Czech Technical University in Prague
Faculty of Electrical Engineering
Department of Circuit Theory

Bachelor's Thesis

Vyhodnocení experimentální léčby epilepsie u laboratorních myší
Evaluation of experimental treatment of epilepsy in laboratory mice

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Study program: Medical Electronics and Bioinformatics

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BACHELOR‘S THESIS ASSIGNMENT

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Vyhodnocení experimentální léčby epilepsie u laboratorních myší

Guidelines:

Epilepsy is a serious chronic neurological disorder affecting 0.5 - 1 % of population in developed countries. About 30% of patients are pharmacoresistant. Therefore, there is an urgent need for novel therapies. One such possibility involves chemogenetics [1]. In chemogenetics, a genetically engineered receptor is delivered to the neurons and activated using an artificial drug which has high affinity to the receptor but low affinity to the natural endogenous receptors. The student will collaborate with the Laboratory of Experimental Neurophysiology at the Second Faculty of Medicine of Charles University where this method treatment is being investigated. Epileptic mice [2] having an inhibitory receptor hM4Di are injected either its agonist deschloroclozapine (DCZ) or saline as a control treatment and multichannel intracranial EEG (iEEG) is recorded. The student will analyze the iEEG of the mice for the presence of seizures and interictal epileptiform discharges (IEDs) [3] and thereby evaluate the efficacy of the treatment. The tasks for the student are:
1) Describe the epileptic EEG activity observed in the epileptic mice (IEDs, seizures).
2) Detect the IEDs using an existing detector. Seizure labels will be provided.
3) Compare the IED rate and seizure rate following the chemogenetic treatment and control treatment.
4) Determine the time course of the treatment efficacy.

Bibliography / sources:

[1] Desloovere et al.: Long-term chemogenetic suppression of spontaneous seizures in a mouse model for temporal lobe epilepsy. Epilepsia (2019). doi: 10.1111/epi.16368
[2] Chvojka et al.: Mouse model of focal cortical dysplasia type II generates a wide spectrum of high-frequency activities. Neurobiol Dis (2023). doi: 10.1016/j.nbd.2023.106383
[3] Janca et al.: Detection of Interictal Epileptiform Discharges Using Signal Envelope Distribution Modelling: Application to Epileptic and Non-Epileptic Intracranial Recordings. Brain Topogr (2014). doi: 10.1007/s10548-014-0379-1

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Supervisor’s signature	Head of department’s signature	Dean’s signature

III. Assignment receipt

The student acknowledges that the bachelor's thesis is an individual work. The student must produce his thesis without the assistance of others, with the exception of provided consultations. Within the bachelor's thesis, the author must state the names of consultants and include a list of references.

.	
Date of assignment receipt	Student's signature

Declaration

I hereby declare that I have completed the submitted work independently and that I have listed all the sources of information used in accordance with the Methodical Guidelines on Adhering to Ethical Principles in the Preparation of University Theses.

In Prague, on May 22nd 2024 .....

Signature of the author

Prohlášení

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V Praze dne 22. května 2024

.....
Podpis autora práce

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Abstract

Epilepsy is a common chronic neurological disorder affecting 0.5 to 1% of the population. One-third of patients are refractory to the best possible currently available treatment. Therefore, there is a high demand for novel strategies. In this thesis, we investigate the efficacy of an experimental chemogenetic treatment strategies of epilepsy on a murine model of focal cortical dysplasia. We used a designer receptor exclusively activated by a designer drug (DREADD), hM4Di, which was delivered by virus injection or was present in the genome of the mice. As a ligand, we used deschloroclozapine (DCZ). The DCZ was administered either by daily injections or continuously by an osmotic pump. Saline served as the control treatment.

Seven mice induced with artificially induced epilepsy underwent both DCZ and saline treatments, allowing for within-subject comparative analysis. Daily seizure frequencies and frequencies of interictal epileptiform discharges (IED) were calculated and analyzed across different treatments.

The results demonstrated no significant reduction in seizure or IED frequencies attributable to DCZ administration. Seizure frequencies exhibited considerable variability without a clear reduction trend during DCZ treatment compared to saline or no drug periods. Similarly, IED frequencies showed overlapping patterns across DCZ, saline, and no drug administration phases, further indicating no efficacy of DCZ in reducing these frequencies.

These findings highlight the necessity for further research to explore the conditions and mechanisms under which DCZ might be effective. Future studies should consider varying dosages, treatment durations, the potential combination of DCZ with other therapeutic agents, or a slight change of research methods to better understand the efficacy of DCZ and optimize its use in epilepsy treatment. Future research should also focus on improving the methods of hM4Di transduction.

Keywords: interictal epileptiform discharge, seizure, epilepsy, deschloroclozapine, osmotic pump

Abstrakt

Epilepsie je časté chronické neurologické onemocnění postihující 0,5 až 1 % z populace. Třetina pacientů je refrakterní k nejlepší možné aktuálně dostupné léčbě. Proto existuje vysoká poptávka po nových léčebných postupech. V této práci zkoumáme účinnost experimentální chemogenetické léčby epilepsie na myším modelu fokální kortikální dysplazie. Použili jsme designový receptor exkluzivně aktivovaný designovým lékem (DREADD), hM4Di, který byl dodán virovou injekcí nebo byl přítomen v genomu myší. Jako ligand jsme použili deschloroklozapin (DCZ). DCZ byl podáván buď každodenními injekcemi nebo kontinuálně osmotickou pumpou. Jako kontrolní léčba byl podáván fyziologický roztok.

Sedm myší s uměle vyvolanou epilepsií podstoupilo léčbu DCZ i fyziologickým roztokem, což umožnilo srovnávací analýzu v rámci subjektu. Denní frekvence záchvatů a frekvence interiktálních epileptiformních záchvatů (IED), byly vypočteny a analyzovány napříč různými způsoby léčby.

Výsledky neprokázaly žádné významné snížení frekvence záchvatů nebo IED, které by bylo možné přičíst podání DCZ. Frekvence záchvatů vykazovala značnou variabilitu bez jasného trendu snižování během léčby DCZ ve srovnání s obdobím podávání fyziologického roztoku nebo bez podávání léků. Podobně frekvence IED vykazovaly překrývající se vzorce napříč DCZ, fyziologickým roztokem a fázemi bez podávání léků, což dále ukazuje na neúčinnost DCZ při snižování těchto frekvencí.

Tato zjištění zdůrazňují nutnost dalšího výzkumu za účelem prozkoumání podmínek a mechanismů, za kterých může být DCZ efektivní. Budoucí studie by měly zvážit různé dávky, trvání léčby, potenciální kombinaci DCZ s jinými terapeutickými látkami, nebo změnu výzkumných metod k lepšímu pochopení účinnosti DCZ a optimalizaci jeho použití při léčbě epilepsie. Budoucí výzkum by se měl také zaměřit na zlepšování metody transdukce hM4Di.

Klíčové slova: interiktální epileptiformní výboj, záchvat, epilepsie, deschloroklozapin, osmotická pumpa

List of Abbreviations

ASM	anti-seizure medicine
CT	computed tomography
DBS	deep brain stimulation
DCZ	deschloroclozapine
DREADD	Designer Receptors Exclusively Activated by Designer Drug
ECoG	intraoperative electrocorticography
EEG	electroencephalography
EpiReC	Epilepsy Research Centre Prague
FCD	focal cortical dysplasia
HFO	high-frequency oscillation
hM4Di	modified muscarinic M4 receptor
iEEG	invasive electroencephalography
IED	interictal epileptiform discharges
ILAE	The International League Against Epilepsy
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
OSEL	Open Signal Explorer and Labeler

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1 Introduction

1.1 Motivation

Epilepsy is one of the most prevalent chronic neurological disorders. It affects 0,5 to 1% of population which constitutes approximately 50 million individuals worldwide[1]. Its impact extends beyond the physiological realm, significantly influencing the socio-economic and psychological aspects of individuals and their families. The primary motivating factor for exploring epilepsy treatment further is the lack of effective treatment options available for some individuals, as there is still approximately one third of patients, who cannot be compensated by an anti-seizure medicine (ASM)[2] . The difficulties that people with epilepsy face in their daily lives are also an enormously important reason why to explore new approaches to the treatment of epilepsy. By analyzing data from interictal epileptiform discharges (IEDs) from an experimental chemogenetic treatment in a murine model of epilepsy, this thesis aims to contribute to the development of the novel therapy which could improve the patient care in the future. The data originated from the investigation of chemogenetic treatment in a murine model of epilepsy due to focal cortical dysplasia (FCD).

1.2 Epilepsy

1.2.1 Definition

Epilepsy represents a chronic neurological disorder characterized by recurrent, unprovoked seizures, always stemming from abnormal electrical activity in the brain. These seizures can manifest in various forms, ranging from brief lapses of consciousness to convulsions and involuntary movements.[3] Epilepsy can have various etiologies, with genetic, developmental, and acquired factors contributing to its onset and progression. Understanding the complexities of epilepsy is paramount for devising effective treatment strategies and mitigating its impact on affected individuals.

1.2.2 Seizures

Epileptic seizures are sudden disturbances in electrical activity within the brain, leading to a temporary alteration of behavior, movement, or sensation. These alterations can manifest in various ways depending on the seizure type and the area of the brain affected. Seizures can range from brief lapses of awareness to full-body convulsions with loss of consciousness. Some seizures are defined as convulsive, meaning involuntary muscle contractions are present in these cases. Of these, one-third begin as generalized seizures (affecting both hemispheres of the brain and causing unconsciousness), the rest begin in smaller brain regions and then propagate further (focal seizures). Understanding the specific seizure type is crucial for diagnosis and treatment planning.[4]

Categorization of seizures recognizes the following types of generalized seizures based on behavioral manifestation:

- **tonic -** characterized by sudden and stiffening muscle contractions.
- **clonic -** defined by abnormal neuromuscular activity characterized by rapidly alternating muscle contraction and relaxation
- **tonic-clonic (grand mal) -** produces bilateral, convulsive tonic and clonic muscle contractions
- **myoclonic -** a muscle, joint, or group of muscles experiences brief, involuntary, irregular twitching, contrasting with clonus, which is characterized by rhythmic or regular movements
- **absence (petit mal) -** defined by a brief loss and return of consciousness not followed by a postictal state (known for drowsiness, confusion, migraine and other symptoms), common in children
- **atonic seizures -** consist of a partial or complete loss of muscle tone, last only a few seconds[5]

1.2.3 Types of epilepsy

Unlike the classification of seizures which focuses on what happens during an epileptic incident, the classification of epilepsies stresses out the underlying causes of this abnormal neural activity. Systematization of both seizures and epilepsies themselves allows for a more effective and hopefully more precise diagnosis assessment in epileptic patient, enabling better treatment.

After some adjustments being made over the years, The International League Against Epilepsy (ILAE) currently defines the epilepsy categories as follows [6]:

1. Unknown cause (mostly genetic or presumed genetic origin)

- a) Pure epilepsies due to single gene disorders
- b) Pure epilepsies with complex inheritance

2. Symptomatic (associated with gross anatomic or pathologic abnormalities)

- a) Mostly genetic or developmental causation
  - i) Childhood epilepsy syndromes
  - ii) Progressive myoclonic epilepsies
  - iii) Neurocutaneous syndromes
  - iv) Other neurologic single gene disorders
  - v) Disorders of chromosome function
  - vi) Developmental anomalies of cerebral structure
- b) Mostly acquired causes
  - i) Hippocampal sclerosis
  - ii) Perinatal and infantile causes
  - iii) Cerebral trauma, tumor or infection
  - iv) Cerebrovascular disorders
  - v) Cerebral immunologic disorders
  - vi) Degenerative and other neurologic conditions

3. Provoked (a specific systemic or environmental factor is the predominant cause of the seizures)

- a) Provoking factors
- b) Reflex epilepsies

4. Cryptogenic (presumed symptomatic nature in which the cause has not been identified)

1.2.4 Syndromes

Epilepsy syndromes are distinct clinical entities characterized by specific seizure types, age of onset, EEG patterns, and prognosis. ILAE classification system categorizes these syndromes based on their clinical and electrophysiological features. This classification offers a structured approach to epilepsy diagnosis, allowing for more targeted treatment strategies.

The process of categorizing epilepsy cases into specific syndromes is more prevalent among children, primarily because, in these syndromes, seizures typically manifest at an early age[7]. Less serious examples include benign *rolandic epilepsy* (the most common type of epilepsy found in childhood, the majority of the affected children simply outgrow it over time). Severe syndromes with diffuse brain dysfunction caused and with frequent seizures resistant
to treatment include for instance *Lennox-Gastaut syndrome* (complex childhood-onset epilepsy syndrome defined by multiple and concurrent seizure types).

1.3 Focal cortical dysplasia (FCD)

Focal cortical dysplasia (FCD) is a common brain development disorder present from birth. In FCD, neurons in a specific brain region do not migrate to their correct positions during fetal development, creating a malformation. This malformation is characterized by aberrant neuronal organization, dysmorphic neurons (abnormally shaped neurons), and abnormal cortical architecture.

FCD is a frequent cause of epilepsy in both children and adults, resulting in seizures that are drug-resistant, which means they do not respond to medication. FCD fosters seizures by inciting an excessively excitable state within the cortex and disturbing the typical functioning of neuronal networks. Understanding how FCD disrupts the brain (pathophysiology) is crucial for developing targeted treatments aimed at controlling these abnormal circuits and reducing seizure frequency. [8]

There are three main types of FCD, each with further subtypes (Figure 1). These subtypes can be distinguished by examining their unique histopathological features[8]

- **Type 1 FCD:**
  - Type 1a: Characterized by abnormal radial cortical lamination.
  - Type 1b: Characterized by tangential radial cortical lamination.
  - Type 1c: Characterized by a spectrum of features, including both radial and tangential cortical lamination.
- **Type 2 FCD:**
  - Type 2a: Characterized by dysmorphic neurons.
  - Type 2b: Characterized by dysmorphic neurons and balloon cells

- **Type 3 FCD:**
  - Type 3a: Characterized by cortical lamination abnormalities in the temporal lobe associated with hippocampal sclerosis
  - Type 3b: Characterized by cortical lamination abnormalities adjacent to a glial or glioneuronal tumor
  - Type 3c: Characterized by cortical lamination abnormalities adjacent to vascular malformation
  - Type 3d: Characterized by cortical lamination abnormalities adjacent to any other lesion acquired during early life

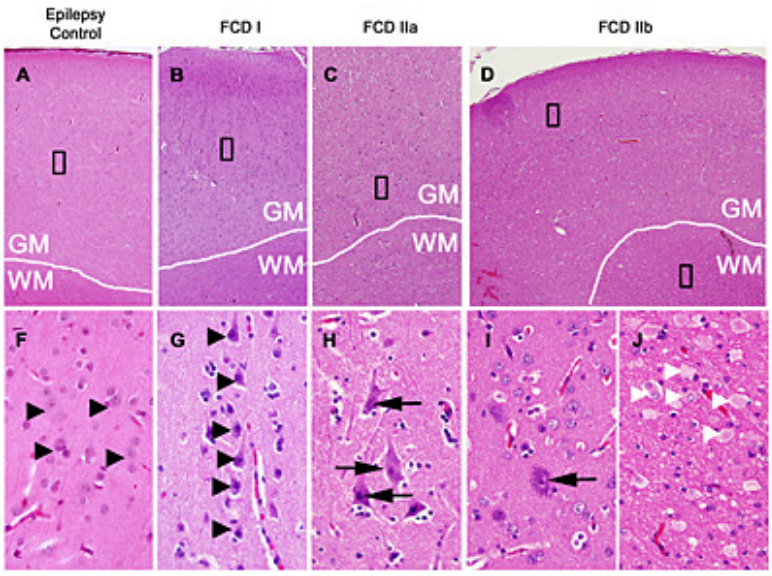

Figure 1: The typical appearance of FCD I, IIa, IIb.
In pictures A and F, irregularly oriented neurons are visible (marked by black arrows).
Pictures B and G show normal-sized dysmorphic neurons.
Tissue with abnormally large dysmorphic neurons is depicted in pictures C and D (adapted from [9]).

The data analyzed in this thesis came from a mouse model of FCDIIa, a specific subtype characterized by dysmorphic neurons. By studying interictal epileptiform discharges (IEDs) in this model, the research aims to enhance our understanding of how FCD disrupts brain function and contributes to drug-resistant epilepsy.

1.4 Electroencephalography (EEG)

1.4.1 Characterization

Electroencephalography (EEG) stands as a cornerstone in the diagnosis and management of epilepsy. By recording the brain's electrical activity through scalp electrodes, EEG offers valuable insights into seizure activity, brain function, and pathological abnormalities.[10] Its high temporal resolution enables the detection of transient changes in brain activity associated with epileptic seizures, aiding in the localization of seizure foci and the characterization of epileptic syndromes.[11] Moreover, EEG plays a crucial role in monitoring treatment response, guiding medication adjustments, and assessing prognosis in individuals with epilepsy.

1.4.2 Invasive electroencephalography (iEEG)

Electroencephalography (EEG) is a common method for examining brain activity. However, scalp EEG has limitations in precisely identifying the source of abnormal activity due to its focus on superficial brain regions and signal distortion caused by intervening tissues.

For patients with focal epilepsy (seizures initially affect only one hemisphere of the brain[12]) involving deep brain areas, intracranial EEG (iEEG) is employed in specialized epilepsy surgery centers to accurately localize the epileptogenic zone.

iEEG involves implanting subdural cortical grids or stereotactically inserting intracerebral electrodes during an invasive neurosurgical procedure under general anesthesia. Patients are monitored for several days to weeks while fully conscious, with 24/7 EEG recordings from up to 256 channels. This detailed data reveals the occurrence of pathological patterns in individual channels during interictal (between-seizure) periods and the onset and propagation of epileptic seizures (ictal).

Experienced neurologists visually evaluate abnormal iEEG to define the precise boundaries of epileptogenic tissue and guide subsequent resective surgery. Moreover, quantitative methods of iEEG analysis are emerging[13][14].

Complete removal of epileptogenic tissue eliminates seizures and cures epilepsy. Surgical treatment aims to remove this tissue without causing permanent damage to the cognitive functions. Therefore, the potential benefits of surgery are carefully weighed against the possible risks of postoperative complications.[15]

1.5 Interictal epileptiform discharges (IEDs)

Interictal epileptiform discharges (IEDs) are abnormal electrical spikes or waves detected in EEG recordings during periods between seizures in people with epilepsy. These brief abnormalities point to underlying epileptic tissue. Clinicians use IEDs to diagnose epilepsy, monitor disease activity, and pinpoint the location of the seizure source (epileptogenic zone). While traditional multichannel intracranial recordings remain the standard technique for studying the organization of epileptic networks, newer methods (such as electrocorticography

grids) offer large-scale recording across broader brain regions, providing more detailed spatial and temporal information. However, this increased data volume presents a challenge – analyzing long recordings from hundreds of channels is time-consuming, prone to human error, and difficult to do manually.[13]

For epilepsy surgery in patients with drug-resistant seizures, clinicians use various imaging techniques like Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) to locate the seizure focus. In some complex cases, they may employ intraoperative electrocorticography (ECoG) during surgery. This involves placing a grid of electrodes directly on the brain surface after removing a skull flap (craniotomy). This allows for real-time recording of IED activity during surgery, which can provide valuable information about the seizure focus, especially since seizures themselves are unpredictable. While IEDs may not perfectly map onto seizure zones, they offer crucial insights that aid surgeons in targeting the epileptogenic tissue for resection.[16]

As presented in Figure 2, IEDs exhibit age-dependent characteristics, necessitating tailored approaches in spike detection. Both human and computer-based spike detectors should adjust thresholds based on patient age. With age, focal IEDs become less sharp, have lower amplitudes, and show reduced slow waves, while becoming more lateralized. They are most prevalent in children and the elderly, with morphology and localization influenced by age. In children, IEDs have higher amplitudes and sharper peaks, which diminish with age. Spike asymmetry, however, remains consistent across all ages. These findings emphasize considering age-related variations in EEG interpretation and diagnostic algorithm design.[17]

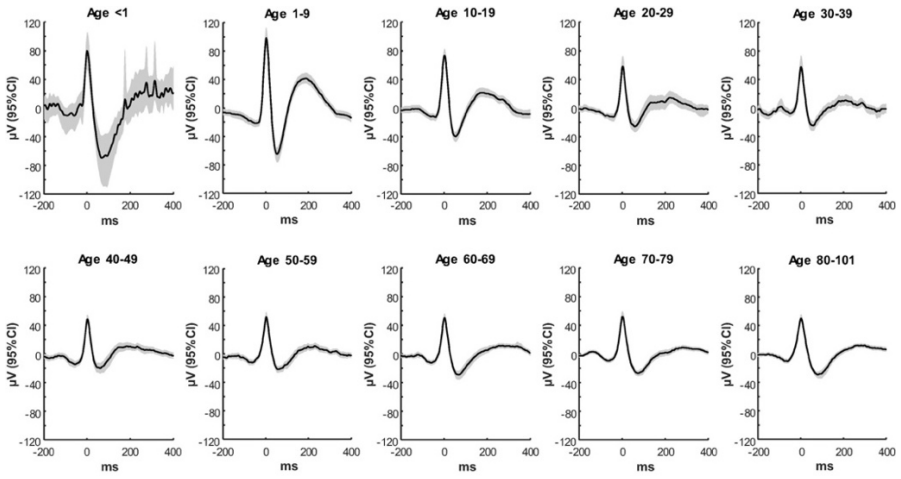

Figure 2: IED morphology by age.
Over time, the spike becomes blunter, slope, amplitude, duration and slow-wave area all depended on age as well.[17]

1.6 Contemporary and emerging approaches to the epilepsy treatment

Epilepsy treatment has seen significant advancements in recent years, offering a wider range of options for managing seizures and improving patient quality of life. Traditional medication remains a mainstay, with newer antiepileptic drugs boasting improved efficacy and tolerability compared to older options. However, for the 25% of patients with drug-resistant epilepsy[18], alternative approaches are crucial.

Surgical resection of the seizure focus offers a potential cure for some patients. Advanced techniques like brain mapping and intraoperative electrocorticography (ECoG) improve the accuracy of pinpointing the epileptogenic zone, minimizing the risk of damaging healthy brain tissue while maximizing seizure control.

Emerging therapies hold promise for the future of epilepsy treatment. Deep brain stimulation (DBS) involves implanting electrodes in specific brain regions to modulate abnormal electrical activity. Gene therapy is being explored as a potential way to inhibit the epileptic neurons e.g. by a genetically encoded ion channel or receptor. Thus said, chemogenetics, a technique that utilizes designer drugs to control specific cell populations, offers a novel approach for targeted seizure control.
These contemporary and emerging approaches offer a more personalized treatment landscape for epilepsy. By combining traditional medications with surgical options and exploring innovative therapies, clinicians can provide patients with a wider range of tools to manage their condition and achieve seizure control.

1.7 Chemogenetics

Chemogenetics offers a powerful tool for manipulating specific cell populations, particularly neurons, with high spatial control. One of the chemogenetic approaches works by introducing designer receptors exclusively activated by designer drugs (DREADDs) into targeted cells. These DREADDs are synthetic receptors not normally found in the body and only respond to specific, synthetic molecules. When administered, a ligand like clozapine or deschloroclozapine (DCZ) binds to DREADDs, triggering a cellular response – typically inhibition, however, an excitatory DREADDs exist as well. This allows researchers to silence or activate specific cell populations with pinpoint accuracy by delivering the ligand and controlling its timing.[19]

Chemogenetics finds numerous applications in neuroscience research, including studying brain circuits involved in behavior, epilepsy, and other neurological disorders. Compared to other experimental treatment approaches, it offers several advantages as it provides deeper brain penetration for targeting structures inaccessible by light (used in optogenetic experiments). Additionally, chemogenetic manipulations are longer-lasting, allowing for extended observation of cellular activity.

Despite current progress in such gene therapy, it has been emphasized that chemogenetics has yet to be utilized in humans as now it serves only as a mean of scientific research.

However, optogenetics (described in subchapter 1.10.) boasts superior temporal resolution, enabling highly precise control over the timing of cellular activity within milliseconds.

1.8 Deschloroclozapine (DCZ)

Deschloroclozapine (DCZ) is a synthetic ligand gaining traction in epilepsy research, particularly chemogenetic studies. Unlike traditional antiepileptic drugs, DCZ does not directly inhibit seizure activity. Instead, it binds to a DREADD which was previously transduced in the targeted cells using e.g. a viral vector[20]. If DCZ binds to the inhibitory DREADD hM4Di in neurons, it triggers hyperpolarization which inhibits the neuronal activity. This can result in suppression of epileptic activity."[21]. This unique approach holds promise of the development of novel therapeutic strategies for epilepsy, potentially offering more localized control of seizures with minimal side effects, since DCZ only affects cells that have been engineered to express the specific DREADD it binds to. Moreover, DCZ can be designed to target a specific DREADD subtype, further minimizing the potential for off-target effects and reducing side effects.

1.9 Osmotic pump

Osmotic pumps, integral components of controlled-release drug delivery systems, function by utilizing osmosis to steadily release medication over time. These pumps (Figure 3) consist of a core containing the drug and an osmogene, enclosed by a semi-permeable membrane with delivery ports. By harnessing osmotic pressure, water permeates the membrane, creating pressure within the system and propelling the drug out through the delivery openings. This mechanism enables sustained release of the drug in a predictable manner (for instance in pain management[22] or hormone replacement studies[23]), independent of gastrointestinal factors, ultimately improving research methodology.[24]

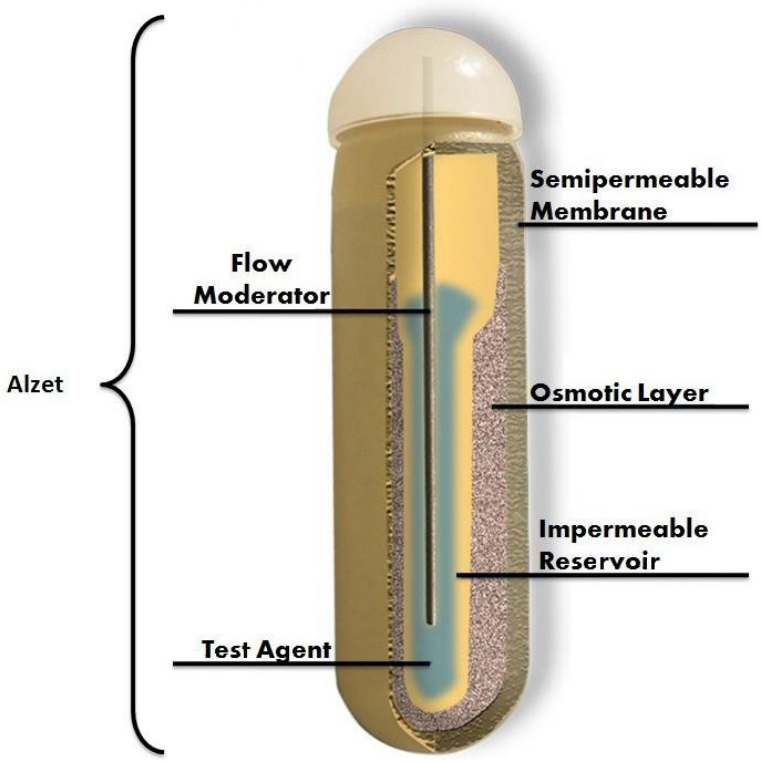

Figure 3: Schematic Diagram of Alzet Osmotic Pump[25]

1.10 Optophysiology

Optophysiology as an alternative approach (however not employed in this thesis) to chemogenetics enables the precise manipulation and observation of neural activity using light, facilitated by the incorporation of light-sensitive proteins through genetic means. Through optogenetic activators such as channelrhodopsin, halorhodopsin, and archaerhodopsin, specific neurons can be controlled with remarkable precision. Conversely, the monitoring of neuronal activity can be achieved using genetically encoded sensors for ions like calcium or membrane voltage. Light acts as the agent, providing unparalleled spatial and temporal resolution across various wavelengths and locations, making it a valuable tool for neuroscientists studying brain function and behavior.[26], [27]

2 Materials and methods

2.1 Software used

The whole practical part of this bachelor's thesis was performed exclusively by using two software tools, namely MATLAB and Microsoft Office Excel.

MATLAB was used for the following tasks:

- signal data visualization using the MATLAB-run research tool (introduced in the subchapter 2.3.)
- IED detection and data filtration for the further analysis
- statistical analysis

Microsoft Excel was used for the following tasks:

- storing the calculated data
- partially for the purpose of the statistical analysis

2.2 Origin of the data

In this thesis I analyze data recorded at the Laboratory of Experimental Neurophysiology at the Department of Physiology at the Second Faculty of Medicine of the Charles University in Prague. The laboratory is part of the consortium EpiReC (Epilepsy Research Centre Prague). This research facility studies the possible causes of epilepsy in humans and tries to propose innovative ways of treatment of this neurological disease. To facilitate required experimentation, EpiReC employs animal models of epilepsy, namely laboratory mice of identifiable lineage, in adherence to the Animal Care and Animal Protection Law of the Czech Republic, which is compatible with Directive 2010/63/EU. All protocols received approval from the Ethics Committees of the Second Faculty of Medicine. The animals presented spontaneous seizures as well as interictal activity consisting of IEDs on some of which was superimposed a high-frequency oscillation (HFO)[28] .

The data discussed in chapters below concern 5 male and 2 female animals with FCD induced by a mutated mTOR gene which was delivered by in utero electroporation in the embryonic day 14[29]. All animals have been administered in multiple day turns with either DCZ or saline at the time.

Preceding the EEG signal monitoring and data recording, this thesis involved two methods of delivering hM4Di, an inhibitory DREADD to specific neuronal populations in mice. In the chemogenetic treatment protocol employing injection (depicted in Figure 2.1.), AAV-PHP.eB–hSyn-DIO-hM4Di-mCherry was utilized for targeted intervention. Specifically, mTORp.L2427P was employed as the indicator of mutation, elucidating the precise genetic alteration. Through electroporation, we established a model of FCD IIa, or alternatively referred to as FCD 2A, to replicate pathological conditions. The introduction of Cre recombinase assumes great significance, conducting the selective activation of hM4Di exclusively within cells harboring the mutated mTOR variant. This strategic use of Cre ensures that the therapeutic

effects are focused within the specified cells carrying the mutated mTOR.

In the second method, using osmotic pumps (Figure 2.2.), the electroporation was conducted on the B6.129-Gt(ROSA)26Sortm1(CAG-CHRM4*,-mCitrine)Ute/J mouse line. This line contains a Cre-inducible hM4Di-mCitrine construct at the ROSA26 locus, which upon electroporation, allows for widespread and controlled expression of hM4Di. The use of osmotic pumps provides continuous delivery of the chemogenetic agent, ensuring sustained modulation of neuronal activity. Both methods are crucial for achieving the precise spatial and temporal control necessary for investigating the role of specific neural circuits in epilepsy.[30]

For the purpose of EEG monitoring, each mouse was at some point implanted with an electrode headstage usually consisting of 4-6 electrodes. The surgical setup and implantation points in this study were similar to the one displayed in Figure 4 (adapted from[31]).

Since I was not only analyzing the provided data but during the semester I became a member of the research team, I gained insight into how the various experiments are performed and approached and towards the end of the semester I learned how to successfully perform the electrode implantation myself.

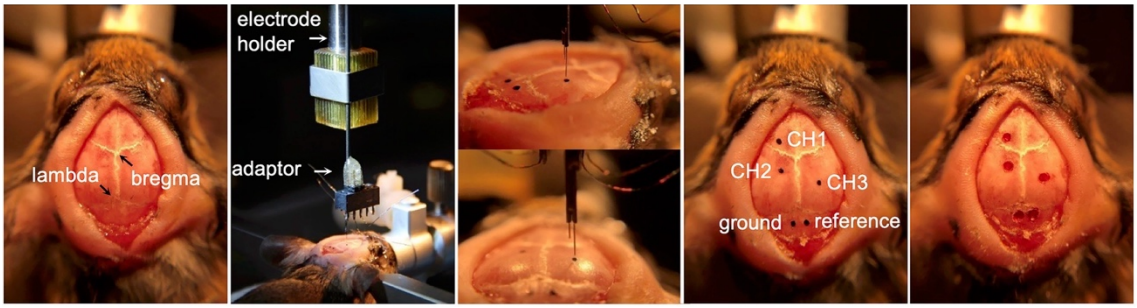

Figure 4: Intracranial electrode implantation in mice.
Figure shows where the electrodes are usually implanted
and the subsequent drilled opening for the electrode placement. (adapted from[31])

For three of the tested animals, the substance has been injected daily only once around 8 AM. In addition, the rest days were introduced, when a certain drug-administration cycle had been finished and the animal was left to rest for several days without being injected or being used for experiments whatsoever. The animals were administered DCZ or saline by intramuscular injections in the thigh muscle[32]. Figure 5, displayed below, shows the injection approach.

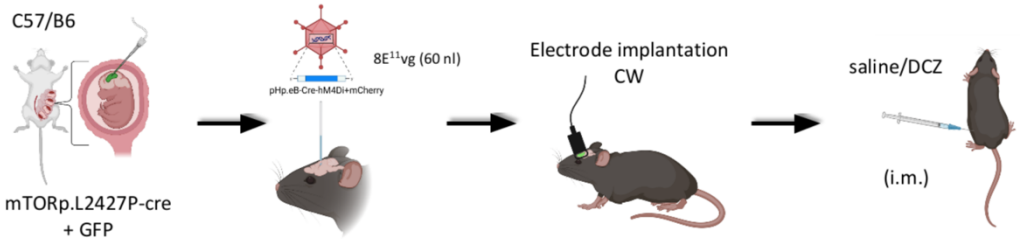

Figure 5: Chemogenetic approach to modulation of neuronal activity - injection approach[30]

For another set of animals (4 mice), a mixed approach was employed. These mice experienced alternating methods of administration: either intramuscular injections of DCZ or saline solution to the thigh muscle, or implantation of osmotic pumps filled with the respective substance, as mentioned earlier. In one instance, the order of drug administration methods was varied (initially employing both types of injections, followed by rounds of the implanted osmotic pump, while for the other three animals, the inverse order was done. This variation aimed to compare the outcomes resulting from different administration sequences. Below, Figure 6 illustrates the method involving the osmotic pump.

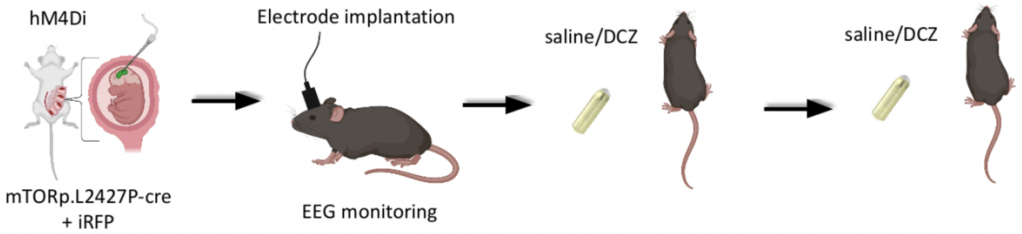

Figure 6: Chemogenetic approach to modulation of neuronal activity - approach using osmotic pump[30]

The data I worked with include recordings from two channels, labeled 'L' and 'C', as explained in section 2.3. Each recording lasts about an hour and was taken at a rate of 250 Hz, though originally the EEG was recorded at 5000 Hz and then decimated. The EEG was recorded over long periods in one-hour chunks, with short breaks in between. The Intan RHD2000 system with 32-channel headstages was used, but only two channels were active; the rest were either grounded or left open. Spike2 software was used with the Intan Talker plug-in to communicate with the hardware. Simultaneously a video was recorded using a webcam. Experts used this to double-check cases where the EEG data was unclear about seizures.

Due to occasional malfunctions of the recording apparatus leading to dropouts in the data, it was needed to account for these gaps when analyzing interictal epileptiform discharge (IED) frequency. Therefore, the IED frequency was standardized by the duration of the valid signal in seconds. This allowed for a more reliable comparison and interpretation of the data despite fluctuations in recording integrity caused by equipment malfunctions.

2.3 Selection of the data used

Following the completion of IED spike detection (introduced in subchapter 2.4.), additional constraints were imposed on the dataset. The analysis focused on the whole provided dataset, including the days without any drug administration which served as a baseline for comparative evaluation. The timing of injections was identified using research data tables. It was subsequently verified visually from the EEG recordings by large artifacts induced by the manipulation with the mouse. To visualize the data (Figure 7), a MATLAB-run program called Open Signal Explorer and Labeler (OSEL) was used. The program was developed and provided by my supervisor (not publicly available).

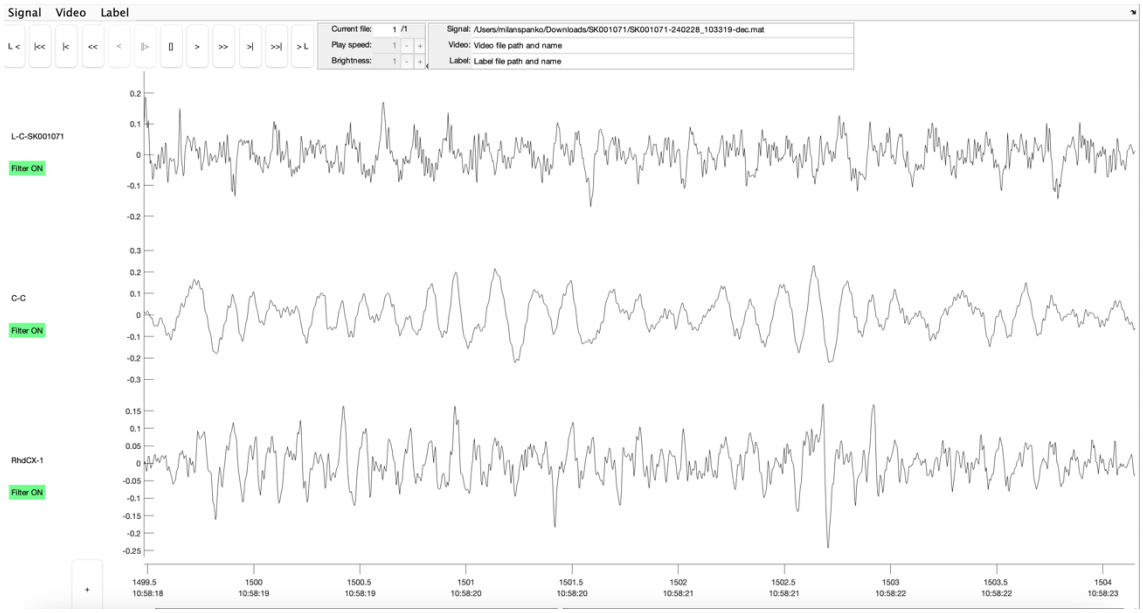

Figure 7: Sample of EEG data analyzed as displayed using Open Signal Explorer and Labeler (OSEL)

Additionally, alongside the EEG data, MATLAB files containing labels for epileptic seizures were provided. They were used for removing spike detections which occurred during seizures since these are not IEDs by definition. To accomplish this task, I wrote a specialized MATLAB function called *"seizureFiltering"*.

The final step of data preprocessing involved sorting the rows in each file based on the start time of IED occurrences using yet another of my custom functions called *"sortRows"*. Each row in the MATLAB IED file corresponded to data collected from two implanted electrodes: electrode 'L' positioned in the parietal bone at the FCD lesion site and electrode 'C' located contralaterally to the FCD lesion. Data from electrode 'C' with start or end times falling within the 100-millisecond time range of any data row associated with electrode 'L' were deemed to originate from the same IED and thus were removed. This filtering process was executed by three more custom functions called *"startFiltering"*, *"endFiltering"* and *"deletingSameRow"*.

Finally, I created one more function called *"hourlyIED"* which was used to detect number of IEDs corresponding to each hour in the given day. From this step on, exclusively the IED data from the "L" electrode were evaluated, as the data from the "C" electrode or the summed IEDs from both channels were deemed to be utterly inconclusive by my supervisor, at least for the purpose of this thesis.

Apart from "L" and "C" channels, Figure 7 displays also the RhdCX-1 channel. It is from the accelerometer placed on the mouse's head and it records the movements which may facilitate in identification of seizures or artifacts, however, it was not further used in the thesis. All custom-made MATLAB functions mentioned above use fairly easy operations with MATLAB tables and manipulations with its columns and rows based on datetime information or the electrode channel number.

Functions written for the purpose of the data analysis are provided in the supplementary material of this thesis.

2.4 iEEG parametrization

2.4.1 IED detection

IEDs have been detected in the EEG recordings by a validated automatic detector written in MATLAB. [13] This MATLAB program has been provided fully provided by the thesis supervisor and is also commonly used for research purposes at EpiReC.
It is a spike detector algorithm designed to detect transients (such as spikes or poly-spikes) in multichannel signals, often used in EEG data analysis for detecting epileptiform activity.

- **Input Parameters:** The function spike_detector_hilbert_v23 accepts three input arguments: the signal d, the sampling frequency fs, and an optional string of settings

- **Signal Processing:** The algorithm initially filters the signal using a bandpass filter in the frequency range of 10 Hz to 60 Hz to eliminate power-line noise.

- **Hilbert Envelope:** Instantaneous envelope is then computed using Hilbert transform. The envelope is then segmented into XX second segments with YY second overlap. Note that the segments are used only for the threshold computation, see below.

- **Thresholding:** Threshold estimation is conducted utilizing the maximum likelihood estimation (MLE) method on the voltage distribution of the envelope within each segment to derive a log-normal model, from which thresholds for outlier detection are computed.

$$th = k_1.[Mode + Median]$$
$$Mode = e^{\mu-\sigma^2}$$
$$Median = e^{\mu}$$

k_1 = coefficient optimized using gold standard spikes[13]
μ and σ^2 are calculated using maximum likelihood estimation

These threshold values from individual segments are interpolated across the entire signal length, yielding an adaptive threshold for comparison with the signal envelope to detect outliers indicative of interictal epileptiform discharges (IEDs).

- • **Local Maxima Detection:** Upon exceeding the threshold, local maxima in the envelope signal are evaluated as potential IED events. This step is pivotal for identifying peaks associated with spike activity.

- **Post-processing:** Detected spike events may undergo post-processing procedures, such as the merging of nearby spikes (polyspike union) and refinement of spike properties.

- **Output:** The algorithm furnishes diverse outputs, encompassing the position, duration, channel, and statistical significance of detected spikes.

- **Optimizations:** The code incorporates optimizations for enhanced efficiency, support for parallel computing and signal decimation to alleviate computational burden.

2.4.2 Seizure detection

Seizures were identified visually using OSEL by experienced researchers. For subsequent analyses I used the seizure labels provided in a spreadsheet. Prevalent approach in seizure recognition within EEG data includes investigating alterations in EEG frequency and amplitude. Such variations, characterized by rhythmic activity at particular frequencies (e.g., theta, alpha, or beta)[33] or abrupt fluctuations in amplitude, collectively serve as discernible markers of epileptic seizures.

2.5 Statistical Analysis

After filtering and preprocessing the data as detailed in subchapter 2.2, the statistical analysis focused on examining the following data procedures:

- daily seizure frequency standardized by the length of daily valid signal in seconds
- IED frequency after the drug injection was delivered (injection approach) and IED frequency in 6-hour time bins (osmotic pump approach)
- mean values of both seizure and IED frequencies
- frequency ratios
- median and interquartile range
- Wilcoxon signed rank test

2.5.1 IED frequency and seizure frequency computation

For each experimental animal, both IED and seizure frequencies are evaluated. In the analysis of the injection drug administration, the IED frequency is displayed in daily datapoints of the sum of the IED rate during 4 hours after the drug was administered.

For the periods of testing the osmotic pump approach, the IED frequency is displayed in 6-hour bins (4 bins per day), as the drug is released gradually over time.

Seizures were already labeled beforehand, and their daily count is known from the laboratory data sheet. Therefore, seizure frequency will be expressed only by this daily seizure count standardized by daily valid signal. Providing information about the daily development of seizures enables the exploration of dependencies between the occurrence of IEDs and labeled seizures.

2.5.2 Mean values of both seizure and IED frequencies

After the aforementioned frequencies are evaluated, all valid datapoint corresponding to the given drug and treatment method were summed and their mean value is calculated. These acquired values are later used for the computation in Wilcoxon signed rank test described in the subsection 2.5.3..

2.5.3 Frequency ratios

For each animal and treatment method, the frequency ratio of seizures and IEDs during DCZ administration to those during saline administration is calculated.

2.5.4 Median and interquartile range

Given the non-parametric nature of the frequency data in this thesis (small sample size of mice, which is less than 30), statistical tools such as the median, interquartile range, and then the Wilcoxon signed rank test (subsection 2.5.5.) were chosen for analysis.

2.5.5 Wilcoxon signed rank test

In this thesis, the Wilcoxon signed-rank test is employed to analyze the data obtained from two different drug administration methods and different drugs administered.
The Wilcoxon signed-rank test is a non-parametric statistical technique used for hypothesis testing, either to test the location of a population based on a sample of data or to compare the locations of two populations using two matched samples. In this case, the related groups are the measurements taken from the same subjects under contrasting drug administration conditions.

For both drug administration methods (injection and osmotic pump), the means of all values corresponding to DCZ and Saline were calculated. The Wilcoxon signed-rank test was then used to determine if the effects of DCZ versus saline were significantly different.

The Wilcoxon signed-rank test works through several key steps:

1. Calculating differences between the paired observations for the two conditions (in this case, the paired observations were means of all valid datapoints corresponding to the given drug administered).

2. Ranking these differences based on their absolute values, disregarding the sign (positive or negative).

3. Reintroducing the signs to the ranks based on whether the post-score was higher or lower than the pre-score.

4. Calculating the W-Statistic by summing the ranks for positive differences (W+) and for the negative differences (W−). The W-statistic is the smaller of these two sums:

$$W = \min(W+, W-)$$

5. Using the calculated W-statistic (p value of the performed statistical test is evaluated using MATLAB "*signrank"* function), assess whether the observed differences are significant, based on a predefined significance level (e.g., p = 0,05).[34]

The significance level was set at the standard p = 0,05, meaning that if the test produces a p-value below this threshold, it supports the hypothesis that the two drugs administered have statistically significantly different effects.
In this thesis, the Wilcoxon signed-rank test was computed using the built-in *"signrank"* MATLAB function. This function facilitates the application of the test by automatically calculating the ranks and the corresponding p-value, ensuring accurate and efficient analysis of the data.

2.6 Hypotheses

The hypotheses outlined below serve as foundational assertions guiding the investigation.

Empirical Hypotheses:

- DCZ treatment will lead to a reduction in the frequency of interictal epileptiform discharges (IEDs), indicative of its antiepileptic potential.
- DCZ treatment will demonstrate a reduction in seizure frequency compared to the periods when saline was administered, supporting its efficacy in mitigating seizures.

3 Results

3.1 Seizure and IED frequency

3.1.1 Mice with the injection drug administration only

As previously mentioned, the experimental animals in this thesis are divided into two main groups. The first group, consisting of one male and two female animals, includes mice FCD024, FCD025, and FCD026, which received the tested substances via injection once daily at approximately 8 AM. Because the expected effect of DCZ on the number of detected IEDs was estimated to last only up to four hours post-injection, only this four-hour window was analyzed and plotted in the graphs. Analyzing the hours leading up to the injection or too long after it was deemed unnecessary.

For some animals, the provided signal files showed considerable periods of time (ranging from a few hours to full days in couple of cases) with the missing signal caused by either system malfunction, animal accidentally detaching from the signal monitoring station, or the animal being involved in some other experiment at the given time. This fact has been accounted for as ignoring this fact would seriously affect the accuracy of the results and the statistics presented. Therefore, all datapoints have been standardized by the number of valid seconds in the given time bin.

Overall, the DCZ administration via injection did not prove efficient in substantially lowering the number of neither daily seizures, nor IEDs in a post-injection period. In some of the figures mentioned below, data sometimes shows a vague decrease in IED frequency during the DCZ administration periods, while in some other places, surprisingly, the saline treatment seems to be more efficient. Based on these findings, the success of lowering the IED and seizure frequency using DCZ administration via injection has showed to be inconclusive. The possible reasons why this approach was not more successful are analyzed in the chapter 4. Discussion.

The discrepancy in the naming style of the tested animals arises from the research facility's change in the system of keeping animal records. Older animals were named "FCD..." whereas newer animals (where both, injections and osmotic pumps were tested) were named "SK...". I use the animal names as they were used at the research facility for easier communication with the rest of the team.

Below, I present the figures of seizure and IED frequency data corresponding to the given mice and with a brief description of the results. Moving average displayed in the figures was calculated using the built-in MATLAB functions *"nanmean"* (calculating the mean of the datapoints, while handling the periods of the invalid data) and *"movmean"* (calculating the moving average, typically with a window size of 5).

- **FCD024**

The top graph in Figure 8 shows the daily seizure frequency for animal FCD024. The data fluctuates significantly with no apparent reduction in seizure frequency following DCZ administration. Peaks and troughs occur roughly every few days, but DCZ does not seem to reduce these fluctuations compared to saline injections or no drug administration. This suggests that DCZ does not effectively inhibit seizures under the conditions tested.

The bottom graph in Figure 8 displays the IED frequency for animal FCD024. The IED frequency shows considerable variability, with no consistent decrease following DCZ administration. The data for DCZ, saline injections, and no drug administration overlap significantly, indicating no clear reduction in IED frequency due to DCZ. These findings suggest that DCZ is not effective in inhibiting interictal epileptiform discharges in this mouse.

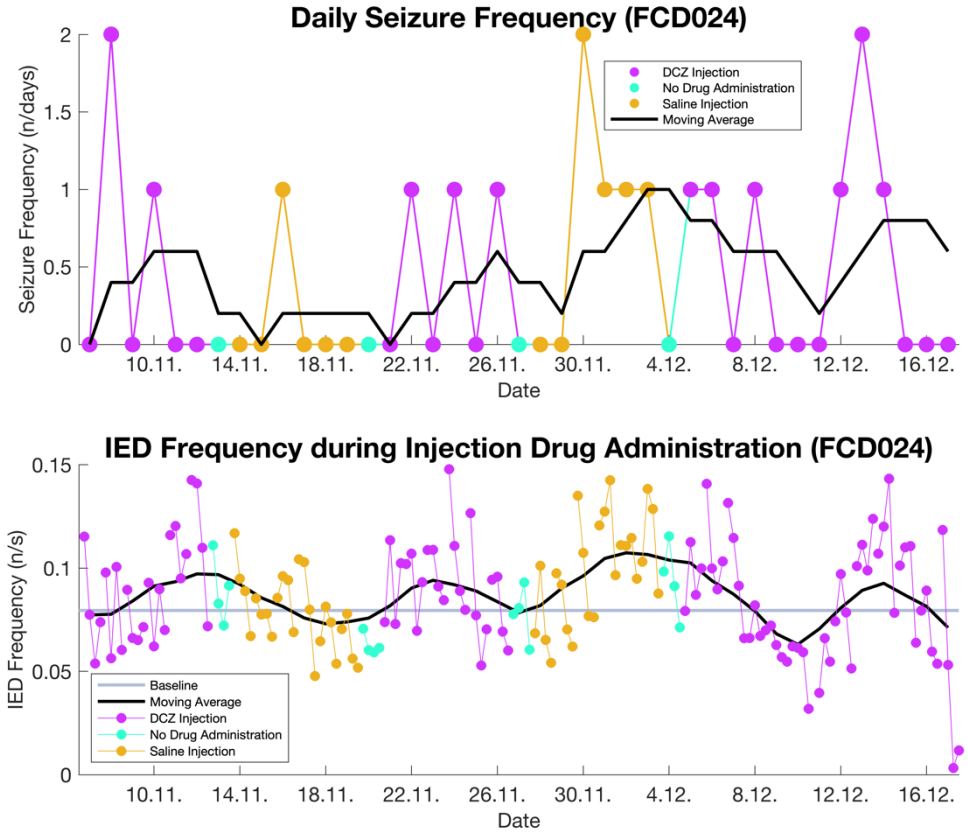

Figure 8: Seizure frequency (n/days) and IED frequency (n/s) of the animal FCD024. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

- **FCD025**

The top graph in Figure 9 presents a significant variability in seizure occurrences over the recorded period. Initially, from 2.11. to about 14.11., seizure frequencies are low with some sporadic increases. However, there is a notable spike around 26.11. where seizure frequency increases significantly, particularly after saline injections, reaching a peak of approximately 20 seizures per day.
Throughout the graph, it is apparent that the administration of DCZ does not consistently reduce the seizure frequency compared to the saline injections or periods of no drug administration.

The IED frequency in the bottom graph in Figure 9 demonstrates considerable variability, with distinct peaks and troughs. Significant gaps between otherwise connected datapoints were caused by invalid signal sometimes spanning a few days, which was omitted during the analysis. Initially, from 2.11. to 8.11., there is a high frequency of IEDs, particularly during DCZ administration. After 8.11., there is a brief period with low IED frequencies corresponding to no drug administration. From 11.11. onwards, the frequency increases again, peaking around 20.11., similar to the pattern observed in the seizure frequency graph. The overlap of data points for DCZ, saline injections, and no drug administration indicates that DCZ does not consistently reduce the IED frequency.

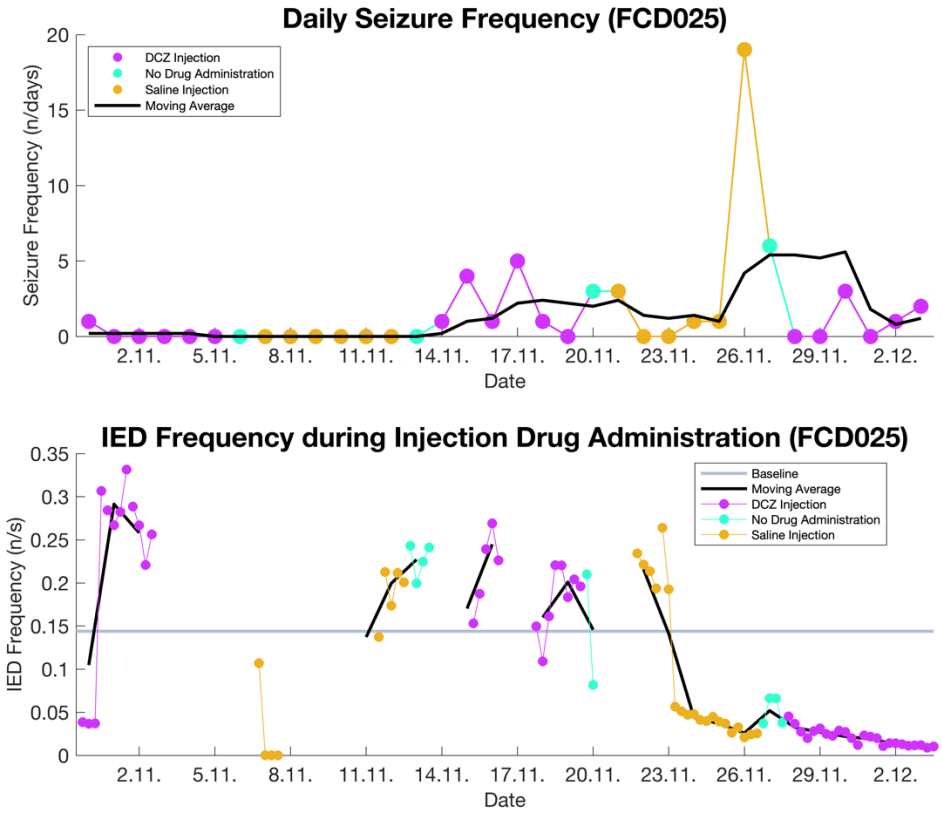

Figure 9: Seizure frequency (n/days) and IED frequency (n/s) of the animal FCD025. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

- **FCD026**

The top graph in Figure 10 displays the daily seizure frequency for animal FCD026. The data reveals that seizure frequency varies over time but remains generally low, with a few notable peaks. On 4.11., there is an initial spike in seizure frequency, which then decreases significantly. Following this, seizure frequency remains low and relatively stable with minor fluctuations especially during saline injections.
The moving average shows a general downward trend with some minor fluctuations, suggesting a reduction in seizure frequency over time. However, there is no clear evidence that DCZ administration effectively reduces seizure frequency compared to saline injections or no drug administration.

The bottom graph in Figure 10 shows variability with occasional peaks and troughs. Initially, from 31.10. to 2.11., the IED frequency is low during DCZ administration. Later, the frequency mainly fluctuates, showing higher variability during saline injections and some periods of no drug administration. Although the IED frequency gradually decreases over time it does not satisfyingly prove the efficacy of DCZ treatment as the frequency drop might relate just to monthly seizure patterns[35] documented in many epilepsy-focused studies (further explained in the chapter 4. Discussion).

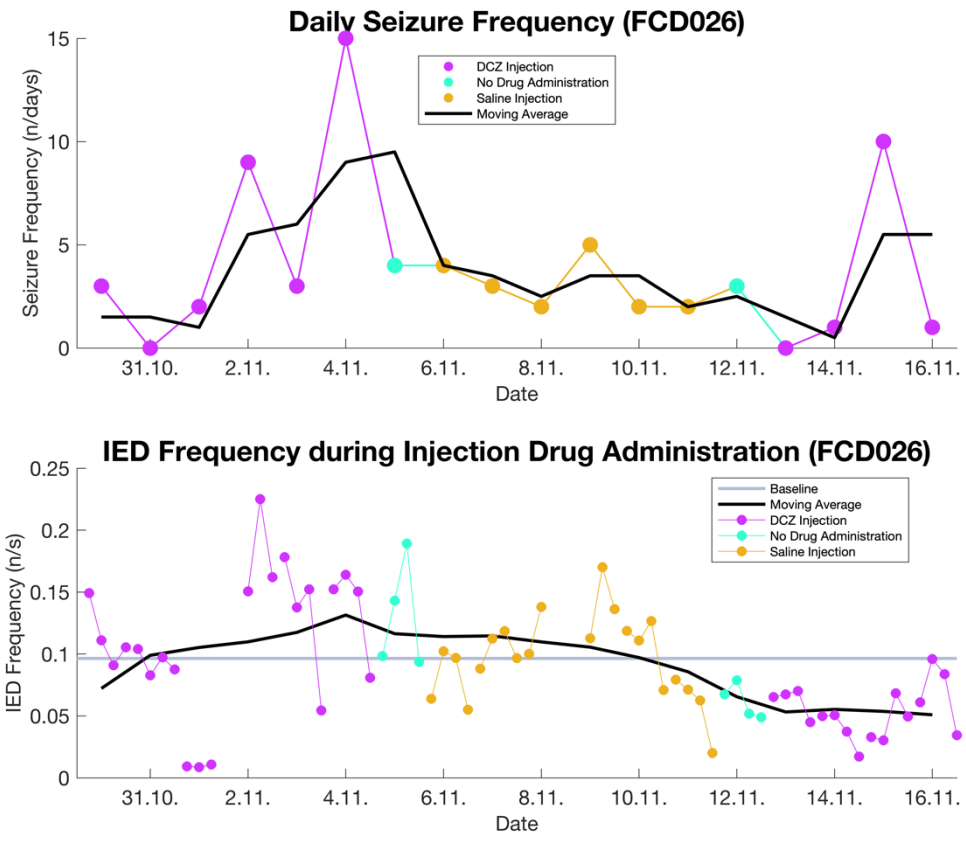

Figure 10: Seizure frequency (n/days) and IED frequency (n/s) of the animal FCD026. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs

3.1.2 Mice with combined drug administration

The second group of animals in this experiment comprises four male subjects: SK000645, SK000652, SK000918, and SK001071. These animals received the tested drugs through both, injections and osmotic pumps. Their monitoring included a considerably long control period (spanning more than 30 days for some) before any drug treatment began. Analyzing the signal data from this control period allowed for a more precise determination of the baseline IED frequency for each animal by averaging the IED rates from the baseline period.

Notably, the order of drug administration and the methods applied were varied among the animals to assess whether different experimental setups produced significantly different results. Furthermore, these mice were monitored for a much longer duration than those in subchapter 3.1.1., undergoing several rounds of drug treatment. Each drug was administered once via osmotic pump (lasting 7 days) and once or twice via injection (lasting 5 days each on average).

Drug administration via the osmotic pump seemed quite promising at first, as it releases the tested substance into the body over a longer period of time, which theoretically promises better inhibitory effect of the drug in question. Although periods of DCZ administration via osmotic pump showed a slight decrease in IED and seizure frequency in some animals, the results were not convincing. Both frequencies generally remained unaffected regardless of the treatment or the method used.

As mentioned in subsection 3.1.1., all data points were standardized by the number of valid seconds in the given time bin. This standardization accounted for occasional signal loss due to animals disconnecting their head bars, malfunctions, or being taken away for other experiments. The following pages will present figures of seizure and IED frequency data for each mouse, along with brief descriptions of the results.

- **SK000645**

For animal SK000645 (Figure 11), both daily seizure frequency and IED frequency exhibit a distinct pattern over time. Initially, frequencies are low and stable, followed by a significant increase during the saline and DCZ pump periods around late December. After this peak, both seizure and IED frequencies drop sharply and remain low during subsequent periods of no drug administration, saline injections and DCZ injections.

The data suggests that while there is a notable rise in seizure and IED frequencies during the "no drug administration" period, these frequencies decrease significantly afterward and remain low regardless of the drug administered. Sudden increase in both seizure and IED frequency in the control period followed by similarly sudden drop of frequency is probably caused by some form of seizure clustering [35].

Overall, the figure indicates that DCZ administration, both via pump and injection, does not consistently reduce seizure or IED frequencies in this animal model, with the observed decreases potentially attributable to factors other than the DCZ administration itself, e.g. spontaneous fluctuations in seizure and IED rates.

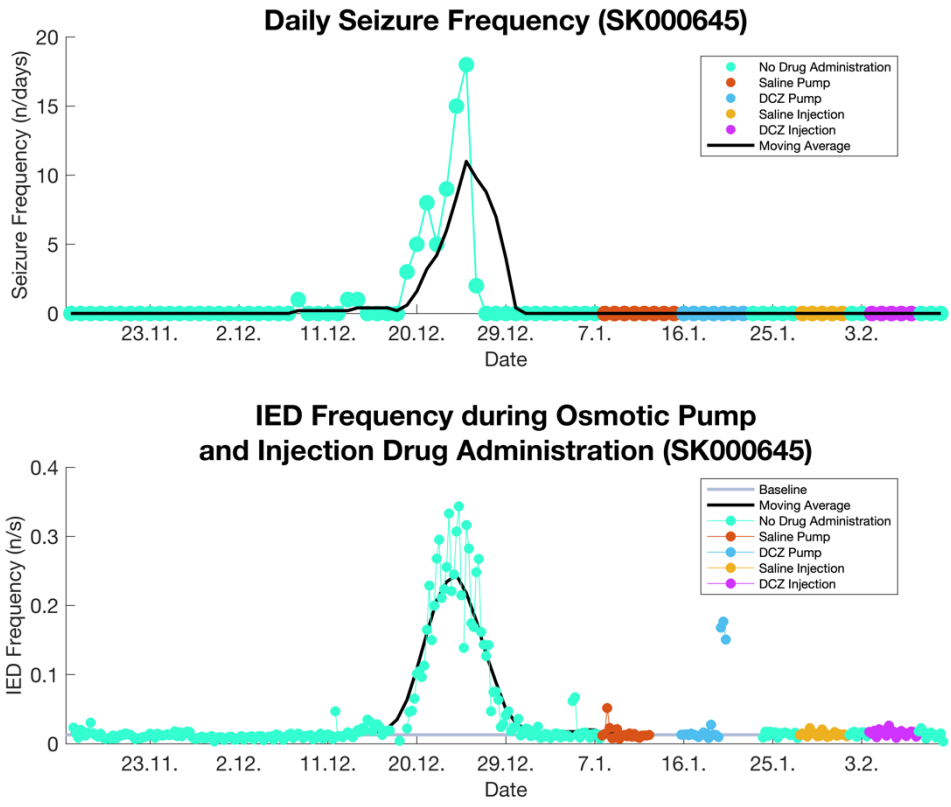

Figure 11: Seizure frequency (n/days) and IED frequency (n/s) of the animal SK000645. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

Figure 12 shows the seizure and IED frequency of SK000645 from the beginning of January onwards in greater detail, however, during this period, no seizures were detected and even IED frequency remains very low:

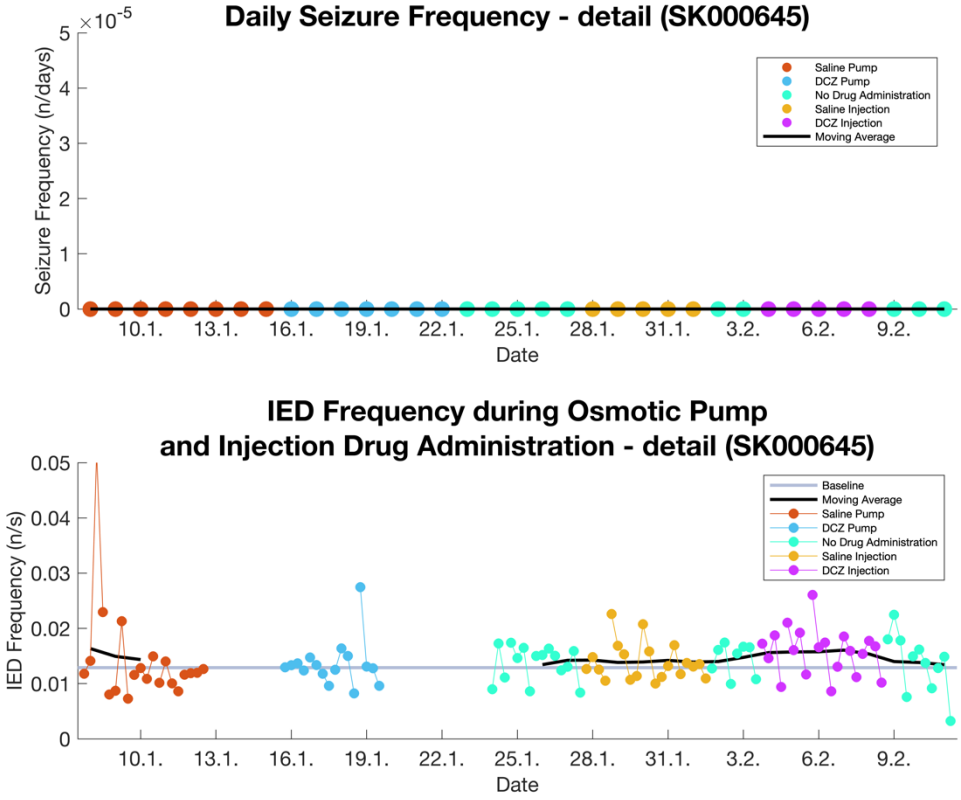

Figure 12: Detail of seizure frequency (n/days) and IED frequency (n/s) of the animal SK000645.

- **SK000652**

For animal SK000652 (Figure 12), both daily seizure frequency and IED frequency exhibit distinct patterns over time. Initially, frequencies are low and stable, followed by significant increases during the DCZ pump and saline pump periods. Peaks in seizure and IED frequencies are observed towards the end of December and early January. Subsequently, frequencies decrease with ongoing fluctuations during the DCZ injection period and stabilize somewhat with smaller variations towards early February.

The data suggests that DCZ administration, whether via pump or injection, does not consistently reduce seizure or IED frequencies in this mouse. The trends indicate that while there are periods of increased frequency associated with both DCZ and saline pumps, the frequencies decrease and stabilize over time regardless of the drug administration method.

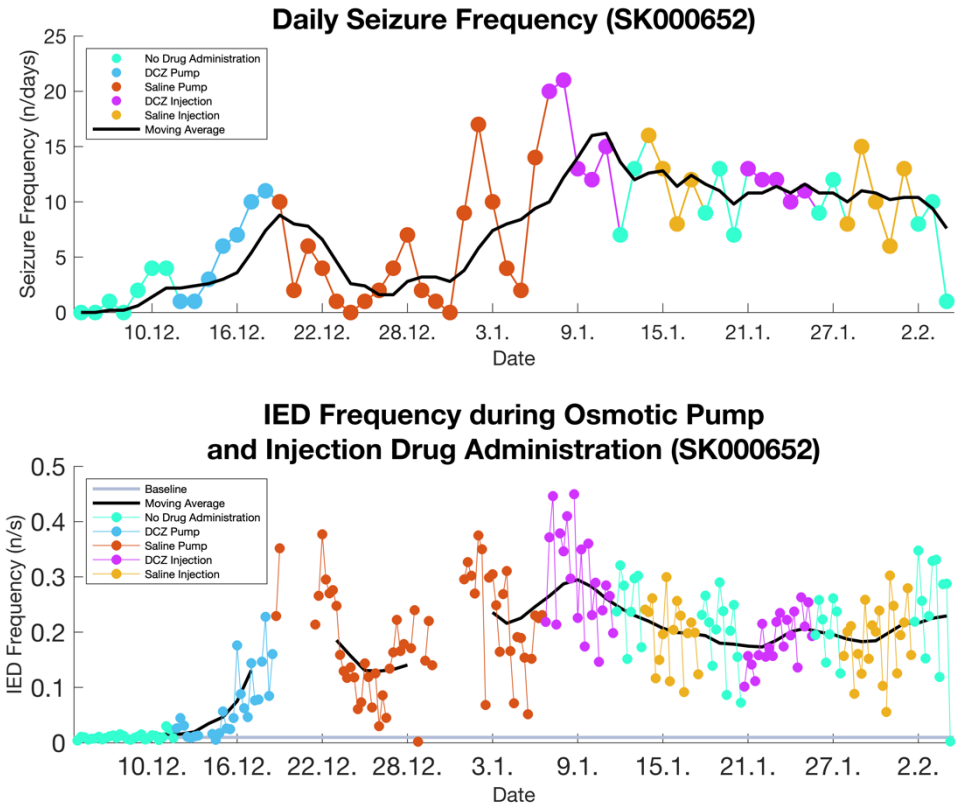

Figure 13: Seizure frequency (n/days) and IED frequency (n/s) of the animal SK000652. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

- **SK000918**

For animal SK000918 (Figure 13), both daily seizure frequency and IED frequency exhibit a certain pattern over time. Initially, frequencies are low and stable during the "no drug administration" period, but they significantly increase during the DCZ pump and saline pump periods. Peaks in seizure and IED frequencies are observed in late January and mid-February. Following this, there is a noticeable decrease in frequencies during the DCZ injection period, with values eventually stabilizing towards the end of February.

The data suggests that DCZ administration, whether via pump or injection, does not consistently reduce seizure or IED frequencies in this animal model. While there are some increases in frequency during both the DCZ and saline pump periods, the frequencies decrease and stabilize over time, regardless of the drug administration method employed. This indicates that DCZ may not have a sustained inhibitory effect on seizure or IED frequencies.

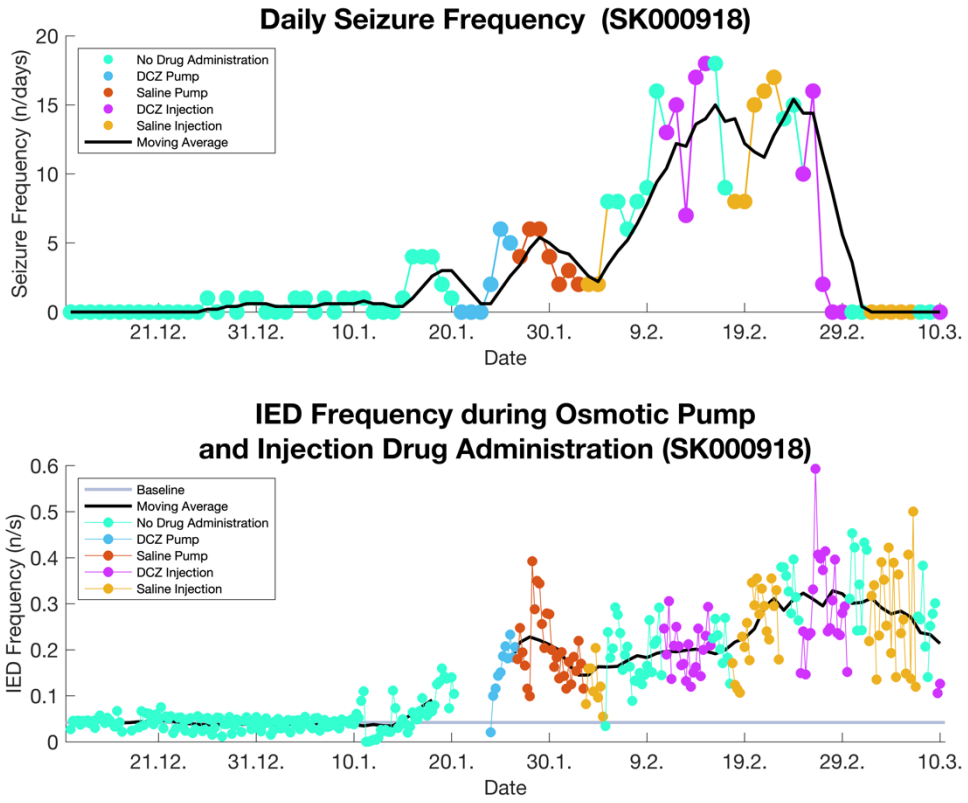

Figure 14: Seizure frequency (n/days) and IED frequency (n/s) of the animal SK000918. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

- **SK001071**

For animal SK001071 (Figure 14), both daily seizure frequency and IED frequency show varying patterns over time. Initially, frequencies are relatively low, with only minor periodical fluctuations during the "no drug administration" period. There is a noticeable increase in seizure and IED frequencies during the DCZ injection period in late February and early March. Peaks are observed during these periods, followed by a slight decrease and stabilization during the saline pump phase. Another increase is seen during the DCZ pump phase towards the end of March.

The data indicates that DCZ administration, whether via injection or pump, does not consistently reduce seizure or IED frequencies in this animal model. Despite fluctuations associated with different treatment periods, there is no clear evidence of a sustained inhibitory effect of DCZ on seizure or IED frequencies, as values increase and stabilize independently of the drug administration method. Interestingly, there is a marked decrease in seizure and IED rates during the saline pump period, which could be attributed to spontaneous fluctuations, the slight increase in salt and water in the mice, the distress caused by the implant, or merely coincidence.

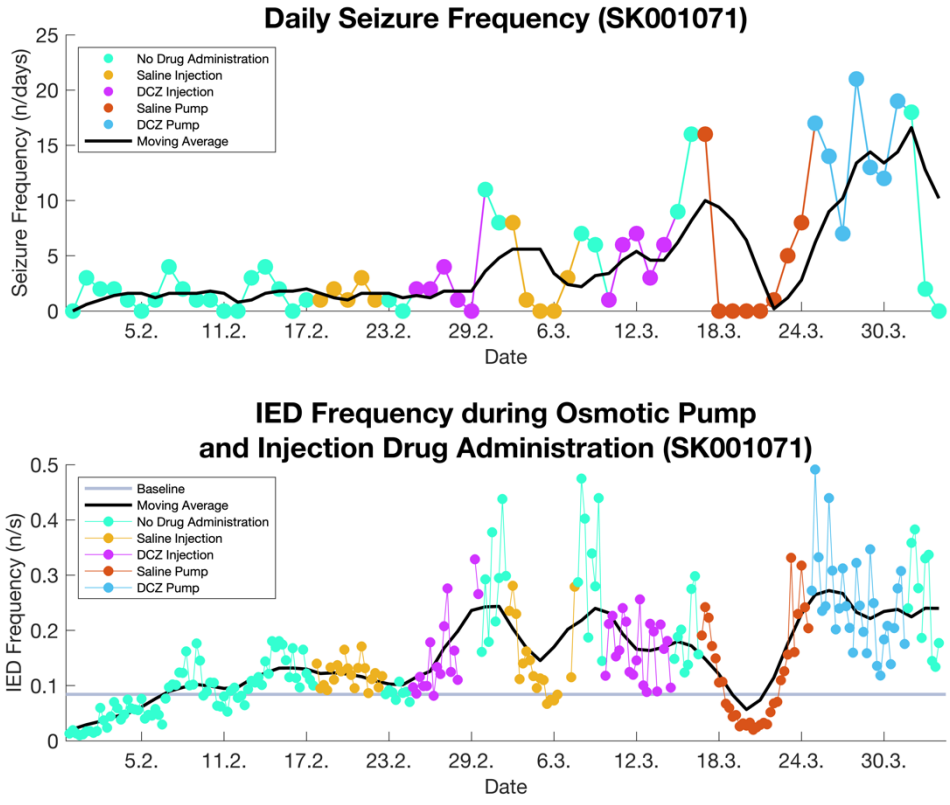

Figure 15: Seizure frequency (n/days) and IED frequency (n/s) of the animal SK001071. Neither of the graphs displayed shows provable efficacy of DCZ in mitigating seizures or IEDs.

3.2 Population graphs

After analyzing the detected seizures and IEDs, the next step involved plotting population graphs to better illustrate the efficacy of the executed experiment. The boxplots presented in paragraphs 3.4.1 and 3.4.2 include the means of all data points (one data point per animal and drug) corresponding to the given drug and administration method. All DCZ administration means were standardized by saline administration means to better present the relative increase or decrease in seizure/IED frequency during DCZ treatment.

These boxplots serve to visually summarize the data, providing a clear comparison between the effects of DCZ and saline treatments. Despite the detailed analysis and graphical representation, the results did not demonstrate any significant efficacy of DCZ treatment. Specifically, the injection DCZ treatment showed no success in mitigating either seizures or IEDs. For osmotic pump administration, only 50% of the tested animals exhibited a decline in both researched frequencies. However, these results still fail to substantiate the pronounced hypotheses regarding the effectiveness of DCZ.

Starting on the following page, you will find the population graph of seizure and IED frequency, displaying the comparison between the DCZ and saline treatment periods. This visual comparison aims to provide a comprehensive overview of the data and facilitate a better understanding of the experimental outcomes.

3.2.1 Population graphs of seizure frequency

The boxplot in Figure 15 illustrates the comparison between DCZ and saline in terms of seizure frequency. The results reveal that seizure frequency is somewhat higher during DCZ administration compared to saline. The data points for DCZ are spread across a wider range and then those for saline, indicating increased seizure activity with DCZ treatment.
The variability within the DCZ data set suggests heterogeneous responses among the subjects, which further complicates the assessment of DCZ's efficacy.

The consistent clustering of lower seizure frequencies around the saline data point underscores that saline seems to be more effective in maintaining reduced seizure frequencies, although this would not probably be the objective interpretation of the statistics. Overall, this boxplot suggests that DCZ not only fails to decrease seizure frequency but seems like intensifying it. However, it is impossible to tell whether the observed increase is due to the treatment or due to the spontaneous slow fluctuations in epileptic activity with the treatment having no effect.

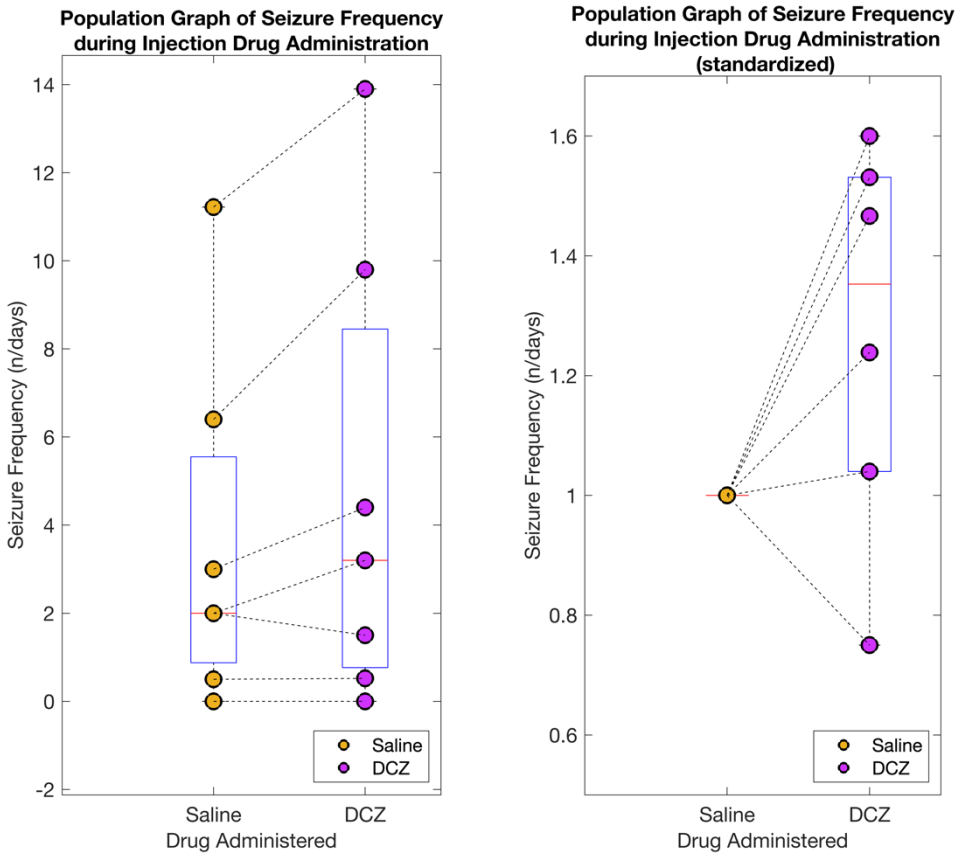

Figure 16: Population graph of seizure frequency during injection drug administration. Boxplot shows a slight increase in number of detected seizures surprisingly during the periods of DCZ administration.

Figure 16 illustrates that seizure frequency during osmotic pump drug administration remained mostly the same in both drug treatments. That suggests, that even DCZ treatment had no notable effect of decreasing the seizure frequency, as the median value stays roughly the same regardless of the drug used.

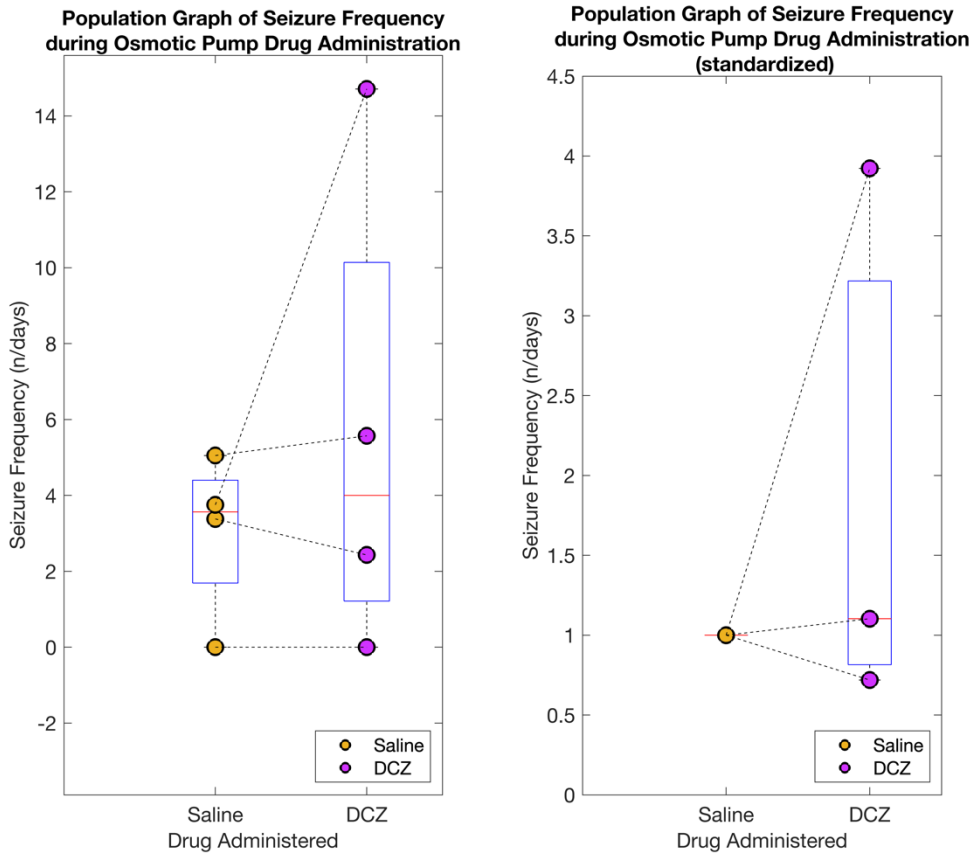

Figure 17: Population graph of seizure frequency during osmotic pump drug administration. Almost all data pairs of both DCZ and saline administration remain around the median, revealing no evident efficiency of DCZ treatment.

Table 1 below provides values of the concerned datapoints and their ratio for all tested animals.

Table 1: Mean values of seizure frequencies and their frequency ratios (DCZ/saline) during both types of treatment.

ANIMAL		FCD024		FCD025		FCD026		SK000645		SK000652		SK000918		SK001071	
ADMINISTRATION METHOD		INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP
MEAN VALUES OF FREQUENCIES	DCZ	0,52		1,50		4,40		0	0	13,90	5,57	9,80	2,43	3,20	14,71
	SALINE	0,50		2,00		3,00		0	0	11,22	5,05	6,40	3,38	2,00	3,75
FREQUENCY RATIO		1,04		0,75		1,47		0	0	1,24	1,10	1,53	0,72	1,60	3,92

Abbreviations: INJ – drug administration via injection, PUMP –drug administration via osmotic pump, DCZ –DCZ treatment, saline –saline treatment

Table 2 includes median of frequency ratios from Table 1, the interquartile range of mean values and medians that correspond with the given drug administration method and the substance tested.

Table 2: Median of mean differences (seizure data) of the contrasting drugs administered, interquartile range and median of mean values corresponding with the given drug administration method and the substance tested.

MEDIAN OF FREQUENCY RATIOS		INTERQUARTILE RANGE				MEDIAN OF FREQUENCY MEANS			
INJ	PUMP	INJ		PUMP		INJ		PUMP	
		DCZ	SALINE	DCZ	SALINE	DCZ	SALINE	DCZ	SALINE
1,24	0,91	7,69	4,68	8,93	2,71	3,2	2	4	3,57

Abbreviations: INJ – drug administration via injection, PUMP –drug administration via osmotic pump, DCZ –DCZ treatment, saline –saline treatment

3.2.2 Population graph of IED frequency

The boxplot in Figure 17 compares the efficacy of the drug used during injection treatment. Each point is standardized by the saline data, providing a clear idea of the relative effects of each treatment. The IED frequency is higher in exactly half of the mice when DCZ is administered, as shown by the data points clustered above the saline treatment values. The absence of any consistent trend indicates that DCZ does not effectively reduce IED frequency. The variability within the DCZ data set, as indicated by the spread of the points, suggests inconsistent responses among the subjects.

The overall inference from this boxplot is that saline appears to maintain slightly lower IED frequencies compared to DCZ, questioning the efficacy of DCZ as a treatment for reducing IEDs.

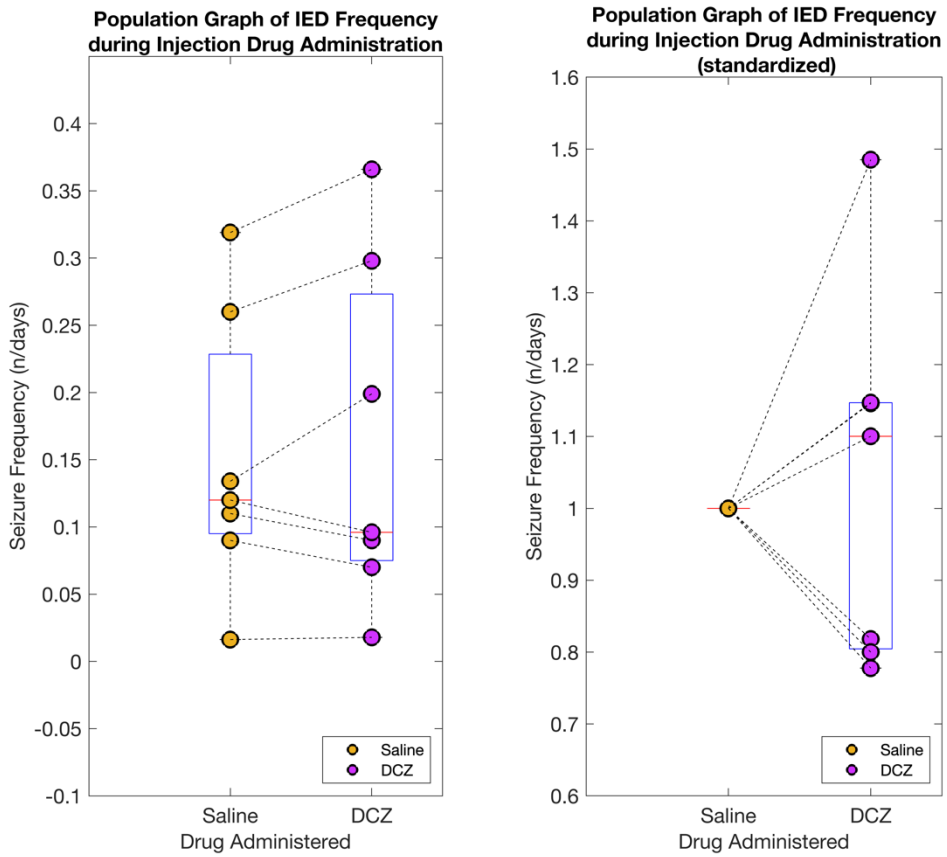

Figure 18: Population graph of IED frequency during injection drug administration. DCZ data means are evenly distributed with three DCZ means showing certain decrease in IED frequency when compared to saline administration period, while other showing the exact opposite and thus supporting an inconclusive statistical result.

The population graph in Figure 18 compares the IED frequency between DCZ and saline treatments when administered via an osmotic pump. The IED frequency is significantly higher with DCZ administration, as indicated by the elevated position of the data points compared to saline. The spread of the DCZ data points shows substantial variability, suggesting inconsistent efficacy among the subjects. In contrast, the saline treatment results in lower IED frequencies in half of the mice. Overall, the boxplot suggests that DCZ, when administered through an osmotic pump, does not reduce IED frequency very effectively, highlighting a limitation in DCZ's potential as a treatment for IEDs.

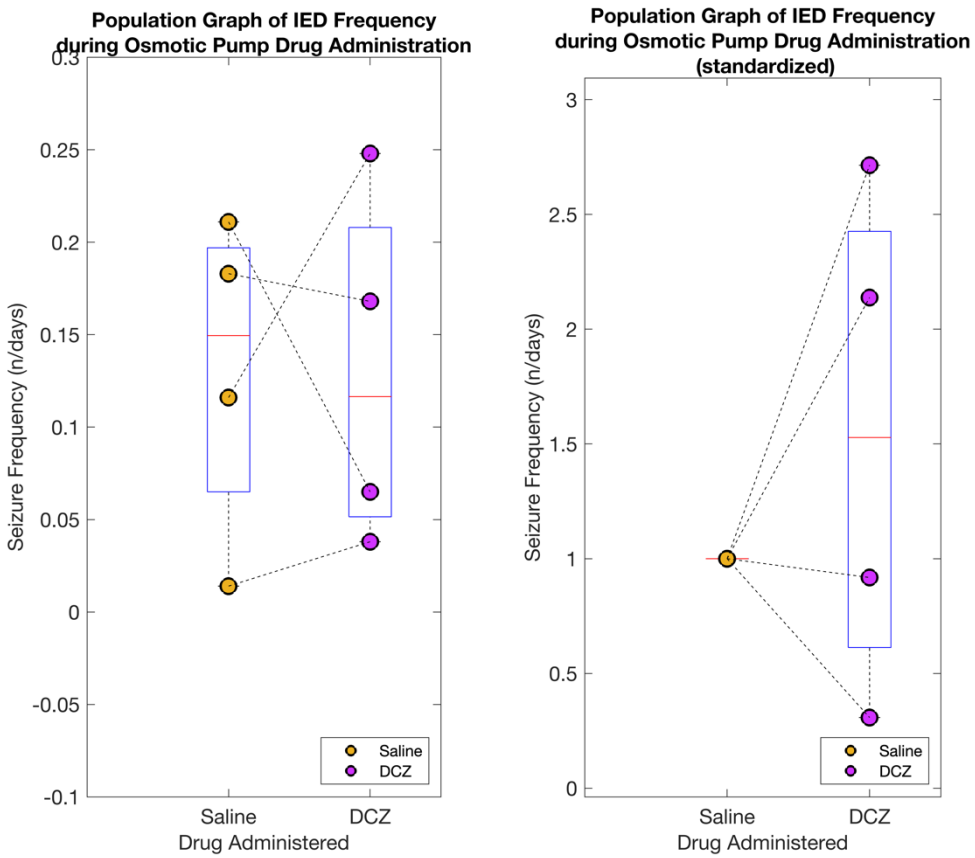

Figure 19: Population graph of IED frequency during osmotic pump drug administration. DCZ data means are evenly distributed around the mean, with only one DCZ mean showing a significant decrease in IED frequency, while 2 other support rather the exact opposite.

Table 3 below provides values of the concerned datapoints and their ratio for all tested animals.

Table 3: Mean values of IED frequencies and their frequency ratios (DCZ/saline) during both types of treatment.

ANIMAL		FCD024		FCD025		FCD026		SK000645		SK000652		SK000918		SK001071	
ADMINISTRATION METHOD		INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP	INJ	PUMP
MEAN VALUES OF FREQUENCIES	DCZ	0,07		0,09		0,09		0,02	0,04	0,29	0,07	0,37	0,17	0,19	0,25
	SALINE	0,09		0,11		0,12		0,02	0,01	0,26	0,21	0,32	0,18	0,13	0,12
FREQUENCY RATIO		0,78		0,82		0,75		1	2,71	1,12	0,33	1,16	0,94	1,46	2,08

Abbreviations: INJ – drug administration via injection, PUMP –drug administration via osmotic pump, DCZ –DCZ treatment, saline –saline treatment

Table 4 includes median of differences from Table 3, the interquartile range of mean values and the median values that correspond with the given drug administration method and the substance tested.

Table 4: Median of mean differences (IED data) of the contrasting drugs administered, interquartile range and median of mean values corresponding to the given drug administration method and the substance tested.

MEDIAN OF DIFFERENCES		INTERQUARTILE RANGE				MEDIAN OF FREQUENCY MEANS			
INJ	PUMP	INJ		PUMP		INJ		PUMP	
		DCZ	SALINE	DCZ	SALINE	DCZ	SALINE	DCZ	SALINE
1,15	1,53	0,20	0,13	0,16	0,13	0,10	0,12	0,12	0,15

Abbreviations: INJ – drug administration via injection, PUMP –drug administration via osmotic pump, DCZ –DCZ treatment, saline –saline treatment

3.3 Evaluation by the Wilcoxon signed rank test

Wilcoxon signed rank test is evaluated by using MATLAB built-in function called *"signrank"* and the computational process is executed using the custom scripts called *"statistics_seizures.m"* (for seizure data) and *"statistics_IED.m"* (for IED data).

For the given drug administration method currently analyzed, the script calculates the ratios of the frequency means during DCZ treatment and during the saline treatment period.

Subsequently, number 1 is subtracted from frequency ratio vector of values to achieve signed vector of ratio values. Lastly, the median of these values is found.

Following this, *"signrank"* function is called on the frequency ratios in the form

```
[p,h,stats] = signrank(___)
```

and consists of the following parts:

- **p:** This is the p-value of the test. The p-value indicates the probability of obtaining test results at least as extreme as the observed results, under the null hypothesis. A low p-value (p = 0,05 used in this analysis) suggests that you can reject the null hypothesis.

- **h:** This is the test decision, returned as 1 or 0.
  If h = 1, it indicates that the test rejects the null hypothesis at the significance level specified (p = 0,05 here).
  If h = 0, it means that the test fails to reject the null hypothesis.

- **stats:** This is a structure containing additional statistics from the test. The structure includes fields like:
  → **zval:** The value of the test statistic.
  → **signedrank:** The sum of the ranks of the positive differences between paired observations

3.3.1 Seizure statistics

As displayed in Table 5, the Wilcoxon signed rank test on seizure frequency means indicated that we failed to reject the null hypothesis (H0), suggesting that the administration of DCZ had no significant seizure-mitigating effect since

$$p_{injection} > p_{H_0}$$

and

$$p_{osmotic\ pump} > p_{H_0}$$

if $p_{H_0} = 0{,}05$ as stated in the subsection 2.5.5..
therefore:

- given $p_{injection} = 0{,}22$, I fail to reject the null hypothesis (H_0). This suggests that there is insufficient evidence to conclude a significant difference between the paired samples. Therefore, the experiment does not demonstrate a statistically significant effect.

- given $p_{osmotic\ pump} = 1{,}00$, I fail to reject the null hypothesis (H_0). This suggests that the observed differences between the paired samples are entirely consistent with random chance, and the experiment does not demonstrate any statistically significant effect."

However, this result does not confirm the null hypothesis; my results are inconclusive, meaning I cannot be certain about the effect of DCZ. Nevertheless, these results are valuable as they highlight the need to repeat and possibly modify the experiment for further investigation. Among the two drug administration methods tested, the injection one seems to have a theoretically at least some seizure-mitigating effect but with 22 % chance of it being only a coincidence, it is still very unconvincing result.

The reasons why the experimental results do not support the null hypothesis (H_0), along with suggestions for improving future experiments, are examined in greater detail in the chapter 4. Discussion.

Table 5: Evaluation of the Wilcoxon signed rank test for seizure frequency means. Given $\boldsymbol{p_{H_0} = 0{,}05}$, neither administration method was proved to be efficient in lowering the seizure frequency.

DRUG ADMINISTRATION METHOD	INJECTION	OSMOTIC PUMP
p	0,16	0,75
h	0	0
TEST STATISTIC	18,00	4,00

3.3.2 IED statistics

As displayed in the Table 6, executing the Wilcoxon signed rank test on seizure frequency means proved, that administration of DCZ had no seizure-mitigating effect, since

$$p_{injection} > p_{H_0}$$

and

$$p_{osmotic\ pump} > p_{H_0}$$

if $p_{H_0} = 0{,}05$ as stated in the subsection 2.5. 5..
therefore:

- given $p_{injection} = 0{,}94$, I fail to reject the null hypothesis (H_0). This suggests that there is very strong evidence to conclude a significant difference between the paired samples. Therefore, the experiment does not demonstrate a statistically significant effect.

- given $p_{osmotic\ pump} = 0{,}63$, I fail to reject the null hypothesis (H_0). This suggests that there is substantial evidence that the observed differences between the paired samples could be due to random chance, and the experiment does not demonstrate a statistically significant effect.

The reasons why the experimental results do not support the null hypothesis (H_0), along with suggestions for improving future experiments, are examined in greater detail in the chapter 4. Discussion.

Table 6: Evaluation of the Wilcoxon signed rank test for IED frequency means. Given $p_{H_0} = 0{,}05$, neither administration method was proved to be efficient in lowering the IED frequency.

DRUG ADMINISTRATION METHOD	INJECTION	OSMOTIC PUMP
p	0,94	0,63
h	0	0
TEST STATISTIC	13,00	7,00

4 Discussion

This study aimed to evaluate the efficacy of chemogenetic treatment using hM4Di and DCZ ligand in reducing seizure and IED frequencies using injection and osmotic pump administration methods. Despite the sophisticated experimental design, the results demonstrated no significant reduction in either seizure or IED frequencies in response to the DCZ treatment. These negative results present a challenge to interpret objectively due to the variety of potential influencing factors.

Several methodological considerations might explain the lack of significant findings. Firstly, it is possible that the treatment was not administered optimally. For instance, there may have been too weak an expression of hM4Di receptors, or the dosage of DCZ might have been insufficient to elicit a therapeutic effect (although Nagai et al. reported behavioral effects at this dose[32]). Moreover, errors in data evaluation could have impacted the results.
The seizure and IED detection algorithms may have had many false positives, or the analysis could have focused on incorrect time periods, skewing the findings (DCZ injections were estimated to have at least some effect approximately during 4 hours after the drug administration[21]).

The results might also be influenced by factors that were not fully accounted for in the thesis. Circadian rhythms can significantly impact epileptic activity, with fluctuations in seizure susceptibility and frequency occurring at different times of the day. Seizure clustering, where seizures occur in rapid succession followed by periods of quiescence, could also obscure the effects of treatment. Additionally, daily and monthly rhythms of epileptic activity might mask or mimic treatment effects, complicating the interpretation of the data.[35], [36]

Efforts were made to mitigate at least some of the interpretational issues.
The methodology employed precise dosing and administration schedules to ensure consistent delivery of DCZ. Trusted algorithms were used to minimize false positives and accurately identify IEDs. However, the complexity of epilepsy and its varying response to treatment highlight the difficulty in obtaining clear results.

The findings suggest that, under the conditions tested, DCZ is not effective in reducing seizure or IED frequencies. This underscores the need for further research to determine the conditions under which DCZ might be effective. While the dosages used in this study were appropriate, the primary challenge appears to be ensuring sufficient expression of hM4Di in the target cell population. Future studies should focus on improving the methods of hM4Di transduction or exploring alternative chemogenetic technologies, such as PSAM-PSEM[37]. Additionally, investigating different drug delivery methods and combining DCZ with other treatments could provide further insights into its potential therapeutic effects.

Moreover, future research should incorporate a more comprehensive methodology to account for the rhythms and other temporal patterns of epileptic activity. This could involve continuous, longer-term monitoring to better capture the variability and clustering of seizures, potentially revealing subtle treatment effects that shorter studies might miss. On the other hand, prolonged expression of the hM4Di receptor may be toxic to cells, potentially leading to the death of the cells carrying the hM4Di. This cell death would render DCZ ineffective due

to the absence of its substrate. Therefore, striking a balance between monitoring duration and receptor expression levels is crucial for optimizing treatment efficacy and minimizing adverse effects.

Overall, while this study does not support the efficacy of DCZ in reducing seizure and IED frequencies, it provides valuable data that can inform future research efforts. By addressing the limitations and potential complicating factors identified here, subsequent studies can build on these findings to develop more effective treatments for epilepsy.

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