Inflammatory reactions and physical activity in humans and animal models of epilepsy

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MATILDA AHL graduated from the medical faculty at Lund University in 2015 with a master's degree in biomedicine. In 2016, she began her doctoral studies in "the department of clinical sciences" were she has started to discern the complex and widespread inflammatory reaction in epilepsy. Her main focus has been the inflammatory response that can be found outside the epileptic focus, such as the blood and eyes both before and after the development of epilepsy. Additionally, physical activity has been investigated as a potential modulator of epilepsy development.

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"*Nothing in life is to be feared, it is only to be understood*"

-Marie Curie

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract
Epilepsy is a chronic neurological disorder affecting 1% of the population worldwide. The main clinical manifestation is the occurrence of seizures, though comorbidities such as depression and neuropsychiatric disorders are common. A proportion of 30-40% of all patients are pharmacoresistant, a number that has not improved over the last 30 years. There are no known prognostic markers, or acute seizure markers in the clinic, making it difficult to predict epilepsy development in high-risk patients, or to confirm seizures retrospectively. These factors together with a high proportion of pharmacoresistant patients empathises the importance of developing new prognostic and diagnostic markers together with new novel strategies for treatments.

The purpose of this thesis is to evaluate inflammatory factors in both the eyes (paper IV, V) and blood (II, III) in search of future prognostic or diagnostic markers for epilepsy. Additionally, exercise was investigated as a potential modulator of epilepsy development (paper I). We identify a significant decrease in epilepsy incidence in a large cohort of Vasaloppet skiers compared to non-participating age- and gender matched controls. Supporting our cohort data, we also determine that voluntary physical activity in a genetic mouse model of epilepsy, implemented before seizure onset gives a robust decrease in the number of animals that develop seizures (paper I). Additionally, we demonstrate inflammatory factors in serum and spleen that are specific to electrically induced focal non-convulsive status epilepticus (fNCSE) in rats compared to a general brain inflammation caused by intrahippocampal lipopolysaccharide injection. Interestingly, fNCSE animals that developed spontaneous recurrent seizures revealed a distinct profile in serum compared to rats with only acute symptomatic seizures (paper II). In paper III we have expanded our search for an inflammatory profile in serum from animal models to patients with epilepsy. Included patients were divided into groups according to diagnosis: temporal lobe epilepsy (TLE), frontal lobe epilepsy (FLE) and psychogenic non-epileptic seizures (PNES). IL-6 was increased in the interictal blood samples in TLE and FLE groups compared to healthy controls, while a higher concentration of ICAM-1 was found in the PNES group compared to controls. We also determined that postictal changes in IL-6, Mip1β, TARC, MDC, INF-γ and ICAM-1 only occurred in the TLE group. Neither interictal nor postictal protein levels could be correlated to parameters associated with disease burden (paper III). To further investigate the extension of the inflammatory reaction in epilepsy and during epileptogenesis we have evaluated retinal inflammation with traditional histology and biochemistry (paper IV) as well as high resolution Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) (paper V). We present a delayed micro,- and macroglial activation in the retina of rats after fNCSE and synaptic alterations with a decrease in the excitatory scaffolding protein PSD-95. The retinal inflammation was modulated and diminished by intracerebroventricular CX3CR1 antibody treatment. Retinal inflammation post-fNCSE was not convincingly detected with high resolution MRI or DTI, though discrete alterations were found implicating that the two techniques used together may add clinical relevance (paper V).

In summary, we have discerned components of the complex and extensive inflammatory reaction in epilepsy in both eyes and blood. Additionally we have identified physical activity as a possible protective factor for epilepsy development. These results is of high clinical relevance and will aid in the search of prognostic or diagnostic biomarkers of epilepsy, toghether with finding new novel treatment targets.

Key words
Epilepsy, inflammation, physical activity, MRI, DTI

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In loving memory of
Anna-Greta and Agne Ahl

Table of Contents

Abstract

Epilepsy is a chronic neurological disorder affecting 1% of the population worldwide. The main clinical manifestation is the occurrence of seizures, though comorbidities such as depression and neuropsychiatric disorders are common. Furthermore, a proportion of 30-40% of all patients are pharmacoresistant, a number that has not improved over the last 30-years. The cause of epilepsy is often unknown, but both genetic and acquired factors such as genetic mutations, brain trauma, stroke or brain infections increase the risk of developing epilepsy. The process initiated after a genetic or acquired factor that eventually leads to epilepsy is termed epileptogenesis. The diagnosis of epilepsy is currently a time and resource demanding process. There are no known prognostic markers, or acute seizure markers in the clinic, making it difficult to predict epilepsy development in high-risk patients, or to confirm seizures retrospectively that has occurred outside the clinical setting. These factors together with a high proportion of pharmacoresistant patients emphasizes the importance of developing new prognostic and diagnostic markers together with new novel strategies for treatments.

The purpose of this thesis is to evaluate inflammatory factors in both the eyes and the blood in search of future prognostic or diagnostic markers for epilepsy. Additionally, exercise was investigated as a potential modulator of epilepsy development. In a large cohort containing long distance skiers participating in Vasaloppet, compared with age- and gender matched controls we reveal a significantly lower incidence of epilepsy in skiers over a 20-year follow up period. Epilepsy incidence was almost reduced by 50% compared to controls and the effect was seen in both genders, and all age groups. We could also establish that the level of fitness in Vasaloppet participants seemed to influence epilepsy incidence, since faster skiers had an even lower incidence of epilepsy compared to the slower skiers. Supporting our cohort data, we also determine that voluntary physical activity in a genetic mouse model of epilepsy, implemented before seizure onset gives a robust decrease in the number of animals that develop seizures. When the voluntary exercise started before seizure onset and continued though the study period most of the exercising animals did not even develop seizures (paper I).

Additionally, we have in this thesis evaluated inflammatory factors in serum and spleen in an electrically induced focal non-convulse status epilepticus model (fNCSE) and compared the inflammatory response with a general brain inflammation caused by lipopolysaccharide injection. Our result show minor acute

alterations in spleen at 6 and 24hrs in proteins associated with leucocytes and astrocytes in the epileptic group, but not in the lipopolysaccharide treated group. Furthermore, no acute changes were seen in serum at 6hr, 24hrs or 1 week after fNCSE. Interestingly, at 4 weeks after fNCSE, when most of the animals had started to experience spontaneous recurrent seizures, a distinct profile in serum with increased MHCII, CD68, galactin-3 expression related to antigenpresentation and phagocytosis, and an increase of CD8, expressed by cytotoxic T-cells, together with a decrease of CD4, expressed by T helper cells, was found. In the lipopolysaccharide treated animals, a similar increase in CD8 was found, but in contrast CD68 expression was decreased, and no other alterations similar to the fNCSE animals was found (paper II). In paper III we have expanded our search for an inflammatory profile in serum from animal models to patients with epilepsy admitted for continuous video and EEG studies. 56 patients were included and divided into groups according to diagnosis: temporal lobe epilepsy (TLE), frontal lobe epilepsy (FLE) and psychogenic non-epileptic seizures (PNES). IL-6 was increased in the interictal blood sample in both TLE and FLE groups compared to healthy controls, while a higher concentration of ICAM-1 was found in the PNES group compared to controls. We also determined that postictal changes only occurred in the TLE group and not in any of the other patient groups. A robust increase of IL-6 was found in TLE patients both at 6 and 24hrs postictally. Similarly, we present an increase of both Mip1β and TARC. Interestingly, at 24 hrs postictally there was an increase of ICAM-1 levels in the TLE group. Neither interictal nor postictal protein levels could be correlated to parameters associated with disease burden (paper III).

To further investigate the extension of the inflammatory reaction in epilepsy and during epileptogenesis we have evaluated the retinal inflammation with traditional histology and biochemistry (paper IV) but also with high resolution Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) (paper V). We present a delayed micro,- and macroglial activation in the retina of rats after fNCSE. No alterations in pericytes or leucocytes could be observed, but synaptic changes with a decrease in the excitatory scaffolding protein PSD-95 was identified. The retinal inflammation was modulated and diminished by intracerebroventricular CX3CR1 antibody treatment. Furthermore, after fNCSE in mice the microglial population were increased in numbers with a more inflammatory morphology, still the retinal inflammation was not as pronounced as in the rat retina. The fNCSE associated inflammation in the mice retina was not convincingly detected with high resolution MRI or DTI, though discrete alterations were found in both the MRI and DTI image analysis, implicating that the two techniques used together may add clinical relevance.

Summary

We identify a significant decrease in epilepsy incidence in a large cohort of Vasaloppet skiers compared to non-participating frequency matched controls. Supporting our cohort data, we also determine that voluntary physical activity in a genetic mouse model of epilepsy, implemented before seizure onset leads to a robust decrease in the number of animals that develop seizures (paper I). Additionally, we demonstrate inflammatory factors in serum and spleen that are specific to electrically induced focal non-convulsive status epilepticus in rats (fNCSE) compared to a general brain inflammation. Interestingly, fNCSE animals that developed spontaneous recurrent seizures revealed a distinct profile in serum compared to rats with only acute symptomatic seizures (paper II). In paper III we expand our search for an inflammatory profile in serum from animal models to patients with epilepsy. Included patients were divided into groups according to diagnosis: temporal lobe epilepsy (TLE), frontal lobe epilepsy (FLE) and psychogenic non-epileptic seizures (PNES). IL-6 was increased in the interictal blood sample in all epileptic patient groups compared to healthy controls, and a higher concentration of ICAM-1 was found in the PNES group compared to controls. We also determined that postictal changes with alterations in IL-6, Mip1β, TARC, MDC, INF-γ and ICAM-1 only occurred in the TLE group. Neither interictal nor postictal protein levels could be correlated to parameters associated with disease burden (paper III). We present the novel finding of retinal inflammation after fNCSE with delayed micro,- and macroglial activation and synaptic alterations in terms of a decrease in the excitatory scaffolding protein PSD-95 (paper IV). High resolution Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) could not convincingly detect the retinal inflammation associated with fNCSE in mice, though discrete alterations were found, implicating that the two techniques used together may add clinical relevance (paper V).

Populärvetenskaplig sammanfattning

Epilepsi är en kronisk neurologisk sjukdom som kan utvecklas i alla åldersgrupper från små barn till äldre. Ca 1% av världens befolkning utvecklar epilepsi med återkommande epileptiska anfall. Epileptiska anfall är en onormal överaktivering av hela eller delar av hjärnan som stör den normala hjärnaktiviteten, och kan ge en stor variation av symptom så som, medvetande påverkan, muskelkramper eller repetitiva okontrollerbara rörelsemönster. Det är även vanligt med olika följdsjukdomar så som depression och ångest i samband med epilepsi. Över en tredjedel av alla patienter med epilepsi blir inte hjälpta med dagens terapier och får trots medicinering återkommande anfall, vilket kallas farmakoresistens. Denna siffra har dessvärre varit nästan oförändrad de senaste 30 åren trots att nya mediciner har utvecklats. Orsaken till sjukdomen är ofta okänd även om man känner till flera riskfaktorer som kan leda till epilepsi. Dessa kan vara både genetiska och förvärvade, så som genetiska mutationer i vissa gener, hjärnskador, stroke eller infektioner i hjärnan. Processen när nervbanor (nätverk) i en frisk hjärna utvecklas till mer anfallsbenägna nätverk kallas epileptogenes.

Det finns idag få mått eller test som kan förutspå hur en patient kommer att svara på behandling, eller vilka patienter som kommer att utveckla sjukdomen efter t.ex. en stroke. Diagnostiseringen av epilepsi är en komplex och resurskrävande process som ofta kräver kontinuerlig video och EEG övervakning i sjukhusmiljö. Det finns i nuläget få markörer som kan mätas i efterhand och visa på att ett anfall har skett. Dessa omständigheter visar vikten av att markörer som kan tala om hur prognosen för en patient ser ut, samt att diagnostiska markörer som visar ifall en patient drabbats av epilepsi eller inte, hittas. Detta tillsammans med nya strategier för behandlingen av patienter är högst efterfrågat.

Ändamålet med denna avhandling var att utvärdera inflammatoriska faktorer både i ögonen och i blodet i sökandet efter markörer för epilepsi, som i framtiden skulle kunna utvecklas för att användas i prognostiskt eller diagnostiskt syfte. Därutöver utforskades träning som ett sätt att skydda sig mot utvecklingen av epilepsi.

En stor grupp av Vasaloppsåkare (fler än 190-tusen) matchades gentemot kön och ålder med kontroller från Sveriges befolkning som inte åkt Vasaloppet. Detta visade att Vasaloppsåkarna under den 20-år långa uppföljningsperioden hade hälften så låg risk att drabbas av epilepsi jämfört med kontrollerna som inte åkt Vasaloppet. Effekten kunde ses i både män och kvinnor, samt i alla åldrar. Vi kunde också visa

att konditionen på Vasaloppsåkarna hade betydelse inom gruppen, då de skidåkare som var snabbast hade ännu lägre risk att utveckla epilepsi jämfört med de skidåkare som var långsammast. För att komplimentera studien med skidåkarna så tittade vi även på hur fysisk aktivitet påverkar anfallsutvecklingen i en genetisk musmodell för epilepsi. När träning introducerades före anfallsutveckling och kontinuerligt under hela studie-tiden stoppades utvecklingen av epilepsi starkt, och de flesta av de fysiskt aktiva mössen utvecklade inte anfall alls (studie I).

Vi har även i denna avhandling utvärderat inflammatoriska faktorer i blod och mjälte in en rått-modell där man med hjälp av en elektrisk impuls utvecklar epileptisk aktivitet i en viss del av hjärnan, en så kallad fokal non-konvulsiv status epilepsimodell (fNCSE). Därefter har vi jämfört hur det inflammatoriska svaret i denna modell skiljer sig ifrån en generell inflammation i hjärnan, som orsakats av bakterietoxinet lipopolysackarid (LPS). Våra resultat visar att direkt efter fNCSE ingreppet sker diskreta förändringar i mjälten i gruppen med inducerad epilepsi i protein som förknippas med vita blodceller och astrocyter, vilka är stödjeceller i hjärnan. Detta kunde inte hittas i gruppen med endast en vanlig inflammation i hjärnan orsakad av LPS. I blodet hittade vi inga akuta förändringar i upp till en vecka efter att djuren inducerats med fNCSE. Vi kunde dock hitta förändringar i blodet 4 veckor senare när djuren med fNCSE börjar utveckla spontana epileptiska anfall utan att stimulering utifrån med elektriska impulser behöver ske. Vi hittade intressant nog protein som MHCII, CD68 och galactin-3 uttryck som man kan koppla till anti-gen presentation hos immunceller samt fagocytos, processen där immunceller äter upp infekterade eller skadade celler, ökade. Samtidigt såg vi en förändring i T-cells populationen där en ökning CD8, uttryckt av cytotoxiska fagocyterande T-celler, och en minskning av CD4, uttryckt av T hjälparceller som är mer kopplade till regleringen av det inflammatoriska svaret. I djuren som var behandlade med LPS som endast ger en generell inflammation i hjärnan hittades inga liknande förändringar (Studie II).

I studie III utökade vi vårt sökande efter inflammatoriska markörer i blod till patienter med epilepsi som blivit inlagda på sjukhuset för en kontinuerlig video EEG utredning pga bekräftad eller misstänkt epilepsi. I studien inkluderades 56 patienter som sedan delades upp i olika grupper beroende på deras specifika epilepsidiagnos: temporal lobs epilepsi (TLE), frontal lobs epilepsi (FLE) eller psykogena icke epileptiska anfall (PNES), vilket är en annorlunda diagnos från epilepsi. Nivåerna av IL-6, ett starkt inflammatoriskt protein i blodet var högre interiktalt, dvs perioden mellan anfall då hjärnaktiviteten är normal och då patienten inte upplever några symptom, i alla patientgrupper med en epilepsidiagnos jämfört med de friska kontrollerna (n=12). Vi fortsatte vår studie med att titta på vad vi kallar post-iktala blodprover, dvs blodproven som tagits kort tid efter att patienten haft ett anfall (iktal aktivitet i hjärnan). Intressant nog så var det bara en av patientgrupperna som hade förändringar i blodet kort tid efter ett anfall, nämligen TLE gruppen. Återigen hittades en kraftig ökning av IL-6 men endast efter ett temporallobs anfall. En

liknande ökning av de inflammatoriska proteinerna kopplade till T-celler och rörligheten hos immunceller hittades av både Mip1β och TARC vid samma tidpunkter och intressant nog även en ICAM-1 ökning in TLE gruppen. Ingen av de interiktala eller postiktlala förändringarna i blodet kunde relateras till sjukdomsbördan, så som anfallsfrekvens, anfallslängd osv (studie III).

För att fortsätta utvärderingen av omfattningen av den inflammatoriska reaktionen vid epilepsi och epileptogenes, så har vi i de två sista artiklarna granskat inflammationen i ögat hos djurmodeller med epilepsi genom att färga ögonen och titta på inflammatoriska celler och proteiner (studie IV), samt genom högupplöst 3-dimetntionell avbildning av ögonen med magnetiskresonanstomografi (MRI) och diffusionstensoravbildning (DTI) (studie V). Vi presenterar i studie IV gjord i råttor en inflammatorisk reaktion i ögat och retina som sker flera veckor efter att djuren inducerats med epileptisk aktivitet. Denna inflammation visade sig som ett ökat antal inflammatoriska celler, s.k. mikroglia celler, som även hade en mer aktiverad form som är mer benägen till fagocytos, uppätande och bortforsling av skadade eller infekterade celler. Vi såg även att stabiliserande Müller cell, en unik celltyp för ögat hade reagerat pga obalans och inflammation. Kanske hade även den minskning av det excitatoriska skaffolding proteinet PSD-95, som finns hos framåtdrivande nervceller som främjar neurala aktivitet betydelse. Inflammationen i ögat kunde även minskas med en antiinflammatorisk CX3CR1 antikroppsbehandling som gavs i ventrikeln (hålrum) i hjärnan, vilket tyder på att vid epilepsi kan det finnas en koppling av det immunologiska svaret mellan hjärnan och ögat. Fortsättningsvis i studie V, efter fNCSE i mus hittades en liknande inflammatorisk reaktion som i råtta, även om den inte var lika stark. Den inflammation i ögat som hittades i mus kunde inte tydligt visualiseras med hjälp av MRI eller DTI. Båda teknikerna gav dock tillsammans överskådliga högupplösta bilder av ögat, med vissa antydningar på de sjukliga förändringar som vi hittat när vi snittat och färgat ögonen.

Abbreviations

ACTH	Adrenocorticotrophic Hormone
Ab	Antibody
AD	Alzheimer Disease
AED	Anti-Epileptic Drug
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BCA	Bicinchoninic Acid
BDNF	Brain Derived Neurotrophic Factor
BRB	Blood Retinal Barrier
BSA	Bovine Serum Albumin
CA	Cornu Ammonis
CNS	Central Nervous System
CRF	Corticotrophin-Releasing Factor
CRP	C-Reactive Protein
CTRL	Control
DAMP	Danger Associated Molecular Patterns
DCX	Doublecortin
CD	Cluster of Differentiation
DG	Dentate Gyrus
DTI	Diffusion Tensor Imaging
EC	Entorhinal Cortex
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Fractional Anisotropy
F-Jade	Fluoro-Jade
FLE	Frontal Lobe Epilepsy
fNCSE	focal Non-Convulsive Status Epilepticus
FTBTC	Focal to Bilateral Tonic-Clonic seizure
GABA	Gamma-Aminobutyric Acid

GAERS	Genetic Abcence Epilepsy Rat from Stratsbourg
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Ganglion Cell Layer
GFAP	Glial Fibrillary Acidic Protein
HPA	Hypothalamic-Pituitary-Adrenal
HPC	Hippocampus
HR	Hazard Ratio
Iba1	Ionized Calcium-Binding Adapter molecule 1
ICAM-1	Cellular Adhesion Molecule-1
ICP	Intracranial Pressure
IL	Interleukin
IBE	International Bureau for Epilepsy
ILAE	International League Against Epilepsy
INF-γ	Interferon-γ
INL	Inner Nuclear Layer
IOP	Intra ocular pressure
i.p	Intraperitoneal
IP-10	Induce Protein-10
IPL	Inner Plexiform Layer
IS	Index Seizure
IQR	Inner Quartile Range
EEG	Electroencephalography
KA	Kainic Acid
KC/GRO	Keratinocyte Chemoattractant/Growth-Related Oncogene
KO	Knockout
LPP	Lateral Perforant Pathway
LPS	Lipopolysaccharide
Map2	Microtubule-Associated Protein 2
MCP	Monocyte Chemoattractant Protein
MDC	Macrophage-Derived Chemokine
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
ML	Molecular layer
MPP	Medial Perforant Pathway
MRI	Magnetic Resonance Imaging
NG2	Neuronal-Glial Antigen 2

NK	Natural Killer Cells
NL	Neuroligin
NMDA	N-Methyl-D-Aspartate
NSC	Non-Stimulated Control
OCT	Optical Coherence Tomography
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PAMP	Pathogen Associated Molecular Patterns
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-Kinase
PNES	Psychogenic Non-Epileptic Seizures
PSD-95	Post Synaptic Density Protein-95
rdKO	Retinal Degeneration Knockout
SGZ	Subgranular Zone
ROI	Region Of Interest
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SD	Sprag Dawley
SE	Status Epilepticus
SRS	Spontaneous Recurrent Seizures
SynIIKO	Synapsin II Knockout
T	Tesla
TA	Temporoammonic Pathway
TARC	Thymus and Activation-Related Chemokine
TBI	Traumatic Brain Injury
TGF-β	Tumour Growth Factor-β
TLE	Temporal Lobe Epilepsy
TNF-α	Tumor Necrosis Factor-α
VCAM-1	Vascular Cell Adhesion Molecule-1
vEEG	Video EEG
WB	Western Blot
WT	Wildtype

Original papers and manuscripts

I. **Ahl M**., Avdic U., Strandberg MC, Chugh D., Andersson E., Hållmarker U., James S., Deierborg T., Ekdahl CT. Physical Activity Reduces Epilepsy Incidence: a Retrospective Cohort Study in Swedish Cross-Country Skiers and an Experimental Study in Seizure-Prone Synapsin II Knockout Mice. *Sports Medicine Open*. 2019, 5:52

II. Avdic U., **Ahl M**., Öberg M., Ekdahl CT. Immune Profile in Blood Following Non-convulsive Epileptic Seizures in Rats. *Front in neurol*. 2019, 10:701

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IV. **Ahl M**., Avdic U., Skoug C., Ali I., Johansson UE., Ekdahl CT. Immune response in the eye following epileptic seizures. *Journal of neuroinflammation.* 2016, 13:155.

V. **Ahl M**., Avdic U., Chary K., Shibata K., Chugh D., Mickelsson PL., Kettunen M., Strandberg MC., Johansson UE., Sierra A., Ekdahl CT. Inflammatory reaction in the retina after focal non-convulsive status epilepticus in mice investigated with high resolution diffusion tensor imaging and magnetic resonance imaging ex vivo. *Epilepsy Research*. 2021, 176: 106730

Introduction

History

The term epilepsy was founded 500 years BC and is derived from the Greek word "epilepsia", which translates to "take hold of". Throughout history epilepsy has often been viewed as a possession or divine manifestation, even if Hippocrates as early as 460-370 BC accurately recognize epilepsy as a disease of the brain. Hippocrates idea slowly spread, and it took over 2000 years for the general opinion of epilepsy to change. Currently the thought of epilepsy as a divine phenomenon is luckily eradicated, even though the long history of superstition still has an impact on smaller communities (de Boer, 2010). During more modern history in the 19th and 20th century, the actual pathophysiology and mechanism behind epilepsy started to unravel with the help of new important discoveries in the field of neuroscience. A breakthrough was the discovery of synapses by Santiogo Ramon Y Cajal who received the Nobel prize in 1906 for his finding. Henceforth, scientists began to understand the essentiality of electrical currents for synapses as a communicative tool between neurons. In 1913, this resulted in a technique where neuronal activity could be measured, the electroencephalography (EEG), the first recording was published in animals by Pravdisch-Neminsky, 1913 cited in (Ahmed and Cash, 2013). The technique rapidly developed and 14 years later, Hans Berger was the first scientist to record the human EEG in Berger, 1929 cited in (Ahmed and Cash, 2013). The invention of EEG is one of the most significant scientific contributions for epilepsy, since it still remains the main diagnostic tool when investigating epileptic seizures.

Even though progress in the pathology of epilepsy had started to discern in the 19th and 20th century, there was a major stigma. When the first European institutions for treating epilepsy appeared, they were secluded from the general public since the inhabitants were seen as "disturbing" , mentally unstable and a shame for any family (de Boer, 2010). In the 20th century several organisations and foundations were established e.g. International League Against Epilepsy (ILAE) founded in 1909, and International Bureau for Epilepsy (IBE) founded in 1961, which significantly improved the view and healthcare of patients with epilepsy (Magiorkinis *et al.*, 2014).

Another important contribution was the evolution of neuroimaging techniques. They have increased the understanding and facilitated the diagnosis of epilepsy. Especially when the Magnetic Resonance Imaging (MRI) scanners started to be

commercially available in the 1980s, consequently making it possible to identify alterations in the brain among patients with epilepsy. Today, the practise of 1.5- and 3 tesla (T) MRI scanners remains the main clinical imaging tool for the diagnosis of epilepsy (Dakaj *et al.*, 2016). Still, to increase sensitivity and resolution, stronger magnets of 7T has started to emerge in the clinical settings (Trattnig *et al.*, 2018).

At present, epilepsy is by most societies accepted as a neurological disorder, even though the general knowledge of epilepsy often remains low, and the stigma around the disease still exists (Fiest *et al.*, 2014). Furthermore, some national studies in high income countries have reported improvements and less stigmatization, though the awareness in developing countries often remain low (Holmes *et al.*, 2019; Chakraborty *et al.*, 2021). The history of epilepsy has influenced how we perceive the disease today. Luckily, knowledge about epilepsy has increased immensely in recent decades, yet it is in our modern time a disease we rarely talk about, surrounded by both stigma and ignorance.

Pathophysiology and epileptogenesis

Epilepsy affects around 1% of the general population worldwide, which makes it one of the most common chronical neurological disorders (Fiest *et al.*, 2017). The disease is characterized by seizures, defined as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher *et al.*, 2005). The transformation of brain activity from an interictal state (brain activity in between seizures) to an ictal state (abnormal seizure activity) is called ictogenesis. All treatments, anti-epileptic drugs (AEDs), currently available are symptomatic, meaning they aim to reduce the occurrence of seizures without modulating the underlying pathology. Furthermore, over 1/3 of all patients are pharmacoresistant and do not gain control of their seizures with the treatments available, a number that has not improved in the last 30 years (Perucca *et al.*, 2020). Especially hard to treat is Temporal Lobe Epilepsy (TLE) that has the highest proportion of pharmacoresistant patients (Choi *et al.*, 2008).

Apart from the unpredictability of seizures, patients with epilepsy commonly suffer from comorbidities. More than 20% of all patients with epilepsy simultaneously suffers from depression (Josephson and Jette, 2017), and the risk of developing epilepsy is significantly higher in patients with neurodevelopmental disabilities such as autism or personality disorders (Ewen *et al.*, 2019). The clinical symptoms of seizures may vary depending on the brain region or regions that are affected. In 2017 the ILAE taskforce updated the classification of epilepsies (Fisher *et al.*, 2017; Scheffer *et al.*, 2017). The seizure classifications are based on seizure onset in parts (focal) or the whole brain (generalized), followed by a definition of the clinical symptoms (e.g motor symptoms). In ***Figure 1***, the expanded version of the ILAE

classification of seizures has been modified from Fisher et al. (2017). Apart for the unknown onset, there are mainly two categories of seizures, focal or generalized. Commonly, a generalized seizure onset in adults give rise to motor symptoms such as tonic-clonic or myoclonic movements. While paediatric generalized epileptic seizure frequently leads to absence seizures with disturbed consciousness, but without motor activation. A focal onset on the other hand can give rise to a plethora of symptoms depending on the activated brain region, and with or without a disturbed consciousness. In focal epilepsy one brain region is always responsible for the seizure propagation, called the epileptic focus. However, the initial activity can spread to larger parts of the brain, in that case causing a Focal to Bilateral Tonic-Clonic seizure (FTBTC).

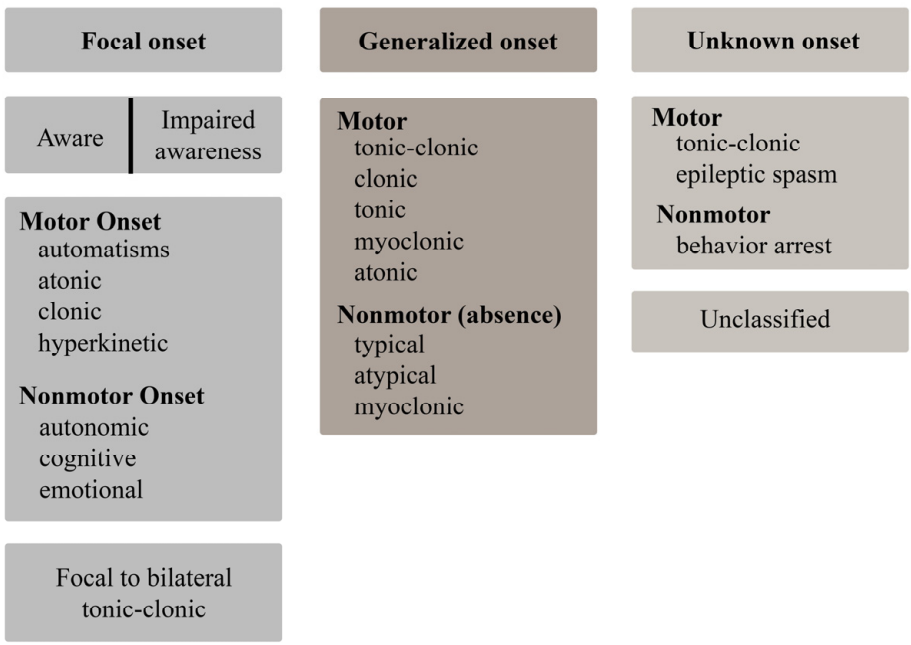


Figure 1: A modified version of the expanded ILAE classification of epileptic seizures Fisher et al. (2017).

Normally a seizure in patients have a duration of seconds to a couple of minutes. While a prolonged seizure, *Status Epilepticus* (SE) defined as >5min can occur in patients both in a generalized convulsive and focal non-convulsive form. Convulsive SE that exceeds 30 minutes in humans leads to a significant neuronal cell death and can be fatal (Leitinger *et al.*, 2019). However, of all cases 20-40% displays a focal Non-Convulsive SE (fNCSE) semiology, which can be challenging to diagnose (Holtkamp and Meierkord, 2011; Trinka *et al.*, 2015; Sutter *et al.*, 2016). Studies in rats found long term pathophysiological changes after fNCSE, represented by neuronal loss, increased inflammation, synaptic rearrangements and behavioural alterations (Krsek *et al.*, 2004; Avdic *et al.*, 2018).

Another clinical challenge is to distinguish epileptic seizures from Psychogenic Non-Epileptic Seizures (PNES), which upon misdiagnosis as epilepsy leads to inappropriate treatments. The relative occurrence of PNES is hard to predict since it depends upon availability of clinical resources, and occurrence of national guidelines for diagnosis and treatment of PNES (Kanemoto *et al.*, 2017).

The cause of epilepsy is often unknown, though there are several risk factors related to its development. A healthy brain can due to genetic or acquired factors, develop a susceptibility to seizures. A genetic predisposition often leads to a debut of epilepsy at an early age. Acquired epilepsies frequently develop in adolescence or adulthood, as a result of a primary insult e.g. Traumatic Brain Injury (TBI), brain infection or a stroke. After a primary insult, patients are without clinical symptoms for months or even years before clinical manifestations expressed as seizures arise. During this time period a process called epileptogenesis is initiated causing inflammation, neurodegeneration and increased excitatory drive in the affected brain area. Seizures themselves also leads to inflammation and neurodegeneration, further actuating the excitatory/inhibitory imbalance and seizure susceptibility (***Figure 2***). The exact mechanism of epileptogenesis is not known, even though both inflammation and increased excitability are two factors that are altered both during epileptogenesis and epilepsy. The epileptogenic process may vary depending on the primary insult (genetic or acquired) and the time passed after the initial injury.

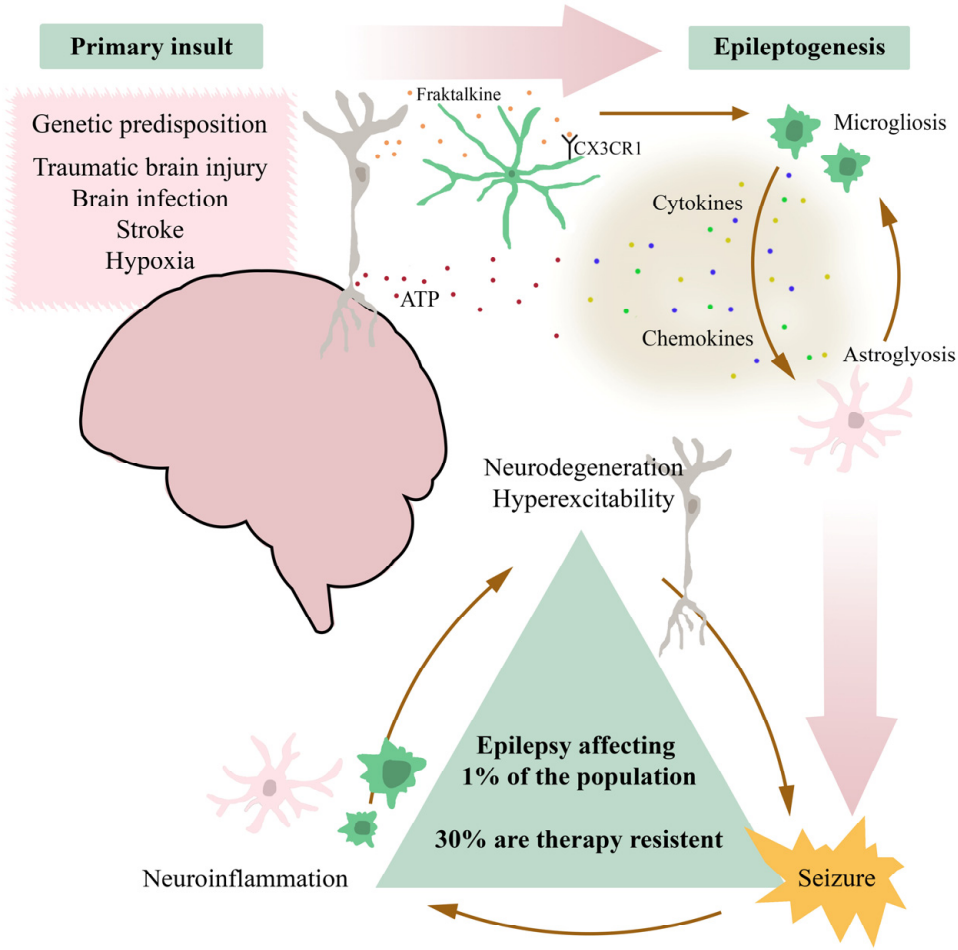


Figure 2: After a primary insult, either genetic or aquired, damaged neurons will signal to immune cells, mainly microglial via fraktalkine or abberant extracellular ATP. This rapidly activtes and induces microglial proliferation and mophological alterations (microgliosis). Microgliosis leads to release of proinflammatory cytokines and chemokines to attract other inflammatory cells such as other microglial cells and astrocytes. Astrocytes are involved in keeping the brain homeostasis by buffering both ions, water and neurotransmitters, and upon a microglial activations these paramentes will be affected, which makes the astrocyte more inflammatory actived. Upon inflammation or injury both microglia and astrocytes promote each others activation by releasing chemokines and cytokines. The prolonged micro- and astroglyosis leads to disruption of the homeostasis and hyperexcitability, and eventually the occurance of the first spontanious seizure. Seizures themselves will also induce neuroinflammation that in turn will lead to more neurdegeneration and increased hyperexcitability.

Animal models of epilepsy

In an attempt to mimic human epilepsy, we have several animal models based on either genetic or acquired factors. In epilepsy research, mice and rats are most frequently used. Animal models are as the name suggests only a model of a disease, meaning they will never represent all characteristics of the human condition. Yet, every animal model will have specific aspects that are consistent with the human disease mechanism and pathology. Furthermore, in acquired models of epilepsy the primary insult might induce seizures, but these need to be regarded rather as acute symptomatic seizures in response to an acute injury, and not epileptic seizures. In animal models and human patients, seizures that develop within a week after a primary insult is frequently viewed acute symptomatic (Beghi *et al.*, 2010). Still, to give an exact time schedule of when a seizure is acute symptomatic, or a spontaneous seizure induced by epileptogenic is not possible. It might be different depending on both the primary insult and the animal itself. It is more accurate to assume that seizures appearing 1 week after the primary insult, and that are reoccurring, can be defined as epileptic Spontaneous Recurrent Seizures (SRSs).

Genetic animal models

Traditionally, mice have most commonly been used for genetic Knock Out (KO) animal models due to their breeding efficiency and relatively fast reproductive cycle. Even so, the WAG/Rij and Genetic Absence Epilepsy Rat from Stratsbourg (GAERS) are two sporadically discovered genetic rat models not uncommonly used for absence or idiopathic generalized epilepsy research (Depaulis *et al.*, 2016; Russo *et al.*, 2016). The use of genetic animal models of epilepsy is often based on mutations found in human epilepsies. In fact, some genetic mutations can reflect several features of a human disease e.g. the CNTNAP2 gene, implicated in cortical dysplasia-focal epilepsy syndrome and autism gives rise to a similar pathology in cortex, and behavioural alterations with autistic features in mice (Strauss *et al.*, 2006; Peñagarikano *et al.*, 2011). In general, genetic models result in an increased seizure susceptibility inducing epileptogenesis and a seizure onset from neonatal to adult age, depending on the mutated gene(s) and the underlying pathology.

In paper I, a genetic mouse model of epilepsy has been used, the Synapsin II Knockout (SynIIKO), which similarly also have been found mutated in human epilepsies (Garcia *et al.*, 2004; Lakhan *et al.*, 2010). Synapsins are proteins that bind vesicles stored with neurotransmitters to the cytoskeleton, regulating synaptic transmission by vesicle release. There are three different genes encoding for synapsin I-III, either most predominantly expressed on excitatory or inhibitory synapses. Synapsin II is most commonly expressed in excitatory glutamatergic synapses, and a KO mutation will in mice give rise to stress-induced presumably focal to bilateral tonic clonic seizures around 2.5-3 months of age, when the synapsin expression is meant to be high (Etholm *et al.*, 2012; Etholm *et al.*, 2013; Chugh *et al.*, 2015). Seizures in SynIIKO mice are spontaneous, but need to be

triggered by increased stress, such as human handling. Therefore, seizures are rarely occurring when mice are undisturbed in their home cage. This makes the synIIKO mice a suitable model to study epileptogenesis, since they have a very specific time window for seizure onset, and the number of seizure triggers can be standardized between animal groups, based on how they are handled. Additionally, SynIIKO mice presents with behavioural alterations and autistic features not uncommonly associated with human epilepsy patients (Greco *et al.*, 2013).

Acquired models

The most common animal models of epilepsy develop an acquired form by either electrical, chemical, or trauma induced epileptogenesis. Frequently, both electrical and chemical animal models of epilepsy are post-status models, meaning the primary insult, either electrical or chemical, induce a prolonged epileptic seizure (SE) that eventually after a latent period of a week or up to months will give rise to SRSs. In humans, epilepsy can be initiated by SE, and it is seen as a risk factor for developing epilepsy (Hesdorffer *et al.*, 1998; Gugger *et al.*, 2020). In the electrical models, and some chemical models, the advantage of a higher seizure susceptibility in certain brain areas are utilized. The hippocampus (HPC), located in the temporal lobe, is a structure with a high seizure susceptibility after a primary insult (Golarai *et al.*, 2001), making TLE the most common form of focal epilepsy. Therefore, the hippocampus is often targeted in electrically induced animal models of epilepsy (As in paper II, IV and V), resulting in a pathogenesis resembling the human TLE.

In chemical models, Kainic Acid (KA), an exogenous glutamate analogue or pilocarpine, acting as an antagonist on cholinergic M2-receptors can be given *intraperitoneal* (*i.p*), in mice or rats to induce acute symptomatic seizures (Vezzani, 2009; Lévesque and Avoli, 2013), giving the drug systemically might be beneficial when investigating Blood Brain Barrier (BBB) dysfunction after acute seizures. A more refined chemical model is the intra-hippocampal KA model, which similarly to the human TLE, and the electrically induced SE model, creates a primary insult, and eventually an epileptic focus in the hippocampus. Lastly, some animal models of epilepsy do not involve the use of exogenous substances (an electrode, or chemicals), and are instead based on trauma induced epileptogenesis, which is a known factor for inducing epilepsy in humans (Stefanidou *et al.*, 2017; Webster *et al.*, 2017). In an attempt to mimic the human mechanism in this aspect, animal models for traumatic brain injury, and post-stroke models are normally used. However, the percentage of animals that do develop SRSs in the traumatic brain injury model are less reliable, and the epileptogenesis can be prolonged (Kharatishvili *et al.*, 2006; Pitkänen *et al.*, 2009). Among the post stroke-models, the photothrombosis model has revealed some relevance in epilepsy research, while others e.g middle cerebral artery occlusion rarely leads to the development of SRSs (Karhunen *et al.*, 2007; Leo *et al.*, 2020). Hence, the benefits for using the trauma-based models are greatest when you want to mimic trauma induced epilepsy in humans.

Inflammation

Our immune system and inflammatory processes are crucial for the ability to protect ourselves from pathogens, and to eliminate damaged cells and tissue before initiation of rebuilding processes. However, inflammation is meant to be an acute process that needs to be tightly regulated, and it can have detrimental consequences if it gets chronically activated. The immune system can be divided into the innate and adaptive immunity. The innate immune system is the first one to react at invading pathogens or cell damage, and it has a more general approach recognizing common pathogen associated or damage associated proteins. Monocytes, or macrophages in the periphery, microglia in the brain, and Natural Killer cells (NK) are all a part of the innate immunity. The adaptive immune system has a more targeted approach, and upon a first infection it takes several weeks to develop a more specific inflammatory defence. It is a combination of antibody producing B-cells, releasing targeted antibodies for a specific pathogen, which the adaptive immune T-cells or other innate immune cells will recognise and eliminate.

Peripheral inflammation

We have several organs involved in the immune system such as bone marrow, the lymphatic system, thymus, and spleen. The bone marrow consists of haematopoietic stem cells surrounded by adipose cells and vascular sinuses, supplying the body with new blood cells, immune cells and lymphocytes. B and T lymphocytes are adaptive immune cells, and their precursors proliferate in the bone marrow similarly to innate immune cells, however maturation of adaptive immune cells has to take place in secondary lymphoid organs such as lymph nodes, thymus or spleen. Usually, the innate immunity reacts first in response to infection or damage, before an adaptive immune response is initiated. Peripheral innate immune cells such as NK cells, monocytes, macrophages or neutrophils all express pattern recognition receptors: Pathogen Associated Molecular Patterns (PAMPs) or Danger Associated Molecular Patterns (DAMPs).

Upon digestion of a pathogen, peripheral innate immune cells can migrate to any of the secondary lymphoid organs to initiate an adaptive immune response and B-cell activation. The primary task for B-cells is the production of specific antibodies against invading pathogens, both to inactivate their ability to bind and infect new cells, but also marking pathogens for elimination to other immune cells. The fully matured B-cell will start to produce antibodies against the presented antigen that presumably comes from a bacteria or virus. Our second innate immune cell is called T-cell. There are several different types of T-cells but simplified you can divide them into two groups: cytotoxic $CD8^+$ cells, and helper T cell that are $CD4^+$. They both descends from the same progenitor cells, however they have two distinctly different functions. Cytotoxic T-cells are able to directly eliminate pathogens, their

main activation pathway comes from Major Histocompatibility Complex (MHC) molecules. MHC class I (MHCI) is expressed on the cell surface by all cells with a nuclei, and it is presenting a fraction of the protein the cell is producing at the time. Via the MHCI molecule T-cells can detect infected virus producing cells. Antigen presenting cells such as dendritic cells in the periphery, and microglia in the brain will express another MHC molecule, class II (MHCII), which is presented at the cell surface mainly to helper T-cells. The MHCII molecules display protein content from cellular components or pathogens that have been phagocytised by the antigen presenting cell. The T-helper cells are upon activation recruiting and activating other immune cells, both innate and adaptive, via cytokine and chemokine signalling (Bruce Alberts, 2008).

In epilepsy there are several studies indicating both a local inflammation within the epileptic focus together with a peripheral or systemic inflammatory response (Vezzani *et al.*, 2013; Varvel *et al.*, 2016). Additionally, proinflammatory cytokines such as interleukin-6 (IL-6) has been shown to increase in cerebrospinal fluid after a generalized seizure in patients with epilepsy (Peltola *et al.*, 2000; Lehtimäki *et al.*, 2004). Chronic levels of immune factors in blood from patients with epilepsy has been identified both as increases of IL-6, and IL-1β (Hulkkonen *et al.*, 2004; Gao *et al.*, 2017), while other studies contradict chronic alterations of these cytokines (Lehtimäki *et al.*, 2007; Uludag *et al.*, 2013). One explanation for different outcomes in different studies might be various seizure frequencies and heterogenous patient groups. Clinical studies have also investigated acute alterations in serum from patients. In a small study IL-6 levels were increased both after a focal and generalized seizure (Lehtimäki *et al.*, 2007). Similarly, an increase of IL-6 was found in focal patients at an individual level, even though these studies did not include a control group (Alapirtti *et al.*, 2009; Bauer *et al.*, 2009; Alapirtti *et al.*, 2018).

Microglia and inflammation in the central nervous system

Microglia is a resident immune cell of the Central Nervous System (CNS), however they are also crucial regulators sensitive to alterations in the brain or eye homeostasis. During the embryogenesis cells originating from the mesodermal cell linage migrates into CNS to create a population of cells that differentiates into a microglial population in the post-natal brain (Ginhoux *et al.*, 2010). Through physiological conditions this is the only residence of microglial cells within the CNS, however during a diseased state increased permeability and disruption of the BBB can occur, leading to an infiltration of monocytes from the blood that in brain parenchyma mature into a second microglial population (Varvel *et al.*, 2016). During homeostasis in both the brain and retina, microglia have their own designated area where they operate, with a ramified or “surveying” phenotype, consisting of a small cell soma, and many long and thin processes. The ramified

state, also called “resting” microglia can be a misguiding term, since ramified microglia are immensely active, always moving their processes to scan the extracellular environment and surrounding neurons (Nimmerjahn *et al.*, 2005). Nevertheless, this phenotype can rapidly change in response to e.g. cellular injury, altered neuronal activity, or the presence of pathogens. The activation process (or rather activation shift) will change the microglia morphology, to an intermediate phenotype with a large cell soma, retracted processes until only a few thicker processes are left, or to a round/ amoeboid appearance that is a fully activated, phagocytic phenotype, with none or very few processes (Ali *et al.*, 2015; Chugh *et al.*, 2015).

Microglia are one of the first cells responding to an injury or infection and depending on the stimulus they can react in different ways. Previously one talked about two distinct phenotypes: The classic “M1” activation considered proinflammatory, releasing cytokines such as IL-6 or IL1-β, and the alternative “M2” activation, considered anti-inflammatory releasing trophic factors such as Tumour Growth Factor-β (TGF-β) and Brain Derived Neurotrophic Factors (BDNF). However, this simplistic way of categorizing microglia does not reflect the variety of microglial phenotypes that can be found in the brain and eyes, hence it is now generally accepted, even though microglia can have pro- or anti-inflammatory properties, it is more likely a scale rather than one or the other. The anti-inflammatory pathway leads to regenerating and survival signals from microglia, which are crucial for synaptic plasticity and neuron survival (Ekdahl, 2012).

Upon injury or disturbed homeostasis, microglia evolve their phagocytic capability to clear tissue debris, damaged or infected cells. They release pro inflammatory factors to recruit other immune cells, such as other microglia, astrocytes or even T-cells. Microglia is an antigen presenting cells and can increase their antigen presenting capacity by upregulating their MHCII expression. In this process activated microglia also signals for increased microglial proliferation, and migration to the injured site, leading to clusters of activated microglia instead of the homeostatic homogeneously spread cells. Microglia have a vast number of “triggering factors”, which makes them very sensitive of discrete extracellular or cellular changes. Therefore, being an immune cell and regulator of the nervous system, the microglial activity needs to be tightly regulated, since prolonged or hyperactivation abruptly can lead to cellular pathology. Indeed, dysfunction of microglia has been implemented in several neurological disorders, including epilepsy, were dysfunctional phagocytosis, motility and pruning properties has been suggested (Abiega *et al.*, 2016; Andoh *et al.*, 2019).

Fractalkine/CX3CR1 signalling

Similar to other innate immune cells in the periphery, microglia recognize PAMPs and DAMPs. Apart from these families of receptors, microglial cells are also sensitive to a variety of other molecules i.e. to altered concentrations of

neurotransmitters, Adenosine Triphosphate (ATP) and/or the release of cytokines and chemokines (Nimmerjahn *et al.*, 2005; Li *et al.*, 2012). One of these important chemokines is fractalkine (also called CX3CL1), expressed by neurons upon injury as a membrane bound, or soluble form. Fractalkines only known receptor, CX3CR1 is primarily expressed on microglia, but also on neurons, and this pathway presents an essential component in the communication between neurons and microglia (Hatori *et al.*, 2002). CX3CR1 is a G-protein coupled receptor and upon binding its ligand fractalkine it can lead to a rapid activation of several intracellular signalling pathways i.e. Phosphoinositide 3-Kinase (PI3K) or Ras signalling in a dose and time dependent manner (Sheridan and Murphy, 2013). The fractalkine/CX3CR1 pathway is involved in microglial activation, survival and proliferation (Boehme *et al.*, 2000; Hatori *et al.*, 2002) and it might have different functions depending on the underlying pathological condition (Pawelec *et al.*, 2020).

The absence of CX3CR1 in knockout studies has suggested induced neurotoxicity after Lipopolysaccharide (LPS) treatment and increased chronic neuronal death after TBI (but not acutely) in mice lacking CX3CR1 (Cardona *et al.*, 2006; Febinger *et al.*, 2015). The same study done by Febinger H. et al. 2015 revealed lesser motor deficits and neuronal death acutely in CX3CR1 KO mice. Additionally, another study has reported beneficial outcomes in CX3CR1 KO mice after stroke by smaller infarction site and milder neurological deficits compared to WT mice (Tang *et al.*, 2014; Febinger *et al.*, 2015). These disperse results might be dependent on the differences in the pathological reactions, time after insult, and the fact that the absence of CX3CR1 signalling leads to alterations in brain development and maturation, which can affect various disease models differently (Zhan *et al.*, 2014; Bolós *et al.*, 2018).

Another approach for blocking the fractalkine/CX3CR1 pathway is the use of antibodies that block either the CX3CR1 receptor, or inhibits fractalkine function. Yeo S.-I. and colleges investigated the effects of manipulating the CX3CR1/fractalkine signalling with antibodies, acutely after pilocarpine induced SE in rats. In fractalkine infused animals there was an increased number of microglia, while antibodies targeting fractalkine or the CX3CR1 receptor led to a decreased number of microglia compared to saline infused animals, suggesting that blocking the fractalkine/CX3CR1pathway had anti-inflammatory effects (Yeo *et al.*, 2011). In another hyperexcitable condition stimulating CX3CR1 signalling *in situ* in rats detected reduced neuronal transmission by diminishing excitatory postsynaptic potentials (Ragozzino *et al.*, 2006). In a more chronic epileptic environment, the intracerebroventricular infusion of a CX3CR1 antibody reduced microglial reactivity (Ali *et al.*, 2015).

Astrocytes and the Blood Brain/Retina Barrier

Astrocytes have as well as microglia an important role in maintaining the brain homeostasis. However, unlike microglia, astrocytes have more prolonged contacts with the same neuron, and they are one of the main cellular components in the BBB/Blood Retinal Barrier (BRB). The BBB/BRB is a highly specialized microvascular unit that sheaths the whole CNS, tightly regulating the passage of molecules, ions and cells between the blood and CNS. In a physiological condition astrocyte endfeet completely covers the micro vessel unit consisting of endothelial cells, and pericytes (Daneman and Prat, 2015). Interacting directly with both neurons and blood vessels, astrocytes are able to adjust deliverance of oxygen and nutrients based on the neuronal activity (Bélanger *et al.*, 2011). Increased BBB permeability is an important function during CNS waste clearance and infection, and BBB dysfunction has been linked to a worse outcome and recovery after stroke and Alzheimer's Disease (AD) (Yamazaki and Kanekiyo, 2017; Nadareishvili *et al.*, 2019). In humans, there is some evidence that disruption of the BBB induces seizures (Marchi *et al.*, 2007). Additionally, more robust findings in animals suggests there is increased BBB leakage both acutely and chronically post SE, and that BBB disruption severity could be linked to seizure frequency (van Vliet *et al.*, 2007).

Apart from involvement in the BBB, astrocytes are important regulators of neuronal activity. They sense increased extracellular concentrations of neurotransmitters such as glutamate, Gamma-Aminobutyric Acid (GABA), ATP and D-serine. The main proportion of synaptic glutamate is taken up by astrocytes that indirectly are regulating both neurotransmitter release and glucose uptake (Bélanger *et al.*, 2011; Bazargani and Attwell, 2016). Astrocytes similarly to microglia are sensitive to homeostatic changes, such as cytokine or chemokine release, and excessive synaptic activity. In epilepsy, astrocyte dysfunction and regulation of synaptic activity has been related to epileptogenesis and seizure progression (Tian *et al.*, 2005; Li *et al.*, 2007; Broekaart *et al.*, 2018; Nikolic *et al.*, 2018).

Synapses and neuronal networks

With a stimulus strong enough seizure activity can be induced in all neuronal networks. However, during epileptogenesis changes on both a synaptic and network level leads to a lower threshold for induction of seizures, and in the end the epileptic network will induce seizure activity spontaneously. Within the brain there are two different principal forces, excitation and inhibition. Excitatory neurons release glutamate and are the driving force of a network, glutamate binds to either N-Methyl-D-Aspartate (NMDA), or α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic (AMPA) receptors in the post synaptic end. Inhibitory neurons (also called interneurons) are the "brake" or fine-tuning force of a neuronal network,

they release GABA on the pre-synaptic end will bind to the GABA receptor on the post-synapse to modulate or inhibit excitatory signals. To stabilize and maintain the synaptic cleft there are a number of adhesion molecules present e.g. neurexins (presynaptic) and neuroligins (NL) (postsynaptic) that binds to each other to form a stable linking of the synaptic connection (***Figure 3***). Furthermore, assembling and binding the neurotransmitter receptors to the cytoskeleton are scaffolding proteins i.e Post Synaptic Density Protein-95 (PSD-95) on excitatory synapses and gephyrin on inhibitory synapses. One of the triggers factors for scaffold protein assembly is the expression of neurexin 1β and NL-1 (Giannone *et al.*, 2013). Excitatory neurons have a natural contribution in seizure activity, and recurrent excitation, when excitatory neurons activate each other such as in the hippocampus, is enhanced in epilepsy (Zhang *et al.*, 2012; Badawy *et al.*, 2013; Andreasson *et al.*, 2020).

On a cellular level, increased firing of excitatory neurons can be affected by their ion channel expression that influences their firing potential (Arnold *et al.*, 2019), On a network level increased excitatory connections contributes to hyperexcitability (Morgan and Soltesz, 2008), still increased hyperexcitability is a combination of increased excitation and decreased inhibition. In patients with epilepsy, alterations in GABA receptor subunits have been identified within the hippocampus (Loup *et al.*, 2000; Loup *et al.*, 2006). Furthermore, alterations on a synaptic level i.e. decreased PSD-95 and increased gephyrin has been identified in animal models of epilepsy, interestingly these alterations also seem to be present in newly formed neurons after SE (Sun *et al.*, 2009; Jackson *et al.*, 2012). On a network level it is suggested that interneurons contribute to seizure activity and ictogenesis by initiating synchronized neuronal firing (Fujiwara-Tsukamoto *et al.*, 2003; Glickfeld *et al.*, 2009; Khazipov, 2016). Yet, certain types of interneurons have been suggested to be extra sensitive to seizure induced neurotoxicity (Marx *et al.*, 2013; Nakagawa *et al.*, 2017). However, in another study, specific vulnerability of interneuron subtypes could not be determined in an acquired epilepsy model of KA or TBI (Huusko *et al.*, 2015). The fully contribution of inhibitory neurons to seizure propagation and their vulnerability in epilepsy is not yet fully understood.

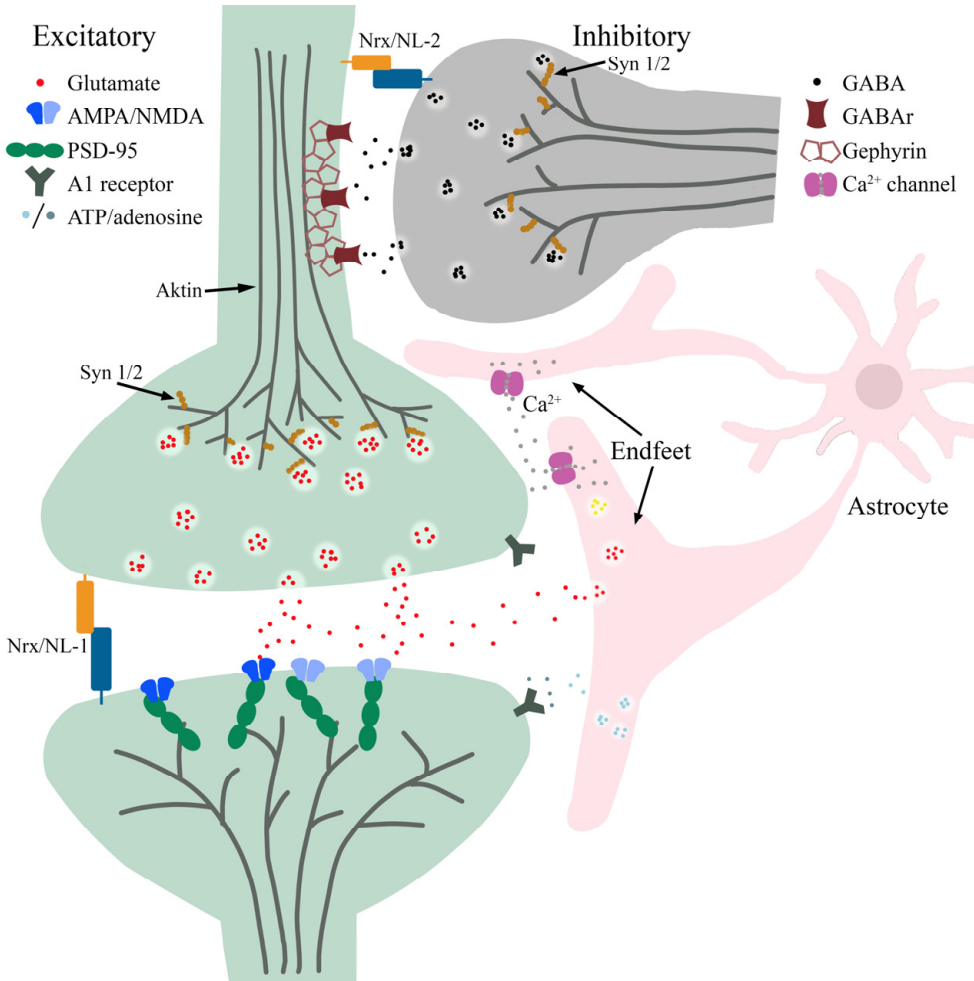


Figure 3: Neurotransmitters are stored in vesicles connected to the cytoskeleton via synapsins. The synaptic cleft is stabilized by the linking of neurexins on the presynapse and neuroligins on the post synapse, their binding also assembles scaffolding proteins such as PSD-95 (excitatory) and gephyrin (inhibitory) which AMPA or NMDA or the GABA receptor will bind to. Upon vesicle release glutamate or GABA concentrations will increase in the synaptic cleft, astrocytic endfeet will take up a majority of the excessive glutamate (or GABA) to regulate the synaptic activity. Increased intracellular astrocytic concentrations of glutamate will trigger both an increased uptake of glucose from the blood to meet the increasing neuronal energy demand, and it will also initiate a negative feedback loop to dampen the excitatory neuron. As another regulator of neuronal excitability Adenosine Triphosphate (ATP) released into the synaptic cleft due to increased synaptic activity will be converted into adenosine, which binds to A1 receptors on both the pre, and post-synaptic cell to reduce neuronal signalling.

The Hippocampus (HPC) consists of a neuronal network with an excitatory tri-synaptic loop originating from the outer layers of Entorhinal Cortex (EC) propagating naturally recurrent excitatory neuronal activity, first to the Dentate Gyrus (DG) then to *Cornu Ammonis* 3 (CA3) and CA1 (***Figure 4***). CA1 pyramidal neurons then signals back into the deeper layers of EC (layer V, VI) to complete the synaptic loop. There are three different connective pathways within the hippocampus, the Perforant Pathway divided into the Lateral (LPP) and Medial Perforant Pathway (MPP) that are important for spatial learning and memory, and the Temporoammonic Pathway (TA), mostly involved in spatial learning. Excitatory mossy fiber cells from the Ganglion Cell Layer (GCL) within the dentate gyrus sends projections to pyramidal neurons in the CA3, CA4 and to interneuons. Temporal lobe seizures leads to neuronal cell death in both CA1, CA3 and CA4 (Schmeiser *et al.*, 2017), but also increased neurogenesis experimentally (Mohapel *et al.*, 2004; Varma *et al.*, 2019). To compensate for lost connections with pyramidal neurons in CA3 and CA4, mossy fibers can synapse aberrantly into the GCL, a phenomenon called mossy fiber sprouting. Additionally, the increased neurogenesis after a temporal lobe seizure will give rise to newly formed progenitors that reinforces the aberrant projections and recurrent excitation of granule cells connecting back to the GCL. There are evidence that mossy fiber sprouting occurs both in humans with TLE and animal models (Parent *et al.*, 1997; Schmeiser *et al.*, 2017; Mo *et al.*, 2019), weather mossy fiber sprouting is amplifying hyperexcitability in the hippocampus is debated, and its exact role is not yet determined (Jakubs *et al.*, 2006; Parent and Murphy, 2008; Cho *et al.*, 2015).

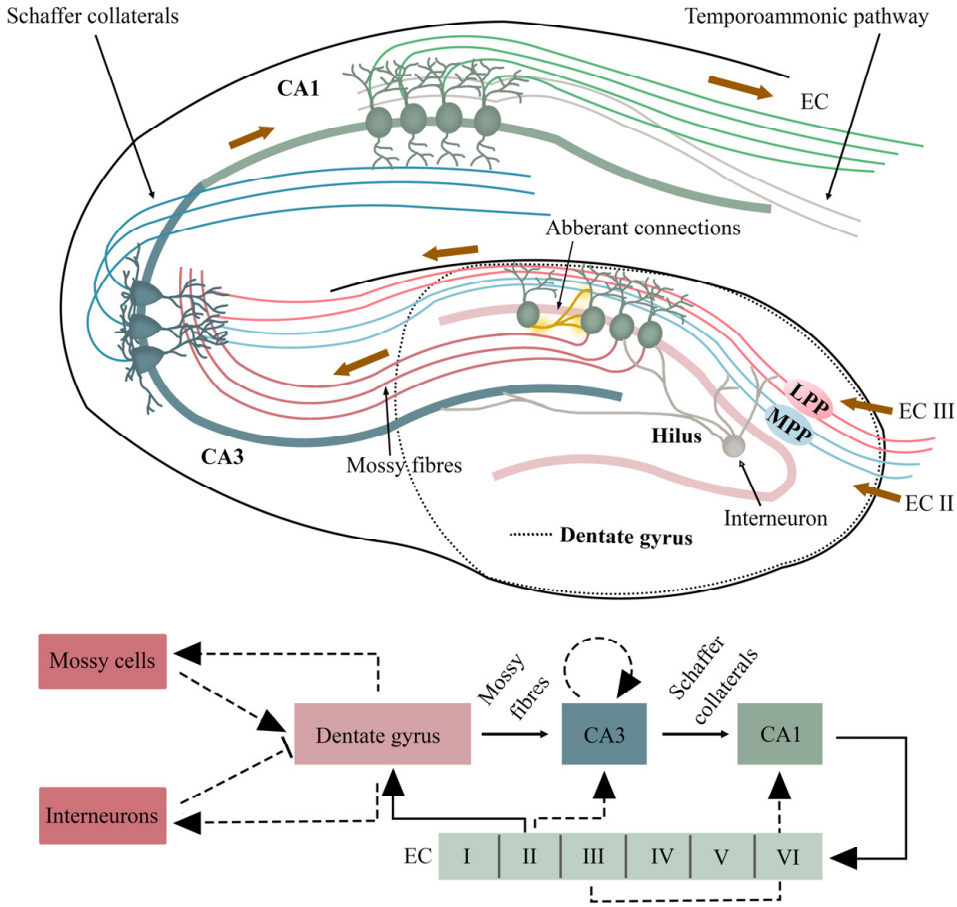


Figure 4: Modified from Deng W. et al. (2010). Signals from entorhinal cortex (EC) propagate activity into Ganglion Cell Layer (GCL) of the dentate gyrus, further signalling to the CA3, CA1 and eventually back to the deeper layers of EC. In epilepsy mossy fibers can aberrantly synapse back into the GCL instead of CA3, a phenomenon called mossy fiber sprouting.

The retina is a neuronal network with a distinct structure. A healthy retina should have well defined layer, starting from the most inner parts: GCL, containing ganglion cells that transfer retinal signals into the optic nerve that eventually reaches the primary visual cortex in the occipital lobe. In the GCL Muller cells, a radial astroglial cell closely interacting with microglial cells (Wang *et al.*, 2011), have their endfeet that are sensitive to homeostatic alterations within the retina. The Inner Plexiform Layer (IPL), containing neuronal fiber tracts, and the majority of the retinal microglial population. Inner Nuclear Layer (INL) consisting of the cellbodies of bipolar cells. Outer Plexiform Layer (OPL) somewhat thinner than the IPL, but similarly consisting of neuronal fibers and microglia. In the most outer layer of the

retina, the Outer Nuclear Layer (ONL) consisting of the photoreceptor cells can be divided into the inner, and outer segments. Inner segments inhabit the photoreceptor cell bodies, and the outer segments contain the light sensitive part of the photoreceptors, rods and cones (***Figure 5***).

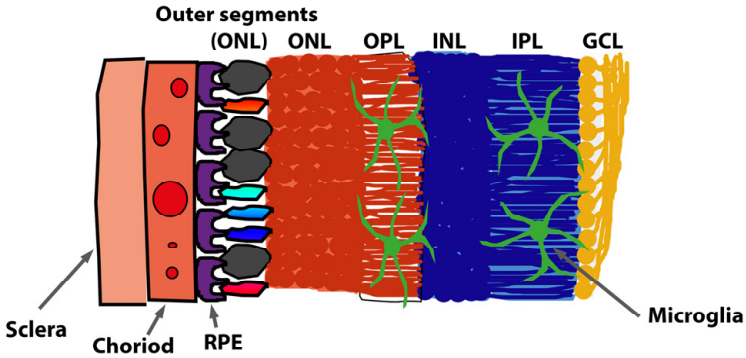


Figure 5: Schematic drawing of the different structural layers in the retina. From left to right: Sclera, choroid containing the vessels supply of the retina, retinal pigment epithelium (RPE), outer segments (OS) of the photoreceptors, outer nuclear layer (ONL) nucleus of the photoreceptors, outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) the ganglion cell layer (GCL).

The brain and exercise

Exercise has showed many beneficial effect and has been suggested as a therapeutic strategy for several diseases (Gubert and Hannan, 2021), however the full extent of exercise and brain related mechanisms are not yet fully understood. The production of free radicals is a naturally occurring process due to the metabolism in all cells, and as a response to avoid oxidative stress cells produce antioxidants. During exercise, muscle cells increase their metabolic rate, which will lead to an increased production of free radicals, such as reactive oxygen species (ROS) or reactive nitrogen species. With physical activity or exercise it's believed there is an upregulation of the antioxidant defence system in response to the free-radical increase (Vargas-Mendoza *et al.*, 2019). However, the relationship between oxidative stress and aerobic vs anaerobic exercise has been hard to establish, since type, duration and persistence might influence the free radical and antioxidant response system, but also since acute and long term effects might be different (Shi *et al.*, 2007; Fisher-Wellman and Bloomer, 2009; Ammar *et al.*, 2020).

Apart from the protection of oxidative stress, there is scientific evidence that exercise also stimulates the hypothalamic-pituitary-adrenal (HPA) axis and decreases systemic inflammation (Bonifazi *et al.*, 2009; Fatouros *et al.*, 2010;

Monteiro-Junior *et al.*, 2018). In rodents, physical activity increase their regenerative capacity of the brain, reducing stress hormones and inflammatory factors (Chennaoui *et al.*, 2002; Makatsori *et al.*, 2003; Chennaoui *et al.*, 2008; Gomes da Silva *et al.*, 2013; Fernandes *et al.*, 2016; de Almeida *et al.*, 2017).

The HPA axis is a stress response system, the main regulator being the hypothalamus, a small region located at the base of the brain, that is a key regulator of hormones. In our stress response system the hypothalamus signals to the closely located pituitary glands via Corticotrophin-Releasing Factor (CRF), which leads to pituitary Adrenocorticotrophic Hormone (ACTH) release into the bloodstream. The ACTH have in turn its target in the adrenal cortex, where it stimulates for synthesis and secretion of glucocorticoids. The main output from increased HPA axis drive is the increase of glucocorticoids. Furthermore, the increase of glucocorticoids, such as cortisol, in the vascular system initiates a negative feedback loop inhibiting hypothalamus production and release of CRF (***Figure 6***). Glucocorticoids are a group of steroid hormones that are released during increased stress as an adaptive response to environmental changes. They increase metabolic rate, cardiovascular tonus and have anti-inflammatory effects. An acute transient increases of glucocorticoids are part of the essential mechanism to handled increased stress or threats, however a more chronic upregulation have been linked to pathological states such as depression, AD and premature aging (Fiocco *et al.*, 2008; Arsenault-Lapierre *et al.*, 2010; Marin *et al.*, 2011; Weng *et al.*, 2016). Physical activity has been shown to induce HPA axis adaptation with an increased sensitivity for glucocorticoids and cortisol. However, some studies suggest that the HPA response, beneficial or detrimental, is dependent on the exercise intensity, duration and circumstantial stress factors (Kraemer and Ratamess, 2005; Hill *et al.*, 2008; Hackney and Walz, 2013; Miller *et al.*, 2018). High intensity or forced exercise in animals fail to initiate these beneficial effects and HPA adaptation (Chennaoui *et al.*, 2002; Droste *et al.*, 2007; Svensson *et al.*, 2016).

Apart from the peripheral benefits from physical activity, such as decreased blood pressure, and decreased inflammation, exercise also induce a number of beneficial processes in the CNS. In rodents, exercise increase neurogenesis and spine formation via BDNF and its receptor Trk pathways (Chen *et al.*, 2017; Ferreira *et al.*, 2019). BDNF is an essential regulator of neuronal survival and brain plasticity by new spine formation and maturation (Zagrebelsky *et al.*, 2018) a process partly regulated by microglia (Parkhurst *et al.*, 2013). Additionally, physical activity decrease cytokine levels in various brain areas in rat (Chennaoui *et al.*, 2008). In animal models of epilepsy, physical activity improves the disease by decreasing seizure frequency (Arida *et al.*, 1999; Vannucci Campos *et al.*, 2017) and increasing the seizure threshold and decreasing the seizure susceptibility (Arida *et al.*, 1998; Arida *et al.*, 2004; Gomes da Silva *et al.*, 2011; Holmes *et al.*, 2015). Studies in humans reveal that patients with epilepsy are less likely to exercise compared to the general population, mostly due to fear of inducing seizures or physical injuries, but

also due to stigma (Cui *et al.*, 2015; Capovilla *et al.*, 2016). However, in line with animal studies, patients with epilepsy benefit from physical activity, commonly with a decrease of seizure frequency (McAuley *et al.*, 2001; Arida *et al.*, 2013; Pimentel *et al.*, 2015). Furthermore, physically active patients with epilepsy suffers less from comorbidities such as cognitive impairment and depression (Camilo *et al.*, 2009).

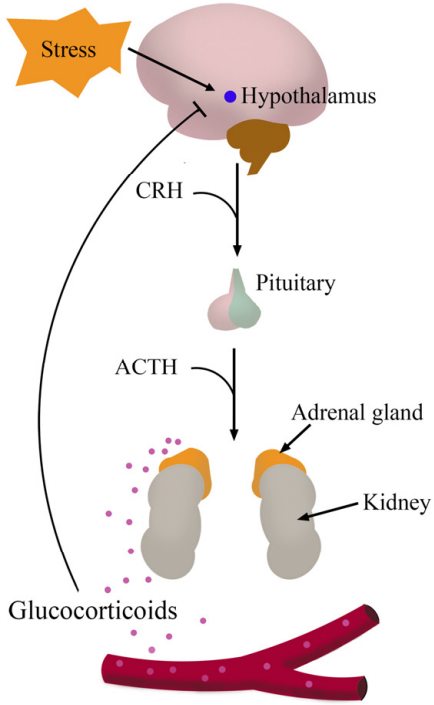


Figure 6: The main regulator of the hypothalamic-pituitary-adrenal (HPA) axis is the hypothalamus. Upon increased stress the hypothalamus responses by producing corticotrophin-releasing factor (CRH), which in turn will signal to the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream. When ACTH reaches the adrenal gland, adrenal cortex starts producing glucocorticoids, which is the main output from an increased HPA axis drive. Glucocorticoids in turn initiates a negative feedback loop that inhibits the hypothalamic production of CRH.

Objectives

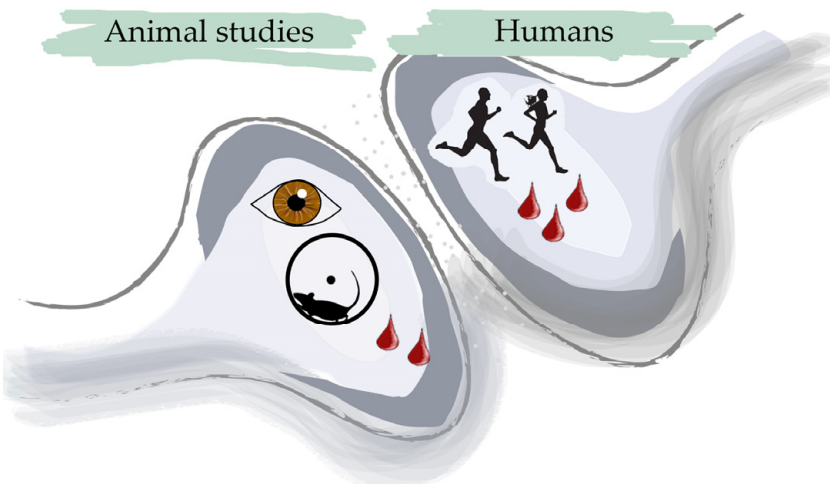
The purpose of this thesis was to evaluate inflammatory factors in both the eyes and blood in search of future prognostic or diagnostic marker for epilepsy. Additionally, to investigate the role of exercise before the development of epilepsy.

The specific aims of the thesis are:

I. To evaluate the role of exercise in both mice and humans for epilepsy development (paper I).

II. To study inflammatory markers in blood both acutely, and chronically in an animal model of temporal lobe epilepsy and to identify inflammatory markers after seizures in human epilepsy patients (paper II, III).

III. Investigate the extent of inflammation in the retina in an animal model of epilepsy with both traditional and non-invasive MRI techniques (paper IV, V).



Methods

Animals

Animals were housed with a 12hr dark/light cycle with water and food in *ad librium*. All animal procedures followed the guidelines set by the local ethical committee of Lund university and were approved by the Swedish board of agriculture.

Genetic models

For paper I the SynIIKO strain was produced by homologous recombination with 10 generations of backcrossing to a C56/bl6 Wildtype (WT) strain. Both males and females were included, and all animals was housed gender wise in pairs with or without free access to a running wheel. The SynIIKO mice develop handling induced focal to bilateral tonic-clonic seizures from 2.5-3 months of age. Dependent on their age and related to their seizure development, voluntary running (running wheels) was introduced and removed at different timepoints (***Figure 7***). For paper V as a positive control for major structural changes in the retina a genetic mouse model, the Retinal Degeneration mice (rdKO) was included in the study. The rdKO mice have a mutation in the gene coding for cGMP phosphodiesterase-6, leading to a degeneration of the photoreceptors and the outer retina with age. Matching control WTs with the same C3H background as the rdKOs was included.

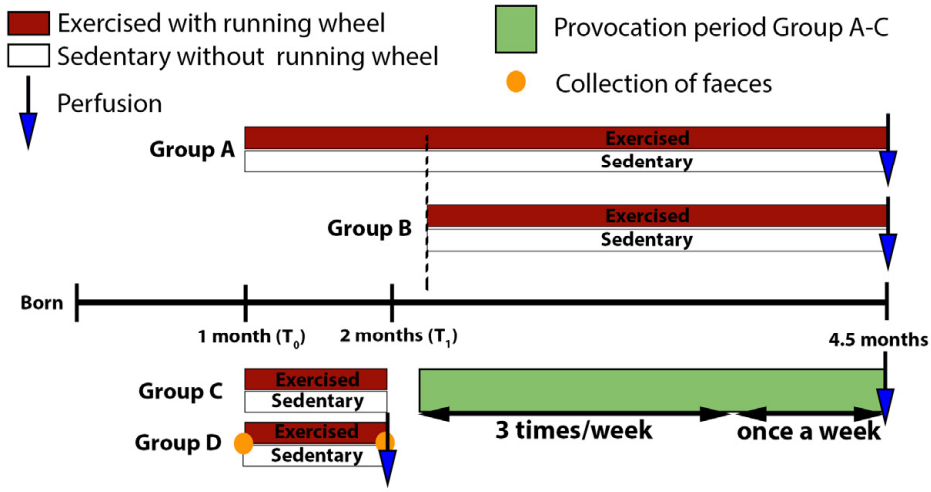


Figure 7: Animal assignment and study design. Group assignment (groups A–D) of the synapsin II knockout (SynIIKO) mice. Group A had running wheels in their home cage from 1 month of age until the end of experiment at 4.5 months of age, with 8 weeks of provocations starting at the age of 2.5 months. Group B had running wheels from 2.5 months of age (expected age of seizure onset) and throughout the 8-week provocation period. Group C had running wheels for 1 month starting at the age of 1 month, followed by 8 weeks of provocation without running wheels. Group D had running wheels for 1 month starting at the age of 1 month and were perfused at 2 months of age, before predicted seizure onset, and received no provocations.

Electrode and canula implantation and SE induction

For paper II, IV and V an acquired model rather than a genetic model of epilepsy was used. Male Sprag Dawley (SD) rats (200-250g) or 2 months old male C56/bl6 mice were anesthetised with 2% isoflourane and implanted with a bipolar electrode into the right ventral hippocampus in the CA1/CA3 region (coordinates rat: 4.8 mm posterior, 5.2 mm lateral from bregma, and 6.3 mm ventral from dura with tooth bar set at -3.0 mm. Coordinates mouse: 2.9 mm posterior and 3.0 mm lateral from bregma; and 3.0 mm ventral from dura, tooth bar set at -3.3 mm) for stimulation and recording. A unipolar electrode was placed between the scull and adjacent muscle to serve as the ground electrode. In addition, for paper III, one group of rats were simultaneously implanted with an intracerebroventricular canula on the ipsilateral side (coordinates 1.0 mm posterior, 1.5 mm lateral to bregma and 3.5mm ventral to the flat scull position) for either saline or CX3CR1 antibody infusion. Following a week of recovery after surgery rats or mice were subjected to electrically induced temporal SE. Before SE induction an individual after-discharge threshold was determined for every animal by stimulation with a square-biphasic pulse (50hz) with a 1s train duration, stating at the intensity of 30μA, and followed by 10μA increase (up to 300 μA) until a 10 s afterdischarge threshold was evoked. The following SE induction duration was 60 min with interruptions every 9th min to record EEG activity for 1 min. After the initial hour, all electrical stimulations were stopped for EEG recordings of a self-sustained focal non-convulsive SE for additionally 2

hours. After 2hrs the fNCSE was stopped with pentobarbital (65 mg/kg i.p). Only animals with the majority of non-convulsive seizure semiology according to Raciness scale (Racine, 1972) originating from the temporal lobe was included in the study i.e. oro-facial twitches, nodding, drooling and unilateral forelimb clonus.

Lipopolysaccharide administration

For paper II, to experimentally induce neuroinflammation the bacterial endotoxin LPS from gram-negative bacteria was used. Naïve animals were anesthetized with isoflurane (2%) and LPS from Escherichia coli, serotype O26:B6 (Sigma-Aldrich, L8274, Sweden; 10 µg in 2 µl of saline) or vehicle (2 µl of saline) was stereotaxtically injected into the right dorsal HPC (coordinates: 4.8mm posterior and 5.2mm lateral from bregma; and 6.0mm ventral from dura, tooth bar set at −3.0mm) using a glass microcapillary (Hamilton syringe). Male SD rats (200-250g) received a single intracerebral injection of LPS or vehicle (Sal).

Tissue preparation

Immunohistochemistry preparation

For immunohistochemical evaluations mice or rats were given an overdose of pentobarbital before transcardially perfusion with ice cold saline (0.9%) and 4% paraformaldehyde (PFA). Brains and eyes were removed for post-fixation at 4°C in 4% PFA for 24 hrs (brains) or 4hrs (eyes). Brains and eyes were thereafter dehydrated in a final concentration of 20% and 25% sucrose, respectively, before using a microtome (Microm HM440E, Thermofisher) to obtain 30 µm thick slices. Eyes were imbedded in Yasulla medium (30% egg albumin, 3% gelatin) before cutting 20µm thick slices using a cryostat (Microm HM 560, USA).

Biochemical and blood sample preparation

For paper II and IV, rats were decapitated after an overdose of phenobarbital, a cardial blood sample was taken, and put at room temperature for 2hrs before centrifugation at 2500rpm and storage in -80 until use. Eyes and spleen were removed, and each brain hemisphere was dissected into cortex, hippocampus and subcortex, all tissue was immediately put on dry ice before storage in -80 until homogenization. Ipsi,- and contralateral eyes (to the electrode), spleen samples and the hippocampus were homogenised on ice in lysis buffer (50.0 Tris-HCl, 150 NaCl, 5.0 CaCl2, 0.02 % NaN3, and 1 % Triton X-100) containing protease inhibitor (Sigma, 1:100) and centrifuged at 17,000g for 30 min before collecting the supernatant and put it in -80 until analysis. For analysis see Western blot, and ELISA subheadings.

Faeces collection and analysis

For paper I mice faeces were collected between 8.00-10.00 AM before (T_0) and 1 month after voluntary running (T_1) before the development of seizures for measuring corticosterone levels (***Figure 7***). The protocol for corticosterone extraction was modified from Touma et al (Touma *et al.*, 2003). Samples were dried at 37 °C overnight, grinded to powder, mixed with 80% methanol at 10% w/v for 30 min on a Vortex, and centrifuged at 2,500g for 15min. Corticosterone levels were analysed by enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Solna, SE) according to manufacturer protocol.

Stainings and image analysis

Immunohistochemistry

Brain sections were stained free-floating while eye sections were stained mounted on gelatine coated slides. In general brain sections stained for intracellular targets were subjected to an additional antigen retrieving solution step (10nM sodium citrate, 0.05% Tween-20, pH 6.0) for 20 min at 90℃ before adding the primary antibodies (***Table 1***). Brain,- and eye sections were incubated with primary antibodies at 4℃ overnight. After washing, sections were incubated in secondary antibodies for 2 hrs in room temperature. For all stainings some brain,- and eye sections went through the protocol without the primary antibody to serve as a negative control. Free-floating brain sections were after protocol completion mounted on gelatine coated glass slides, all slides (including eye sections) were coversliped in DABCO (Sigma) containing 1:1000 hoescht for nuclear stain.

Flouro-jade and hematoxylin-eosin staining

For paper IV and V eye sections were washed with potassium phosphate buffer saline, hydrated and pretreated with 0.06% potassium permanganate for 15 min, rinsed with distilled water and treated with 0.001% Fluoro-Jade (F-Jade) (Histo-Chem, Jefferson, AR, USA) for 30 min or hematoxylin-eosin for 1 min. Slides were then washed with distilled water, dehydrated by ethanol and xylene treatment, and cover slipped with PERTEX mounting medium (HistoLab, Sweden).

Epifluorescence and confocal microscopy

All morphological analysis were performed using a Cell Sense Olympus software and Olympus BX61 epifluorescence microscope. For paper I quantifications of the immunostainings were performed bilaterally in temporal lobe structures, hippocampus and EC, in 3–4 brain sections/animal as previously described (Ali *et al.*, 2015; Avdic *et al.*, 2015; Chugh *et al.*, 2015) by a researcher blinded to treatment conditions. Number of Ionized Calcium-Binding Adapter Molecule 1 ($Iba1^+$) microglial cells/sections in dentate gyrus, GCL, and Molecular Layer (ML) of the hippocampus and EC were quantified manually due to low numbers of cells. Morphological analysis of microglia (ramified/surveying; small soma with several long processes, intermediate/activated; larger cell soma with fewer, thicker and retracting processes, and round/amoeboid/phagocytic; large soma with no processes) was performed in a subset of 120 $Iba1^+$ cells/animal in dentate hilus, GCL and ML, separately, and 80 $Iba1^+$ cells/animal in EC. Glial Fibrillary Acidic Protein (GFAP) expressed by astrocytes, Microtubule-Associated Protein 2 (Map2) expressed by neurons, BDNF, and TrkB expression were analysed by intensity measurements as the mean grey value (ImageJ software, NIH, USA). Numbers of $Map2^+$ processes were manually counted in layer II of EC. Mean numbers of Doublecortin (DCX^+) newborn cells/section were quantified manually in the GCL and Subgranular Zone (SGZ) in the hippocampus.

For paper IV and V quantification and analysis of the eyes were done in 8–12 regions of interest (ROIs) within 4–6 sections/eye, located in the peripheral retina, approximately 500 μm from the ora serrata (the junction between the retina and the ciliary body). The morphological phenotype of $Iba1^+$ microglial was determined in a subset of 120-240 cells in the whole retina. Number of Iba1, Cluster of Differentiation-68 (CD-68), CD-45, Neuronal-Glial Antigen 2 (NG2) and GFAP positive cells were quantified manually, while in paper IV PDS-95, IL-6, IL-4, IL1b intensity (mean grey value) was measured using a confocal laser scanning microscope (Zeiss, Germany) with a x63 oil-immersion objective and 5x digital zoom.

Western blot

For paper II, protein samples were denatured at 99°C for 5 min in 2x laemmeli sample buffer (Biorad, Germany). A total of 50 μg spleen, 100μg HPC or serum diluted 1:50 was loaded on a precast 4-15% mini-PROTEAN TGX sodium dodecyl sulfate polyacrylamide gel (Biorad) and transferred using the Trans-Blot turbo mini nitrocellulose transfer pack (Biorad). Membranes were blocked in Tris-Buffered Saline containing 0.2% Tween 20 in (TBST) and 5% non-fat dried milk for 2hrs in room temperature. Following this, membranes were incubated with primary antibodies (***Table 1***) diluted in TBST containing 0.5% Bovine Serum Albumin (BSA) (Sigma, Germany) overnight at 4°C. Next day membranes were washed and

incubated for 2 hrs in room temperature with the corresponding secondary antibody: (1:5000) Horseradish peroxidase conjugated anti-mouse, anti-goat, or anti-rabbit (Sigma). Membranes were washed and protein immunoreactive bands were visualized by application of enhanced chemiluminescence and imaged using Chemidox XRS+ system (Biorad). Band intensities were quantified using ImageJ software. Western blot (WB) analysis compared the relative protein expression towards controls, normalized to the loading control, aktin or Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) in spleen and HPC and transferrin in serum.

Table 1:Overview of primary antibodies

	Primary antibodies			
Antigen	**Host**	**Staining**	**Dilution**	**Company**
B-actin	mouse	WB	1:10,000	Sigma
BDNF	sheep	IHC	1:100	Santa Cruz
DCX	rabbit	IHC	1:200	Abcam
CD4	mouse	WB	1:200	Biorad
CD8	mouse	WB	1:200	Biorad
CD45	rabbit	IHC	1:100	Santa Cruz
CD45	rabbit	WB	1:500	Santa Cruz
CD68/ED1	mouse	IHC	1:200	Serotec
CD68/ED1	mouse	WB	1:500	Biorad
CX3CR1	rabbit	WB	1:500	Abcam
E-cadherin	mouse	WB	1:500	Abcam
Galactin-3	mouse	WB	1:500	Abcam
GAPDH	rabbit	WB	1:2000	Cell Signaling
GFAP	mouse	IHC	1:400	Sigma
GFAP	mouse	WB	1:500	Sigma
Iba1	rabbit	IHC	1:1000	Wako, Japan
IL-1β	goat	IHC	1:100	Santa Cruz
IL-4	rabbit	IHC	1:100	Santa Cruz
IL-6	rabbit	IHC	1:400	Abcam
Intergin-αM	goat	WB	1:500	Santa Cruz
Map2	rabbit	IHC	1:200	Santa Cruz
NeuN	mouse	IHC	1:500	Santa Cruz
NG2	mouse	IHC	1:200	Millipore
RT1B	mouse	WB	1:500	Biorad
S100-β	rabbit	WB	1:2000	Abcam
Tubulin III	rabbit	IHC	1:1000	Abcam
Transferrin	goat	WB	1:1000	Abcam
TrkB	rabbit	IHC	1:100	Santa Cruz

MRI

9.4T DTI

After perfusion, mice brains and both eyes were imaged *ex vivo* in a 9.4 Tesla (T) magnet (Oxford Instruments PLC, Abingdon, UK). Diffusion Tensor Imaging (DTI) data was acquired using a spin-echo-echo-planar-imaging sequence using a TR/TE = 1000/35 ms, echo spacing = 0.664 ms, number of shots = 6, bandwidth = 250000 Hz, number of averages = 1, FOV = 16 × 16.5 × 18 mm^3, spatial resolution = 125 × 125 × 125 μm^3, number of diffusion directions = 42, b-value = 3039.6 s/mm^2, number of minimally diffusion weighted images = 1, gradient amplitude = 35.11 G/cm, gradient duration (δ)/separation (Δ) = 6/11.50 ms, acquisition time = 10 h 48 min. Data was processed with in-house codes using Matlab (ver. R2012b, MathWorks, Natick, Massachusetts, U.S.A.) to reconstruct diffusion weighted images. Each diffusion weighted dataset was corrected for residual eddy current induced geometric distortions using the relevant toolbox in Explore DTI (Leemans and Jones, 2009) (www.exploredti.com). In order to increase anatomical contrast, diffusion weighted data was sampled by a factor of two using B-spline interpolation, and a voxel-wise fit of the diffusion tensor model was performed to generate Fractional Anisotropy (FA) maps.

7T T_1 MRI

Due to disturbances of the cavity left by the electrode in the hippocampus on the ipsilateral side, only the contralateral eye was imaged using a single loop coil in a horizontal bore 7T-magnet (Bruker Pharmascan). 3D T_1W-FLASH (GE without fluid attenuation) data were acquired using a TR/TE = 150/7.2 ms, number of averages = 4, FOV = 1.17 × 1.17 × 1.17 mm^3, spatial resolution = 26 × 26 × 26 μm^3, and acquisition time = 12 h 32 min.

Patients

Vasaloppet Skiers and matched controls

Vasaloppet is an annual long-distance ski-race that takes place in Sweden. In paper I from year 1989-2010 126,362 males (62%) and 77,447 females (38%) participated in at least one Vasalopp race. The majority (55%) of skiers participated in the 90-km race distance, and the rest (45%) participated in the 30/45 km race. All skiers were randomly frequency matched to a person from the general population in Sweden (controls). They were matched according to gender, age (with a 5-year interval), region and year of race. Individuals with severe disease (stroke and chronic neurological diseases) before study entry were excluded from both the Skiers and controls.

Video and EEG study design and patient inclusion

In paper III patients admitted to a continuous video-EEG (vEEG) study either at Lund University hospital or Uppsala University hospital were offered study enrolment. Inclusion criteria were age ≥18 and suspected or confirmed diagnosis of epilepsy and/or PNES. Exclusion criteria were age <18, intraparenchymal EEG monitoring, head trauma or surgery within the last 6 months, presence of active chronic systemic immune diseases, and regular intake of medication targeting the immune system. Prior to study inclusion a written informed consent was signed by all participant (patients and controls) according to the Declaration of Helsinki. At the enrolment a questionnaire regarding seizure frequency, neuropsychiatric-psychiatric conditions, exercising habits together with a patient-reported medical history was answered. Age and gender matched healthy individuals served as controls (n=12) and upon enrolment they submitted the same questionnaire as the vEEG patients. Included patients underwent 3-5 days of continuous video and vEEG monitoring with 24hrs medical supervision. Seizure semiology was classified in accordance with the ILAE seizure classification system of epileptic seizures, and non-epileptic seizure semiology was based on a systematic review suggesting a classification of non-epileptic seizures (Fisher *et al.*, 2017; Asadi-Pooya, 2019). The electrodes for the EEG used the international 10-20 electrode placement consisting of 32 or 64 electrodes recorded as a bipolar montage. Based on the vEEG recordings patients were divided into the following patient groups: TLE, Frontal Lobe Epilepsy (FLE), TLE+PNES or only PNES. Medical records and previous EEG recordings were used to establish or exclude epilepsy diagnosis in patients that exhibited PNES exclusively during the vEEG monitoring.

Patient blood sample collection

A baseline blood sample was taken at the time of study enrolment (interictal), and 2 postictal samples were taken at 2 occasions: 6-8hrs and 24hrs after a seizure. Based on the results from the ongoing vEEG, a swift verification of seizures was allowed before postictal blood sampling after an Index Seizure (IS). Healthy controls only provided the baseline blood sample. After sample collection in a 5 ml BD Vacutainer serum tube the blood was incubated at room temperature for 1hr before centrifugation at 2500rpm for 15 min. The separated serum was aliqoted and put in -80 until use.

ELISA

For paper III serum concentrations of several cytokines and chemokines was determined via Mesoscales Multiplex ELISA Proinflammatory panel 1 (Interferon-γ (INF-γ), IL1-β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and Tumor Necrosis Factor-α (TNF-α), chemokine panel 1 (Eotaxin, Eotaxin-3, Macrophage Inflammatory Protein-1α (MIP-1α), MIP-1β, Thymus and Activation-Related Chemokine (TARC), INF-y Induce Protein-10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP-1), MCP-4 and Macrophage-Derived Chemokine (MDC)) and vascular injury panel 2 (Serum Amyloid A (SAA), C-Reactive Protein (CRP), Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intra Cellular Adhesion Molecule-1 (ICAM-1)) human kits from Mesoscale. The keratinocyte chemoattractant/growth-related oncogene (KC/GRO) and fractalkine concentration was determined via RD systems, human Quantikine plates, both kits according to the manufacturer's instruction. For paper IV the total protein concentration was determined in whole eye samples by Bicinchoninic acid (BCA) protein assay (BCA, Pierce, Rockford, IL) as per manufacturer's instructions. Levels IL-1β, TNF-α, INF-γ, IL-4, IL-5, IL-6, IL-10, IL-13, and KC/GRO were measured by Multiplex ELISA Proinflammatory Panel 2 rat kit. Briefly, human serum samples (duplicates) were diluted 1:2, 1:4 or 1:1000, or loaded undiluted (singlets), into the plate together with the assigned standard. Eye rat samples were loaded at 2µg/µl in duplicates. Plates were either incubated on a shaker with samples at 4°C overnight, or for 2 hours in room temperature. Plates were then washed, detection antibody was added for 2hrs at room temperature or at 4°C. Plates were washed again before adding read buffer. Plates were read using the MSD MESO QuickPlex SQ and the protein concentration was calculated. The Quantikine plates were analysed instantly after stop solution was added using the ASYS Expert 96 plate reader at 450nm. All samples run in duplicates with a CV% constant above 40 were excluded from all comparisons.

Statistical analysis

ELISA data is expressed as mean with standard deviation, or median with Interquartile Range (IQR) upon scattered distribution. All data were tested for normality using the Shapiro-wilks test. Upon non-normal distribution of data comparison of 2 groups were performed with Mann-Whitney and ≥3 groups Kruskal Wallis. All normally distributed data was compared among 2 groups with an unpaired student's t-test or 2-way ANOVA with Bonferroni post hoc test in case of ≥3. In paper I when comparing the epidemiological datasets, a log-rank test with unadjusted Hazard Ratio (HR) from the Cox model was used and presented as Kaplan-Meier curves or as confidence intervals (incidence in s kiers and controls of diagnostic sub-codes for epilepsy). HR was later adjusted for previous alcohol diagnosis. Seizure onset in SynIIKO mice and binary data in paper III was compared

with a Fisher's exact test. In paper II and III, continuous variables such as seizure frequency was correlated with protein levels with a non-parametric Spearman correlation. Interictal protein samples were compared pairwise with postictal protein levels from the same patient with a Wilcoxons paired test. All p-values <0.05 were considered statistically significant.

Results

Physical Activity Reduces Epilepsy Incidence: a Retrospective Cohort Study in Swedish Cross-Country Skiers and Experimental Study in Seizure-Prone Synapsin II Knockout Mice (Paper I)

Robust clinical and experimental studies on whether the development of epilepsy (epileptogenesis; e.g. time window before first spontaneous seizure) is affected by physical activity are few. We therefore investigated the incidence of epilepsy in a uniquely large cohort of physically active participants in a Swedish long-distance ski race (Vasaloppet) and compared it to the incidence in a non-participating matched control group. To further investigate the timing of initiation of physical activity and epileptogenesis, we studied epilepsy-prone genetically modified mice lacking Synapsin II. The SynIIKO mice were provided with running wheels for voluntary physical exercise at different time points during both epilepsy development and progression.

Epidemiological data

A cohort of 395,369 individuals was divided into two groups, participating skiers and non-participating controls. Both groups had equal numbers of men and women, age distribution and year of subject recruitment. We found a significantly lower incidence of epilepsy (epilepsy diagnosis codes 345 or G40) among skiers following up to 20 years after their participation in the ski race compared to non-participating controls (n =424 in the skiing group compared to n = 789 in control group, p>0.001). Since none of the individuals were diagnosed with epilepsy before entering the study, the data imply a reduced incidence of epilepsy of almost 50% at 20 years (***Figure 8***).

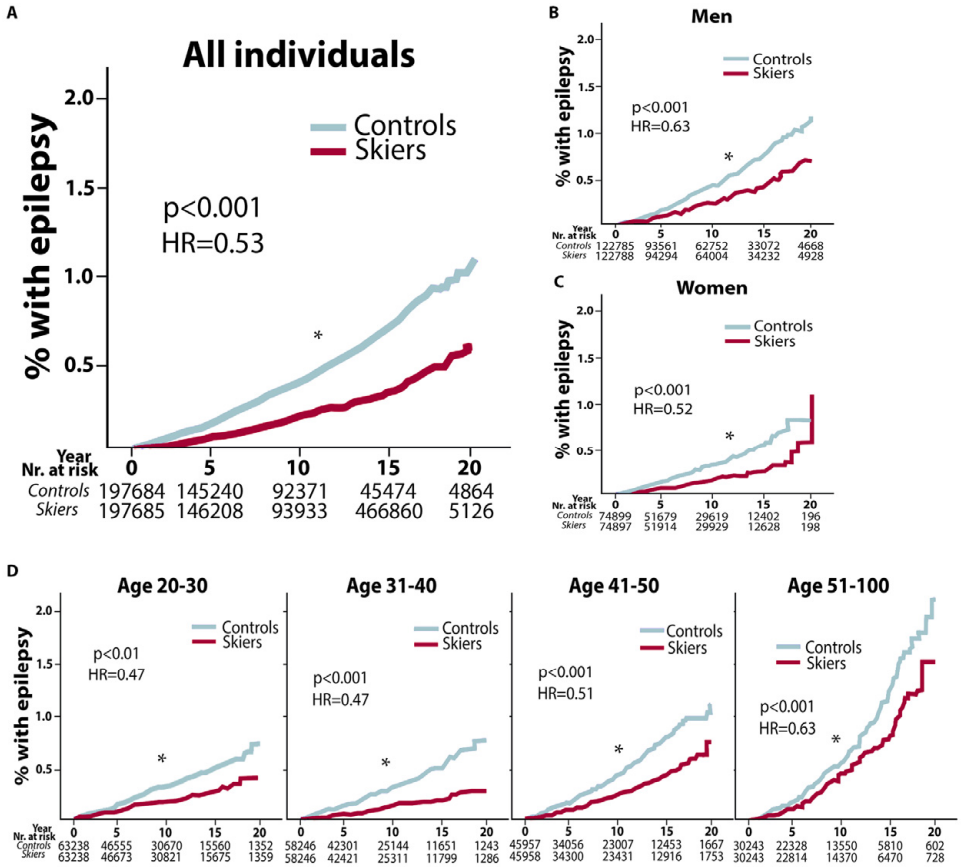


Figure 8: Epilepsy incidence divided according to gender and age groups. Data presented with Kaplan-Meier curves, with unadjusted hazard ration (HR). Y-axis represents incidence in %, and the X-axis presents follow-up time in years and number at risk. Overall incidence of epilepsy diagnoses at 0, 5, 10, 15 and 20 years after completed ski race in skiers compared to matched non-participating controls (a), in men (b) and women (c), in age groups 20–30, 31–40, 41–50 and 51–100 years (d). *p < 0.05

The epilepsy incidence was further reduced among faster skiers, defined as skiers with a race time below the median, compared to slower skiers with a race time above the median (***Figure 9***). The actual reduction between faster and slower skiers was relatively small (about 20%). Interestingly, when dividing epilepsy incidence in subgroups of epilepsy with International Classification of Diseases (ICD-10) diagnosis codes G40.0-9, we observed significant differences in 40.2 (partial symptomatic epilepsy and epilepsy syndromes with complex partial seizures) and G40.9 (unspecified epilepsy), even after the exclusion of previous or current alcohol diagnosis in the G.40.9 subgroup.

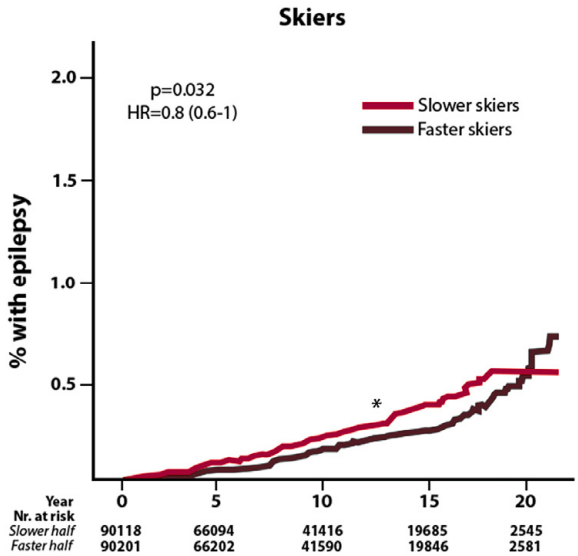


Figure 9: Epilepsy incidence in faster compared to slower skiers. Data presented with Kaplan-Meier curves, with unadjusted hazard ration (HR). Y-axis represents incidence in %, and the X-axis presents follow-up time in. Skiers where divided into slower skiers, with a finishing time below the median and faster skiers, with a finishing time above the median. *p < 0.05.

Experimental data

Voluntary running starting at the age of 1 month, hence before the expected seizure onset (at 2.5 months), and continuing until 4.5 months of age significantly delayed seizure onset in the SynIIKO mice (***Figure 10***, group A; p=0.04). The majority of exercising SynIIKO mice in group A did not develop seizures at all during the provocation period (with seizures: exercised 3 out of 11 compared to sedentary 9 out of 12). Running wheels introduced later, at the expected seizure onset (at 2.5 months of age) and continuously present during the provocation period, had no effect on seizure development or frequency (***Figure 10c***, group B; p > 0.99). In group C, the SynIIKO mice received access to running wheels for a period of only 1.5 months before expected seizure onset (at 2.5 months) followed by no access during the 8-week provocation period (***Figure 10e***, group C; p = 0.01). During the first 4 weeks of provocations, the percentage of mice in group C that developed seizures in the exercised group was again less than 20% (with seizures: exercised 3 out of 13 compared to sedentary 8 out of 10). The percentage started to rise during the last 4 weeks of provocations but remained at 40% at the end of the experiment (with seizures: exercised 5 out of 13 compared to sedentary 9 out of 10).

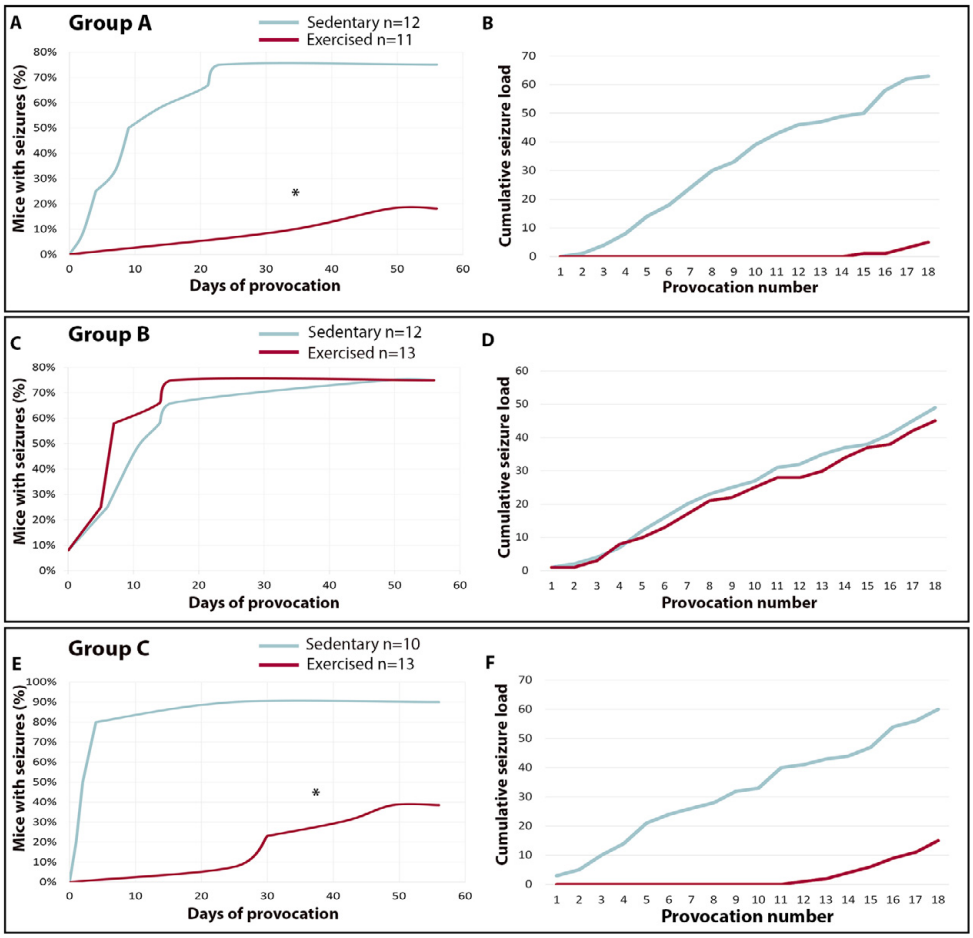


Figure 10: Delayed seizure onset in synapsin II knockout (SynIIKO) mice following voluntary running. Percentage of SynIIKO mice with or without voluntary running (exercised and sedentary group) exhibiting provoked seizures (a, c, e) and cumulative seizure load during the provocation period (b, d, f). Group A starting 1.5 months before the 8-week long provocation period (a, b). Group B voluntary running for 2 months starting concurrently with seizure provocations (c, d). Group C voluntary running for in total 1 month starting 1 month before provocation period (e, f). Corticosterone level in both the sedentary and exercised group was reduced at 2 months compared to 1 month of age. However, no differences were observed in corticosterone levels in faeces before (T0; mice 1 month of age) and after (T1; mice 2 months of age) 1 month of voluntary running between sedentary and exercised mice (g). *$p < 0.05$, a–c Fisher exact test. Group A; n sedentary = 12 and n exercised = 11, group B; n sedentary = 12 and n exercised = 13, group C; n sedentary = 10 and n exercised = 13

Searching for the mechanism behind the inhibitory effect of epilepsy development, we investigated corticosterone levels in faeces and the level of inflammation in the brain after 1 month of voluntary running, before seizure onset to avoid confounding factors from epileptic seizures themselves. No differences were observed in corticosterone levels in faeces before (T0; mice 1 month of age) and after (T1; mice 2 months of age) 1 month of voluntary running between sedentary and exercised mice (***Figure 11***).

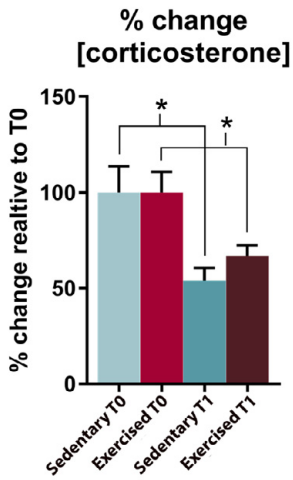


Figure 11: At 2 months of age a decreased in corticosterone was found in both the sedentary and exercised group. T_0= At 1 moth of age before voluntary running, T_1= At 2 months of age after 1 month with or without voluntary running. *p < 0.05 with a paired Student's t test. n sedentary = 12 and n exercised = 12.

Neither could any differences in the microglial number or morphology could be detected in the hippocampus nor entorhinal cortex (***Figure 12a-d***; EC: 124±5 in sedentary vs 114±10 cell/brain section in exercised group). Astroglial activity measured by GFAP intensity in the dentate gyrus and entorhinal cortex was not affected (***Figure 12e***; EC: mean grey value 14.0±0.7 sedentary vs 13.7±0.3 exercised group), and neuronal dendrite intensity measured by map2 intensity in the dentate gyrus (mean grey value in hilus; 23.4±0.9 sedentary vs 23.4±0.8 exercised, GCL; 18.5±0.9 sedentary vs 20.1±0.7 exercised, ML; 42.4±0.7 sedentary vs 41.8±1.1 exercised) or number of map2^{+} processes in entorhinal cortex (50±1 sedentary vs 44±4 exercised) remained unaltered. However, investigating the number of newborn neurons we found a higher number of DCX^{+} cells within the sub-granular zone in the dentate gyrus in exercised compared to sedentary mice. Furthermore, no differences in BDNF or TrkB expression could be found in either the hippocampus or entorhinal cortex (***Figure 12f-m***).

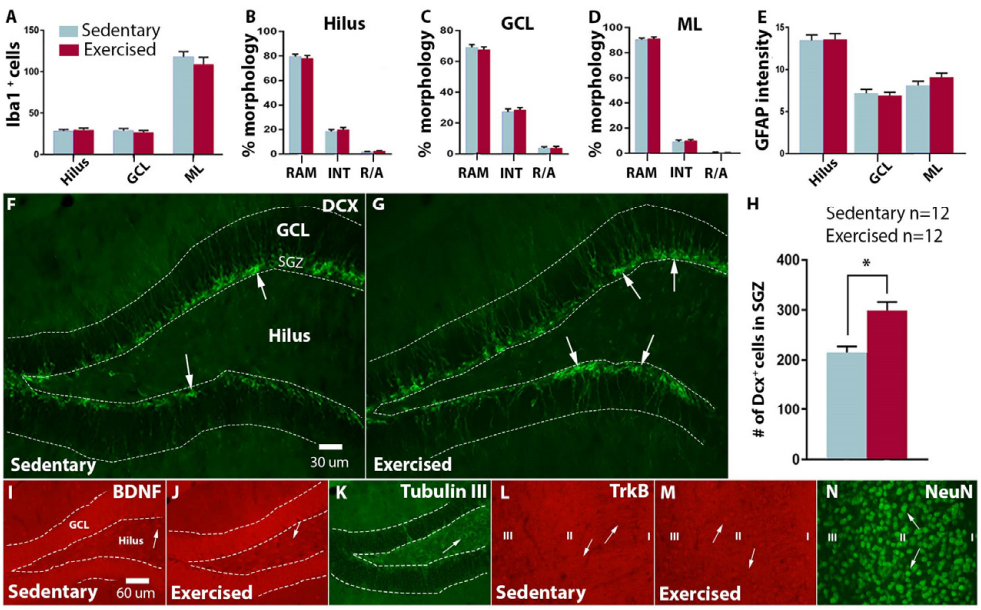


Figure 12: Number of Iba1^{+} cells in the hippocampus (a). Percentage of ramified (RAM), intermediate (INTER) and amoeboid/round (A/R) morphologies of Iba1+ cells in the hilus (b), GCL (granule cell layer (c)) and ML (molecular layer (d)). Intensity measurements of GFAP immunohistochemical staining in astrocytic processes within the hilus, GCL and ML of the hippocampus (e) Numbers of newborn neurons in the hippocampus and BDNF/TrkB expression in hippocampus and entorhinal cortex of SynIIKO mice after 1 month of voluntary running before seizure onset Distribution of DCX^{+} cells within the subgranular zone (SGZ) of the dentate gyrus in the hippocampus in exercised mice as compared to sedentary SynIIKO mice (Group D) (f-g). Number of DCX^{+} cells as mean number of cells / brain section (h). BDNF expression in the dentate hilus, GCL, and ML of the hippocampus in sedentary and exercised SynIIKO mice (i and j), with representative region of interest delineated also in tubulin III (k). TrkB expression in the entorhinal cortex in sedentary and exercised SynIIKO mice (l and m), with representative region of interest indicated also in NeuN staining (n). *p<0.05, unpaired student's t-test, n sedentary=12 and n exercised=12.

Immune Profile in Blood following Non-Convulsive Epileptic Seizures in Rats (Paper II)

In paper II and III, we focused on defining an immunoprofile during epilepsy development and seizures. In animals both acutely and chronically after fNCSE inflammatory factors were investigated in brain, serum and spleen, using LPS treated rats as a positive control for brain inflammation without epileptogenic origin. In paper III, the study of acute seizure markers, and an inflammatory profile in serum continued in patients with epilepsy.

Acute transient changes in cytokines and chemokines in Serum following fNCSE and LPS injection

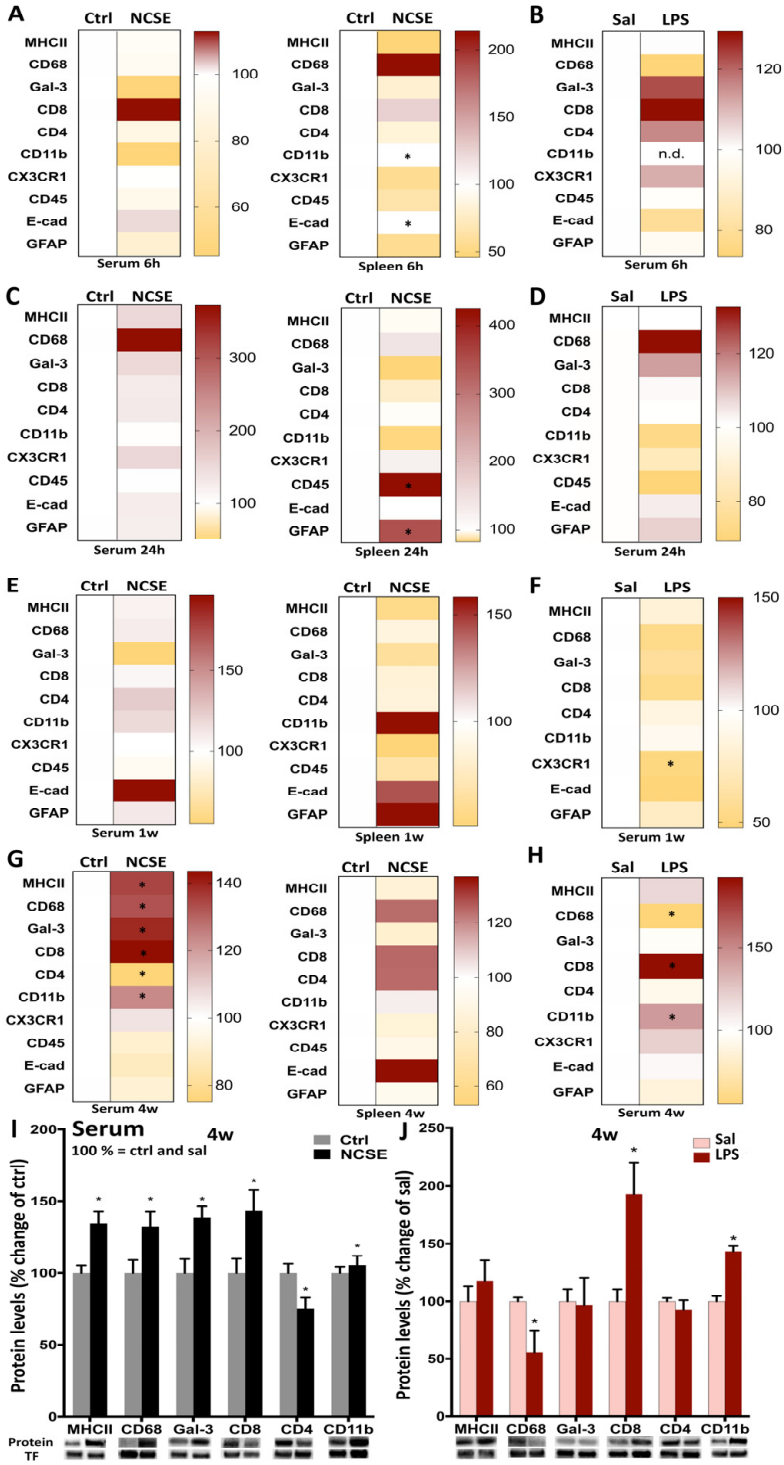
At 6hrs following fNCSE showed increased serum levels of IL-6, and KC/GRO. These acute changes could not be confirmed in spleen tissue from the same animals, instead the same alterations could be detected in hippocampal brain tissue from the epileptic focus. At 24hrs after fNCSE the increased IL-6 and KC/GRO had subsided together with a small decrease of TNFα. No alterations were found with ELISA at 1 week or 4 weeks post fNCSE. Representing brain inflammation without the epileptic insult LPS-treated animals revealed increased KC/GRO and TNFα in serum 6hrs after LPS injection. The initial LPS-induced serum profile was no longer present at 24hrs and KC/GRO levels decreased below control levels. At 1 week post LPS injection the TNFα levels were again increased. Similarly, to the fNCSE animals no alterations was found 4 weeks after LPS injection.

Chronic immune profile in serum 4 weeks following fNCSE and LPS injection

To further study adaptive and innate immune responses additional components were analysed. In contrast to the rapid pro-inflammatory release of cytokines and chemokines in serum at 6 and 24 hrs, proteins such as surface antigens (CDs) on immune cells and proteins related to phagocytosis, were not altered in serum at these early time points (6 and 24hrs) after fNCSE (***Figure 13***). In contrast, modest alterations of CD11β and E-cadherin was found in spleen acutely 6hrs after fNCSE, followed by increased CD45 and GFAP expression at 24 hrs. At 1 week post fNCSE no changes were observed in either serum or spleen. In LPS treated animals no acute alterations were found 6hrs, 24 hrs and 1 week, apart from a small decrease of chemokine receptor CX3CR1 in serum 1 week after LPS injection. In the more chronic state at 4 weeks post fNCSE, more substantial changes were detected. Levels of MHCII, CD68 and Galectin-3, predominantly associated with microglia, macrophages and antigen presenting cells, were markedly increased in serum

compared to non-stimulated controls (***Figure 13 g,i***). Levels of CD4, primarily expressed by T helper cells were decreased, and in contrast CD8, primarily expressed by cytotoxic T cells were upregulated together with CD11b, present on leucocytes (***Figure 13 g,i***). None of these changes could be confirmed in spleen tissue after fNCSE. The increase in MHCII, CD68 and galectin-3 in serum 4weeks post fNCSE was not mimicked at the same timepoint after LPS injection, CD68 serum levels were even decreased in LPS compared to the saline treated group. However, CD8 and CD11b was as in the fNCSE animals upregulated in serum 4 weeks after LPS injection (***Figure 13 h***).

Figure 13: Protein levels of immune factors plotted in heat maps from NCSE rats and LPS-injected rats in serum samples and spleen 6 h, 24 h, 1w, and 4w after the insult. (a–h) Score magnitudes are shown as gradient colors from yellow to dark red, where white color bar on the left side in each heat map represents Ctrl for NCSE and saline-injected control group (Sal) for LPS-treated rats, respectively. Expression of each protein was normalized to transferrin (75 kDa) and set to 100%. Serum protein levels of MHCII (34 kDa), CD68 (110 kDa), galectin-3 (30 kDa), CD8 (34 kDa), CD (51 kDa), and CD11b (170 kDa) 4w post-NCSE, and LPS injection are presented relative to their respective controls. (i,j) Data are presented as mean ± standard error of mean: Ctrl; n = 7–12, NCSE; n = 7–16, Sal; n = 5, LPS; n = 5. *$p < 0.05$, unpaired t-test.


A
Ctrl NCSE
MHCII
CD68
Gal-3
CD8
CD4
CD11b
CX3CR1
CD45
E-cad
GFAP
Serum 6h
Spleen 6h
B
Sal LPS
n.d.
C
Serum 24h
Spleen 24h
D
E
Serum 1w
Spleen 1w
F
G
Serum 4w
Spleen 4w
H
I
Serum
4w
100 % = ctrl and sal
Protein levels (% change of ctrl)
Protein
TF
J
Protein levels (% change of sal)

Immune profile in blood following fNCSE correlates with the development of subsequent spontaneous seizures

To elucidate if the systemic changes were associated with the development of spontaneous seizures after fNCSE, rats in the 4-week post fNCSE-group were divided into rats with only fNCSE (NCSE) and rats with fNCSE that experienced Spontaneous Seizures (SS). Similar to the measurements shown for the entire fNCSE group, cytokines and chemokines levels in serum were not changed in fNCSE rats with or without the development of spontaneous seizures. However, the alterations in serum levels of MHCII, CD68, galectin-3 and CD4 that was identified after fNCSE were only significantly altered in rats that also developed spontaneous seizures (***Figure 14 a***). WB analysis of hippocampal brain tissue from the epileptic focus from the same animals showed increased expression of CD68 and CD4, while levels of MHCII and Galectin-3 remained unaltered in both groups (***Figure 14 b***). Levels of MHCII, CD68, Galectin-3 and CD4 in serum did not correlate with the total number or duration of spontaneous seizures, nor was there a correlation between the immune profiles and the latency to develop the first spontaneous seizure. In ***Figure 14c*** a summary of the alterations found overtime in fNCSE compared to Ctrls in both the brain, blood and spleen is displayed.

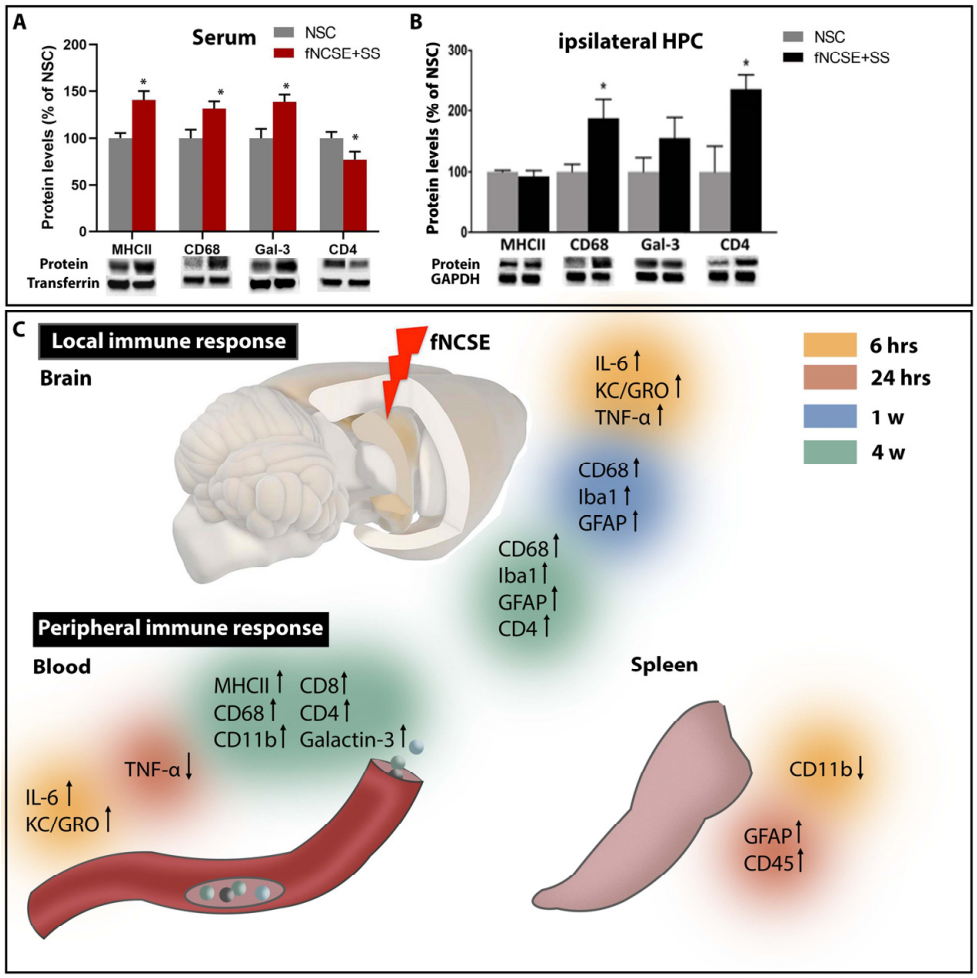


Figure 14: At 4weeks post fNCSE increased levels of MHCII, CD68, Galactin-3 and a decrease of CD4 was found in rats with developed spontaneous seizures compared to NSC (a). In the brain a similar change of CD68 was found, but CD4 levels were in contrast increased (b). A schematic summary of acute and chronic alterations both locally and peripherally. At 6 hrs acute changes with increased levels of IL-6 and KC/GRO was found both in the brain and serum. At 1 week inflammatory changes was only found in the brain with increased levels of Iba1, CD68 and GFAP. In the chronic timepoint at 4 weeks increased levels of CD68 was the only common characteristics between brain and serum. Additionally, in the brain Iba1, GFAP together with a CD4 increase was found. In contrast CD4 was decreased in serum together with increases of MHCII, CD68, CD11b, galactin-3 and CD8 was found. Few alterations were found in spleen or at 24 hrs overall (c).

Acute and chronic immune response in serum from patients with epileptic and non-epileptic seizures (paper III)

vEEG patient inclusion and demographics

In total, 63 patients were recruited from either Skane University hospital (n=56) or Uppsala University hospital (n=7). Six admitted patients had to be excluded due to undefined epilepsy diagnosis (n=3), generalized epilepsy (n=2) or ongoing infection (n=1) leaving 56 patients for study inclusion. The remaining patients were divided into: TLE (n=28), FLE (n=13), TLE with PNES (n=5) and PNES (n=10) (***Figure 15***).

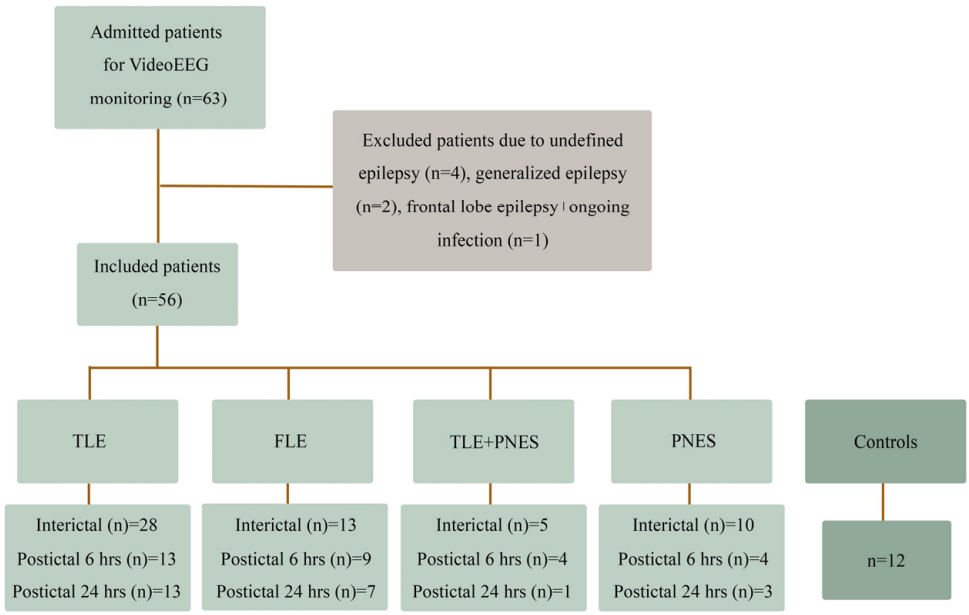


Figure 15: Study flow chart indicating the enrolment of patients with epileptic and psychogenic non-epileptic seizures (PNES) for blood sampling on hospital admission (interictal) and after video-EEG verified seizures (postictal). TLE = temporal lobe epilepsy, FLE = frontal lobe epilepsy.

Demographics, patient self-reported medical history and seizure characteristics are presented in ***Table 2***. The age of patients and controls ranged from 18-64 years, with no median differences between any groups. Women were most prevalent in all groups except for the FLE group that had a significantly higher number of men compared to the TLE+PNES (p=0.003) and PNES group (p=0.005), which exclusively included women. Yet, no patient group expressed gender differences compared to the control group. Self-reported comorbidities were higher in TLE

(p=0.002), FLE (0.032) and PNES (p=0.003) patients compared to controls. Comorbidities included neuropsychiatric disorders (autism, ADD, ADHD), psychiatric disorders (depression, anxiety, personality disorders), type 1 diabetes, inactive rheumatoid arthritis, inactive multiple sclerosis or hypothyroidism. MRI findings were common in all patient groups and included hippocampal sclerosis, cortical dysplasia, previous ischemic brain infarction, cortical heterotopia, congenital artero-venous malformations, benign cysts, ganglioglioma and dysembryoplastic neuroepithelial tumour or cavities after previous brain surgery. The majority of patients reported no physical activity e.g. no regular physical activity for 30 min/ week where the heart rate exceeds 100 beats/min (equivalent to a quick walk). Furthermore, the reported seizure frequency ranged from 0.25 to 11 seizures/week, both night and day, but with more nocturnal seizures in the FLE group compared to PNES (p=0.024) and TLE+PNES group (p=0.036). The time since the last self-reported seizure on admission was on average 1 week for all patient groups.

Table 2

Variable	TLE (28)	FLE (13)	TLE with PNES (5)	PNES (10)	Control (12)	P-value
Demography						
Age: median years (range)	31 (19-63)	25.5 (20-51)	38 (36-63)	34.0 (18-64)	31.5 (22-50)	0.48[2]
Gender: women % (n)	57 (16)	21 (3)	100 (5)	90 (9)	58 (7)	0.005[1]
Medical history						
Current AEDs: median no (IQR)	2.0 (1.0-2.75)	2.0 (1.0-3.0)	2.0 (1.5-3.0)	1.0 (0.0-1.25)	-	0.008[2]
Reported co-morbidities: % (n)	36 (10)	15 (2)	20 (1)	60 (6)	0 (0)	0.004[1]
MRI findings: % (n)	57 (16)	54 (7)	50 (3)	20 (2)	-	0.21[1]
Reported sleep disturbances: % (n)	14 (4)	7 (1)	0 (0)	10 (1)	0 (0)	0.86[1]
Reported physical activity grade 1-3: median (IQR)	1 (0-1.75)	0 (0-1)	0 (0-1)	1 (0-2)	1 (0-2)	0.48[1]
Reported seizure characteristics on admission						
Seizure semiology last 6m incl. bilateral convulsive % (n)	Fo_IA_NM =25 (7) Fo_IA_M =4 (1) Fo_IA_Mauto =18 (5) Fo_IA_BC= 46 (13) Fo_IA= 4 (1) Fo_M= 4 (1)	Fo_A= 8 (1) Fo_A_Mtonic= 8 (1) Fo_A_Mhyperkinetic= 8 (1) Fo_IA_NM= 8 (1) Fo_IA_M= 23 (3) Fo_IA_Mtonic= 15 (2) Fo_M= 8 (1) Fo_Mhyperkinetic= 15 (2) Fo_Mtonic= 8 (1)	A=20 (1) HM=60 (3) SS=20 (1)	A=30 (3) HM=40 (4) SS=20 (2)	-	-
Seizure frequency last 6m: median no/week (IQR)	0.75 (0.25-2.0)	5.5 (0.88-11)	4 (2.1-5)	1.5 (0.25-10.5)	-	0.022[2]
Time since last seizure: median days (IQR)	7 (7-30)	7, 1-7	7, 1-30	7, 1-30	-	0.21[2]
Seizures during night time: % (n)	55 (12)	82 (9)	20 (1)	25 (2)	-	0.038[1]

Recorded seizure semiology and EEG patterns

During vEEG monitoring both focal and focal to bilateral convulsive seizures were recorded. In the PNES group a normal background alpha activity was observed during seizures, and no interictal activity was detected during the monitoring. The observed seizure semiology of the index seizure is presented in ***Table 3***. The FLE and TLE group hade the shortest, and longest median seizure duration, respectively. A majority of the TLE patients had focal seizure with impaired awareness (94%), and 31% of the TLE patients exhibited focal to bilateral tonic clonic index seizures. FLE patients mostly experienced motor index seizure e.g. tonic, myoclonic, hyperkinetic and bilateral tonic clonic and 78% of their index seizures were nocturnal. The observed seizure semiology in the PNES patient group during vEEG recordings all occurred during wakefulness and included varying amounts of motor activity with hypermotor seizure (25%), akinetic seizures (50%) and subjective symptoms (25%).

Table 2: Demography, medical history and reported seizure characteristics in patients on hospital admission for continuous videoEEG monitoring. TLE = temporal lobe epilepsy, FLE = frontal lobe epilepsy. AEDs = Anti-Epileptic Drugs. Comorbidities = neuropsychiatric disorders (autism, ADD, ADHD), psychiatric disorders (depression, anxiety, personality disorders), type 1 diabetes, inactive rheumatoid arthritis, inactive multiple sclerosis or hypothyroidism. MRI findings = hippocampal sclerosis, cortical dysplasia, previous ischemic brain infarction, cortical heterotopia, congenital artero-venous malformations, benign cysts, osteoma, ganglioglioma and dysembryoplastic neuroepithelial tumor or cavities after previous brain surgery. Physical activity = 30 min episode of physical activity with heart rate above 100 beats / min) grade 1 = never, grade 2 = 1-3 times / week, grade 3 = >3 times / week. Reported epileptic seizure semiology included focal onset (Fo), aware (A) or impaired awareness (IA), motor (M) or non-motor (NM), with automatisms (auto), tonic movements (tonic) or hyperkinetic movements (hyperkinetic), or evolving into bilateral convulsive tonic-clonic seizures (BC). Non-epileptic seizure semiology included akinetic (A), hypermotor (HM) or subjective symptoms (SS). 1=Fisher, 2=Kruskal Wallis, p-values ≤0.05.

Tabell 3: Confirmed epileptic and non-epileptic seizures in patients during vEEG monitoring Index seizure = the seizure from which 6-8 or 24 hours was calculated until blood sampling. Number of index seizures for each patients group are reported on the top row (n at 6 hours), including n=3 TLE patients from which only 24 hrs (not 6 hrs) blood samples were achieved. Number and time points of additional seizures occurring after index seizure but before blood sampling are also reported. Epileptic seizure semiology included focal onset (Fo), aware (A) or impaired awareness (IA), motor (M) or non-motor (NM), with automatisms (auto), tonic movements (tonic) or hyperkinetic movements (hyperkinetic) or evolving into bilateral convulsive tonic-clonic seizures (BC). Non-epileptic seizure semiology included akinetic (A), hypermotor (HM) or subjective symptoms (SS). Interictal activity was graded as 0= no interictal activity, 1=sparse interictal activity, 2= moderate interictal activity, and 3=abundant interictal activity.

	TLE ($n=13_{6\ hrs}+3_{24\ hrs}$)	**FLE (n=9)**	**TLE + PNES (n=4)**	**PNES (n= 4)**
Semiology Index seizure	Fo_A_NM= 6 (1) Fo_IA_NM= 13 (2) Fo_IA_M= 6 (1) Fo_IA_Mauto= 44 (7) Fo_IA_BC= 31 (5)	Fo_A_Mtonic= 11 (1) Fo_A_Mhyperk= 22 (2) Fo_A_Mmyoclonic= 11 (1) Fo_IA_NM= 11 (1) Fo_IA_Mtonic= 22 (2) Fo_IA_Mhyperk= 11 (1) Fo_IA_BC= 11 (1)	A= 25 (1) HM= 25 (1) SS= 50 (2)	A= 50 (2) HM= 25 (1) SS= 25 (1)
Duration of index seizure: median sec (IQR)	90 (67-143)	50 (13-68)	75 (45-131)	73 (16-298)
Index seizure during sleep % (n)	40 (6)	78 (7)	0(0)	0 (0)
Seizures between Index and postictal blood sampling: median no (range)	6 hrs: 1 (0-4) incl. n=4 FBTCS 24 hrs: 1.5 (0-4) incl. n=1 FBTCS	6 hrs:1 (0-4)	6 hrs: 0 (0-1)	0.5 (0-2)
Time between last seizure and postictal blood sampling: median hrs (IQR)	4.3 (2.2-6.5)	3.6 (0.6-5.9)	6.1 (6.0-6.2)	1.0 (0.8-5.5)
Interictal activity grading 0-3: median (IQR)	1 (0-2)	1 (0-3)	0 (0-1.5)	0 (0-0)

Interictal serum levels altered in epileptic and non-epileptic patients

No interictal alterations in serum was seen between groups in CRP, Eotaxin, Eotaxin-3, Fractalkine, IL-2, IL-4, IL,8, IL-10, IL12p70, IL-13, INF-γ, IP-10, KC/Gro, MCP-1, MCP-4, Mip1α, Mip1β, SAA, TARC, TNFα or VCAM (data not shown). An increase of interictal levels of IL-6 was seen in all epilepsy patient groups (TLE, FLE, and TLE with PNES group) compared to controls (***Figure 16 a***). In contrast, increased interictal ICAM-1 levels was found in PNES patients compared to the controls (***Figure 16 b***). The interictal MDC levels was also altered in PNES patients compared to TLE (median, IQR pg/ml; PNES 1270, 1000-1440 vs TLE 770, 700-1010 p=0.01) but no difference in patient groups could be observed in MDC compared to controls (median, IQR pg/ml: control 1030, 930-1120; FLE 1040, 650-1350; TLE with PNES 1300, 700-1330). Interictal IL-6 concentrations could not be correlated to interictal activity, time-duration since the last self-reported seizure, or any of the self-reported comorbidities. Additionally, interictal IL-6 and ICAM-1 showed no correlation to either reported seizure frequency, reported focal to bilateral tonic clonic, MRI findings or the number of AEDs.

Postictal alterations 6 and 24hrs after temporal lobe seizures

Postictal protein levels at 6-8 and 24hrs were pairwise compared with the interictal protein levels presented as a ration (postictal/interictal). No postictal alterations in FLE, TLE with PNES or the PNES group was found. Instead, a postictal immune response was observed in the TLE group exclusively, both at 6 and 24hrs post the index seizure. A significant increase of IL-6 was seen in the 6-8 hrs postictal sample (p=0.003), which persisted for 24 hrs (p=0.002). Similarly, Mip1β and TARC were significantly increased at 6-8 and 24 hrs, respectively ($p_{Mip1b\ 6\text{-}8hrs}$=0.04, $p_{Mip1b\ 24hrs}$=0.0005, $p_{TARC\ 6\text{-}8hrs}$=0.005 and $p_{TARC\ 24hrs}$=0.0007) (***Figure 16 d,e***). A transient alteration was seen in MDC and INF-γ with an increase in MDC (p=0.0062) and a decrease of INF-γ (p=0.007) after 6-8 hrs, both proteins were back to interictal levels within the 24 hrs postictal sample (***Figure 16 f,g***). ICAM-1 had a significant increase not detectable until the 24hr postictal sample (p=0.005) (***Figure 16 h***). The postictal alterations of IL-6, TARC, MDC, INF-γ or ICAM-1 did not correlate to any of the seizure characteristics in ***table 3***, except for Mip1β, which was positively correlated to the number of bilateral to tonic clonic seizures before the first postictal sample.

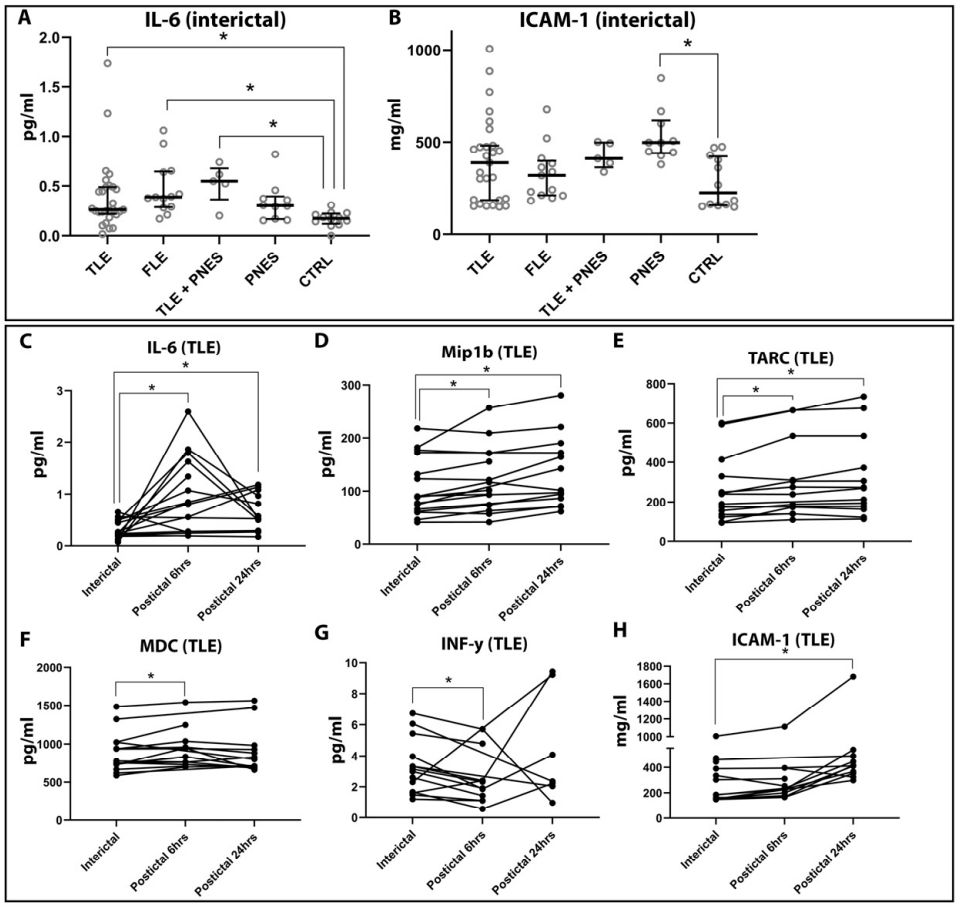


Figure 16: Interictal and postictal immune reaction in serum from patients with epileptic and non-epileptic seizures. Interictal IL-6 levels were increased in epileptic patients compared to controls (a). Interictal ICAM-1 was elevated in psychogenic non-epileptic (PNES) patients compared to controls (b). All postictal alterations were found in the Temporal lobe (TLE) epilepsy patient group. IL-6, Mip1β and TARC was elevated both a 6 hrs and 24 hrs post a temporal lobe seizure (c-e). In MDC there was a discrete increase at 6hrs (f). The INF-γ levels were decreased in the 6 hrs postictal sample but remained unaltered at 24 hrs (g). ICAM-1 protein levels were increased first at 24hrs postictally (h)

Immune response in the eye Following Epileptic Seizures (Paper IV)

We have previously reported microglial activation in both cortical and,- subcortical brain areas, and peripheral tissue such as serum and spleen. Therefore, the relationship between brain inflammation and epileptogenesis might be further understood by studying the immune response in remote areas to the epileptic foci that are not known to either generate or propagate seizures. In the present study, we therefore decided to explore whether a post-seizure immune response can extend beyond an epileptic focus within the temporal lobes and, hence, be detected as far as the retina of adult rats. We also investigated whether a seizure-induced retinal immune response may be modulated by intracerebroventricular infusion of an Antibody (Ab) against the chemokine receptor CX3CR1.

Lack of acute changes in the expression of immune mediators, glial activation, and cell death in the eyes after fNCSE

The quantification of F-jade$^+$ cells never exceeded 3 cells per eye, acutely 6hrs post fNCSE in the retina in neither SE nor the Non-Stimulated Control (NSC) group. The number of Iba1$^+$ microglial cells were not increased, and their morphology was not altered. Biochemical analysis of pro and anti,- inflammatory mediators in the protein homogenates from the eye tissue excluding the lens revealed no changes in contrast to the epileptic focus were several immune factors are upregulated acutely after SE.

Lack of subacute changes in the expression of immune mediators, glial activation, and celldeath in the eyes after temporal status epilepticus

Again, none or <3 F-jade$^+$ cells were observed in the retina 1 week post fNCSE in any of the experimental groups. The microglial population remained continuously unaltered in both number and morphology. The expression of immune mediators was unaltered, apart from a small decrease in IL-10 in the ipsilateral eye.

Delayed glial activation in the retina after temporal status epilepticus

We hypothesized that a seizure-induced tissue injury may be delayed in the retina compared to other brain structures, and therefore, we extended our analyses to a later timepoint at 7 weeks after fNCSE. No celldeath or <3 F-jade$^+$ cells were observed per eye. The immune response in the epileptic foci is often characterized by a prominent activation of microglial cells, which can be detected both acutely and chronically after fNCSE. In the retina Iba1$^+$ microglia was mainly observed in

the synaptic layers (OPL and IPL) in both NSC and SE animals. Interestingly the number of Iba1$^+$ cells were increased bilaterally (***Figure 17 d***) in the SE rats, with a few aberrant cell clusters in the INL and the occurrence of processes in the ONL (***Figure 17 b***). Accordingly, the morphology of the Iba1$^+$ microglia significantly changed. The SE group showed a relative decrease in ramified and an increase in round/amoeboid microglial phenotype with a significant interaction between groups and morphology in both the ipsi-, and contralateral retina (***Figure 17 e***).

Biochemical analysis of eye homogenates showed increased KC/GRO levels in the ipsilateral eye (***Figure 17 f,g***). Intensity measurements of immunohistochemical stainings for 3 pro,- and anti-inflammatory markers, showed regional alterations in IL-1β, with an increase in IPL (***Figure 17 h***) in the ipsilateral eye, while the intensity of IL-6 and IL-4 remained unaltered (***Figure 17 i,j***). No other changes could be observed in IL-1β, IL-6 or IL-4 intensity in INL, OPL and ONL.

In order to evaluate a possible systemic contribution of leucocytes to the immune response in the retina after fNCSE, numbers of CD45$^+$ cells were evaluated at 7 weeks. Again, very low cell numbers were found with no difference between NSC and SE. At both 1 and 7 weeks, CD45$^+$ cells were primarily located in the subretinal layers, not overlapping with the Iba1 staining (***Figure 17 l***). In an attempt to define whether the immune response in the retina was associated with a subtle microvascular disturbance, we also analysed the number of vascular NG2-expressing pericytes. NG2$^+$ cells were found in the same retinal layers as the Iba1$^+$ cells, but their expressions did not overlap (***Figure 17 m***). Often the NG2$^+$ cells were aligned in clusters of 2-3 cells, as if embracing a micro vessel (Figure 17 m inset). However, the numbers did not differ between SE and NSC group neither 1 nor 7 weeks post-fNCSE.

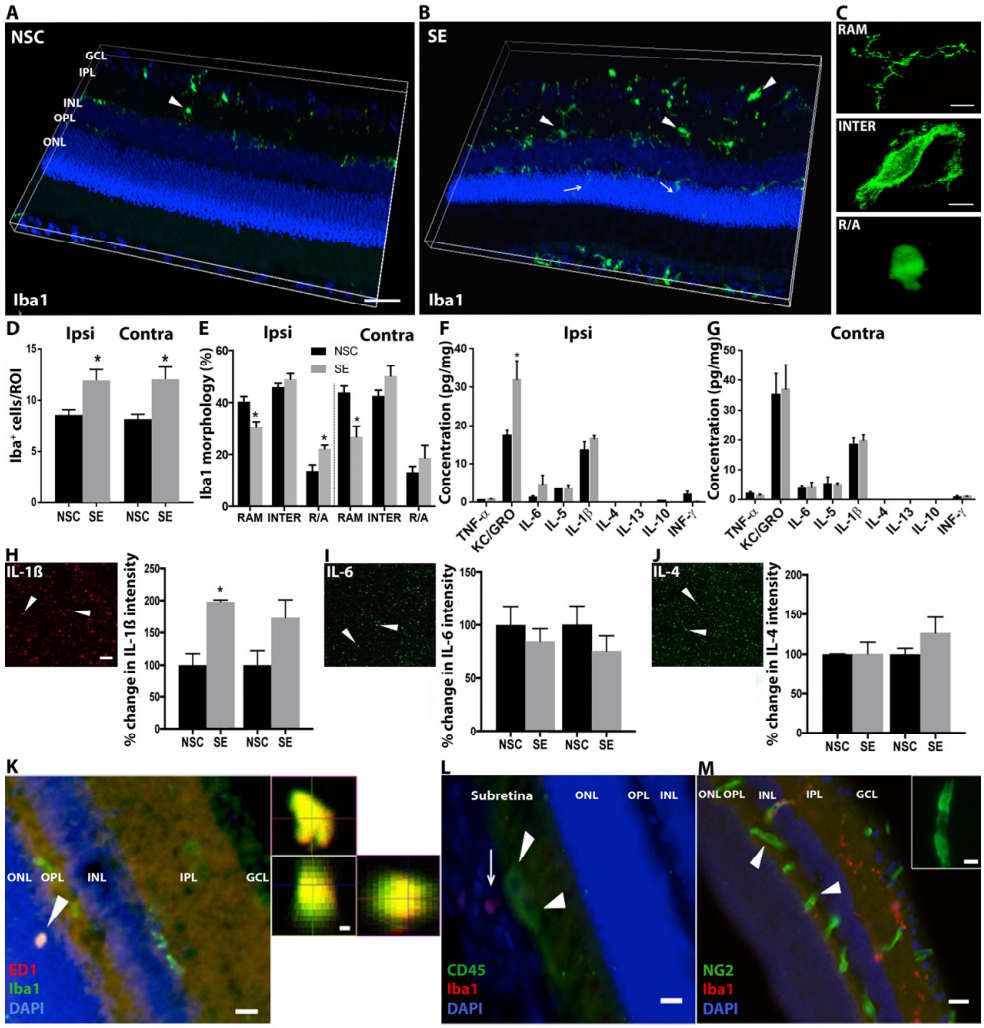


Figure 17: Delayed microglial activation in the retina 7 weeks after temporal SE. Representative confocal photomicrographs of microglial activation in non-stimulated controls NSC (a) and 7 weeks after SE (b). Arrow heads in (a) and (b) depict Iba1⁺ cells in IPL and GCL. Arrows in B mark Iba1⁺ processes in the ONL. Representative images of different Iba1⁺ cell morphologies, including ramified (RAM), intermediate (INT), and round/ amoeboid (R/A) (c). Note the elongated cell soma and thicker proximal processes in INT compared the RAM cells. Quantification of numbers of Iba1⁺ cells in the ipsi- and contralateral retina 7 weeks following SE compared to NSC showed an increase after SE (d). Quantification of the relative percentage of microglia with the three different morphologies revealed a relative reduction in ramified and an increase in amoeboid morphology in the SE group (e). Biochemical analysis detected a SE-induced increase in chemokine KC/GRO levels in ipsilateral retina (f), but no changes in the contralateral eye (g). Representative pictures and intensity measurements in the IPL of cytokine IL-1β (h), IL-6 (i), and IL-4 (j) immune staining showed increased levels of IL-1β only. Confocal images of Iba1 and ED1 immunostaining of the retina (left) and orthogonal projection of an Iba1+ ED1+ cell (right) (k), Iba1 and CD45 immunostaining of the retina (l), and Iba1 and NG2 immunostaining of the retina with NG2⁺ cells in higher magnification in inset (m). Data are presented as means ± SEM, n = 5 NSC and n = 5 SE for ELISA, n = 9 NSC and n = 6 SE for cell quantification and evaluation. *p ≤ 0.05 un-paired t test in (d) and (i, j), 2-way ANOVA in (e). Scale bars are 500 µm for (a) and (b), 10 µm for (c) 5 µm for (h-j), 25 µm for (k-m), and insets 3 µm for (k) and 5 µm for (m)

Apart from microglial activation, seizure-induced gliosis in the brain is also associated with a strong astrocytic reaction, which is readily evaluated with the typical upregulation of GFAP. In the retina, Müller cells and retinal astrocytes, together referred as macroglia, are responsible for injury-induced gliosis. In fNCSE rats at 7 weeks post-SE, no difference in $GFAP^+$ Müller cell intensity in end feet/processes and astrocytes were found in ILM and GCL. We graded the number of $GFAP^+$ Müller cell processes in the IPL, INL+OPL and ONL, respectively, and found extensive $GFAP^+$ staining in the ILM and GCL and, occasionally also in the Müller cell end feet in the outer limiting membrane outside ONL. GFAP expression in Müller cell processes was significantly increased in the IPL contralaterally and with a trend towards and increase ($p=0.07$) ipsilaterally to the epileptic focus. The increase was also significantly increased in the INL and OPL in the contralateral eye. No differences were found in the ONL. The high variation in glial activation in the SE group did not correlate with SE severity (total time a rat exhibited generalized processes was not evident at either 6hrs or 1 week post SE.

Altered synaptic protein expression in the retina after temporal status epilepticus

The activation of micro- and macroglia may indicate an ongoing synaptic/ neuronal dysfunction. We have previously shown that levels of the scaffolding protein PSD-95, expressed primarily in excitatory synapses, may change due to an immune response in the brain. Here, we measured the protein levels of PSD-95 in the eye 6hrs and 1-week post-SE, but no differences were found. However, intensity measurements of PSD-95 clusters 7 weeks post-SE in the different retinal layers revealed a small decrease in the ONL ipsilateral to the epileptic focus.

Intracerebroventricular CX3CR1 antibody infusion decreases micro- and macroglial activation in the retina after temporal status epilepticus

In order to evaluate possible similarities between the immune reaction in the retina and within the epileptic focus, an antibody against the chemokine receptor CX3CR1 was infused intracerebroventricularly during 6 weeks starting 1 week after SE. We have previously shown that inhibition of CX3CR1 decreases microglial activation within the temporal epileptic foci. In the eyes, intracerebroventricular CX3CR1 ab treatment we observed an almost 25% significant decrease in number of $Iba1^+$ cells in the contralateral retina compared to SE animals without ab treatment (***Figure 18 a-c***). The morphology of $Iba1^+$ cells was also changed with a higher percentage of ramified and less intermediate and round phenotypes in the CX3CR1 ab-treated SE group on both the ipsi- and contralateral side (***Figure 18 d***). In addition, the number of $GFAP^+$ Müller cell processes was decreased in the CX3CR1 ab-treated group, with fewer processes in the IPL in both ipsilateral and contralateral retina (***Figure 18 e-g***). The differences in the IPL were not due to the differences in seizure severity during the SE.

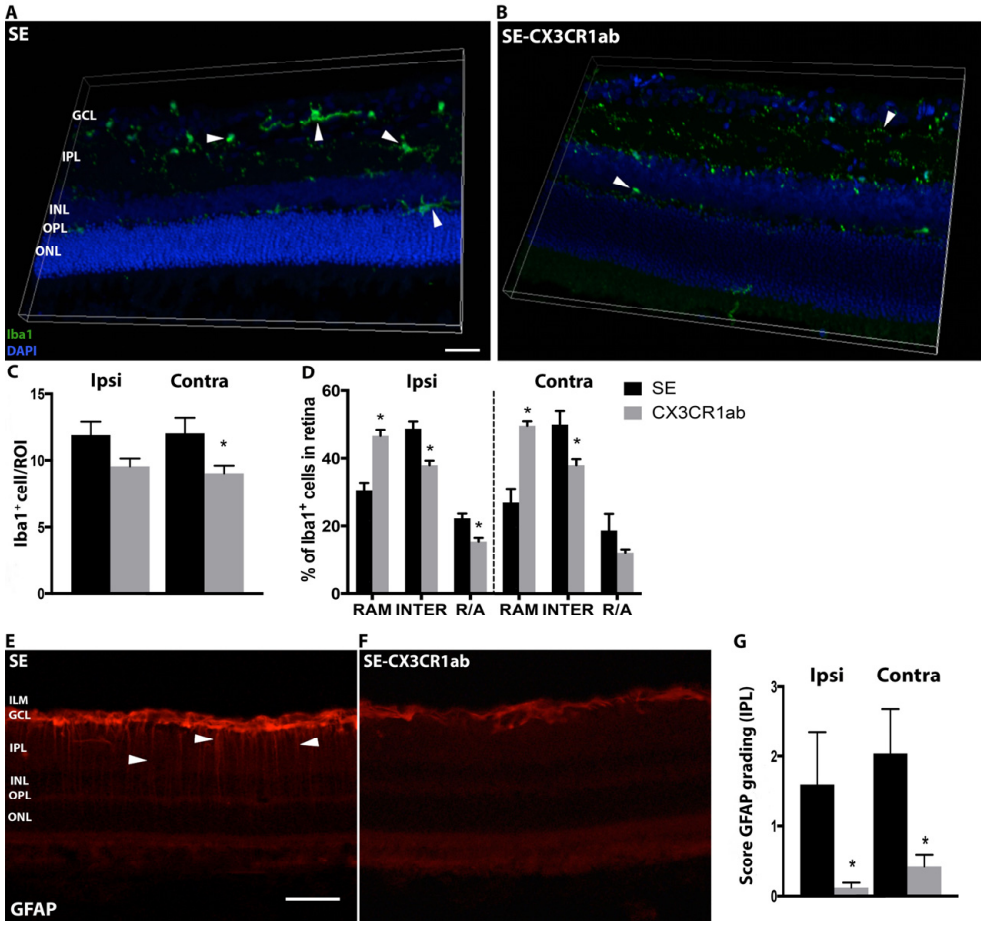


Figure 18: Decreased seizure-induced glial activation in the retina after CX3CR1 antibody treatment. Representative photomicrographs of the retina 7 weeks after SE (a) and CX3CR1 antibody-treated SE (b). Arrow heads depict Iba1+ cells in the IPL and OPL. Quantification of numbers of Iba1+cells in the ipsi- and contralateral retina at 7 weeks revealed a decrease after CX3CR1 treatment (c). Quantification of the relative percentage of microglia with different morphologies at 7 weeks showed a relative increase in ramified and a reduction in intermediate and amoeboid morphologies (d). Representative images of GFAP expression in macroglia in the retina 7 weeks post-SE (e) and post-SE with CX3CR1-treatment (f). GFAP+ processes in the IPL are marked with arrow heads in (e). Quantification of GFAP+ Müller cell processes in the IPL showed reduced numbers after SE (g). Data are presented as means ± SEM in (c) and (d), and as median range with upper quartile range in (g), n = 6 SE and n = 7 CX3CR1-treated SE group. *p ≤ 0.05 un-paired t test in (c), 2-way ANOVA in (d), Mann-Whitney's rank sum test in (g). Scale bars are 500 µm for (a) and (b), and 50 µm for (e) and (f).

High resolution diffusion tensor imaging and magnetic resonance imaging of ex vivo retina from mice with focal non-convulsive status epilepticus and retinis pigmentosa (paper V)

Activated microglia in the retina of focal non-convulsive status epilepticus and rdKO mice

Due to prominent cell death, structural differences and prominent neuronal degeneration in the outer layers of the retina of rdKO mice, quantification of $Iba1^+$ cells were only performed in the IPL where increased numbers were found in rdKO compared WT mice ($p=0.0002$). Further analysis of microglial morphology showed a modest, but significant interaction between $Iba1^+$ cell phenotypes ($p=0.02$) in the IPL, where a decrease in ramified morphology was detected in the rdKO mice. Analysis of $Iba1^+$ cell numbers in the fNCSE group revealed a significant increase in both IPL and OPL in the ipsilateral retina ($p_{ipsiIPL}=0.006$, $p_{ipsiOPL}=0.03$), but no changes in the contralateral retina ($p_{contraIPL}=0.14$, $p_{contraOPL}=0.57$). Morphological evaluation of $Iba1^+$ cells showed, bilaterally, a decrease in ramified and an increase in intermediate phenotype ($p_{ipsi}=0.001$, $p_{contra}=0.006$), suggesting an on-going immune response. Analysis of GFAP intensity was performed in the GCL and did not differ in either rdKO mice or fNCSE mice compared to their respective control groups ($p_{rdKOvsWT}=0.06$, $p_{fNCSEvsCTRL}=0.42$ (ipsi), 0.79 (contra)). $GFAP^+$ Müller cell processes that extend out to the OPL were evident in the rdKO mice compared to WT mice ($p_{No.}=0.03$, $p_{lenght}=0.03$, $p_{thickness}=0.03$). Though this phenomenon has previously also been demonstrated following fNCSE in rats (Paper IV), it was absent in after fNCSE in mice.

Neuronal death in retina of rdKO mice while preserved neuronal structures following focal non-convulsive status epilepticus

The total number of $F\text{-}Jade^+$ cells was significantly increased in rdKOs compared to WT ($p=0.04$). No differences in numbers of $F\text{-}Jade^+$ cells were observed between fNCSE and CTRLs ($p_{ipsi}=0.64$, $p_{contra}=0.26$).

Discrete heterogeneity in FA maps of the mouse retina after focal non-convulsive status epilepticus

FA values in the rdKO retina were significantly lower compared to the WT animals (***Figure 19 a, b***), while no changes were observed in the distal part of the optic nerve

(***Figure 19 c***). When FA values in the entire retina of fNCSE were compared to CTRLs, no alterations were observed in either ipsi- or contralateral eye (***Figure 19 d-g***) or in the optic nerve (p_{ipsi}=0.34, p_{conta}=0.06) (***Figure 19 g***). Neither did the percentage of voxels with FA values <0.1 differ between fNCSE and CTRL animals (p=0.55, CTRL 43±3% vs fNCSE 46±4%). When sub-dividing the retina into lateral and medial part, the FA values were significant decreased in the lateral part of the ipsilateral retina ($p_{lateral}$=0.02, p_{medial}=0.21) (***Figure 19 h***), which is mostly innervated by the ipsilateral brain hemisphere with the electrode and epileptic focus. No differences were found in FA values in the lateral or medial part of the contralateral retina ($p_{lateral}$=0.87, p_{medial}=0.88) (***Figure 19 i***).

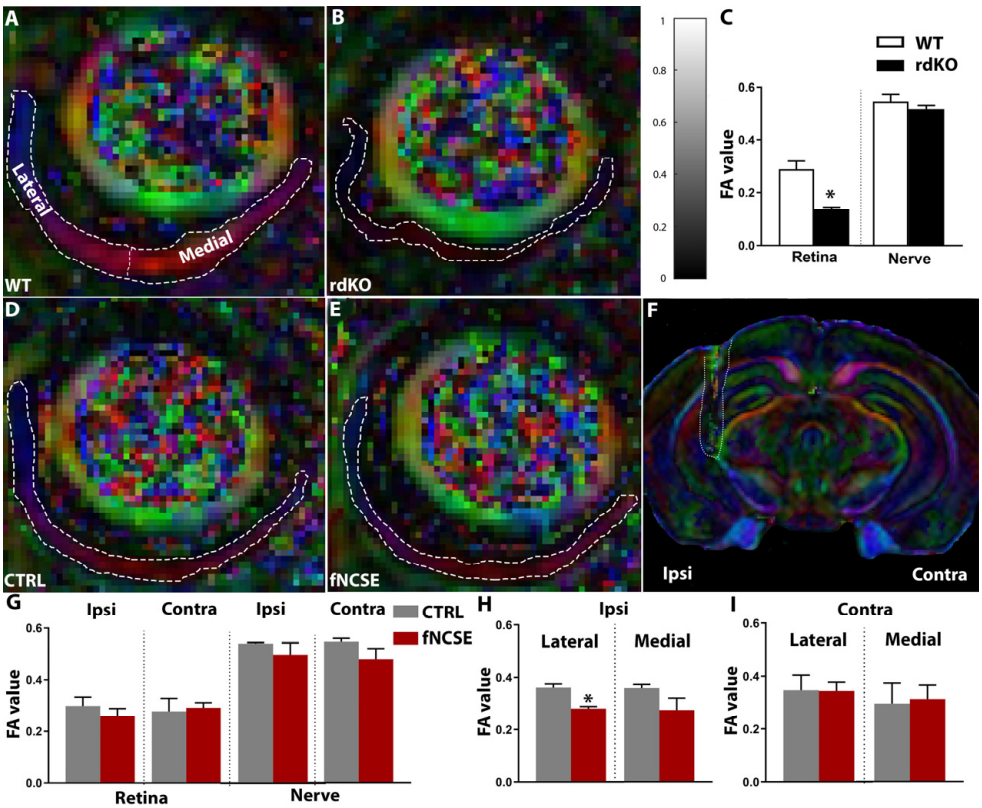


Figure 19: Representative sections of colour coded FA maps in WT (a) and rdKO with a greyscale bar of FA values (b). (c) Mean FA values in rdKO retina were significantly lower compared to WT, no changes in the optic nerve. Representative sections of CTRL (d) and fNCSE (e) that on occasion displayed darker patches. (f) Cavity from an electrode in the brain in a dorsal-ventral direction. (g) fNCSE showed no differences in non-colour FA-values in the retina or the optical nerve compared to CTRL. (h, i) When dividing the retina into medial and lateral parts, a significant decrease in FA values in the ipsilateral retina on the lateral side was observed. *p>0.05. nWT=4 vs nRd1=4 and nCTR=3 vs nfNCSE=5.

Minor changes in T_1-weighted retinal images after focal non-convulsive status epilepticus

Visual analysis of T_1-weighted images suggested a thinner and darker retina in rdKO compared to WT mice (***Figure 20 a***) and the vitreous body also displayed a darker colour. Statistical analysis revealed a lower vitreous intensity in rdKO animals compared to WT (mean grey value normalized to lens intensity WT 5.4±0.15 vs rdKO 4.5±0.14, p=0.01), an observation that may indicate retinal and/or ocular pathology. However, intensity measurements of the distal optic nerve showed no differences between groups (WT 6.1±0.23 vs rdKO 5.6±0.18). Analysis of the heatmaps representing the T_1 intensity through the different retinal layers displayed a thinner retina in rdKOs (156 µm in all mice) compared with WTs (312-338 µm), though, no significant alterations in intensity could be found (***Figure 20 b, c***). In the fNCSE and CTRL mice, no significant differences were detected in the choroid mean intensity (contralateral eye: medial part CTRL=7.7±0.62 vs fNCSE 8.8±0.24, p=0.09; lateral part CTRL 7.5±0.54 vs fNCSE 7.8±0.31, p=0.065). Analysis of the heatmaps (thickness 286-312µm) showed no differences in lateral or medial part of the contralateral retina, but when subdividing the retinal layers into outer (ONL+OPL) and inner layers (INL+IPL) fNSCE mice exhibited a minor increase in the T_1 intensity at position 0 and 130 µm of the lateral retina compared to CTRL ($p_{lateral}$=0.004, p_{medial}=0.22) (***Figure 20 d-h***). No intensity changes in the optic nerve were observed in fNCSE compared to CTRL mice (contralateral nerve CTRL: 5.9±0.41 vs fNCSE 5.5±0.28, p=0.08).

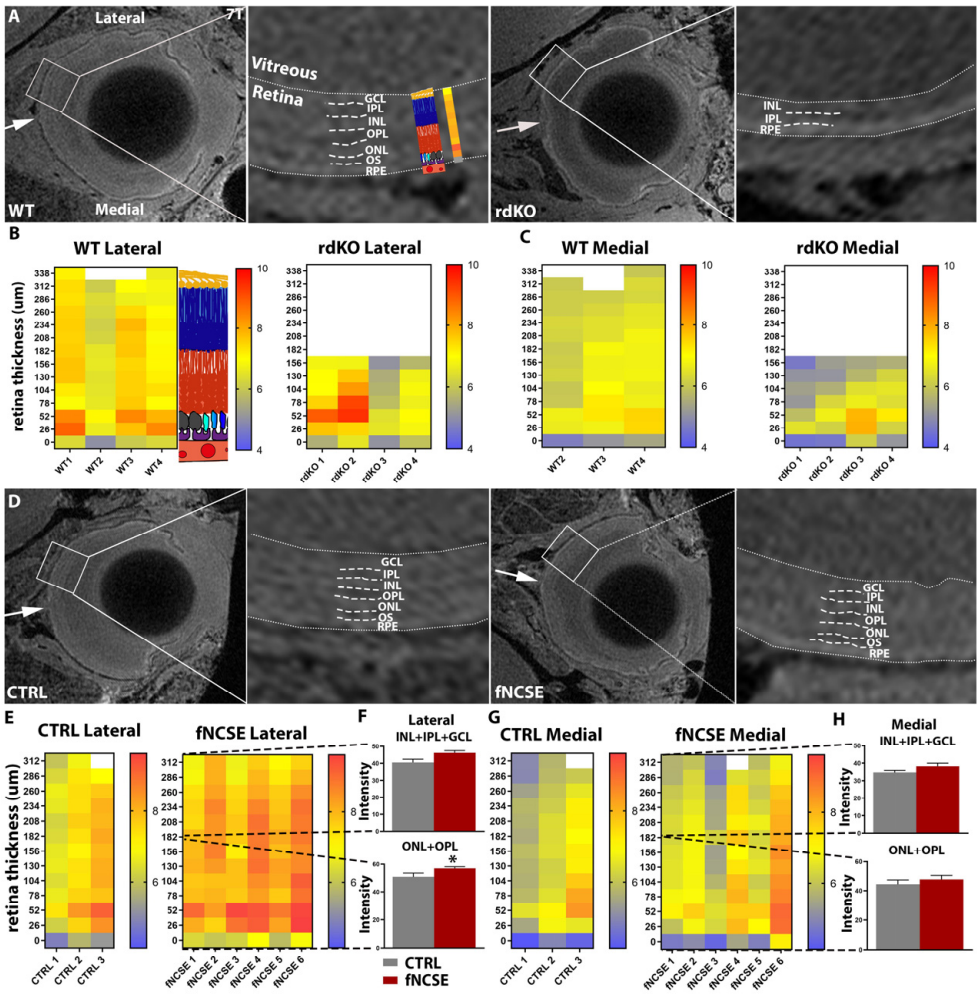


Figure 20: Panel (a), represents T_1-weighted images of WT and rdKO eyes. Arrows indicate the placement of the optic nerve. To the right a magnification of the retina (see dotted lines) and a display of the retinal layers. For clarification of retinal layering see schematic and heatmap inset. (b, c) Heatmaps represent mean intensity of 10 lines from position 0-338 µm (Y-axis), for each animal (X-axis). The heatmaps of rdKO mice were shorter than WT maps on both the lateral and medial side, reflecting rdKOs pervading pathology. No difference in intensity was found in the remaining rdKOs retinal layers (INL, IPL and RPE) from position 0 to 156 µm compared to WT. In panel (d), representative T_1-weighted images of the contralateral eye of CTRL and fNCSE Visually fNCSE mice exhibited similar retinal layering as CTRLs, apart from occasional heterogenous areas where the retinal layers were harder to distinguish. (e-h) Statistical analysis of the heatmap on the lateral and medial side of the retina, subdividing the retina into outer and inner retinal layers, identified a significant increase in T_1 intensity on retinal position 0 to 182 µm (ONL+OPL) in the lateral retina in fNCSE animals compared to CTRLs. *p>0.05. n_{WT}=4 vs n_{Rd1}=4 and n_{CTR}=3 vs n_{fNCSE}=6.

Discussion

Exercised induced prevention of epilepsy

We have reported a possible inhibitory effect on epilepsy development in both humans and a genetic mouse model of epilepsy. In humans the effect was observed in a large cohort of Vasaloppet skiers in both genders, independently of age until retirement, educational or occupational level. Interestingly, the incidence of epilepsy was further decreased in faster compared to slower skiers in the cohort. Subdividing epilepsy diagnosed skiers and controls into different sub diagnoses of epilepsy revealed a reduced incidence in skiers mainly in partial and unspecified epilepsy. In our genetic model the SynIIKO, a strong inhibitory effect was seen on seizure development if physical activity was implemented before seizure onset at 1 month of age. The strongest effect was seen during continuous exercise, yet running wheels introduced at 1 month and taken away at 2 months of age continuously decreased seizure development even after running wheel removal. The inhibitory effect on seizure development due to exercise did not affect microglia or astrocytes or BDNF/TrkB expression, however an increased neurogenesis was observed in the SGZ.

The beneficial effect we have observed in skiers are most likely connected to their lifestyle. From a survey it is determined that Vasaloppet participants have a healthier lifestyle and exercise more regularly compared to the general population in Sweden (Carlsson *et al.*, 2007). Additionally, faster skiers, presumed to be exercising regularly, have an even stronger inhibitory effect on epilepsy development compared to the slower skiers. Supporting our data is a study on 1.2 million participants from the basic military service in Sweden, that revealed a lower incidence of epilepsy during a 37-year follow up period in subjects with a high vascular fitness at the age of 18 compared to subjects with a low vascular fitness at the military enrolment. In the synIIKO mice, the lack of anti-convulsive effects was unexpected, though previous studies in other experimental epilepsy models have shown anti-convulsive effects (Arida *et al.*, 1999; Gomes *et al.*, 2014; de Almeida *et al.*, 2017). The various results might be due to the different animal models, and the severity and initiation of seizures. In SynIIKO mice seizures arise as a result of gradually predisposing features associated with the genetic mutation. In the previous articles they have all used the pilocarpine SE model, relying on a severe acute insult to initiate epileptogenesis and seizure propagation. The inflammatory reaction previously identified in the SynIIKO mice before seizure development (Chugh *et*

al., 2013) was not altered due to exercise, suggesting involvement of more specific inflammatory pathways, or other predisposing features. Voluntary exercise has been seen to increase number of glutamate transporters, which would help clear the excessive glutamate seen synaptically in the synIIKO mice (Medrihan *et al.*, 2015; Graban *et al.*, 2017).

Lastly, physical activity has been shown to increase neurotrophic factors and neurogenesis (van Praag *et al.*, 1999; Vivar *et al.*, 2013; Ahn *et al.*, 2016; Sun *et al.*, 2018). One month of voluntary running did significantly decrease the corticosterone levels in feces, however sedentary animals had a similar decrease suggesting voluntary exercise is not the main contributor of the decreased corticosterone levels. Yet, we found an effect with increased number of newly formed cells in the exercised compared to sedentary mice. Whether increased neurogenesis during the epileptogenic phase before seizure onset reflects or is associated with the inhibition of epilepsy development remains to be clarified.

Systemic inflammation as a biomarker for seizures?

We have in both a rodent model and human patients demonstrated a peripheral immune response after seizures. In rats with fNCSE, the immediate immune response (6 hrs post-fNCSE) within the epileptic focus was mimicked acutely in serum, but not in spleen, with increased IL-6 and Kc/GRO levels. At 4 weeks when a large proportion of fNCSE animals had started to develop SRSs, the brain pathology consisted of neuronal death and glial activation. In the periphery a selective immune response emerged in animals that had developed SRSs, consisting of altered levels of MHCII, CD68, Galactin-3, and CD4 in serum. These changes could not be observed in rats that did not develop SRSs.

In patients interictal levels of IL-6 were elevated in TLE, FLE and TLE with PNES patients compared to controls. Moreover, interictal levels of ICAM-1 was increased in patients with PNES compared to controls, but remained unaltered in the other patient groups. Post-ictal serum levels were only significantly changed in the TLE group. Six to eight hrs after a temporal lobe seizure postictal serum levels of IL-6, Mip1β, TARC, and MDC levels were elevated, together with a decrease of INF-γ. In the 24hr postictal temporal seizure sample MDC and INF-γ levels were not significantly altered from the interictal sample, however, IL-6, Mip1β and TARC were continuously increased. Additionally, in the 24hrs post ictal serum sample ICAM-1 levels were significantly upregulated in the TLE group. Both the results from animals and patients suggest a inflammatory profile in serum following seizures and presumably during epileptogenesis. Larger patient groups are necessary for it to be developed into a relevant clinical tool and a possible biomarker for epileptic seizures.

Peripheral response in rats

The acute cytokine release observed in our animal model of fNCSE supports emerging clinical evidence of early and transiently increased levels of IL-6, IL-17A, INF-γ, total number of leucocytes, neutrophils and lymphocytes in plasma and serum from patients with TLE (Bauer *et al.*, 2008; Gao *et al.*, 2017; Alapirtti *et al.*, 2018). In our study, a similar acute peripheral response was observed following an intracerebral LPS injection and we could not provide a seizure specific immune response within the first 24hrs. In addition, the acute IL-6 elevation did not correlate with parameters from the fNCSE episode. At the subacute and chronic timepoints post-fNCSE pathology in the brain suggested gliosis and increased microglia with phagocytic capacity. Indeed, changes related to phagocytosis such as MHCII and CD68 are well documented in other studies regarding epilepsy and SE (Shaw *et al.*, 1994; Tooyama *et al.*, 2002; Boer *et al.*, 2006; Luo *et al.*, 2016). The immune reaction after seizures in the brain and inflammation following intracerebral LPS administration share similarities, typically by activation of microglia and production of cytokines (Herber *et al.*, 2006; Jakubs *et al.*, 2008; Erickson and Banks, 2011; Chugh *et al.*, 2013).

Nevertheless, the peripheral immune response observed in our study differed at 4 weeks between rats that developed spontaneous seizures after fNCSE and rats receiving LPS injection. Alterations in proteins normally situated in the cell membrane may in the present study translate to either increased protein expression on individual cells, increased number of cells or increased soluble proteins as a result of cleavage/cell disruption. The occurrence of SRSs may have initiated the changes observed in antigen-presenting cell markers in serum such as: MHCII, CD68, and Gal-3. Alternatively, both seizures and the peripheral immune response are the results of the underlying emerging brain pathology during epileptogenesis after fNCSE. Regardless of the mechanism these findings might have prognostic clinical value in patients after fNCSE. Galactin-3 has previously been reported to be elevated in serum from patients with intractable focal epilepsy (Tian *et al.*, 2016). Despite the systemic changes in cytokine and immune protein levels in serum, we found very few changes in spleen tissue with ELISA and WB analysis. Earlier experimental studies have described dense innervation of spleen tissue with receptors on macrophages and/or lymphocytes that in turn modulate the immune response (Bellinger *et al.*, 1989; Rosas-Ballina *et al.*, 2008; Andersson and Tracey, 2012). In addition, splenectomy reduces seizure associated mortality in rodents with convulsive SE (Marchi *et al.*, 2011). The acute transient decrease of CD11b may suggest increased recruitment to/from the spleen and increased levels of CD45 may reflect mobilization of leucocytes at 24hrs post fNCSE. Changes in GFAP level in the spleen at 24hrs post fNCSE and LPS injection might reflect early astrocytic activation in the brain due to bidirectional neuroimmune interaction.

Peripheral response in patients

We found a robust increase of interictal levels of IL-6 in patients with epilepsy, including patients with temporal lobe seizures. Previous reports have presented an increased baseline of IL-6 in serum from TLE patients compared to healthy controls (Uludag *et al.*, 2015). Pharmacoresistant patients with epilepsy might be offered surgery in an attempt to resect the epileptic focus in the brain to interrupt the initiation of seizures. These patients often have a strong inflammatory response before surgery due to failure of seizure control. Furthermore, elevated IL-6 levels before surgery were decreased both 1-2 years post resection surgery (Lorigados Pedre *et al.*, 2018). Other studies have identified increased IL-6 levels in serum from temporal lobe patients compared to extra temporal lobe patients (Alapirtti *et al.*, 2018). Earlier studies have failed to correlate interictal IL-6 levels to the duration of epilepsy, lateralization or reported seizure frequency (Bauer *et al.*, 2009; Uludag *et al.*, 2015).

Here, similarly to previous studies we did not find a correlation between reported seizure frequency, reported lateralization, or reported last seizure and the increased IL-6 levels. However, there are evidence that after resection surgery IL-6 levels decrease in serum in patients that become seizure free compared to patients that has a continuation of seizures (Lorigados Pedre *et al.*, 2018), suggesting that seizure frequency might indirectly influence the IL-6 levels in serum. Furthermore, interictal ICAM-1 levels were significantly increased in PNES patients without epileptic seizures. Another study done by Gledhill J. and colleagues tried to define pathological differences between epileptic seizures and PNES, and in line with our study they found increased plasma levels of ICAM in PNES patients compared to patients with epilepsy (Gledhill *et al.*, 2021). They created an algorithm that identified the use of several inflammatory factors as potential diagnostic markers for PNES compared to epilepsy. To increase the sensitivity even more when using inflammatory factors as a marker of PNES, comorbidities were taken into account (including depressive disorders, migraines, or trauma) as a risk factor for PNES.

In our study, interictal levels of ICAM-1 were altered in PNES patients and not in patients with epilepsy, furthermore patients with epilepsy had an interictal increase of IL-6, which was not observed in the PNES patients. The potential for these two factors together as a tool to distinguish PNES from epilepsy in an interictal state will have to be determined in future studies. However, apart from interictal changes we also observed several postictal alterations at both 6-8 and 24hrs after a temporal lobe seizure, which could add additional value when diagnosing TLE and differentiate it from PNES patients. Despite the higher seizure frequency in FLE patients, the postictal IL-6 increase was only detected in the TLE group. Furthermore, the IL-6 increase could not be explained by factors related to seizure burden such as duration of index seizure, number of seizures before blood sample, time from latest seizure before postictal blood sample or occurrence of FTBTC

seizures. Other studies have similarly tried to identify factors that are correlated to a postictal IL-6 increase and have in contrast to our study found an association between IL-6 increase and FCBTC seizures (Alapirtti *et al.*, 2018).

The exact role of IL-6 in epilepsy remains unclear, still it has been shown that polymorphisms in IL-6 affects the pathogenesis and treatment response in epilepsy, although this has not been confirmed in in clinical studies (Tiwari *et al.*, 2012; Gök *et al.*, 2014). It is still not clear which intrinsic factors in TLE that may contribute to the postictal IL-6 increase. Apart from IL-6 we also found a postictal increase of TARC in TLE patients. A previous study has investigated chronic TARC levels in plasma in patients with focal epilepsy, identifying a trend towards an elevation in serum in these patients, and discerning epilepsy patients from controls was done by their TARC/cICAM5 ratio (Pollard *et al.*, 2012). However, this study did not take the timing of the blood sample into account or make any distinction between different focal epilepsies, making it harder to interpreting the results in relation to our study.

As with TARC, few studies have been made on the role of MDC in epilepsy. However, studies in children with autism, has found a strong correlation between TARC and MDC expression in serum (Al-Ayadhi and Mostafa, 2013). If the TARC and MDC expression is indeed affecting each other postictally after TLE remains to be investigated. Another interesting alteration after temporal lobe seizures was the decrease of INF-γ. Previous studies have in contrast reported an increase of INF-γ after TLE seizures (de Vries *et al.*, 2016; Gao *et al.*, 2017). It remains unclear if we indeed have found a transient decrease of INF-γ 6hrs after temporal lobe seizures that previous studies have failed to detect. The INF-γ levels were harder to detect compared to other cytokines. We currently do not know if another detection method could give more stable results, or if the INF-γ levels has a faster degradation then other cytokines making it harder establish it's concentration in serum

Lastly, ICAM-1 levels were increased at 24 hrs but not at 6 hrs after a temporal lobe seizure. A previous study has identified a sICAM-1 increase in serum from patients with a drug-refractory epilepsy compared to drug responsive epilepsies, and newly diagnosed epilepsies were treatment response cannot be determined (Luo *et al.*, 2014). However, this study does not distinguish epilepsy patients according to different seizure types, which makes the result harder to correlate to our study were an ICAM-1 increase was only seen postictally in the TLE patient group. The relationship between a chronic elevation of ICAM-1 in PNES patients and an acute/postictal increase of ICAM-1 after temporal lobe seizures need to be clarified in future studies to estimate its clinical relevance. Our study suggests different peripheral immune responses after seizures in temporal lobe epilepsy and frontal lobe epilepsy, stressing the need for diagnostic specificity in patients with focal epilepsy when conducting future studies. Additionally, the specific mechanism behind the increase of IL-6 after temporal lobe seizures and not the other patient groups needs to be determined.

Retinal pathology in epilepsy

We have provided with the first evidence that epileptic seizures originating within the temporal lobe of the adult rodent brain, leads to a glial activation in the retina. In rats with fNCSE a delayed activation of both micro- and macroglia was found in the ipsi- and contralateral eye, with more prominent inflammation in the ipsilateral retina. In mice subjected to fNCSE microglial activation was found in the ipsilateral eye, and more discrete inflammatory changes in the contralateral eye, furthermore no delayed activation of macroglia was found. Additionally, intracerebroventricular infusion of CX3CR1 antibody in rat significantly reduced the retinal pathology in both the ipsi,- and contralateral eye.

Our result suggests a common immunological reaction in the retina and the epileptic focus in rats and mice. However, no neurodegeneration was found in the retina, as compared with the epileptic focus where a more extensive degeneration of cells can be found. Interestingly, the pathological profile was more subtle in the mouse retina compared to rats, with no indication of macroglia activation, and more modest microglial alterations. The discrepancies we observe in terms of the pathological response after fNCSE might emerge from the different rodent species. The same pathological insult in rats and mice may lead to different pathophysiology due to cerebrovascular anatomy, or cerebral blood flow (Boswell *et al.*, 2014). Additionally, differences in animals with acute symptomatic vs SRSs might differentiate in terms of systemic pathological response (paper II). However, in paper IV and V we did not distinguish between animals with epileptic or acute symptomatic seizures. It would be interesting to investigate if animals with developed SRSs have an alternative inflammatory reaction both in the brain and eyes, and if this could give rise to the observed differences in retinal pathology.

Suggested mechanism

There are at least 4 possible scenarios, that might elucidate the underlying mechanism and retinal pathology after fNCSE in rodents. First, fNCSE initiate an acute hippocampal immune reaction that may spread through the BRB. We have demonstrated that inflammatory mediators are upregulated in blood and spleen both 6 and 24hrs post fNCSE (paper II), however evaluating the same markers in the eyes acutely after fNCSE (paper IV) did not suggest a common pathogenesis. Instead, a delayed more subtle alteration in cytokine and chemokines was observed in the eyes. This supports the idea of a systemic inflammatory reaction that give rise to a delayed pathological response. However, we did not observe any alterations in infiltrating leucocytes or vascular pericytes, which would be expected during a systemic reaction spreading through the blood.

Secondly, seizures might change the intracranial pressure (ICP) and thereby affecting the intraocular pressure (IOP). An imbalance between the anterior IOP and

the posterior ICP would create a pressure on the optic nerve, possibly leading to abnormal function and nerve damage in the GCL and ONL, which is frequently observed in glioma (Berdahl *et al.*, 2008; Weinreb *et al.*, 2014). Supporting this, we present and altered PSD-95 expression together with gliosis 7weeks post fNCSE in the rat retina.

Thirdly, the immune response in the epileptic focus may spread to other brain areas involved in the seizure network, including subcortical structures (Avdic *et al.*, 2018), ultimately leading to inflammation in the lateral geniculate nucleus, a thalamic structure that connects the brain with the optic nerve. That kind of scenario would likely induce a stronger immune response were the optic nerve fuses with the eye, though we found no evidence that the retina close to the nerve was especially affected. Furthermore, in eye sections where the most anterior part of the optic nerve was included, we could not observe any inflammatory reaction in the microglial or astrocyte population. However, to fully evaluate this scenario a more detailed investigation of the nerve fibre tract is required, to enable detection of more subtle and local alterations. Supporting this suggestion are studies in AD patients were pathogenic factors spreads from the limbic structures into visual cortex, the optic nerve and the retina (Hill *et al.*, 2014; Pogue *et al.*, 2014).

Lastly, epileptic seizures may induce retrograde currents through the optic nerve, thereby initiating an excitatory/inhibitory imbalance in the retinal network and commencing glial activation. It is to our knowledge not known if retinal neurons might exhibit properties such as abnormal synchronized firing, similar to what can be observed within the epileptic focus. However, the activated muller cell processes might contribute to epileptic discharges, in the same way as suggested for astrocytes in the brain (Crunelli *et al.*, 2015).

Heterogeneity and lateralization

In mice and rats the seizure-induced glial activation was evident in both the ipsi- and contralateral eye. Nevertheless, in both species the pathogenesis seemed more pronounced in the ipsilateral retina compared to the contralateral. Interestingly, a previous case report on Rasmussen's encephalitis, which is a more severe epileptic encephalopathy only engaging one brain hemisphere, described an ipsilateral ocular inflammation in the patient (Fukuda *et al.*, 1994). The authors speculate if the immune reaction in the eye after focal seizures may be associated with clinical manifestations. In paper IV we did not differentiate between lateral and medial parts of the retina, hence we cannot elucidate if the pathology observed is lateralized and correlate with the innovation from the ipsilateral brain hemisphere. Furthermore, in paper V, the medial-lateral orientation of the eye was preserved during the MRI. Analysis of FA-maps identified a decrease on the lateral side of the ipsilateral eye, which is mostly innervated from the ipsilateral brain hemisphere. The fNCSE induced pathology was less pronounced in mice compared to rats, however in the

ipsilateral eye of mice, microglia increased both in number and percentage of morphological intermediate phenotype. Indeed, we found an alteration in the DTI of the ipsilateral eye, however for the IHC analysis, the medial-lateral orientation was lost, hence a correlation between microglial changes and decreased FA-values after fNCSE have to be interpreted with caution.

Utility of MRI detection of fNCSE induced retinal pathology

In paper V, we continued our previous study to evaluate if high-resolution T_1, and DTI MR-techniques can detect the fNCSE induced retinal inflammation that we previously observed. As a positive control for retinal pathology the Retinis pigmentosa model, rdKO mice, was included showing extensive degeneration of the outer retinal layers in adulthood. We could confirm that even a small structure such as the mice retina could be visualized with both 7T T_1 MRI and 9.4T DTI *ex vivo*. The rdKO mice had a clear alteration in FA-map intensity, and T_1 images revealed a thinner retina with a darker vitreous, hence the pathology could be confirmed both with T_1 and DTI techniques. Additionally, the examination of fNCSE mice that expressed modest retinal alterations mainly in the plexiform layers, did not conclude any convincing evidence for the possibility of using T_1 and DTI with a resolution of 26, and 125 μm respectively for detection of fNCSE induced pathology. Differences in pathological severity, and heterogeneity of tissue re-organisation seems as two important factors when using MRI as an investigative tool of the mice retina. Furthermore, a more feasible approach might be the use of Optical Coherence Tomography (OCT) imaging, or alternatively DTI with a higher resolution, for detection of discrete regional alterations in the retina after fNCSE.

Future perspective

Epilepsy is devastating and complex disorder with several pathological features. One of these is the ongoing inflammatory reaction both in animal models of epilepsy and patients. To discern the complex and extensive inflammatory reaction in epilepsy gives the opportunity to develop new novel treatments strategies and tools for developing prognostic or diagnostic biomarkers, a clinical tool that is currently absent.

In this thesis physical activity was shown to have beneficial effects by delaying seizure onset in both humans and animals. Future studies will have to evaluate when physical activity need to be initiated to enable this inhibitory effect on epileptogenesis, and if certain types of physical activity are more beneficial than others remains to be clarified. We could not identify the mechanism behind this phenomenon, which makes the effect and relevance of the finding harder to interpret. Presumably physical activity would reduce inflammation and affect the HPA axis. Even though we in our study could not identify such a phenomenon, a more complex investigation of microglial motility or the expression of cell surface markers might reveal differences between physically active and sedentary animals. In humans it is from our study hard to interpret which factor that influences epilepsy incidence the most. Vasalopp skiers are more physically active, with a higher consumption or fruits and vegetables, together with a lower number of smokers. If all these factors combined e.g. physical activity, diet and smoking habits reduce the risk of developing epilepsy, or if physical activity alone is enough to delay epilepsy onset needs to be further investigated. In the end, with more research, the question if exercise could be used as a prevention of epilepsy development in patients with risk factors for developing epilepsy could be answered.

Furthermore, discerning the inflammatory reaction growing evidence has identified peripheral inflammation both chronically and acutely after seizures. We have tried to stratify the peripheral reaction in rats to establish a profile in serum and spleen, which can be associated with epileptogenesis and interictal activity. Rats that developed SRSs had a distinct profile with upregulation of proteins associated to antigen presentation, phagocytosis and a disrupted balance in the T-cell population. With our current method we cannot identify if there is an increased number of cells expressing the antigen or if cells already positive for the antigen has upregulated their expression. To get more detailed information of how the balance between different inflammatory cells in the periphery gets disrupted during epileptogenesis

future studies could use a more complex analysis of blood plasma using Fluorescence-Activated Cell Sorting (FACS). The ultimate goal for future studies would be to establish if any of the identified proteins could be used as prognostic markers or new novel treatment targets for inhibiting epileptogenesis. We continue our study of the peripheral immune response in patients with epilepsy in search of an acute inflammatory profile in serum after epileptic seizures. To increase the clinical value of these finding creating larger patient groups is essential. Especially important will be to extend the PNES patient in order to evaluate if any acute differences between TLE and PNES patients exists. Another interesting aspect would be to establish why temporal seizures seems to initiate a stronger inflammatory post-ictal reaction compared to base line interictal levels than frontal lobe seizures. Are there specific mechanisms in TLE that acutely leads to more inflammation, such as BBB disruption or dysfunctional and more reactive microglia?

Furthermore, in the CNS we have shown that the inflammatory reaction reaches as far as the retina outside the closest proximity of the epileptic focus. For future studies, the investigation of the eyes and the retina of patients with epilepsy would be essential, and to establish if the inflammation can be associated with minor visual disturbances in either animals or patients. The origin of the delayed retinal reaction after fNCSE could not be established in our studies. Even so, the mechanism might answer if this could be used as a prognostic marker in epilepsy. In addition, clinical tools to non-invasively investigate the retinal pathology after fNCSE in humans also need to be stratified. The use of MRI as an imaging tool might be less attractive compared to the use of OCT. However, to fully establish the use of OCT investigating the retinal associated inflammation after seizures, future studies will have to investigate its use in both animals and humans.

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