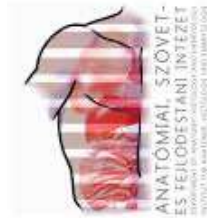Doctoral School of Theoretical Medicine, University of Szeged, Hungary

Albert Szent-Györgyi Medical School, University of Szeged, Hungary

Department of Anatomy, Histology and Embryology

Characterisation of Hippocampal Sclerosis and the Assessment of the Kynurenic Acid Analogue SZR104 in a Murine Model of Pilocarpine-induced Epilepsy

Short summary of the Ph.D. Thesis

Adrienne Mátyás, M.Sc.

Supervisor:

András Mihály, M.D., Ph.D., D.Sc.

Professor Emeritus

Szeged

2024

Doctoral School of Theoretical Medicine, University of Szeged, Hungary

Albert Szent-Györgyi Medical School, University of Szeged, Hungary

Department of Anatomy, Histology and Embryology

Characterisation of Hippocampal Sclerosis and the Assessment of the Kynurenic Acid Analogue SZR104 in a Murine Model of Pilocarpine-induced Epilepsy

Short summary of the Ph.D. Thesis

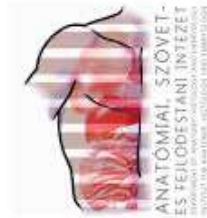**Adrienne Mátyás, M.Sc.**

Supervisor:

András Mihály, M.D., Ph.D., D.Sc.

Professor Emeritus

Szeged

2024

I dedicate this thesis to my parents, my husband, and my daughters.

Introduction

Epilepsy is a chronic, noninfectious disease of the brain that affects people of all ages worldwide, and is also a complex social and economic problem. Epilepsy has now become the second most common neurological disease globally after stroke. In epilepsy, abnormal electrical activity of the brain leads to seizures that can recur, often spontaneously and without warning. Current evidence suggests that hypersynchronous neuronal discharges play a crucial role in the development of seizures, which eventually cause significant destruction of the sensitive neuronal cell population. The combined abnormal interaction between the excitatory and inhibitory systems is crucial in the generation and termination of epileptic seizures. Understanding this complex interplay of excitatory and inhibitory systems in the brain is essential to develop effective treatments for epilepsy and manage seizures in affected individuals. The hippocampus has been shown to be one of the most sensitive areas of the brain in epilepsy and is central to the disease. Temporal lobe epilepsy corresponds to complex partial epilepsy according to the officially adopted classification based on the recommendation of the International League Against Epilepsy. Even under physiological conditions, the hippocampus undergoes significant development and maturation during postnatal ontogenesis. Probably the rapid changes in the hippocampus during this critical developmental window may make it vulnerable to the initial triggers that can ultimately lead to medial temporal lobe epilepsy. The most affected cells in the disease are the pyramidal cells, the granule cells and the mossy cells. Concerning epileptic reorganisation, at the level of neuronal networks, intercellular connections are altered, and axons die or sprout, leading to the death or sprouting of pathways, thus altering the connections between brain areas. At the cellular level, morphological changes, cell death, gliosis, and neurochemical changes are detectable. As a consequence, these processes together lead to hippocampal sclerosis, which is a condition characterised by the shrinkage of the hippocampus. Clinical symptoms in patients with epilepsy can include seizures, memory disturbances, and other neurological symptoms. Histological examinations can detect the minimum criteria of hippocampal sclerosis, such as neuronal loss, thickening of the granular cell layer, signs of gliosis, and other characteristic lesions at the microscopic level in different regions of the hippocampus. Synaptic reorganisation affects the mossy fibres and often also the supragranular layer of the dentate gyrus. Living with epilepsy can not only be a challenge for patients and their families, but can also put a great burden on health care system. However, with appropriate medical care, support, and management strategies, many people with epilepsy can lead a full and productive life. The economic, social and personal burden of this disorder

underscores the need for more research efforts that lead to new approaches for the diagnosis, treatment, and prevention of epilepsy and its consequences. There are two main pillars of these research efforts: On the one hand, researchers are focusing on how to reduce seizure activity and minimize its impact on life quality; on the other hand, more forward-looking experiments are being conducted targeting the prevention of convulsions. This preventive approach can also be achieved through pharmacotherapy, but there are many attempts to develop more targeted and less expensive therapeutic instruments to prevent seizures. Although human studies would be optimal, they are rarely feasible due to obvious ethical, statistical, and financial constraints. In conclusion, preclinical studies employing animal models to study and test new therapeutic mechanisms remain indispensable. *In vivo* animal models are based on the induction of convulsions induced by external epileptogenic effects. Chronic models are used to study epileptogenesis and interictal epileptogenic lesions. *Status epilepticus* is a condition in which several seizures occur repeatedly and last for at least half an hour. During *status epilepticus*, mild to severe respiratory failure associated with cerebral hypoperfusion is observable. These abnormalities lead to hypoxia and hypoglycaemia, to which neurons are very sensitive. In *status epilepticus* animal models, the surviving animals show morphological changes that are resemble for the characteristics of medial temporal lobe epilepsy in humans. The most dramatic neuronal loss is detectable the in parts of the *cornu ammonis* (CA*)*, specifically in the CA1, CA3, and subiculum areas in hippocampal formation, which resembles hippocampal sclerosis. Spontaneous recurrent convulsions are also observed in experimental animals after a latent period.

The murine model of pilocarpine-induced epilepsy is one of the most widely used animal models in research to study the underlying mechanisms of human medial temporal lobe epilepsy. In this model, after systemic administration of pilocarpine, generalised motor convulsions develop that typically last more than 1h. Most animals have status epilepticus with significant mortality, but the surviving animals enter a latent phase without apparent symptoms, showing recovery and normalisation of vital signs. However, after this latent period, in the third chronic phase, the pilocarpine-treated animals exhibit spontaneous recurrent seizures.

The complex neuropathological phenomena of hippocampal sclerosis coincide with significant cognitive disturbances in human patients. Functional and morphological damage to the hippocampus in drug-induced preclinical rodent models can be assessed by not only neuropathological but also behavioural studies, as the hippocampus is a key brain structure involved in memory formation and spatial navigation. There are numerous behavioural tests to assess learning and memory in rodents. By their nature, rodents are motivated to avoid open

spaces and bright lights. The Barnes maze is based on this aversion, which encourages them to search for shelter in the escape box. This test is best suited to the lifestyle of rodents.

One of the potential preventive pharmacotherapeutic approaches that we investigated was the potential neuroprotective effect of the synthetic kynurenic acid analogue SZR104 in preclinical epilepsy models. Kynurenic acid plays a key role in various neuroinflammatory and neurodegenerative disorders of the central nervous system. Kynurenic acid affects the immune system in the brain, particularly through the regulation and modulation of effector immune cells, such as microglial cells. These cells play a crucial role in the immune response and are known to be involved in the progression of various neurological disorders, including epilepsy. Our interest focused on the potential anticonvulsant effect of SZR104, thus we tested it as a potential drug candidate in Naval Medical Research Institute (NMRI) strain mice (inbred albino mouse line).

The specific aims of the present studies were the following:

1) We set out to characterise in detail the neuropathology of hippocampal sclerosis that develops over the course of 3.5 months of pilocarpine-induced spontaneous recurrent seizures in NMRI mice, as such data in the literature were sparse/lacking despite that the NMRI mouse is a widely used strain in epilepsy research.

2) We were especially interested in studying parvalbumin-immunopositive and calretinin-immunopositive interneurons in our hippocampal sclerosis model, as the integrity of these interneurons is crucial not only for normal hippocampal functions, but also for seizure-induced pathological alterations.

3) We also aimed to study the effects of pilocarpine-induced spontaneous recurrent seizures on spatial learning and memory processes in the NMRI mouse strain.

4) We investigated the acute pharmacological effects of the synthetic kynurenic acid analogue SZR104 on seizure-induction and microglial activation 24 h after in our murine pilocarpine-induced epilepsy model.

Materials and Methods

Treatment of the experimental animals

In these studies, adult, male, six-week-old NMRI strain mice were used. The experiments were performed in the morning. In the first study, the experimental animals were divided into two groups. Treated animals (n=40, bwt=28-36g) were injected intraperitoneally (i.p.) with a single dose of pilocarpine hydrochloride (195 mg/kg). Control animals received the same volume of physiological saline, the solvent of the pilocarpine (n=10). With this dose of pilocarpine, most of the animals exhibited *status epilepticus*. 90 min after *status epilepticus* onset, the animals were injected with diazepam (Seduxen) to stop convulsions. Approximately half of the animals that had seizures died on the day of treatment. After a survival period of 3.5 months, the animals were anaesthetised, transcardially fixed, and the brains were removed for further processing. In the second study, we tested the effects of SZR104 pre-treatment in temporal lobe epilepsy. Convulsions were induced and stopped as described above. Animals were divided into four groups (n=10/each groups). Control animals (*group1*) received physiological saline i.p. The animals in *group2* were injected i.p. with a single dose of pilocarpine-hydrochloride. SZR104 was dissolved in distilled water and administered i.p. at a dose of 358 mg/kg (*group3*) and animals treated with SZR104+pilocarpine (*group 4*) received a single dose of i.p. SZR104 40 min before pilocarpine injection. One day (24 h) after the treatments, the surviving animals were sacrificed and the brain and blood were further processed.

Spatial learning test

Three months after injections, the experimental animals in study 1 (control n=9, pilocarpine-treated n=9) were tested for 4 consecutive days in a hippocampus-related learning paradigm, the Barnes maze, to measure spatial learning and memory capabilities. The essence of the test is that the animals should find the escape box during 5 min in the open and brightly lit arena. Those animals who failed to find the escape box were gently guided to the target and could spend 15 sec there. The latency of both animal groups was measured and statistically compared.

Tissue preparation

The animals were sacrificed 3.5 months (first study) or 1 day (SZR104 study) after injections. The animals were deeply anaesthetised with diethyl ether and perfused through the ascending aorta with sodium sulphide in phosphate buffer and then with 4% formaldehyde in phosphate buffer. The brains were dissected and cryoprotected overnight in sucrose in phosphate buffer.

Coronal plane brain sections were cut on a freezing microtome at a thickness of 24 μm on the following days. The sections used for immunohistochemical analysis were stored in phosphate buffer containing sodium azide in a refrigerator until processing.

Immunohistochemistry

Free-floating sections were treated with 0.5% Triton X-100 and 3% hydrogen peroxide in 0.1 M Tris-HCl; pH 7.6, and then with normal swine serum (1/10). The following primary antisera were used: rabbit anti-neuropeptide-Y (NPY; 1/10000); mouse anti-neuronal N (NeuN; 1/8000); rabbit anti-Iba1 (Iba1; 1/8000), goat anti-calretinin (CALR; 1/20000), mouse anti-parvalbumin (PARV; 1/40000). All incubations were carried out in plastic vials with continuous agitation at room temperature overnight. After careful washing, the sections were incubated with the corresponding biotinylated secondary antibodies (1/400) for 1h at room temperature, then peroxidase-labelled streptavidin (1/6000) for 1h. The site of immunoreaction was visualised with 3,3'-diaminobenzidine (DAB), and in case of NPY staining, DAB+Ni was used.

Morphometry and evaluation of data

The immunostained sections were scanned, then, according to the area of interest, the software calculated a staining density score on the digital images based on the proportion of positive and negative pixels. In the NeuN, and Iba1 immunostainings, the values of density score measurements were averaged per animal and used for statistical processing. The size of the hippocampal formation was also measured in the sections stained with NeuN. The mathematical mean of the data was statistically evaluated to illustrate the shrinkage of the hippocampal formation in pilocarpine-treated animals. The PARV- and CALR-immunostained cells were counted by using the digitised images and then the number of these cells was corrected to 1 mm^2 area of the hippocampal formation. For the evaluation of results of immunohistochemistry and the behavioural test, a Student's t-test for independent samples was used. In the second study, four Iba1-stained sections from each animal were analysed. The software counted the number of immunostained cells in the entire area of interest, and then normalised to 1 mm^2. Thereafter, the surface area of the single microglial cells was also measured. Ten microglial cells were selected from the hippocampi of each experimental group, and the cell area was expressed as μm^2 values. Data were expressed as mean ± SEM, differences were calculated with ANOVA, post hoc test Bonferroni ($p < 0.05$ was significant). GraphPad Prism8 software (version 8.4.3, GraphPad Software, LLC, San Diego, CA, USA) was used to statistically analyse the results of the immunohistochemical measurements.

Results and Discussion

In the present studies, we employed the established murine model of pilocarpine-induced epilepsy. We made novel observations regarding the long-term functional consequence on spatial learning and the neuropathological appearance of the sclerotised hippocampus. Moreover, using the same model, we identified an interesting novel short-term effect of the kynurenic acid analogue SZR104 on activation and morphological changes in microglia. The major specific findings in the dorsal hippocampus are as follows:

1) Pilocarpine-induced high mortality due to *status epilepticus* and spontaneous recurrent seizures in surviving animals persisted over the previously unassessed course of 3.5 months observation period in NMRI mice. Epileptic mice displayed severe disturbances of spatial learning and memory indicating the development of severe hippocampal dysfunction (Fig. 1).
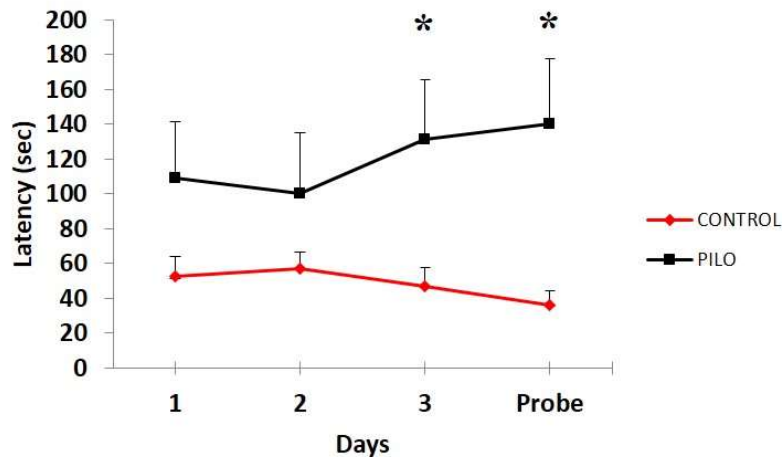

Figure 1. Performance of the control and pilocarpine-treated animals in the Barnes maze for 4 consecutive days. Two trials were performed on each day, except for the probe day. The average latencies of the control and pilocarpine-treated animals (n = 9/ each groups) were plotted against time. The SEM values were depicted. The controls showed an improved performance as a function of time, whilst the performance of the pilocarpine-treated animals was becoming worse. Using the paired t-test, the statistical analysis showed that the variances of the mean latencies in the two groups were significantly different on day 3 and day of the probe (* p = 0.043 and 0.025, respectively).

2) All animals developed severe hippocampal sclerosis characterised by severe neuronal damage that could culminate in the near-total destruction of the hippocampus, as shown by NeuN immunohistochemistry. Parallel to it, microglial cells are activated rapidly

following pathological changes of the brain in epilepsy confirmed by Iba1 immunostaining. In addition, reactive sprouting of mossy fibres has been demonstrated using NPY immunohistochemistry. These findings are in agreement with previous data using shorter observation periods (Fig. 2).
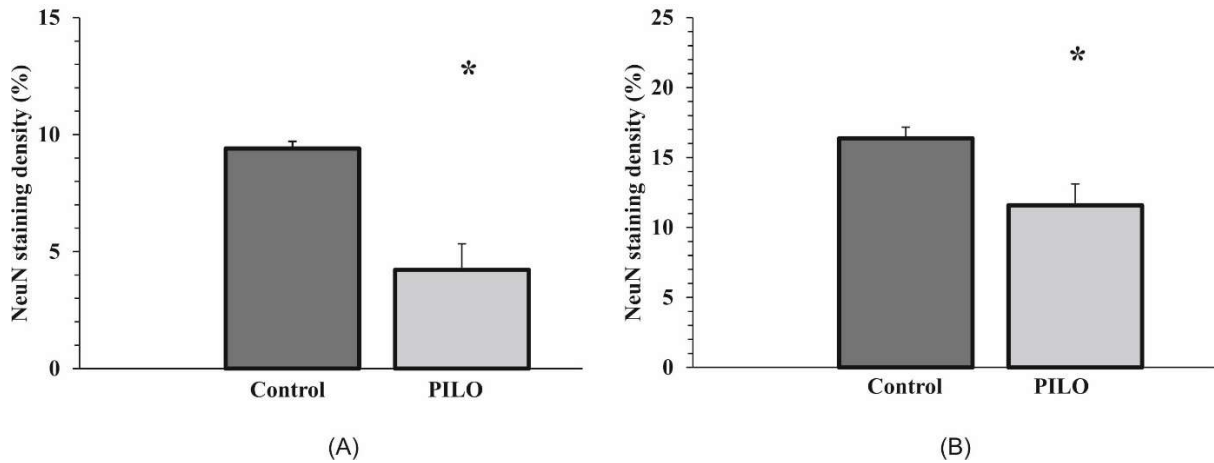

(A)

(B)

Figure 2. Densitometric analysis of NeuN-immunostained hippocampi: NeuN staining density throughout the CA region in control and pilocarpine-treated animals (n=9/ each groups; paired t-test, *p = 0.001) (**A**). NeuN staining density in the entire area of the DG in control and pilocarpine-treated animals (n=9/each groups; paired t-test, *p = 0.016) (**B**).

3) The specific loss of CALR-immunopositive and PARV-immunopositive interneurons in the hippocampus has also been confirmed in our model (Fig. 3), and moreover PARV-immunopositive astrocyte-like cells were novelly detected in the sclerotised hippocampus.
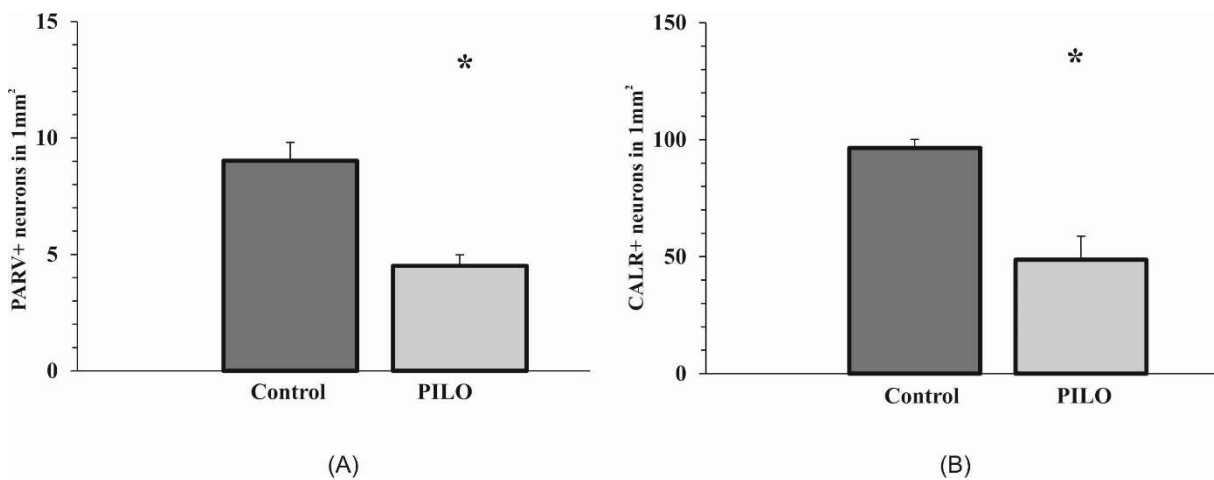

(A)

(B)

Figure 3. The number of PARV-containing neurons in the hippocampal formation in control, and pilocarpine-treated animals (paired t-test, *p <0.0001) (**A**). The number of CALR-containing neurons in hippocampal formation in control, and pilocarpine-treated animals (paired t-test, *p <0.001) (**B**).

4) The potentially neuroprotective, novel kynurenic acid analogue SZR104 did not reduce pilocarpine-induced seizures or seizure-induced mortality, however, it potently ameliorated the acute (24h) neuroinflammatory response detected by the Iba1 immunopositive microglial cell count and morphological changes (Fig. 4).
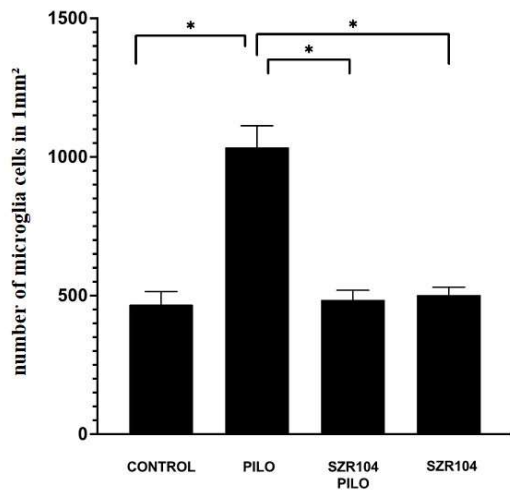

Figure 4. Analysis of Iba1-positive microglial cell counts in the entire hippocampal formation of epileptic, and control mice (n=4 sections / each groups; mean±SEM). Statistical differences were detected between the control, pilocarpine-treated, SZR104+pilocarpine-treated, and SZR104-treated animals (* p <0.05).

The pilocarpine model has been one of the more widely used models of drug-induced chronic epilepsy. Our findings confirm and further advance the understanding of the neuropathological and neurochemical characteristics (Fig. 5) and pathomechanistic aspects of hippocampal sclerosis that are prominently developed in this model.
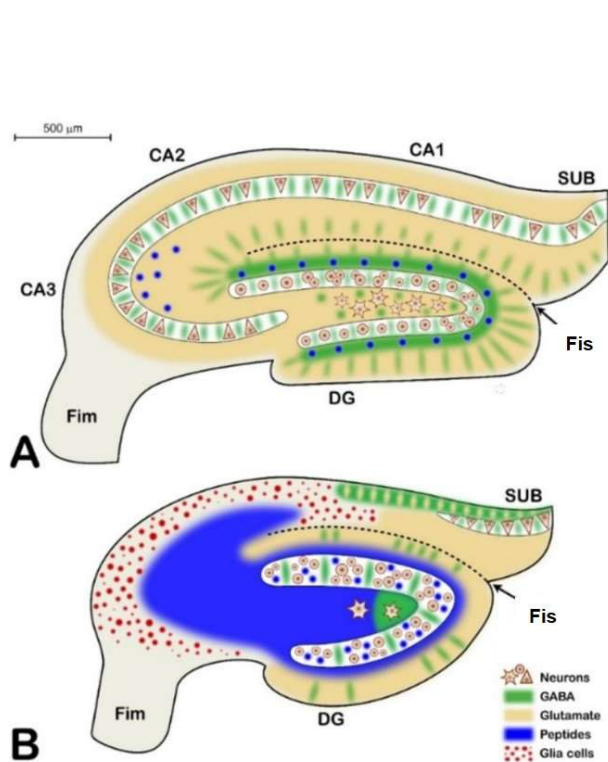

Figure 5. Scheme of neurochemical parcellation of the hippocampus in the control (**A**) versus pilocarpine-treated (**B**) animals. Principal neurons are represented by triangular, circular, and multipolar shapes. GABAergic transmission is depicted in green, Glu transmission is beige, and peptide transmitters are in blue. Glia is represented by red dots. The pilocarpine-treated hippocampus is not only shrunken, but also invaded by peptidergic axons, some of which did not have postsynaptic cells, and the peptide release was diffusing toward unknown targets. This neurochemical alteration drastically changed the function of the hippocampus, as shown by the Barnes maze experiments. Abbreviations: **CA1**, **CA2,** and **CA3** are regions of the *cornu ammonis*; **DG**=dentate gyrus; **SUB**=subiculum; **Fim**=fimbria; **Fis**=hippocampal fissure; Scale bar: 500 μm.

Conclusions

The most important novel findings in our studies were the following:

1. Pilocarpine-induced chronic epilepsy in NMRI mice is an excellent preclinical model of chronic temporal lobe epilepsy that can simulate all the major features of the human pathology. This includes severe hippocampal dysfunction and structural alterations.

2. The novel long-term observation period enables the detection of large-scale neuronal loss including both principal neurons and selected populations of GABAergic interneurons, reactive axonal sprouting, and microglial activation, all the hallmarks of severe hippocampal sclerosis.

3. SZR104 was shown to selectively ameliorate the acute microglial response, suggesting the involvement of kynurenine metabolites in this response.

Acknowledgements

First of all, I would like to express my deepest gratitude to my family and friends, for their emotional support, unconditional love, tolerance, and for always being there for me. Thank you for accepting lost evenings, weekends, and holidays.

Officially, I would like to thank my supervisor, Prof. Dr. András Mihály, who provided me the opportunity to join his research group and gave access to the laboratory and research facilities. I would like to express my special thanks to Prof. Dr. Antal Nógrádi, the head of the Department of Anatomy, Histology, and Embryology, for supporting to finish my PhD thesis.

I would like to thank the following two people without whom this thesis would not have been possible. I would like to express my deepest gratitude to Dr. Emőke Borbély, whose support, generous help, and friendship were essential to the completion of my doctoral work and PhD thesis over the past years. I wish to express my sincere and heartfelt gratitude to Dr. Ferenc Domoki for his personal guidance, professional advice and, even more importantly, for his patience, continuous support, and encouragement.

I owe a debt of gratitude to Prof. Dr. Ferenc Bari for his kind support and believing me all the time during this long journey.

Special thanks go to Mónika Kara for her technical assistance during the experiments, and to Dr. Endre Dobó for his guidance in the laboratory work.

I must mention my former teachers, who have played an immense role in bringing me to this point. First of all, I would like to mention Zsuzsanna Berzai Trencséniné, my former high school teacher of biology and chemistry. She made me fall in love with these two subjects and eventually I got my degree as a result at the Attila József University. I have also had the pleasure of meeting some excellent professors of the university who have set an example both professionally and as people. I would like to thank Prof. Dr. Éva Fekete, Prof. Dr. Katalin Halasy, Prof. Dr. József Toldi, Prof. Dr. Csaba Visy, Dr. Irma Tari Dr. Miklósné Görgényi, Dr. Mária Simon Dr. Lehoczkiné, Prof. Dr. István Hannus, Prof. Dr. Gyula Farkas, and Dr. Zoltán Galbács for their dedicated work, from whom I have learnt a lot.
I also would like to thank my colleagues who have supported me in any way during my work. Last but not least, a special thanks goes to all the mice who gave their lives for my PhD.

Publications related to the subject of the thesis *(cumulative IF: 11.524)*

I.   **Mátyás A**, Borbély E, Mihály A. Hippocampal Sclerosis in Pilocarpine Epilepsy: Survival of Peptide-Containing Neurons and Learning and Memory Disturbances in the Adult NMRI Strain Mouse. **Int J Mol Sci.** 2022; 23 (1):204. 19 p. (**IF (2022): 5.6**, SJR: **Q1**)

II.   Lajkó N, Kata D, Szabó M, **Mátyás A**, Dulka K, Földesi I, Fülöp F, Gulya K, Vécsei L, Mihály A. Sensitivity of Rodent Microglia to Kynurenines in Models of Epilepsy and Inflammation In Vivo and In Vitro: Microglia Activation is Inhibited by Kynurenic Acid and the Synthetic Analogue SZR104. **Int J Mol Sci.** 2020; 21 (23):9333. 15 p. (**IF (2020): 5.924**, SJR: **Q1**)