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Comprehensive study on the involvement of vitamin D in the physiological and biochemical mechanisms of psychiatric manifestations associated with epilepsy

DOCTORAL THESIS SUMMARY

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CONTENT

List of abbreviations

FDA- Food and Drug Administration
BBB- blood-brain barrier
BP- phenobarbital
CBZ- Carbamazepine
CT- Computer Tomography
EEG- electroencephalogram
JME- juvenile myoclonic epilepsy
FAE- levetiracetam
INM- Montreal Neurological Institute
MRI- Magnetic Resonance Imaging
CSF- cerebrospinal fluid
TBI- traumatic brain injury
LIÎE- International League Against Epilepsy
MAE- antiepileptic drugs
WHO- World Health Organization
FIP- feline infectious peritonitis
PTZ- pentylenetetrazole
ROS- reactive oxygen species
SE- Status epilepticus
CNS- central nervous system
RCTs - randomized controlled trials
VDR- vitamin D receptor
Vit D- Vitamin D
VPA- Valproic acid
SOD- superoxide dismutase
GPx- glutathione peroxidase
MDA- malondialdehyde
EIAED- liver enzyme inducing antiepileptic drugs
NEIAED- non-liver enzyme-inducing antiepileptic drugs

Keywords

Epilepsy, seizures, psychiatric comorbidities, vitamin D, valproic acid, Carbamazepine

Introduction

Epilepsy is a chronic neurological disorder characterized by the brain's long-term predisposition to generate epileptic seizures (fits) through its excessive electrical activity (Thijs et al., 2019; Beghi, 2020). This condition is the result of at least two unprovoked seizures in 24 hours (Kaculini et al., 2021). These seizures are based on neurological, psychiatric, cognitive, and social considerations (Fisher et al., 2017; Beghi, 2020; Falco-Walter et al., 2019). Epileptic seizures are divided according to the impairment of the patient's consciousness, whether or not they involve multiple symptoms (involuntary movement, uncontrolled urination), and according to the physiological location of their onset in the brain. The latter, in turn, can be divided into three categories: focal (starting from a specific part of the brain), generalized (starting from both cerebral hemispheres) or their onset is unknown. (Fisher et al., 2017; Falco-Walter et al., 2018; Parck., 2019; Thijs et al., 2019). Low cognitive performance such as memory and learning disorders are frequently observed in epileptic patients (Kundap et al., 2017).

Depending on the type of epilepsy and seizures, different concentrations of antiepileptic drugs are administered. There are people who are resistant to a certain type of antiepileptic drugs or who are recommended to take two types of drugs to be effective against epileptic seizures. Antiepileptic drugs are classified into two categories depending on their metabolism in the body, thus certain drugs are considered liver enzyme inducers (EIAED – phenytoin, phenobarbital, primidone, carbamazepine, oxcarbamazepine, topiramate) inducing the catabolism of certain substances and antiepileptic drugs that do not induce liver enzymes (NEIAED – gabapentin, valproic acid, lamotrigine, levetiracetam, pregabalin, tiagabine) being considered to have a small effect on nutrients (Ensrud et al., 2008; Soltani et al., 2016).

Increasing the function of cytochrome p-450 enzymes through hepatic enzyme-inducing antiepileptic drugs leads to an inactivation of the active form of vitamin D and thus will also reduce calcium absorption in the body (Teagarden et al., 2014; Shen et al., 2014; Soltani et al., 2016).

Chapter 1 The current state of knowledge

1.1 History

The concept of religion and medicine in ancient times made the basis of the definition of an epileptic seizure from a magical explanation to a scientific one. The first description of this kind dates back to 2500 BC in Sumerian documents from Mesopotamia, so the person with epileptic condition with focal tonic seizures of our time was then considered the "disease of the fall" being the person who was linked to both sin and the moon of God.

However, the definitions that lead to today's description of epilepsy date back to the Hippocratic and post-Hippocratic era. Hippocrates (460-370 BC) based medicine on the hypothesis that nature was made up of four basic elements, thus these elements are effective in the body in four bodily fluids or humours, health being the harmonious connection between the four humours resulting in the state of eucrasia (Bujalkova et al., 2001; Kalachanis and Michailidis, 2015; Patel and Moshé, 2020). Thus, Hippocrates was the first to attribute epilepsy to the brain and to suggest that it is hereditary rather than contagious (Iftimovici 1995; Kalachanis and Michailidis, 2015; Kaculini et al., 2021).

In the Middle Ages, there was a regression in scientific knowledge about epilepsy, dominating the thinking about demonic possession, but also madness reflected by the light of the moon and the spirits related to it. At that time there was a confusion between epilepsy and mental disorders (Tamkin, 1994, Patel and Moshé, 2020). However, there were people who continued the scientific research of epilepsy, thus a Persian physician Avicenna considered that the clinical manifestation of a seizure can be associated with its origin in the brain, stomach, spleen, but also with changes in behaviour, temperament and psychoneurological dysfunctions (anxiety, strong excitement) before the seizures (Dadmehr, 2018; Patel and Moshé, 2020).

At the end of the 19th century, the electroencephalogram (EEG) appeared, which helped to advance the understanding of epilepsy, through clearer clinical descriptions. Thus, Frederic Andrews Gibbs, together with his colleagues Erna Leonhardt Gibbs and William Lennox, distinguished that there are different EEG patterns for each major type of clinical seizures such as generalized absence seizures, psychologic seizures, focal temporal lobe seizures. They discovered through the EEG that most often there are abnormalities in the interictal period, which will help the clinician to realize the type of seizure without having to observe the patient having a seizure (Wolf, 2014; Gibbs et al., 2002; Patel and Moshé, 2020).

In 1964, the International League Against Epilepsy (ILAE) emerged as a response to the need for a standardized system to classify seizures. This was possible under the leadership of Gastaut. Seizures were divided into partial,

generalized, unilateral or predominantly unilateral in children, irregular in the unborn, and unclassified. The terms partial, focal, and local have been used interchangeably, but Gastaut believed that partial is the oldest and most commonly used term, being a way of defining the neuronal population that is localized by widespread discharges in a specific region of the brain (Gastaut et al., 1972; Patel and Moshé, 2020). These in turn are divided according to frequency into isolated, repeated, prolonged, or repetitive (Caveness and Radermecker 1964; Patel and Moshé, 2020).

In 1981, a revision of the classification of epileptic seizures was proposed, which helps to study the semiology of seizures through video-EEG recordings, which will help to develop a common language of terms (Angeles, 1981; Patel and Moshé, 2020). In 2001, a task force was proposed within the ILAE to introduce a standard glossary of terminology for ictal semiology, they proposed a five-axis diagnostic scheme (Angeles, 1981; Patel and Moshé, 2020). However, in 2010, the classification was majorly revised by Berg and his collaborators. Thus, terms that were considered to be misused focal (previously synonymous with partial) were dropped, the terms simple and complex were eliminated, and their replacement with focal seizures with/or impairment of consciousness/awareness was recommended), the replacement of secondary generalized seizure with evolution to a bilateral, convulsive seizure, neonatal seizures were no longer considered a separate entity, the subclassification of absence seizures was simplified, and spasms were declared as a type of seizure. However, this latest revision was criticized as being unnecessary or more complicated than the previous one, being difficult to use in daily clinical practice (Chang et al., 2017; Ferrie, 2010; Blume et al., 2001; Patel and Moshé, 2020).

The current international classification was approved in 2017 by the IELTS, which also used public comments, criticisms, and separate expert consultations to revise previous observations. This resulted in what is currently known about the diagnosis. In 2019, the World Health Organization (WHO) together with its partners (the International League Against Epilepsy and the International Bureau for Epilepsy) produced the first global report on epilepsy, followed by the adoption of the Global Intersectoral Action Plan for Epilepsy and Neurological Disorders 2022-2031, which recognizes preventive, pharmacological, and psychosocial approaches to epilepsy and other neurological disorders. These are aimed at raising awareness of epilepsy, strengthening public and private efforts to improve care, and reducing the impact that this condition can have on people (World Health Organization, 2019).

1.2 Prevalence and epidemiology

According to the World Health Organization, active epilepsy affects approximately 50 million people in the general population, with a prevalence of 4-10 per 1000 people depending on the area. With an estimated 5 million new

cases diagnosed annually, it is one of the most common neurological diseases worldwide (World Health Organization; 2019; Chen et al., 2023; Fiest et al., 2017).

Because epilepsy can be acquired or diagnosed during life, there are differences between the prevalence of epilepsy during life and the prevalence of active epilepsy (of a period of time that is conditioned by the survival of the individual). Two meta-analyses conducted in the last 10 years have been identified in the literature, one of which is summarized in research from Africa on patients with epilepsy (children and adolescents), and the other is a systematic review of international studies (Table 1.1). The differences that exist between the two meta-analyses are the possible higher reported risk factors for epilepsy in Africa (parental, neonatal infections, neuroparasites of the central nervous system, traumatic brain injuries). In the meta-analysis that used results from African countries, it was not possible to calculate the incidence rate because the estimates of incidence rates were one, two or even three years in some studies.

Previous research has shown that epilepsy is not a contagious disease, which is why the large number of people with this condition is worrying, in approximately 50% of people with this condition globally there is no certain cause, it remains unknown. The causes that could be determined were divided into five categories: structural in the brain (tumor, hippocampal sclerosis, tuberous sclerosis, cortical developmental malformation, focal cortical dysplasia, cerebral palsy, neurofibromatosis), genetic (certain genetic syndromes, some of which are hereditary), infectious (brain infection: meningitis, encephalitis, neurocysticercosis, brain abscess), metabolic and immune (autoimmune diseases) (World Health Organization, 2019).

Table 1.1 Results on the prevalence and incidence of patients with epilepsy from meta-analyses

	Total prevalence	Average prevalence on life course	The average prevalence of active epilepsy	The average prevalence of unclassified epilepsy	Cumulative incidence	Incidence rate	Average incidence rate
Fiesta et al. 2017	7.6 per 1000 people	7.06 at 1000 people	2.83 to 1000 people	-	67.77 per 100,000 people	61.44 per 100,000 people	56.79 per 100,000 people
Number of eligible items	56 studies	56 studies	11 studies	-	14 studies	13 studies	13 studies
Biset et al. 2024	17.3 per 1000 children	18.6 per 1000 children	6.8 per 1000 children	45.5 per 1000 children	250 per 100,000 children	-	-
Number of eligible items	42 studies	42 studies	42 studies	42 studies	6 studies	-	-

1.3 Diagnostic criteria

Epilepsy can be diagnosed quite simply, but various doubts may arise in order to avoid clinical errors. In order to reach this diagnosis, the account of the seizure episode must be known from both the patient's and the witnesses' point of view so that the clinician can understand whether it was a non-epileptic event or a seizure, as well as its type (Van Donselaar et al., 2006). The next step consists of additional screening investigations such as electroencephalogram (EEG), magnetic resonance imaging (MRI), computer tomography (CT), these can

determine the etiology that will help clarify the epileptic syndrome, subsequently the patient can be guided and the decision can be made to start a treatment with the selection of antiepileptic drugs (AEDs) (Van Donselaar et al., 2006).

There are three diagnostic levels defined by the International League Against Epilepsy in 2017 and accepted by the WHO. These are neonatal and infant onset levels representing children up to 2 years of age; childhood onset syndromes; and syndromes that may begin in later life (Scheffer et al., 2017).

Infants with epilepsy face significant cognitive and behavioural comorbidity, presenting an increased risk of drug resistance and mortality (Zuberi et al., 2022).

Childhood-onset epilepsy syndromes are classified for children between the ages of 2 and 12. They are divided into three broad categories: self-limited focal epilepsies, generalized epilepsy syndromes (thought to have a genetic basis), and developmental and epileptic encephalopathies that can have both focal and generalized seizures (Specchio et al., 2022).

1.4 Therapeutic interventions

In the last 30 years, approximately 15 third-generation antiepileptic drugs have appeared, being selected according to the type of seizure, but also according to the patient's medical history, but there is currently no drug that prevents the onset of the disease before the first epileptic seizure is triggered (Kaculini et al., 2021; Ghosh et al., 2021).

Glutamate is an excitatory neurotransmitter responsible for stimulating increased calcium and sodium conductance in ligand-gated ion channels. In animal models, two antagonists (AMPA, NMDA) have been shown to have anticonvulsant properties. Consequently, at the onset of epileptic seizures, activity-dependent plasticity of glutamate receptors becomes an important feature of the epileptic brain. In mice, it has been observed that those with a mutation in a subunit of AMPA receptors accumulate receptors with increased calcium permeability, a neurological phenotype of cell death and various severe seizures (Kumar et al., 2002; Salpietro et al., 2019; Ghosh et al., 2021). Glutamate is released at the synapse acting on metabotropic and ionotropic receptors, being responsible for stimulating and escalating convulsive activity, but also reduced expression of glutamate transporters can induce seizures, thus it has been observed that genetic manipulation related to the functioning of glutamate receptor proteins in rat models can increase the seizure threshold (Ghosh et al., 2021).

The GABA receptor plays an important role in the mechanism and management of epilepsy. Synaptic inhibition by GABA alters the regulation of neuronal excitability that is linked to epilepsy. GABA-energetic functions have shown abnormalities in acquired and genetic animal models of epilepsy, and in human epileptic tissue, changes in GABA receptor densities and GABA

concentrations have been identified, with a reduced number of GABAα receptors in human epileptic hippocampal tissue. There are studies showing that GABA antagonists are proconvulsant. Therefore, some effective drugs can cause seizures because they inhibit GABA synthesis. There are anticonvulsant drugs that are closely related to GABA synthesis by means such as increasing GABA-mediated inhibition (barbiturates, benzodiazepines), extending the time of chloride channel opening (barbiturates), enhancing the rate of chloride channel opening by improving GABA binding to its receptors (benzodiazepines), increasing synaptic GABA by decreasing GABA catabolism or reuptake (vigabatrin, tiagabine) (Ghosh et al., 2021).

Cholinergic receptors, through their structural and functional diversity, perform modulatory functions throughout the mammalian brain (Ghosh et al., 2021). Disturbance of cholinergic mechanisms can lead to disorders such as epilepsy, Parkinson's disease, dementia, schizophrenia, autism, Alzheimer's disease. Functional nAChRs are dispersed in the central nervous system (CNS) as follows: on dendrites, axon terminals, cell bodies, thus mediating synaptic neurotransmission. Therefore, in several studies it has been observed both in animal models and in epileptic patients that nAChR is altered in different types of epilepsy (juvenile myoclonic epilepsy, autosomal dominant nocturnal frontal lobe epilepsy), but also that its disruption can give rise to epilepsy (Bertrand et al., 2002; Ghasemi and Hadipour-Niktarash, 2015; Ghosh et al., 2021).

Serotonin through its neurotransmission has a potential role in epilepsy. Serotonin receptors (5-HTR) have been shown to be promising targets for the development of new antiepileptic drugs, as they can regulate a wide variety of focal and generalized seizures. Long-term administration of selective serotonin reuptake inhibitors is one of the causes of decreased serotonin synthesis and increased susceptibility to seizures (Singh et al., 2017; Ghosh et al., 2021).

There are other approaches that underlie the inhibition of epileptic seizures such as: vagus nerve stimulation, progressive muscle relaxation, yoga, cognitive-behavioural therapy, ketogenic diet, vitamin D, herbal treatments (Ghosh et al., 2021).

1.5 Association of psychiatric comorbidities

In the literature, there are frequent situations in which it is indicated that patients with epilepsy may present psychiatric comorbidities such as anxiety (5-25%) (Wiglusz et al., 2018) and depression (20-80% Domínguez-Aguilera and Muñiz-Landeros, 2017), personality disorders such as these may precede or progressively develop over time after the diagnosis of epilepsy (Elger et al., 2017; Domínguez-Aguilera and Muñiz-Landeros, 2017; Wiglusz, 2018). There are certain guidelines for psychiatric comorbidities in adults with a chronic health problem that provide an algorithm that can be applied in the context of epilepsy. These tools can be used for screening or quantifying psychiatric symptoms

through self-assessment such as: personality trait assessment questionnaires Neurobehavior Inventory (NBI), Revised Personality Inventory (NEO-PI-R); assessment of symptoms - interactive during the last months Interictal Dysphoric Disorder Inventory (IDDI), questionnaires for depression symptoms - Beck Depression Inventory (IBD), Beck Depression Inventory II (IBD II), Inventory of Neurological Depression Disorders for Epilepsy (ITDN-E); HASEM anxiety and depression scale, anxiety assessment questionnaires State-Trait Anxiety-Questionnaire, Disorder-7Items (PHQ-GAD-7) or using SEMM IV criteria through structural clinical interviews for disorders SEMM IV axis I (SCID I) or SEMM IV axis II (SCID II), MINI International Neuropsychiatric Interview (MINI) (Elger et al., 2017; Gurgu et al., 2021).

Epilepsy characterization indicates that most psychiatric comorbidities are a consequence of seizure disorders (Sadock et al., 2015; Duță et al., 2024). However, there is a complex relationship between psychiatric comorbidities and epilepsy, as individuals with epilepsy are at increased risk of developing psychiatric disorders, while patients with primary disorders are also at increased risk of developing epilepsy. Epidemiological studies suggest bidirectional relationships (Duță et al., 2024).

This situation leads to the effects of psychiatric comorbidities on the evolution and management of seizure disorders, manifested at several levels, such as I) a history of mood disorders occurring before the onset of epilepsy, which is associated with an increased risk of treatment-resistant epilepsy (Hitiris et al., 2007; Petrovski et al., 2010; Kanner, 2017; Duță et al., 2024); II) a personal or family psychiatric history that is associated with a higher risk of non-psychiatric and psychiatric adverse events (Mula et al., 2003; Kanner, 2017; Duță et al., 2024); III) a current or past history of mood and anxiety disorders that may facilitate the occurrence of seizures in stressful situations (Haut et al., 2003;Haut et al., 2007; Kanner, 2017; Duță et al., 2024).

Lifetime psychiatric history preceding the onset of epilepsy can have a significant impact on the lives of people with epilepsy, such as: I) a current mood or anxiety disorder is a strong predictor of poor quality of life (Cramer et al., 2003; Johnson et al., 2004; Kanner, 2017; Duță et al., 2024); II) an active mood disorder is associated with increased use of health services, resulting in higher costs for the patient, family and society (Cramer et al., 2003; Kanner, 2017; Duță et al., 2024); III) psychiatric comorbidities, especially substance abuse and mood disorders, are associated with an increased risk of premature death from external causes, such as accidents and suicides (Kanner, 2017; Christensen et al., 2007; Fazel et al., 2013; Duță et al., 2024).

1.6 The role of vitamin D in epilepsy

There are many scientific studies that indicate that vitamin D deficiency is more common in people with epilepsy compared to people without this

condition. Although there are limited studies, they can demonstrate the importance of periodically analysing the amount of vitamin D in the human body, especially in patients with epilepsy, but also the decrease in the number of epileptic seizures after optimizing the amount of vitamin D in the body (Duță, et al., 2024).

Vitamin D is a fat-soluble vitamin that is considered a neuroactive steroid of the brain that acts through both its nuclear and membrane receptors (Sizar, O. et al., 2021, Anjum, I., et al., 2018). The role of this vitamin in the body is given by bone metabolism, antioxidant and anti-inflammatory functions, being associated with improving mood by modulating the biosynthesis of neurotransmitters. Studies have been identified in the specialized literature that prove the importance of vitamin D through the benefits it brings to the body, but there are also studies that support the opposite, namely that this vitamin cannot have any significant impact (Jamilian et al., 2019; Duță et al., 2024).

The human body naturally synthesizes vitamin D through ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). The ways of synthesizing vitamin D in the body are dermal in nature by exposing the skin to ultraviolet rays of the sun (vitamin D2 and D3) (Sizar, O. et al., 2021), but also the synthesis of dietary intake (vitamin D3) synthesized in the liver into 25-hydroxyvitamin D2 (25-OH-D2) and 25-hydroxyvitamin D3 (25-OH-D3) by the enzyme 25-hydroxylase (CYP25A1) and then hydrolysed in the kidneys by 1-alpha-hydroxylase (CYP27B1) in their active state, in the form of vitamin D (1, 25 hydroxyvitamin D) (Sizar et al., 2021, Christakos et al., 2017). One of the most important physiological roles of vitamin D is calcium homeostasis through regulatory actions in calcium-handling organs, but most cells respond and express the vitamin D receptor (VDR) (Voutsadakis 2020; Trivedi et al., 2021) to help genes maintain calcium and phosphate balance in specific tissues (Meyer et al., 2020). It belongs to a family of nuclear transcription factor receptors (Voutsadakis, 2020; Trivedi et al., 2021) (Figure 1.1).

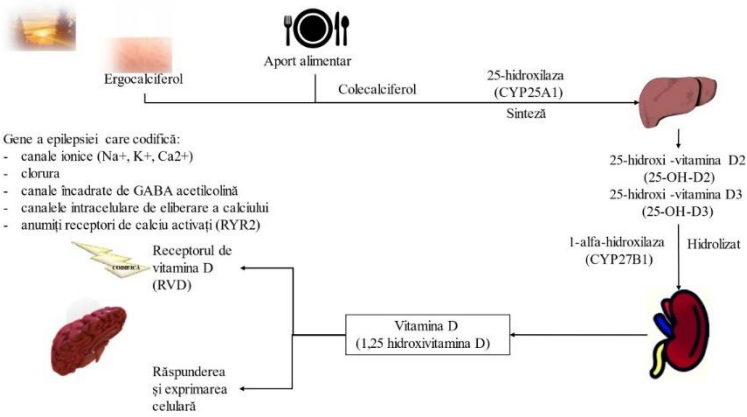


Figure 1.1. Schematic representation of vitamin D synthesis in the human body

For the forms of epilepsy there are a number of mutant genes that encode voltage- or ligand-gated ion channels. These mutations have been identified in Na+, K+, Ca2+, chloride channels, GABA-gated acetylcholine channels and intracellular calcium release channels, but also in certain activated calcium receptors such as ryanodine (RYR2), these major biological networks are influenced by the VDR (Bansal et al., 2020).

Vitamin D can be synthesized and metabolized locally in the central nervous system (CNS) by the presence of 1α-hydroxylase in neurons and glia in the brain, but it can also be found in the CNS and its metabolites can cross the blood-brain barrier. VDR is also localized in the hippocampus, cortex, limbic systems, somatosensory system (Cui et al., 2017) thus helping to regulate neurotransmitters, neuronal differentiation, axial growth, calcium channel stress sensitivity, neurotrophic factors and reactive oxygen species (ROS) - through which they are affected by proper neuronal function (Cui et al., 2017). Several studies have shown that there are a variety of nucleotide polymorphisms in genes that express elements of vitamin D metabolism, such as CYP2R1, CYP27B1, CYP24A1, VDR, GC, and are associated with the prediction of serum 25(OH)D levels (Mpandzou et al., 2016).

The main hallmarks of the cerebral inflammatory response (common source in epilepsy) are: traumatic brain injury (TBI), blood-brain barrier (BBB) dysfunction such as microglial and astrocyte activation and migration. Neutrophils mediate early pathogenesis by stimulating edema and oxidative stress, and the release of inflammatory factors such as cytokines, chemokines and ROS (Pendo et al., 2016; Webster et al., 2017). The most well-known cytokines released after TBI include tumor necrosis factor (TNF-α), transforming growth factor-β (TGF-β) and interleukin-1β (IL-1β), -6, -10 (Pendo et al., 2016; Vezzani et al., 2017). These, in turn, can recruit additional neutrophils and monocytes

from the blood into the damaged tissue, thus spreading an inflammatory cascade (Pendo et al., 2016).

Chapter 2 Epilepsy in animals

Epilepsy is not an exclusively human condition, as animals are also affected by it (Mínguez et al., 2019; Mínguez et al., 2021; Löscher, 2022), especially in cases of inbreeding when trying to repopulate the species. The main characterization of this condition is the persistent predisposition to generate spontaneous recurrent epileptic seizures. Status epilepticus is a common form of neurological emergency in both humans, being strikingly similar to naturally occurring epilepsy in domestic animals (Leppik et al., 2011; Löscher, 2022). Epilepsy and recurrent seizure disorders (RCDs) have been the subject of veterinary research for the last 75 years in companion animals (O'Neill et al., 2020). This can also be induced in various animal models for scientific research purposes (Dudek and Staley, 2012; Pitkänen et al., 2015).

The process of epileptogenesis is a chronic process by which previously normal neuronal networks are functionally altered by genetic or acquired factors to lead to increased seizure susceptibility in animal models (Dudek and Staley, 2012; Pitkänen et al., 2015). Existing evidence suggests that this process is a continuous and prolonged one that may have an increased frequency after the first unprovoked or spontaneous seizure (Bertram and Cornett, 1993; Hellier et al., 1998; Nissinen et al., 2000; Williams, 2009; Kadam et al., 2010; Pitkänen et al., 2015).

2.1 Native epilepsy in animals

2.1.1 Epilepsy from laughter

The Iberian lynx (Lynx pardinuss) can present epileptic episodes, both idiopathic and focal, manifested by hypersalivation (sialorrhea), facial twitching (rapid movement of the ears and/or eyelids), unilateral eye blinking, repeated head jerking, followed by tonic-clonic seizures, lying in a lateral position. Incidentally, episodes of anxiety, restlessness, as well as fear or disorientation reactions were also observed. The complete biochemical profile of blood, infectious disease panel, brain MRI and cerebrospinal fluid analysis confirmed the diagnosis of epilepsy. Epilepsy has also been shown to be a genetically recessive condition and is successfully treated with phenobarbital (Mínguez et al., 2019; Mínguez et al., 2021).

2.1.2 Canine epilepsy

In canine epilepsy, a correct diagnosis is made on a broad sample of clinical signs, age of onset and underlying causes (Löscher, 2022). Brain imaging in animals with epilepsy suggests that the brain region that acts as a network system called the limbic system, which includes the hippocampus and cingulate

gyrus, is often affected in dogs, which can often indicate anxiety-like behavioural manifestations or cognitive changes (Löscher, 2022).

The three categories of epileptic seizures presented by the International League Against Epilepsy according to their onset: focal, generalized (motor and non-motor) and unknown (ilae) are also found in dogs (Löscher, 2022). These being an important factor for the prognosis of therapy (Bhatti et al., 2015; Löscher, 2022). Most of the time, idiopathic epilepsy with focal onset has a better prognosis than that of structural epilepsy with focal onset, being associated with a period of altered behaviour in which the dog seems nervous, hides or looks for its owner, these are manifested due to the fact that focal type seizures can be subtle, so that the owner of the four-legged friend does not notice, but they can also be complex, manifesting themselves through bizarre behaviour compared to the period of convulsive onset through unprovoked aggression, uncontrolled running or rhythmic barking (Löscher, W., 2022; De Risio et al., 2015).

One or more episodes of canine status epilepticus (CSE) are observed in approximately 59% of dogs with epilepsy, with a shorter average lifespan than canines with epilepsy without CSE (Leppik et al., 2011). The prognosis for these dogs is quite poor, with approximately 25% of affected dogs not surviving to hospital discharge (Löscher, 2022). As in humans, the main topics to achieve the objectives are: identifying the cause of the seizures, managing any complications that may occur, and preventing subsequent seizures, so that drugs for the prevention of epileptic seizures effective in SE in dogs may be a form of translation for human therapeutic trials being used in human patients with SE (Löscher, 2022; Leppik et al., 2011; De Risio et al., 2015). The goal of this therapy is an ideal to completely eliminate seizures, but it is not achievable, so a secondary goal is formed to reduce seizures by their number and duration. Human antiepileptic drugs cannot all be used by dogs due to the fact that in terms of pharmacokinetic differences they are eliminated more quickly by canines. Thus, there is only primidone used in the USA, and in Europe there are three drugs phenobarbital, imepitoin and potassium bromide; the last being used only if the first two have failed.

Thus, a capacity of the canine organism to have resistance to antiepileptic drugs has also been observed, through scientific studies that have demonstrated that two MAEs have failed to make an organism of certain animals become or remain seizure-free. In epileptic dogs, the percentage of drug resistance may be higher than in humans (Leppik et al., 2011).

In the evaluation of antiepileptic drugs, these treatments are not approved by the Food and Drug Administration (FDA) for CSE, but there could be approval of the placebo-controlled study, the advantages being related to: their adverse effects could be easily highlighted, especially those of cardiac arrhythmia, hypotension and behavioural changes; because the volume of

distribution in dogs is similar to that in humans and compared to smaller animal models (mice, rats, zebrafish), thus providing more efficient information on the dose of drugs with a useful effect and target plasma concentrations that are also suitable for humans (Leppik et al., 2011).

2.1.3 Epilepsy in domestic cats

In recent years, studies have emerged showing that there is a population of cats that meet clinical criteria for a diagnosis of genetic (primary) epilepsy. This is described as having an onset between 1 and 7 years of age, with a neurological examination between seizures, metabolic screening, magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) examinations, all excluding other underlying causes. Their incidence in cats, compared to dogs, is much lower (Moore, 2014). However, there is structural (secondary) epilepsy, including infectious etiologies such as feline infectious peritonitis (FIP), toxoplasmosis, non-infectious inflammatory disease and ischemic or Hemorrhagic infarcts (Moore, 2014). Or reactive seizures that do not trigger the condition epilepsy, but may occur due to systemic, toxic or metabolic abnormalities such as diseases: hepatic encephalopathy, severe uremia of end-stage renal disease, severe hypoglycemia, severe hyperthyroidism, severe systemic hypertension, polycythemia vera, hypoglycemia, hypertriglyceridemia or exposure to organophosphates (Moore, 2014).

Antiepileptic drugs (AEDs) used in cats are similar to those used in dogs, but there is a difference in toxicity and metabolism. The most commonly used are first-line: phenobarbital (PB), levetiracetam (FAE), zonisamide, but these have less frequent adverse effects, but which are more serious in cats than in dogs, such as PB including acute toxicity, idiosyncrasy of the liver or bone marrow that can occur within the first four weeks of administration, or acquired myelo-hepatic fibrosis after prolonged administration of the drug (Moore, 2014; O'Neill, et al, 2020).

2.1.4 Epilepsy in horses

Although there are not many studies that clarify epileptic seizures in horses, this is due to the fact that there are disorders similar to these seizures such as attacks, strokes, grad mal seizures. According to the classification made for epilepsy in humans, the epileptic syndrome is not found in horses, the diagnosis being based on ictal phenomenology, classification by type, classification by etiology, as well as an optional indication of the degree of impairment caused by the epileptic status (Lacombe, 2015).

Ictal phenomenology consists of the description of the behaviour and clinical signs during the periods before (preictal), between (interictal) or after (postictal) seizures, being characterized by epilepsy (Lacombe, 2015). These seizures have a wide range of manifestations such as variation between mild changes in consciousness, muscle fasciculations that can lead to the diagnosis of

generalized seizures in the entire cerebral cortex being a bilateral motor activity or focal seizures that originate from a site of the peripheral cerebral cortex (Lacombe, 2015).

The diagnosis of epilepsy in horses is difficult to make, due to the limitations of brain imaging techniques, the etiology of intra- and extra-cranial diseases, thus being expensive and time-consuming (Lacombe, 2015). In order not to confuse epileptic seizures with other types of seizures, a physical examination and anamnesis are performed to help exclude them. Blood and cerebrospinal fluid analyses are bioclinical tests that help the clinician to exclude metabolic abnormalities that may lead to seizures such as complete blood count, fibrinogen, viral infections, tests for bile acids, ammonia and blood glucose (Lacombe, 2015).

2.2 Animal models of epilepsy

2.2.1 Modelling epilepsy in rodents

Mice and rats are an animal model often used in scientific research to induce and analyse various conditions. They are used in behavioural, physiological and genetic studies. In epilepsy research, they are used as a wild-type/Wistar model (where most often the induction of epileptic seizures is based on pentylenetetrazole, kainic acid) (Abdel-Wahab et al., 2017; Momeni et al., 2019; Sahin et al., 2019), WAG/Rij type (genetic epileptic model with depressive comorbidities) (Aygun et al., 2019; Sitnikova., 2024), GAERS type (genetic absence epilepsy) (Sitnikova, 2024.).

In the case of these models, there are scientific studies that demonstrate the role that vitamin D can have in controlling epileptic seizures, which will help prevent cognitive impairment and minimize the number of adverse effects (Abdel-Wahab et al., 2017; Momeni et al., 2019; Sahin et al., 2019; Aygun et al., 2019).

2.2.2 Modelling epilepsy in zebrafish

The zebrafish (*Danio rerio*) is a model organism that has gained more space in biomedical research. In neurological research, it is used for its central nervous system architecture similar to mammals and its physiological and genetic properties homologous to humans, making it a good model due to the ease of absorption of compounds used in research, as well as the ease of genetic manipulation techniques compared to rats (Kalueff et al., 2014; Sassen and Köster, 2015; Chitolina et al., 2023). Several studies have shown that over half of the protein-coding genes in the human genome have a counterpart in the zebrafish genome, and 84% of human diseases have a homologue in diseases that can be acquired by zebrafish (Howe et al., 2013; Chitolina et al., 2023). It is also used in studying various physiological and biochemical mechanisms of epilepsy, as well as studying old and new drugs that are used to reduce/prevent epileptic seizures (Zang et al., 2022; Chitolina et al., 2023).

Over time, it has been proven that zebrafish are a good and effective experimental model for epileptic seizures induced chemically by means of seizure-inducing agents: pentylenetetrazole, pyroxene allylglycine, kainic acid, kainate at different administered concentrations (Baraban et al., 2005, Williams et al., 2009; Leclercq et al., 2015, Yang, et al., 2017; Chitolina et al., 2023).

Behavioural, biochemical and genetic testing have made a great contribution to the discovery of new compounds or finding substances as potential candidates for anticonvulsant drugs capable of reducing or eliminating epileptic seizures that mimic human epileptic seizures and minimizing adverse effects on the body.

Chapter 3 Research hypothesis and objectives of the paper

In most research articles, epilepsy is considered a condition as a whole, but it varies depending on the heterogeneous encephalopathies that have a common symptom: epileptic seizures that occur and can be identified most easily through appropriate imaging modalities, but also through electroencephalogram, medical history. To these types, certain psychiatric comorbidities can be added that can occur at different intervals from seizures, respectively depending on the epileptic onset.

I wanted to demonstrate the multifactorial nature through which vitamin D, from a physiological and biochemical point of view, plays an essential role in psychiatric disorders associated with epilepsy using Danio rerio animal models.

Objective 1. Studying and systematically combining the results of studies in the specialized literature on cases of juvenile myoclonic epilepsy on the part responsible for processing information in the human brain.

Objective 2. Understanding the effect of valproic acid across multiple doses in animal models of epileptic seizures that can mimic neuropsychiatric manifestations

Objective 3. Establishing the effect of a dose of vitamin D and doses of antiepileptic drugs in *Danio rerio animal models.*

Chapter 4 Research methodology

The purpose of this doctoral thesis is divided into two parts: 1) to bring new information on cases of juvenile myoclonic epilepsy from the literature 2) by using zebrafish (*Danio rerio*) that exemplary animal in study role VITAMIN D in epilepsy in function of the effect of two antiepileptic drugs (valproic acid, Carbamazepine). In order to achieve this goal, 1) we performed a meta-analysis that helps to observe the level of gray matter in patients with epilepsy and 2) we used epilepsy modelling by chemical methods - pentylenetetrazole (PTZ), but also the administration of four substances: valproic acid in the form of a powder specific for experiments, valproic acid in the form of tablets, Carbamazepine in the form of an oral solution, and vitamin D in the form of pill.

4.1 Experimental study I

4.1.1 Scope

The meta-analysis aims to systematically synthesize and combine the results of independent studies on cases of juvenile myoclonic epilepsy that observe changes in gray matter.

4.1.2 The design experimental

A search of all online articles based on patients with juvenile myoclonic epilepsy and healthy controls was conducted, identified in the PubMed, Web of Science, Brain Map, and Cochrane libraries. All results were generated based on the following keywords: juvenile myoclonic epilepsy (JME), voxel-based, morphometry (VBM) and have former REPORTED of by Institute neurologically FROM Montreal (MNI) or AREA Talairach.

4.1.3 Statistical analysis of dates

For this analysis saddle used SOFTWARE Seed-based of Mapping (SDM) (Albajes-Eizagirre et al., 2019), version 6.21. demographic data and peak coordinates were tracked. The full width at half maximum was set at 20 mm, and the statistical threshold was $p < 0.005$. These were used because previous studies have shown that the values have the best control for false positive rates, being able to optimize the balance between sensitivity and specificity (Seidman et al., 2011). A *jackknife sensitivity analysis was also used,* for which one study was omitted and the mean analysis and a heterogeneity analysis were repeated, using a random-effects model with Q statistics (Seidman et al., 2011, Pan et al., 2012).

4.2 Experimental study II

4.2.1 Scope

The specialized literature presents numerous studies, publications or cases in which discusses the ability of valproic acid (VPA) to reduce or stop seizures in both people and various animals suffering from epilepsy, as well as the various psychiatric comorbidities associated with this disorder. conditions.

This study was to highlight the differences at the behavioural level but also at the biochemical-enzymatic level that exist between different amounts of valproic acid administration (5,000, 7,5000, 10,000 µM) in both the epileptic model and the healthy control.

4.2.2 The design experimental

Danio rerio species (zebra fish) purchased from a local authorized producer in Iași County were used as animal models. After the acclimatization stage, the zebra fish were divided into 5 Groups equal in mode SHUFFLE (n=10) in AQUARIUMS we of 10 IT (using 5 IT of water) for another 5 days of adaptation to the new experimental conditions. The temperature in the laboratory, respectively from aquariums was constant of ± 26 ^{0} C throughout the entire experiment, the water having a pH of 7.5. A 14-hour light/10-hour dark cycle was maintained throughout the experiment. The fish were feed of two OR on day with TetraMin Flakes, and water A former change daily. animals have been maintained and treated strictly in accordance with the EU Commission Recommendation (2007), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on guidelines for the accommodation, care and protection of animals used for experimental purposes. After the acclimation period, the fish were analysed to observe their initial behaviour before the administration of the 2 substances.

The doses used in this experiment were 10 mM pentylenetetrazole (PTZ), 5 respectively. 7,5 and 10 mM VPA, all have former PREPARATIONS daily of *new* and MANAGED on a period of 26 days. The administration of the substances was carried out directly in specific containers that contained 20 mL of solution for 20 minutes/fish. Behavioural tests were performed 5 times over the 26 days, with an interval of 4 days between tests (Figure 4.1).

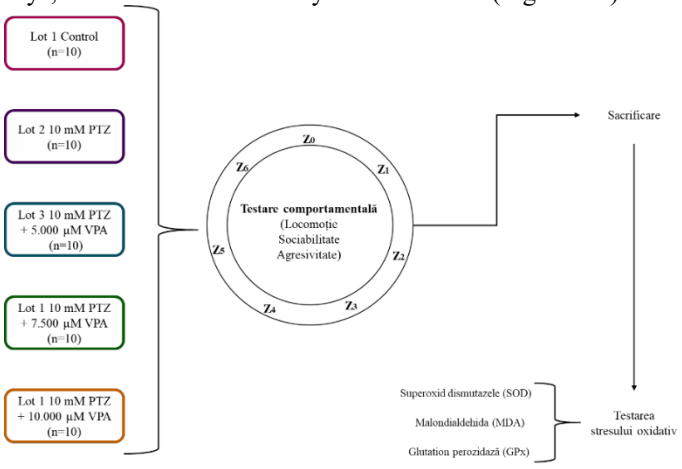


Figure 4.1 Scheme of the experimental study protocol II

After the 26 days of treatment, at the end of the experimental period, the fish were sacrificed by immersion in ice water, a protocol approved by the European Union Commission, and stored at -80 ^{o}C until subsequent biochemical analyses.

4.2.3 Test behavioural

New aquarium tests

The test is performed in a new 10 L aquarium (filled with 6 L of water, for 4 minutes) compared to the one in which the fish are usually housed. The following aspects are monitored during the test: latency to reach the upper half of the aquarium, time spent in the upper half of the aquarium, number of entries into the upper half of the aquarium, average duration of entries, and number and duration of freezing. Freezing was defined as complete lack of movement, with gill and eye movements, for two seconds or more. Increased anxiety is usually accompanied by reduced exploratory behaviour (Figure 4.2).

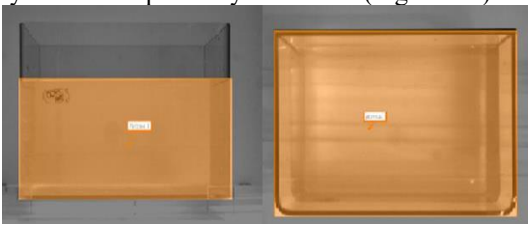


Figure 4.2. Representation of the new aquarium test calibration in Etho Vision XT 14 software.

Social test

The social test is performed with a cross maze transformed into a T-maze by closing one arm with a colourless plastic slot, thus the maze is composed of two arms, one left, one right and a main arm in which the starting box is located. In behavioural science, the T-maze is used both for social studies, through which the subject is offered a direct choice, this will help to study how to stimulate spatial and non-spatial navigation, through the monitored parameters. To determine social behaviour, two zebrafish will be placed in a box in the left arm of the maze (the box will be made by placing a colourless slot). Another fish will be placed in the starting box, exploration of the maze and the time spent in the left arm of the maze will prove the accentuated social behaviour of the fish (Figure no. 4.3).

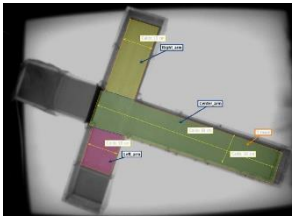


Figure 4.3. Representation of the social T-test calibration in Etho Vision XT 11.5 software

The test of aggression

Aggression is a feature complexity which maybe be difficult of measured with accuracy. These manifestations of struggle with the opponent in the mirror, chronic unpredictable stress, social challenge, predator presence test, dominant-subdominant encounters, the pattern of aggression can be observed with this test, being related to the psychiatric disorders manifested. Even if the fish cannot recognize themselves in the mirror, they can recognize that their opponent is exactly the same size and makes exact same movements that and them (Zabegalov, KN and etc., 2019). Various types of aggression have been identified. The aggression test is performed with a cross maze transformed into a T maze by closing one arm with a slot with a mirror-like surface, thus the maze is composed of two arms, one left, one right and a main arm in which the starting box is located. Each fish will spend 4 minutes in the maze where several parameters will be analysed with the Etho Vision XT 11.5 software. In behavioural science, the T maze is used both for social studies and to test aggression (Figure 4.4).

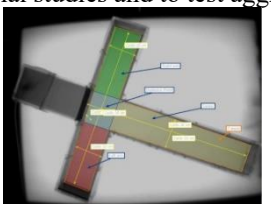


Figure 4.4. Representation of the calibration of the T-test of aggression in the Etho Vision XT 11.5 software.

4.2.4 Oxidative stress assessment

After the experiment was completed, each group was subjected to oxidative stress analysis. For this, several steps were taken: the first of these consisted of homogenizing each fish in cold saline solution (0.90% NaCl; Hemofarm, Romania) and centrifuging at 5500 rpm for 10 min at 4º C according to the method of Jin et al. (Jin et al., 2015). After centrifugation, the supernatant of each sample was transferred to a clean PE tube and divided equally for the analysis of total proteins, SOD, GPx and MDA.

SOD (kit 19160-1KT-F), GPx (kit CGP1-1KT) and MDA (kit MAK085-1KT) levels, as well as total protein concentrations in tissues (kit

TP0300-1KT) were determined using test kits from Merck, Germany. Each parameter was measured according to the manufacturer's protocols. A spectrophotometer (SPECORD 210 Plus manufacturer Analytik Jena, Germany) was used to determine the amount of total protein, SOD, GPx and MDA levels. Data were expressed as mean ± SEM.

4.2.5 Statistical analysis of data

All data were analysed using excel spreadsheets (Microsoft Office) and Originpro v9.3 software (2016, Originlab corporation, USA), the latter being used for one-way ANOVA followed by post-hoc Tukey Honest Significant Difference test of paired group means and the generation of the presented graphs.

4.2.6 Ethics statement

This protocol was approved by the Ethics Committee of the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași, with registration number 364/04.02.2022.

4.3 Experimental study III

4.3.1 Scope

The purpose of this study will help us understand and demonstrate the multifactorial nature of vitamin D, its physiological and biochemical role in psychiatric disorders associated with epilepsy in Danio rerio animal models. The importance of this vitamin both in what includes epilepsy at the motor and biochemical levels and its existence as an aid to the two drugs known to be inducers of CYP450 enzymes thus accelerating the catabolism of vit D into its inactive polar metabolites (Siniscalchi, A., 2020).

4.3.2 Chemicals used in study

Pentylenetetrazole known under names such as 1,5-Pentamethylenetetrazole, Metrazole, was purchased from Sigma Aldrich, product code P6500, having a molecular weight of 138.17 g/mol. This is a substance that induces epileptic seizures and is widely used in animal models as a good proconvulsant. A stock solution was made consisting of 0.6909 PTZ + 1000 mL distilled water to bring the stock solution to 5 mM PTZ.

Calciferol or popularly called vitamin D was purchased from a local pharmacy, being a product intended for adults in the form of a dietary supplement. The dose administered was 400 IU/L of vit. D.

Valproic acid is a product intended for people with epileptic conditions and those with bipolar disorders, this is a drug. A single tablet contained 333 mg of sodium valproate and 145 mg of valproic acid, equivalent to 500 mg of sodium valproate. The dose administered was 50 mg/L of VPA.

Carbamazepine is a drug used for the treatment of certain types of epilepsy, but also as an adjuvant in the control of mood disorders, being a good substitute for lithium treatment. It was purchased in the form of an oral suspension. Each 5 mL of suspension contains 100 mg of carbamazepine. The

dose administered was 40 mg/L of CBZ.

4.3.3 The experimental design

In this experimental study, 200 wild zebrafish were used as animal models, purchased from a local authorized producer in Iași County. After the acclimation stage, the zebrafish were divided into 10 equal groups (n=20) in new 10 L aquariums (using 5 L of water) for another 10 days of adaptation to the new experimental conditions. The temperature in the laboratory, respectively in the aquariums, was constant at ±26°C throughout the experiment, with the water having a pH of 7.5. A 14-hour light/10-hour dark cycle was maintained throughout the experiment. They were fed twice a day with TetraMin Flakes, and the water in each experimental tank was changed daily. The animals were maintained and treated strictly in accordance with the EU Commission Recommendation (2007), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, normative on guidelines for the accommodation, care and protection of animals used for experimental purposes.

Before the administration of the substances, behavioural tests were carried out to be able to observe the exact changes that the fish will show following the experimental treatments.

Over a period of 11 days, 5 mM pentylenetetrazole (PTZ) was administered for 15 minutes per day to all fish from the first 6 batches, being tested on day 12. After testing, we continued the administration with the same dose in the same time interval, for another 15 days. During this period, the administration was started with a supplement of 2000 IU of vitamin D3 and the anticonvulsants 500 mg Valproic Acid, 200 mg Carbamazepine which were dissolved in 5L of water (Figure 4.5). The fish were kept in the treatment aquariums for two hours, after which they were moved to aquariums with clean water.

Thus, the studied batches were as follows: Batch 1 Pentylenetetrazol, Batch 2 Pentylenetetrazol + Vitamin D, Batch 3 Pentylenetetrazol + Valproic Acid, Batch 4 Pentylenetetrazol + Carbamazepine, Batch 5 Pentylenetetrazol + Valproic Acid + Vitamin D, Batch 6 Pentylenetetrazol + Carbamazepine + Vitamin D, Batch 7 Control, Batch 8 Vitamin D, Batch 9 Valproic Acid, Batch 10 Carbamazepine. The behavioural tests were performed 3 times in the first 6 batches in which PTZ was administered as follows: before, on day 12, and on day 26, respectively, and in the other 4 batches these tests were performed 2 times before and after the administration of the 15 days of treatment.

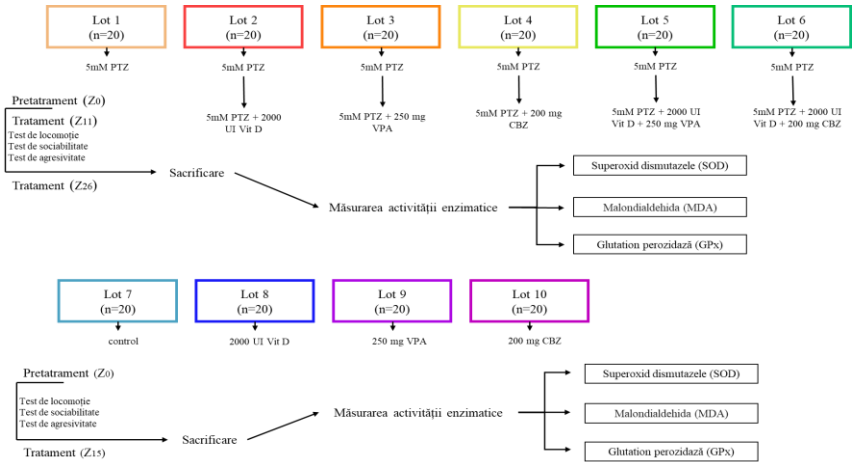


Figure 4.5. Experimental stage III protocol scheme

The groups were grouped according to the substance administered for each parameter analysed in the three behavioural tests. Thus, the three days (Initial, day 11 of exposure, respectively day 26 of exposure) were analysed for the 6 groups that were exposed to Pentylenetetrazole (PTZ), and for the other 4 groups, two days were analysed (day 11 of exposure/initial and day 26 of exposure (day 15 of treatment) and day 15 of treatment) (Table 4.1). Group 1 is made up of all the positive batches (in which only one substance was administered) (Lot 1 PTZ, Lot 7 Control, Lot 8 Vit D, Lot 9 VPA, Lot 10 Carba), group 2 contains all the batches in which vitamin D was administered (Lot 2 PTZ+Vit D, Lot 5 PTZ+VPA+Vit D, Lot 6 PTZ+Carba+Vit D, Lot 8 Vit D), group 3 includes the batches with Valproic Acid (Lot 3 PTZ+VPA, Lot 5 PTZ+VPA+Vit D, Lot 9 VPA), respectively group 4 which had Carbamazepine as treatment (Lot 3 PTZ+Carba, Lot 6 PTZ+Carba+Vit D, Lot 10 Carba).

Table 4.1 Administration of substances to experimental groups

LOT	Z_1 -Z_{11}	Z_1 -Z_{15}	Z_{11} -Z_{26}
Lot 1 Pentylenetetrazole	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole
Lot 2 Pentylenetetrazole + Vitamin D	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole + 2000 IU/5L Vit D
Lot 3 Pentylenetetrazole + Valproic Acid	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole + 250 mg/5L VPA
Lot 4 Pentylenetetrazole + Carbamazepine	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole + 200 mg/5L CBZ
Lot 5 Pentylenetetrazole+ Valproic Acid + Vitamin D	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole + 250 mg/5L VPA + 2000 IU/5L Vit D
Lot 6 Pentylenetetrazole + Carbamazepine + Vitamin D	5mM Pentylenetetrazole	-	5mM Pentylenetetrazole + 200 mg/5L CBZ + 2000 IU/5L Vit D
Lot 7 Control	-	-	-
Lot 8 Vitamin D	-	2000 IU/5L Vit D	-
Lot 9 Valproic Acid	-	250 mg/5L VPA	-
Lot 10 Carbamazepine	-	200 mg/5L CBZ	-

4.3.4 Behavioural tests

New aquarium tests

The test is performed in a new aquarium compared to the one in which the fish are usually housed, 10L (filled with 6L of water, for 4 minutes. The following aspects are monitored during the test: latency to reach the upper half, time spent in the upper half, number of entries in the upper half, average duration of entries and, number and duration of "freezing". Freezing was defined as total lack of movement. Except for the gills and eyes, for 2 s or more. Increased anxiety is usually accompanied by reduced exploratory behaviour (Figure 4.6.).

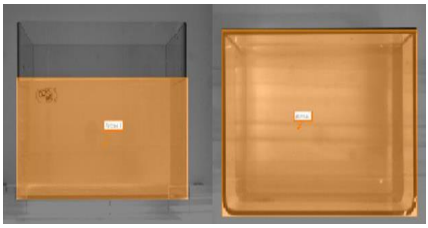


Figure 4.6. Representation of the new aquarium calibration test in the Etho Vision XT 14 software.

Social test

The social test is performed with a cross maze transformed into a T-maze by closing one arm with a colourless plastic slot, thus the maze is composed of two arms, one left, one right and a main arm in which the starting box is located. In behavioural science, the T-maze is used both for social studies, through which the subject is offered a direct choice, this will help to study how to stimulate spatial and non-spatial navigation, through the monitored parameters. To determine social behaviour, two zebrafish will be placed in a box in the left arm of the maze (the box will be made by placing a colourless slot). Another fish will be placed in the starting box, exploration of the maze and the time spent in the left arm of the maze will prove the accentuated social behaviour of the fish (Figure 4.7).

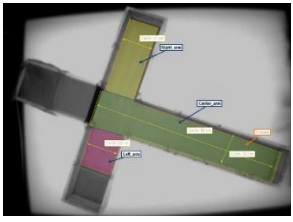


Figure 4.7. Representation of the social T-test calibration in Etho Vision XT 11.5 software

The test aggressiveness

Aggression is a complex trait that can be difficult to measure accurately. These manifestations of mirror-firing, chronic unpredictable stress, social

challenge, predator presence test, dominant-subdominant encounters can be observed with this test, the pattern of aggression being related to the manifested psychiatric disorders. Even though fish cannot recognize themselves in the mirror, they can recognize that their opponent is exactly the same size and makes exactly the same movements as them (Zabegalov et al., 2019). Different types of aggression have been identified. The aggression test is performed with a cross maze transformed into a T-maze by closing one arm with a slot with a mirror-like surface, thus the maze is composed of two arms, one left, one right and a main arm in which the starting box is located. Each fish will spend 4 minutes in the maze where several parameters will be analysed with the Etho Vision XT 11.5 software. In behavioural science, the T-maze is used both for social studies and to test aggression (Figure 4.8).

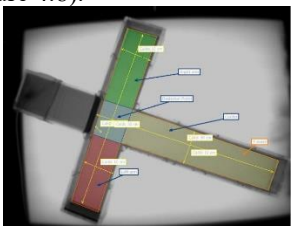


Figure 4.8. Representation of the calibration of the T-test of aggression in the Etho Vision XT software
11.5.

4.3.5 Stress assessment oxidative

After the experiment was completed, each group was subjected to oxidative stress analysis. For this or crossing may much stages. received from these findings in homogenizing each fish in cold saline solution (0.90% NaCl; Hemofarm, Romania) and centrifuged at 5500 rpm for 10 min TO 4º C conformable method his Jin and of. (Jin and etc., 2015). After centrifugation, the supernatant of each sample was transferred to a clean Eppendorf tube and divided equally for analysis of total proteins, SOD, GPx and MDA.

SOD (kit 19160-1KT-F), GPx (kit CGP1-1KT) and MDA (kit MAK085-1KT) levels, as well as tissue protein concentrations (kit TP0300-1KT) were determined using assay kits from Merck, Germany. Each parameter was measured according to the manufacturer's protocols. A spectrophotometer (SPECORD 210 plus, manufacturer Analytik Jena, Germany) A former use for A determine quantity of PROTEIN total, levels of SOD, GPx and MDA. Data were expressed as mean ± SEM.

4.3.6 Statistical analysis of dates

The normality and distribution of behavioural data were determined by the Shapiro-Wilk test using Graph Pad Prism software (v 9.1.0.221, San Diego, CA, USA). Subsequently, comparisons between groups were performed with one-way ANOVA followed by post-hoc parametric tests, such as Tukey HSD.

Graphical analysis using OriginPro 9.0 with which the evolution of the distance travelled by each group in each arm of the social and aggressive type test can be compared.

4.3.7 Ethics statement

This protocol was approved by the Ethics Committee of the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași, with registration number 3480/24/10/2022.

Chapter 5 Results and Discussion

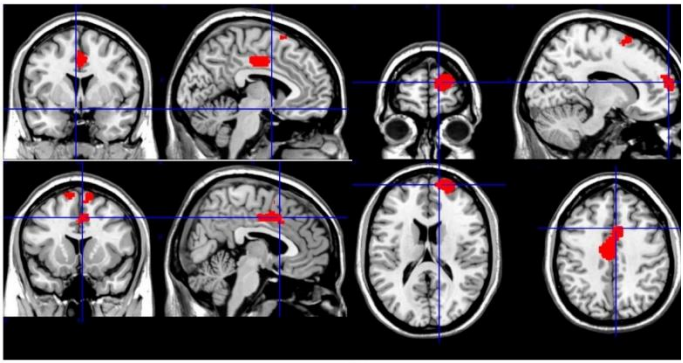
5.1 Experimental study I

5.1.1 Meta-analysis results

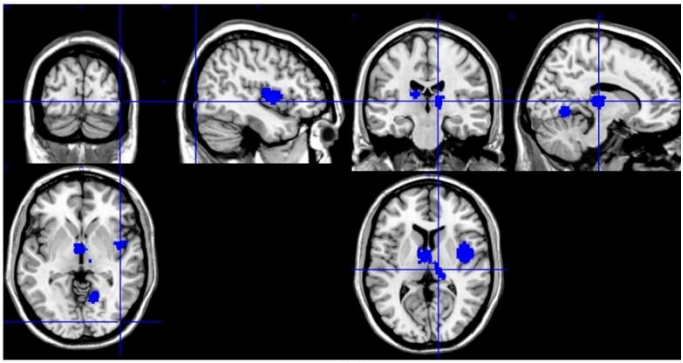
The initial search yielded 31 studies, which were filtered by reviews, no control group, and no well-detailed peak coordinates. For this meta-analysis, 12 studies with a total of 325 patients with juvenile myoclonic epilepsy (JME) resulted, and the control group was 357 witnesses healthy.

This meta-analysis showed an increase in gray matter in patients with JME compared to controls in the left medial cingulate or paracingulate gyrus (Brodmann area 23, SMD-Z: 1.404, where the statistical difference was p=0.0002, Voxels: 820 MNI coordinates; -4, -6, 42) frontal gyrus HIGH justice (area Brodmann 10, SMD-Z; 1,446, where p=0.0002, voxels 186, MNI coordinates : -20, -20, 66) right supplementary motor area (Brodmann area 6, SDM-Z: 1.135, where p=1.139, Voxels: 152, MNI coordinates: 8 10, 60) and left supplementary motor area (Brodmann area 6, SDM-Z: 1,139, where difference significantly A former p=0.0013, Voxels: 72, MNI coordinates: -8, 12, 68).

Thus, patients with JME have present a volume may low of material gray in thalamus left (SDM-Z: -1.1875, where p=0.00001, Voxels: 970, coordinates NIM: -12, -6, 8) and in island left (area Brodmann 48), SDM-Z: -1,372, where p=0.0008, Voxels: 553, coordinates MNI? 42, 2, 6)
(Figure 5.1).



(A)



(B)

Figure 5.1. Gray matter changes in patients with juvenile myoclonic epilepsy
(A) Areas with increased gray matter volume in patients with JME
(B) Areas of reduced gray matter volume in patients with JME compared to controls.
Source: Kazis et al., 2021

There was no significant evidence of publication bias by visual inspection of funnel plots and Egger's tests. Because there were both robust, repeatable positive and negative peaks, a one-study omission was performed each time using jackknife analysis, and a repeat of the mean analysis (Figure 5.2).

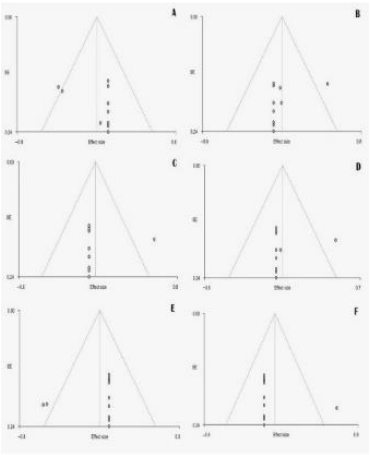


Figure 5.2. Fundel plot diagrams on all areas that showed statistically significant differences between patients with JME and controls.
(A) Thalamus, (B) Left medial cingulate cortex,
(C) Left precentral gyrus, (D) Left supplementary motor area,
(E) Right Island, (F) Right superior frontal gyrus.
Source: Kazis et al., 2021

This meta-analysis showed that there is increased gray matter volume in frontal regions of the brain, consistent with studies using other modalities to highlight differences in these brain regions in juvenile myoclonic epilepsy compared to healthy controls.

This lobe plays an important role in working and prospective memory in executive functions. Sqartz and his collaborators showed through two studies that there is a difference in visual working memory between patients with juvenile myoclonic epilepsy (JME) and patients with frontal lobe epilepsy (FLE), respectively, and control patients. Sqartz and his team conducted the study to assess visual working memory and observed that patients with JME had a lower level of memory, but also a reduced 18FDG uptake in the ventral premotor cortex, caudal, bilateral dorsolateral prefrontal cortex and left premotor area (Lee et al., 2020; Swartz et al., 1994). This has been demonstrated by several groups of researchers such as Devinsky et al., Pascalicchio et al., and Kim et al. (Swartz et al., 1996; Devinsky et al., 1997; Pascalicchio et al., 2007). However, there are studies that show the opposite, with comparable performance on working memory tasks in patients with JME and controls (Kim et al., 2019; Vollmar et al., 2012; Savic et al., 2004; Iqbal et al., 2015; Kazis et al., 2021).

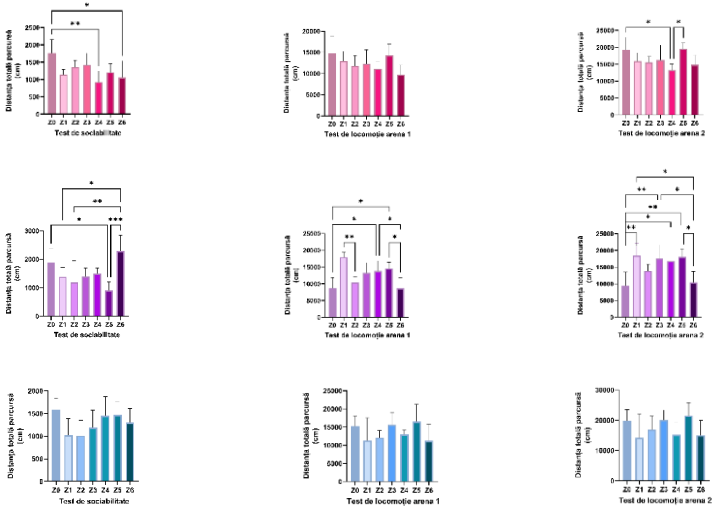
5.2 Experimental study II

5.2.1 Swimming performance

The graphs below show the performance of zebrafish in the locomotion

test, as well as in the sociability test. Differences can be observed between the control group and the groups in which PTZ was administered alone or together with different concentrations of VPA, their results being presented as mean ± SEM. Thus, during the 4 minutes of each test, statistical differences in the total swimming distance can be observed. These parameters were calculated according to the arenas, so arena 1 represents the lateral filming in which the swimming preference in the upper and lower parts of the aquarium can be observed, respectively arena 2 represents the vertical filming in which the swimming perimeter seen from above compared to the front part, where the lateral and posterior camera of the aquarium was fixed.

In the groups in which PTZ and VPA of different concentrations were administered, there were statistical differences in the average total swimming distance. In group 3, where 5,000 µM VPA was administered, there were no statistically significant differences, but in group 4 with administration of 7,500 µM VPA, an increase in the average total swimming distance in the sociability test was observed between Z_1 and Z_6 (p=0.0457). In the last group in which the two substances were administered and VPA was at a concentration of 10 mM, significant differences in the total swimming distance were observed only in the locomotion test, thus in arena 1, two increases in the average Z_2 and. Z_4 (p= 0.0037), Z_2 and. Z_5 (p= *0.0037), respectively in arena 2 two increases in the mean* Z_0 and Z_1 (p= 0.0022), Z_0 and. Z_6 (p= *0.0005)* were observed (Figure 5.3).



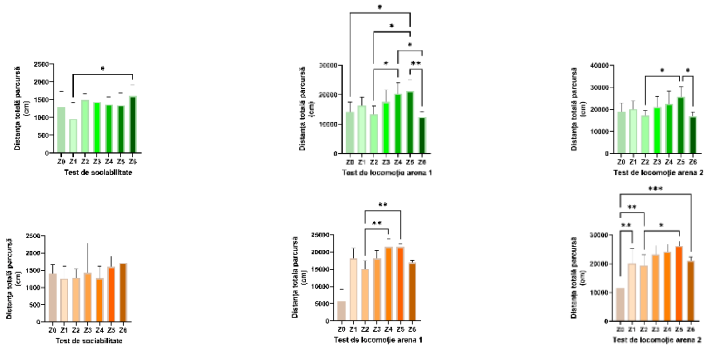


Figure. 5.3. Total swimming distance by experimental groups (cm) depending on the testing day of the sociability and locomotion test. Results are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The average speed of the fish is definitely and directly related to the total swimming distance, but it can provide information about their condition at the time of testing according to the statistically significant differences that can be observed during the administration of the proconvulsant (PTZ) and the anticonvulsant (VPA) in the three different concentrations. Thus, significant differences were observed in the average speed travelled in the control group, all differences in the sociability test were related to the pretreatment day and decreased along the way, such as Z_0 and Z_1 (p=0.0301), in the locomotion test in arena 1 (lateral) only one significant difference was observed by decreasing the average speed between Z_0 and. Z_6 (p=*0.0370). In group 2 where PTZ was administered during the sociability test, a decrease in the average speed* Z_1 and Z_6 (p=*0.0496)* was observed. During the locomotion test the following were observed: in arena 1 there was an increase between Z_0 and. Z_1 (p=0.0001) and a decrease in the average speed Z_1 and. Z_6 (p=0.0001), respectively in arena 2 (vertical) there was the same increase between Z_0 and Z_1 (p=0.0025), but there was a decrease in the average speed Z_1 and. Z_6 (p=0.0006) (Figure 5.4).

In group 3 where PTZ and 5,000 µM VPA were administered, it was observed that there were no statistically significant differences in the average speed as well as the parameter of the total swimming distance. Thus, in group 4 where 7,500 µM VPA was administered, in the sociability test, a single significant increase in the average speed between Z_1 and Z_6 (p=0.0459) could be observed, in the locomotion test, no statistical significance was identified between the 3 days that we considered the most relevant. In the last group, statistically significant differences were observed only in the locomotion test: in arena 1, an increase in the average speed z_0 and z_1 (p=0.0134) was observed, and in arena 2 (vertical) two significant increases in the average were observed: z_0 and z_1 (p=0.0010), z_0 and z_6 (p=0.0360) (Figure 5.4).

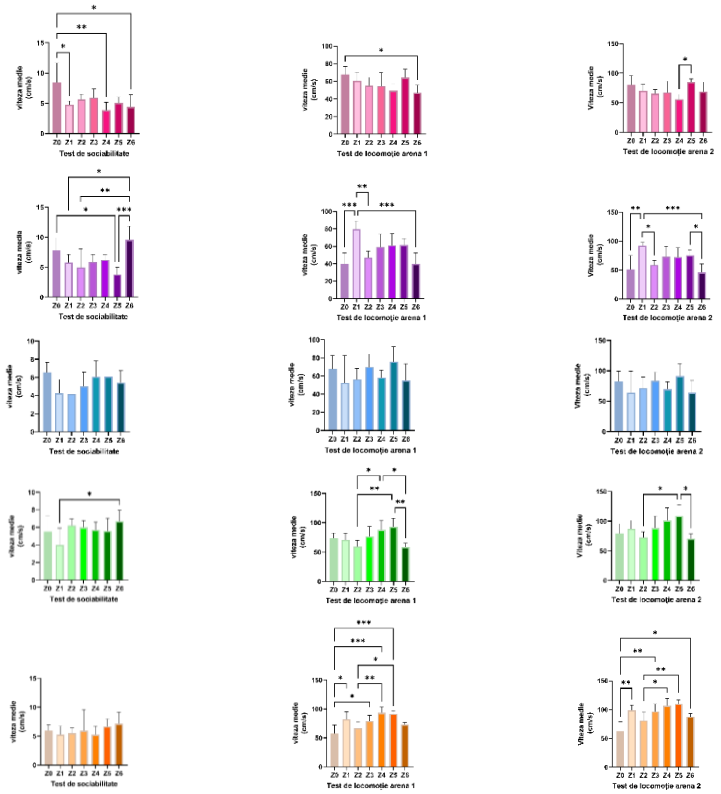


Figure. 5.4 Average speed travelled by the experimental groups (cm/s) depending on the testing day of the sociability and locomotion test. Results are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the locomotion and sociability tests, swimming performance was also taken into account in terms of the rotation frequency that the fish achieved during the tests, but also how long they had periods of immobility, which can lead us to detect situations of states such as dissociation, self-breakage, or acceptance of suffering that seems inevitable.

It was observed that in all groups there were no statistically significant differences in the sociability test. In contrast, in the locomotion test, significant differences were noted in the control group and in the group in which PTZ and 7,500 µM VPA were administered. Thus, the rotation frequency had differences in the control group in arena 1 (lateral) there was a significant decrease in the number of rotations Z_0 and. Z_6 (p=0.001), in arena 2 (vertical) there were no statistically significant differences. In group 4, statistically significant differences were observed only in the case of arena 2, where an increase in the number of

rotations was found between the pretreatment day compared to the first day of testing during treatment Z_0 and. Z_1 (p=0.0364), and a decrease in the average frequency of rotations between the first day of locomotor testing under the influence of the drug compared to the last day of locomotor testing Z_1 and. Z_6 (p=0.0294) (Figure 5.5).

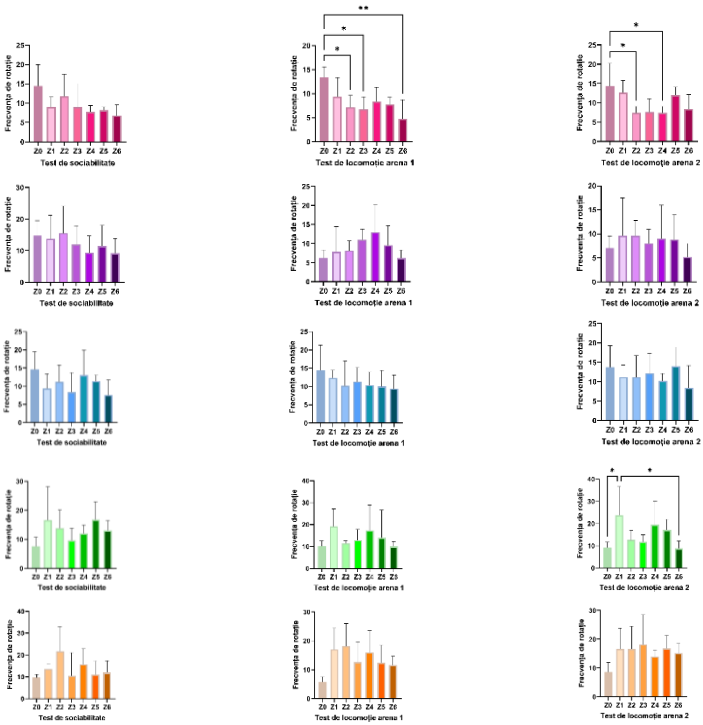


Figure. 5.5 Frequency of rotations completed by the experimental groups depending on the day of testing of the sociability and locomotion test. Results are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the case of the fish's resting period of the freezing period during the sociability test, statistically significant differences could be identified in group 4 in which PTZ and 7,500 µM VPA were administered, this difference exists between the pretreatment day and the last day of treatment testing: Z_0 and Z_6 (p=0.0175). In the case of the locomotion test, there were statistically significant differences in the group with PTZ administration (lateral) and the group with PTZ + 7,500 µM VPA administration (arena 1). Thus, in group 2 on the pretreatment day they spent more time in the stationary period compared to the first day of treatment, the significant differences in the average decrease compared to the pretreatment day Z_0 and. Z_1 (p=0.0069), and then there is an increase in the

average of fish stationary time on day 6 of the locomotion test compared to the first day of testing under the influence of the treatment Z_1 and. Z_6 (p=0.0069). In group 4 a decrease was observed compared to the pretreatment day Z_0 and Z_1 (p=0.0170) (Figure 5.6).

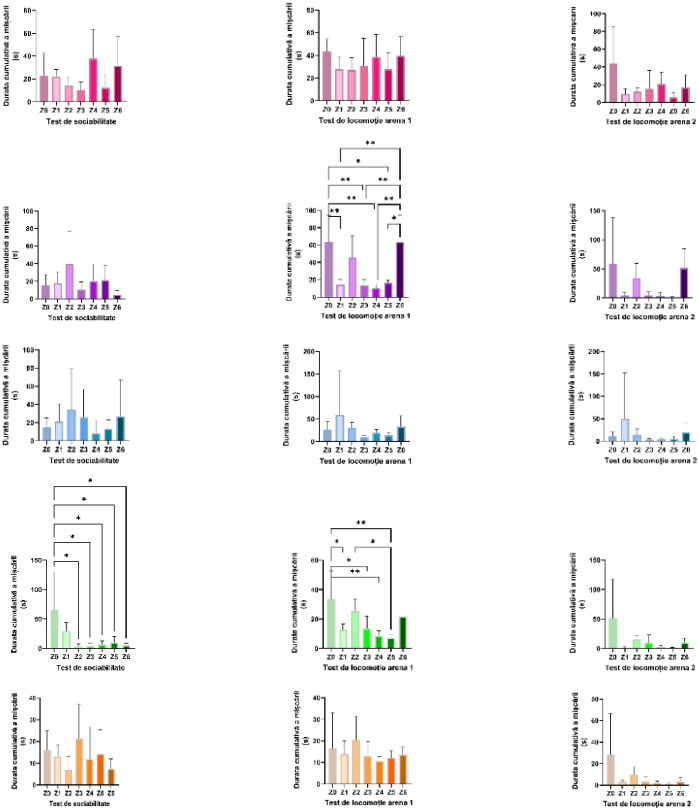


Figure. 5.6 Stationary time obtained by the experimental groups (s) depending on the day of testing of the sociability and locomotion test. Results are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the sociability test, the preference for swimming in one of the three arms could suggest whether the zebrafish depending on the experimental group wanted to explore more the arm (left) where two other fish were located behind a transparent slit. In terms of the cumulative time spent in each arm, fish from all groups had a greater preference to explore the central arm of the T test, but it was observed that in the control group an increase in the preference for socialization was seen in the first two days of behavioural testing, after which it began to fluctuate every day, reaching the highest value of the 6 days of testing on day 6 (Figure 4.7. a). In the group that was administered PTZ for 26 days, it can be seen

that, similar to the control group, the social preference of the fish increased in the first two days, but in this group, exploration of the central arm began more from day 2 to day 6 of testing, thus decreasing the time spent in the left arm (Figure 4.7. b). A constant fluctuation of the preference in the left arm was observed in the fish in group 3 (PTZ+ 5 mM VPA), where it was observed that compared to before treatment and the first day of treatment there was an increase in social preference (left arm), but also in exploration of the right arm, and z1 of social testing compared to the last day (z_6) it can be seen that the interest in exploring the central arm was greater than in the other two arms (Figure 3.7. c). In the group in which the administration was of PTZ+7.5 mM VPA, an increase in the swimming preference of the left arm over the central arm or the right arm can be observed in the first two days of behavioural testing, on day 3 and day 6 there is a decrease in the exploration of the left arm compared to the previous days, but an increase in swimming in the right arm (Figure 3.7. d). In the last group, a significant increase in swimming performance in the left arm could be observed compared to the day before treatment and the first day of behavioural testing after the start of treatment (Figure 3.7. e).

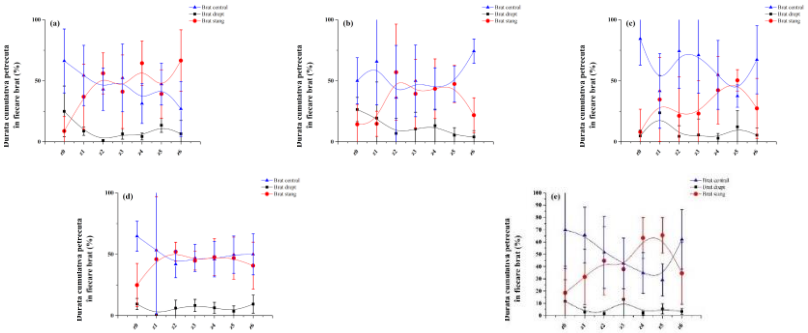


Figure 5.7. Social behavioural fluctuations over time

(a) Lot 1 Control;
(b) Lot 2 10mM Pentylenetetrazole;
(c) Lot 3 10mM Pentylenetetrazole + 5000µM VPA;
(d) Lot 4 10mM Pentylenetetrazole + 7500µM VPA;
(e) Lot 5 10mM Pentylenetetrazole + 10000µM VPA.

5.2.2 The effect of substances on aggressive behaviour

In the aggression test, differences in parameters such as total swimming distance, average speed, rotation frequency, and periods of inactivity of movement were monitored. In the control group, statistical significance was observed in three of the four parameters in which one-way ANOVA was used, so that in total swimming distance a statistically significant decrease was observed between Z_0 and Z_1 (p=0.0018).

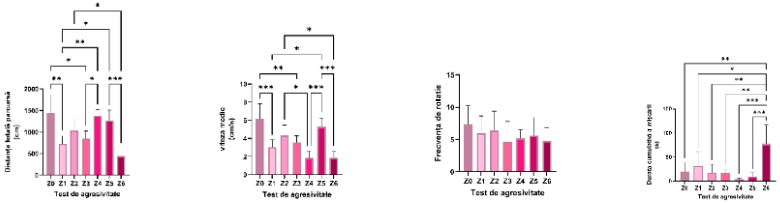
At the average speed, five decreases in average speed were seen, which led to statistical differentiation between Z_0 and Z_1 (p=0.0008). And at the

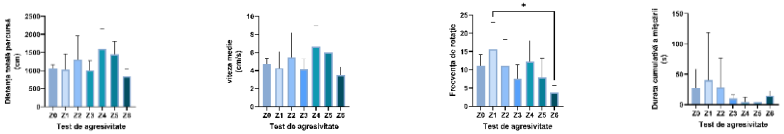
inactivity parameter of the fish in the control group, it was observed that on the 6th day they were less active than on the first day before the start of the treatment and after the start of Z_0 and Z_6 (p=0.0042), Z_1 and Z_6 (p=0.0352) (Figure 5.8).

In the groups where PTZ was administered, a statistically significant difference was observed, more precisely in Group 3 where 5 mM VPA was also administered, this difference was between the first day of treatment and the last day of treatment in the parameter indicating the frequency of rotation, where the average of these clockwise rotations decreased. z_1 compared to z_6 (p=0.0217) (Figure 5.8).

In the aggression test, the time spent in each arm was also monitored, especially in the left arm, where a mirror was attached to the wall of the arm that helps reflect the fish's movements, inducing a type of visual aggression. In all groups, it can be seen that the preference was in the central arm, except for the control group and group 5. Thus, in the control group, a change was observed from one testing day to another in both the left arm and the central arm, in the right arm there were small changes in the time spent (Figure 5.9. a).

In the case of the group that was administered 10 mM Pentylenetetrazole, an increase in swimming preference on days 1 and 2 of behavioural testing, the exploration of the other two arms being in decline compared to the day before treatment. On the last day of aggression testing, a decrease in swimming performance was observed in both the left and right arms (Figure 5.9. b). In the groups that were administered PTZ together with 5 mM VPA (Figure 5.9. c), respectively 7.5 mM VPA (Figure 5.9. d) a significantly greater increase can be observed in the central arm on days 4 and 5 of behavioural testing, followed by a sudden decrease on day 6, thus increasing the swimming preference of the fish in the left arm where the mirror is placed. In group 5 it can be observed that between the day before treatment and the first day of treatment there is an increase in exploration of the arm where the mirror is attached, thus decreasing the preference to swim in the central and right arm. On day 3 to day 6 one can observe concomitant fluctuations in the time spent in the right arm, respectively the central arm, but an increase in exploration of the left arm (Figure 5.9. e).





Total swimming distance | Movement speed | Rotation frequency | Total rest

Figure. 5.8. Parameters obtained by the experimental groups depending on the day of aggression testing. Results are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the case of the group that was administered 10 mM Pentylenetetrazole, an increase in swimming preference on days 1 and 2 of behavioural testing, the exploration of the other two arms being in decline compared to the day before treatment. On the last day of aggression testing, a decrease in swimming performance was observed in both the left and right arms (Figure 5.9. b). In the groups that were administered PTZ together with 5 mM VPA (Figure 5.9. c), respectively 7.5 mM VPA (Figure 5.9. d) a significantly greater increase can be observed in the central arm on days 4 and 5 of behavioural testing, followed by a sudden decrease on day 6, thus increasing the swimming preference of the fish in the left arm where the mirror is placed. In group 5 it can be observed that between the day before treatment and the first day of treatment there is an increase in exploration of the arm where the mirror is attached, thus decreasing the preference to swim in the central and right arm. On day 3 to day 6 one can observe concomitant fluctuations in the time spent in the right arm, respectively the central arm, but an increase in exploration of the left arm (Figure 5.9. e).

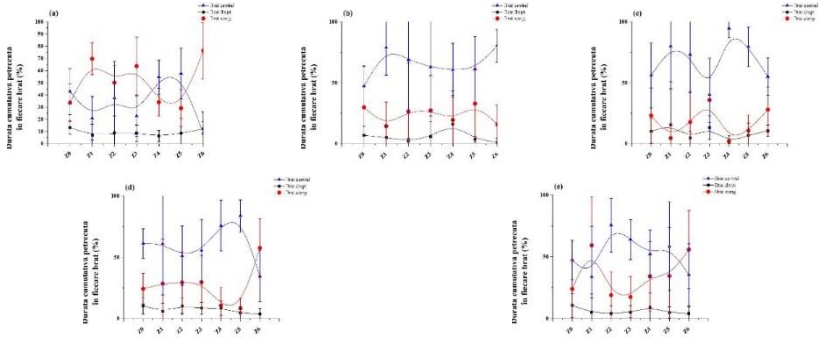


Figure 5.9. Aggressive behavioural fluctuations over time

(a) Lot 1 Control;
(b) Lot 2 10mM Pentylenetetrazole;
(c) Lot 3 10mM Pentylenetetrazole + 5000µM VPA;
(d) Lot 4 10mM Pentylenetetrazole + 7500µM VPA;
(e) Lot 5 10mM Pentylenetetrazole + 10000µM VPA.

5.2.3 Effect of substances on oxidative stress

PTZ is a proconvulsant that generates oxidative stress in the body of animal models. After performing the three tests, a significant decrease in the SOD level of the control group (LOT 1) was observed with statistical significance compared to LOT 3 (p=0.0489), LOT 4 (p=0.0002) and LOT 5 (p=0.0004), respectively a decrease in lots 2 and 3 compared to lots 4 and 5 (LOT 2 and LOT 4 (p=0.0024), LOT 2 and LOT 5 (p=0.0067), LOT 3 and LOT 4 (p=0.0150), LOT 3 and LOT 5 (p=0.0445), where SOD had the highest values, this aspect suggests that in the first three groups there is oxidative stress. Enzymatic activity led to an increase in the amount of MDA compared to the control group (LOT 1 and LOT 2 (p=0.0082), LOT 1 and LOT 3 (p=0.0015), LOT 1 and LOT 4 (p=0.0012), LOT 1 and LOT 5 (p=0.0007) GPx activity showed low levels in 4 groups, which represents the existence of oxidative stress in these groups (Figure 5.10).

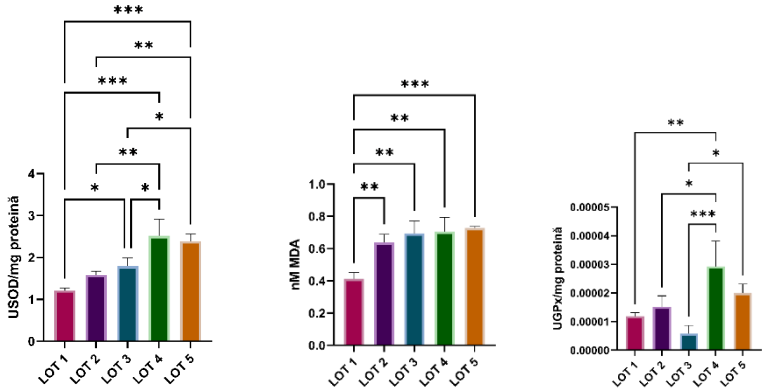


Figure. 5.10. Results of oxidative stress parameters presented in the form mean ± SEM; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

5.2.4 Discussions

Zebrafish (Danio *rerio)* have become an excellent model for the study of neurodegenerative disorders in recent years due to their broad repertoire of behavioural benefits (Vaz et al., 2018; Kalueff et al., 2013). They are inherently collective species that live in schools, and thus display a variety of social behaviours. They possess well-documented expressions of fear and anxiety and can learn complex associations (Loeschke and Currie, 2007).

The administration of the proconvulsant Pentylenetetrazole (PTZ) led to reliable results that can lead us to the convulsant effect, showing an imbalance in most of the parameters analysed in the behavioural tests (locomotion, sociability, aggression), but this imbalance was more pronounced in the groups where valproic acid was administered in concentrations of 7.5 mM and 10 mM, respectively, and no significant change was observed in the group where PTZ and 5.mM VPA were administered.

In order to ensure the safety of the PTZ effect, repeated administration for 26 days was able to determine the differences that existed in the 6 days of behavioural testing compared to the pretreatment day (z_0) and the effect of VPA as an anticonvulsant was equally important to understand the ability of this drug to stop epileptic seizures, but also to stop the behaviour induced by the proconvulsant (PTZ). This was observed by other researchers in zebrafish larvae, after the start of PTZ administration, locomotor changes could be observed that lasted from a few seconds to the end of the administration, but these can also be observed after administration (Gawel et al., 2020; Li et al., 2018).

Robea et al. conducted a study on zebrafish larvae at 6 days post-fertilization (dpf) exposed to 48 μM VPA for 24, 48 and 72 hours. The group exposed to VPA for 72 hours spent most of the time near the mirror (Robea et al., 2021). There is also some controversy on this subject because Zimmermann et al. (Zimmermann et al., 2015) contradict these findings after the administration of 48 μM. VPA influences the social component, anxiety and locomotion more than aggressive behaviour. We highlighted the absence of any indicator indicating the existence of a neurological disorder. This condition was complementary to social behaviour, but combined with high aggression. Liu et al. (Liu, et al., 2016) provided relevant evidence on how 20/100 μM VPA for 7 hours per day for 6 days in 24-hour pf larvae. This substance causes a deficit in social preference, while acute exposure affects locomotor activity. Valproic acid administration did not significantly alter the behavioural response to light, nor did it inhibit the anxiety response, thus linking it to chronic exposure that did not alter locomotor activity.

In the results of the aggression test presented above, it can be observed that there are statistical differences in the same batch, but depending on the days of administration of valproic acid with Pentylenetetrazole. These most often indicate the preference that the fish acquired during the exploration of the T aquarium, but also the dominance of aggression.

Throughout all behavioural tests, no resting periods of a few seconds were observed in the behaviour of zebrafish that would be associated with a physical, emotional, and cognitive paralysis response, which leads us to think that regardless of the concentration of VPA in the antiepileptic treatment, they did not have sudden anxiety of a few seconds of the autonomic nervous system.

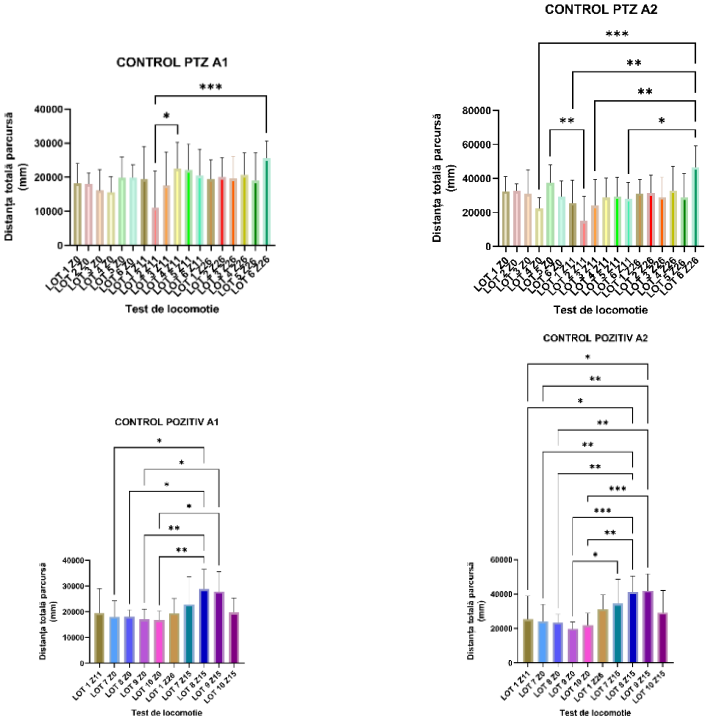
5.3 Experimental study III

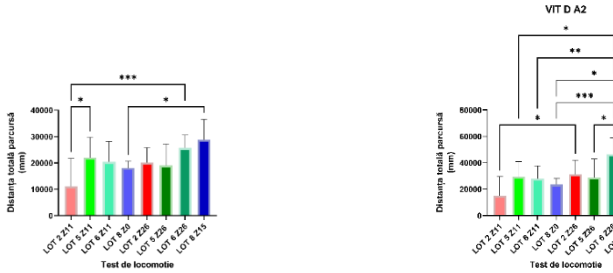
In order to identify the behaviour of zebrafish as concisely as possible, the locomotion test was used in a new aquarium, in which we added one litter of water in addition to the aquariums in which they were kept. The data analysis was performed on each of the two arenas, which represent the vertical and lateral sides of the angle at which the behaviour was filmed. Thus, in the graphs below, an analysis using ANOVA will be observed, where we used a single variable per

batch. In cases where statistical significance was identified, these were noted as asterisks representing comparisons with a p value ≤0.05.

5.3.1 Swimming performance

In the parameter measuring the total swimming distance, statistical significance was observed in the group with the lots where PTZ was administered as follows: on arena 1 (lateral) a significant increase was observed between LOT 2 Z_{11} and LOT 4 Z_{11} (p=0.0367), and on arena 2 (vertical) significant increases in differentiation were observed between LOT 6 Z_{11} and LOT 6 Z_{26} (p=0.0420), in the groups where all lots were considered control for the other groups (positive lots) an increase in statistical differentiation was observed in each arena, thus on arena 1 lot 9 had a difference between Z_0 and Z_{15} (p=0.0219) and in arena 2 lot 8 had a difference between Z_0 and Z_{15} (p=0.0045). In the rats where Vit D was administered as an anticonvulsant, two significant increases were observed on arena 1: LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0262), LOT 8 Z_0 and LOT 8 Z_{15} (p=0.0303), and in arena 2, four significant increases were observed: LOT 2 Z_{11} and LOT 2 Z_{26} (p=0.0358), LOT 6 Z_{11} and LOT 6 Z_{26} (p=0.0098), LOT 8 Z_0 and LOT 8 Z_{15} (p=0.0159), LOT 5 Z_{26} and LOT 6 Z_{26} (p=0.0165) (Figure 5.11).

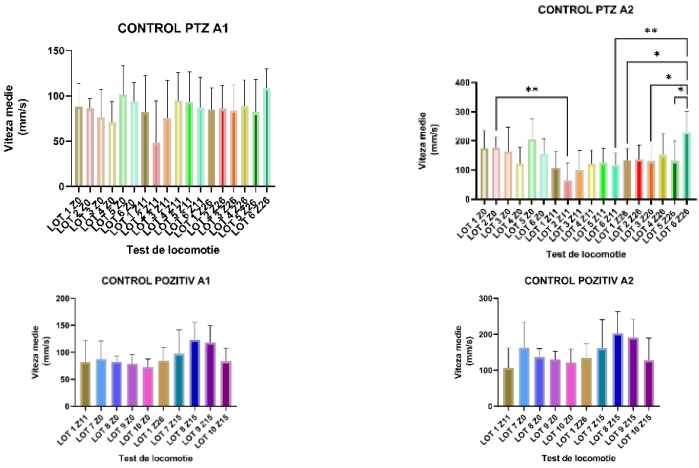


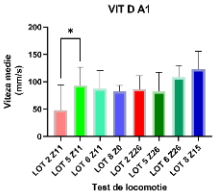


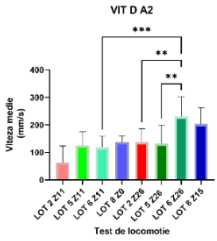
Arena 1 (side shot) Arena 2 (vertical filming)

Figure 5.11 Total swimming distance (mm) in untreated zebrafish (n=10) over 26 days *- p <0.05; ** - p <0.01; ***- p <0.10

The average speed (mm/s) obtained by zebrafish during the locomotion test differed depending on the chamber used. Thus, in the group in which PTZ was administered, increasing statistically significant differences were observed only in arena 2 (vertical) LOT 6 Z_{11} and LOT 6 Z_{26} (p=0.0046), LOT 1 Z_{26} and LOT 6 Z_{26} (p=0.0408), LOT 3 Z_{26} and LOT 6 Z_{26} (p=0.0252), LOT 5 Z_{26} and LOT 6 Z_{26} (p=0.0281). In the groups where vitamin D was administered, it was observed that there were increases in the average, which led to statistical differences, in arena 1 (lateral) LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0384), respectively in arena 2 LOT 6 Z_{11} and LOT 6 Z_{26} (p=0.0006), LOT 2 Z_{26} and LOT 6 Z_{26} (p=0.0076) LOT 5 Z_{26} and LOT 6 Z_{26} (p=0.0038). In the groups with positive lots, no statistical differences were found depending on the average speed (Figure 5.12).



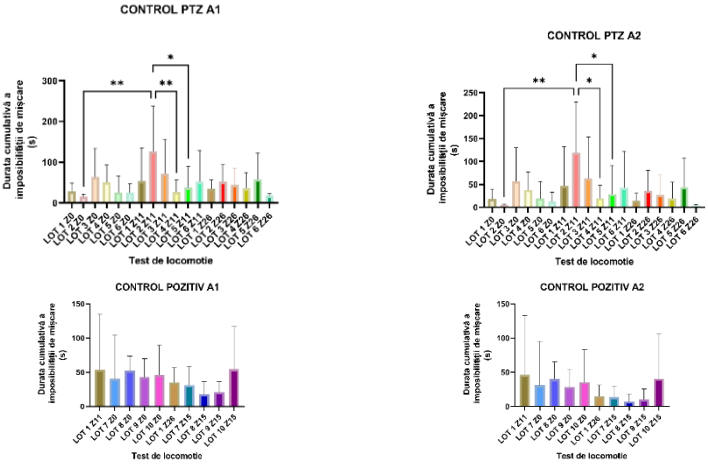




Arena 1 (side shot) Arena 2 (vertical filming)

Figure 5.12 Average running speed (mm/s) in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10;

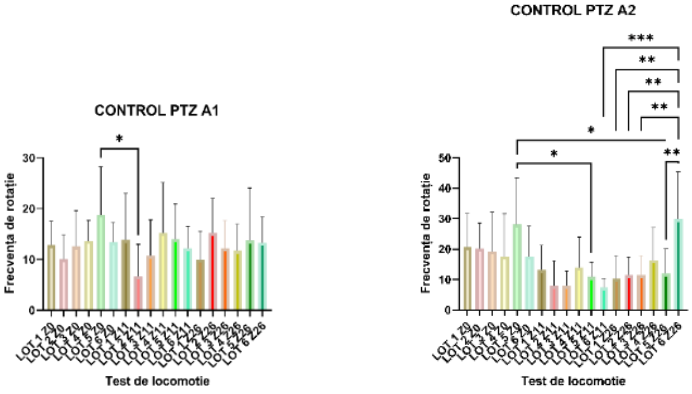
Episodes of inactivity were found in approximately all fish in all batches. In the groups with PTZ administration in arena 1 (lateral) an increase in the average duration of LOT 2 Z_0 and LOT 2 Z_{11} (p=0.0016) and two significant decreases in LOT 2 Z_{11} and LOT 4 Z_{11} (p=0.0097), LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0390) were observed, and in arena 2 (vertical) an increase in LOT 2 Z_0 and LOT 2 Z_1 (p=0.0016) and two significant decreases in LOT 2 Z_{11} and LOT 4 Z_{11} (p=0.0151), LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0482) were observed. In the groups where vitamin D was administered, a differentiation of the resting average was observed, this being decreasing in each arena, thus arena 1 LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0247) and arena 2 LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0282) (Figure 5.13).

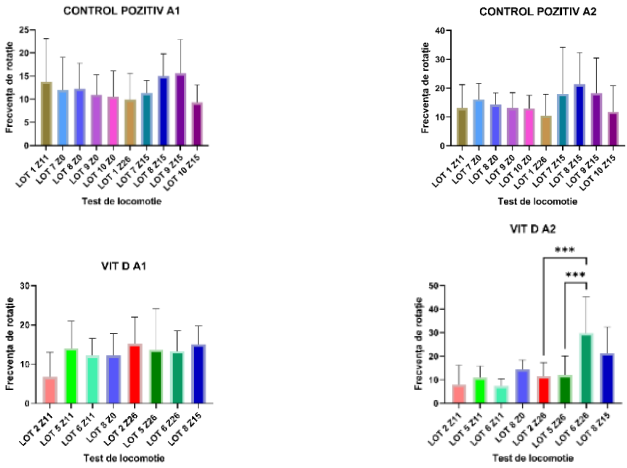


Arena 1 (side shot) Arena 2 (vertical filming)

Figure 5.13 Accumulated resting time (s) in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

For the locomotion test, the number of tendencies the zebrafish had to turn clockwise was analysed. Thus, in arena 1 (lateral) no statistically significant differences were observed, but in arena 2 (vertical) differences in the average rotation frequency were identified in the groups in which PTZ was administered, being significantly decreasing for LOT 5 Z_0 and LOT 5 Z_{11} (p=0.0161), LOT 5 Z_0 and LOT 5 Z_{26} (p=0.0342) and significantly increasing for LOT 6 Z_{11} and LOT 6 Z_{26} (p=0.0001), LOT 1 Z_{26} and LOT 6 Z_{26} (p=0.0023), LOT 2 Z_{26} and LOT 6 Z_{26} (p=0.0061), LOT 3 Z_{26} and LOT 6 Z_{26} (p=0.0066), LOT 5 Z_{26} and LOT 6 Z_{26} (p=0.0085). In the groups where VIT D was administered, two significant increases in the mean frequency were observed LOT 2 Z_{26} and LOT 6 Z_{26} (p=0.0002), LOT 5 Z_{26} and LOT 6 Z_{26} (p=0.0003), respectively in the groups where CBZ was administered, an increase in the mean and a decrease were observed LOT 4 Z_{26} and LOT 6 Z_{26} (p=0.0339), LOT 6 Z_{26} and LOT 10 Z_{15} (p=0.0016) (Figure 5.14).

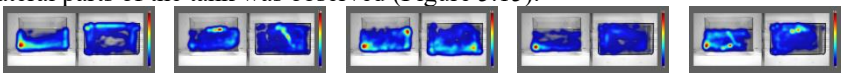




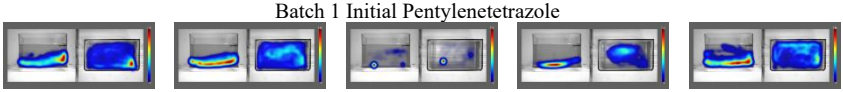
Arena 1 (side shot) Arena 2 (vertical filming)

Figure 5.14 Turnover frequency in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

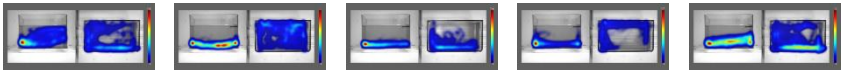
During this time, thermographic maps were made in which the time spent by the zebrafish in the new aquarium can be observed. Depending on the intensification of the colour, it is possible to understand their preference to stay or explore. During the pretreatment experiments, they spent most of their time towards the bottom of the aquarium, exploring part or all of the surface. Some of them also analysed one of the sides of the aquarium. After the 11 days of exposure to PTZ, in the 6 groups, a similarity was observed as in the previous test day, but with an intensification of the anxiety state by examining the aquarium only on one side of the lower part. During the 15 days of treatment with anticonvulsant drugs, it was observed that in four of the five positive batches (Lot 7 Control, Lot 8 Vitamin D, Lot 9 Valproic Acid, Lot 10 Carbamazepine) the zebrafish were more active in the upper and lateral parts of the aquarium, whereas in the positive batch, Lot 1 Pentylenetetrazole, it was observed that the fish maintained a constant tendency to explore the new aquarium. In the batches that were administered the proconvulsant pentylenetetrazole for 26 days, but also anticonvulsant treatments for 15 days, a constant examination of the lower and lateral parts of the tank was observed (Figure 5.15).



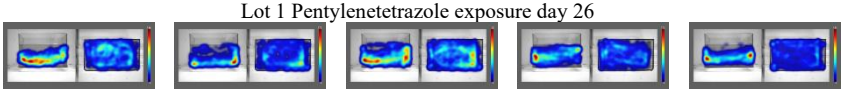
Batch 1 Initial Pentylenetetrazole



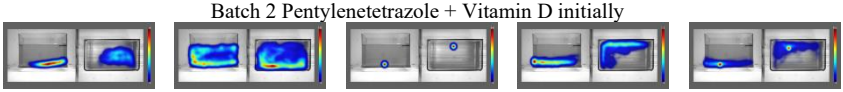
Lot 1 Pentylenetetrazole exposure day 11



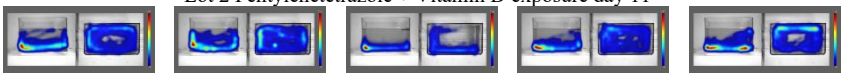
Lot 1 Pentylenetetrazole exposure day 26



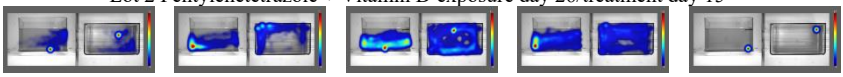
Batch 2 Pentylenetetrazole + Vitamin D initially



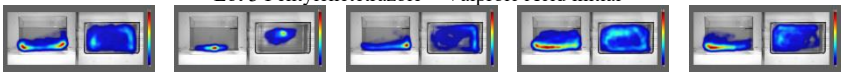
Lot 2 Pentylenetetrazole + Vitamin D exposure day 11



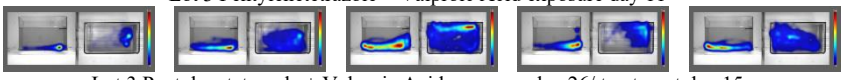
Lot 2 Pentylenetetrazole + Vitamin D exposure day 26/treatment day 15



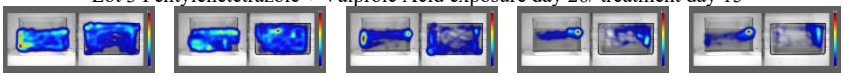
Lot 3 Pentylenetetrazole + Valproic Acid initial



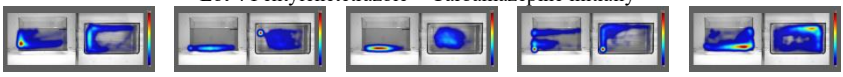
Lot 3 Pentylenetetrazole + Valproic Acid exposure day 11



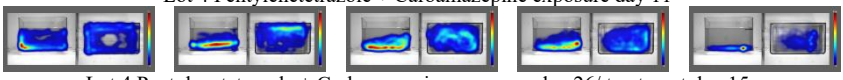
Lot 3 Pentylenetetrazole + Valproic Acid exposure day 26/ treatment day 15



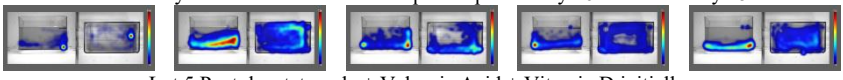
Lot 4 Pentylenetetrazole + Carbamazepine initially



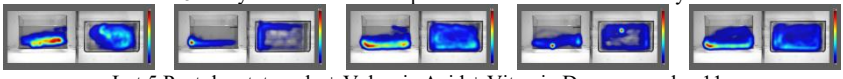
Lot 4 Pentylenetetrazole + Carbamazepine exposure day 11



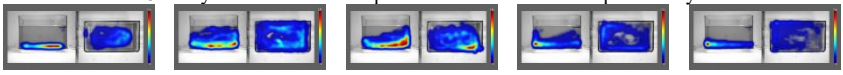
Lot 4 Pentylenetetrazole + Carbamazepine exposure day 26/ treatment day 15



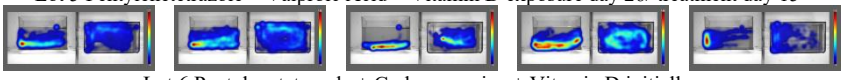
Lot 5 Pentylenetetrazole + Valproic Acid + Vitamin D initially



Lot 5 Pentylenetetrazole + Valproic Acid + Vitamin D exposure day 11



Lot 5 Pentylenetetrazole + Valproic Acid + Vitamin D exposure day 26/ treatment day 15



Lot 6 Pentylenetetrazole + Carbamazepine + Vitamin D initially

Figure 5.15. Thermographic map pattern in (un)treated zebrafish (n=10) during the 26 days of exposure/15 days of treatment

During the sociability tests, two zebrafish from outside the group were added to the left arm of the T-shaped aquarium to observe how sociable they could be from the beginning of the interaction. Thus, the time (in seconds) they spent in each arm, each group, respectively the total swimming distance (centimetres), the frequency of clockwise rotation and the average speed (cm/s) across the five groups depending on the treatment applied were monitored.

During the 26 days of Pentylenetetrazole administration, a decrease in the time spent in the left arm was observed in the sociability tests, but an increase in the central arm in the groups where there was treatment from day 11 to day 26, except for group 6, where Pentylenetetrazole + Carbamazepine + Vitamin D was administered, in which there was a small increase in the time spent in the left arm.

In the positive groups, the same decreases in the fluctuation in the left arm were observed for the control group, but also for the group where vitamin D was administered, respectively a slight increase in the left arm for the group where valproic acid was administered and in the group with Carbamazepine administration (Figure 5.16).

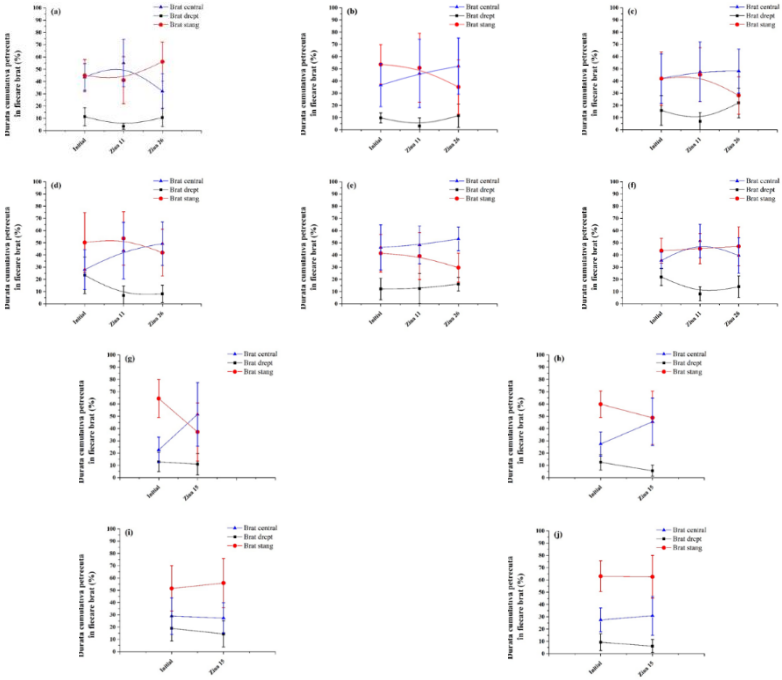


Figure 5.16. Social behavioural fluctuations over time in untreated zebrafish (n = 10) over 26 days

(a)	Lot 1 Pentylenetetrazole	(b)	Lot 2 Pentylenetetrazole + Vitamin D
(c)	Lot 3 Pentylenetetrazole + Valproic Acid	(d)	Lot 4 Pentylenetetrazole + Carbamazepine
(e)	Lot 5 Pentylenetetrazole + Valproic Acid + Vitamin D		
(f)	Lot 6 Pentylenetetrazole + Carbamazepine + Vitamin D		
(g)	Lot 7 Control	(h)	Lot 8 Vitamin D
(i)	Lot 9 Valproic Acid	(j)	Lot 10 Carbamazepine

The results of the sociability test travelled by the fish of the 10 batches were calculated in parameters such as total swimming distance (cm), average speed (cm/s) and rotation frequency. For the first calculated parameter, it can be seen that there are no statistically significant differences in the average in the groups with PTZ administration, positive control, but in the batches where vitamin D was administered there are two significant increases in the average distance LOT 4 Z_{26} and LOT 6 Z_2 (p=0.0339), LOT 6 Z_{26} and LOT 10 Z_{15} (p=0.0016) (Figure 5.17).

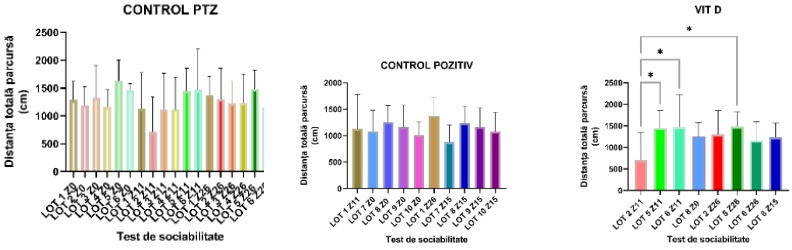


Figure 5.17. Total swimming distance (cm) in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

In the case of the average speed parameter, no significant differences in the average can be noted in the groups in which PTZ was administered, respectively the groups where there was control, thus a constant average level can be observed. However, in the groups in which the treatment was with VIT D, two significant increases in the average were observed LOT 2 Z_{11} and LOT 5 Z_{11} (p=0.0242), LOT 2 Z_{11} and LOT 6 Z_{11} (p=0.0192). (Figure 5.18).

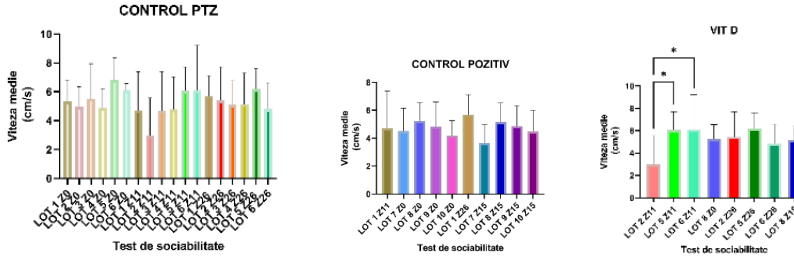


Figure 5.18. Average running speed (cm/s) in untreated zebrafish (n=10) over 26 days *-
p<0.05; ** - p<0.01; ***- p<0.10

The frequency of the central point rotating clockwise may indicate epileptic behaviour, before, during or after an epileptic seizure. This rotation tendency was present in all fish in each group, but there was no significant difference between the groups in the three situations (Figure 5.19).

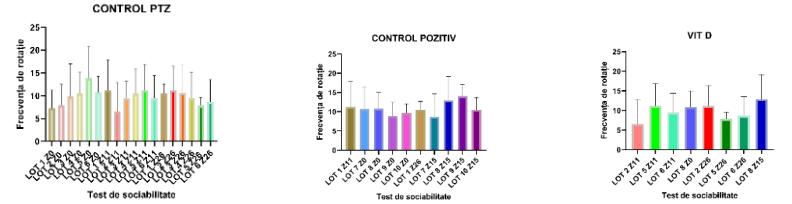


Figure 5.19. Turnover frequency in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

The last test performed was the aggression test, which measured how many times the fish swam to the left arm, where a mirror was placed on the

outside wall of the T-shaped aquarium. The parameters monitored in this test were the distance travelled and the time they spent in each arm, and the difference in five groups between the total swimming distance, velocity, inactivity, and frequency of clockwise rotations throughout the test.

The time that zebrafish spent in the three arms during the aggression tests differed depending on the day of administration. In all groups, an increase in the time spent in the central arm was observed, but in five groups (Lot 1 PTZ, Lot 2 PTZ+Vit D, Lot 4 PTZ+CBZ, Lot 7 Control, Lot 8 Vit D) an increase of over 50% of the time spent in this arm was observed. On the last day of treatment, only in Lot 3 PTZ+VPA and Lot 9 VPA was a slight increase in the time spent in the left arm, where the mirror was present, suggesting a slight increase in aggressive behaviour (Figure 5.20).

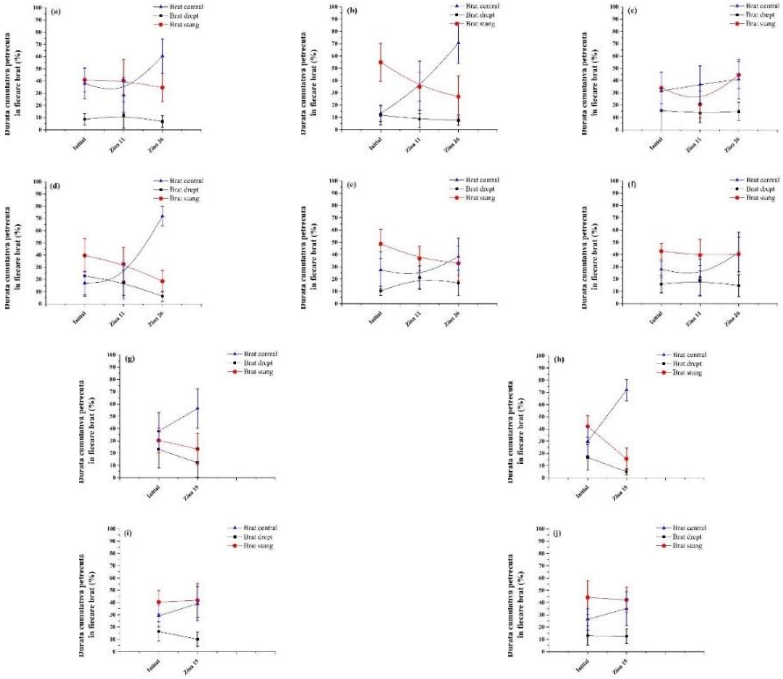


Figure 5.20. Aggressive behavioural fluctuations measured over time in untreated zebrafish (n = 10) over 26 days

(a)	Lot 1 Pentylenetetrazole	(b)	Lot 2 Pentylenetetrazole + Vitamin D
(c)	Lot 3 Pentylenetetrazole + Valproic Acid	(d)	Lot 4 Pentylenetetrazole + Carbamazepine
(e)	Lot 5 Pentylenetetrazole + Valproic Acid + Vitamin D		
(f)	Lot 6 Pentylenetetrazole + Carbamazepine + Vitamin D		
(g)	Lot 7 Control	(h)	Lot 8 Vitamin D
(i)	Lot 9 Valproic Acid	(j)	Lot 10 Carbamazepine

5.3.2 The effect of medications on aggressive behaviour

Aggression is defined by the zebrafish's ability to approach, raise their

fins, make undulating body movements, open their mouths towards the "enemy", change colour, bite, chase and spin in circles. These behaviours may occur due to the fact that a territory must appear (territorial behaviour), males can become aggressive to protect their females or other resources that need to be protected, but also to establish their dominance, territory, all of which were observed in the examined fish.

In the aggression test, a statistical difference was observed in the parameter of total swimming distance in the group in which the positive lots were found, such as LOT 2 Z_0 and LOT 1 Z_{11} (p=0.0440), LOT 4 Z_0 and LOT 1 Z_{11} (p=0.0032), LOT 4 Z_0 and LOT 3 Z_{11} (p=0.0346) where an increase in the average was observed. However, there were no statistical significances between the other groups with PTZ, VIT D, VPA or CBZ administration (Figure 5.21).

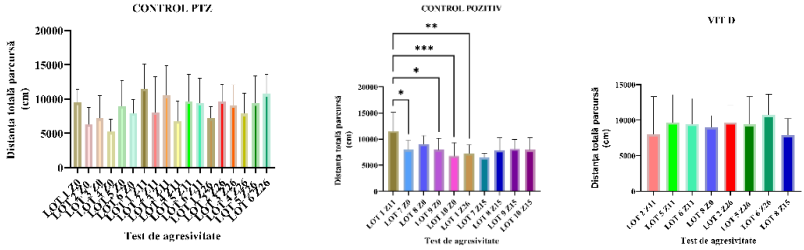


Figure 5.21. Total swimming distance (cm) in untreated zebrafish (n=10) over 26 days
*- p <0.05; ** - p <0.01; ***- p <0.10

At the average speed, statistical significance was found in the group with PTZ administration LOT 1 Z_{11} and LOT 1 Z_{26} (p=0.0023) and in the group with the positive lots LOT 1 Z_{11} and LOT 10 Z_0 (p=0.0005), both being in decreasing average (Figure 5.22).

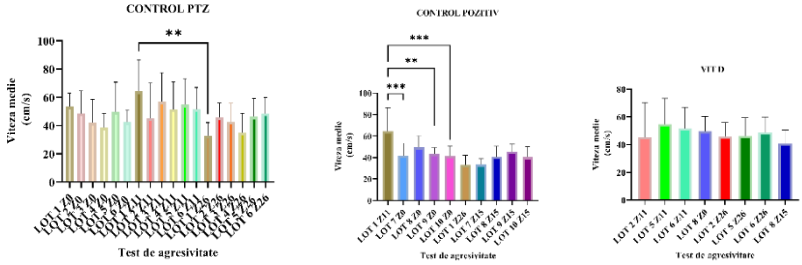


Figure 5.22. Average running speed (cm/s) in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

There were moments of inactivity, which were short-lived (between 0.1-26.70s), but these did not exist throughout all aggression tests, thus there was no statistically significant difference in any group of batches (Figure 5.23).

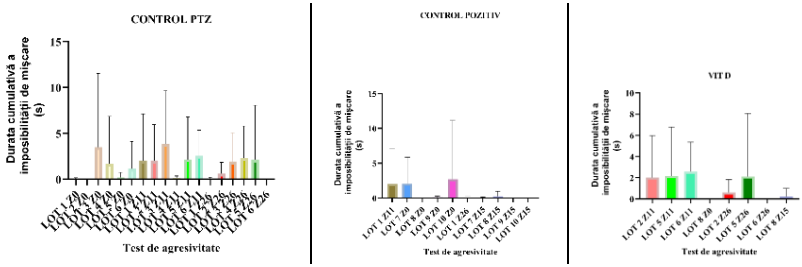


Figure 5.23. Episodes of inactivity in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

The clockwise rotation trend existed in the group with Pentylenetetrazole administration, being a decrease in the mean LOT 1 Z_0 and LOT 1 Z_{26} (p=0.0060), LOT 1 Z_{11} and LOT 1 Z_{26} (p=0.0369), in the group with positive lots there was a statistically significant decrease in the mean LOT 1 Z_{11} and LOT 1 Z_{26} (p=0.0024), respectively two increases in the mean that led to statistical significance LOT 1 Z_{26} and LOT 10 Z_{15} (p=0.0037), LOT 7 Z_{15} and LOT 9 Z_{15} (p=0.0379). Thus, in the case of the groups in which VIT D, VPA or CBZ were administered, there were no statistical differences (Figure 5.24).

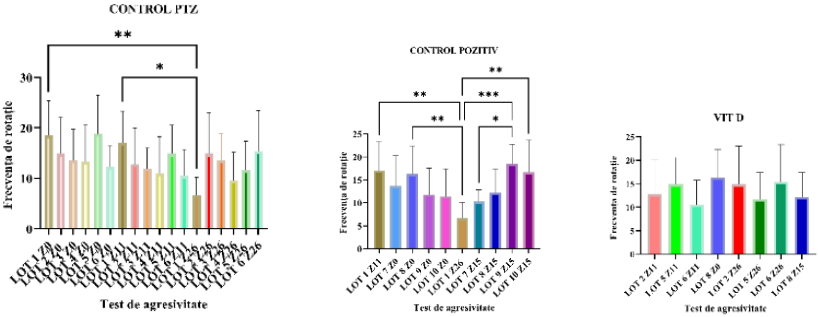


Figure 5.24. Turnover frequency in untreated zebrafish (n=10) over 26 days
*- p<0.05; ** - p<0.01; ***- p<0.10

5.3.3 Effect of drugs on oxidative stress

These results prove that Pentylenetetrazole was a factor of oxidative stress. After performing the three tests, it was possible to observe enzymatic activity, it can be observed that there is oxidative stress by increasing the level of SOD in certain groups, it was found that the level of oxidative stress is lower compared to group 1 in which only the proconvulsant was administered (group 2 PTZ+VIT D p=0.0001, group 3 PTZ+VPA p=0.0008, group 5 PTZ+VIT D+VPA p=0.0036, group 8 VIT D p=0.0145, group 9 VPA p=0.0079), respectively group 2 PTZ+VIT D indicated a significant increase compared to group 4 PTZ+CBZ p=0.0037 and group 7 CONTROL p=0.0145, the same can be observed between group 3 PTZ+VPA and group 4 PTZ+CBZ p=0.0240. GPx activity is elevated

only in Lot 4 PTZ+CBZ where the existence of oxidative stress is lower and there are statistically significant differences with lot 1 PTZ (p=0.0068), lot 2 PTZ+VIT D (p=0.0355) and lot 9 VPA (p=0.0371), this may lead us to think that SOD replaces GPx activity in the other lots.

The increased levels of MDA in 8 batches indicate that there is an oxidative stress activity, batch 1 PTZ shows a small amount of MDA with statistical significance compared to batch 4 PTZ+CBZ (p=0.0091), batch 5 PTZ+VIT D+VPA (p=0.0012), batch 6 PTZ+VIT D+CBZ (p=0.0027), respectively batch 8 VIT D (p=0.0379), but also in batch 3 with a small amount of MDA compared to batch 5 PTZ+VIT D+VPA (p=0.0189), respectively lot 6 PTZ+VIT D+CBZ (p=0.0408) (Figure 5.25).

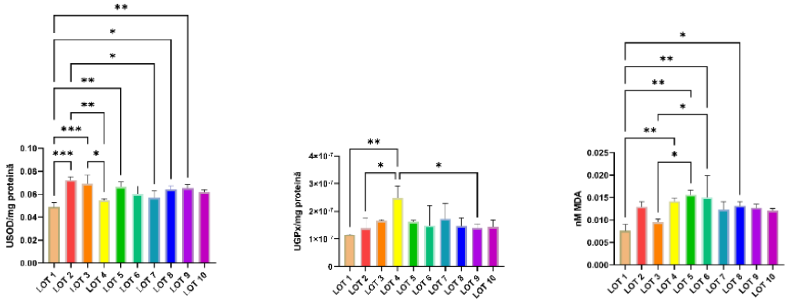


Figure 5.25. Results of oxidative stress parameters after treatment exposure presented as mean ± SEM *- p<0.05; ** - p<0.01; ***- p<0.10

5.3.4. Discussions

Locomotion, social, aggression tests were performed before, during, and after administration of the proconvulsant pentylenetetrazole, respectively before and after treatment with the anticonvulsants: Vitamin D, valproic acid, Carbamazepine. Therefore, it was observed that there is a degree of visibility through which vitamin D can be correlated with other anticonvulsant drugs, so as to reduce the number of epileptic seizures, but also associated psychiatric comorbidities.

The concentrations of the drugs used in the experiment had the role of antiepileptic protection; through them we tried to observe the differences that may exist comparatively

with the groups in which the proconvulsant was administered, but also in the positive control groups in which only the key substance was administered. Vitamin D had the role of observing whether this vitamin could have positive effects in fish that were administered PTZ and whether it could support the treatment of two of the drugs that inhibit vit D in the body.

Canzian and his collaborators showed that PTZ caused acute convulsions in adult fish, but also that they were subjected to a stress factor at the same time, they presented aggressive, anxious behaviour, but also their memory

was affected, being a disruptor of fish school cohesion after the recovery period (Canzian et al., 2021). We also observed this in the locomotion test where the fish had preferences to stay in the same place or to swim only in a part of the aquarium where they felt safer, without visualizing the entire aquarium, but also in the other two tests, where periods of freezing were observed.

It is not known exactly whether the concentration of PTZ administered is directly proportional to the reaction it produces, thus triggering hindbrain stimuli (Pineda et al., 2013). Which will cause the pharmacological medication to atrophy the stimulant connections made by the proconvulsant. Thus, Valproic Acid can generate an inhibition of GABA transaminase, thus reaching a partial attenuation of seizures (Pineda et al., 2013; Pieróg et al., 2021).

A marker that reflects oxidative stress is MDA. Lipid peroxidation through the autocatalytic reaction is dangerous because it initiates cellular damage. In studies conducted on humans, it was shown that the MDA level increased in patients with epilepsy compared to patients in the control group (lr and Demir, 2024). Thus, in this study, the oxidative stress in the group in which only the proconvulsant was administered (5 mM PTZ) had a lower amount of MDA compared to the other groups in which only vitamin D was administered or PTZ was administered together with Vit D and anticonvulsants valproic acid or Carbamazepine.

In studies conducted on epilepsy with antioxidants, it was observed that patients with this condition have a lower level of SOD, in refractory epilepsy this may be due to the fact that lipid compounds accumulate in large quantities in the brain (Yilgor and Demir, 2024). In this study, it was observed that in the group in which only PTZ was administered, respectively PTZ + CBZ, this antioxidant decreased compared to the other groups in which PTZ was administered or not, but there was a large amount of SOD in the groups in which PTZ and Vit D, respectively PTZ and VPA were administered.

General conclusions

Epilepsy is a neurological disease that affects approximately 50 million people worldwide, but can also occur naturally in animals. It is often associated with various psychiatric comorbidities. There are three types of epilepsy, which differ depending on the area of the brain that is prone to generate electrical activity called seizures.

Depending on the nature of the seizures and their onset, the neurologist prescribes different antiepileptic drugs. These drugs can have a negative role on metabolism by inducing the catabolism of certain nutrients such as vitamin D.

There are epileptic seizures that are produced through the reduction of gamma aminobutyric acid (GABA). Thus, vitamin D plays a physiological role through its biochemical functions of synthesizing, and helping to form vitamin D receptors (VDR) which help to generate GABA receptors.

In the first experiment in which we described the meta-analysis, we could observe the correlation that exists between the brain region and juvenile myoclonic epilepsy in patients who are diagnosed with it compared to control patients who do not have juvenile myoclonic epilepsy.

There are studies that prove the role of vitamin D in epilepsy and in animal models such as mice and rats. There are no studies on zebrafish models, but this may be relevant because we showed in the chapter on research materials, the importance of zebrafish studies for biomedical research and metabolic control, being more accessible to observe brain-organ communication

In the second experiment, it was possible to observe the differences that may exist in the behavioural tests, but also in the tests on oxidative stress enzymes between the groups in which three different concentrations of pure valproic acid were administered, together with the proconvulsant, a control group and a Pentylenetetrazole control group. Thus, the locomotor and sociability testing showed statistically significant differences only in the groups in which a high concentration of valproic acid was administered (7500 μM and 10000 μM), and in the aggression testing it was observed that there are statistical differences in the control group and the proconvulsant control group.

The parameters chosen for the three behavioural tests revealed that there are statistically significant differences in the positive groups (in which only one substance was administered) and in the groups in which the proconvulsant was administered for 26 days. We cannot conclude whether there is a difference in terms of locomotor, social and aggressiveness in the population suffering from epilepsy with valproic acid or Carbamazepine medication who take vitamin D supplements.

The results presented above indicate that vitamin D supplementation in patients suffering from psychiatric comorbidities associated with epilepsy would play an important role compared to the population without seizures. Further research is essential, especially from a biochemical point of view to understand the links that exist between antiepileptic drugs and metabolism, to diversify a more appropriate diet, but also from a molecular point of view to stop the production of new epileptic seizures.

These preliminary results may help in potential therapeutic strategies between vitamin D and pharmacological drugs that are already used in various epileptic therapies, due to the responses on zebrafish which are an important animal model.

The study limitations were as in another scientific research:

- in animal models: the number of concurrent batches, biochemical and physiological testing on different sections of the zebrafish body, being living beings, each one reacts differently, so the exclusion of outliers from each batch

had to be calculated and precise;

- budgetary constraints for the acquisition of zebrafish with genetic polymorphisms or mutations that exhibit native epilepsy, reagents, materials and software;

- there were no data collected from human subjects to strengthen the research goal that vitamin D plays an important role on epilepsy associated with various psychiatric comorbidities.

Selective bibliography

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Dissemination of results

List of published works containing results of the doctoral thesis

Articles published in ISI Web of Science ranked journals with impact factor :

Kazis, D., Petridis, F., Chatzikonstantinou, S., Karantali, E., Jamali, R., Chowdhury, R., **Duta, R** ., Luca, AC, Ciobica, A. and Mavroudis, I., Gray matter changes in juvenile myoclonic epilepsy. A voxel-wise meta-analysis, Medicine - Lithuania, 57(11) 2021 p.1136. DOI:10.3390/medicina57111136 (FI = 2.948, AIS = 0.523)

Ilie, OD, **Duta, R.** , Balmus, IM, Savuca, A., Petrovici, A., Nita, IB, Antoci, LM, Jijie, R., Mihai, CT, Ciobica, A. and Nicoara, M., . Assessing the neurotoxicity of a sub-optimal dose of rotenone in zebrafish (Danio rerio) and the possible neuroactive potential of valproic acid, combination of levodopa and carbidopa, and lactic acid bacteria strains. Antioxidants, 11(10) 2022, p.2040. DOI:10.3390/antiox11102040 (FI = 7, AIS = 0.946), 6 citations

Articles published in journals indexed in the International Database (IDB)

Duță, R ., Visternicu, M., Miler, A. and Ciobîcă, A., 2024. The impact of vitamin d on psychiatric and physiological mechanisms in the context of epilepsy. Bulletin of Integrative Psychiatry, (4). DOI:10.36219/BPI.2024.4.01

Kinda, PT, Guenne, S., Basile Tindano, NOUFOU, Ouedraogo, NO, Zerbo, P., **Duta, R.** , Ciobica A., And Kiendrebeogo, M., 2021. Studying some neuroprotective effects of Calotropis procera extracts against scopolamine-induced neuropsychiatric comorbidities in a rodent model of epilepsy. Romanian Biotechnological Letters., 26, p.3114. DOI:10.25083/rbl/26.6/3114-3119

List of papers presented at international and national conferences

Ecology and Protection of Ecosystems" (EPE) XIV Edition, 2023, Bacau, Romania **, RE Duță** , A. Săvucă, AS Ciobîcă, R. Lefter, MN Nicoară– "Preliminary results regarding the effects of antiepileptic drugs in zebrafish", 2-4.11.2023 www.epe.ub.ro

List of published works outside the topic of the doctoral thesis but in the same research field

Singeap, AM, Minea, H., Petrea, O., Robea, MA, Balmuș, IM, **Duta, R** ., Ilie, OD, Cimpoesu, CD, Stanciu, C. and Trifan, A., Real-world utilization of corticosteroids in severe alcoholic hepatitis: eligibility, response, and outcomes. Medicina, 60(2) 2024, p.311. DOI:10.3390/medicina60020311 (FI = 2.4, AIS = 0.558), 2 citations

Ilie, OD, **Duta, R.** , Nita, IB, Dobrin, I., Gurzu, IL, Girleanu, I., Huiban, L., Muzica, C., Ciobica, A., Popescu, R. and Cianga, P., A Comprehensive Overview of the Past, Current, and Future Randomized Controlled Trials in Hepatic Encephalopathy. Medicine, 59(12),

2023. p.2143. DOI:10.3390/medicina59122143 (FI = 2.4, AIS = 0.558),

Ilie, OD, **Duta, R.** , Jijie, R., Nita, IB, Nicoara, M., Faggio, C., Dobrin, R., Mavroudis, I., Ciobica, A. and Doroftei, B., Assessing anti-social and aggressive behaviour in a zebrafish (Danio rerio) model of Parkinson's disease chronically exposed to rotenone. Brain Sciences, 12(7) 2022., ps.898. DOI:10.3390/brainsci12070898 (FI = 3.3, AIS = 0.772), 11 citations