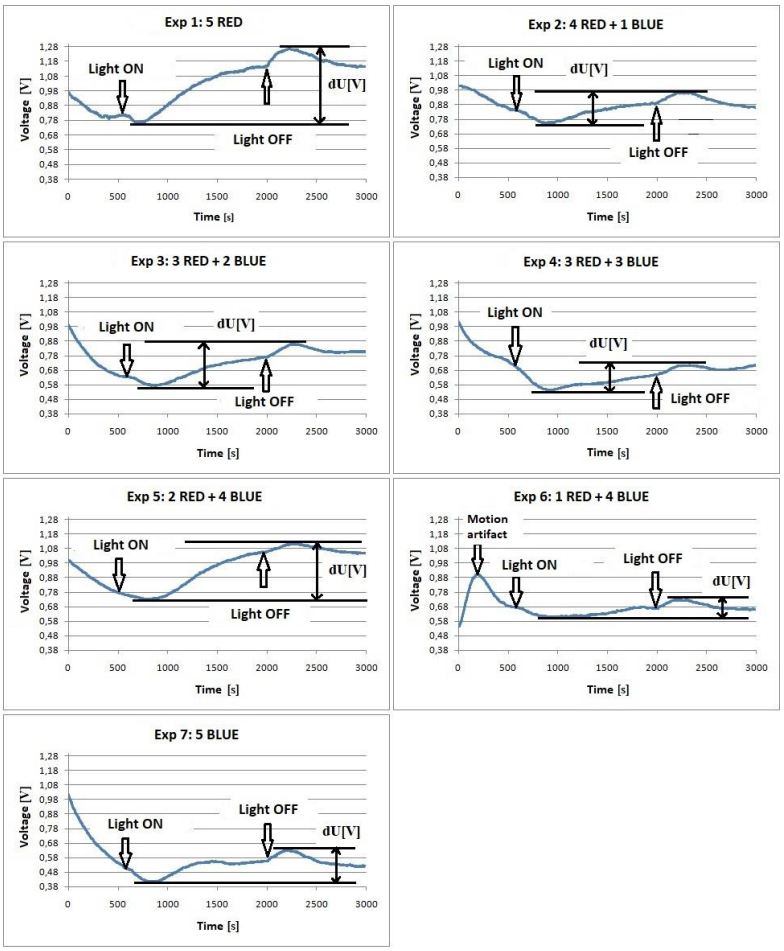

Fig. 5. The variation of the biopotentials depending on the lighting state

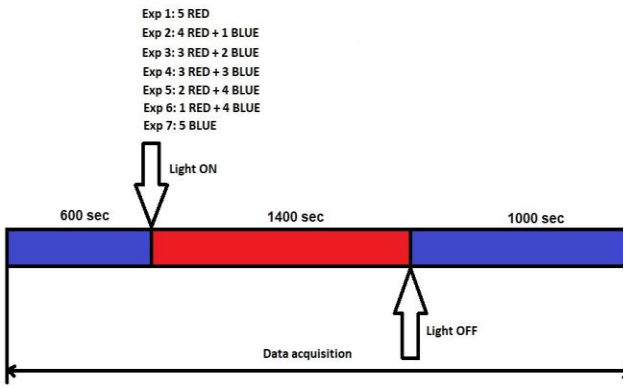

Fig. 4. Experimental protocol

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RESULTS AND DISCUSSION

Figure 5 shows the graphical records of the biopotentials collected from the same leaf for all 7 experiments.

The maximum potential difference is recorded, the moment of starting and stopping the lighting, respectively.

The temperature in the room where the experiments were performed was in the range of 22 - 230 C, and the humidity was around 50%. The room is equipped with aluminum blinds that allow almost total reduction of exterior light. A UT383 luxmeter was used to adjust the light intensity and normalize the values of the experimentally determined potentials.

Table I shows the values of the maximum potential difference recorded during each experiment, the light intensity for each experiment and the normalized value of the potential difference.

Table I. Processed experimental data

Experiment	LED association	Raw potential difference ΔU [V]	Light intensity [lux]	Correction factor	Corrected potential difference ΔU_{cor} [V]
1	5R	0.51	651	1.79	0.91
2	3R+1B	0.2	385	3.03	0.6
3	3R+2B	0.29	407	2.87	0.83
4	3R+3B	0.16	495	2.36	0.37
5	2R+4B	0.38	625	1.87	0.71
6	1R+4B	0.12	722	1.62	0.19
7	5B	0.21	1170	1	0.21

Graphically representing of the values of the potential differences corrected for all seven experiments we can deduce which are the combinations of LEDs, in other words what is the optimal spectral composition for the process of photosynthesis under the conditions given to the plant crassula ovata. In figure 6. these values are represented, as well as the acceptable range of potential difference values.

It is observed that this parameter registers the best values for lighting: 100% red LEDs, and association of 60% red LEDs and 40% blue LEDs.

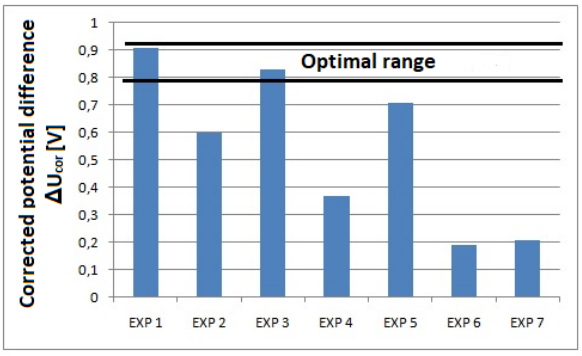

Fig. 6. Identifying the optimal spectral composition of light

In the case of all experiments it is observed that there is a latency between the lighting is turned on and the increase of the electric potential of the leaf, which can be explained by the slower propagation of the action potential in plant tissues, but also by the fact that the first stage of photosynthesis, photochemical stage it doesn't start practically instantly. Also, the potential increases for a period and after the lighting is turned off because the energy accumulated during the photochemical stage is sufficient to maintain for a period the biochemical transformations in plant cells, respectively the reduction of carbon dioxide to sugars. And this process generates a potential difference that is recorded in the form of an increase the potential after the lighting is turned off. The lighting aspect in all the 7 experiments is presented in Figure 7.

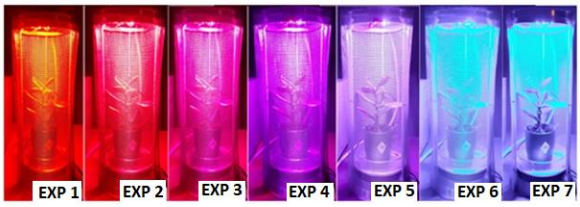

Fig. 7. Experimental light aspect

CONCLUSIONS

Experiments show that in order to facilitate the process of photosynthesis in the case of plants grown under artificial light, the presence of red light radiation in the proportion of about 60% is necessary, but the presence of blue light radiation in a proportion of about 40% is also useful. Most likely, lighting strictly with red light radiation is not beneficial in the long therm, taking into account that the absorption spectrum of chlorophyll has a maximum absorption in its blue region and not only in the red one.

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DISPOZITIV EXPERIMENTAL PENTRU STUDIUL PROCESULUI DE FOTOSINTEZĂ

REZUMAT
În prezenta lucrare este prezentat un dispozitiv experimental electrofiziologic care permite determinarea spectrului luminos optim pentru cultura plantelor în condiții artificiale. Aparatul conține o sursă de lumină formată din leduri roșii și albastre care sunt aprinse în proporții variabile, un amplificator de biosemnale și un sistem de achiziție digitală a datelor. Se măsoară potențialul electric cules la nivelul frunzelor față de tulpina plantei ca răspuns la expunerea la lumina de un anumit spectru. S-au comparat aceste potențiale generate de frunzele plantei Crassula ovata iluminate cu diverse asocieri de leduri roșii și albastre. S-a constatat că radiația luminoasă care conține 60% lumină roșie și 40% lumină albastră este cea mai favorabilă pentru desfășurarea procesului de fotosinteză.
Cuvinte cheie: fotosinteză, biopotențial, amplificator, spectru luminos, optimizarea creșterii plantei.

NEW CONCEPTS IN CEREBELLAR PHYSIOLOGY

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ABSTRACT

The cerebellum is well known for its role in movement coordination, but its impact on cognitive function has been recently emerged. The cerebellum has an intricate organization system with subunits with a sagittal disposition. The classic view over the cerebellum functions places its role as a regulator of the cerebral cortex's motor areas through the cerebellothalamic network. Recently, studies conducted in animal models by electrophysiological and histological methods, and in humans by magnetic resonance imaging, have been suggested that the cerebrocerebellar circuit is also a regulator of the non-motor regions of the brain. The involvement of the cerebellum in motor adaptation has been partly studied using non-invasive brain stimulation techniques. The cerebellum lesions cause movement incoordination syndromes such as ataxia and dystonia. Functional neuroimaging and electrophysiological studies revealed new data regarding cerebellum-related pathologies and justified a careful reconsideration of the traditional cerebellar organization model. We summarize here the research on potential mechanisms of neurological and behavioral abnormalities, providing novel insight into how cerebellum functions. The physiologic mechanisms of cerebellar functioning are still the object of ongoing research.

Keywords: cerebellum, oscillations, feedforward mechanism, modulator, motor diseases

THE CLASSIC UNDERSTANDING OF CEREBELLUM PHYSIOLOGY

For a while, some believed that cerebellum was not essential for movement and deficits could be compensated by other brain regions. However, the literature indicates otherwise since cerebellar agenesis is associated with severe movement deficits [1,2]. Afterwards, the cerebellum has been thought to be involved in fine-tuning of movements. This classic view was based on the clinical observation that cerebellar lesions were causing ataxia, a lack of movement coordination, especially in the control of antagonistic muscles during rapid movement tasks (dysdiadochokinesis), intention tremor and inability to acquire new motor skills [3]. The anatomical structure and cerebellum connections sustain its role as a significant center for motor control and generation of corrective signals for motor output [4]. The cerebellum comprises two hemispheres (neocerebellum) positioned lateral to a medial structure called vermis (paleocerebellum), and the flocculo-nodular lobe (archicerebellum or vestibulo-cerebellum), located anterior and inferior to the vermis. Based on cerebellum surface folding, it is divided into lobules numbered from I to X, both for the vermis and the corresponding lobules of the hemispheres.

Furthermore, based on gene expression and afferent fiber type, the cerebellum is organized into transverse zones that partially align with the lobules, providing functional zones [5]. Histologically, the cerebellum is composed of the cerebellar cortex and several clusters of gray matter centrally located in the white matter core, called deep cerebellar nuclei (DCN). In the cerebellar cortex, five types of neurons have been described: 1) large GABA (gamma-aminobutyric acid)-ergic neurons called Purkinje

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cells that represent the output of the cerebellar cortex; 2) the granule cells, glutamatergic neurons that synapse with Purkinje cells dendrites; 3) the Golgi cells, inhibitory interneurons of granule cells; 4) the stellate and basket cells, interneurons that inhibit Purkinje cells [6], and 5) the unipolar brush cells, small glutamatergic neurons with intrinsic activity located mainly in the vermis and the flocculo-nodular lobe, that synapse with granular cells [7]. The cerebellar cortex is formed of three layers that cover the white matter core. The molecular layer consists of stellate cells, basket cells, the axons of granular cells that form the parallel fibers, and the dendritic tree formed of Purkinje cells. The Purkinje layer contains the neuronal bodies of Purkinje cells. The granular layer comprises granular cells, Golgi cells, and unipolar brush cells [5]. The cellular connections within the cerebellar cortex are illustrated in Figure 1.

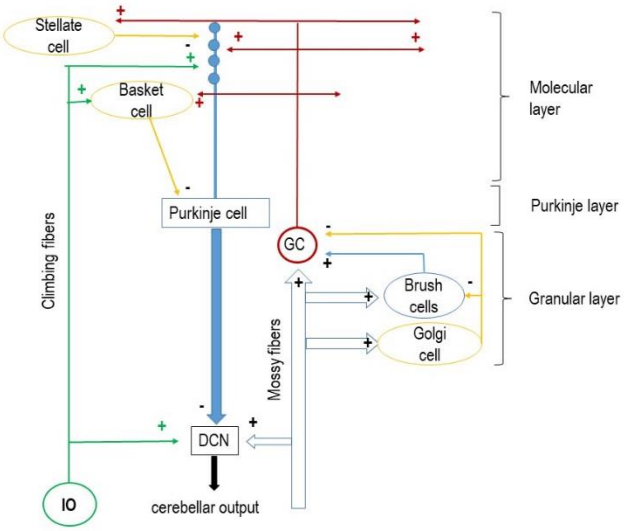

Fig. 1. Schematic representation of the cerebellum microcircuitry structural organization. IO inferior olivary nucleus; GC granular cell; DNC deep cerebellar nuclei; + excitatory synapse; - inhibitory synapse.

Inputs from the cerebral cortex, inferior olive, vestibular nuclei, colliculi, reticular formation, and the spinal cord through spinocerebellar tracts, cerebro-ponto-cerebellar pathway and the cerebro-olivo-cerebellar pathway are ending in the cerebellar cortex as excitatory mossy fibers and climbing fibers. The climbing fibers deliver information only from the inferior olive and form synapses with the neurons in the DCN and the cerebellar Purkinje cells [8]. Mossy fibers, afferents from various cerebral and spinal structures, form excitatory synapses with neurons in DCN and with granule cells and unipolar brush cells of the cerebellar cortex. Flocculo-nodular lobe receives sensory inputs from the vestibular system involved in the vestibulo-ocular processing, whereas the cerebellar vermis and the pars intermedia of the cerebellar hemispheres (paravermis) receive sensory inputs from the face and homolateral spinocerebellar systems. The lateral part of the cerebellar hemispheres processes information from the cortico-ponto-cerebellar pathway [9].

The Purkinje cells integrate all the sensory inputs from mossy and climbing fibers, and the modulatory signals of interneurons and granule cells, and send their outputs only through the DCN. The cerebellar output through cerebello-rubral, dentato-thalamic, and fastigio-reticular tracts projects to various brainstem nuclei and to the cerebral cortex. Inhibitory, GABA-mediated Purkinje cell output determines a reduction of excitatory output from DCN to the thalamus and, lastly, to the motor cortex, and thus regulates the modulation of movement. Animal studies and non-invasive cerebellar stimulation techniques in humans revealed that the cerebellum connects with both inhibitory and excitatory neurons in the motor cortex [10].

The observation that the cerebellum contains the majority of brain neurons, in a ratio of 3.6 cerebellar neurons to each neuron in the cerebral cortex, raised the idea that the cerebellar functions exceed the well-understood role in motor control [11]. Since 1986, Leiner suggested that the cerebellar circuitry may serve as a fast information-processing adjunct of the association cortex and contribute to mental functions [12].

CEREBELLUM - A MODULATOR OF BRAIN OSCILLATIONS

The oscillatory electrical cortical activity is characterized by the frequency of component waves and by their location. We cannot associate a particular oscillatory activity with a cognitive function, just as we cannot associate a single cognitive function with a frequency. In the literature, several oscillatory rhythms have been defined. In this work, we are particularly interested in the beta oscillations (13-30 Hz) and gamma oscillations (30-100 Hz) during motor tasks.

Even though growing evidence indicates that the cerebellum works together with the cortex and basal ganglia (Figure 2), the exact nature of the reciprocal connections between these three brain regions remains unclear. The cerebellum plays a wide range of critical roles through the cerebello-basal ganglia-thalamo-cortical network over various motor and cognitive functions [13]. Also, recent studies suggest that the cerebellum has an influence on downstream targets through the rubrospinal tracts and also an action on upstream centers (Figure 3) [14,15]. The cerebellum is connected via two synapses with a relay in the thalamus to the neocortex in the sensory, motor, and premotor cortical regions [16,17].

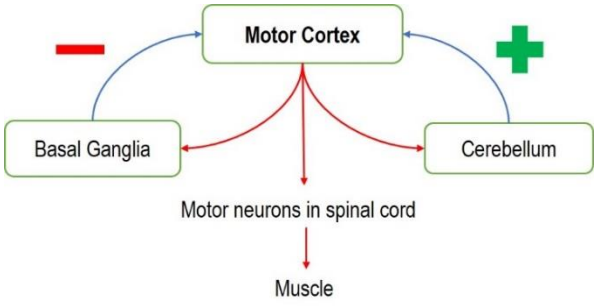

Fig. 2. Influence of basal ganglia and cerebellum over the motor cortex.

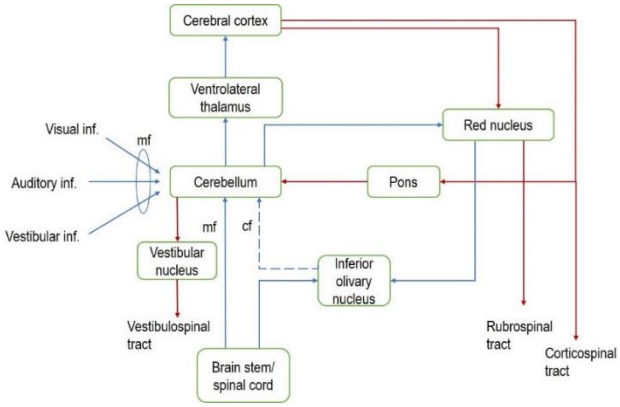

Fig. 3. Cerebellum main anatomical connections. Here, cf=climbing fibers; mf=mossy fibers; inf=information

Middleton et al. (2008) have shown in *in vitro* studies that the cerebellum is capable of generating gamma rhythms at the same frequency as the cortical areas but with different mechanisms [18]. Popa et al. studied the cerebellum's role in the sensorimotor treatment of exploratory behavior in rats [19]. During this behavior, they observed peaks of consistency, mainly in the theta, beta, and gamma frequency bands at the primary motor and sensory cortex levels. After the inactivation of the cerebellum by injecting the GABAergic antagonist muscimol, the rats' exploratory behavior was not modified, but the gamma coherence was reduced. The rate of cell discharge recorded in the motor thalamus and the motor cortex was decreased.

The cerebellum exerts an overwhelming influence on cortical gamma activity via the cerebellar-thalamic pathway. The cerebellum's contribution to rapid cortical rhythms via the cerebellum-thalamic pathway had already been shown in anesthetized cats. The thalamocortical neurons present spontaneous oscillations (30 - 80 Hz) whose pattern follows the rapid depolarizations evoked by the stimulation of the cerebellum-thalamic axons. After the damage to the cerebellar thalamic neurons, this spontaneous activity decreases. The activity of thalamocortical neurons, whether spontaneous or evoked by stimulation of the cerebellar thalamic axons, is synchronized with the field potentials recorded in the motor cortex [20,21].

In monkeys, a synchronization of oscillations in the frequency band of 20 to 40 Hz between the deep nuclei of the cerebellum and the contralateral primary motor cortex has been described during a grasping task. The monkeys had to grab a lever between their thumb and forefinger, with one or two hands depending on the previous visual cue. Then, they had to move and hold this lever for a second, a force opposing the movement. This oscillatory synchronization in the gamma band at the cerebellum level and primary motor cortex (M1) is undoubtedly involved in sensorimotor processing [22,23]. In humans, Dalal et al. carried out a magnetoencephalography (MEG) study on 12 young, healthy, right-handed volunteers. Subjects performed a task of pressing a response button with their right hand's index finger and then their left hand. Subjects should have pressed it at an interval of approximately 4 s. During the movement's preparation and execution, there was a decrease in the beta frequency in the contralateral sensorimotor cortex, which extended to the ipsilateral cortex. This decrease was accompanied by an increase in gamma oscillations (65-90 Hz) at the level of the contralateral sensorimotor cortex and the cerebellum when the button was pressed [24].

Other research has indicated that oscillations with frequencies between 4 and 25 Hz of the cerebellar cortex granule cell layer can organize communication at multiple levels [23]. By modifying the Purkinje cell simple spikes, these oscillations can influence cellular networks that then change the cerebellar cortex output. Also, the synchronization of granule cells in time (as they work as functional units) can take specific shapes for a certain spatial-aware motor task. Finally, the granule cells can also organize the communication between the cerebellum and cerebral cortex during a particular task's performance. In conclusion, these oscillations can assist in propagating information across the sensorimotor system [25–27].

THE CEREBELLUM AND MOTOR CONTROL - FEEDFORWARD MECHANISM

As mentioned above, the cerebellum is involved in learning new motor skills and in modulating the movements. Motor adaptation is a process in which the cortico-cerebellar system learns to improve motor performance in a constant environment, and predicts and corrects precise motor tasks despite the changes in environmental coordinates [28]. There are two algorithms for motor adaptation, the error-based learning that involves the cerebellum and the reward-based learning in the basal ganglia, motor commands being updated continuously [29]. Because timing, the ability to generate specific intervals between movement sequences, and the coordination of

antagonistic muscles are two parameters that need real-time adjustment during the motor task, the delayed sensory feedback is an inappropriate control mechanism [30]. It was hypothesized that movement is controlled by a forward internal model that predicts the sensory consequences of a motor command, compares the predicted result with the real sensory feedback, and generates prediction errors used to adjust movements and drive learning (Figure 4) [31]. A mathematical model of communication between the cerebral cortex, cerebellum, and basal ganglia during motor learning sustains the hypothesis that error-based motor adaptation strength decreases with the degree of visual perception alteration. It will also switch on the reward-based learning when there is a severe alteration of visual sensory afferents that blocks the cerebellum error-based algorithm [32]. The internal forward model's neural substrate is still an ongoing research subject, but all studies point to the role of olivo-cerebellar and cortico-cerebellar systems. Patients with cerebellar dysfunctions lose predictive muscle activities [33], and non-invasive cerebellar stimulation during a task deviates the hand trajectories from the target [34].

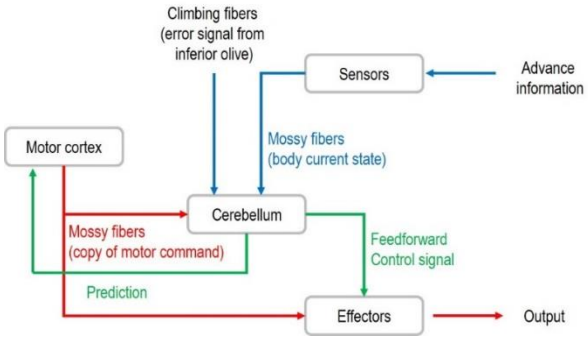

Fig. 4. Cerebellum functions as a feedforward filter

The mossy fiber-granule cell synapses and the massive climbing fiber input to each Purkinje cell are involved in motor learning [35]. Axons of olivary neurons, the climbing fibers, are direct excitatory projections to Purkinje cells, forming with them functional ensembles. The firing of adjacent olivary neurons increases sensory-driven complex spike synchrony of Purkinje cells, in sagittal oriented microbands that project to a specific set of cells in the DCN [36]. This signaling of the occurrence of movement errors through the climbing fibers is called supervised learning [13]. The bursting activity of DCN (dentate nucleus) during voluntary movement of the limb is related to mossy fiber afferents that synapse both on DCN neurons and granule cells. In the cerebellar cortex, the input from mossy fibers to Purkinje cells is facilitated feedforward through excitatory granular cells and the facilitatory effect of unipolar brush cells. The granule cells augment the number of unique representations that can be learned by Purkinje cells due to their increased number, expanding and decorrelating the cerebellar input [13]. However, there is also an inhibitory loop through Golgi cells, stellate cells, and basket cells. These parallel pathways are a competitive way to inhibit or facilitate the activity of Purkinje cells (Figure 5). Ishikawa et al. [37] observed that Purkinje cells activity is suppressed before the onset of wrist movements, and the dentate nucleus neurons released from tonic inhibition of Purkinje cells increase burst activity.

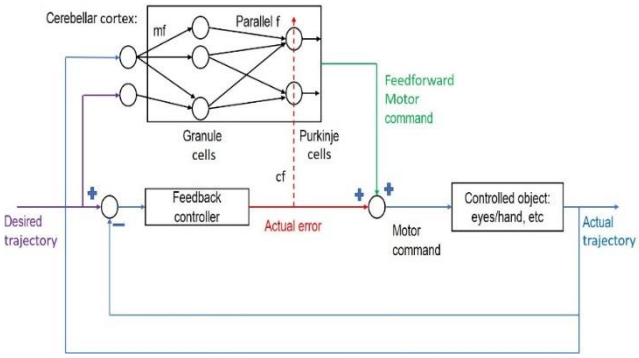

Fig. 5. Cerebellar feedback-error-learning model (modified after [66]). Feedforward reflexes and feedback of information during movement. Here, cf=climbing fibers; mf=mossy fibers; f=fibers.

Electrophysiological recordings and mathematical models revealed that the cerebellar output could predict the cerebellar input in the future. In the computing model of the cerebellum, Purkinje cells generate a predictive state from the current body state based on mossy fibers input (predictive computation), dentate cerebellar nucleus compares the predicted state generated by Purkinje cells with the sensory feedback from mossy fibers (filtering computation) and predicts a future input to the cerebellum (cerebellar prediction) [31].

CEREBELLAR DYSFUNCTION - EVIDENCE FROM DISEASE STATES

In the classical view, the cerebellum function is exclusively participating in motor activity through the influence on the primary motor cortex, after receiving input from other cortical areas [38]. Recently, new research has resulted in a change of paradigm. It is now clear that the cerebellum's output reaches thalamus and from there multiple regions of the neocortex [39].

Cerebellar disturbances trigger motor symptoms such as dysmetria, hypotonia, tremor, and dysarthria. These symptoms were first described by Sir Gordon Holmes in 1939 [40]. Nevertheless, cerebellar dysfunction of the Purkinje cell's anatomy or function affects the precision of

movements, which is generally described as ataxia. In ataxic mice and humans, Purkinje cell abnormalities seem to be common, irrespective of the type of ataxia and appear to be present before symptoms [41–43]. Indeed, in mice with ataxia, it has been proposed that improper cell firing of Purkinje cells is the main modification that causes motor abnormalities [44].

Two-way interactions between the cerebellum and the basal ganglia are essential for motor and cognitive functions [45]. Through these connections, the abnormal activity can be transmitted from one region to the other. These pathways contribute to the understanding of cerebellar contribution in two pathologies: dystonia and Parkinson's disease [13,45].

Dystonia is a heterogeneous movement disorder characterized by involuntary, prolonged muscle contraction that causes repetitive or abnormal movements [46–48]. Every muscle of the body can be affected, such as palpebral muscles (blepharospasm in the eyelids) [49], writer's cramp [50], torsion dystonia of the trunk [51]. Even though dystonia has multiple manifestations, dystonia consists of faulty communication in the circuit formed by the cerebral cortex, thalamus, basal ganglia, and brainstem [52]. The role of the cerebellum was recently demonstrated by several research groups [53–55]. Currently, the general agreement is that in dystonia, the communication is disturbed in two circuits: the cerebello-thalamo-striatal circuit and cerebello-thalamo-cortical circuit [56,57]. Calderon et al. recently showed that rapid-onset dystonia-parkinsonism that defects in either the basal ganglia or the basal ganglia could trigger the disease [58]. In rodents, the cerebellum's surgical elimination stops the dystonic attacks, suggesting the idea that the cerebellum can induce dystonia [59,60].

Altered metabolic activity within the cerebellum, associated with the deterioration of the midbrain dopaminergic neurons is found in another motor disease, Parkinson's disease. Parkinson's disease is one of the major neurodegenerative disorders characterized by bradykinesia, rigidity, and tremor [61]. The damage of these neurons has vast functional implications in the basal ganglia and elsewhere, especially in the motor cortex and, lately, in the cerebellum [62]. Also, cerebellar tremor is a symptom that is associated with cerebellar damage. The pathophysiology of this tremor type is not well understood [63].

Also, cerebellar abnormalities have been found to play a role in many developmental disabilities, such as autism, attention deficit-hyperactivity disorder, and progressive dyslexia. Damage to the cerebellum during early development can potentially cause long-term consequences on movement, cognition, and even emotional regulation [64], [65]. Table I illustrates a few disorders in which the cerebellum was proven to be involved in the disease's pathophysiology.

Table I. Non-exhaustive list of diseases with cerebellar involvement [67].

Motor diseases	Non-motor diseases
Ataxia	**Autism spectrum disorders**
Huntington's	**Sleep apnea**
Dystonia	**Dyslexia**
Parkinson's	**Fetal alcohol syndrome**
Tourette's	**Obsessive-compulsive disorder**
Tremor	**Schizophrenia**
Multiple sclerosis	**Vertigo**

CONCLUSION

The data presented here support that the pathogenic mechanisms of the motor diseases provide new insights into cerebellar connectivity. New technologies such as tracing of axonal pathways developed to gather information, visualizing climbing and Purkinje cell function by means of magnetic resonance imaging, together with development of relevant animal models, could further broaden the knowledge of the cerebellum physiology. However, even though the precise relationships between different cortical areas and brainstem nodes with the cerebellum were described, the cerebellum's contribution to the information processing in these structures is far more complex and more studies are required in order to be fully revealed.

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NOI ASPECTE PRIVIND FIZIOLOGIA CEREBELULUI

Rezumat
Cerebelul este binecunoscut pentru rolul său în coordonarea mișcărilor, dar recent a fost evidențiat impactul sau în funcțiile cognitive. Cerebelul are un sistem complex de organizare, cu subunități anatomo-funcționale cu dispoziție sagitală. Concepția clasică asupra funcțiilor cerebelului îi plasează rolul de modulator al ariilor motorii ale cortexului cerebral prin rețeaua cerebelo-talamică. Recent, studiile efectuate pe modele animale prin metode electrofiziologice și histologice, precum și studiile clinice de neuroimagistică funcțională, au sugerat că circuitul cerebro-cerebelar este, de asemenea, un modulator al activității regiunilor non-motorii ale creierului. Implicarea cerebelului în adaptarea motorie a fost studiată parțial folosind tehnici non-invazive de stimulare a creierului. Leziunile cerebeloase provoacă sindroame de incoordonare motorie, cum ar fi ataxia și distonia. Studiile de neuroimagistică funcțională și electrofiziologice au scos la iveală date noi privind patologiile legate de cerebel și au justificat o reconsiderare atentă a modelului tradițional de organizare cerebeloasă. Rezumăm cercetările privind mecanismele potențiale ale anomaliilor neurologice și comportamentale asociate disfuncției cerebeloase, oferind o perspectivă nouă asupra modului în care funcționează cerebelul. Mecanismele fiziologice ale funcționării cerebelare rămân o temă actuală de cercetare.
Cuvinte cheie: cerebel, oscilații, mecanism de feedforward, modulator, afecțiuni motorii

CRYOPRESERVATION OF NATURAL KILLER CELLS

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ABSTRACT
Cryopreservation is a method used to preserve cells and tissues for a long period of time. The ideal procedure should keep the structure of the cells intact and should not affect their function. Although the use of cryopreservation dates from the begging of research, the cryobiological response is different for every type of cells so an optimization of the process is needed. The progress made in the past years to understand the biology and function of natural killer cells led to the use of the cells in immunotherapy. The manufacturing strategies of NK cell products are highly costly and time consuming due to the complex materials needed and small number of specialized institutions. Cryopreservation of the cells can lower the costs for quality tests and facilitate the shipping transport of the products for having an "on-demand" available therapy. The article discusses the effects of different cryopreservation methods on NK cell's viability and function, highlighting the importance of cytokine IL-2.
Key words: NK cells, cryopreservation, cytokines

NATURAL KILLER CELL BIOLOGY

Natural killer cells are granular lymphocytes, part of the innate immune system, that can kill tumoral and virus infected cells. Their phenotype is characterized by expression of CD56 and CD16 surface antigens and lack of CD3/TCR (Campbell & Hasegawa 2013). Two types of NK cells populations can be identified depending on the density of CD56 and CD16 receptor: CD56 bright CD16 dim with high cytokine production, and CD56 dim CD16 bright with high cytotoxic activity (De Maria *et al.* 2011; Campbell *et al.* 2001).

On the surface of natural killer cells are several activating and inhibitory receptors which can be triggered by soluble factors (cytokines IFN alpha, IFN beta, TNF, IL-12, IL-15) in the environment or by ligands on the surface of target cells. The activating receptors (AR) can be C-type-lectin-like activating receptors like NKG2D or natural cytotoxicity receptors (NCR) like NKp46, NKP30, NKp44. Two groups of inhibitory receptors are described until now. The inhibitory-receptor superfamily (IRS) comprises the C-type-lectin inhibitory receptor (CLIR) and killer-inhibitory receptor (KIR). NK cells express several KIR specific for different MHC molecules or for closely related MHC molecules.

The activating receptors on natural killer cell bind to the unusual molecules on the potential target cell, which act like a red flag indicating the fact that the cell has been stressed, sending an activation signal for the lysis of the cell. If the inhibitory receptors encounter normal levels of MHC I molecules expressed on the surface of the cell, then the inhibitory signal overrides the activation signal.

The viral or tumoral transformation of the cell cause downregulation of MHC class I molecules and the upregulations of the stress ligands on the surface of the cells. Upon the encountering of NK cells, the inhibitory receptors will not engage with the MHC class I molecules and the activating receptors will bind to the stress ligands determine a killing signal for the target cell. The NK cell will kill the tumoral cell by releasing the cytoplasmic vesicles with perforin and granzyme. The tumor cell apoptosis can be induced also by expressing FasL (TNF–family member) and TRAIL (TNF-related apoptosis inducing ligand) which will bind to their respective receptors. However, not only the lack of inhibitory signal due to missing MHC class I molecules, but the balance of the signals received by the inhibitory and activating receptors determine the recognition and killing of the target cell.

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The activation of natural killer cells to kill the target cells is made in an unrestricted HLA fashion, enabling the use of allogenic source of NK cells to treat malignant disease without triggering graft-versus-host diseases (Miller *et al.* 2005, Blazar *et al.* 2018).

ADOPTIVE TRANSFER

The use of activated and expanded allogenic NK cells had led to promising results in immunotherapy compared to autologous NK cells, due to the higher possibility of mismatch between KIR-donor HLA that will not trigger the inhibitory receptors as in the case of autologous cells (Ruggeri *et al.* 2015, Miller *et al.* 2005, Rubnitz *et al.* 2010, Rezvani & Rouce 2015)

The amount of allogenic NK cells used in clinical trials is higher than the actual number obtained from donor-derived leukapheresis products (Lapteva *et al.*, 2014). This call the need for expansion of NK cells from peripheral blood mononuclear cells (PBMCs) with the use of feeder cells. The feeder cells can expand NK cells from PBMC by 21.6 fold in 7 days (Fujisaki *et al.*, 2009), or from cryopreserved apheresis products by 70-fold in 8 days (Lapteva *et al.*, 2014) Enrichment of NK cells is necessary to generate high purity needed for therapy use (Zeng *et al.* 2017).

ALTERNATIVE SOURCES

Although the majority of clinical trials are focusing more on primary NK cells obtained from PBMC, some other alternative sources from bone marrow, human embryonic stem cells (hESC), induced pluripotent stem cells (iPSC) and from umbilical cord blood started to get into attention (Woll *et al.* 2009; Chouaib *et al.* 2014). Generation of NK cells from umbilical cord blood passed the experimental stage to clinical trials with promising results (Spanholtz *et al.* 2011).

The option of generating NK cells from umbilical cord can be an "off-the-shelf" source of NK cells due to the high numbers of NK cells present in the cord blood and naïve T cells that have a lower risk of causing GVH D (Shah *et al.* 2013; Shaim & Yvon 2015). Studies showed low expression of adhesion molecules of CB-NK that determine weaker synapses with target cells (Dalle *et al.* 2005; Tomchuck *et al.* 2015). CB-NK have high expression of lectin-like inhibitory receptors CD94/NKG2A and low expression of KIR, which suggest an immature phenotype (Della Chiesa *et al.* 2011).

A new strategy for generation of functional CB-NK using an aAPC platform obtained aAPC-expanded CB-NK cells with a phenotype similar with PB-NK cells, which were able to form strong immune synapses with target cells (Shah *et al.* 2013). A number of phase I/II clinical trials are underway to test the feasibility and efficacy of CB-NK cell adoptive therapy in patients with hematologic malignancies (NCT01619761, NCT01729091 NCT02280525, NCT01914263, and NCT00412360).

HUMAN NK CELL LINE

Another source of NK cells that can be taken into considerate are human NK cell lines. First of all the products are more available and easy to expand then other donor-derived NK cells and second the lack of expression of most KIRs makes the NK cell lines less immunogenic and with higher chances of antitumor activity *in vivo* (Pittari *et al.* 2015, Tonn *et al.* 2013). The most characterized NK cell line is NK-92 (Fig.1) used in most of preclinical and clinical trials (Pittari *et al.* 2015, Tonn *et al.* 2013, Klingemann *et al.* 1996). NK-92 are cells of malignant origin that need to be irradiated for their use in immunotherapy (Klingemann *et al.* 1996).

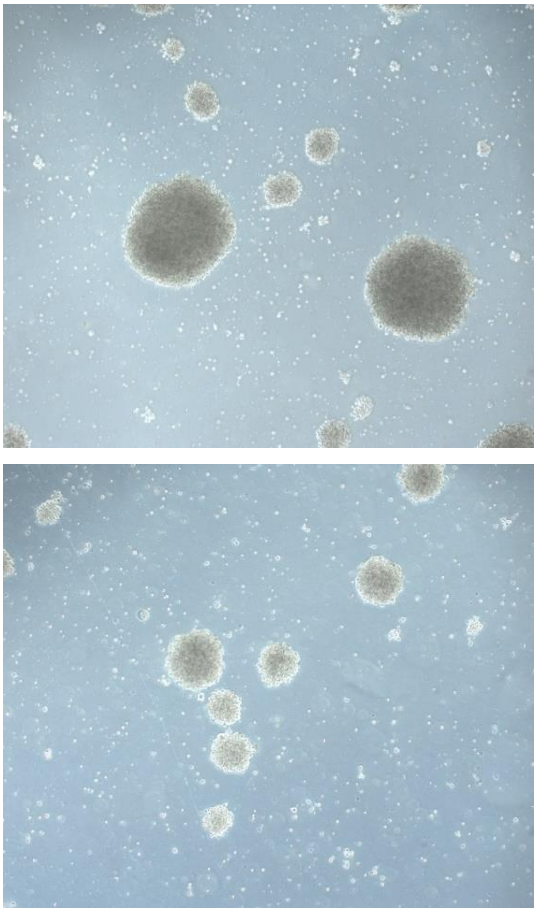

Fig. 1. NK-92 cells in culture at (A) low and (B) high density (magnification 50x). The aggregates indicate high viability and good recovery after thawing.

IL-2 IMPORTANCE IN CRYOPRESERVATION. PHENOTYPE AND FUNCTION

Mata *et al.* 2014 studied the differences in ADCC and NK activity of cryopreserved PBMC that were thaw/overnight rested and thaw/not rested to the freshly isolated PBMC from the same donors. The cytotoxic activity was measured by using two different methods ^{51}Cr release assay and CD107a expression analyzed on flowcytometer. They observed that in frozen/not rested $CD56^{dim}$ the expression of CD107a was upregulated when non-stimulated compared to the fresh or frozen/rested overnight cells. The CD107a expression of frozen/not rested cells is not significantly differently from the fresh and frozen/rested NK cells when stimulated by target cells. In each case of treatment the cell populations had similar expression of receptors with no significant difference. The viability of cells upon thawing was 80% and after overnight resting 70%.

Standardized protocols and panels for flowcytometry assay were used by (Veluchamy *et al.* 2017) to study the effect of different culture conditions on PBMC samples. They tested the phenotype and function of NK cells from fresh, cryopreserved PBMC from which samples were split into two fractions of non-activated and cytokine-activated overnight with 1000 U/ml IL-2 and 10ng/ml Il-15. They did not perform any depletion of the cells to obtain NK cells, but they adjusted the numbers needed for cytotoxicity assay to the number of target cells. The results showed no significant changes in the phenotype of NK cells from cryopreserved PBMC compared to the fresh samples exposed to A41 cells alone and A431 cells treated with CET. Expression levels of markers NKG2A, NKG2C, NKG2D, KIR2D, NKp44 showed no significant difference between non-activated fresh and cryopreserved NK cells from PBMC either activated or non-activated with cytokines. They observed that when cryopreserved samples activated with cytokines were exposed to A431 alone the CD16 expression levels were significantly reduced in total NK cell CD16 population and in CD56dim CD16+ NK cell subpopulation. However the levels of CD16 in NK cells from PBMC exposed to A431+CET were not different in fresh and cryopreserved samples. Fresh PBNK cells stimulated with cytokines expressed higher levels of CD25 when exposed to A431 alone, but this did not affect the degranulation status of cells. The expression levels of CD107a did not differ significantly between fresh and cryopreserved samples.

Another study performed by (Koehl *et al.* 2013) analyzed the effect of IL-2 stimulation of NK cells before cryopreservation. After the depletion and enrichment from PBMC, NK cells were cultured for 10-12 days in 1000 rhIL-2 and then cryopreserved. The viability of IL-2 stimulated NK cells after unfreezing was 2x higher compared to unstimulated NK cells. The effect of IL-2 stimulation was also significant for NK cell recovery: a media of 84% for IL-2 stimulated cell was obtained compared to 22% for unstimulated cells. The IL-2 stimulation of NK cells determined an increase in expression levels of surface activating receptors.

Expansion of NK cells with different sources of feeder cells is a possibility to produce large numbers of functional effector cells. Torelli *et al.* 2015 showed that expansion of NK cells with irradiated (35 Gy) autologous monocytes, T and B cells as feeders and Il-2, IL-15 for 14 days upregulates the activating receptors NKG3D, DNAM1, NKp30, NKp44 compared to fresh samples. To identify changes that can occur due to cryopreservation of expanded NK cells they analyzed the degranulation capacity and cytolytic activity of fresh and cryopreserved expanded effector cells. The CD107a expression levels were similar in case of fresh 84+-3.6% and cryopreserved NK cells 87.6+-2.5% when stimulated with PMA and ionomycin. In presence of K562 the cryopreserved expanded NK cells had a mean percentage of 31.2%+-8.7 at an E:T ratio of 1:1. No significant difference was observed for cytolytic activity of fresh 80.1% ±3.4 and cryopreserved 72.9% ±5.8 expanded NK cells in presence of K562 cell line. Viability of cryopreserved expanded NK cells after thawing was high with a media of 80-90%.

Lapteva *et al*. 2012 established a cryopreservation method with a 90% recovery of expanded NK cells. Even though the recovery rate was high, the expanded NK cells had low cytolytic activity against K562 if not let to rest overnight. An important finding was the fact that the recovery of cells after thawing depends on the donor with a variability of 51-95%.

The results of Berg *et al.* 2009 showed that cryopreserved feeder-expanded NK cells require IL-2 culturing after thawing to restore their cytolytic activity. They observed that incubation of thawed expanded NK cells in 500 IU/mL Il-2 medium for 6 hours increased with 50% their function and receptor expression of TRAIL and NKG2D. By culturing the NK cells for 16 hours in Il-2 media the expression of TRAIL and NKG2D, together with their cytolytic activity, restored to the same levels as in freshly isolated cells. The viability of cells declined from 93-97% after thawing to 38-50% after 16 hours cultivation in IL-2.

Two types of NK cells products: freshly activated with 1000 U/mL IL-2 (FA-NK) and expanded NK cells with K562 cells expressing membrane bound IL-15 and 41BB-L and 10 U/mL IL-2 (EX-NK) were cryopreserved and tested for their viability and function (Miller et al. 2014) . To recover the cells they cultured the EX-NK and FA-NK overnight in IL-2 medium. Viability of cells after overnight culture was different 20% viable cells in case of EX-NK and 73% in case of FA-NK. The cytolytic activity of cryopreserved cells against K562 tumoral cells tested immediately after thawing was low, but increased after overnight culture in IL-2 medium. The viability of both types of cells after thawing was 90%, but when the cells were infused into the mice immediately after thawing they observed lower numbers of cells present than in the case of non-frozen NK cells. Surprisingly the FA-NK cultured expanded at the same level like fresh FA-NK in mice given IL-15.

CRYOPRESERVATION OF DIFFERENTIATED NK CELLS

Nham *et al.* 2018 showed that expanded CB-NK derived from fresh, short-term, long-term cryopreserved cells produce similar levels of pro-inflammatory cytokines like IFN-γ and TNF-α when stimulated with a cocktail of IL-12, IL-15, and IL-18. Also they did not observe any difference between fresh and cryopreserved CB-NK when tested their cytotoxicity against breast cancer cell lines TNBC Cell Line, MDA-MB-231 and primary breast cancer cells. More, the percentage of lysed tumoral cells increased when CB-NK cells were stimulated with IL-12/IL-15/IL-18 for 24 hours. The increase in cytotoxicity can be explained by higher percentages of surface activation markers like CD69, NKp30, NKp44, CD25 in expanded CB-NK.

Cryopreservation of NK cells differentiated from CB CD34+cells did not affect their phenotype and cytotoxic activity when compared to fresh differentiated cells (Domogala *et al.* 2016). The cryopreserved NK – CB CD34+ cells had the same proliferation rate like fresh NK-CB CD34+ cells and PB-NK control when stimulated with IL-2 or IL-15. Although the function of the NK-CB CD34+ was not affected by cryopreservation, the recovery rate after thawing the cells was low. In their work of differentiating NK cells from CBCD34+ (Domogala *et al.* 2016) proposed that cryopreservation might select for the most potent CBCD34+ cells which can lead to higher numbers of NK cells at the end of the culture without having compromised phenotype and function.

In their study, Zeng *et al.* 2017 observed that expanded PBC-iPSC-NK maintain their function after cryopreservation, but had low percentage of viability. They showed that PBC-iPSC-NK remain expandable after cryopreservation. A solution proposed by Zeng *et al.* 2017 is to cryopreserve the cells after differentiation and expand them only when used after thawing, which will lead to higher numbers of cells.

IMPROVEMENTS IN CRYOPRESERVATION AND THAWING PROCEDURE

In cryopreservation the main issue is the cryoinjury of cells due to intracellular and extracellular formation of ice crystals and osmotic changes (Mazur 1970)(Pegg 2002). To prevent the ice crystal growth and water transport is important to use a cryoprotectant. Depending on the cell type the cryopreservation media is prepared using different concentration of cryo-agents, which will replace the water in the cytoplasm during the slow freezing and protect the cell (Karlsson & Toner 1996)(Gao & Critser 2000). The most used cryo-agent is Dimethylsulfoxide (DMSO) due to lower cytotoxicity than other cryo-agents, low cost and best results in protection against freeze injury of cells (Jang *et al.* 2017).

Pasley *et al.* 2017 developed a DMSO-free cryo-agent for cryopreservation of NK-92 cells. The cells were compared with the "off the-shelf" NK-92 cryopreserved in DMSO in terms of viability, cytotoxic activity against K562 and CD107a degranulation. They obtained 70% viable cells with the same behavior like the control cells. In case of CD107a degranulation the NK-92 cryopreserved in DMSO-free media performed better than the controls. The DMSO-free media dextran and ectoine cryo-agents, eliminates the wash step in thawing procedure and avoids cell loss.

Hønge *et al.* 2017studied the effect of different thawing procedures on the viability of cryopreserved PBMC. They found that the composition and temperature of thawing medium, the centrifugation force, centrifugation duration, and on the incubation duration influenced the viability of PBMC cells. The thawing medium in which the cells had the higher viability was 20% FBS and 80% RPMI. Warm medium is recommended for thawing the cells.

The freshly collected cells are preferred because cryopreservation can affect their viability and functionality, but this causes donor-variability (Lapteva *et al.* 2012) and inter-laboratory variation, present in multi-center clinical trials (Hensley-McBain *et al.* 2014; Stevens *et al.* 2007; Trück *et al.* 2014; Hønge *et al.* 2017). However, cryopreservation of NK cells and batch production limits the drawbacks of real time analysis of fresh samples over different time points, thus reducing instrument variability and allowing generation of more consistent results (Veluchamy *et al.* 2017). It is considered that an ideal product for NK cell therapy should have a high percentage of viability, purity and potency and should be available in time for more than one infusion of the same product. (Lapteva *et al.* 2014)

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CRIOCONGELAREA CELULELOR NATURAL KILLER

REZUMAT

Criocongelarea este o metodă folosită pentru conservarea celulelor și a țesuturilor pentru o perioadă mai îndelungată de timp. Procedura ideală ar trebui să păstreze intactă structura celulelor și să nu afecteze funcția acestora. Cu toate că criocongelarea datează de la începutul cercetării, răspunsul criobiologic este diferit pentru fiecare tip de celulă fiind nevoie de o optimizare a procesului. Progresul realizat în ultimii ani pentru a înțelege biologia și funcția celulelor natural killer a dus la folosirea acestora în imunoterapie. Strategiile de producere a batch-urilor de celule NK sunt costisitoare și consumatoare de timp datorită materialelor complexe necesare și a numărului mic de instituții specializate. Criocongelarea celulelor poate diminua costurile și poate facilita transportul produsului pentru a avea o terapie valabilă la cerere. Prezentul articol discută efectele diferitelor metode de congelare asupra viabilității și funcției celulelor NK, accentuând importanța citokinei IL-2.

Cuvinte cheie: celule NK, crioprezervare, citokine

THE MICROBIOTA-GUT-BRAIN AXIS – A COMPLEX SYSTEM CROSS-TALK

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ABSTRACT
There is growing evidence suggesting a bidirectional interaction between the central nervous system, the enteric nervous system, and the gastrointestinal tract. Recent studies show that a pivotal factor for these gut-brain interactions is the gut microbiota. The microbiome was recently associated with modulation of behavior, cognition, and mental health. Multiple mechanisms, including nervous, endocrine, immune, and metabolic pathways are involved in the gut microbiota-brain axis, and conversely, the brain can also regulate the gut microenvironment. A more thorough understanding of the relationship between gut microbiota and the brain could advance our knowledge about the physiopathology of neurological diseases and help identify new target therapies via modulation of the microbiome.
Keywords: microbiome-gut-brain axis, central nervous system, enteric nervous system, autonomic nervous system, hypothalamic-pituitary-adrenal axis, neurological disorders

INTRODUCTION

The human gut microbiome is a complex collective community of bacteria, archaea, and unicellular eukaryotes [1], which acts as an adaptive interface with the environment. Most gut bacteria are facultative anaerobes spanning 7 different phyla (*Bacteriodetes, Firmicutes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria*, and *Actinobacteria*) [2]. The *Firmicutes* and *Bacteroidetes* phyla harbor the most abundant species in the gut microbiome, gathering approximately 90% of the adult human microbiome [2,3]. The intestinal microenvironment is a dynamic entity, its diversity and composition modifying with human development. It colonizes the gastrointestinal tract immediately after birth and during the first year of life undergoes continuous change [4]. Moreover, it can be influenced by numerous factors (Figure 1) [5], such as diet, antibiotic use, and stress, which were found to change the microbiome and significantly affect the health of individuals [6–8]. Aging is also associated with a decline in the diversity of gut flora, which correlates with the host health and life span [9–11].

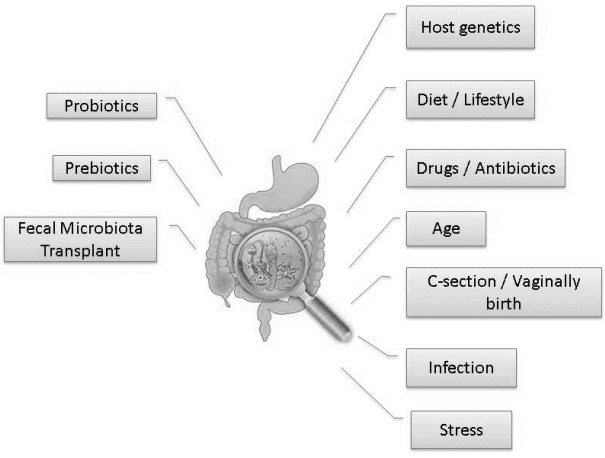

Fig. 1. Putative factors modulating gut microbiome.

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Knowledge about microbiota and its modulatory effects on the host has expanded rapidly in the recent period and interest has grown in the role of the human gut microbiome in the physiopathology of diseases. Alteration of the composition of the flora, known as dysbiosis, contributes to the development of numerous disorders such as irritable bowel syndrome (IBS) [12], obesity and metabolic dysfunction [13], intestinal cancers [14], allergies [15], and type I diabetes [16]. Emerging evidence points to the impact of gut bacteria on neurological outcomes, by influencing the brain's development, behavioral, and cognitive functions [17].

Currently, the exact mechanisms of interactions between microbial communities and the brain have not yet been fully elucidated and understood. The communication between gut microbiome and brain is referred to as the microbiota-gut-brain axis (MGBAx) and it's a bidirectional communication (Figure 2) [18,19]. This interaction network includes the nervous systems, such as central (CNS), autonomic (ANS), and enteric nervous system (ENS), the hypothalamic-pituitary-adrenal axis (HPA), the gut microenvironment, and the blood-brain barrier (BBB), communicating through neurotransmitters, hormones, neuropeptides, and microbial-derived metabolites [20]. The enteric bacterial community expands its influence on the brain via numerous neuro-immune-endocrine pathways (Figure 3) [21].

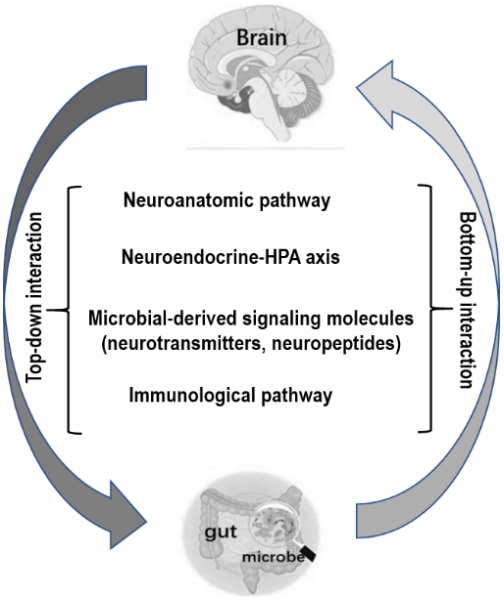

Fig. 2. Schematic representation of the bidirectional microbiome-gut-brain axis.

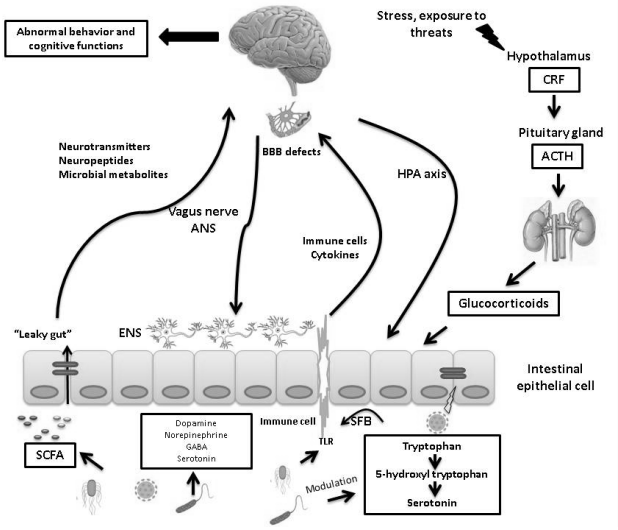

Fig. 3. Potential relationships between the microbiota and the brain. The CNS can interact with the microbiome through neuroanatomical pathways such as the ANS, ENS, and vagus nerve. The CNS can also communicate, through the HPA axis, with the gut microbiota. HPA axis is activated in response to stress and is finalized with the release of glucocorticoids from the adrenal glands. The microbial production of metabolites (SCFAs), and neurotransmitters (dopamine, norepinephrine, GABA, serotonin) can cross the "leaky gut" and can affect brain function. The serotonin synthesis is also modulated by the bacterial community. The enteric microbiome can activate gut immune cells through TLR, which can release cytokines into the bloodstream. SFB can restore the function of B and T lymphocytes. An impaired BBB favors the circulation of substances to the brain. CNS, central nervous system; ANS, autonomic nervous system; ENS, enteric nervous system; HPA, hypothalamic–pituitary–adrenal; SCFAs, short-chain fatty acids; GABA, γ-aminobutyric acid; TLR, Toll-like receptor; SFB, segmented filamentous bacteria; BBB, blood-brain barrier; CRF, corticotropin-releasing factor; ACTH, adrenocorticotropic hormone.

NEUROANATOMICAL ROUTES BETWEEN GUT AND BRAIN

The gut microbiome-brain interplay relies on two neuroanatomical pathways. The first one is the direct communication between the gut and brain via the autonomic nervous system (ANS) and vagus nerve (VN) in the spinal cord. To support this, vagotomized mice didn't exhibit the neurochemical and behavioral effects determined by specific bacteria (*Lactobacillus* strain) in non-vagotomized rodents, thus, identifying the vagus as a major communication pathway in the MGBAx [22]. Using a rodent model of chronic colitis, it was proved that anxiety-like behavior was a consequence of the gut inflammation and probiotic treatment (*Bifidobacterium longum*) managed to efficiently improve the altered anxiolytic effect. In this study,

the effect of probiotic was suppressed by vagotomy, implying that vagal routes are critical for the intercourse between the gut microbiome and brain [23].

The other pathway of interaction is between the enteric nervous system (ENV) in the gut and the ANS and VN in the spinal cord [24]. The ENS is a branch of the ANS that regulates the gastrointestinal motility, its local blood flow, mucosal transport and secretions, and also the immune-endocrine functions [25]. The microbiota was found to influence the functional activity of the ENS, either through Toll-like receptor 2 signaling [26] or by stimulating macrophages to secrete bone morphogenetic protein 2 (BMP2) and, subsequently, to modify the pattern of smooth muscle contractions [27].

The enteric microbiome also induces the maturation of ENS by discharging serotonin and activating its receptors [28]. Germfree (GF) mice (raised without any microbial exposure) are useful tools to assess the MGBAx [29]. GF mice were found to have an immature ENS and colonization with microbiota from conventionally raised mice managed to modify the anatomy of ENS in a serotoninergic manner [28]. Serotonin is mainly produced in the gut (approximately 95%), where it plays a key role as an important signaling molecule throughout fetal and adult life [30]. Based on its connections with the ENS, CNS, and microbiome, serotonin may be a pivotal link in the MGBAx.

THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS – THE NEUROENDOCRINE PATHWAY

The hypothalamic-pituitary-adrenal (HPA) axis represents the major neuroendocrine component that regulates the brain responses to threatening stimuli [31]. The most important regulator of the HPA is the corticotropin-releasing factor (CRF) released from the paraventricular nucleus (PVN) of the hypothalamus, which induces the release of adrenocorticotropic hormone (ACTH) into the bloodstream and subsequently the secretion of glucocorticoids [31].

Chronic stress leads to abnormalities of the HPA and an increased level of glucocorticoids, responsible for the development of anxiety and depression. Latest research points to a direct interaction between the microbial community and the neuroendocrine system. A link between these two is suggested by the fact that episodes of anxiety and depression may be experienced more frequently in patients with IBS [32].

GF mice exhibited elevated plasma ACTH and glucocorticoid levels relative to specific pathogen-free (SPF) mice in response to stress, concentrations that were reversed by the reconstitution of gut flora with probiotics (*Bifidobacterium infantis)* [33]. This finding is suggestive for increased activation of the HPA in response to stress as a result of the absence of enteric microbial colonization. Furthermore, when compared to SPF mice, GF mice revealed a lower level of brain-derived neurotrophic factor (BDNF) expression in the hippocampus and cortex, an effect also shifted by the administration of probiotics [33]. BDNF has several roles in neuronal survival and growth, serving as a neuromodulator and being essential for learning and memory [34]. The exaggerated HPA response to stress in GF mice was partly reversed by a fecal transplant from SPF only at an early stage, and not later. This indicates a critical time window for exposure to bacteria in order for the HPA system to become fully maturated [33].

THE GUT-IMMUNE INTERACTION

The gut, due to its large epithelial surface and functioning as an open system, is the place where the immune system meets a huge amount of antigenic structures, both dietary components and bacterial antigens. The gut-associated immune system has the role to discriminate between antigens, and to decide whether or not to generate an immune response against some of them, in order to maintain immune tolerance towards the commensal bacteria, and to protect the host from invasion by foreign pathogens.

Several structural features of the gut mucosa prevent the entry of viruses, bacteria or parasites: 1) the gut epithelial cells are linked through tight junctions and covered by a mucus layer; 2) the gut-associated lymphoid tissue (GALT) is well represented as intra-epithelial lymphocytes (IELs) or organized as Peyer's patches; 3) there are epithelial M cells, which select antigens from the lumen and deliver them to the lymphoid tissue through antigen-presenting cells; 4) at the base of the crypts there are the Paneth cells that can identify microbial products and secrete defensins, antimicrobial proteins, thus contributing to innate immunity; 5) in the lamina propria layer of the gut mucosa, there are antigen-presenting cells called dendritic cells that constantly check the content of the gut lumen and activate IELs, including immunoglobulin A-producing plasma cells [35].

The innate immune receptors mediate both the tolerance to microbiota and the defense immune responses to pathogens, by binding molecules released from healthy or injured tissues, referred to as safety or danger signals respectively [36]. The receptors involved in identifying molecular motifs of microorganisms are the pattern recognition receptors (PRRs), membrane-bound receptors of the innate immune system expressed on the surface of epithelial cells and gut immune cells, with high expression in the distal parts of the small bowel (jejunum, ileum) and weak expression in the duodenum [37]. PRRs include toll-like receptors (TLRs), Nuclear Oligomerization Domain-like receptors (NLRs; NOD-like receptors), and Retinoic acid Inducible Gene-like receptors (RLRs). The safety signals, food components, bacterial metabolites, and other signaling molecules from healthy mucosa, limit

inflammatory reactions to commensal microflora, and support long-term gut colonization [36].

The immune-gut interaction is bidirectional. The intestinal immune system maturation and development depend on the gut microbiota [20] and the immune system modulates the microbiota composition. Polysaccharide A produced by the Bacteroides fragilis, a commensal bacteria, modulates T lymphocytes-dependent immune responses and in the GF mice, restores the T helper cells balance thus preventing intestinal inflammation [38]. The development of Candida albicans can be limited by the immune system in response to a tryptophan metabolite, indole-3-aldehyde derived from Lactobacillus reuteri [39]. GF mice lack immune activity, but colonization with certain gut microbiota restores their function [40]. Segmented filamentous bacteria found in the human intestinal tract have the ability to restore the proper function of B and T lymphocytes [41]. Furthermore, IL-15 decreases the number of butyrate-producing bacteria, promoting intestinal dysbiosis and the development of celiac disease and inflammatory bowel disease [42]. Altered microbiota composition could contribute to allergic rhinitis and asthma. In children with asthma, fecal IgE levels were positively correlated with abundant Clostridium genus bacteria, and in children with rhinitis genus Dorea was less abundant [43]. Gut microbiota by modulating T-cell differentiation may have a role in tumorigenesis of gastrointestinal tract cancers and could influence the response to cancer therapy [44].

ROLE OF GUT MICROBIOTA ON BRAIN DEVELOPMENT

During the early stages of development, both the gut microbiota, as well as, the brain develops in a rapid manner. The colonization of the gut runs in parallel with brain development, thus, disruptions of the enteric microbiome during this critical period may impair brain growth and function.

Maternal microbiome is of paramount importance, influencing the offspring's brain programming through two processes. Brain development is a process that demands high amounts of energy and the maternal enteric microbiota provides the substrates and metabolites necessary for this growth. Vertical transmission of maternal microbiome during birth and the subsequently colonization of the offspring's gut is essential for a proper metabolism, immune system and neurodevelopment. The mode of delivery can also influence the offspring's microbiome. An infant born via C-section has a microbial community rich in *Staphylococcus*, the maternal cutaneous microbiome, whereas the vaginally born infants have an abundant *Lactobacillus* community.

Microbes were found to produce and consume a wide range of neurotransmitters, including dopamine, norepinephrine, serotonin, or gamma-aminobutyric acid (GABA) [45]. The enteric microbial community may shape brain development through these neurotransmitters, as well as through the production of bacterial metabolites, such as short-chain fatty acids (SCFA). SCFA such as butyrate, propionate, and acetate are produced in the gut by anaerobic bacterial fermentation of starches and dietary fibers [46]. SCFAs exert their effect on enteroendocrine cells and promotes signaling to the brain through systemic circulation and vagal routes, inducing the secretion of local enteric hormones such as glucagon-like peptide 1 and peptide YY, as well as γ-aminobutyric acid, and serotonin [47]. In the CNS, SCFAs modulate neuroinflammation by shaping glial cell morphology and function and by regulating BDNF levels [47,48].

Infants with higher alpha diversity of the enteric microbial community had lower cognitive scores when compared to the ones with high levels of *Bacteroides* [49]. Consequently, the microbial community of the human gut at one-year of age could predict the cognitive function at two-year of age [49].

The formation of an intact BBB is also mandatory during the proper development of the CNS. BBB matures progressively during fetal life and is formed by an elaborate system of tight junctions (TJ) which regulates what goes to the brain [50]. TJ proteins restrict paracellular transport and are mainly formed of transmembrane proteins such as claudin-5 and occludin [51]. Emerging evidence suggests that the enteric microbiome affects the permeability of BBB in both fetal and adult life. The lack of gut microbial community in GF mice resulted in increased permeability of BBB, an effect reversed after the administration of fecal transplant from conventionally raised mice [52]. Moreover, fetal mice with GF mice also exhibited a more permeable BBB and a reduced closure of BBB when compared to SPF mice [52]. Furthermore, the expression levels of claudin-5 and occludin were lower in fetal and adult GF mice relative to SPF mice, reinforcing the implication of a bacterial colonized gut [52]. As we mentioned, GF mice exhibit a high glucocorticoid level in response to stress. A permeable BBB during development may favor the transport of plasma glucocorticoids to the growing brain, impairing neurogenesis and the production of BDNF.

GUT MICROBIOTA IN AUTISM AND DEPRESSION

Disruptions of the abovementioned bidirectional interactions between indigenous microorganisms and the nervous system are highlighted in neuropsychiatric disorders such as autism, depression, bipolar disorder, dementia, schizophrenia, Alzheimer's and Parkinson's disease [53–55]. In the following part, we will focus on two of the major disorders that are directly affected by microbial

imbalance and greatly impact daily life, and these are autism and depression.

Autism spectrum disorder (ASD) is a multifaceted set of neurodevelopmental conditions characterized by altered social interaction together with stereotyped behaviors. Amongst all subtypes of ASDs, autism embodies the primary type. In individuals with a genetic predisposition, the condition seems to be related to different factors, such as environment, genetics, or diet [56]. Recently, a contributor to these abnormal behaviors may be the microbiota which appears to impact the development of the nervous system [57].

A significant number of individuals with ASDs have gastrointestinal disturbances, especially chronic abdominal pain, distorted bowel habits which come together with their neurological abnormalities [58]. What is more, these gastrointestinal symptoms in these subjects appear to link with the severity of their ASD [59]. Several studies have demonstrated a significant modification of the makeup of the gut microbiota in children with autism and indicated that these symptoms might be a manifestation of an inflammatory process [60]. Dysbiosis is connected with a disruption of the mucosal barrier and, as a consequence, there is an increased absorbency of exogenous peptides from the food ingested or from the local bacteria [61].

One early hypothesis when exploring the connection between ASD and the microbiota was the extensive use of antibiotics that can result in an increase of Clostridium that might expose affected individuals to elevated levels of bacterial metabolites that can be neurotoxic.

The earliest studies exploring the relationship of the microbiota and children with ASD proposed that excessive antibiotic use led to an overgrowth of spore-forming Clostridium, which researchers hypothesized might be exposing these children to. Improvement of symptoms seemed to relate to vancomycin treatment. In the study, 11 children with a severe form of ASD were given vancomycin for 8 weeks and then 4 weeks of probiotics. 73% of the children showed an improvement of social behavior, but the improvement did not last [62]. Furthermore, fluorescence *in situ* hybridization techniques helped to identify increased Clostridia in stool samples and correlated the gastrointestinal symptoms with ASD [63].

Other studies provided support of the hypothesis that children with ASD and gastrointestinal disturbances have increased immune response and dysbiosis. Transforming growth factor (TGF) beta 1 and regulatory cytokines had a distorted balance in these children [64]. Inflammatory diseases of the gastrointestinal tract are known to aggravate dysbiosis. Moreover, Luna et al. demonstrated that increased cytokines that reflect the mucosal immunity, including interleukin 6, IL-1, IL-17A, and interferon-gamma, have been linked to abdominal pain and the rise in the Clostridium in ASD [65].

Animal studies bring other evidence to these connections between ASD and microbiota. Sharon et al. relocated gut microbiota from human individuals with ASD into germ-free mice. The colonization with this microbiota was sufficient to induce autistic symptoms. Also, treatment of these mice with microbial metabolites improved the abnormalities previously developed [66].

A close relationship between the microbiota and major depression disorders was also explained [67]. The administration of broad spectrum antibiotics in mice triggered depressive symptomatology by changing the neuronal firing in the hippocampus. Interestingly, the phenotype could be reversed after probiotic treatment with Lactobacillus [68]. Moreover, GF mice can presented with altered levels of the serotonin in the hippocampus [69]. In addition, a depression-like phenotype was obtained in mice when transplanting gut microbiota from depressed humans. The symptomatology included anxiety and was believed to happen only due to the new microbiota, as transplanted mice were germ-free or microbiota – deficient at the moment of the operation [70,71]. However, human studies were not conclusive regarding whether bacterial taxonomic categories are useful in preventing depression. The minimal consensus was reached regarding whether microbial diversity or abundance could influence the disease [67].

CONCLUSION

Gut microbiota complex composition has an important role in modulating nervous, immune and endocrine systems. Dysbiosis is increasingly involved in T-lymphocytes linked pathologies such as allergies, inflammatory bowel syndrome, cancers, and other autoimmune disorders. The increased absorbency of exogenous peptides including neurotoxic substances or signaling molecules, and the neurotransmitters availability alterations associated with intestinal dysbiosis, connect gut microbiota to brain development and neuropsychiatric disorders. Unraveling microbiome-gut-brain interactions holds the promise of discovering potentially novel etiologies and therapies for neurological disorders.

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AXUL MICROBIOM-INTESTIN-CREIER – UN SISTEM COMPLEX DE COMUNICARE

REZUMAT

Există tot mai multe dovezi care sugerează o interacțiune bidirecțională între sistemul nervos central, sistemul nervos enteric și tractul gastro-intestinal. Studii recente arată că un factor esențial pentru aceste interacțiuni intestin-creier este microbiota intestinală. Microbiomul a fost recent asociat cu modularea comportamentului, a funcțiilor cognitive și a sănătății mentale. Mai multe mecanisme, incluzând căi nervoase, endocrine, imune și metabolice sunt implicate în axul microbiom-intestin-creier. La rândul lui, creierul poate modela, flora intestinală. O mai bună înțelegere a relației dintre microbiota intestinală și creier ar putea aduce noi informații cu privire la fiziopatologia bolilor neurologice și ar ajuta la identificarea unor noi terapii țintite, prin modularea microbiomului.

Cuvinte cheie: axa microbiom-intestin-creier, sistemul nervos central, sistemul nervos enteric, sistemul nervos autonom, axa hipotalamo-hipofizo-suprarenaliană, tulburări neurologice

EATING DISORDERS. AN UPDATED DESCRIPTIVE REVIEW ON PATHOPHYSIOLOGY

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ABSTRACT
The purpose of this article is to make an accurate and succinct evaluation of the literature on the pathophysiology in Alignment Disorders. Eating disorders are a complex pathology, which often has fluctuations in evolution, with long periods of remission, followed in 35-50% cases by relapses and persistence of the disease. The onset of the pathology can be long before the diagnosis, so is important to know the pathophysiological substrate as correctly as possible to intervene early, but also to develop more effective methods of intervention to prevent relapses. **Methods**: Forty original or review studies on the searched topics were selected. Of these, 20 were included in the present study. **Results**: We identified eight central topics in the pathophysiology of the ED disorders in the scanned literature, we selected the most accurate and the ones that presented accurately the link between the pathology and the elements that are important as specific traits in ED. we identified eight central topics as factors implied in the pathophysiology of ED disorders in the scanned literature; (1) factors outside the body: social environment, physical environment, nutritional factors, (2) biological factors inside the body: metabolic and endocrine system, microbiota, immune system, brain, sleep. **Conclusions**: Detailed knowledge of the pathophysiological substrate and each case personalised appropriate interventions make the difference between a curative intervention and a relapsing condition (failure) that may increase the disability degree of a certain patient.
Keywords: eating disorders, anorexia nervosa, bulimia nervosa, binge-eating disorder, microbiota, pathophysiology, genetics, Genome-wide-association studies (GWASs)

INTRODUCTION

Eating Disorders represent a complex pathology that needs a multidisciplinary intervention for an effective recovery. The characteristic of EDs consists of persistent disrupted eating and weight-related behavior, leading to changes in feeding, physical activity, and social behavior, specifically influencing the dietary intake, generating the alteration of the psychosocial and physical health [1]. Another important element is the connection with neurobiology and how they influence each other [2,3]. The most frequent eating disorders reported are anorexia nervosa (AN), bulimia nervosa (BN), and binge-eating disorder (BED) [1]. Eating disorders implies multiple areas of affectation – somatic, physiological, psychiatrical, nutritional, social, familial – and the onset may be long before the onset of symptoms. There are often cases in which the symptoms reappear after long periods of remission [4]. Recently there are data available that states that the brain neurophysiology impairment in ED may reside in altered interaction between the classical pathogenetic paths – endocrine-metabolic - and the digestive's system permanent guests – the gut microbiota [5]. It is emphasized the role of the environmental and nutritional factors, of genetic factors related to the microbiome, the role of the metabolic and endocrine system [6], the involvement of the immune system, and the brain - in addition to phenotypical traits of EDs [1]. **Less is known** about the endogenous and endogenous premorbid traits, how the persons can be aware of their predisposition of developing EDs, and which are the solutions for developing strategies of prevention and early intervention [7]. **Aim of the study:** to overview the recent progress in the pathophysiology of ED (especially AN and BN) to aim new therapeutic targets for early intervention, fast recovery, and decrease of relapse in AN and BN by literature screening.

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DATA SELECTION

We made an accurate search in the main sources of Databases (PubMed, BMC Public Health, Global Health, Cross Ref, Scopus, Web of Science, Google Scholar and Medline) refined on the last four years (2016-2020 up to date) on the keywords: **TOPIC:** (pathophysiology) *OR* **TOPIC:** (genetics) *OR* **TOPIC:** (nutrigenomic) *AND* **TOPIC:** (eating disorders) *OR* **TOPIC:** (anorexia nervosa) *OR* **TOPIC:** (bulimia nervosa) *OR* **TOPIC:** (Binge-Purge). **Results:** our search retrieved 5959 papers **timespan:** All years. on Web of Science Core Collection (7,650) MEDLINE® (6,829) Current Contents Connect (5,305) Derwent Innovations Index (230), Data citation index (207) SciELO Citation Index (137) KCI-Korean Journal Database (43) BIOSIS Citation Index (3,795) **refined by PUBLICATION YEARS:** (2020 OR 2019 OR 2018 OR 2017 OR 2016) AND *Databases= WOS, BCI, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, 2016-2020.*

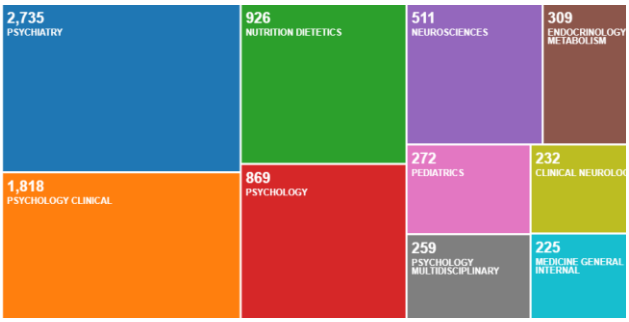

Fig. 1. Results Analysis Treemap for Web of Science Categories Field

Table I. Results Analysis Table for Web of Science Categories Field

Field: Web of Science Categories	Record Count	% of 5,959
Psychiatry	2,735	45.8 %
Psychology Clinical	1,818	30.5 %
Nutrition Dietetics	926	15.5%
Psychology	869	14.5 %
Neurosciences	511	8.5 %
Endocrinology Metabolism	309	5.2 %

Fourty original or review studies on the searched topics were selected. Of these, 20 were included in the present study.

RESULTS AND DISCUSSIONS

20 articles of central importance on the topic were identified in a systematic search on eight databases, articles selected on the searched topics; we identified eight central topics as factors implied in the pathophysiology of ED disorders in the scanned literature (Table I). (1) factors outside the body: social environment, physical environment, nutritional factors, (2) biological factors inside the body: metabolic and endocrine system, microbiota, immune system, brain, sleep.

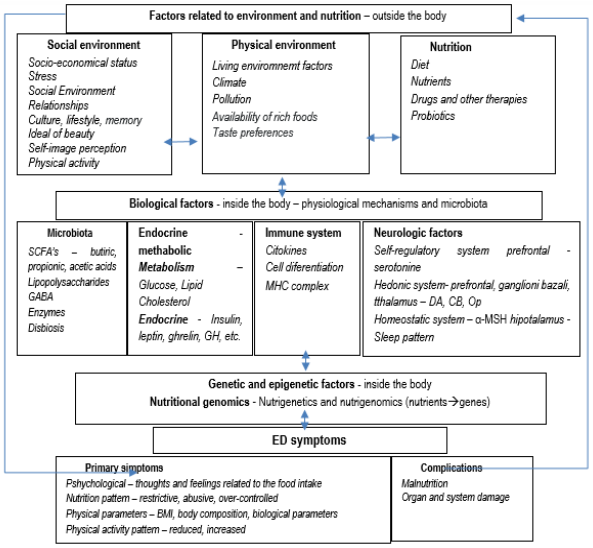

Fig. 1. Info-graph on pathophysiological model of eating disorders. SCFAs, short chain fatty acids; GABA, gamma-aminobutyric acid, MHC, major histocompatibility complex; 5-HT, serotonin; DA, dopamine; CB, cannabinoid; Op, opioids; α-MSH, melanocyte-stimulating hormone (after Himmerich, 2019)

Factors outside the body in the pathogenesis of ED

Socio-economical status

Limiting access to food with an adequate caloric index, poverty but especially the adverse events experienced in early childhood are risk factors that determine. A recent study of the National Epidemiological Survey on Alcohol and Related Conditions III (NESARC-III) (AOR = 2.98 [95% CI = 1.56-5.71]) and BED (AOR = 2.95 [95% CI = 1.73-5.03]) [i] showed that children which are malnourished are at higher risk of developing ED's comparing with the general population [5,6,7, 28].

Stress, Social Environment, Relationships

Not infrequently, people with eating disorders have been exposed in the past to fairly severe and annoying episodes of bullying. it is necessary to be aware of these aspects, to observe the sensitivity of the most fragile people, and to understand that for some people jokes are not auspicious and can do them more harm than good. Also, intra-family relationships can be strained, the lack of a secure base of attachment can be another element encountered, and very often there can be a strained mother-daughter relationship,

a competition that maintains the patient's need to lose weight. The parental model is an essential element in the development and maintenance of certain food patterns, reporting to the meal ritual, and a healthy/balanced diet [8].

Culture, lifestyle, Ideal of beauty, Self-image perception

Following sensitivity experiments, it has been observed that there is an intrinsic deficit of interoception and proprioception in Anorexia Nervosa and Bulimia Nervosa [9]. There is a complex link between interoception, proprioception, and the vestibular system – all of them creating the inner body perception. It is a domain that is usually affected in eating disorder and it is perceived as a discrepancy between how the body is perceived and how it is [10].

Multiple studies practice (Riva & Dakanalis, 2018; Dakanalis & al., 2016; Serino & Dakanalis, 2017) promote that there are new methods to evaluate the body perceptions and its particularities through virtual reality and analyze the variation of the parameters mentioned [11].

Developing a new therapeutic instrument based on the evaluation of body perception and which are the specific traits in EDs can be a good topic for future research.

Physical environment – the role of the physical activity of the ED patients

There are two categories of people who can be diagnosed with an eating disorder - people who are sedentary but still try to control their body image through food intake and resort to purgative behaviors, and on the other side of the barricade there are people who they exercise excessively to maintain their body weight and have greater control over the underlying pathology [12].

Nutritional factors

Diet

In the last two decade in the literature is described a new term, nervous orthorexia (NO) which is represented by the excessive preoccupation with healthy eating and the concentration of activities around this subject - compulsive and exaggerated behaviors that promote healthy eating, strict diets that amplify the state of anxiety and anxiety and maintains a restrictive behavior, as well as the elimination from the diet of many dietary principles that cause weight loss, but without a desire in this regard. It frequently associates psychiatric comorbidities such as eating disorders, obsessive-compulsive disorder, and psychotic disorders, but can also cause them. Dunn and Bratman made up a self-administered questionnaire about Orthorexia Nervosa, but frequently in the history of the patient exist a psychiatric pathology or at least a tendency [13,14].

Nutrients

Unfortunately, in our country, there is no adequate education in the sense of knowing the main nutrients, vitamins, and minerals necessary for the installation in people's lives of eating behavior that satisfies their metabolic needs and promotes health. The lack of these guidelines, dominated by tradition and poverty, often causes nutritional deficiencies and decreased appetite or changes in it that may be the precursors of eating disorders [15].

Zinc is an essential mineral, involved in various brain mechanisms - especially in regulating glutamatergic function, and in the literature, there is described an association with AN. Deficiency reduces the regulation of NMDA receptors and thus to their deficient function with overactivation and overactivation and upregulation. This results in elevated pathological levels of glutamate with intracellular calcium influx and disorders in synaptogenesis and neuroplasticity. The effectiveness of zinc treatment in AN is not yet proven, but trials exist and are worth trying in the future. In the short term, ketamine can be used to regulate the glutamatergic system [16].

Medication and drugs

There are patients who, after specific intervention in AN with external stimulation - tDCS (transcranial direct current stimulation) end up developing disorders of glucose metabolism and even diabetes. Rigorous investigations are needed before we can start various specific treatments and know exactly the characteristics of the patient in question, but also the attestation of this hypothesis on larger population groups to evaluate the impact of the technique on glucose/insulin metabolism. The proposed technique helps to improve the perception on one's own body and an important step in starting the process of increasing body weight. [17].

Factors inside the body in the pathogenesis of EDs

Probiotics

There are repeated studies regarding the possibility that in AN there is an imbalance of the intestinal flora that maintains the psychic symptomatology and the low need for food ingestion. The inner body, permenent host living bacteria and their metabolic outproducts influence the immune and metabolic system and also the endocrine and brain systems. The exact evaluation of the microorganisms involved in this pathology can be a starting point for innovative methods of treatment of AN, namely probiotics. Some studies have tested the genetic material of bacteria in the intestinal flora and have shown the existence of intestinal dysbiosis in both people with AN and those with depression or anxiety. This information can be a new starting point in subsequent interventions [18].

The metabolic, genetic and endocrine areas

Studies show changes in eating disorders of cholesterol and lipid metabolism, with hormonal markers released into the bloodstream - ghrelin, insulin, leptin [19]. In BN are described polymorphisms in the genes associated with ghrelin and those for adipose tissue and obesity and in AN polymorphisms of NKRD50 gene [20] .

In the literature, there are described **genetic changes** in AN at the level of the fetal raphe and ventral tegmental areas, with the observation on animal models of the changes in the expression of the Rps26 and Dalrd3 genes. There is particular genetics in ED and subcortical appetitive circuits are affected [13]. There is mentioned a 10 fold greater lifetime risk in persons with first degree relatives with a AN pathology compared with the absence of heredocolaterality but is not clear if the imprint is due to genetic or environmental factors of the family [22,23].

On the screened literature we found no data available now regarding the nutrigenomic of the ED, even though are a promising approach towards the effects of diet on health [24].

Recent research suggests that changes in **blood levels of leptin and ghrelin** are correlated with particular eating behavior, depression, impulsivity; there is also a correlation with the luteal phase corresponding to premenstrual dysphoric disorder (PMDD) - this rise can prone to a predisposition to obesity. In comparison with the control sample, women with PMDD frequently associated higher BMI, sweet cravings and uncontrolled eating, and also depression and emotional eating [25].

The **low availability of opioid receptors** described in AN may be another cause that explains specific traits and difficulties in emotional regulation, with an exaggerated desire for control that is manifested by exaggerated attention to body weight - reward/aversion system imbalances, but also intense fear not to gain weight, clear anxiety from the pathological spectrum maintained by the neurobiochemical substrate. Hypercorticism is also associated [26].

Immune system and microbiota

The field of research related to the role of the microbiota in the development of EDs started from the assumption that there are autoantibodies that interact with neuropeptides that regulate satiety, such as α-melanocyte-stimulating hormone (α-MSH) in the arcuate nucleus in hypothalamic neurons. An anorexigenic protein – bacterial antigen - secreted by *Escherichia coli* has recently been discovered, namely caseinolytic protease B (ClpB). ClpB is mimetic of α-MSH triggering synthesis of targeted cross-reactive auto anticorps, generating Ag-Ac immune complexes with α-MSH (α-MSH – Ac Anti α-MSH complexes) that chronically activete melanocortin system generating increased satiety and anxiety. Disorders present in the immune system, microbiota (increased gut *Enterobacteriaceae*) and the brain melanocortin system regulating feeding can maintain EDs, which is why they can be considered as therapies. etiological, to correct dysbiosis (Figure 4) [27].

The immune system is affected by both stress and diet and substances secreted by the intestinal microbiota, which will cause a modified secretion of TNF- α and IL-6, but also other factors involved in cell differentiation. It can be stated that AN and BN have as substrate an imbalance between gut microbiota and host immune and neuroendocrine systems that regular feeding behaviors [28].

Specific therapies - α-MSH auto Abs in ED - that attack autoantibodies produced to specific bacterial antigen mimetic of α-MSH - autoantibodies that create circulating immune systems with α-MSH that stimulates the melanocortin system that influences eating behavior - are worthy of view in future interventions [29].

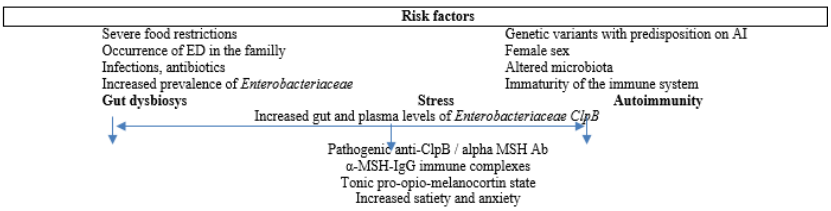

Fig. 2. Role of microbiota in the pathophysiology of eating disorders. (after Current Opinion in Pharmacology)

Brain and the neuroanatomy of EDs - AN

At the structural level, there may be cerebral atrophy or even decreased brain activity in different areas of the brain, which will lead to a neurocognitive decline that will maintain the difficulties of a collaboration of the affected person revealing altered reward dependence behaviors that have impaired brain reward circuitry.

It is well documented in animals and humans the dopamine release cascade in the reward site of the brain - the "Brain Reward Cascade" (BRC) – involving chemical messengers - serotonin, enkephalin, and GABA that work in unison to provide the release of dopamine (DA) at the nucleus accumbens (NAc). Any impairment due to either genetics or environmental influences on this cascade will result in a reduced amount of dopamine release in the brain reward site (normal "happy" or hypodopamminergic "unhappy" brain state). Manipulation of the BRC can be done using neuro-nutrient therapy by nutrigenomic principles. Prospective studies have shown a very high recovery capacity of these deficits in the context of balanced food intake, the highest recovery being in the binge-purge subtype.

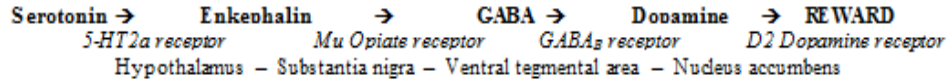

Fig. 3. Sequence in the dopamine satiety cascade

Cardi *et* al. conducted a study in 2019 that sought to evaluate neural circuits related to eating anxiety. They performed multiple fMRIs in both a group with AN and normal ones and in those with AN they noticed a lower reactivity in the anterior cingulate cortex. [20] After specific therapy applied to people with AN, there were no changes in the anterior cingulate. cortex, but changes in the dorsolateral prefrontal cortex and decreased activity in the superior parietal lobe were observed. The higher the degree of activation of the island area before the intervention, the lower the fear of eating AN after the intervention. Changes in neuronal activation - the middle temporal gyrus and lateral parietal cortex - have also been observed. Practically, exposure to food has improved emotional regulation and increased the ability to concentrate.

Sleep

A quality sleep with a balanced diet significantly improves the physiology and somatic balance of the body; there are multiple correlations in the literature that argue that sleep deprivation can promote or sustain the development of psychiatric pathologies [30,31]. Most people with eating disorders report associated sleep disorders, even if the topic is not sufficiently researched. Sleep regulation can improve the recovery process from eating disorders and increase the quality of life by lowering the relapse rate [32].

CONCLUSIONS

Promoting a healthy but balanced diet and avoiding imbalances is essential when it comes to nutrition. Excesses never lead to satisfactory results and that is why it is necessary to promote healthy eating behaviors early on to prevent the further development of imbalances that are much harder to control. Knowledge of information about the social environment, family environment, exposure to stress, cultural model, nutritional factors, body image perception, genetic/endocrine/metabolic substrate, immune system and microbiota, neuroanatomy, and sleep patterns are defining elements to be kept. the account in the intervention of EDs, along with psychoeducation, specific psychotropic treatment, and psychotherapy, to ensure patients' quality and long-term recovery.

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TULBURĂRILE DE ALIMENTAȚIE. O EVALUARE ACTUALĂ A LITERATURII DE SPECIALITATE PE TEMA FIZIOPATOLOGIEI

REZUMAT

Scopul acestui articol este sa evalueaze lucrarile de referinta din literatura de specialitate pe tema fiziopatologiei existente in Tulburarile de Alimentatie. Tulburarile de alimentatie reprezinta o patologie complexa, care are deseori fluctuatii in evolutie, cu perioade lungi de remisie, urmate in unele cazuri de recaderi. Debutul patologiei poate fi cu mult inaintea diagnosticului, tocmai de aceea este important sa se cunoasca cat mai corect substratul fiziopatologic pentru a putea interveni precoce, dar si pentru a se dezvolta metode mai eficace de interventie, care sa previna recaderile. Metode: patruzeci de review-uri originale pe temele cautate au fost selectate, dintre care 20 incluse in acest studiu. Rezultate: am identificat opt teme centrale in domeniul fiziopatologiei tulburarilor de alimentatie si le-am sintetizat din articolele cele mai relevante pe tema respectiva pentru a le descrie in acest articol. Concluzii: importanta unei abordari terapeutice adecvate este esentiala in tulburarile de alimentatie. Cunoasterea substratului fiziopatologic si a interventiilor necesare si adecvate fiecarui caz in parte sunt esentiale si fac deseori diferenta dintre o abordare salvatoare de viata si una care creste gradul de dizabilitate al persoanei respective.

Cuvinte cheie: tulburări de alimentație, anorexia nervosa, bulimia nervosa, microbiomul, fziopatologie, genetică, GWASs