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Bc. Alika Chernova

Identification of the impact of generalized seizures on brain electrical activity during development using EEG correlates

Identifikace dopadu generalizovaných záchvatů na elektrickou aktivitu mozku během vývoje pomocí EEG korelátu

Diploma thesis

Supervisor: Mgr. Grygoriy Tsenov, Ph.D.

Advisor: doc. RNDr. Jiří Novotný, DSc.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 08.08.2024

Podpis …….

Poděkování

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Abstrakt

Tato studie jako první provádí záznam EEG a spektrální analýzu výkonové spektrální hustoty v konkrétních frekvenčních pásmech (delta, theta, alfa, beta, gama) v pozdních fázích vývoje po aplikaci flurothylu u nezralých potkanů. Tato studie předpokládá, že epileptogenní proces po flurothylem indukovaných opakovaných generalizovaných záchvatech u nedospělých potkanů vykazuje specifický EEG vzorec závislý na vývoji a pohlaví, který bude vhodným biomarkerem pro diagnostiku a sledování progrese onemocnění.

Tato pozorování ukazují záchvatovou aktivitu (výskyt elektrografických záchvatů i interiktálních příhod) v časných postnatálních stadiích (P12, P15, P25) s významnými rozdíly specifickými pro pohlaví, s vyšší frekvencí záchvatů u samců. Navíc, absence "tiché fázi" svědčí o rychlém rozvoji chronické epilepsie po flurotylem indukovaných opakovaných záchvatů. Spektrální analýza EEG dat ukázala změněnou neuronální excitabilitu u flurothylových skupin ve srovnání s kontrolními zvířaty, což naznačuje potenciální biomarker založený na EEG korelátech, který by mohl sloužit jako vhodný nástroj pro další výzkum a klinické využití.

Klíčová slova: novorozenecké záchvaty, rekurentní generalizované spontánní záchvaty, nedospělí potkani, flurothyl, EEG, výkonová spektrální hustota

Abstract

This study is the first to perform EEG recording and spectral analysis of the Power Spectral Density across specific frequency bands (delta, theta, alpha, beta, gamma) during late stages of development after flurothyl treatment in immature rats. This study hypothesizes that epileptogenic process followed flurothyl-induced repetitive generalized seizure in immature rats exhibit specific developmental and sex-dependent EEG pattern, which will be a suitable biomarker for diagnosis and monitoring of disease progression.

Our findings detected seizure activity (both electrographic seizure and interictal epileptiform events occurrence) in early postnatal stages (P12, P15, P25), with significant gender-specific differences, with higher seizure rates in males. Moreover, it reveals no silent phase, indicating rapid development of chronic epilepsy after recurrent seizure induction. Spectral analysis of EEG data showed altered neuronal excitability in flurothyl-treated groups compared to non-treated animals, indicating a potential biomarker based on the EEG correlates, that could serve as a tool for further research and clinical application.

Keywords: neonatal seizures, recurrent generalized spontaneous seizures, immature rats, flurothyl, EEG, Power Spectral Density

Index

Abbreviations

ATR Alpa to Theta Ratio

Ch1/Ch2 Channel 1/2

DEE Developmental and Epileptic Encephalopathy

EDF European Data Format

EEG Electroencephalogram

FFT Fast Fourier Transform

GABA γ-aminobutyric acid

GABA()R γ-aminobutyric acid receptor

GDP Giant Depolarizing Potential

GTR Gamma to Theta Ratio

HIE Hypoxic-Ischemic Encephalopathy

ILAE International League Against Epilepsy

KA Kainic Acid

KCC Potassium-Chloride Cotransporter

MES Maximal Electroshock Seizures

NKCC Sodium-Potassium-Chloride Cotransporter

NMDA N-methyl-d-aspartate

P Postnatal day

PSD Power Spectral Density

SD Standard Deviation

SEM Standard Error of the mean

SNR Substantia Nigra pars Reticulata

SNR(a) Substantia Nigra pars Reticulata anterior

SNR(p) Substantia Nigra pars Reticulata posterior

Introduction

Epilepsy is a chronic neurological disease manifested by recurrent unprovoked seizures. Seizures are disorders of motor, autonomic, sensory, or mental functions caused by abnormal activity and synchronization of neurons in the cerebral cortex. Epilepsy is not a single disease, but is subdivided into separate forms characterized by different electro-clinical features, characteristic symptoms, therapeutic options, etc.

The prima facie classification divides paroxysms into focal (obsolete name partial) and generalized forms. Focal forms are considered to be seizures with a clear cortical epileptogenic focus, which subsequently spreads to other parts of the brain, which is confirmed by electroencephalographic (EEG) data and neuroimaging of the local genesis of the paroxysm. In the case of generalized forms, seizures have properties of bilateral synchronous genesis with spread to both hemispheres of the brain. Generalized seizures manifest bilaterally motorically with characteristic ictal and inter-ictal EEG discharges. Typical patterns of EEG include mainly normal activity of the baseline EEG recording, the presence of generalized 3-6 Hz spike-and-wave bursts, irregular polyspike waves, and slow activity sharp-slow waves Drury & Henry, 1993).

The first two days from birth are critical in the development of neonatal seizures. The presence of seizures is associated with increased morbidity and mortality. Most neonatal seizures are acute (provoked, occasional, reactive), caused for example by hypoxic-ischemic encephalopathy (HIE), infection, stroke, or haemorrhage (Glass & Sullivan, 2009). In addition, neonatal seizures can be comorbid with various neurologic, somatic, and endocrine diseases. Neonatal seizures carry a serious risk of subsequent generalized recurrent seizures in infancy, as well as neurologic disability and/or neuropsychiatric developmental impairment such as developmental and epileptic encephalopathy (DEE). A key element is that these seizures are currently still difficult to identify and effectively treat clinically.

Neonatal seizures

Classification

In 1981, the ILAE (International League Against Epilepsy) established a basic classification of epileptic seizures into focal and generalized seizures. Since neonatal seizures have exclusively focal onset, there is no need to divide into focal and generalized paroxysms. The ILAE defines neonatal seizures as an electrographic event with a pattern characterized by sudden, repetitive, evolved stereotyped waveforms with a beginning and end Pressler et al., 2021). However, in early neonatal encephalopathies with tonic focal or asymmetric seizures, there is a possibility of developing generalized recurrent seizures in infancy. Neonatal seizures are usually clinically subtle and difficult to distinguish from normal interictal behaviour or physiologic phenomena. There is no recognizable postictal state as well. The newest classification of seizure types will be presented below (Table 1), which is based on the predominant clinical manifestation (motor and non-motor phenomena) rather than the localization of seizure onset, which will more readily have clinical relevance to aetiology.

TABLE 1. Integration with the 2017 ILAE Classification of Seizures and considerations for neonates (Pressler et al., 2021).

Type	Description	Special consideration	Clinical context of seizure type
Automatism	A more or less coordinated motor activity usually occurring when cognition is impaired. This often resembles a voluntary movement and may consist of an inappropriate continuation of preictal motor activity.	Typically oral in neonates. Behaviour in term and preterm infants may mimic ictal automatisms, thus EEG / aEEG mandatory.	Seen in HIE and preterm infants. Often part of sequential seizures.
Clonic	Jerking, either symmetric or asymmetric, that is regularly repetitive and involves the same muscle groups.	Seizure type, which is more reliably diagnosed clinically.	Typical seizure type in neonatal stroke or cerebral haemorrhage. May be seen in HIE.
Epileptic spasm	A sudden flexion, extension, or mixed extension–flexion of predominantly proximal and truncal muscles that is usually more sustained than a myoclonic movement but not as sustained as a tonic seizure. Limited forms may	Brief in neonates, thus may be difficult to differentiate from myoclonic seizures without EMG channel. May occur in clusters.	Rare. May be seen in inborn errors of metabolism or early-infantile DEE.

	occur: Grimacing, head nodding, or subtle eye movements.		
Myoclonic	A sudden, brief (<100 msec) involuntary single or multiple contraction(s) of muscles(s) or muscle groups of variable topography (axial, proximal limb, distal).	Clinically difficult to differentiate from non-epileptic myoclonus, requires EEG, ideally with EMG channels.	Typical seizure type in inborn errors of metabolism and preterm infants. May also be seen in early-infantile DEE.
Tonic	A sustained increase in muscle contraction lasting a few seconds to minutes.	Focal, unilateral or bilateral asymmetric. Generalized tonic posturing not of epileptic origin	Typical seizure type early-infantile DEE and genetic neonatal epilepsies.
Autonomic	A distinct alteration of autonomic nervous system function involving cardiovascular, pupillary, gastrointestinal, sudomotor, vasomotor, and thermoregulatory functions.	May involve respiration (apnoea). EEG / aEEG mandatory.	Rare in isolation. Seen in intraventricular haemorrhage as well as temporal or occipital lobe lesions. Also described in early-infantile DEE.
Behavioural arrest	Arrest (pause) of activities, freezing, immobilization, as in behaviour arrest seizure.	EEG / aEEG mandatory.	Rare as an isolated seizure type. More commonly seen as part of sequential seizure.
Sequential seizure	This term is used in the instruction manual for the ILAE 2017 operational classification of seizure types for events with a sequence of signs, symptoms, and EEG changes at different times.	No predominant feature can be determined, instead the seizure presents with a variety of clinical signs. Several features typically occur in a sequence, often with changing lateralization within or between seizures.	Often seen in genetic epilepsies such as self-limited neonatal epilepsy or KCNQ2 encephalopathy.

Electrographic -only seizure	Subclinical, without clinical manifestation.	EEG / aEEG mandatory.	Often seen in preterm infants, HIE (particularly in those with basal ganglia/thalamus injury), critically ill and neonates undergoing cardiac surgery.
Unclassified seizure type	Due to inadequate information or unusual clinical features with inability to place in other categories.	EEG / aEEG mandatory.	

Abbreviations: aEEG, amplitude-integrated EEG; EMG, electromyography; msec, milliseconds.

Epidemiology and risk factors

The incidence of paroxysms in the neonatal period ranges from 1 to 3 per 1000 live births of preterm infants with a prevalence of approximately 1.5% (Vasudevan & Levene, 2013). Pediatric research reported that children with a history of neonatal seizures have as greater than 50% chance of developing epilepsy in childhood (Pisani et al., 2012).

Risk factors for neonatal seizures are categorized based on their origin: infant-dependent, maternal-dependent, and intrapartum-dependent. Low birth weight (up to 1.5 kg) is a notable risk, along with cerebral anomalies, chromosomal abnormalities, asphyxia, intraventricular haemorrhage, cystic periventricular leukomalacia, and hypoxic-ischemic encephalopathy (Fukao et al., 2023). Neonatal seizures also show a gender disparity, with boys having a higher predisposition than girls (Kohelet et al., 2004). Premature birth is a significant risk, with seizures occurring more frequently in babies born before 31 weeks of gestation (Bergman et al., 1983). Maternal factors include advanced age (over 40 years) and conditions like diabetes mellitus, intrapartum fever, or infections, which increase seizure risks (Fukao et al., 2023). Drug-related factors during pregnancy, such as intoxication or withdrawal from narcotics, disrupt placental metabolism and increase oxidative stress, enhancing the risk. Significant intrapartum events like fetal distress, placental abruption, uterine rupture, or cord prolapse, are closely linked to seizures in term infants, mainly due to hypoxic brain damage (Glass et al., 2009).

Etiology

The neonatal stage in humans can present a diverse array of seizure types and epileptic conditions. In the neonatal phase, seizures frequently stem from acute medical issues, including hypoxia, hypoxic-ischemic encephalopathy (HIE), perinatal stroke, intracranial haemorrhages, infections, brain malformations, and metabolic disorders. Seizures caused by HIE or hypoglycaemia usually begin in the first 24 hours of life, while infections, congenital malformations, and other metabolic disorders usually cause seizures after 48 hours of life. Therefore, early identification of the etiology factor is essential for both the choice of optimal management and the estimation of prognosis (Ziobro & Shellhaas, 2020). During infancy and the early years of childhood, fevers are a known trigger for seizure (Spiciarich & Moshé, 2018). The roots of epilepsy during these stages are often traced back to HIE, genetic, structural, or metabolic causes.

Metabolic epilepsy typically arises from congenital metabolic defects such as hypoglycaemia, hypocalcaemia, hypernatremia. Genetic predisposition can influence the abnormal formation and functioning of the nervous system, which in turn can lead to seizures. Familial febrile convulsion (West syndrome) is a monogenic autosomal dominant disorder (mutations in voltage-gated potassium channel genes) where the defective gene is responsible for the development of epilepsy. Many genes responsible for ion transport in neurons may be associated with epilepsy. For example, mutations in genes encoding subunits of potassium (KCNQ2 and KCNQ3), sodium (SCN1A, SCN2A, and SCN3A) (Pijpers et al., 2023) or calcium channels (CACNA1G) (Carvill, 2019) can cause abnormalities in brain electrical activity, predisposing to seizures. Notably, some neonatal epilepsy cases develop into more persistent and changing epileptic syndromes over time, indicating possible shared pathways in epileptogenesis.

Epileptogenesis in neonatal brain

Epileptogenesis is the process of pathologic development of the normal brain into the epileptic brain. There are three phases of epileptogenesis: **(i)** the latent period which is the time from an acute injury or other predisposition before seizures occur; **(ii)** the occurrence of recurrent seizures; and **(iii)** the development of refractory epilepsy (in about 30% of patients). Genetic or acquired etiologic factors are involved in the development of the epileptogenesis, which subsequently affect the structural and functional changes in the brain, leading to the development of spontaneous seizures. Up to 27% of infants with a history of neonatal seizures develop epilepsy and/or cognitive and behavioural deficits in later life (Ronen et al., 2007). Neonatal seizures, as well as their etiologies, can affect the maturation of the developing brain, neuronal circuitry, neurogenesis, and network activity. Mechanisms of seizure generation (ictogenesis) and the development of recurrent unprovoked seizures are age- and sex-

specific, which is reflected in seizure susceptibility. Such mechanisms of ictogenesis and epileptogenesis may include excitatory-inhibitory imbalance and sex-specific network maturation during brain development.

Excitatory-Inhibitory imbalance during development

The development of the brain is a complex interplay between excitatory and inhibitory processes, which is crucial for synaptic formation, neuronal growth, and overall brain maturation. This balance is pivotal in preventing seizures and providing normal brain development. The progression from neonatal period to adulthood illustrates a dynamic shift in excitatory and inhibitory balance. In the neonatal period, excitation predominates over inhibition in neuronal networks across the cerebral cortex and limbic structures (Ben-Ari et al., 2007). This excitatory surge results from the fact that neurons are undergoing differentiation and formation of functional synapses that are responsible for establishing communication patterns between neurons during the critical period of brain development. However, the intrinsic hyperexcitability of immature networks, and-or an imbalance between excitatory and inhibitory processes increases the risk of seizures in early life. In this section will be discussed GABA signalling and its alterations, along with the synergistic actions of GABA and NMDA receptors in the context of epileptogenesis during development.

While GABA is inhibitory evoking hyperpolarization of the postsynaptic neuron in adult brains, this neurotransmitter is excitatory and produces depolarization during early stages of life (Rheims et al., 2008). This immature period is due to a characteristic developmental sequence where excitatory GABAergic synapses mature earlier, followed by glutamate synapses. This is supported by a precocious increase in synapse and spine density in the first two weeks of life in (Rheims et al., 2008) and through the first year in humans (Huttenlocher et al., 1982). Concurrently, expression levels of excitatory ion channels and transporters are elevated enhancing excitation while inhibitory mechanisms are less mature compared to later in life (Figure 1).

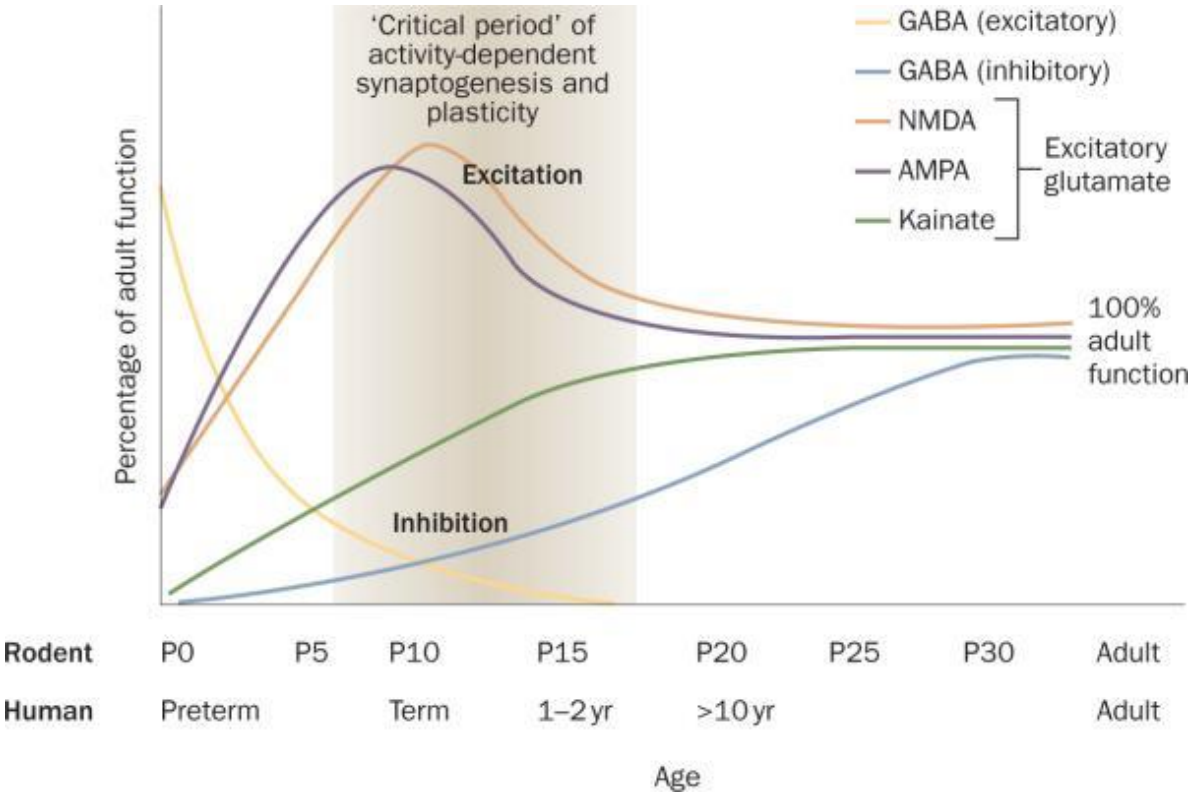

Figure 1. Schematic depiction of maturational changes in glutamate and GABA receptor function in the developing brain (Rakhade & Jensen, 2009)

Equivalent developmental periods are displayed for rats and humans on the top and bottom x-axes, respectively. Activation of GABA receptors is depolarizing in rats early in the first postnatal week and in humans up to and including the neonatal period. Functional inhibition, however, is gradually reached over development in rats and humans. Before full maturation of GABA-mediated inhibition, the NMDA and AMPA subtypes of glutamate receptors peak between the first and second postnatal weeks in rats and in the neonatal period in humans. Kainate receptor binding is initially low and gradually rises to adult levels by the fourth postnatal week. Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; GABA, γ-aminobutyric acid; NMDA, N-methyl-d-aspartate; P, postnatal day.

The GABA released from axonal terminals binds to ionotropic receptors $GABA_A$ and $GABA_C$ and metabotropic $GABA_B$ receptors. Both $GABA_A$ and $GABA_C$ receptors are pentameric, consisting of five subunits that form a Cl^- channel. Upon activation, both $GABA_A$ and $GABA_C$ receptors typically cause hyperpolarization due to the influx of Cl^-, which underlines their inhibitory function in mature neurons. During first two postnatal weeks, in immature neurons, $GABA_A$ and $GABA_C$-mediated responses can be depolarizing. This is due to the higher intracellular Cl^- concentration such that upon receptor activation, Cl^- efflux occurs which depolarizes the cell (Figure 2). The elevation of intracellular Cl^- is largely due to the presence of sodium-potassium-chloride cotransporters (NKCCs) (Plotkin et al., 1997) and they outnumber the potassium-chloride cotransporters (KCCs), that expel Cl^- from the cell, bringing down its intracellular levels. By the second week after birth in rodents, GABA begins to exert an inhibitory influence, coinciding with the expression of a cotransporter (KCC2) that lowers the internal chloride concentration (Dzhala et al., 2005). Similarly, NKCC1 expression is highest in the human

cortex at term, whereas KCC2 is not expressed until the middle of the first year of life (Dzhala et al., 2005). This shift is prompted by an increase in the density of glutamatergic synapses, necessitating robust inhibition to avert the onset of seizures.

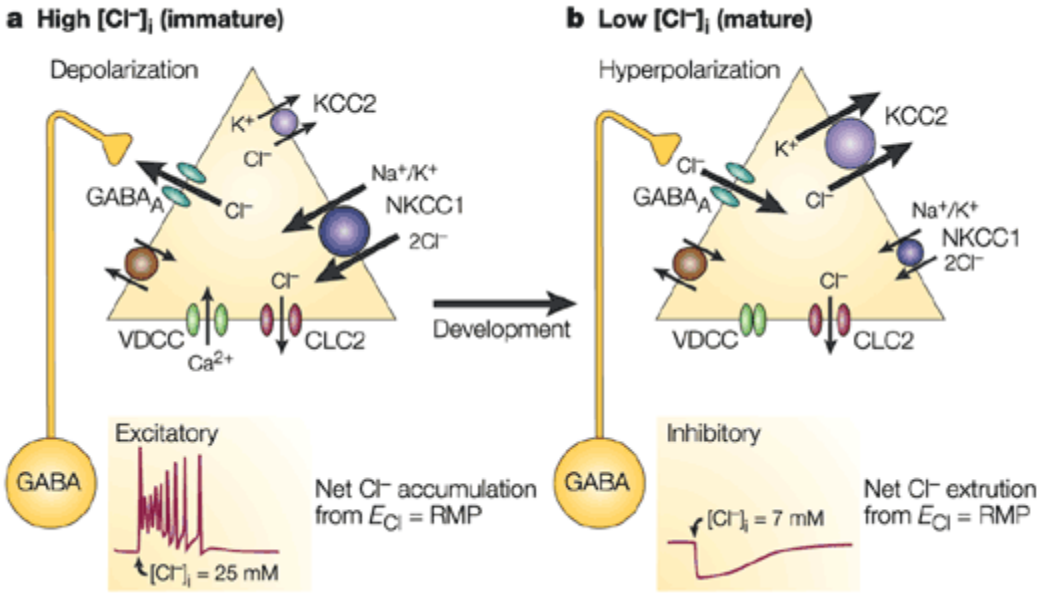

Figure 2. Early expression of NKCC1 and late expression of KCC2 determines developmental changes in [Cl–] (Ben-Ari, 2002).

Schematic diagram depicting the Na^+–K^+–$2Cl^-$ cotransporter NKCC1, the K^+–Cl^- co-transporter KCC2 and voltage-gated calcium currents, as well as the gradients of chloride ions. (a) NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride $[Cl^-]_i$ is relatively high. (b) KCC2 expression predominates in mature neurons. Note that the activation of GABA (γ-aminobutyric acid) type A receptors generates an efflux of chloride and an excitation of immature neurons, and an influx of chloride and an inhibition of adult neurons. CLC2, voltage-gated chloride channel 2; ECl, chloride reversal potential; RMP, resting membrane potential (V_{rest}); VDCC, voltage-dependent calcium channel.

During the neonatal period, stimulation of GABA receptors induces calcium flows by directly triggering voltage-gated calcium channels (Yuste & Katz, 1991). The depolarization resulting from GABA receptor activation effectively lifts the voltage-dependent magnesium blockade on NMDA (Leinekugel et al., 1995), altering the NMDA channels' magnesium binding affinity and leading to a rise in intracellular calcium concentration. This rise in intracellular calcium concentration is necessary for neuronal development and the creation of synapses, while simultaneously preventing the harmful effects of glutamate.

Immature hippocampal neurons, including CA3 and CA1 pyramidal neurons, granule cells and interneurons, generate Giant Depolarizing Potentials (GDPs) until the end of the 12th postnatal day (Ben-Ari et al., 1989), with extensive polysynaptic activity owing to the synergistic action of GABA and

NMDA receptors. This mechanism facilitates the establishment of synapses by allowing immature neurons to coordinate their activity with mature ones. Growth of the density of glutamate synapses results in the abolition of the GDPs and replacement by GABA as the primary inhibitory transmitter in the developing hippocampus (Represa & Ben-Ari, 2005). The brain in early development is predisposed to seizures as the GABAergic system matures slowly and GABA continues to exhibit excitatory effect, along with the late maturation of other inhibitory mechanisms, such as $GABA_B$ receptors and potassium channel activation.

First, in the hippocampus and other structures of the immature rats` brain, $GABA_A$ receptor signalling has depolarizing effects, whereas excitatory NMDA transmission peaks early in development (Brady et al., 1994). Both these characteristics may significantly contribute to the increased epileptogenicity of the immature brain. Thus, the delayed onset of functional GABAergic inhibition may contribute to the enhanced excitability of the immature brain. Moreover, it was demonstrated, that partial deficit in KCC2 increases the susceptibility to seizures and seizure-related hippocampal injury (Delpire & Mount, 2002). Seizures in the early stages of life can impair or disrupt $GABA_A$ signalling system by decreasing the subunit composition (α1 and γ2 subunit) of its (Xi et al., 2020) or causing abnormal reversal of the polarity of $GABA_A$ receptor currents (Akman et al., 2014). This alteration can contribute to epileptogenesis or cognitive and neurodevelopmental deficits.

Sex-specific network maturation during ontogenesis

The development of brain networks is a sophisticated process, which is modulated by several factors, among them by sex hormones. These are responsible for creating anatomical and functional dissimilarities between male and female brains by affecting the maturation and function of critical brain structures including the substantia nigra pars reticulata (SNR). The SNR was demonstrated to differentially respond to epileptogenic triggers in a region-specific, age-specific, and sex-specific manner (Velíšek et al., 2006) by exposing rodents to flurothyl-induced seizures (a model of generalized clonic and tonic–clonic seizure).

The SNR is divided into anterior (SNRa) and posterior (SNRp) regions, each with unique responses to GABA-mimetic drugs. In adulthood, the SNRa mediates anticonvulsant effects of the GABA-mimetic drugs; the SNRp mediates proconvulsant effects in males, whereas there is no proconvulsant region in females. During development, the functional differentiation between SNRa and SNRp emerges postnatally, with an earlier maturation (≥P25) in females compared to males (≥P30) (Velíšek et al., 2006). In male rats, neonatal castration changes the SNR phenotype to that seen in females (Velísek et al., 2006a), showing a profound and lasting effect of gonadal hormone exposure on the development of

anti- and proconvulsant brain networks and their vulnerability to epileptogenesis. Moreover, increased KCC2 mRNA expression has been shown in the SNR in female than in male between P15 (rat infantile stage) and P30 (rat prepubertal stage) (Galanopoulou & Moshé, 2003). In male PN15 rat SNR neurons, the $GABA_A$ receptor agonist muscimol triggered depolarizing currents, and increased calcium intracellularly, whereas in female P15 SNR neurons, muscimol caused hyperpolarizing currents and failed to activate calcium signalling (Galanopoulou & Moshé, 2003)

The SNR is able to process information via "dynamic gating", which has been suggested to suppress excitation through GABAergic projection neurons to the basal ganglia and prefrontal (Spiciarich & Moshé, 2018). It therefore represents a site where the epileptogenic process might modulate brain functions during development, with potentially significant implications, especially in the context of its SNR's role in controlling seizures.

Models of generalized seizures in immature rats

Animal models of epileptic seizures remain essential in studying the basic mechanisms of ictogenesis, epileptogenesis, and the development of new antiepileptic drugs. The predominant species for animal models of epilepsy are rodents. Epileptogenesis in the immature brain during the first two weeks in rodents has similar mechanisms to those of brain development in humans during the neonatal and infant period. Thus, it has been suggested that postnatal (P) 8-10 rodents are developmentally equivalent to human newborn babies (Table 2), because the rate of brain growth, and its DNA, cholesterol and water contents resemble those of a human full-term neonate (Dobbing & Sands, 1979). The infantile stage continues until P21 with peaks in myelination rate and synaptic density in P20-21 (Semple et al., 2013), the juvenile age at P21-32 in females and P21-35 in males, early puberty occurs around P32- 36 in females and P35-45 in males, and finally adulthood begins at P60 in both sexes (Table 3).

TABLE 2. Species equivalency for developmental milestones (Galanopoulou & Moshé, 2011).

MILESTONES	RODENTS	HUMANS
DURATION OF GESTATION	23 days	40 weeks
FULL-TERM NEONATE[A]	P8–13	39–40 weeks
EYE OPENING	P13–15	Right after birth (~26 weeks)

WEANING FROM MOTHER/END OF BREASTFEEDING	P21	6th month or later
AMBULATION	2 weeks	>1st year of life
LIFE EXPECTANCY	2 years	~80 years in USA

P is the postnatal day

TABLE 3. Developmental stages in rodents based on maturation of hypothalamo-pituitary-gonadal axis (Semple et al., 2013).

STAGE	FEMALE	MALE
NEONATAL	P0–6	P0–6
INFANTILE	P7–21	P7–21
JUVENILE	P21–32	P21–35
EARLY PUBERTAL	P32–36	P35–45
PUBERTY	P34–38	P45–60
ADULT	P>60	P>60

P is the postnatal day

From a practical point of view, animal models can be used to perform invasive methods, such as epidural electrode recording, which allows for more accurate examination of functional components of cortical areas; to perform long-term continuous EEG monitoring (for days, weeks) in chronic epilepsy models (except for immature rats); to create the necessary epilepsy model depending on the induction method. Epilepsy models can be divided into acquired (symptomatic) and into idiopathic (genetic) epilepsies. Healthy immature rats are used to reproduce symptomatic generalized seizures by electrical or chemical induction. This approach is based on the desire to reveal the nature and mechanism of epileptogenesis, synaptic reorganization, and functional damage to the developing hippocampal/limbic network, without the prima facie presence of pathology. Examples of chemically-induced and electrically-induced models of generalized seizures in immature rats are presented below:

Electrically-induced seizures

There are two types of seizures induced by electrical stimulation: those elicited by stimulation of the whole brain (maximal electroshock seizures) and those induced by local stimulation of a defined brain area (kindling model).

- *Maximal electroshock seizures (MES).* For MES, a device capable of delivering a current of up to 300 mA is required. The current is delivered through ear clip electrodes, and the normal range

is 150-200 mA for seizure induction in rats (Löscher et al., 1991). Maximal electroshock delivered via ear-clip electrodes immediately produces tonic-clonic convulsions with the loss of righting. The rat is usually restrained during stimulation. This model provides insight into the drug's ability to prevent the spread of seizures when all neural circuits in the brain are maximally active.

- *Kindling.* Delivery of short (1–2 s) trains of alternating current (either sinusoid or rectangular pulses), at frequencies 50–60 Hz, at regular intervals, which is initially subthreshold for generalized seizure discharge and eventually leads to evoked seizures, is called kindling. The principle of kindling consists in delivery of subthreshold stimulus for motor seizures. With repetitions of this stimulus at appropriate intervals (4–48 h in adult rats) the seizures develop, become more and more severe, and also permanent. While initial stimulations produce focal electrical discharges (focal afterdischarges must be induced by the kindling stimulus), repeated stimulations lead to more severe stages of motor seizures, which represent secondary generalization (McNamara, 1986).

Chemically-induced models

Generalized seizures (primary or secondary) can be induced in freely moving animals by systematic application of chemical drugs (convulsant). Intraperitoneal injection is commonly used. Subcutaneous injections are administrated into the skin fold on the dorsal surface of the neck. Convulsants can also be injected intravenously into the tail vein. Despite its simplicity, the administration of convulsant drugs by injection causes severe stress, which can be difficult to standardize in experimental groups. An alternative method of inducing generalized seizures is the application of flurothyl by inhalation.

- *Pentylenetetrazol* is a $GABA_A$ receptors blocker (blockage via the TBPS site in the chloride channel). Seizures are provoked mainly by a bolus dose (single injection of a relatively large dose) of a subcutaneous or intraperitoneal aqueous solution of pentylenetetrazol, ranging from 40-120 mg/kg (Marescaux et al., 1984). During induction, whole-body myoclonic jerks occur, accompanied by individual spikes on the EEG (often superimposed on motion artifacts). Clonic convulsions of the forelimbs and face then develop. These convulsions last up to tens of seconds and manifest on the EEG as a series of spike-wave discharges. Finally, when high doses of convulsants or longer intervals with systematic induction are used, generalized tonic-clonic seizures with a loss of righting reflex (Velisek et al., 1992). These characteristic seizures are common with drugs that block $GABA_A$ receptors (bicuculline, picrotoxin, and flurothyl).
- *Bicuculline and Picrotoxin.* Both drugs block $GABA_A$ receptors with the difference that bicuculline competitively binds to GABA at its binding site, whereas picrotoxin non-competitively binds to its own binding site in the associated chloride channel. Bicuculline is

usually dissolved in 0.1 N HCl and pH is titrated back with 0.1 N NaOH (de Feo et al., 1985) to allow it to cross the blood-brain barrier. In addition, the final pH is acidic and extremely painful when injected into an area with high density of nerve endings; therefore, the drug is administered intravenously (about 2 mg/kg) or intraperitoneally (6-8 mg/kg) (Velíšková et al., 1990). In the second case, the dosage is significantly increased to circumvent the "first pass" effect. In female rats, a higher concentration of progesterone is correlated with a heightened seizure threshold to bicuculline-induced convulsions (Borowicz, 2009).

- *Kainic acid (KA)* is a neurotoxic analog of glutamate with a preferential effect on the limbic system. Bolus doses (10-20 mg/kg) are administered intraperitoneally. KA-induced seizures are termed "limbic seizures" (with focal onset and secondary generalization), whereas tonic-clonic seizures are extremely rare. However, the clonic phase can last up to several hours, progressing to status epilepticus. The severity of epileptic seizures correlates with the degree of neuronal damage in the hippocampus (Sutula et al., 1998).
- *Strychnine.* Seizures are provoked by the blockade of chloride channels controlled by glycine, which are present in large quantities in the spinal cord and brain stem(Singh et al., 1990). Strychnine sulphate is dissolved in saline (at 1 mg/ml) and administered subcutaneously in doses of 2-3 mg/kg. This drug produces myoclonic twitches and tonic-clonic seizures with loss of righting.
- *N-methyl-D-aspartate (NMDA)* is an agonist of the glutamate NMDA receptor subtype. NMDA can be dissolved in saline solution up to 50 mg/ml. Systematic doses that induce seizures in adult rats are 150-300 mg/kg (Mareš & Velišek, 1992). The seizure-inducing doses of NMDA in immature rats are lower, partly because of the better permeability of the blood-brain barrier in the immature brain. On the other hand, the higher sensitivity to NMDA may be due to a transient increase in NMDA receptor density in the developing brain, as well as a different subunit composition of NMDA receptors in infancy, i.e. high NR2B subunit content (Jantzie et al., 2015). In NMDA-induced seizures, the tonic phase follows the clonic phase, and usually the appearance of the tonic phase indicates a fatal seizure.
- *Flurothyl* is described in detail below.

Flurothyl model of recurrent generalized tonic-clonic seizures in neonates

Flurothyl, bis-2,2,2-trifluoroethyl ether, is a liquid convulsant, which quickly evaporates and eliminates through the lungs. The mechanism of action involves antagonism of $GABA_A$ receptors and activation of sodium channels, which stimulates the central nervous system and triggers epileptic activity. It was observed that flurothyl shows anesthetic properties at high concentrations (Koblin et al., 1981) or even

could be lethal due to the fact that it causes asphyxia (with lower mortality in immature rats due to their resistance to hypoxia), whereas lower doses induce behaviour phenomena such as freezing, repetitive myoclonic jerks and swimming movements (Velíšek, 2006). Flurothyl is commonly used to recreate recurrent generalized tonic-clonic seizures in immature rats. Flurothyl-induced seizures are brief and last from 15 to 60 seconds. Seizures are administrated five times a day over the course of 5–10 days (usually P0–P9) with intervals of at least 2 hours (Huang et al., 1999). The major advantage of flurothyl application is that the animal is not subjected to painful injections or stressful restraint, resulting in minimal interference by stressor. Moreover, frequent flurothyl kindling is perfect to mimic natural epileptogenesis in neonates.

The equipment consists of an airtight glass cylinder that serves as an inhalation chamber, and a fume hood for expelling excessive fumes of the ether. Flurothyl is commonly administrated using a constant infusion rate pump to the filter paper at the top of the chamber, where it evaporates. Latency of seizure onset could be explained by the size of the chamber related to the size of the animal or flow rate (Sperber & Moshé, 1988).

After 8-9 minutes of administration of 20 µl/min of flurothyl, rats usually exhibit myoclonic twitches followed by a clonic seizure that progresses to a tonic-clonic phase. The specificity and sequence of the seizures are very similar to the effect of administration of the above-mentioned $GABA_A$ antagonizing drugs. With the difference that the seizures induced by flurothyl are significantly more severe. Clonic seizures are often accompanied by tonic flexion and frequently also extension of components in the axial muscles and may involve all four limbs simultaneously, which may lead to temporary loss of posture. Often the tonic phase is accompanied by the appearance of cyanosis and acidosis, which, however, disappear within 5 minutes after the end of seizures. Starting from the third postnatal week in rats, the EEG shows rhythmic spindle-shaped discharges (Figure 3a), which are associated with fixed gaze and freezing behaviour. In young rats, these discharges very loosely correlate with myoclonic twitches; the correlation improves with age (Velíšek, 2006). The seizure on EEG begins with a prolonged period of sharp waves and spike-and-wave complexes (Figure 3b), where bilateral clonus is associated with high-frequency polyspike discharges. The tonic phase is associated with high frequency of the spikes. This activity is observed in the cortex in adult rats, whereas in immature rats the discharges occur in both cortex and hippocampus.

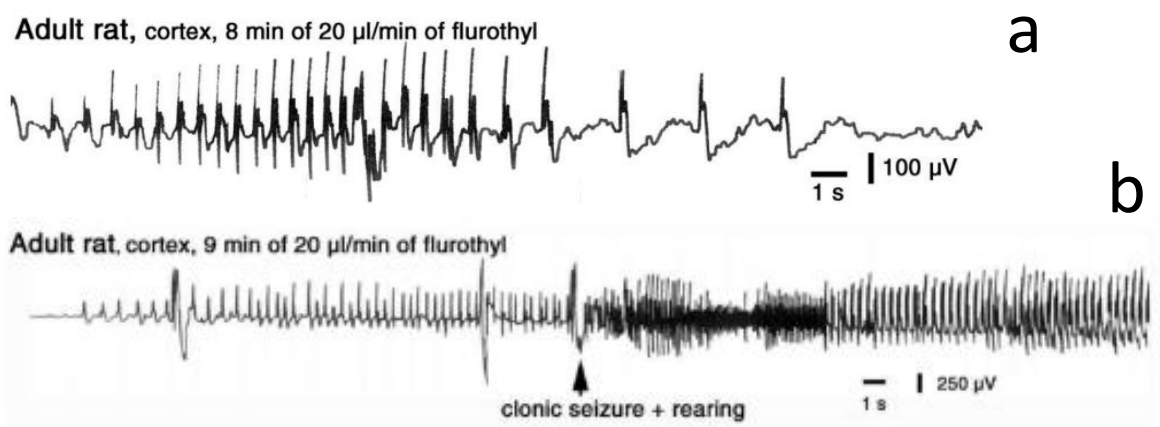

Figure 3. (a) Rhythmic, spindle-shaped discharge induced by inhalation of flurothyl in sensorimotor cortex of an adult Sprague-Dawley rat. This discharge was associated with freezing behaviour. Bipolar recording from the sensorimotor cortex is shown; (b) Spike-and-wave and polyspike discharges induced by inhalation of flurothyl in sensorimotor cortex of an adult Sprague-Dawley rat. Arrow indicates onset of clonic seizure and rearing. The onset of this discharge significantly preceded the onset of motor seizures. However, the occurrence of high-frequency polyspike discharges was associated with the onset of clonic seizure(Velíšek, 2006).

Flurothyl-induced seizures are age-specific; it was demonstrated that clonic seizures in immature rats evolve into tonic-clonic seizures (with tonic extension of all four limbs followed by prolonged clonus) with a much shorter latency period of seizure onset as compared to mature groups (Sperber & Moshé, 1988). However, clonic seizures can be either unilateral or asymmetric and asynchronous. There are no significant age differences in the EEG, although the frequency of discharges is slower, and the discharges are less synchronized than in adult rats.

The role of gender-specific features has also been noted. It has been shown that males have a lower threshold for the development of tonic-clonic seizures induced by flurothyl than females. These studies confirm the role of prepubertal and peripubertal increases in testosterone in differential development (Velísek et al., 2006b), which excludes the hypothesis of the influence of female hormones and estrous cycle on protective abilities during flurothyl-induced tonic-clonic seizures (Velísek et al., 1999).

Recurrent seizures elicited with flurothyl during early development caused a highly significant long-term detrimental effect in cognitive function (Lenck-Santini & Holmes, 2008). Rats exposed to recurrent flurothyl seizures in the neonatal period took more time in the Morris Water Maze, which is a hippocampal-dependent spatial memory test, in comparison with controls. Extratemporal areas can also be affected by seizures. Increased prefrontal cortex thickness, alterations in synaptic plasticity, and decreased behavioural flexibility have been documented following chronic epilepsy (Hernan et al.,

2013). The data are indicative that similar prefrontal cortex modifications may underlie the consistent cognitive impairments in adult humans with a history of infant or juvenile seizures (Isaeva et al., 2013).

Taken together, the flurothyl-induced seizure model in immature rats is essential due to its age- and sex-dependent parameters in investigating the complex interactions among seizures, brain maturation, and neuroendocrine functions. The essential vastness of the model includes not only the ability to target the fundamental processes of epileptogenesis but also to lay an evidence-based foundation for the development of specific age- and sex-specific therapies against epilepsy. Thus, considering that different ages and sexes demonstrate various sensitivity and progression of seizure, this will help researchers to focus their studies to obtain more elaborate information about this essential neural disorder and to develop effective personalized therapeutic tactics.

Aims

This study investigates age-specific changes in the amplitude and frequency patterns of EEG during the early ontogenetic phases following repeated postnatal generalized seizures induced by flurothyl, which could serve as age-specific indicators for possible diagnosis and therapy.

The primary aims of this study are: **(1)** to compare cortical excitability across different age groups using EEG analysis; **(2)** to determine whether there is gender-specific difference in the paroxysmal activity during early ontogenetic phases (in postnatal days P12, P15, and P25).

To fulfill the goals two types of analysis methods were used:

1. Seizure Detection: This method focuses on seizure incidence among determined age groups after flurothyl treatment, seizure frequency, duration of detected electrographic seizures, and number of paroxysmal events. It helps in understanding the general seizure dynamics and their variation between groups differing in age and gender.

2. Power Spectral Density (PSD) analysis: This method examines the power spectral density of individual EEG bands of each animal and time profile of PSD changes in time. It provides a detailed view of the changes in brain electrical activity associated with seizures and helps identify specific patterns that could serve as diagnostic indicators.

We hypothesize that epileptogenic process followed flurothyl-induced repetitive generalized seizure in immature rats exhibit specific developmental and sex-dependent EEG pattern, which will be a suitable biomarker for diagnosis and monitoring of disease progression. Our results could lead to new diagnostic methods and possible treatment options intended for specific age groups and potentially genders, improved diagnostic tools for use in hospitals, and an enhanced understanding of epileptogenesis and its age-specific manifestations. This study aims to provide insights that can advance both clinical practice and scientific knowledge in the field of early childhood epilepsy.

Methods

Animals

Outbred Wistar rats of both sexes (Institute of Physiology of the Czech Academy of Science, Prague) were used in the experiments. Animals were maintained under controlled temperature (22±1°C) and humidity (50 to 60%) with a 12/12 h light/dark cycle (lights on at 6:00 AM). Food and water were provided *ad libitum*. Animals were bred in our animal room and at postnatal day five (P5) the number of pups was reduced to 12-14 animals in each litter. Each litter consisted of both males and females. Day of birth was defined as day 0.

To prevent a litter effect, only a limited number of animals from the same litter were used. Each litter was randomly divided into control and flurothyl-exposed animals. In total eight litters were used for this study and each treatment interval group was composed of animals from at least their litters.

The number of animals is indicated for each experiment. Experiments were approved by The Central Committee for Animal Care of the Czech Academy of Science (approval #15/2018). Animal care and experimental procedures were also conducted according to the guidelines of the European Community Council directives 86/609/EEC. 2010/63/EC).

Seizure induction

At P6 animals in each litter were randomly assigned to treatment groups. Five brief seizures two hours apart were induced daily with flurothyl inhalation starting at P6 till P10, i.e. in total, animals experienced 25 seizures. For seizure induction, animals were placed into a small enclosed Plexiglass chamber (21x20x8 cm). Liquid flurothyl (BIS-2,2,2-trifluoroethyl ether; Sigma-Aldrich, St. Luis, Mo., USA) was delivered with a syringe pump (Harvard Apparatus) at the rate of 50µl/min. After 2-3 minutes animals developed generalized trembling that was followed by repetitive myoclonic jerks and swimming movements which progressed to generalized tonic-clonic seizures with tonic extension of both forelimbs and hindlimbs. After seizures, animals were left to rest at a healed pad (32±2°C) for 10 minutes and after that, they were returned to their dams. Except for flurothyl exposure, all manipulations were the same in controls and experimental animals.

Surgery

Surgery preparations were always performed on the day of the experiment, which corresponded to 12, 15, and 25 postnatal days (P12, P15, and P25). Each age group consisted of 6-15 animals of each sex. Diethyl ether served as the anaesthetic during the surgical procedures. Two silver registration electrodes were implanted epidurally over the left hemisphere (Figure 4). First electrode (Ch1) was located over the sensorimotor cortex (AP=0; L=2.5 mm), and second electrode (Ch2) was over the parietal cortex (AP=3; L=3 mm) on the basis of the bregma-lambda distance (on the average 8 mm in adult rats, 4 mm in 12-day-old animals). Coordinates were recalculated for each animal (BÜTTNER-ENNEVER, 1997). The ground and reference electrodes were placed into occipital bone over the cerebellum. Animals at P12, lacking thermoregulation, were maintained on a heated pad (32±2°C) throughout the surgical preparations and experimental procedures. The recovery period from the surgical procedure for each animal was approximately one hour.

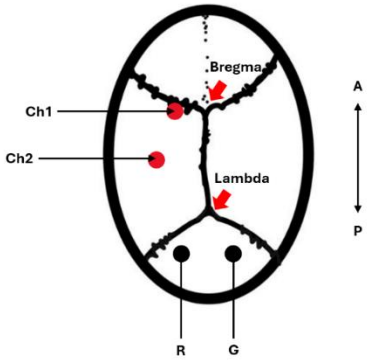

Figure 4. The image shows the placement of electrodes on the skull of a rat for EEG recording—dorsal view. Two silver registration electrodes were implanted epidurally over the left hemisphere: Channel 1 is located over the sensorimotor cortex (AP=0; L=2.5 mm), and Channel 2 is over the parietal cortex (AP=3; L=3 mm). These coordinates are based on the bregma-lambda distance (8 mm in adult rats and 4 mm in 12-day-old rats). The ground and reference electrodes were placed in the occipital bone over the cerebellum. Abbreviations: Ch1 – electrode located over frontal cortex; Ch2 – electrode located over parietal cortes; G – ground; R – reference; A – anterior; P – posterior direction.

EEG recording

Animals implanted with electrodes were placed in individual plastic boxes to provide consistent conditions during EEG recording. The setup is schematically depicted in the Figure 5. Electrodes were connected via a cable to a low-impedance preamplifier (RA4PA 4-channel Medusa Preamp). The

amplified signal from two channels was sent to the Pentusa Base Station (model RX5), a digital signal processor that converted the analog signal to digital. EEG recordings were stored and processed using LabChart 8 Reader (AD Instruments) for digitization and analysis.

The P12 and P15 groups were monitored for spontaneous seizures for 4 hours. The study's limitation stemmed from the immature stage of development, which affected the animals' ability to self-regulate temperature and required maternal care. In contrast, the P25 group, capable of independent thermoregulation and post-weaning, was recorded for 12 hours.

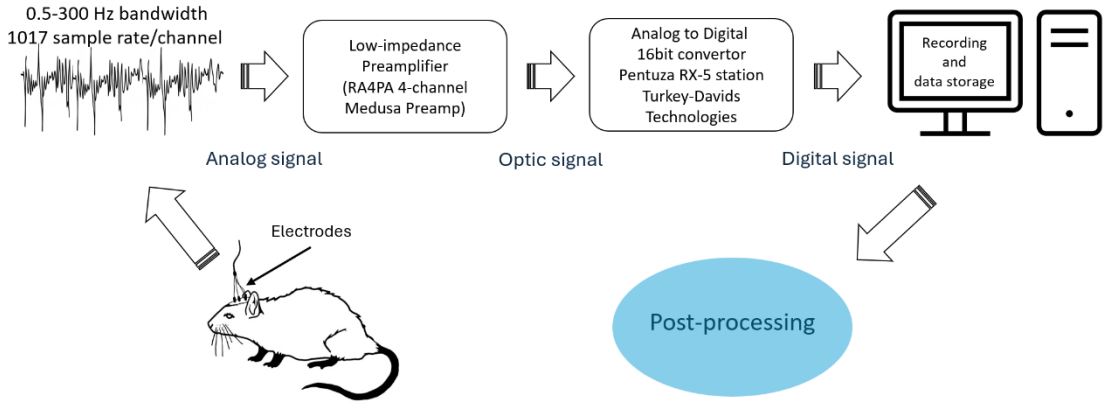

Figure 5. Schematic illustration of the setup for recording EEG signals. The analog signals captured by the electrodes are transmitted via a connecting cable to a low-impedance preamplifier (RA4PA 4-channel Medusa Preamp, Turkey-Davids Technologies) that amplifies the signals with a gain factor of 4x. These amplified signals are then sent as optic signals to the Pentusa Base Station RX5 (Turkey-Davids Technologies, USA), a digital signal processor that converts the analog signals into digital form. The digital signals are stored on a PC for further analysis. Biopotentials within a frequency range of 0.5-300 Hz at were recorder at sampling rate of 1017 samples per second per channel.

Data acquisition

All raw data (Figure 6) were collected in the Turkey-Davids Technologies Format from Pentusa Base Station and subsequently exported to the European Data Format (EDF). The recorded signals were processed using LabChart Reader (AD Instruments). The notch filter (50Hz) was applied to eliminate power-line noise, and a band-pass filter ranging from 0.5 to 70 Hz was used to limit the frequencies of interest. The EEG data could only be processed after the application of these filters. These steps ensured the data were free from noise and artifacts, improving the accuracy and reliability of further analyses.

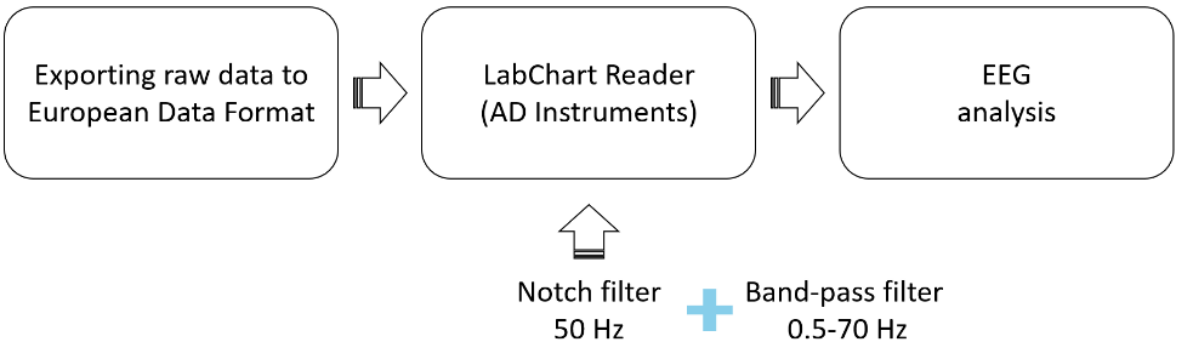

Figure 6. The diagram illustrates the processing steps for EEG data collected from animals. Initially, all raw data are collected in the Trusted Data Format (TDF) and subsequently exported to the European Data Format (EDF). The recorded signals, sampled at 1017 Hz, are processed using LabChart Reader (AD Instruments). During this process, a 50 Hz notch filter is applied to eliminate power-line noise, and a band-pass filter ranging from 0.5 to 70 Hz is used to isolate the field of interest.

The filtered signals were subjected to analysis for the presence of seizures and related paroxysmal events. Additionally, the Power Spectral Density (PSD), including delta, theta, alpha, beta, and gamma bands, was examined (Figure 7). For the PSD analysis, we developed custom scripts in MATLAB (MathWorks, USA) to calculate the relative PSD (refer to Spectral Analysis below for details). The processed data were subsequently analysed statistically to identify significant differences between animal groups.

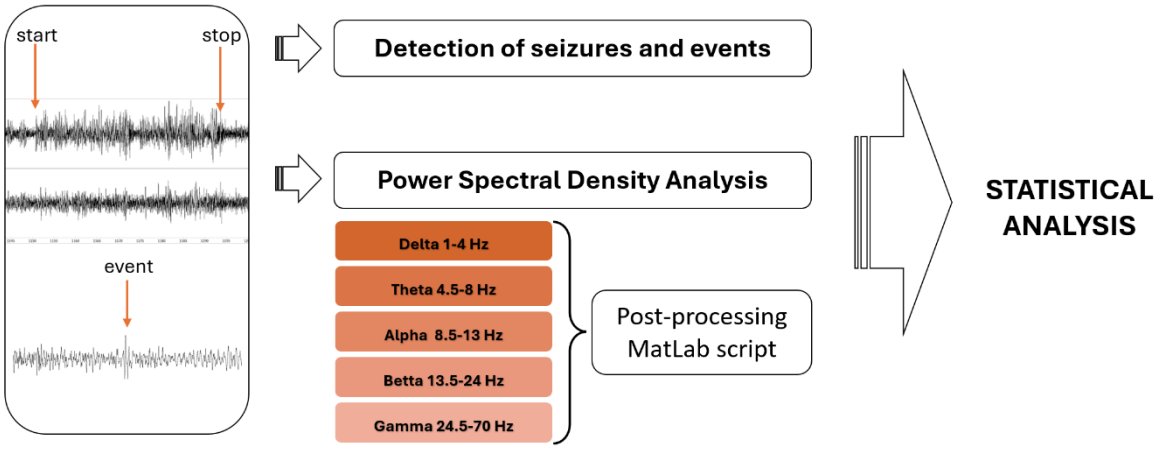

Figure 7. This schematic picture illustrates the steps in the analysis of EEG recordings. After filtering, the recordings are analysed for the presence of seizures and events, and for Power Spectral Density (PSD) of the main neuronal oscillations, including delta (1-4 Hz), theta (4.5-8 Hz), alpha (8.5-13 Hz), beta (13.5-24 Hz), and gamma (24.5-70 Hz) waves. Custom scripts created in MATLAB (MathWorks, US) are used for calculating the relative PSD during the spectral analysis. All processed data are then statistically analysed.

Seizure detection and analysis

All EEG data files were analysed for an incidence of spontaneous seizures and their occurrence in time, average and total duration of seizures, and incidence of the paroxysmal epileptic-like events.

Detected spontaneous seizures are characterized by frequency activity in the range of 5-9 Hz, at least double amplitude compared to basal EEG activity level of each subject, with a minimum duration of 5 s and a maximum interval of 1 s between two separate seizures. The epileptiform events were defined with the same frequency rate (5-9 Hz) and amplitude but with a duration in the range of 2-4.5 s. See Figure 8 for more details.

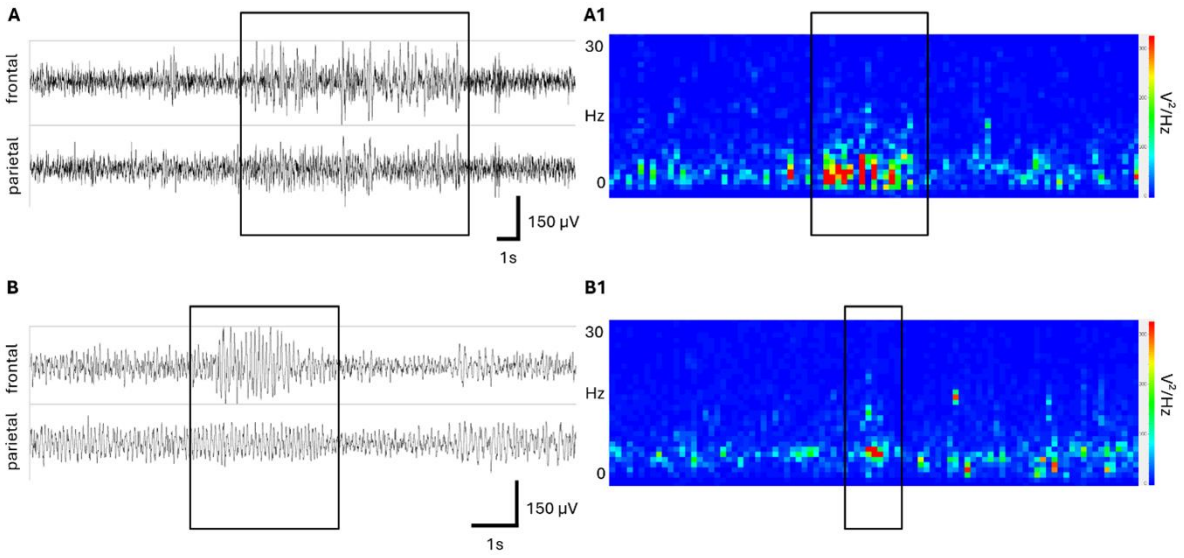

Figure 8. Examples of detected seizures and epileptiform events. Electrographic seizures and epileptiform events (left) were always represented in both channels (either in Frontal (Ch1) and Parietal (Ch2) cortical regions). EEG: x-axis time (s), y-axis amplitude (V). On the right side are spectrograms with the Hann (cosine-bell) data window 1K (1024) without overlap, with x-axis time, the left y-axis of band-pass of 0-30 Hz, and the right y-axis (V^2/Hz). The electrographic seizure and the epileptiform event are marked within a black rectangle. All pictures are taken from the P25 flurothyl-treated female. ***A****: The seizure duration was 15.97, presented in both channels.* ***A1****: Corresponding spectrogram showing Power Spectra of the related electrographic seizure.* ***B****: representation of the paroxysmal event.* ***B1****: Corresponding spectrogram showing the Power Spectra of the epileptiform event.*

Spectral analysis

To calculate the PSD, we applied Fast Fourier Transform (FFT) with the Hann (cosine-bell) data window 1K (1024) without overlap, but with removing zero-frequency, to represent the frequency domain for every EEG band per 5s windows of the total EEG recording. Using the LabChart Reader (AD Instruments) program the calculation for Delta 1-4 Hz, Theta 4.5-8 Hz, Alpha 8.5-13 Hz, Beta 13.5-24 Hz, and Gamma 24.5-68 Hz had been performed and exported to Matlab (MathWorks, US).

Using the MATLAB environment, we programmed the script, which leveraged FFT from the previous calculations and computed the magnitude-squared of the FFT output to determine the PSD with measurements labelled in $\mu V^2/Hz$. Relative PSD for individual EEG band for every single animal had been calculated for each 5s window for the whole period of the recording using the following formula:

$$Relative\ PSD = {\int_{Freq\ start}^{Freq\ stop} PSD(i)_{5sec}} \Big/ {\sum PSD\{Delta; Theta; Alpha; Beta; Gamma\}_{5sec}}$$

Each relative PSD(i) is the Amplitude^2 of individual EEG band determined by frequencies (start-stop) as described above (Figure 7). This procedure was performed for general normalization and reducing the entropy of the result, which may have been caused by the surgery, the resistance of electrodes and connecting cables, etc.

Furthermore, to explore the dynamic relationship between the different EEG frequency components in our dataset, we computed the ratio in between relative PSD for alpha/theta and gamma/theta bands.

Statistical analysis

For statistical analysis, collected data for seizure detection (including the number of unprovoked electrographic seizures, average seizure duration, and average number of events) were processed using an ordinary One-Way ANOVA test followed by Sidak post-hoc test in the GraphPad Prism software. This statistical analysis was chosen to identify significant differences between the control and flurothyl-treated animals. For the number of seizures visualisation, data are presented as mean ± SD; however individual number of events are presented as mean ± SEM.

The PSD data were processed using GraphPad Prism software by ordinary Two-Way ANOVA test followed by Tukey`s multiple comparisons test. This analysis was chosen to identify differences among the following groups within the age groups: control-female vs. flurothyl-female; control male vs. flurothyl-male; control-female vs. control male; and flurothyl-female vs. flurothyl-male. Another factor to be included in the total relative PSD was the comparison between the EEG bands. In the relative PSD

based on 15-minute intervals, the second comparison factor was time. All data are presented as mean ± SD.

The level of significance was defined as * $p < .05$; ** $p < .01$; *** $p < .001$.

Results

The short-term EEG monitoring was performed on 109 animals of three different ages (P12, P15, and P25 respectively), in both sexes and two treatment groups. The group details are summarized in Table 4. Seizure detection analysis for all age groups was evaluated for a 4-hour period. For the P25 group, the first 4 hours of the total 12-hour recording were used for the seizure detection analysis. Six animals (1x P12 flurothyl male, 3x P25 flurothyl females, and 2x P25 flurothyl males) were excluded from the analysis due to technical problems during EEG recording (e.g. higher impedance of electrodes, high noise-signal ratio, severe movements artefacts).

TABLE 4. Summary of animal groups for short-term EEG monitoring.

	P12		*P15*		*P25*	
	Control	Flurothyl	Control	Flurothyl	Control	Flurothyl
Female	8	7	10	8	6	15
Male	7	8	8	10	7	15

Seizure detection

Electrographic seizures were always represented in both channels (either in Frontal (Ch1) and Parietal (Ch2) cortical regions). Considering the negligible differences, on the order of hundreds of milliseconds, between the start and end of the seizure in Ch1 and Ch2, we describe the results only for the Frontal Cortex (Ch1).

Overall incidence rate of spontaneous generalized seizures among age groups

Initially, we measured the total incidence rate for each animal group to provide a general overview (Figure 9). Electrographic generalized seizures occurred in all seven animals treated with flurothyl in both sexes of the P12 group, with spontaneous electrographic seizure activity also observed in the control groups. A similar trend was observed in the P15 group, where recurrent seizures were exhibited by all eight females and all ten males following flurothyl application. Additionally, in P15 control were noted in 2 of 8 males with electrographic seizures, possibly due to acute inflammation induced by surgery.

The P25 group exhibited a lower incidence rate in the experimental group, with unprovoked electrographic seizures occurring in 1 of 12 females and 4 of 13 males, whereas no seizure activity was observed in any control animals of either sex.

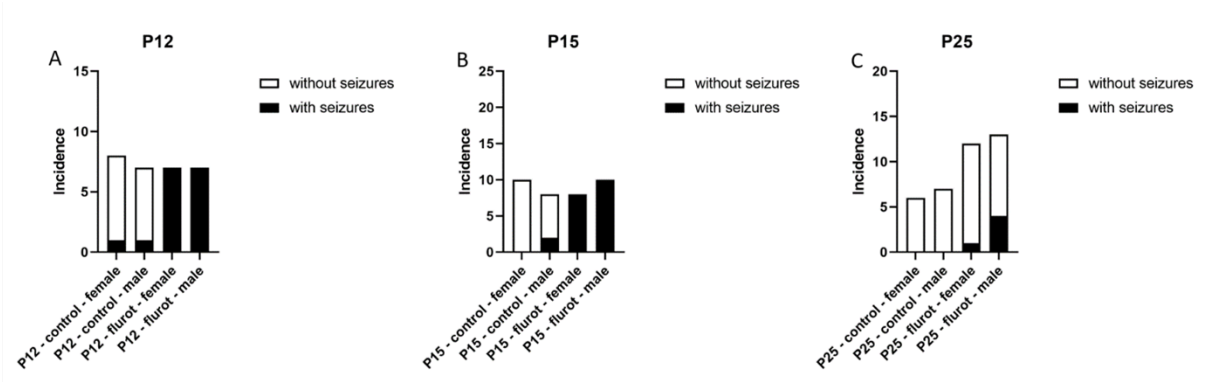

Figure 9. The graphs illustrate the total incidence rates of electrographic seizures across different animal groups at three postnatal days: P12, P15, and P25. (A) At P12, electrographic seizures occurred in all seven animals treated with flurothyl, affecting both sexes, and spontaneous seizure activity was also observed in the control groups. (B) In the P15 group, all eight females and ten males treated with flurothyl exhibited recurrent seizures, with additional seizures noted in 2 of 8 control males, possibly due to acute inflammation from surgery. (C) By P25, the incidence rate of seizures in the experimental group was lower, with unprovoked seizures occurring in 1 of 12 females and 4 of 13 males, while no seizure activity was observed in the control animals of either sex. This data provides a clear overview of seizure incidence across different age groups and treatment conditions.

Individual numbers of seizures among age groups

P12 group.

Individual numbers of seizures were measured for each animal group at postnatal day P12 (Figure 10). In P12 group, One-Way ANOVA results following by Sidak post-hoc test indicates significant differences between the control and flurothyl-treated animals ($F_{3,25}$=11.85, p<.001). Specifically, the control females had an average seizure incidence of 0.25 ± 0.71 compared to 15.43 ± 11.94 in the flurothyl-treated females (p<.001). Similarly, control males had a mean number of seizures of 0.14 ± 0.38, whereas flurothyl-treated males had an incidence of 13.14 ± 5.21 (p<.01). There were no significant differences in seizure incidence between flurothyl-treated females and males (p>.05). This data highlights the pronounced effect of flurothyl treatment on seizure incidence in both sexes compared to the control groups.

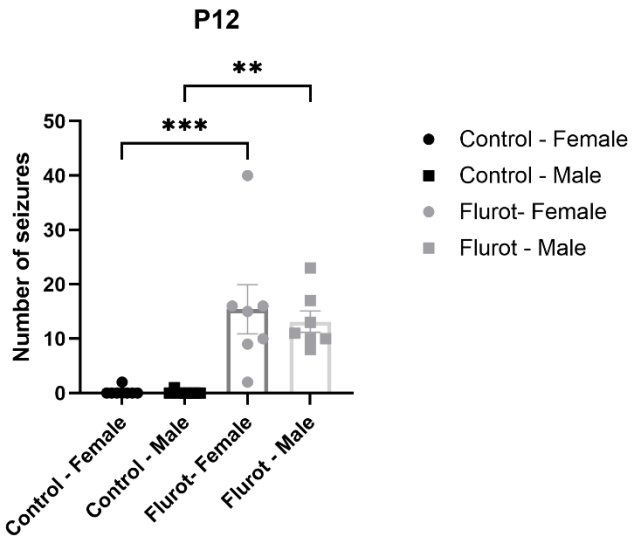

*Figure 10. The graph illustrates the individual numbers of seizures for each animal group at postnatal day P12 (Mean ± SD). There were significant differences between the control and flurothyl-treated animals. For females, the mean seizure number was 0.25 ± 0.71 for control females vs 15.43 ± 11.94 for flurothyl-treated females (***p<.001). Similarly, control males had a mean seizure number of 0.14 ± 0.38, whereas flurothyl-treated males had a mean value of 13.14 ± 5.21 (**p<.01). There was no significant difference in the incidence of seizures between flurothyl-treated females and males (p>.05).*

P15 group.

As indicated by One-Way ANOVA test the differences between the control and flurothyl-treated animals are significant ($F_{3,32}$=31.63, p<.001). Interestingly, males treated with flurothyl had a higher average seizure frequency with the mean number of the seizures of 17.90 ± 6.49 per animal versus females treated with flurothyl that only had 10.38 ± 6.95 (p<.01). This would suggest that male animals treated with flurothyl have a greater susceptibility to seizures than females as well as the control groups that displayed little seizure activity (Figure 11).

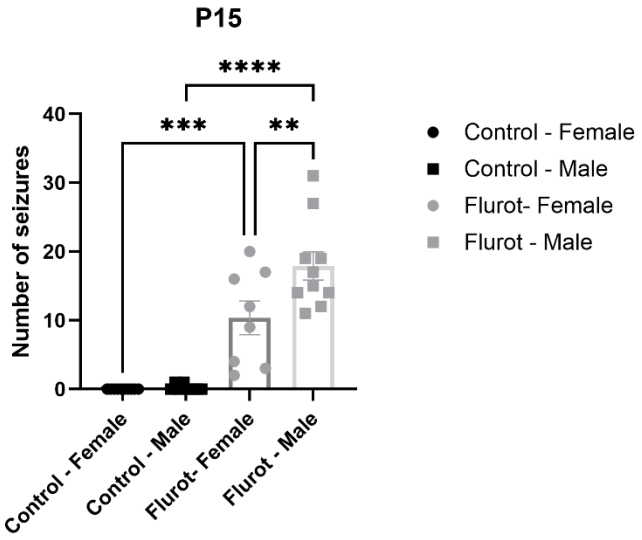

*Figure 11. The graph illustrates the individual numbers of seizures for each animal group at postnatal day P15 (Mean ± SD). Significant differences were detected between the control and flurothyl-treated groups for both sexes (*** p<.001). Notably, the flurothyl-treated males exhibited a higher mean seizure occurrence, with an average of 17.90 ± 6.49, compared to 10.38 ± 6.95 in flurothyl-treated females (** p<.01).*

P25 group.

In P25 group, no statistical significance was found for all animal groups tested in this cohort ($F_{3,34}$=1.48, p>.05). While flurothyl-treated males did experience a slightly higher incidence than flurothyl-treated females, with a final mean seizure amount of 0.46 ± 0.78 for males versus 0.17 ± 0.58 for females, these differences were not significant (p>.05). This indicates that by P25, the impact of flurothyl on seizure incidence has diminished, with overall seizure activity in treated groups not significantly different from that in control groups (Figure 12). This suggests that as development progresses, animals may exhibit reduced susceptibility to seizures.

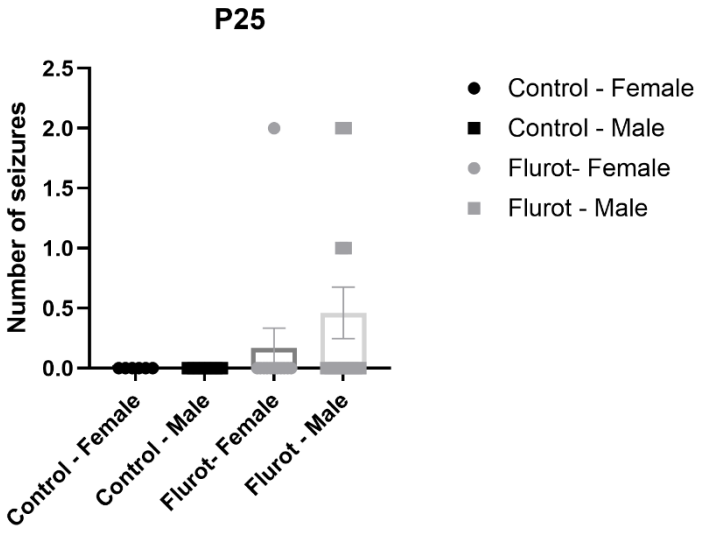

Figure 12. The graph illustrates the individual numbers of seizures for each animal group at postnatal day P25 (Mean ± SD). Flurothyl-treated males exhibited a slightly higher seizure incidence with a mean of 0.46 ± 0.78 compared to 0.17 ± 0.58 for flurothyl-treated females. However, these differences were not significant (p>.05). All control groups did not show any presence of the seizure activity, but no significance between control groups versus flurothyl-treated groups was observed.

Average seizure duration among age groups

The average duration of detected seizures for each animal at various postnatal stages, measured in seconds summarized in Figure 13.

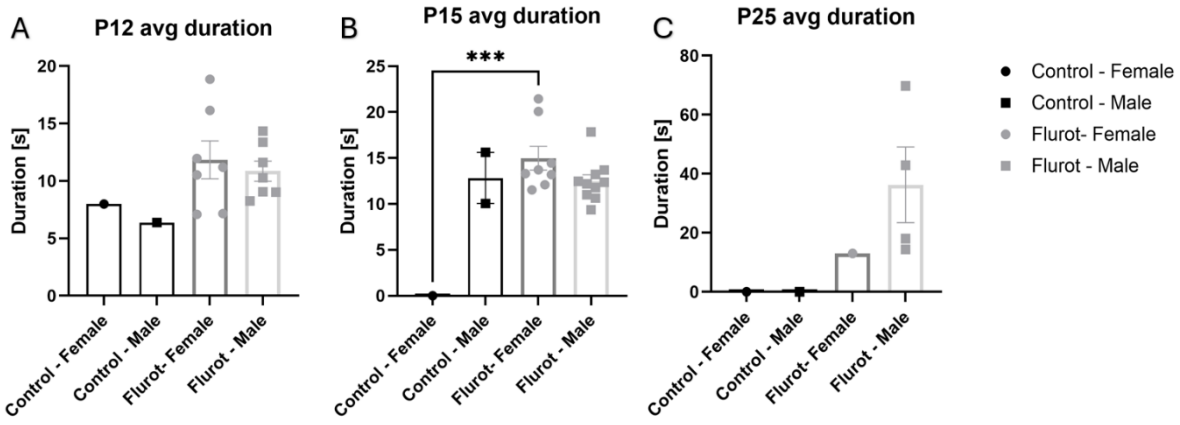

*Figure 13. The graphs illustrate the average seizure duration for each animal at P12, P15, and P25 postnatal days, measured in seconds, [s]. Data are presented as mean and. (A) At P12, there were no significant differences among the animal groups. Control-females averaged 7.99 ± 0.00 vs. 11.84 ± 1.65 for flurothyl-treated females, and 6.3 ± 0.00 for control males vs. 10.85 ± 0.88 for flurothyl-treated males. (B) At P15 group, the only significance was detected between the control group of females averaged 0.00 seconds compared to 14.98 ± 1.31 for flurothyl-treated females (*p<.05). No significant differences were found between control males and flurothyl-treated males (12.83 ± 2.78 vs. 12.46 ± 0.72; p>.05). (C) At P25, flurothyl-treated males showed a noticeable increase in seizure duration compared to flurothyl-treated females (36.26 ± 12.84 vs 13.02 ± 0.00), but it did not reach statistical significance (p>.05).*

P12 group.

There was no significant difference between the groups, that was tested by One-Way ANOVA following by Sidak post-hoc ($F_{3,12}$=0.95, p>.05). Even the control animals, which did not receive flurothyl inhalation, had similar average durations of spontaneous electrographic seizures. The mean duration was 7.99 ± 0.00 for control females versus 11.84 ± 1.65 for flurothyl-treated females, and 6.3 ± 0.00 for control males versus 10.85 ± 0.88 for flurothyl-treated males.

P15 group.

In this group, a significant difference was found between the control and flurothyl-treated animals ($F_{3,17}$=7.27, p<.01). A notable difference was observed between control females and flurothyl-treated females (0.00 seconds versus 14.98 ± 1.31, respectively). No significant differences were found between the male groups (12.83 ± 2.78 for control versus 12.46 ± 0.72 for flurothyl-treated) or between the treated groups of both sexes (p>.05).

P25 group.

There was no significant difference between the treated and non-treated groups ($F_{3,3}$=0.94, p>.05). Although there was an apparent increase in seizure duration in flurothyl-treated males. However, this increase did not reach statistical significance (p>.05).

Individual numbers of events among age groups

P12 group.

Individual numbers of events were measured for each animal group at postnatal day P12 (Figure 14). Significant differences were detected only between control groups and treated groups of both sexes ($F_{3,25}$=10.28, p<.001). Control females had decreased mean number of events of 18.25 ± 3.45 compared to flurothyl-treated females (35.57 ± 6.74), with a significance of p<.05. Similarly, control males had a mean number of events of 14.14 ± 1.96 versus the flurothyl male group of 42.29 ± 3.24 (p<.001). There was no significant difference between flurothyl-treated female and male groups (p>.05). These data indicate a pronounced increase in the numbers of events due to the application of flurothyl in both sexes compared to control groups.

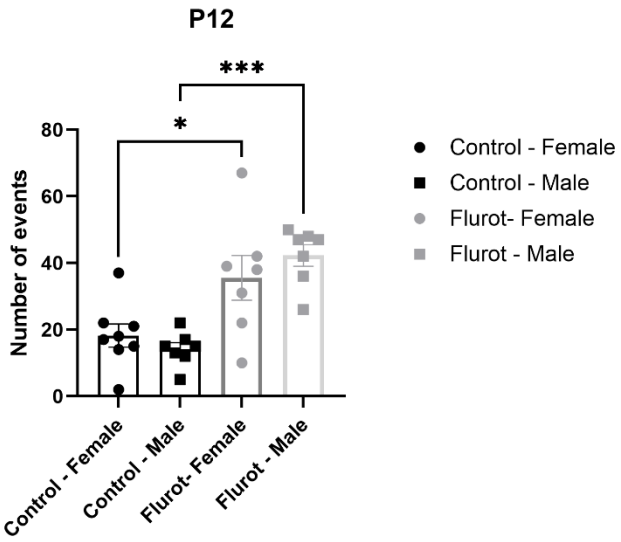

*Figure 14. The graph illustrates the individual numbers of events for each animal group at postnatal day P12. Data are presented as mean ± SEM. Significant differences were detected between the control and flurothyl-treated groups for both sexes. Control females had a mean number of events of 18.25 ± 3.45, significantly lower than the 35.57 ± 6.74 observed in flurothyl-treated females (*p<.05). Similarly, control males had a mean number of events of 14.14 ± 1.96, while flurothyl-treated males showed a mean of 42.29 ± 3.24 (***p<.001). There was no significant difference in the number of events between flurothyl-treated females and males (p>.05).*

P15 group.

In P15 group, there was a significant difference between the treated and non-treated groups ($F_{3,32}$=17.02, p<.001). Interestingly, the occurrence of events in the female groups was quite similar (18.70 ± 2.50 for non-treated females versus 26.38 ± 3.19 for flurothyl-treated females; p>.05). However, a significant difference was observed in the male groups (13.88 ± 3.01 for non-treated males versus 42.30 ± 3.49 for flurothyl-treated males; p<.001), as well as between flurothyl-treated females and males, with a significance of p<.01. These data indicate, that flurothyl treatment significantly increases the event number in male group more than in females (Figure 15).

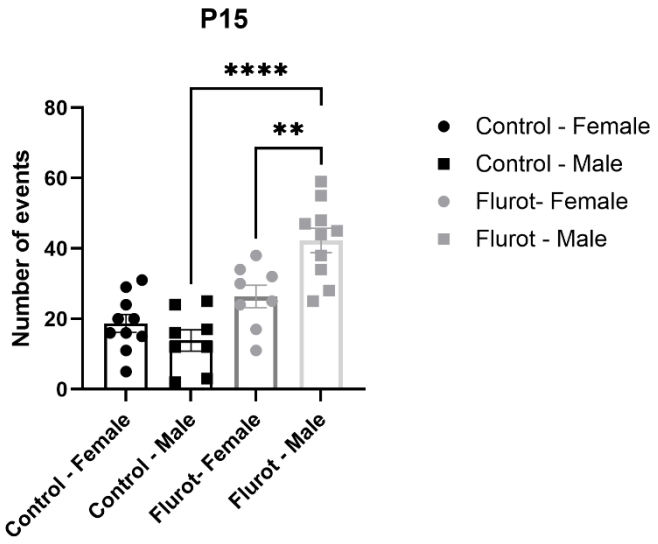

*Figure 15. The graph illustrates the individual numbers of events for each animal group at postnatal day P15. Data are presented as mean ± SEM. Control females had 18.70 ± 2.50 the mean number of events, while flurothyl-treated females had 26.38 ± 3.19 the mean number of events (p>.05). Non-treated males had 13.88 ± 3.01 the mean number of events, significantly fewer than the 42.30 ± 3.49 in flurothyl-treated males (***p<.001). There was also a significant difference between flurothyl-treated females and males (**p<.01).*

P25 group.

The graph below depicts the numbers of individual events for the animals in each group at postnatal day P25 (Figure 16). In this group, a significant difference was found between the control and flurothyl-treated animals ($F_{3,34}$=14.22, p<.001). None of the animal in control group of both sexes exhibited events during the first 4 hours of EEG recording. However, a significant difference was detected in the groups, which were treated by flurothyl inhalation (p<.05). The mean number of events for treated females was 3.08 ± 0.56, whereas the mean number of events for treated males was slightly higher (5.92 ± 0.95), indicating an increased event rate in treated males.

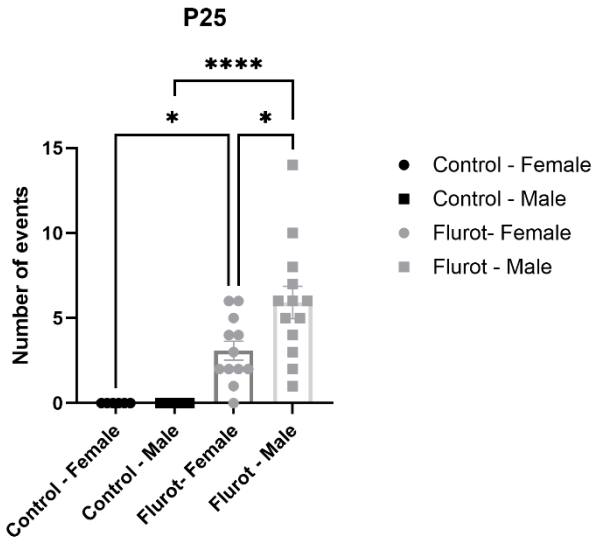

*Figure 16. The graph illustrates the individual numbers of events for each animal group at postnatal day P25. Data are presented as mean ± SEM. Control groups had no events in the first 4 hours of EEG recording. Flurothyl-treated groups showed a significant difference (*p<.05): treated females had 3.08 ± 0.56 the mean number of events, and treated males had 5.92 ± 0.95 for the same value.*

Spectral analysis

Power Spectral Density analysis for all age groups was evaluated for 3.5 hours in P12 animals, 4-hour period for P15, and ~16-hour period for the P25 group. However, the representation of relative PSD for 15-minute intervals in the P25 group was shortened to the first four hours.

Electrographic seizures were always represented in both channels (either in Frontal and Parietal Cortical regions). Considering the negligible differences, on the order of hundreds of milliseconds, between the start and end of the seizure in Ch1 and Ch2, we describe the results only for the Frontal Cortex (except total relative PSD for the general view).

Total relative Power Spectral Density

Initially, we measured the total relative PSD of five bands (delta, theta, alpha, beta, gamma) for each animal groups to provide a general overview. The relative PSD is presented as ($\mu V^2/Hz$) and calculated for both channel: channel 1 over the sensorimotor cortex, channel 2 over the parietal cortex. For the females and males, the values in the PSD for both the control and flurothyl-treated groups are compared in the frontal and parietal cortices separately. The box plots (Figure 17) show a mean of the relative PSD and whiskers from min to max.

P12 group.

Our data suggest that for the frontal (Figure 17A), there are no significant differences in relative PSD between treated and non-treated groups of both sexes ($F_{3,13}$=2.04, p>.05), including flurothyl-treated females and males, in any of the frequency bands ($F_{4,13}$=4.99, p>.05). For the parietal (Figure 17B) there is no statistical significance for treatment groups ($F_{3,13}$=1.30, p>.05) nor EEG bands ($F_{4,13}$=1.18, p>.05).

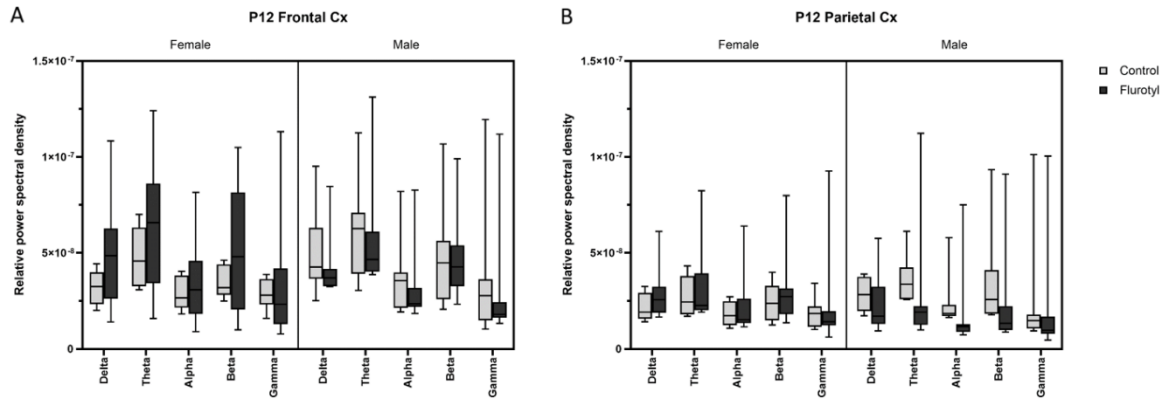

Figure 17. The graphs illustrate total relative PSD, $\mu V^2/Hz$, of the frequency bands (delta, theta, alpha, beta, gamma) for each animal group at postnatal day P12. Data are presented in boxes and whiskers from min to max. (A) Frontal Cortex: No significant differences in PSD between control and flurothyl-treated females for all frequency bands (p>.05). Similarly, no significant differences are detected in PSD between control and flurothyl-treated males (p>.05). The PSD did not show any significant differences between the flurothyl-treated female and male groups in any of the frequency bands (p>.05). (B) Parietal Cortex: No significant differences in PSD values between control and flurothyl-treated females (p>.05). No significant differences are found in PSD between control and flurothyl-treated males (p>.05). There were no significant differences in PSD between flurothyl-treated females and males across all frequency bands (p>.05). Abbreviation: cx – cortex.

P15 group.

We detected no significant differences in the frontal cortex for treatment groups ($F_{3,16}$=2.54, p>.05), whereas EEG bands comparisons were significant ($F_{4,16}$=30.72, p<.001) (Figure 18A). However, in the parietal cortex both sources of variations were significant ($F_{3,16}$=4.06, p<.01 for treatment groups and ($F_{4,16}$=22.29, p<.001 for EEG bands). In the group of flurothyl-treated females there was significant reduction in theta band in comparison to control females at P15 (Figure 18B). A significant difference in the theta band is noted with a value of 8.895 ± 1.12 PSD in control females compared to a value of 5.484 ± 6.85 in flurothyl-treated females (p<.01). No other significant differences are found among the groups, including the flurothyl-treated females versus males.

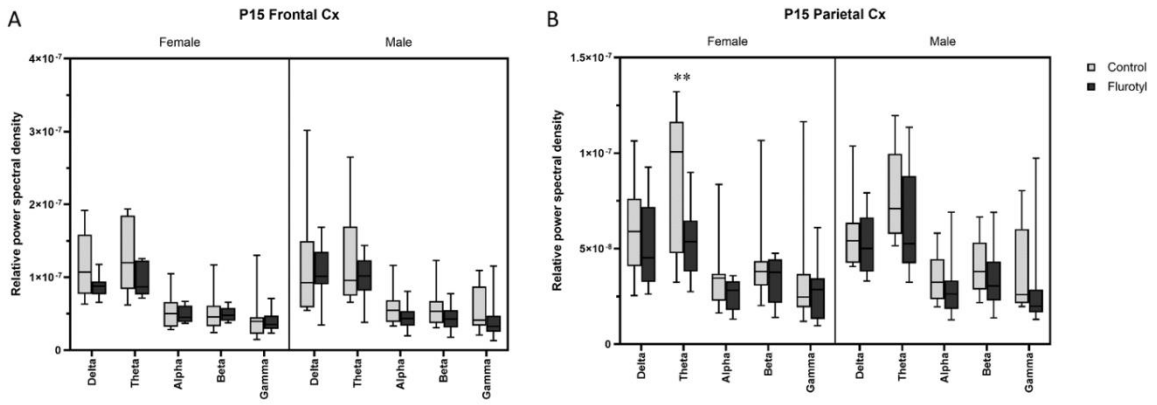

*Figure 18. The graphs illustrate total relative PSD, $\mu V^2/Hz$, of the frequency bands (delta, theta, alpha, beta, gamma) for each animal group at postnatal day P15. Data are presented in boxes and whiskers from min to max. (A) Frontal Cortex: No significant differences in PSD between control and flurothyl-treated females for all frequency bands (p>.05). Similarly, no significant differences are detected in PSD between control and flurothyl-treated males (p>.05). The PSD did not show any significant differences between the flurothyl-treated female and male groups in any of the frequency bands (p>.05). (B) Parietal Cortex: A significant difference in the theta band is noted with a value of 8.895 ± 1.12 PSD in control females compared to a value of 5.484 ± 6.85 in flurothyl-treated females (**p<.01). There are no significant differences between the rest of the frequency bands. No significant differences are found in PSD between control and flurothyl-treated males (p>.05). There were no significant differences in PSD between flurothyl-treated females and males across all frequency bands (p>.05). Abbreviation: cx – cortex.*

P25 group.

We did not detect any significant differences among groups at postnatal group P25 in either the frontal (Figure 19A) or parietal cortices (Figure 19B), nor between the control and flurothyl-treated groups ($F_{3,195}$=2.44, p>.05 for the frontal cortex and $F_{3,195}$=1.12, p>.05 for the parietal). The EEG bands` ratios were significant in both cortices ($F_{4,195}$=20.60, p<.001 for the frontal cortex and $F_{4,195}$=25.52, p<.001 for the parietal). However, in the frontal cortex, the only significant difference is between control females and control males in the theta band PSD (1.470 ± 5.34 for control females compared to 2.780 ± 2.29 in control males, with the significance of p<.05).

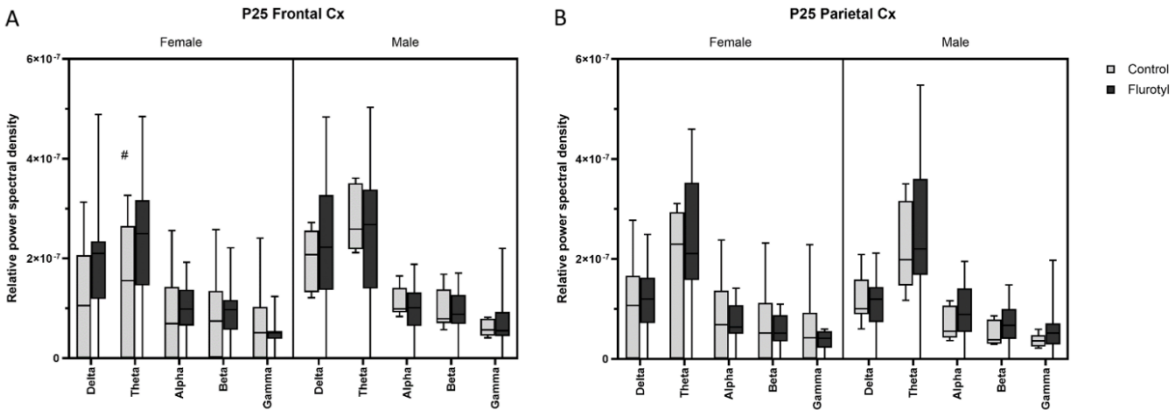

*Figure 19. The graphs illustrate total relative PSD, $\mu V^2/Hz$, of the frequency bands (delta, theta, alpha, beta, gamma) for each animal group at postnatal day P25. Data are presented in boxes and whiskers from min to max. (A) In the frontal cortex, significant differences in PSD between control and flurothyl-treated females were not observed for all the frequency bands (p>.05). There was the same result in PSD between control and flurothyl-treated males for all frequency bands (p>.05). No significant differences between the studied parameters of PSD were also found between flurothyl-treated female and male groups in any of the frequency bands (p>.05). However, the only significant difference is between control females and control males in the theta band PSD (1.470 ± 5.34 for control females compared to 2.780 ± 2.29 in control males, with the significance of *p<.05) (B)* Parietal Cortex: *No significant differences in the values of PSD between control and flurothyl-treated females are revealed (p>.05). ANOVA results revealed no significant differences in PSD between control and flurothyl-treated males (p>.05). There was no difference in PSD found to be significant for females compared to males at any frequency band for the flurothyl-treated group (p>.05). Abbreviation: cx – cortex.*

Relative Power Spectral Density based on 15-minute intervals

P12 group.

In addition, detailed analysis using 15-minute time bin intervals was performed (Figure 20). For the delta band, Two-way ANOVA followed by Tukey`s post-hoc test indicated significant differences in both factors, treatment and time ($F_{3,350}$=29.49 p<.001 and $F_{13,350}$=7.58 p<.001, respectively). Details of the post-hoc test for each time interval and each frequency band are described in the tables in **Annex A.**

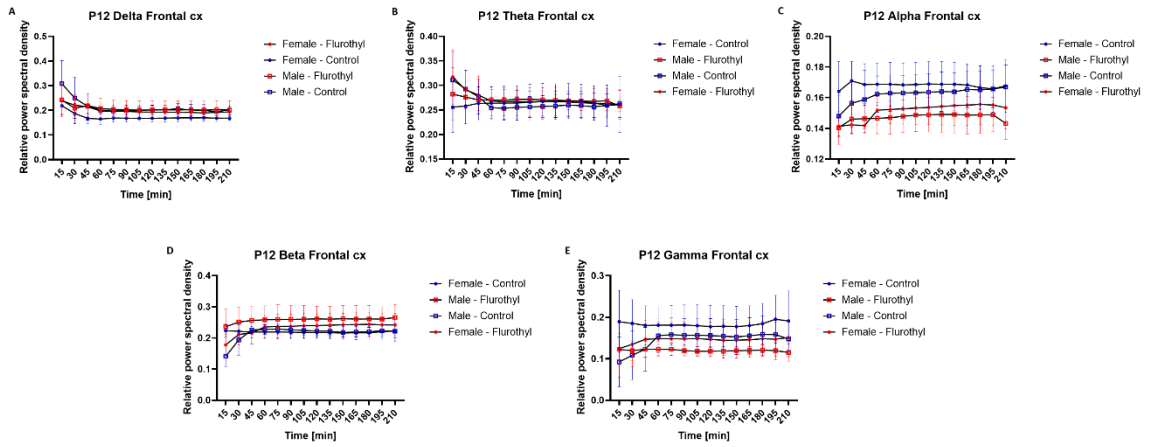

Figure 20. The graphs illustrate relative PSD, $\mu V^2/Hz$, based on 15-minute intervals of the specific frequency bands in Channel 1 over the frontal cortex for the P12 group. Each point is presented as mean ± SD. Round marks represent females, and squares denote males. Blue indicates control groups, and red indicates flurothyl-treated groups. ***(A) Delta band (1-4 Hz).*** *Two-way ANOVA indicated significant differences for both treatment ($F_{3,350}$=29.49, p<.001) and time ($F_{13,350}$=7.58, p<.001).* ***(B) Theta band (4.5-8 Hz).*** *Significant for time ($F_{13,346}$=1.94, p<.05), but not for treatment ($F_{3,346}$=1.64, p>.05).* ***(C) Alpha band (8.5-13 Hz).*** *Significant for treatment ($F_{3,346}$=58.71, p<.001), but not for time ($F_{13,346}$=1.68, p>.05).* ***(D) Beta band (13.5-24 Hz).*** *Significant for both treatment ($F_{3,346}$=33.90, p<.001) and time ($F_{13,346}$=3.50, p<.001).* ***(E) Gamma band (24.5-70 Hz).*** *Significant for treatment ($F_{3,346}$=37.04, p<.001), but not for time ($F_{13,346}$=0.63, p>.05). Abbreviation: cx – cortex.*

P15 group.

For this group, Two-Way ANOVA test followed by Tukey`s post-hoc test had been also performed (Figure 21). In the delta frequency band, significant differences were identified for both treatment and time factors ($F_{3,502}$=11.59 p<.001 and $F_{15,502}$=11.25 p<.001, respectively). Details of the post-hoc test for each time interval and each frequency band are described in **Annex B.**

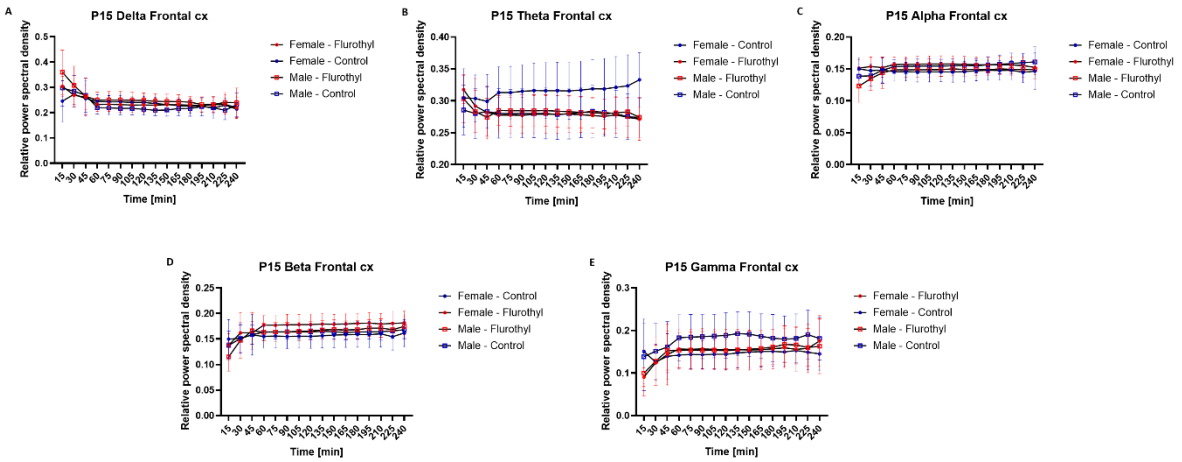

Figure 21. The graphs illustrate relative PSD, $\mu V^2/Hz$, based on 15-minute intervals of the specific frequency bands in Channel 1 over the frontal cortex for the P15 group. Each point is presented as mean ± SD. Round marks represent females, and squares

denote males. Blue indicates control groups, and red indicates flurothyl-treated groups. ***(A) Delta band (1-4 Hz).*** *Two-way ANOVA indicated significant differences for both treatment ($F_{3,502}$=11.59, p<.001) and time ($F_{15,502}$=11.25, p<.001).* ***(B) Theta band (4.5-8 Hz).*** *Significant for treatment ($F_{3,502}$=33.47, p<.001), but not for time ($F_{15,502}$=0.37, p>.05).* ***(C) Alpha band (8.5-13 Hz).*** *Significant for both treatment ($F_{3,502}$=15.50, p<.001) and time ($F_{15,502}$=2.04, p<.01).* ***(D) Beta band (13.5-24 Hz).*** *Significant for both treatment ($F_{3,502}$=15.37, p<.001) and time ($F_{15,502}$=5.23, p<.001).* ***(E) Gamma band (24.5-70 Hz).*** *Significant for both treatment ($F_{3,502}$=13.14, p<.001) and time ($F_{15,502}$=2.507, p<.01). Abbreviation: cx – cortex.*

P25 group.

In P25 group (Figure 22), for the delta band, Two-way ANOVA followed by Tukey`s post-hoc test indicated significant differences for both treatment and time factors ($F_{3,544}$=62.62 p<.001 and $F_{13,544}$=7.53 p<.001, respectively). Details of the post-hoc test for each time interval and each frequency band are described in **Annex C.**

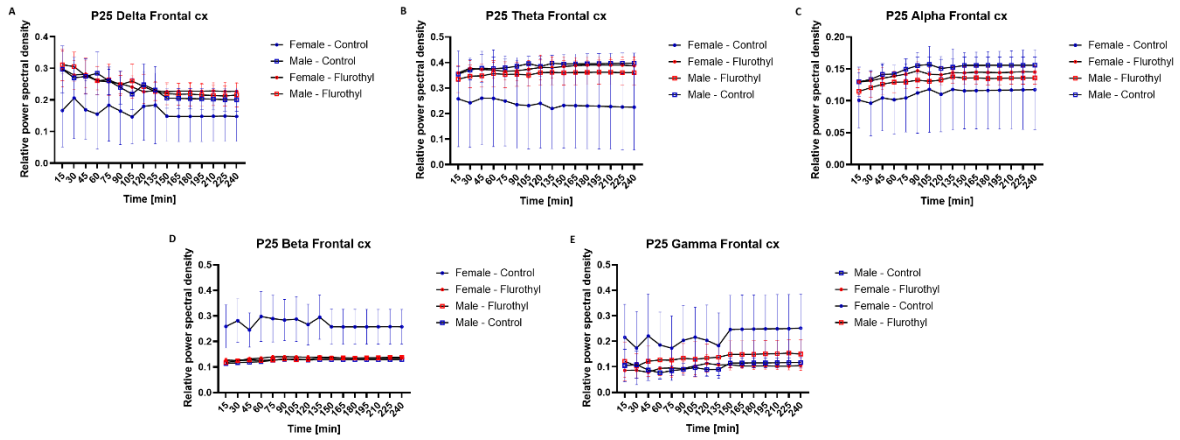

Figure 22. The graphs illustrate relative PSD, μV^2/Hz, based on 15-minute intervals of the specific frequency bands in Channel 1 over the frontal cortex for the P25 group. Each point is presented as mean ± SD. Round marks represent females, and squares represent males. Blue indicates control groups, and red indicates flurothyl-treated groups. ***(A) Delta band (1-4 Hz).*** *Two-way ANOVA indicated significant differences for both treatment ($F_{3,544}$=62.62, p<.001) and time ($F_{13,544}$=7.53, p<.001).* ***(B) Theta band (4.5-8 Hz).*** *Significant for treatment ($F_{3,544}$=88.03, p<.001), but not for time ($F_{15,544}$=0.13, p>.05).* ***(C) Alpha band (8.5-13 Hz).*** *Significant for both treatment ($F_{3,544}$=47.23, p<.001) and time ($F_{15,544}$=2.38, p<.01).* ***(D) Beta band (13.5-24 Hz).*** *Significant for treatment ($F_{3,544}$=90.03, p<.001), but not for time ($F_{15,544}$=0.18, p>.05).* ***(E) Gamma band (24.5-70 Hz).*** *Significant for both treatment ($F_{3,544}$=84.02, p<.001) and time ($F_{15,544}$=1.77, p<.05). Abbreviation: cx – cortex.*

Alpha/theta ratio changes

Our results demonstrated age and sex-specific patterns in the Alpha to Theta ratio (ATR) in controls see Figure 23-25, furthermore the treatment factor had a specific age pattern too. In general, the flurothyl-

treated animals had lower ATR than controls except for females in the P15 group. See more detailed descriptions in the individual legend for figures.

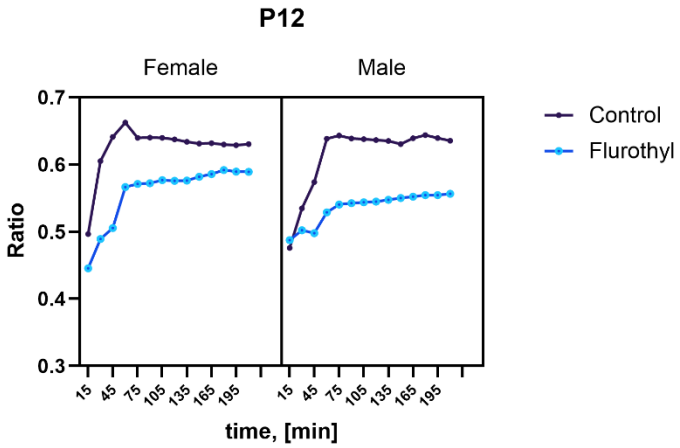

*Figure 23. Changes of Alpha to Theta ratio in 15-minute intervals in P12 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,39}$=73.65, *** p<.001). Specifically, flurothyl-treated females showed decreased ATR mean compared to control female group (0.56 ± 0.05 vs 0.62 ±0.04, respectively with ***p<.001). Similarly for the male group, with the mean of 0.54 ±0.02 for flurothyl-treated versus 0.61±0.05 for non-treated (***p<.001). A significant difference was detected between flurothyl-treated animals of both sexes (*p<.05).*

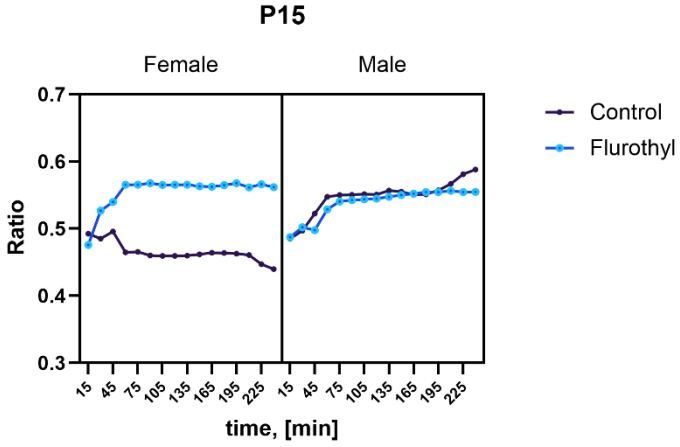

*Figure 24. Changes of Alpha to Theta ratio in 15-minute intervals in P15 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,45}$=69.95, ***p<.001). Flurothyl females had significantly increased ATR mean of 0.55±0.02 compared to controls with a mean of 0.46±0.01 (***p<.001), whereas no significance was detected between flurothyl-treated and non-treated males (0.54±0.02 vs. 0.55±0.03, p>.05). Moreover, there is no significant difference between flurothyl groups of both sexes (p>.05).*

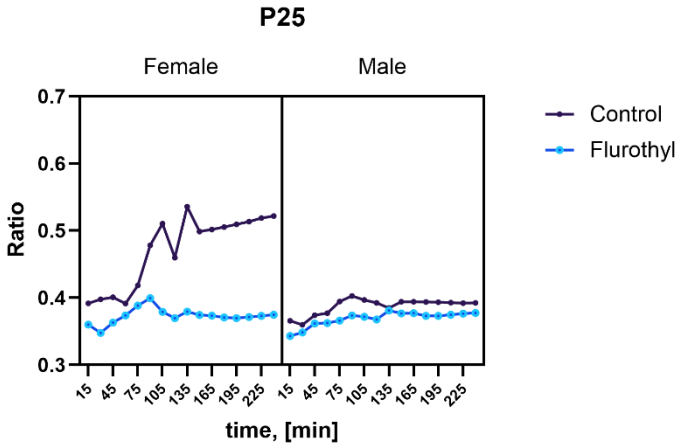

*Figure 25. Changes of Alpha to Theta ratio in 15-minute intervals in P25 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,45}$=66.95, ***p<.001). Flurothyl females had significantly decreased ATR mean of 0.37±0.01 compared to controls with a mean of 0.47±0.05 (***p<.001). In contrast, no significance was detected between flurothyl-treated and non-treated males (0.37±0.01 vs. 0.39±0.01, p>.05). Moreover, there is no significant difference between flurothyl groups of both sexes (p>.05).*

Gamma/theta ratio changes

Our results demonstrated age and sex-specific patterns in the Gamma to Theta ratio (GTR) in controls see Figure 26-28, furthermore the treatment factor had a specific age pattern too. In general, the flurothyl-treated animals had lower GTR than controls except for females in the P15 group and for males in the P25. See more detailed descriptions in the individual legend for figures.

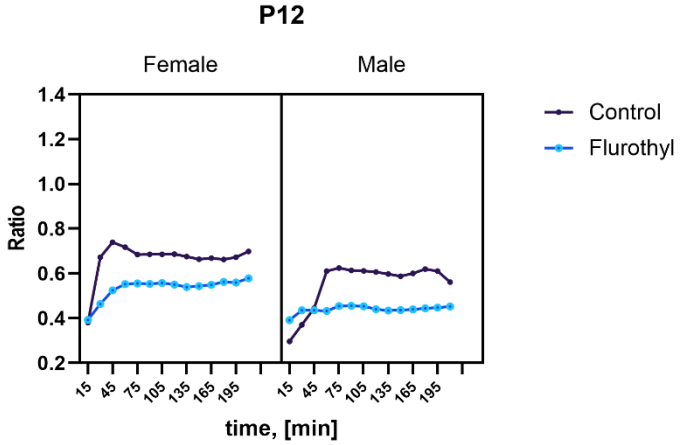

Figure 26. Changes of Gamma to Theta ratio in 15-minute intervals in P12 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test

*followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,39}$=49.63, ***p<.001). In addition, flurothyl-treated females had decreased GTR mean compared to the control female group (0.53 ± 0.05 vs 0.66 ±0.08, respectively with ***p<.001). Similarly for the male group, with the mean of 0.44 ±0.02 for flurothyl-treated versus 0.55±0.10 for non-treated (***p<.001). A significant difference was detected between flurothyl-treated animals of both sexes (***p<.001).*

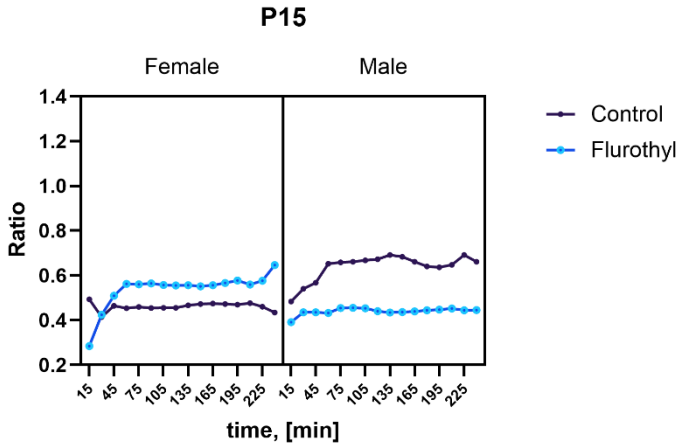

*Figure 27. Changes of Gamma to Theta ratio in 15-minute intervals in P15 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,45}$=331.1, ***p<.001). We detected a significantly increased GTR mean in flurothyl-treated females (0.54±0.08) compared to non-treated females (0.46±0.02), ***p<.001. In contrast for the male group, the GTR mean was decreased in flurothyl-treated animals compared to controls (0.44 ± 0.02 vs 0.64 ±0.06, respectively with ***p<.001). Moreover, a significant difference was detected between flurothyl-treated animals of both sexes (***p<.001).*

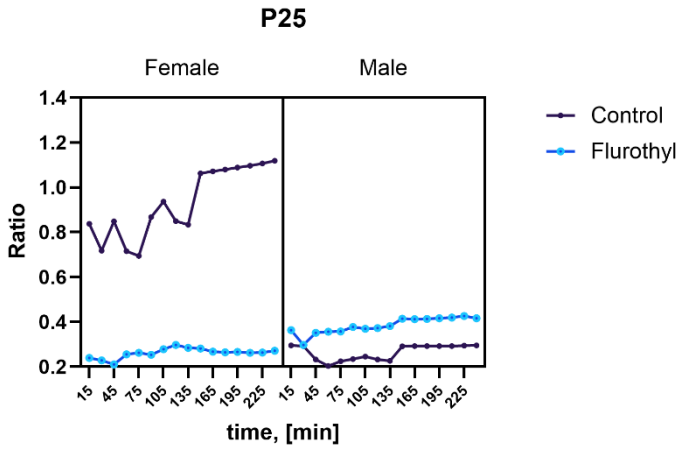

*Figure 28. Changes of Gamma to Theta ratio in 15-minute intervals in P25 group. Data presented as a Mean. Light blue – flurothyl, dark blue – controls, on X – time in minutes, on Y – ratio. As indicated by the results of Two-Way ANOVA test followed by the Tukey`s post-hoc test a significant difference was detected in the treatment factor ($F_{3,45}$=74.82, ***p<.001). There is a significantly decreased GTR mean in flurothyl-treated females (0.26±0.02) compared to non-treated females*

*(0.93±0.16), ***p<.001. In contrast for the male group, the GTR mean was increased in flurothyl-treated animals compared to controls (0.38 ± 0.04 vs 0.27 ±0.03, respectively with ***p<.001). Moreover, a significant difference was detected between flurothyl-treated animals of both sexes (***p<.001).*

Discussion

Seizure detection

This study investigates the development of spontaneous seizure activity during early ontogenesis in both genders. The specific aims of this research were (1) to compare cortical excitability across different age groups using EEG analysis; and (2) to determine whether there is a gender-specific difference in paroxysmal activity during early ontogenetic phases (postnatal days P12, P15, and P25).

This study is the first to focus on EEG screening after flurothyl exposure during the early postnatal days (P6-P10). The primary aim was to identify and describe EEG patterns following this exposure. However, previous researchers were focused not on EEG screening in later stages, but mostly on EEG recording during seizure induction and related behavioural phenomena such as freezing, repetitive myoclonic jerks and swimming movements followed by tonic-clonic seizures with tonic extension of both forelimbs and hindlimbs (Sperber & Moshé, 1988; Velíšek, 2006; Huang et al., 1999). Findings from studies using in vitro voltage-clamp techniques suggested that neonatal seizures evoked by flurothyl treatment induce long-lasting alterations in both CA1 and CA3 neurons (Villeneuve et al., 2000) and neocortical pyramidal neurons of layer 2/3 of the somatosensory cortex (Isaeva et al., 2010), leading to increased excitability and altered inhibitory-excitatory balance in these critical brain regions.

Our observations indicate that all animals within the specified age groups (P12, P15, and P25) exhibited electrographic seizure activity after flurothyl treatment. It implies that there is no silent period during these early stages of life. Similar findings were observed in the mouse model (C57BL/6J and DBA/2J strains), where seizure induction by flurothyl kindling occurred at approximately 7-8 weeks of age (Kadiyala & Ferland, 2017). In contrast with the pilocarpine model of chronic epilepsy (repetitive seizures and Status Epilepticus) where the seizures begin to appear at a mean of 15 days after pilocarpine-induced seizures (Mello et al., 2005). However, comparing these two models is incorrect due to the different mechanisms of action and distinct long-term consequences induced by these treatments. Despite this fact, this observation indicates a rapid progression from the acute phase of seizure induction into a chronic form of epilepsy. Second, the absence of a silent period might reflect different processes underlying mechanisms of neuronal damage, synaptic reorganization, and network excitability. This potentially means that the process by which the brain develops epilepsy is different with flurothyl than with pilocarpine. Clinically, in the future, this could influence the choice of treatment protocols and underscore the need for more frequent monitoring using the EEG.

The presence of seizures in a few control animals can be explained by the effects of acute electrode implantation surgery, which may lead to inflammation and seizure-like electrographic activity. Our

results demonstrate a significantly higher number of animals with seizures in short intervals after the end of flurothyl exposure and fewer animals with seizures in longer intervals.

The higher incidence rate in P12 flurothyl-treated animals can be attributed to the recent flurothyl treatment and increased seizure susceptibility due to GABAergic excitatory transmission. In contrast, the P25 age group, with the maturation of GABA to inhibitory neurotransmission, and lower acute effects of flurothyl, exhibited fewer seizures.

In the P15 group, flurothyl-treated males exhibited a significantly higher number of seizures compared to females. This finding is also consistent with previous research indicating that males have a lower threshold for the development of seizures induced by flurothyl than females, confirming the role of prepubertal and peripubertal increases in testosterone in differential development (Velísek et al., 2006).

The average duration of seizures showed no significant differences between flurothyl-treated males and females. However, when comparing across different age groups, a slight increase in seizure duration was observed. This trend can be attributed to several developmental factors, including neuronal maturation and myelination. The maximal rate of myelin accumulation in rats occurs at about 20 days of age (Downes & Mullins, 2014). This process enhances the speed and coordination of neural transmission, which can affect seizure characteristics.

Paroxysmal events are characterized by brief intense bursts of brain electrical activity abnormalities with the associated disruption in brain function, and non-paroxysmal events may be triggered by external stressors or physiological factors (Mostacci Barbaraand Di Vito, 2019). Interictal events, whether paroxysmal or non-paroxysmal, can be influenced by other factors like surgery or acute stress. In our study, these events were predominantly increased in male subjects, further supporting the hypothesis of greater susceptibility to seizures in males (Velísek et al., 2006).

The Flurothyl model of recurrent generalized seizures mimic neonatal seizures in humans, which do not cause morphological damage but can alter normal development, resulting in synaptic reorganization of the axons and terminals of the mossy fibers of the dentate granule cells, reduction in the number of newly born neurons in the dentate gyrus and hilus, altered GABA subunit composition, and significant long-term cognitive deficits that manifest in later stages of life (Hernan et al., 2013; Huang et al., 1999; Isaeva et al., 2013; Lenck-Santini & Holmes, 2008; Rheims et al., 2008).

This study highlights the importance of frequent EEG screening for children with a history of neonatal seizures. On the one hand, due to the risk of recurrence of spontaneous seizures or even due to non-convulsive seizure occurrence. In addition, regular monitoring can help to detect early signs of refractory epilepsy development. Moreover, it is crucial for developing targeted treatment protocols and improving long-term outcomes for patients with a history of early-life seizures.

Spectral analysis

Power Spectral Density is a crucial calculation in signal processing used to analyse a signal's frequency content. This analysis provides a detailed view of the power present in various frequency bands. The PSD of a signal is estimated using methods such as the Fast Fourier Transform (FFT), which transforms EEG signals from time-based into frequency-based and continues with feature extraction to take characteristics from each filtering signal using the mean of each EEG signal (Matousek & Petersén, 1983) (Novitasari et al., 2019). The result of PSD is a plot displaying power as a function of frequency, usually in units of magnitude-squared of the FFT per Hertz ($\mu V^2/Hz$). In the present study the relative PSD was measured for general normalization and reducing the entropy of the result, which may have been caused by the surgery, the resistance of electrodes and connecting cables, etc. Seizures are characterized by abnormal rhythmical electrical activity in the brain. The PSD is a tool, that helps to identify and quantify this activity by representing the PSD of specific frequency bands. In addition, PSD analysis could be used as a potential biomarker, that precedes seizure onset or even helps in understanding the pathophysiology of epilepsy by revealing how different brain regions interact and synchronize during normal and epileptic states.

In the present study, we measured relative PSD for individual EEG frequency bands (delta, theta, alpha, beta, gamma). Relative PSD was calculated for each 5s window for the whole period of the recording for every single animal. Data were collected from both channels (Frontal and Parietal Cortical regions). Total relative PSD represents the means of each five bands. This analysis was provided to show general dynamics across treatment groups and genders. In the P12 group of the frontal cortex, each relative PSD of five bands in flurothyl-treated females was increased compared to control female group. This observation might mean, that the increased PSD across all frequency bands assumes higher neural excitability and increased electrical activity (Rektor et al., 2011). However, these data were without significance, reflecting a possible requirement for larger animal cohorts or more refined experimental conditions. For the P15 group of the parietal cortex, non-treated females had significantly higher relative PSD of the theta band compared to the flurothyl-treated females. The theta band is associated with normal brain activity related to behaviours such as exploration and REM sleep (Kramis et al., 1975). The significantly lower theta relative PSD in flurothyl-treated females could indicate that flurothyl treatment disrupts normal theta activity, possibly leading to cognitive and behavioural changes. This could be in confirming that recurrent seizures elicited with flurothyl during early development caused a highly significant long-term detrimental effect on cognitive function (Lenck-Santini & Holmes, 2008). Rats exposed to recurrent flurothyl seizures in the neonatal period took more time in the Morris Water Maze, which is a hippocampal-dependent spatial memory test, in comparison with controls. For the P25 group of the frontal cortex, flurothyl-treated animals of both sexes had slightly increased (however, without significance) theta relative PSD in comparison with control animals. Moreover, a significant

difference was detected between non-treated males and females, whereas such a difference was absent between flurothyl-exposed groups of both sexes. The absence of a significant difference between flurothyl-treated males and females suggests that flurothyl treatment might have a levelling effect, reducing the natural sex-related differences observed in the control group. However, further research is essential, accompanied by the need for a larger cohort size, to enhance the validity and reliability of the findings.

To observe time-dependent dynamic changes in the relative PSD of each band, we applied a 15-minute interval analysis. We detected high activity in each frequency band across different treatment and even age groups during approximately the first hour. This activity could be potentially caused by diethyl ether anaesthetic exposure, acute surgery, unfamiliar place with new conditions, and maternal separation effect (Gramsbergen, 1976; Nagamura & Iwahara, 1968). The same tendency was observed in animals of P12 and P15 groups in the last 30-40 minutes of the 4 hours EEG monitoring. This is presumably associated with maternal craving, and physiological need for food.

In the P12 group`s frontal cortex, flurothyl treatment affects the relative PSD differently across various frequency bands, with notable changes in the delta and beta bands over time. For the P15 group, delta band was significantly increased in flurothyl-treated animals during the first time interval, which might reflect an acute response, characterized by an increase in a slow-way activity. The same observation was detected in P12 males and P25 females with flurothyl treatment. This could be caused by neuronal inhibition by diethyl ether or longer recovery processes due to acute surgery. However, over time the delta activity decreases, normalizing towards the levels seen in control animals.

In addition to the above, ratios alpha / theta, and gamma / theta were calculated for the frontal cerebral cortex to observe a time-depended dynamic of power ratio changes. A common analysis measure for neuro-electrophysiological recordings is to compute the power ratio between two frequency bands. Band ratio measures are often used in EEG studies investigating possible physiological correlates (Matousek & Petersén, 1983), cognitive development and aging (Clarke et al., 2001), reward processing (Schutter & Van Honk, 2005), and affect (Putman et al., 2010).

One of the most consistent lines of research in this area focuses on the theta / beta ratio as a potential biomarker for executive function, and in particular attentional processing (Angelidis et al., 2016; Gordon et al., 2018). Other work using EEG experiments have explored ratio measures in learning and memory using the theta / beta ratio (Trammell et al., 2017), and memory impairment using the theta / gamma ratio (Moretti et al., 2009). Similar work in animals has investigated the theta / delta ratio in hippocampal recordings during associative learning paradigms in rabbits (Nokia et al., 2008) and rats (Kim et al., 2016). In addition, changes in theta / alpha ratio are likely predictive of cognitive impairment (Klimesch, 1999) in old adults, but this relationship may depend upon age. Similarly, the theta/gamma ratio was investigated as early marker of cognitive decline (Moretti et al., 2009).

In the context of epilepsy, the band ratio measures in quantitative EEG analysis are noted too. Existing studies investigated band ratios in mesial temporal lobe epilepsy in adults, comparing interictal abnormalities between patients with temporal lobe epilepsy and control group. Higher spectral power in the delta and theta frequency bands with significantly lower alpha-theta ratios was found in the affected hemisphere in patients, especially during sleep (Bonacci et al., 2024; Fonseca et al., 2022; Pellegrino et al., 2017).

In our research we observed age and sex-specific patterns in the alpha / theta ratio in controls and flurothyl treated animals. Particularly, flurothyl induced repeated generalized seizures results in lower alpha / theta ratio in comparison with controls except P15 flurothyl females. Likewise, age and sex-specific patterns in the gamma / theta ratio was identified. Furthermore, the flurothyl-treated animals had lower gamma / theta ratio in comparison with controls except P15 flurothyl females and P25 flurothyl males. Both findings bring new insights into a recognition of epileptogenesis process during early brain development. Our results show the possibility of effectively using these EEG biomarkers due to their age and sex specify.

Conclusion

This study is the first to present insight into the process of development and progression of chronic epilepsy following flurothyl-induced seizures at early postnatal stages. Using EEG recording and its spectral analysis with PSD of different frequency bands (delta, theta, alpha, beta, gamma), we observed significant electrographic seizure activity in the early postnatal days (P12, P15, P25) with no silent phase. This finding suggests a rapid transition from acute to chronic epilepsy, implying different mechanisms involved in epileptogenesis compared to other epilepsy models. Higher incidence in seizure occurrence in P12 flurothyl-treated animals highlights increased seizure susceptibility due to GABAergic excitatory neurotransmission. In contrast, the P25 age group, with the maturation of inhibitory GABA transmission, showed fewer seizures. Gender-dependent differences were also found to be present. Males exhibited more seizures compared to females; this finding agreed with previous studies that associate testosterone to be associated with lower seizure thresholds. Spectral analysis indicated higher PSD values in flurothyl-treated females, especially in the P12 group, which was an indication of heightened neural excitability; this disrupted normal theta activity seen in P15 flurothyl-treated females, which suggests the potential impact of flurothyl treatment on cognitive function and neural activity. In addition, dynamic changes in relative PSD over time were observed, which were influenced by several factors such as anaesthesia and maternal separation in the first and last hours of EEG recording. Flurothyl-induced repeated generalized seizures resulted in lower alpha / theta ratio in comparison with controls except P15 flurothyl females. Likewise, age and sex-specific patterns in the gamma / theta ratio was identified. Furthermore, the flurothyl-treated animals had lower gamma / theta ratio in comparison with controls except P15 flurothyl females and P25 flurothyl males.

Given the challenges associated with frequent EEG monitoring in clinical practice for children with a history of neonatal seizures, this study explores the potential of using EEG biomarkers specific to age and sex. Moreover, identifying such patterns in EEG could serve as a useful tool in understanding the effects of antiseizure medications and offer new potential biomarkers for pharmacological treatment of patients with epilepsy. Quantitative EEG screening with targeted treatment protocols may improve long-term outcomes, particularly in relation to developmental delays and cognitive impairments.

Further studies are needed to confirm these findings and clarify the underlying mechanisms.

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Legend to tables

Numbers from 15 till 210/240 represent time interval in minutes.

* – significance level between flurothyl-treated and non-treated animals;

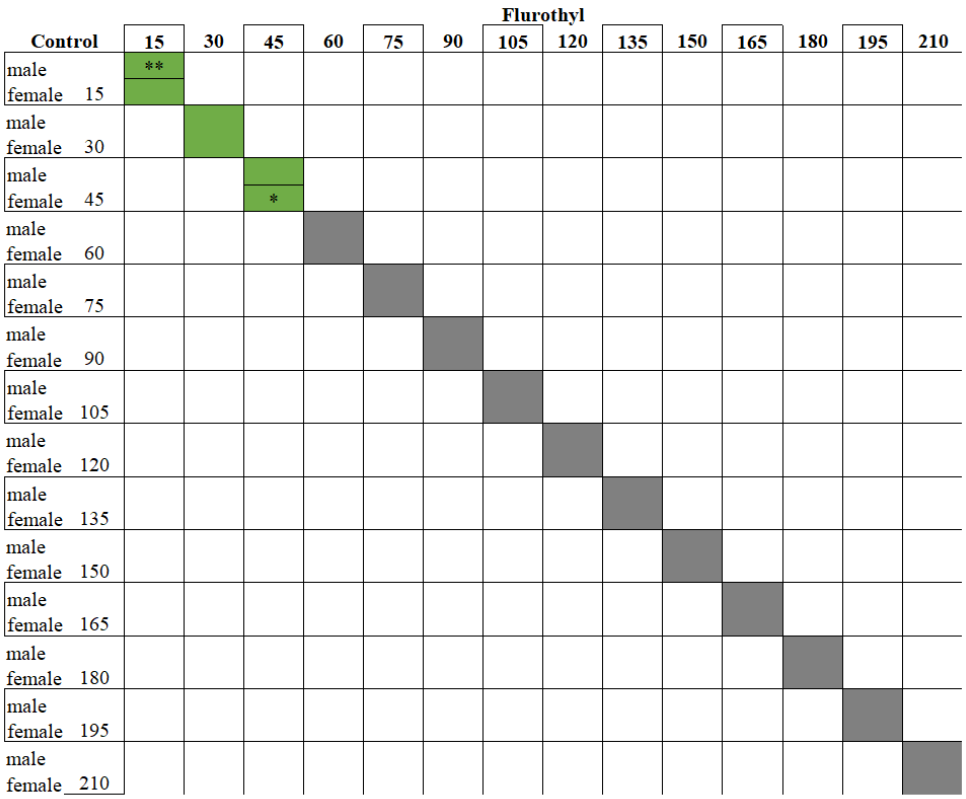
Blue – significant differences between flurothyl-treated animals of both sexes;

Green – significant differences between control animals of both sexes;

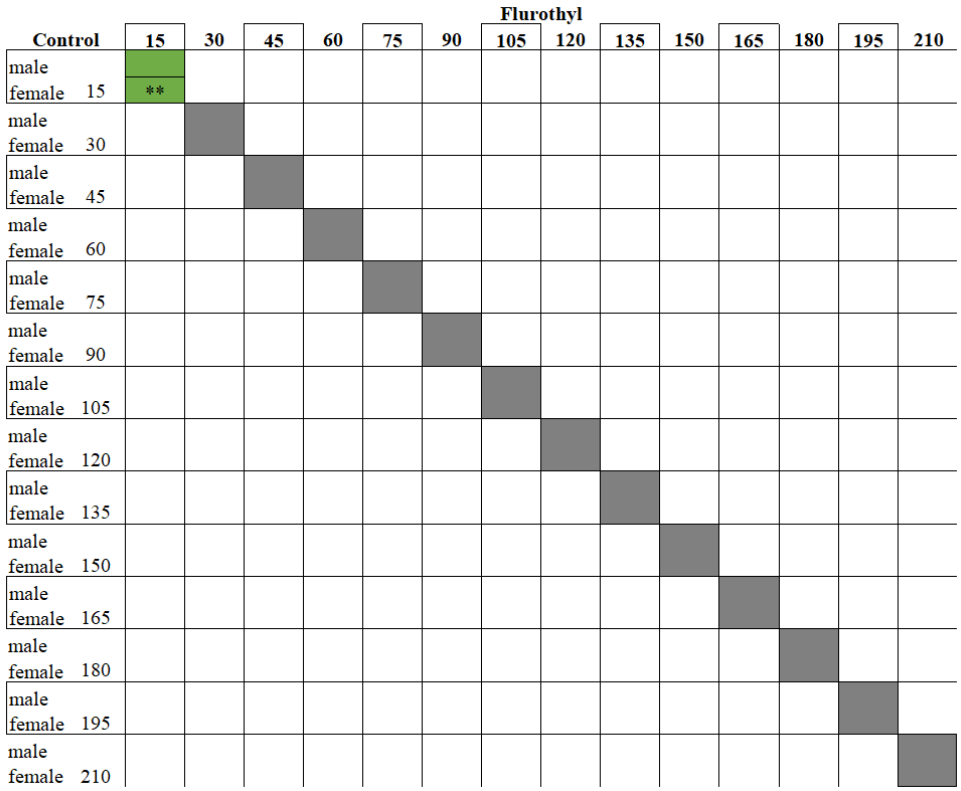
Grey – without significance.

Annex A

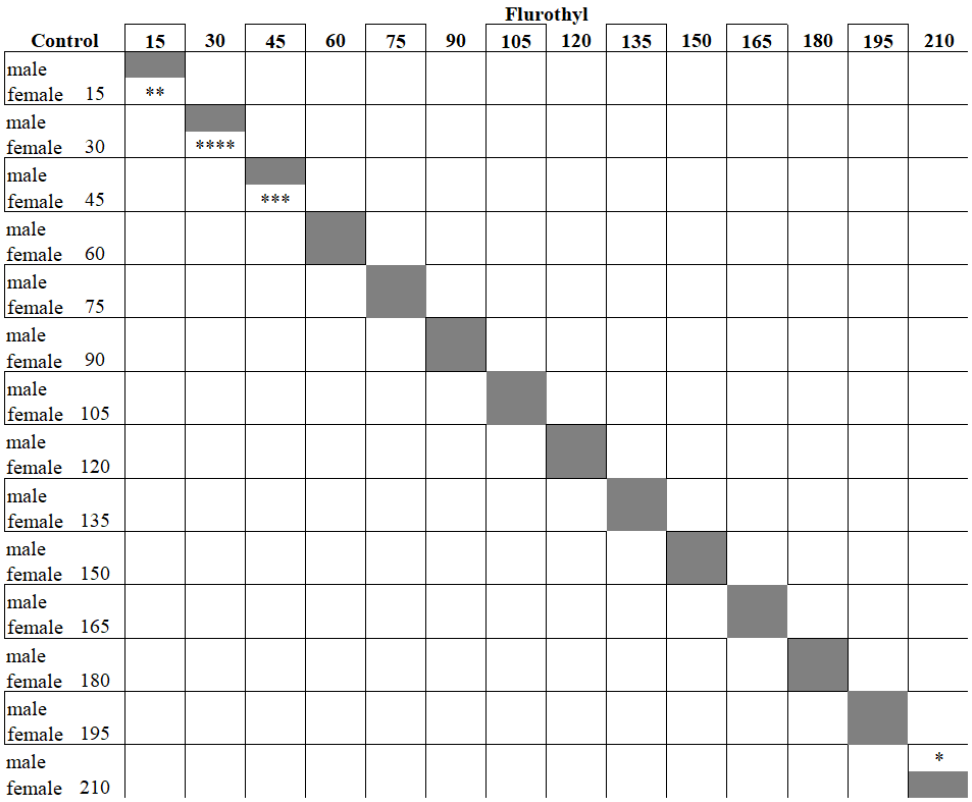
P12 delta band:



P12 theta band:



P12 alpha band:



P12 beta band:

		Flurothyl													
	Control	15	30	45	60	75	90	105	120	135	150	165	180	195	210
male		***													
female	15	*													
male			**												
female	30														
male															
female	45														
male															
female	60														
male															
female	75														
male															
female	90														
male															
female	105														
male															
female	120														
male															
female	135														
male															
female	150														
male															
female	165														
male															
female	180														
male															
female	195														
male															
female	210														

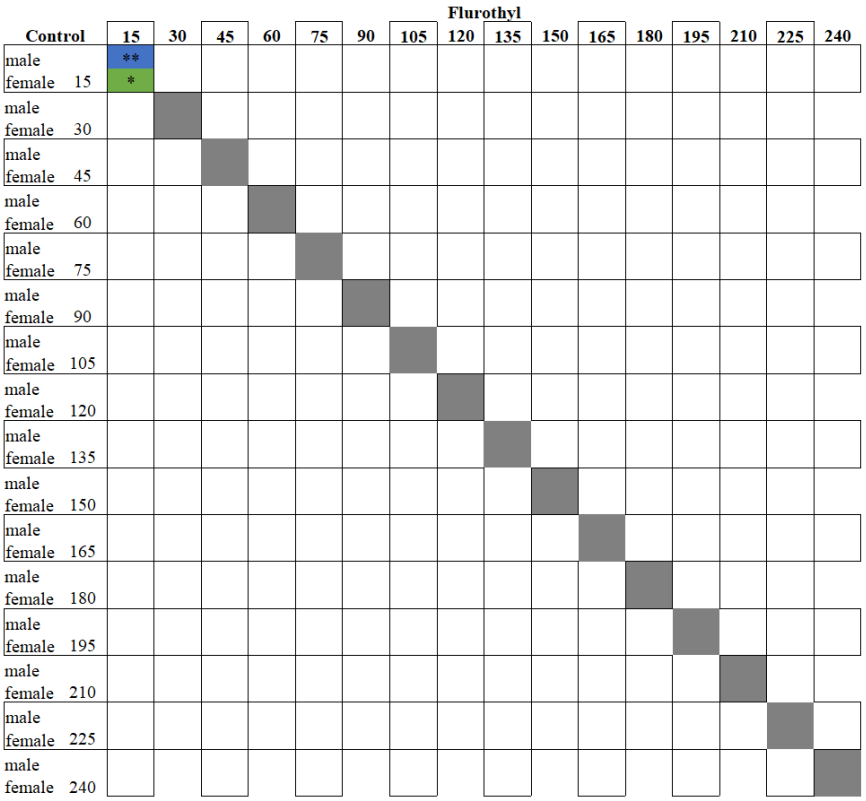
P12 gamma band:

		Flurothyl													
	Control	15	30	45	60	75	90	105	120	135	150	165	180	195	210
male															
female	15	*													
male															
female	30														
male															
female	45														
male															
female	60														
male															
female	75														
male															
female	90														
male															
female	105														
male															
female	120														
male															
female	135														
male															
female	150														
male															
female	165														
male															
female	180														
male															
female	195														
male															
female	210														

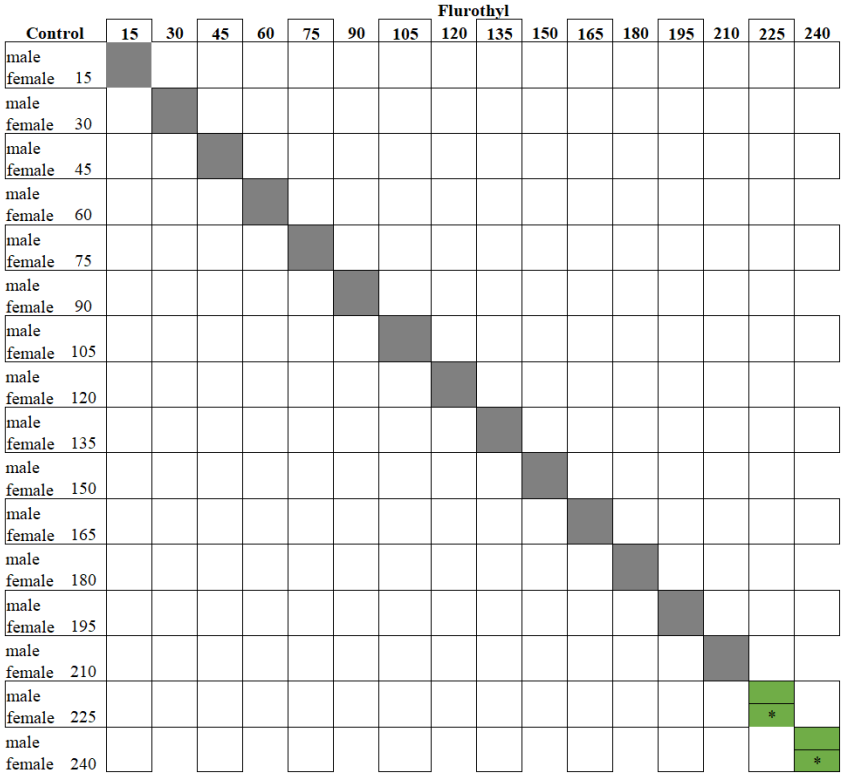
Click to return to 44

Annex B

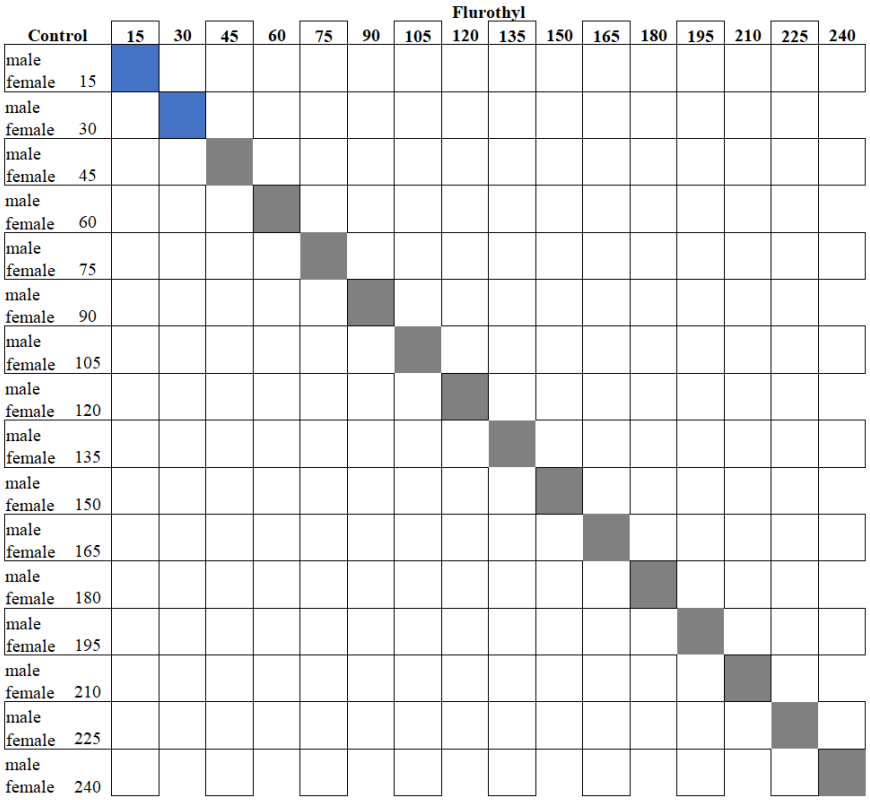
P15 delta band:



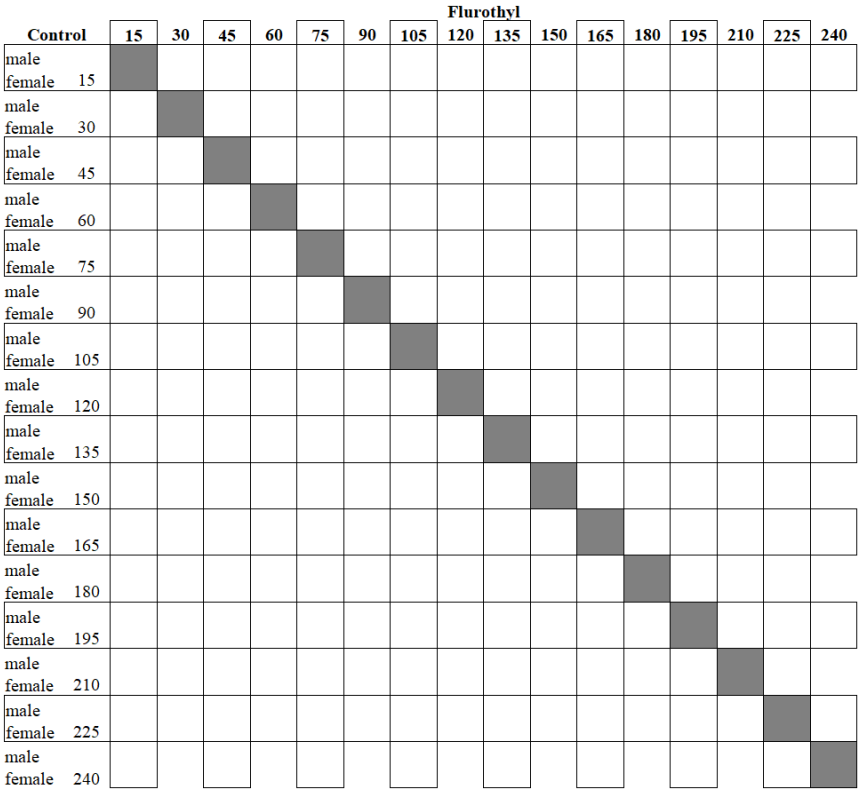
P15 theta band:



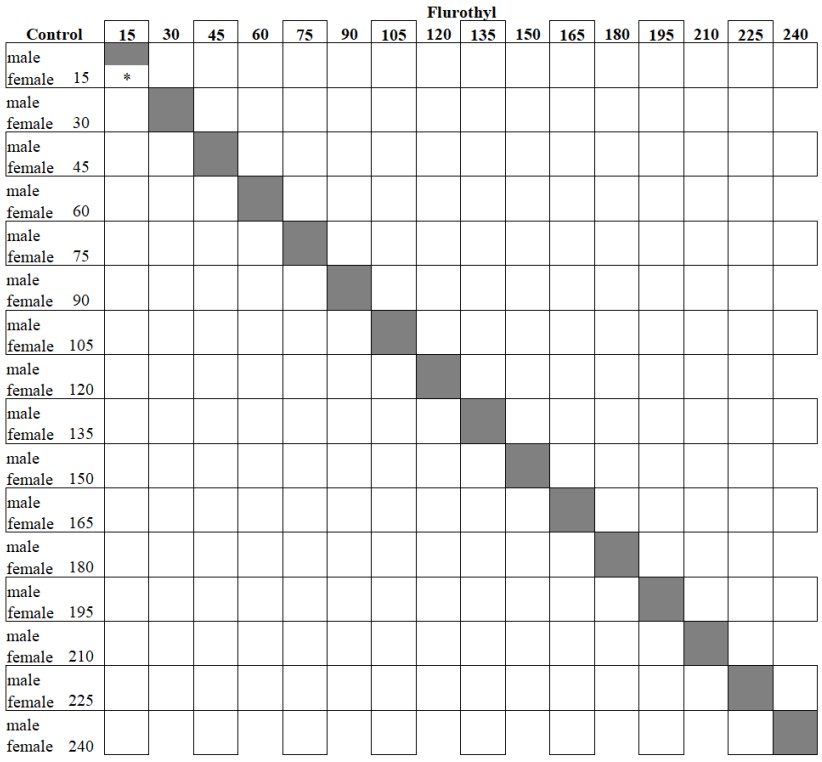
P15 alpha band:



P15 beta band:



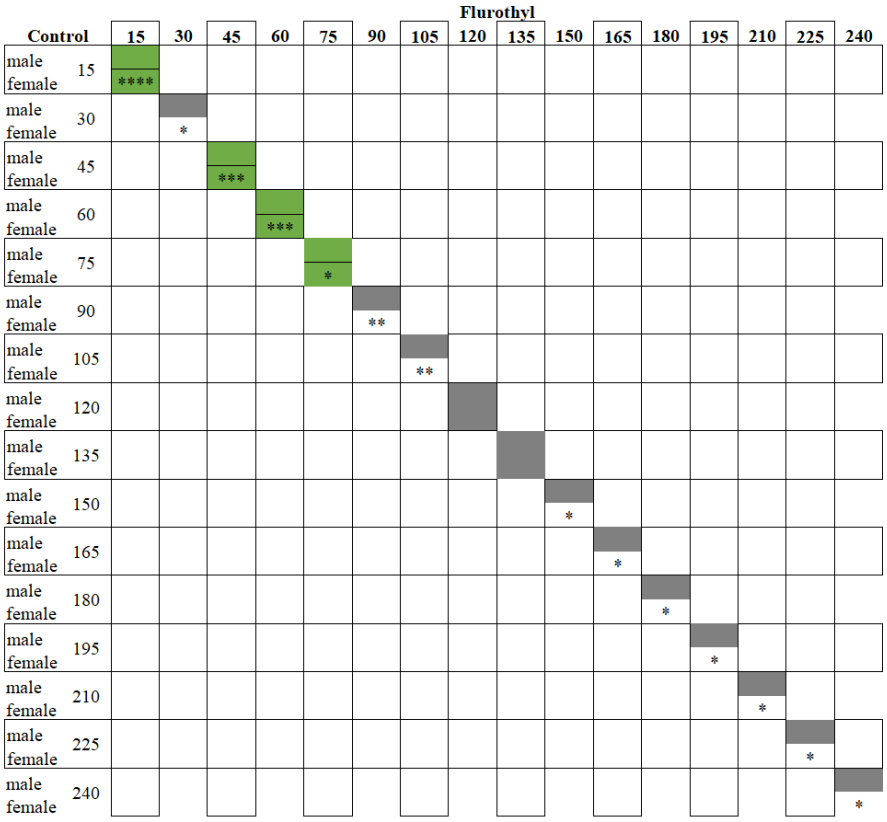
P15 gamma band:



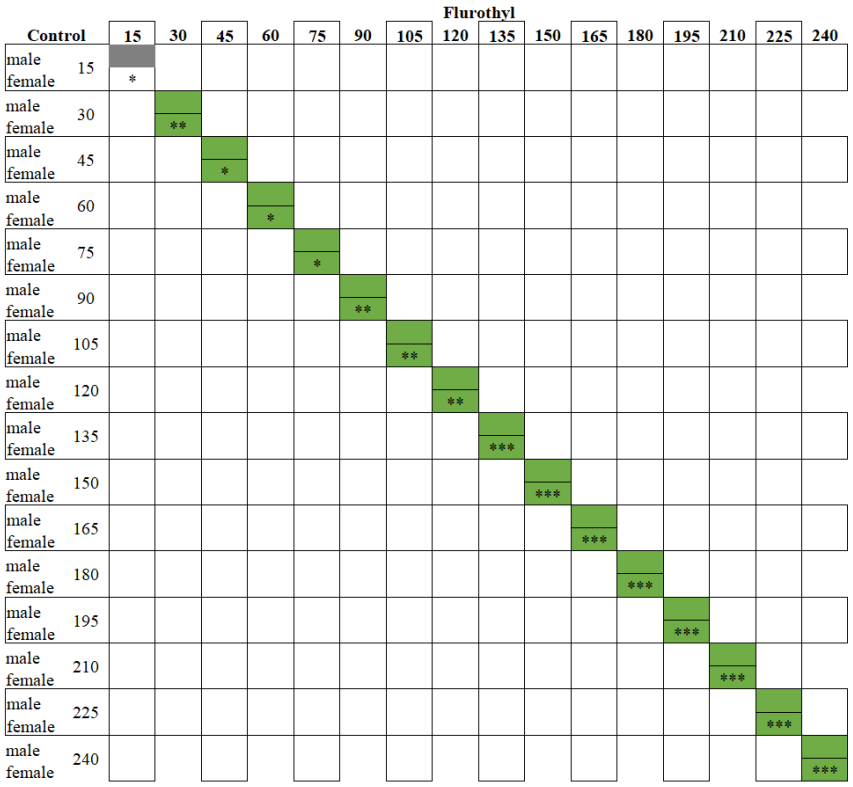
Click to return to 45

Annex C

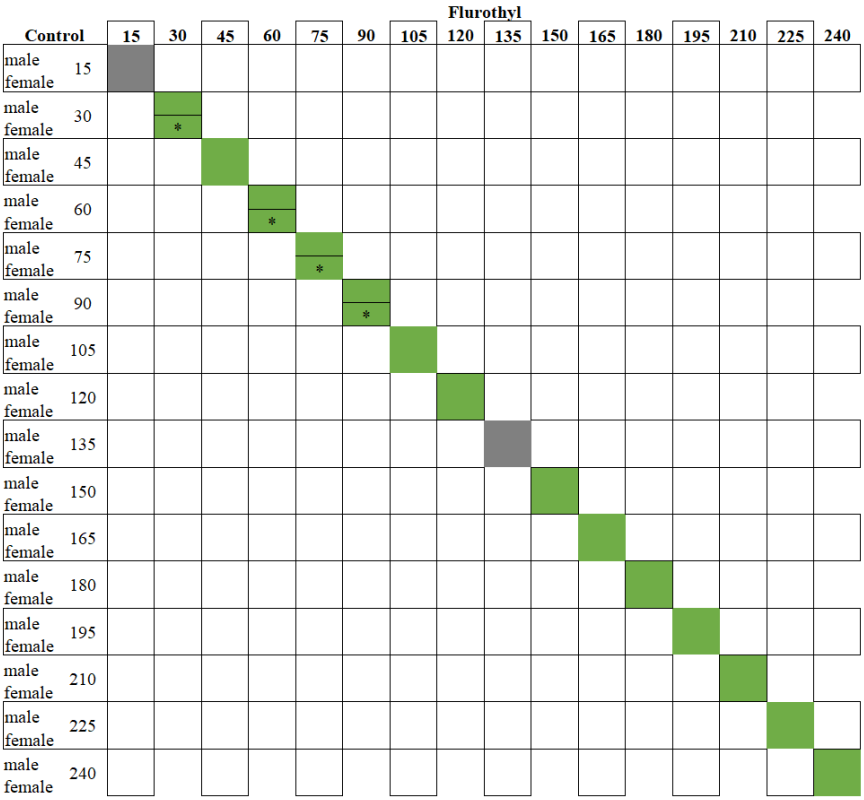
P25 delta band:



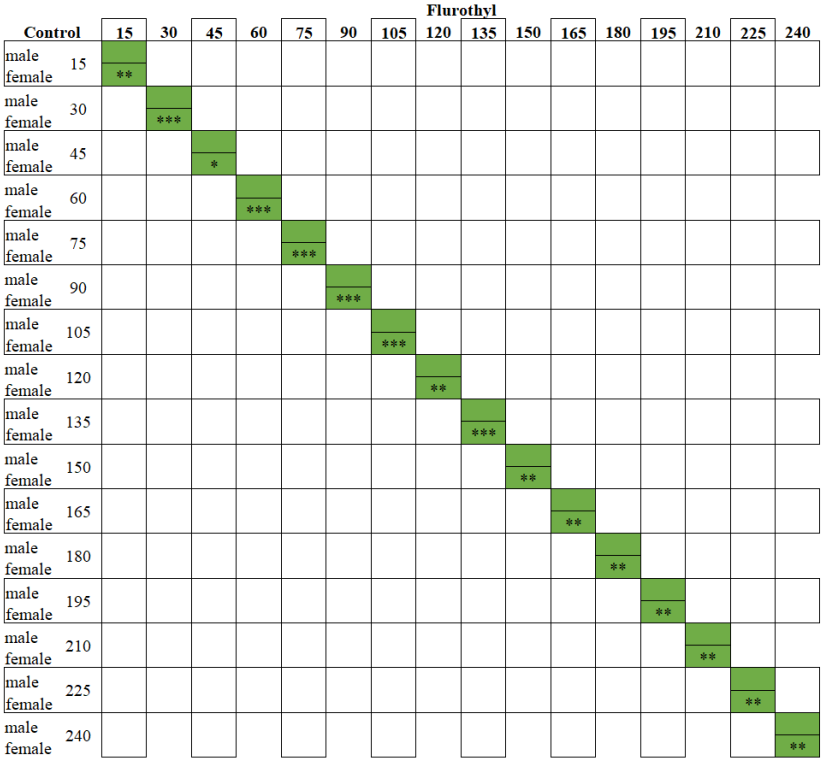
P25 theta band:



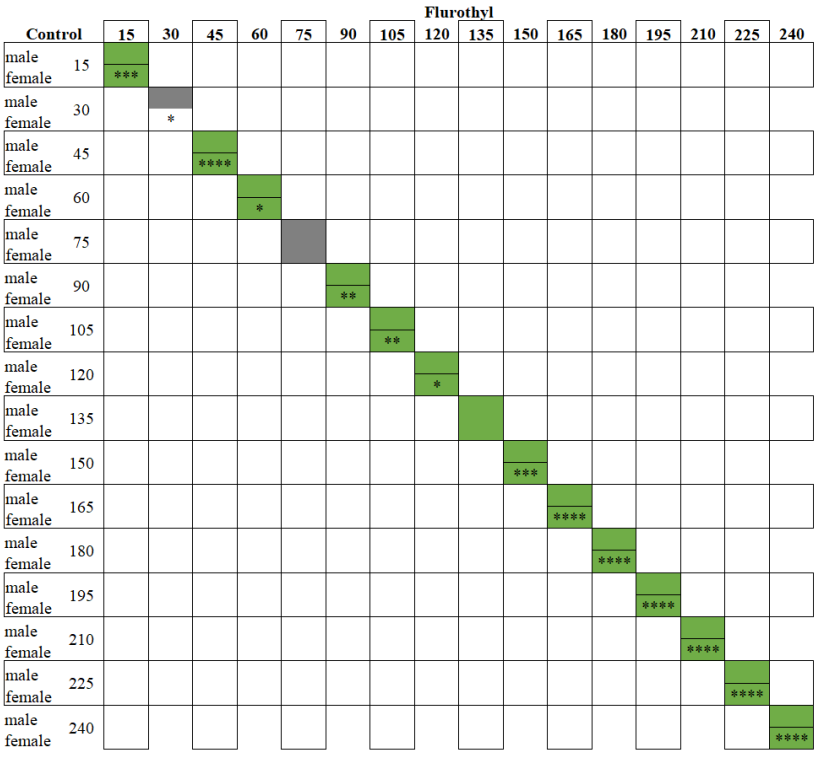
P25 alpha band:



P25 beta band:



P25 gamma band:



Click to return to 46