Immune responses and synaptic protein expression in epilepsy

Avdic, Una

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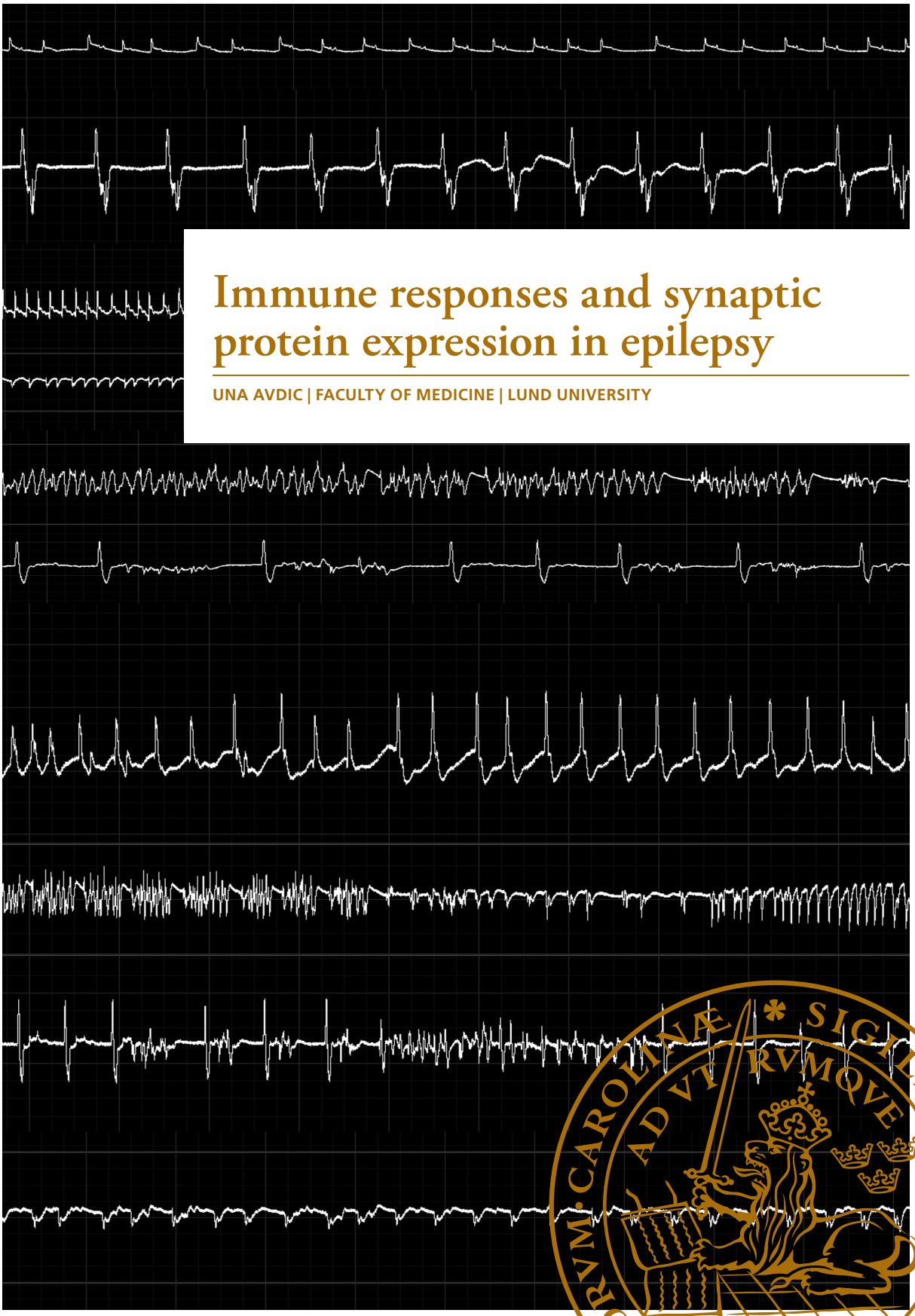

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Abstract

Epilepsy is a chronic neurological disorder that affects approximately 1% of the world population and is characterized by recurrent spontaneous seizures. Current antiepileptic treatment is only symptomatic and fails to adequately control seizures in almost 40% of patients. Many studies postulate inflammation and an excitatory/inhibitory imbalance as key driving forces. Common causes of epilepsy include brain trauma, stroke and status epilepticus (SE). Thus, increased understanding of the molecular pathways underlying the development of epilepsy (epileptogenesis) and further stratifying the pathology will aid in developing new diagnostic/prognostic and therapeutic strategies for this debilitating disease.

The main objective in this thesis is to characterize the pathology associated to non-convulsive SE (NCSE) and the development of epilepsy. We observe an acute release of pro- and anti-inflammatory cytokines in the epileptogenic focus and blood, and show a developing chronic activation of microglia and astrocytes with subsequent neuronal loss in the epileptic focus (Paper IV, V). We also identify transient changes in excitatory and inhibitory synaptic protein levels in the hippocampus, before the onset of spontaneous seizures, possibly suggesting seizure- promoting mechanisms. Moreover, we demonstrate that mice lacking the receptor for Il-1β, exhibit increased microglial activation in the hippocampus and an altered synaptic protein expression, suggesting an important role of the IL-1R1/Il-1β signaling pathway in maintaining physiological conditions in both neurons and microglia (Paper I). In addition, we provide the first evidence of pathology in the retina following epileptic seizures (Paper II), where a delayed glial activation is detected, and blocking the immune response by modulating the putative fractalkine/CX3CR1 pathway reduces the seizure-induced pathology detected in the retina. Moreover, in animals treated with CX3CR1 antibody, we show altered levels of PSD-95 on newly formed neurons in the hippocampus along with reduced microglial activation in the dentate hilus (Paper III). Finally, we characterize the pathology in rats with NCSE following monotherapy with levetiracetam, and with levetiracetam combined with intracerebral infusion of an antibody against the excitatory adhesion molecule N-cadherin (Paper VI). We show that both neurodegeneration and microglial activation are reduced in the hippocampus, along with altered levels of PSD-95 in the dentate hilus.

In conclusion, this thesis provides evidence of a widespread and developing immune profile in brain, eyes and blood following NCSE. We also describe changes in synaptic protein expression associated to the excitatory/inhibitory balance. These results provide evidence for a model that is highly clinically relevant for future investigations of the mechanisms behind the brain pathology associated with NCSE and treatment-resistant epilepsy. They also suggest new diagnostic/prognostic strategies with direct clinical value as predictors of subsequent epilepsy development and disease progression.

Key words

NCSE, epilepsy, synaptic proteins, inflammation, microglia, immune system

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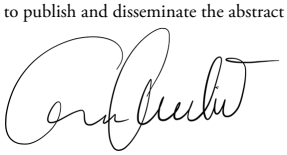
Signature 

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Una Avdic

LUND
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To my family

'Success is walking from failure
to failure with no loss of enthusiasm'

– Winston Churchill

CONTENTS

ABSTRACT

Epilepsy is a chronic neurological disorder that affects approximately 1% of the world population and is characterized by recurrent spontaneous seizures. Current antiepileptic treatment is only symptomatic and fails to adequately control seizures in almost 40% of patients. Many studies postulate inflammation and an excitatory/inhibitory imbalance as key driving forces. Common causes of epilepsy include brain trauma, stroke and status epilepticus (SE). Thus, increased understanding of the molecular pathways underlying the development of epilepsy (epileptogenesis) and further stratifying the pathology will aid in developing new diagnostic/prognostic and therapeutic strategies for this debilitating disease.

The main objective in this thesis is to characterize the pathology associated to non-convulsive SE (NCSE) and the development of epilepsy. We observe an acute release of pro- and anti-inflammatory cytokines in the epileptogenic focus and blood, and show a developing chronic activation of microglia and astrocytes, with subsequent neuronal loss in the epileptic focus (Paper IV, V). We also identify transient changes in excitatory and inhibitory synaptic protein levels in the hippocampus, before the onset of spontaneous seizures, possibly suggesting seizure- promoting mechanisms. Moreover, we demonstrate that mice lacking the receptor for Il-1β, exhibit increased microglial activation in the hippocampus and an altered synaptic protein expression, suggesting an important role of the IL-1R1/Il-1β signaling pathway in maintaining physiological conditions in both neurons and microglia (Paper I). In addition, we provide the first evidence of pathology in the retina following epileptic seizures (Paper II), where a delayed glial activation is detected, and blocking the immune response by modulating the putative fractalkine/CX3CR1 pathway reduces the seizure-induced pathology detected in the retina. Moreover, in animals treated with CX3CR1 antibody, we show altered levels of PSD-95 on newly formed neurons in the hippocampus along with reduced microglial activation in the dentate hilus (Paper III). Finally, we characterize the pathology in rats with NCSE following monotherapy with levetiracetam, and with levetiracetam combined with intracerebral infusion of an antibody against the excitatory adhesion molecule N-cadherin (Paper VI). We show that both neurodegeneration and microglial activation are reduced in the hippocampus, along with altered levels of PSD-95 in the dentate hilus.

In conclusion, this thesis provides evidence of a widespread and developing immune profile in brain, eyes and blood following NCSE. We also describe changes in synaptic protein expression associated to the excitatory/inhibitory balance. These results provide evidence for a model that is highly clinically relevant for future investigations of the mechanisms behind the brain pathology associated with NCSE and treatment-resistant epilepsy. They also suggest new

diagnostic/prognostic strategies with direct clinical value as predictors of subsequent epilepsy development and disease progression.

SUMMARY

Epilepsy is a chronic neurological disorder that affects approximately 50 million people worldwide. Patients have excessive and abnormal neuronal activity that manifests as spontaneous seizures. Current antiepileptic treatment options are only symptomatic and fail to adequately control seizures in almost 40% of patients. Although the pathophysiological mechanisms are not fully elucidated, many studies postulate inflammation and an excitatory/inhibitory imbalance as key driving forces. Common causes of epilepsy include brain trauma, infection, stroke and status epilepticus (SE). Thus, increased understanding of the molecular pathways underlying the development of epilepsy (epileptogenesis) and further stratifying the underlying pathological mechanisms will aid in developing new diagnostic/prognostic and therapeutic strategies for this debilitating disease.

In this thesis, we characterize the inflammatory response and demonstrate a developing pathology associated to non-convulsive SE (NCSE) and epilepsy, in an experimental rodent model that presents similar EEG patterns and semiology to patients with complex partial NCSE, that subsequently leads to the development of spontaneous seizures. We observe an acute release of pro- and anti-inflammatory cytokines in both the epileptogenic focus and blood, and report a developing chronic activation of microglia and astrocytes with subsequent neuronal loss in the epileptic focus (Paper IV, V). In addition, we identify transient changes in excitatory and inhibitory synaptic protein levels in the hippocampus, at 1 week following NCSE before the onset of spontaneous seizures, possibly suggesting seizure- promoting mechanisms.

Moreover, we provide the first evidence of pathology in the retina following epileptic seizures (Paper II), where a delayed and substantial glial activation is detected, without changes in structural cytoarchitecture. Long-term retinal changes in the synaptic scaffolding protein PSD-95 were also evident. Interestingly, blocking the immune response by modulating the putative fractalkine/CX3CR1 pathway reduced some the seizure-induced pathology detected in the retina such as glial activation. Further studies will need to address the question of any possible functional retinal deficiencies associated to the pathological, subclinical findings. Moreover, in animals treated with CX3CR1 antibody, we present a decreased PSD-95 expression on newly formed neurons in the hippocampus along with reduced microglial activation in the dentate hilus (Paper III). These results warrant further studies on the functional role of the changes in terms of integration and seizure burden. When further studying the link between immunological alterations and the expression of synaptic proteins, we demonstrate that mice lacking the receptor for Il-1β, exhibit increased microglial activation

in the hippocampus and an increased expression of PSD-95 with subsequent decrease in gephyrin, suggesting an important role of the pro-inflammatory IL-1R1/Il-1β signaling pathway in maintaining physiological conditions in both neurons and microglia. Future studies need to address if these changes are associated with an imbalance in the excitatory/inhibitory signaling in conditions such as epilepsy, where dysregulation in Il-1β is particularly pronounced.

Finally, we characterize the pathology in rats with NCSE following monotherapy with levetiracetam, and with levetiracetam combined with intracerebral infusion of an antibody against the excitatory adhesion molecule N-cadherin (Paper VI). We show that both neurodegeneration and microglial activation are reduced in the hippocampus, and that the expression of PSD-95 is modulated in the dentate hilus. However we were not able to detect changes in epileptogenesis and seizure burden, suggesting that a reduced seizure burden may manifest at a later time point, a speculation that remains to be confirmed.

In conclusion, in this thesis we have characterized the immunological response, where we show a distinct developing immunological profile in brain, eyes and blood following NCSE and epilepsy. We also describe changes in synaptic protein expression associated to the excitatory/inhibitory balance, and that modulating these, and the immune system, might be a feasible therapeutic strategy. However, the functional implications of changes in synaptic protein expression in an epileptic brain, needs further validation. Our studies aid in underpinning the developing pathophysiology associated to seizure activity and epileptogenesis, and stratifying these mechanisms further will offer insight into how the immune system can help in propagating the pathology and seizure activity. Notably, the pathophysiological changes described here are promising for future clinical studies and hold great value in terms of prediction of diagnostic/prognostic biomarkers and therapeutic strategies for epilepsy.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Epilepsi är en neurologisk sjukdom som drabbar ca 1% av Sveriges befolkning. Ett epileptiskt anfall orsakas av övergående elektriska urladdningar i nervceller och beror på en elektrisk obalans i hjärnan. Anfallen varierar i grad och sjukdomen yttrar sig olika beroende på var i hjärnan den epileptiska aktiviteten uppstår och kan, i bland annat temporallobsepilepsi, yttra sig som återkommande partiella krampanfall, där symptomen kan vara automatiska rörelsemönster, förnimmelser, förvirring och medvetandepåverkan. De partiella anfallen kan också spridas till andra delar av hjärnan och leda till generaliserade krampanfall med upprepade muskelsammandragningar, så kallade toniskt-kloniska anfall. Om anfallen pågår i mer än 30 min, vilket enligt gammal definition kallas status epilepticus (SE), är de direkt livshotande pga. den ökade metabolismen och svårigheter med bl.a. andningen. Antiepileptisk behandling består huvudsakligen av medicin som ökar den inhibitoriska aktiviteten i hjärnan och därmed sänker hyperexcitabiliteten. Dessvärre har många av dessa läkemedel oönskade biverkningar och är huvudsakligen symptomatiska, dvs. symptomlindrande utan någon inverkar på den underliggande orsaken till problemet. Dessutom är ca 40% av alla patienter resistenta mot behandling. Behovet av både bra biomarkörer och effektiv behandling är således mycket stort.

I många fall föranleds epilepsi av en skada, såsom skallskada, infektion eller stroke. Detta leder i sin tur till ett antal strukturella och molekylära förändringar i hjärnan, ett förlopp som kan omvandla en i övrigt normal hjärna till en som är hyperexcitabel och mer benägen att drabbas av anfall. Denna latenta period, även kallad epileptogenes, innebär subtila förändringar i hjärnan och kan fortlöpa allt från ett par månader till flera år efter den ursprungliga skadan. Genom att närmare studera händelseförloppet under epileptogenesen och de mekanismer som kan leda till epilepsi, kan vi försöka modifiera sjukdomsförloppet och i bästa fall även förhindra utvecklingen av epilepsi helt och hållet.

I denna avhandling har vi huvudsakligen karakteriserat den inflammatoriska reaktionen i hjärna och blod i samband med epileptiska anfall i råttor. Då epilepsi även kännetecknas av förändringar i de molekyler som styr synapsens aktivitet, där de stabiliserar, integrerar och förankrar cellerna till dels omgivningen och dels till varandra, har vi även utvärderat det synaptiska proteinuttrycket efter anfall. Vi identifierar patologiska förändringar i en modell av icke-konvulsivt status epilepticus i form av nervcellsdöd, mikroglia- och astrocyt-aktivering i hjärnan. Eftersom det i kliniska sammanhang saknas konsensus kring hur skadlig denna typ av anfall är, har denna studie ett tydligt kliniskt värde med potential för nya behandlingsstrategier. Vi beskriver även ett specifikt immunologiskt svar i blodet efter epileptiska anfall och visar att den systemiska immunologiska profilen förändras över tid och

skiljer sig från det systemiska svaret efter en intracerebral infektion. Utöver detta, visar vi att den immunologiska responsen sprids till ögonen efter temporallobsanfall i råtta där både mikroglia- och makrogliaaktivering reduceras med hämmandet av en mikroglia receptor, CX3CR1 - ett resultat med potentiellt prognostiskt kliniskt värde.

Vidare, då denna avhandling även berör de molekylära förändringar i hjärnan i en inflammatorisk miljö, har vi utvärderat det synaptiska proteinuttrycket i en musmodell som saknar den pro-inflammatoriska proteinreceptorn interleukin (IL)- 1 och beskriver en mikroglia-aktivering samt förändring av synapsproteinuttrycket. Dessa resultat tyder på en viktig roll för IL-1 receptorn under normala fysiologiska förhållanden i nervceller och hjärnan. Våra resultat visar även att uttrycket av synaptiska protein förändras i nybildade nervceller i samband med epileptiska anfall och påverkas av intracerebral tillförsel av CX3CR1 antikropp. Resultatet visar alltså att den inflammatoriska reaktionen efter anfall påverkar de synaptiska proteinernas uttryck på nya nervceller. Vi identifierar även en kritisk period innan utvecklingen av spontana anfall då en rad proteiner i hjärnan förändras till följd av ett långt icke-konvulsivt status epilepticus. Ytterligare studier behövs för att kartlägga de långsiktiga effekterna av dessa förändringar. Slutligen undersöker vi hur intracerebral tillförsel av en antikropp mot adhesion molekylen N-cadherin i kombination med ett antiepilepticum (Keppra) kan påverka den inflammatoriska miljön, synapsproteinuttrycket och anfallsfrekvensen i råttor med epileptiska anfall. Resultaten tyder på att varken behandling med Keppra eller tillägg av N-cadherin antikropp minskar anfallsfrekvensen hos råttorna. Vi observerade däremot en minskning av de patologiska förändringarna i samband med behandlingen.

Sammanfattningsvis, i denna avhandling har vi kartlagt det immunologiska svaret och våra resultat visar en tydlig immunologisk profil i hjärna, ögon och blod hos råttor med epileptiska anfall. Sammantaget bidrar denna studie till en ökad förståelse för de mekanismer och patofysiologiska förändringar förknippade med epilepsi och icke-konvulsivt status epilepticus. Vi beskriver även en förändring i synaptiskt proteinuttryck i hjärnan och ytterligare validering krävs för att förstå dess betydelse i en epileptisk hjärna. Eftersom karakterisering av det immunologiska svaret kan komma att underlätta framtida diagnostisering vid epilepsiutredningar, har dessa resultat ett tydligt klinisk intresse för diagnos/prognos och behandling.

LIST OF ARTICLES & MANUSCRIPTS

I. **Avdic U**, Chugh D, Osman H, Chapman K, Jackson J, Ekdahl CT. Absence of interleukin-1 receptor 1 increases excitatory and inhibitory scaffolding protein expression and microglial activation in the adult mouse hippocampus. *Cell Mol Immunol.* 2015 Sep;12(5):645-7.

II. Ahl M*, **Avdic U***, Skoug C, Ali I, Chugh D, Johansson UE, Ekdahl CT. Immune response in the eye following epileptic seizures. *J Neuroinflammation*. 2016 Jun 27;13(1):155.

III. Ali I, **Avdic U**, Chugh D, Ekdahl CT. Decreased post-synaptic density-95 protein expression on dendrites of newborn neurons following CX3CR1 modulation in the epileptogenic adult rodent brain.*Cell Mol Immunol*. 2017 Oct 30. doi: 10.1038/cmi.2017.112.

IV. **Avdic U**, Ahl M, Chugh D, Ali I, Chary K, Sierra A, Ekdahl CT. Non-convulsive status epilepticus in rats leads to brain pathology. *Epilepsia*, 2018 May;59(5):945- 958.

V. **Avdic U**, Ahl M, Öberg M, Ekdahl CT. Immune profile in blood following non-convulsive epileptic seizures in rats, *submitted to Scientific Reports*

VI. **Avdic U**, Ahl M, Andersson M, Ekdahl CT. Levetiracetam and N-cadherin antibody treatment counteract brain pathology without reducing early epilepsy development after non-convulsive status epilepticus, *manuscript*

ABBREVIATIONS

AED	anti-epileptic drug
ANOVA	analysis of variance
BBB	blood brain barrier
BSA	bovine serum albumin
DTI	diffusion tensor imaging
EEG	electroencephalogram
E/I	excitatory/inhibitory
GABA	γ- aminobutyric acid
Gal-3	galectin-3
GCL	granular cell layer/ganglion cell layer
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
IL	interleukin
IFN	interferon
iML	inner molecular layer
INL	inner nuclear layer
IPL	inner plexiform layer
INTER	intermediate
KC/GRO	keratinocyte chemoattractant/growth related oncogene
KPBS	potassium phosphate-buffered saline
LPS	lipopolysaccharide
ML	molecular layer
NCSE	non -convulsive status epilepticus
N-cad	N-cadherin
NeuN	neuron-specific nuclei
NF	neurofascin
NFL	nerve fiber layer
NMDA	N-methyl-D-aspartate
NL	neuroligin
NPY	neuropeptide Y
oML	outer molecular layer
ONL	outer nuclear layer
OPL	outer plexiform layer
PFA	paraformaldehyde
PSD-95	post synaptic density – 95

RAM	ramified
R/A	round/amoeboid
SE	status epilepticus
SEM	standard error of mean
Syn-I/II	synapsin I/II
TGF-β	transforming growth factor - β
TLE	temporal lobe epilepsy
TNF	tumor necrosis factor

INTRODUCTION

Epilepsy

Epilepsy is a chronic neurological disorder affecting approximately 1% of the general population (Ngugi et al., 2010, Bell et al., 2014) and is characterized by recurrent spontaneous seizures. A general definition is presented in Fisher et al 2005 and states that *'epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure.'* It is often referred to as a family of disorders, highlighting the heterogeneous and complex nature of the disorder. Notably, the classification of epilepsy rests on understanding phenotypic patterns and the underlying mechanisms, and because of the continuously emerging knowledge from clinical and preclinical studies, continuous efforts are made to refine and re-evaluate classification of the epilepsies. An epileptic seizure in turn, is characterized by an abnormal and excessive activation and synchronization of cortical neurons in the brain (Margineanu, 2010). Again, a general conceptual definition states that a seizure is *'a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. The term transient is used as demarcated in time, with a clear start and finish'* (Trinka et al., 2015, Fisher et al., 2005). The clinical manifestations of seizures include a wide range of sensory phenomena and are usually classified as partial (focal) or generalized seizures. Focal seizures originate from a confined part of the brain and present with various symptoms depending on which particular brain region is affected, and often include symptoms such as altered consciousness, automatism and subtle motor activity, without any convulsive features. Generalized seizures on the other hand engage both hemispheres, including cortical and subcortical structures that propagate seizure activity (Chang et al., 2017). Again, symptoms can vary and patients may experience disturbed consciousness, loss of postural tone, bilateral tonic-clonic muscle movements including convulsive movements of all four limbs and facial muscles, and respiratory arrest. Epilepsy is associated to a lower quality of life, and besides the risk of injury in patients and even premature death, the physiological and socioeconomic aspects also present significant burdens.

Pathogenesis

A larger proportion of epilepsy cases are unknown, also referred to as cryptogenic. However, although the etiology of the disorder varies, epilepsy is often preceded by a

precipitating injury and may arise due to various insults to the brain and perturbations of the parenchymal integrity. TLE is often initiated by a traumatic event such as status epilepticus (SE), trauma or febrile seizures (Pitkanen and Sutula, 2002, de Lanerolle et al., 2003, Sharma et al., 2008). This latent process, also termed epileptogenesis, is characterized by pathological molecular and functional alterations that reorganize neural tissue and render it more susceptible to epileptic activity and spontaneous seizures. Epileptogenesis, which may take several years to develop, can be triggered by insults such as stroke, infection, traumatic brain injury or SE. Although the epileptogenic mechanisms still remain elusive, the insults are often accompanied by neurodegeneration, brain inflammation and excitatory/inhibitory (E/I) imbalance that may underlie the development of epileptiform activity and ultimately epilepsy (Ravizza et al., 2011). It has been widely accepted that the epileptogenic phase is not only limited to the period that precedes the onset of spontaneous seizures, but underlies its development and can thus propagate the pathological phenotype in parallel with the progression of the disease (Fig 1) (Pitkanen and Engel, 2014). Stratifying epileptogenesis and the mechanisms that render the brain susceptible to spontaneous seizures may identify the optimum time window for potential modulation and treatment regimes, which may either have significant disease-modifying effects or prevent the development/progression of the disorder altogether. The timing of any therapeutic intervention is thus critically important to the outcome in terms of pathology and the development of epilepsy.

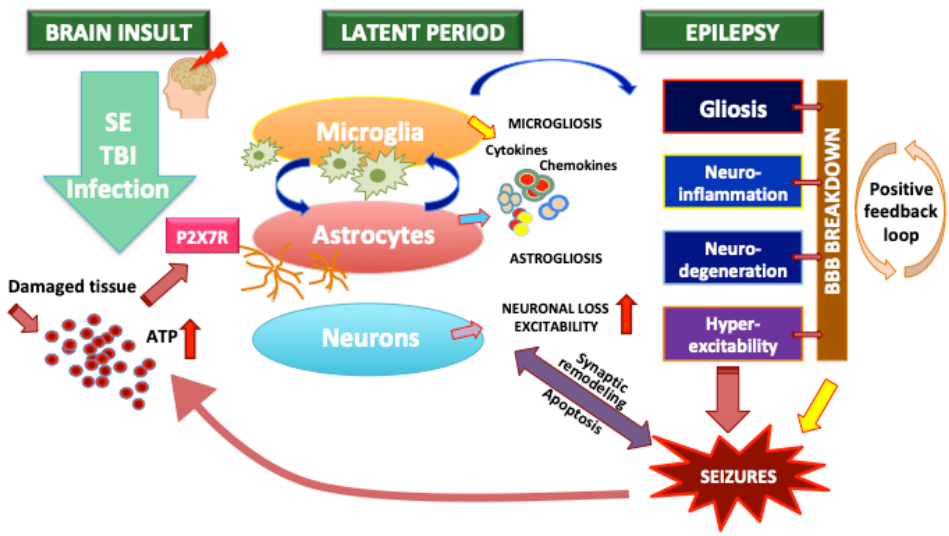

Fig 1. Schematic representation of the epileptogenic process following a precipitating event. Molecular and functional alterations are major features of epileptogenesis, where microglial and astrocytic activation, neuronal death, increased BBB permeability and network remodeling trigger hyperexcitability and epilepsy development. *Modified from (Beamer et al., 2017).*

One of the most common forms of epilepsy, temporal lobe epilepsy (TLE), originates from the temporal lobe and is characterized by complex partial seizures. Although the seizures are limited to a specific area, secondary generalizations are commonly observed. TLE often includes structural degeneration of the hippocampus, including reactive gliosis, atrophy and loss of neurons in particularly the sub-fields CA1, CA3 and dentate hilus (Margerison and Corsellis, 1966, O'Dell et al., 2012), and may even display other pathological features such as aberrant mossy fiber sprouting in both animals models and resected human tissue (Pitkanen and Sutula, 2002, Sharma et al., 2007, Crespel et al., 2002, Sutula et al., 1989, Zheng et al., 2011). The common clinical pattern includes lack of responsiveness, mouth or hand automatisms or alteration of consciousness (Blair, 2012).

The hippocampus is considered as a critical structure in epileptogenesis and TLE, an area believed to be the epileptogenic zone. The hippocampus is divided into distinct regions (Fig 2), which guides information in one direction, giving it a distinctive connectivity that is believed to underlie the increased vulnerability to seizure activity (Dudek and Sutula, 2007). The glutamatergic principle cells, together with a large number of interneurons, populate the hippocampus and govern overall activity and synchronicity in the structure. Both inhibitory and excitatory synapses are altered in epilepsy and can initiate self-sustaining activity. Several studies show that an abnormally enhanced glutamatergic activity is the key pathophysiological feature in epileptogenesis, which initiates neural hyperactivity (Barker-Haliski and White, 2015), whereas others demonstrate prominent loss of hilar interneurons that cause a dysregulation in network connectivity and increase propensity for hyperexcitability. Thus, the hippocampus is an important area to study further, in terms of seizure initiation, propagation and epileptogenesis, and stratifying the mechanisms that render the hippocampal formation prone to epileptic seizures may pave way for new therapeutic strategies.

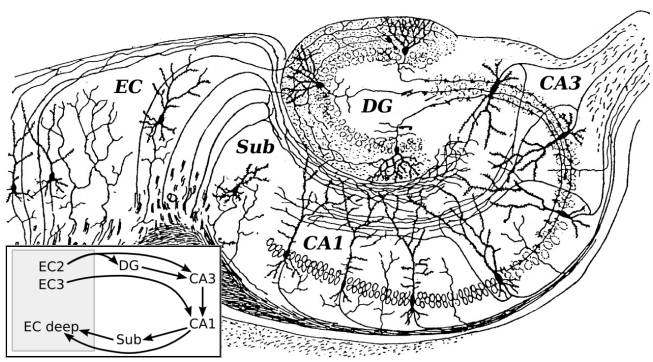

Fig 2. Schematic representation of the hippocampal circuits. Information via the perforant path from the entorhinal cortex enters the granule cells of the dentate gyrus and is relayed to CA3 via mossy fibers that send the signal to CA1 via Schaffer collaterals. The CA1 projections in turn send the information to the subiculum and back to the entorhinal cortex. *Adapted from the work of Santiago Ramón y Cajal (1911). © Public Domain, Wiki Commons: https://commons.wikimedia.org/wiki/Category:Histology_of_hippocampus#/media/File:CajalHippocampus_(modified).png*

Status epilepticus

In general, seizure duration varies with seizure type and most seizures are self-limited and last only a few minutes. If however, the intrinsic anticonvulsant mechanisms fail and the epileptic activity continues without regained consciousness for a period of 5 min or more, SE develops. SE is a medical emergency that may be life threatening if not interrupted. A proportion of patients with epilepsy will develop SE at least one time, although SE can develop without any underlying pathology or lesion (Nandhagopal, 2006). The most frequent manifestations of SE are convulsive tonic-clonic seizures with distinct motor features. However, 20-40% of all SE cases originate in the temporal lobes and lack overt convulsive events and instead display subtle and heterogeneous semiology termed non-convulsive SE (NCSE) (Holtkamp and Meierkord, 2011, Trinka et al., 2015, Walker, 2007). Symptoms typically include altered consciousness, automatism and minor motor activity such as lip-smacking and orofacial/arm/hand movements (Holtkamp and Meierkord, 2011, Williamson et al., 1985). Convulsive SE can be fatal and leads to substantial neuronal death in the brain. In addition, hypertension, disturbed electrolytic balance and cardiac failure are serious consequences if convulsive SE is not rapidly treated. In contrast, the pathology associated to NCSE is somewhat unclear and there is no general consensus regarding the magnitude of brain damage associated with it. In addition, the diffuse and subtle symptoms that NCSE displays, pose significant clinical challenges and treatment is often delayed. Further studies need to address the heterogeneity of SE in order to map out the mechanisms responsible for epilepsy development and the appropriate treatment strategies.

Pharmacological intervention and therapy

Pharmacological interventions i.e. anti-epileptic drugs (AEDs), used to terminate epilepsy and SE include strategies that alter and dampen the excessive neuronal activity in the brain. More specifically, modulating and potentiating GABAergic tone and increasing overall inhibitory signaling, reducing glutamatergic transmission by inhibiting NMDA receptors (Sagratella, 1995, Ghasemi and Schachter, 2011) and hence altering the balance and conductance of sodium, potassium and calcium (Shorvon and Ferlisi 2011) is the most common way to treat patients. Other drugs, including levatiracetam, used widely in the clinic as an anti-convulsive/epileptic agent, target synaptic proteins such as synaptic vesicle protein 2A (SV2A), inhibit presynaptic calcium channels, and hence reduce general synaptic transmission in the brain (Mazarati et al., 2004, Abou- Khalil, 2008, Surges et al., 2008). However, AEDs are primarily symptomatic, rather than being disease modifying and do not address the neuropsychiatric comorbidities often observed in these patients (Walker et al., 2002). Moreover, AEDs are often associated with severe side effects and many patients are not effectively treated with the available drugs. While a clearly stratified and identifiable focus in subjects provides possible candidates for surgical resection of the afflicted tissue (Engel et al.,

2003), complete recovery is seldom observed, and cognitive impairment and psychiatric comorbidities often follow surgical intervention (Markand et al., 2000), making the need for new and more effective treatment strategies highly warranted.

Clinical challenges

Clinically, epilepsy still remains a therapeutic challenge. Few patients have complete seizure control with monotherapy, and often more AEDs are utilized in order to improve seizure management. This however is associated with more adverse effects, which poses a problem in terms of everyday life for patients. Moreover, many epilepsy patients remain refractory to current AED treatments and new therapeutic targets and strategies are warranted. Although epilepsy is of a complex and heterogeneous nature, inflammation is recognized as a major component of the epileptic brain. However, despite the prominent role of the immune system, which has been shown to propagate hyperexcitability and pathology in epileptic conditions, there are few medications targeting the immune response. Thus, there is an urgent need for the development of therapy that targets immune mechanisms and a future challenge to characterize specific immune mediators involved in the epileptogenicity in chronic epileptic tissue. Furthermore, one of the major challenges in epilepsy is early prevention following a precipitating injury that may progress to the development of spontaneous seizures. Hence, early stratification of the disease process is crucial as this might identify and validate biomarkers that will help predict the development of an epilepsy condition and perhaps even measure the progression of the disease, both locally and peripherally. In addition, diagnostic biomarkers that provide prediction about the progression and severity of epilepsy and information about seizure-type and seizure burden, will ultimately help clinicians to stratify patients and allow for individually tailored, dose-adjusted treatments.

Experimental research to identify reliable prognostic and diagnostic biomarkers are primarily conducted on rodents and there are several animal models that are used in the search for measurable changes associated to the development of spontaneous seizures. These are briefly described below.

Animal models of epilepsy

Today, there are a number of animal models that are used in research to study epilepsy and SE. The models should recapitulate mechanisms underlying the disease and phenotypic symptoms associated to the human epileptic condition, as well as allow for observable and quantifiable measures of the pathology. The range of heterogeneity of the disease makes it practically difficult to fully mimic the pathophysiology and mechanisms in epilepsy in a single

model. Equally, it is important to distinguish between animal models of acute seizures from models that develop chronic spontaneous seizures, i.e. epilepsy. Several animal models are typically utilized to mimic and monitor the biochemical, neuroanatomical and behavioral aspects in epilepsy development as closely as possible.

Models of acquired epilepsy

There are several animal models of acquired epilepsy, where rodents display a precipitating injury afflicting the hippocampus and/or temporal lobe, a latent period between the injury and manifestation of spontaneous seizures and histopathological changes, all of which mimic the pathology characteristic of TLE. One of the ways to model epilepsy or epilepsy-like conditions is with electrical stimulation. Kindling, repeated application of short electrical stimulation to limbic brain regions such as the amygdala and hippocampus, progressively decreases focal seizure threshold and increases seizure severity (Goddard, 1967). Overtime, it leads to the development of spontaneous seizures and is widely used as a model of TLE because of its similarities to the clinical condition in terms of complex-partial and secondary generalized seizures (Sato et al., 1990). Other electrical models include post- SE epilepsy models induced by sustained electrical stimulation of intracerebral electrodes in the hippocampus or amygdala, and are characterized by recurrent spontaneous seizures after a latency period as well as displaying pathological changes that are often encountered in patients with TLE (Lothman et al., 1989, Loscher, 2002). SE can also be induced by chemical toxins such as pilocarpine and kainate (Loscher, 2002, Krsek et al., 2001, Ben-Ari, 1985, Riban et al., 2002, Leite et al., 2002, Loscher, 1984), administered either systemically or microinjected focally into the hippocampus or amygdala. As with the electrical models a latent period follows SE, after which the animals develop spontaneous recurrent seizures. Rodents with chemically-induced SE typically manifest more extensive damage and lesions in the hippocampus, with significantly compromised cortical regions and generally have a higher mortality compared to their electric counterparts (Kandratavicius et al., 2014). Other animal models of post-injury epilepsy include the traumatic brain injury (TBI) (Pitkanen et al., 2009), in which rodents develop epilepsy following a mechanical damage to the brain, that equates to TBI in humans and recapitulates the human epileptic condition which may develop after a traumatic brain injury. However, unlike the chemical and electrical models, this *de-novo* epilepsy model is highly time consuming, expensive and presents with a long latent phase and low yield of epileptic animals (Kandratavicius et al., 2014).

Models of idiopathic and genetic epilepsy

There are a number of animal models of epilepsy in which genetic mutations result in spontaneous seizures. The 'Genetic Absence Epilepsy Rat from Strasbourg' (GAERS) model

with spontaneous spike-wave discharges is widely used to study absence epilepsy (Marescaux and Vergnes, 1995, Loscher, 1984). In addition, knockout (KO) models are also used for the study of epilepsy and seizure activity, where genes encoding for ion channels and presynaptic vesicle proteins are altered/removed in order to mimic the human condition (Meisler et al., 2001, Rosahl et al., 1995). One example is the deletion of the synapsin II (synII) gene that produces handling-induced tonic-clonic seizures in mice (Garcia et al., 2004, Lakhan et al., 2010, Etholm et al., 2012, Corradi et al., 2008). Studies on idiopathic epilepsies in rodents, without any externally applied toxins such as kainate and pilocarpine, or perturbations to the brain, make them good candidates for the study of epileptogenesis and the development of spontaneous seizures, as any alterations would only be from seizure-related brain pathology and not associated with the initial insult/toxin.

Neuroinflammation

The CNS immune system is primarily comprised of innate immune cells and is considered an 'immune privileged' site, a concept that stems from the notion that it lacks a strong immune response when challenged, and a lymphatic drainage. Due to the vascular blood brain barrier (BBB) that regulates infiltration of blood cells into the brain parenchyma, there is a restricted exchange of contents between the vascular system and the brain. When the BBB is compromised in pathological conditions, the permeability increases and peripheral innate and adaptive immune cells, including monocytes, neutrophils, T cells and B cells, infiltrate the tissue. The brain-resident immune cells of the CNS, i.e. microglia and astrocytes are highly diverse cells that serve as the first line of defense and respond to a variety of pathological conditions. They are also crucial for a number of different physiological processes and neuronal functions such as guiding migration during development, modulating synaptic function and plasticity, and regulating the extracellular microenvironment by buffering neurotransmitters and ion concentrations. They contribute to the permeability of the BBB and help maintain homeostasis in the CNS. The fundamental basis of maintaining homeostasis is the transformation of glial cells from a resting state to an activated phenotype in response to an injury, where pro-and anti-inflammatory mediators control the outcome of the inflammatory response. The glial response needs to be tightly controlled following a perturbation, where immune mediators released by glial cells should resolve the inflammatory tissue response and limit the injury. However, if this mechanism fails, uncontrolled activation of glial cells can be detrimental to normal neuronal function (Vezzani et al., 2011) and may cause imbalance in glia-mediated regulation of ions and neurotransmitters, leading to hyperexcitability, synchronization and ultimately seizures. Indeed, increasing evidence implicates inflammatory mechanisms in seizures and epileptogenesis (Vezzani et al., 2011, van Vliet et al., 2018). Clinical data shows that steroids and other anti-inflammatory treatments

have an anti-convulsive effect in drug resistant epilepsies (Riikonen, 2004, Wirrell et al., 2005, Wheless et al., 2007). Several studies suggest that inflammatory mediators and their receptors can mediate neuronal cell loss and become detrimental to the already epileptic tissue (Vezzani et al., 2013, Devinsky et al., 2013). The pro-inflammatory cytokine IL-1β can directly act on neurons and affect their excitability threshold at a cellular and network level (Vezzani et al., 1999, Vezzani et al., 2000). In addition, glial buffering of neurotransmitters is particularly important following increased excitatory synaptic activity, where glutamate uptake prevents cross talk between neighboring cells and the activation of peri-synaptic glutamate receptors (Devinsky et al., 2013), preventing further spread of the activity. Since neuroinflammation in epilepsy is not a simple epiphenomenon, but indeed contributes reciprocally to the neuropathology, hyperexcitability and progression of the disease, the mechanisms underlying its activation and propagation need to be addressed. Novel antiepileptic strategies should be considered with the intent of modulating the glial and inflammatory responses and identifying immunosuppressant and disease-modifying strategies that may delay or arrest the epileptic process.

Microglia

Microglia are the innate immune cells of the CNS, and are homologous to macrophages found in the periphery. They are of mesodermal/mesenchymal origin and migrate as monocytic, amoeboid cells during early fetal development from peripheral progenitors originating in the bone marrow (Alliot et al., 1999). Studies suggest that a second population of microglial cells invade the CNS from blood-borne monocytes that enter the brain parenchyma soon after birth (Ling et al., 1980). After invading the brain they change morphological phenotype into a ramified morphology, with a small cell soma and highly branched cellular processes. In adults, little exchange between blood and brain parenchyma takes place with respect to microglial migration and once present in the brain, the pool of microglia is capable of self-renewal and does not depend on infiltrating circulating monocytes (Ajami et al., 2007). In the healthy CNS, these cells are in their resting, ramified state and actively scan their environment. The motile processes are in constant motion, monitoring the extracellular space, interpreting environmental cues and surveying any changes in structural and functional integrity (Nimmerjahn et al., 2005). Upon activation in response to disturbances in brain homeostasis, they quickly respond by changing their morphological phenotype into an activated intermediate or amoeboid shape. When activated, microglia release a plethora of cytokines and chemokines that orchestrate the immune response and recruit the innate immune cells to the site of injury. In addition, receptors on microglia involved in pathogen recognition are upregulated, followed by removal of cells/debris by phagocytosis. The role of microglial phagocytosis is not only critical in a pathological milieu, but it also serves as an important mediator of synapse architecture and reshaping dendritic

trees and networks during development (Stevens et al., 2007). Microglia have been shown to make physical contacts with neurons and synaptic structures and monitor the functional state of synapses, suggesting that, while microglia themselves are not excitable, they play a crucial role in modulating the structure and function of excitable neurons, hence changing neuronal brain circuits and synaptic transmission (Wake et al., 2009, Tremblay et al., 2010).

Long and abnormal activation of microglia has been implicated in the pathogenesis and progression of several diseases (Colonna and Butovsky, 2017). In epilepsy, microglial activation has been found in animal models of TLE (Bonde et al., 2006, Ekdahl et al., 2003a, Ali et al., 2015) and in surgically resected tissue of epilepsy patients (Liu et al., 2014b, Sosunov et al., 2012). The release of pro-inflammatory mediators is postulated to underlie the pathophysiology of epilepsy and the driving force of epileptogenesis (Abraham et al., 2012, Aronica et al., 2017), and microglia-derived factors have been shown to affect synaptic transmission. Pro-inflammatory cytokines released from microglial cells can modulate neuronal activity and viability by either promoting release of neuro-modulatory molecules from glia or directly activating neuronal receptors in the brain (Vezzani et al., 2011, York et al., 2018, Bechade et al., 2013). One such cytokine is IL-1β, which is rapidly up-regulated in response to an injury and is generally considered to increase neuronal excitability by reducing GABAergic inhibition (Wang et al., 2000, Viviani et al., 2003). Studies show that intrahippocampal IL-1β infusion prolongs kainate-induced seizures in rats (Vezzani et al., 1999) and blocking the IL-1R/IL-1β pathway during epileptogenesis reduces neuronal damage in animal models of TLE (Noe et al., 2013), suggesting that the IL-1R/IL-1β pathway enhances glutamatergic neurotransmission and facilitates neurodegeneration.

Furthermore, receptors present on the cell membrane of microglial cells detect pathological alterations and are involved in motility, phagocytosis and activation of microglia. The chemokine receptor, CX3CR1 and its ligand fractalkine, have been shown to be involved in intercellular communication between neurons and microglia and regulate activation, migration and phagocytic activity of microglial cells (Harrison et al., 1998, Fuhrmann et al., 2010, Noda et al., 2011). Recent studies suggest a role of the fractalkine-CX3CR1 pathway in the pathogenesis of epilepsy and the associated cell death (Yeo et al., 2011, Xu et al., 2012). Increased expression of both fractalkine and CX3CR1 has been reported in hippocampal sections from epileptic patients as well as in animal models of TLE (Yeo et al., 2011, Xu et al., 2012). In addition, the fractalkine/CX3CR1 pathway has been shown to alter GABAA currents in human TLE (Roseti et al., 2013), suggesting a possible role for the pathway in epilepsy and seizure propagation. Because of the highly dynamic and heterogeneous properties of microglia, future studies need to further address the functional diversity of the cells (Zhang et al., 2005, Hanisch, 2013) as well as activation in pathological conditions and characterize the underlying mechanisms and inflammatory responses associated to the diseases. Thus, the functional subpopulations of microglia that coexist in the CNS have to be stratified in order to

modulate microglial activity and function in neurological diseases and thus identify new therapeutic strategies.

Astrocytes

Astrocytes are the most abundant cells in the brain that regulate several aspects of normal brain function, including supporting neural tissue, BBB formation and structure, and maintaining general homeostasis (Markiewicz and Lukomska, 2006). One of the most important functions of astrocytes includes the regulation of synaptic integrity and function. Astrocytic processes envelop essentially all synapses (Brown and Ransom, 2007) and maintain fluid, ion and transmitter homeostasis of the synaptic interstitial fluid in order to provide the correct synaptic activity. They also make extensive contact with the vasculature and have multiple bidirectional interactions with blood vessels, and affect the blood flow by regulating blood vessel diameter (Gordon et al., 2007). Astrocytic processes are connected through gap junctions that facilitate direct cytoplasmic communication between neighboring cells. The astrocytic end-feet, covering endothelial cells of the blood capillaries, help astrocytes to control the BBB and regulate the flow of blood in the CNS and in response to fluctuations in homeostasis such as alterations in neuronal activity, astrocytes rapidly adapt to change local blood flow (Koehler et al., 2009). In addition, astrocytes are critical for rapid glutamate clearance, maintaining glutamate at physiological levels at the synapse (Rothstein et al., 1994, Huang et al., 2004), and for buffering K^+ ions at sites of intense neuronal activity (Kielian, 2008, Anderson and Swanson, 2000).

In response to an injury, astrocytes undergo a series of morphological changes known as reactive astrocytosis (Pekny et al., 2014, Pekny and Pekna, 2014). The most prominent hallmarks of astrocytosis is hypertrophy of cellular processes and upregulation of intermediate filaments, in particular the glial fibrillary acidic protein (GFAP), which is the main component of the intermediate filament system in astrocytes (Pekny and Pekna, 2004). Together with microglia, they release various cytokines and pro-inflammatory mediators that orchestrate an immune response in the brain parenchyma. Astrocytes have been implicated in the pathophysiology of epilepsy and evidence has shown that they can modulate synaptic plasticity and excitability in both excitatory and inhibitory synaptic compartments (Bowser and Khakh, 2004, Bonansco et al., 2011, Henneberger et al., 2010). A growing number of studies suggest that disturbances of neuron-astrocyte cross-talks contribute to the pathology in epilepsy (Tian et al., 2005, Fellin et al., 2006, Angulo et al., 2004). Notably, hypertrophic astrocytes are a prominent feature of several experimental epileptic models and of human TLE (Krishnan et al., 1994, do Nascimento et al., 2012), and seizures are shown to frequently initiate within or in proximity to gliotic brain tissue (McKhann et al., 2000). Astrocyte abnormalities have been linked to pro-convulsant activity and increased astroglial Ca^{2+} signaling was found to potentiate excitatory synaptic strength (Alvarez-Ferradas et al., 2015). The pro-inflammatory

cytokines IL-1β and TNFα are also reported to act on astrocytes via autocrine pathways and can reduce glutamate uptake, while simultaneously increasing glutamate release in cells, suggesting that cytokines mediate the pro-convulsant effects described in astrocytes (Ye and Sontheimer, 1996). Astrocytes seem to be key players in regulating brain tissue homeostasis and neuronal excitability, and clarifying the specific role they have in epilepsy and epileptogenesis could unveil novel anti-epileptic targets and drugs.

Peripheral immune cells

Under normal conditions, the BBB strictly regulates the entry of peripheral components into the brain parenchyma. Upon injury, several immune factors such as adhesion molecules i.e. E-selectin, intracellular adhesion molecule-1 (ICAM) and vascular cell adhesion moleucle-1 (VACM), are upregulated on endothelial cells of the vasculature, facilitating the extravasation of leukocytes and lymphocytes into the site of injury in the brain (Fabene et al., 2008, Librizzi et al., 2007). Immune and BBB factors are emerging as new candidates and targets in the pathogenesis of epilepsy and they seem to play a crucial role in the initiation and maintenance of epileptic activity. The innate immune reaction in the brain that follows seizures and epilepsy leads to changes in BBB permeability and recruitment of systemic immune cells (Fig 3). BBB disruption can be triggered by direct insults to the endothelium or by systemic factors that activate circulating leukocytes, which release mediators that increase vascular permeability (Librizzi et al., 2012). Infiltration of $CD4^+$ and $CD8^+$ T cells into the brain parenchyma following a single seizure has been reported (Bauer et al., 2008, Silverberg et al., 2010), as well as a chronic monocyte and lymphocyte infiltration in both clinical and experimental studies of epilepsy (Varvel et al., 2016). Moreover, infiltration of $CD45^+$ and $CD3^+$ lymphocytes has been demonstrated in sclerotic tissue from patients with therapy-resistant TLE and in experimental kainic acid- SE models (Zattoni et al., 2011). Altered levels of circulating cytotoxic $CD8^+$ T lymphocytes have also been observed in serum in experimental models of SE (Marchi et al., 2007, Marchi et al., 2009) and clinical studies describe acute increased levels of pro-inflammatory cytokines, neutrophils and cytotoxic T lymphocytes in serum and plasma from patients after temporal lobe seizures (Bauer et al., 2008, Gao et al., 2017, Alapirtti et al., 2018).

The exact role of the adaptive immune response in epilepsy is however unclear. Bauer et al (2008) showed that cytotoxic $CD4^+$ T cells were decreased while *total* lymphocytes and natural killer cells were increased in patients with temporal lobe epilepsy. In a recent study, the frequency of circulating $CD4^+$ and $CD8^+$ T lymphocytes was not reported to be different in patients and healthy individuals, but instead patients displayed a different cytokine expression profile (Rosa et al., 2016). Interestingly, another study reported that mice lacking T- and B-

lymphocytes displayed more neurodegeneration following kainate-induced epilepsy and an earlier onset of spontaneous seizures (Zattoni et al., 2011), suggesting that infiltrating lymphocytes have a neuroprotective effect. Conversely, recent data demonstrated significant infiltration of the brain parenchyma by activated $CD4^+$ and $CD8^+$ T lymphocytes along with an increased number of pro-inflammatory IL-17 -producing T lymphocytes in the epileptogenic zone that positively correlated with seizure severity, in drug-resistant pediatric epilepsy. Moreover, they showed that the cytokine IL-17 caused increased neuronal hyperexcitability in hippocampal pyramidal neurons, suggesting a seizure-promoting effect of infiltrating lymphocytes (Xu et al., 2018). In addition, $CD3^+$ T cells, natural killer cells and B cells were also observed in the resected epileptogenic center. Whatever the function of infiltrating immune cells is, they actively participate in the pathophysiology of seizures and epilepsy which warrants further studies on their immunoregulatory responses in neuronal hyperexcitability, viability and epileptogenesis.

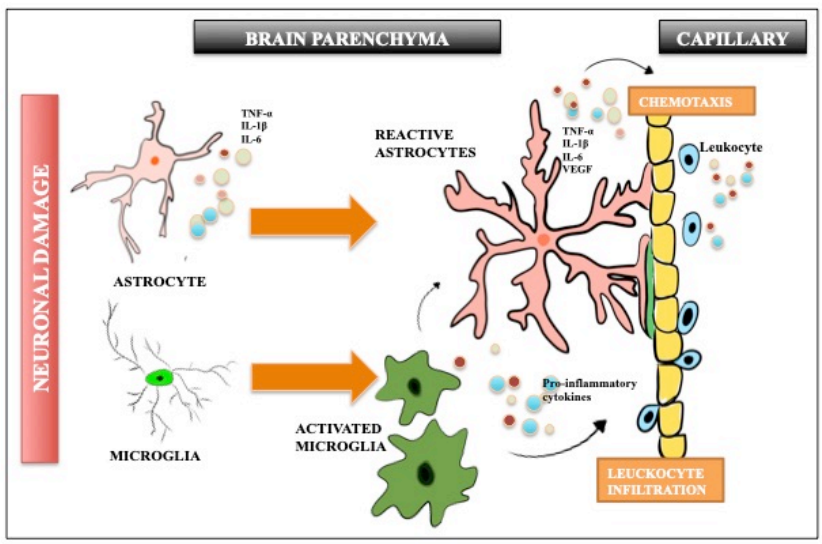

Fig 3. Schematic representation of the blood brain barrier and glial cells. Astrocytes enwrap the endothelial cells of the blood vessel and provide support and stability, while ramified microglia scan the environment. When activated by neuronal damage, pro-inflammatory cytokines are released from glial cells, as well as factors that help permeate the blood brain barrier that induce chemotaxis and recruit peripheral leukocytes, which subsequently further promotes the inflammatory response in the brain.

Excitatory/inhibitory balance

The electrical activity in an epileptic network is associated with an excitatory/inhibitory (E/I) imbalance that leads to excessive synchronization in neuron populations. The E/I

balance, also postulated as a mechanism underlying the pathophysiology that follows SE, involves a disruption of the delicate balance between excitatory and inhibitory neuronal pathways, which are normally regulated by a number of proteins within the neuronal synapses, including scaffolding proteins and adhesion molecules such as neuroligins and cadherins. They are known to regulate synaptic establishment, spine shape, synaptic transmission and strength and thus regulate overall network excitability (Dalva et al., 2007, Arikkath and Reichardt, 2008, Sudhof, 2008). Furthermore, neurons must not only form, but also maintain stable connections with specific synaptic markers in order to ensure proper synaptic transmission. Scaffolding proteins are crucial for functional organization of synapses. They enable accumulation of neurotransmitter receptors at the post-synaptic membrane and provide physical constraints by interacting with the cytoskeleton for maintaining receptors at the synapse. Previous studies have indicated altered expression in synaptic proteins in both patients and animal models of epilepsy and synaptic dysregulation is closely related to both neuronal death and glial activation (Ben-Ari, 2001, Farber and Kettenmann, 2005). Mapping out the mechanisms involved in E/I imbalance and the changes that may be underlying epileptogenesis might aid in elucidating the underlying mechanisms essential to hypersynchronizing the network and thus determining if the changes are predictive of epilepsy. A schematic illustration of the molecular organization of pre- and postsynaptic proteins on excitatory and inhibitory synaptic terminals is illustrated below in Fig 4.

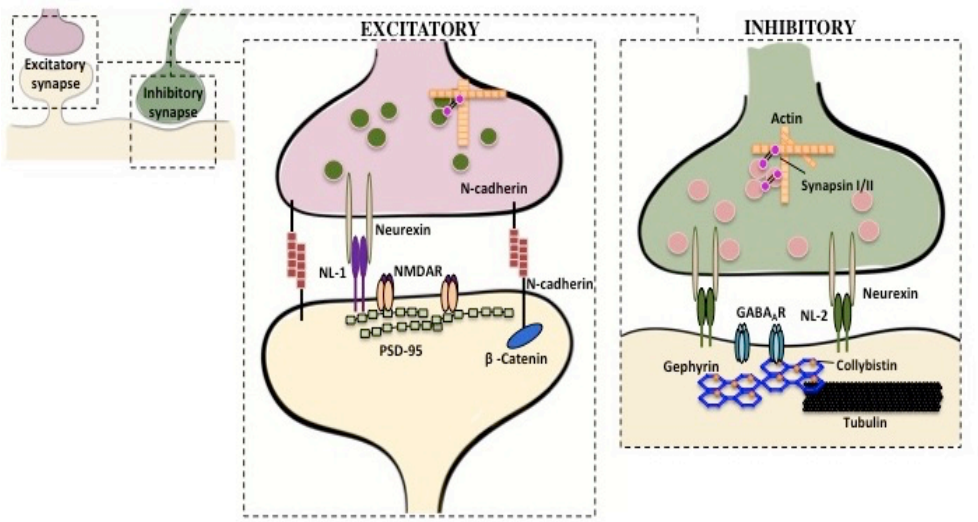

Fig 4. Schematic representation of the organization of excitatory and inhibitory synaptic compartments.

Neuronal transmission relies on excitatory and inhibitory signals passing from distinct synaptic areas. For this contact to happen, pre- and postsynaptic compartments must be in close apposition. Cell adhesion molecules expressed on the neuronal surface play crucial roles in maintaining the trans-synaptic contact and structural integrity of synapses, regulate neuronal migration and synaptic plasticity, and are in general critical for fine- tuning the synaptic response (Arikkath and Reichardt, 2008, Washbourne et al., 2004, Shen et al., 2004). Contact and synapse formation in synaptogenesis also requires stabilization of both pre- and post-synaptic elements, where adhesion molecules aid in anchoring (Scheiffele, 2003, Biederer et al., 2002, Sytnyk et al., 2002, Fu et al., 2003). Scaffolding proteins ensure proper subcellular location of the receptor machinery and serve as an anchor that maintains long-term stability of synapses despite the continuous turnover of individual components. In order to understand the regulatory mechanisms of this trans-synaptic balance in the neuronal network, which may result in hyperexcitability if disrupted, an effort has to be made to elucidate the mechanisms involved in synaptic protein mediated reorganizations.

Adhesion molecules

N-cadherin

Cadherins are transmembrane cell adhesion molecules important for the formation of cell-cell adhesion and recognition, mediated by calcium ions. They are composed of an extracellular part, which mediates the interaction between cadherin molecules, a transmembrane and cytoplasmic part. Neuronal (N)- cadherin was first identified in 1982 (Grunwald et al., 1982) and is widely expressed on both pre- and post-synaptic terminals in the CNS (Takeichi, 1995, Fannon and Colman, 1996). At immature synapses in vivo, N-cadherin is evenly distributed along the synapse, whereas at mature hippocampal synapses, cadherin is localized to regions that border the mature active zone and the postsynaptic density (PSD) of glutamatergic sites (Uchida et al., 1996, Fannon and Colman, 1996). At later stages of development, N-cadherin is progressively lost from inhibitory synapses and retained and concentrated at excitatory glutamatergic compartments (Benson and Tanaka, 1998). The cytoplasmic domain of N-cadherin interacts with the intracellular cytoskeleton by binding to α- and β-catenin molecules, which are proposed to regulate cadherin function and motility (Derycke and Bracke, 2004). The function of N-cadherin/β-catenin complex involves a number of events that control axonal growth, guidance to synapse formation and synaptic plasticity during development and synaptogenesis (Doherty and Walsh, 1996). In addition to its adhesive role during early development, it plays an important part in spine morphology, synaptic remodeling and synaptic assembly (Togashi et al., 2002, Bamji et al., 2003, Zhang and Benson, 2001, Bozdagi et al., 2004).

The functional properties of N-cadherin/β-catenin complex in the adult brain are still not well understood. N-cadherin KO mice die early during embryonic development due to heart failure formation (Radice et al., 1997), but once synapses are established blocking N-cadherin assembly has very little effect on the structure and function of synapses. Although *in vitro* studies show altered short-term plasticity at glutamatergic synapses in mice with conditionally ablated N-cadherin (Jungling et al., 2006, Bozdagi et al., 2004), the number and shape of dendrites and spines, as well as the number of synapses remain the same (Nikitczuk et al., 2014). Studies show that synaptic stimulation can modify N-cadherin conformation and demonstrate that synaptic adhesion is a dynamic and complex process that can be modulated by synaptic activity (Tanaka et al., 2000), suggesting a malleable and dynamic structure, which may be important for maintenance of neuronal circuits and normal synaptic function. Nikitczuk et al further reported that the selective loss of N-cadherin at excitatory synapses increased the density of inhibitory synaptic markers, decreased levels of the AMPAR subunit GluA1 and PSD-95, and decreased severity of hippocampal seizures (Nikitczuk et al., 2014), suggesting that the N-cadherin/β-catenin complex is important for normal synaptic transmission and cognitive function.

Neuroligins

The most common trans-synaptic signaling involved in synaptogenesis involves interaction between the presynaptic neurexins and postsynaptic neuroligins. Three genes have been described for neurexin, each of which encodes α- and β-neurexin, that can be found throughout the CNS. Neuroligins are predominantly expressed in the brain, and mammals express four neuroligin genes (NLG 1-4) that bind to α- and β-neurexins depending on the splice variant (Chih et al., 2006, Boucard et al., 2005). Evidence suggests that the neuroligin-neurexin complex mediates both excitatory and inhibitory synapse formation *in vitro* and that they take part in determining the specificity and differentiation of synaptic contacts. Both neurexin and neuroligin contain an intracellular PDZ binding domain that interacts with scaffolding proteins and promotes bidirectional synapse assembly and allows for postsynaptic NMDA receptor recruitment. Changes in expression in neuroligin result in alterations in synapse number and dendritic spine density, respectively (Prange et al., 2004, Levinson et al., 2005, Chih et al., 2005, Graf et al., 2004). Recent work suggests that adhesion between neurexins and neuroligins may be a generic initiator of synapse formation (Scheiffele et al., 2000).

The control of inhibitory or excitatory synapse formation depends on the type of neuroligin and neurexin subtypes involved. Neuroligin (NL) 1 is predominantly localized to glutamatergic excitatory synapses (Song et al., 1999) and promotes the formation of excitatory synaptic connections (Graf et al., 2004, Chih et al., 2005). Conversely, NL-2 is primarily found on inhibitory synapses (Varoqueaux et al., 2004) and induces formation of inhibitory

synaptic connections (Graf et al., 2004, Chih et al., 2005). NL-2 has also been shown to regulate GABAergic functions and thus balance excitation and inhibition in the brain (Sun et al., 2013). Jedlicka et al demonstrated impaired $GABA_AR$-mediated miniature inhibitory postsynaptic currents and an overall increased excitability in the dentate gyrus in NL-2 deficient mice (Jedlicka et al., 2011). NL-3 is localized at both excitatory and inhibitory synapses (Budreck and Scheiffele, 2007) and NL-4 is primarily found at glycinergic inhibitory postsynapses in the retina (Hoon et al., 2011). Knockdown of all three neuroligin genes *in vitro*, decreases frequency of inhibitory, but not excitatory, miniature synaptic currents, suggesting that neuroligin signaling is important in inhibitory synaptogenesis *in vitro*. Moreover, neuroligins bind PSD-95 through their intracellular PDZ binding domain, an important component of the excitatory synaptic machinery that recruits transmitter receptors, and clusters other postsynaptic proteins (Rao et al., 1998, Friedman et al., 2000, Gerrow et al., 2006). The interaction with PSD-95 is also important for the distribution of neuroligins to excitatory or inhibitory synapses, making this an important mechanism for setting the excitatory/inhibitory balance in neurons (Levinson et al., 2005, Prange et al., 2004). In addition, NL- 1 and PSD-95 act as a presynaptic recruitment machinery, suggesting that this interaction determines where the synapse forms (Gerrow et al., 2006). Consequently, the specific extracellular and intracellular domains determine the particular synaptogenic function *in vitro*. Interestingly, despite normal synapse number in mice lacking NL 1-3, the respiratory centers are impaired and mice die shortly after birth due to respiratory failure. This suggests that removal of neuroligins results in synapse maturation deficits, causing imbalance in excitatory and inhibitory cues in the respiratory centers (Varoqueaux et al., 2006). Similarly, genetic mutations in neuroligins have also been associated with autism disorder and epilepsy, where an imbalance in the E/I balance has been implicated (Chih et al., 2004, Laumonnier et al., 2004, Jamain et al., 2003). These findings implicate the neuroligin/neurexin complex in synapse development and basic cognitive function.

Scaffolding proteins

PSD-95

The postsynaptic density (PSD), a glutamatergic machinery at excitatory synapses that underlies and maintains synaptic transmission, is localized beneath the post-synaptic membrane (Kennedy, 2000, Sheng, 2001), where release of glutamate from synaptic terminals elicits fast excitatory postsynaptic potentials by activating NMDA and AMPA receptors. In the highly dynamic PSD, multiple proteins serve as scaffolds, clustering and organizing neurotransmitter receptors, adhesion molecules and signaling molecules to the postsynaptic membrane. One of the major components at the postsynaptic density is PSD-95, a scaffolding protein that belongs to the membrane-associated guanylate kinase (MAGUK) family, and

contains three PDZ domains that bind proteins and help stabilize the PSD complex. *In vitro* studies show that overexpression of PSD-95 increases postsynaptic clustering and activity of glutamate receptor, dendritic spine formation and the maturation of pre-synaptic terminals in cultured hippocampal neurons (El-Husseini et al., 2000). Similarly, studies conducted in cortical pyramidal neurons demonstrate increased amplitude of AMPAR-mediated evoked excitatory post-synaptic currents (Beique and Andrade, 2003), most likely due to enabling accumulation of AMPAR at synapses, stabilizing the receptor complex and the synaptic transmission (Ehrlich et al., 2007). Ehrlich et al further show that PSD-95 is involved in remodeling and modifying strength of synaptic connections by altering the spine density and morphology, increasing spine turnover (Ehrlich et al., 2007). Recent studies suggest that PSD-95 is crucial for anchoring AMPA and NMDA receptor complexes to the postsynaptic density (Chen et al., 2015, Nair et al., 2013), as well as for dictating the ratio of excitatory-to-inhibitory synaptic contacts by binding to NL-1 (Prange et al., 2004). Interestingly, overexpressing PSD-95 induces a shift in the redistribution of NL-2 from inhibitory to excitatory synapses, altering the E/I balance (Levinson et al., 2005). Interestingly, changes in PSD-95 expression have been observed in several pathological conditions such as schizophrenia, where a substantial reduction in PSD-95 was observed in the CA1 region of the hippocampus (Matosin et al., 2016). In pentyleneterazol (PTZ)- induced seizures in mice, PSD-95 expression was increased (Rakhade et al., 2012), and an increased co-assembly of PSD-95 with NMDA receptor subunits was reported in patients with focal cortical dysplasia that also present epileptic seizures (Ying et al., 2004), suggesting that PSD-95 is critical for molecular organization and synaptic stability and consequently cognitive functions.

Gephyrin

Fast synaptic inhibition is mainly mediated by the neurotransmitters GABA and glycine. $GABA_AR$ are ion channels assembled from at least 18 homologous subunits and composition of the receptor governs physiological, pharmacological and targeting properties. Both $GABA_AR$ and glycine receptors are anchored postsynaptically by gephyrin, a 93-kDa scaffolding protein located beneath the plasma membrane, that interacts with the cytoskeleton and provides structural and functional regulation of the inhibitory postsynaptic compartment (Luscher and Keller, 2004, Kneussel and Loebrich, 2007, Kirsch, 2006). Alternative splicing can generate multiple isoforms of gephyrin, which is highly conserved across species and is comprised of three structural domains (G,C and E). Gephyrin is crucial for glycinergic receptor clustering (Kirsch, 2006), whereas the role of gephyrin in $GABA_AR$ still remains elusive. Interestingly, studies involving deletion of gephyrin or $GABA_AR$ subunit genes, show that gephyrin-independent $GABA_AR$ clusters can occur and that the clustering of $GABA_AR$ with gephyrin depends on neuronal and synapse type, suggesting that gephyrin is not essential to all $GABA_AR$ clustering. Blocking gephyrin by gene targeting or siRNA affects GABA

receptor clustering and inhibitory postsynaptic currents (Essrich et al., 1998, Yu et al., 2007, Fritschy et al., 2008). However, post-synaptic clustering of gephyrin at inhibitory synapses seems to depend on $GABA_A$Rs, which was demonstrated in $GABA_A$R knockout animals where clustering of gephyrin was prevented (Gunther et al., 1995, Kralic et al., 2006, Studer et al., 2006). They observed intracellular aggregates of gephyrin instead of postsynaptic clusters, in neurons lacking certain $GABA_A$ receptor subunits *in vivo* (Kralic et al., 2006, Studer et al., 2006). Post-synaptic clustering of gephyrin is dependent on collybistin, a protein capable of translocating gephyrin from cytoplasmic aggregates to submembrane compartments (Kins et al., 2000), and NL-2 (Harvey et al., 2004, Papadopoulos et al., 2007). Targeted deletion of the gene encoding collybistin impairs gephyrin and $GABA_A$R clustering and GABAergic transmission (Papadopoulos et al., 2007). Studies have also shown that the adhesion molecule NL-2 drives the formation of gephyrin and $GABA_A$R clusters (Poulopoulos et al., 2009) and rodents lacking NL-2 display selective loss of gephyrin and $GABA_A$R clusters at synapses of principal cells in the hippocampus (Poulopoulos et al., 2009, Jedlicka et al., 2011). Faulty post-synaptic clustering of $GABA_A$R and glycine receptors may result in receptors with altered properties that contribute to changes in transmission and ultimately in hyperexcitability and seizures. Indeed, disrupted clustering of gephyrin has been reported in both patients with epilepsy and in experimental models (Gonzalez et al., 2013, Fang et al., 2011) and a reduced expression of gephyrin has been reported in the latent period in a mouse model of temporal lobe epilepsy (Gonzalez, 2013, Knuesel et al., 2001). Studies have also shown that both gephyrin and collybistin increase the risk of epileptic seizures and psychiatric symptoms (Lionel et al., 2013, Shimojima et al., 2011). In addition aberrant alternative splicing of gephyrin has been reported in patients with TLE, affecting the post-synaptic clustering of $GABA_A$R (Forstera et al., 2010). More specifically, they show abnormally spliced gephyrin, lacking axons in their G terminal, which possibly disturbs the activity of gephyrin. These findings suggest that gephyrin plays a crucial part in post-synaptic assembly, stability and specialization of inhibitory synapses, and suggests modulation of gephyrin as a possible therapeutic target.

Synapsins

The synapsins are a group of highly conserved phosphoproteins that are widespread in both the central and peripheral nervous system (Greengard et al., 1993). Three mammalian synapsin genes have been identified; *SynI, SynII* and *SynIII,* with alternative spicing that generates many synapsin isoforms (Kao et al., 1999). They are localized to synaptic vesicles within nerve terminals and modulate neurotransmitter release in the pre-synaptic terminals by tethering synaptic vesicles to the actin cytoskeleton (Cesca et al., 2010, Hilfiker et al., 1999). Synapsins are generally not expressed in non-neuronal cells and they are absent from cells involved in sensory transduction such as photoreceptors and bipolar cells in the retina (Haas et

al., 1990, Mandell et al., 1990). SynI and II expression rapidly increases during the differentiation of neurons and is particularly high during synaptogenesis (Lohmann et al., 1978). They have been linked to synapse formation and synapse rearrangement whereas synIII shows a distinct expression pattern in the brain, peaking during embryonic development and declines during adulthood. It is highly expressed in cell bodies and processes of immature neurons in neurogenic regions such as the subgranular zone of the hippocampus and subventricular zone in the walls of the lateral ventricles (Pieribone et al., 2002). In *synIII* $^{-/-}$ a reduction in the proliferation and survival of neuronal progenitors in the dentate gyrus was observed (Kao et al., 2008) and loss of synI and II respectively, causes no overt defects in the development of the nervous system but impairs synaptic transmission and overall excitability of the neuronal network. *SynII* $^{-/-}$ mice develop epileptic seizures due to a general impairment of the inhibitory network, shifting the balance towards excitability (Gitler et al., 2004, Chiappalone et al., 2009).

OBJECTIVES

The primary objective of this thesis is to understand the pathological changes occurring in response to NCSE and during epilepsy development, including local and peripheral immune responses and the link to the expression of synaptic proteins important for the E/I balance in epilepsy.

The specific aims of this thesis are:

I. To study the role of IL-1R1/IL-1β pathway in terms of microglial activation and synaptic protein expression at physiological conditions (Paper I)

II. To describe and characterize local and peripheral immune responses in a model of non-convulsive status epilepticus (Paper II, IV, V)

III. To study the effect of immune modulation on seizure-induced brain and retinal pathology (Paper II, III)

IV. To study changes over time in synaptic protein expression, associated with excitatory and inhibitory activity, in a model of non-convulsive status epilepticus (Paper IV)

V. To assess the effects of levetiracetam treatment in combination with intracerebral infusion of N-cadherin antibody on epileptogenesis and the associated pathology following NCSE (Paper VI)

METHODS

Animals

Adult male Sprague-Dawley rats (200- 250 g) and wild-type male C57/BL mice (20-30g), procured from Charles River (Germany) were used in Paper I-VI. IL-1R1 KO mice (4-5 months old) were kindly provided by Dr Emmanuel Pinteaux, University of Manchester, UK (Paper I). All animals were housed individually after surgical intervention and housed with a 12-hour light/dark cycle and *ad libitum* food and water. Experimental procedures followed guidelines set by the Malmo-Lund Ethical Committee in Sweden for use and care of laboratory animals. Animals were randomly divided into the different experimental groups and analyzed by researchers who were blind to the treatment conditions.

Electrode and cannula implantations

Animals were anesthetized with 2 % isofluorane and implanted with a bipolar-insulated stainless steel electrode (Plastics One, Roanoke, VA) into the right ventral CA1/ CA3 region of the hippocampus (coordinates 4.8 mm posterior and 5.2 mm lateral from the bregma and 6.3 mm ventral from the dura, tooth bar set at –3.0 mm) for stimulation and recording. A unipolar electrode was placed between the skull and adjacent muscle to serve as ground electrode. A subset of animals were implanted with a brain infusion cannula (Brain Infusion Kit 1, Alzet) in the fornix (coordinates: 1.0 mm posterior and 1.1 mm lateral to bregma; and 3.8 mm ventral to the flat skull position with bregma as reference) (Paper VI) or into the lateral ventricle (coordinates: 1.0 mm posterior and 1.5 mm lateral to bregma; and 3.5 mm ventral to flat skull position) in the ipsilateral hemisphere (Paper III).

Induction of status epilepticus

Following a week of recovery after surgery, rats were subjected to electrically induced temporal SE. Prior to SE induction, an after-discharge thresholds was determined for each rat by applying a square-biphasic pulse (50Hz) of 1 s trains duration starting at an intensity of 10 μA, followed by an increase of 10 μA/min until a 10 s after-discharge is evoked. Stimulation continued for 60 min, with interruptions every 9th min to record electroencephalogram (EEG)

activity for 1 min (Fig 5). Only rats that displayed self-sustained ictal EEG activity for 2 h in the temporal lobe and mainly partial seizure semiology e.g., oralfacial twitches, nodding, drooling, and unilateral forelimb clonus, according to Racine's scale (Racine, 1972), were included in this study. Behavioral symptoms and ictal EEG activity were completely interrupted after 2h of self-sustained SE by the administration of pentobarbital (65 mg/kg, intraperitoneal (i.p.) injection). Electrode- and cannula-implanted non- stimulated rats served as controls.

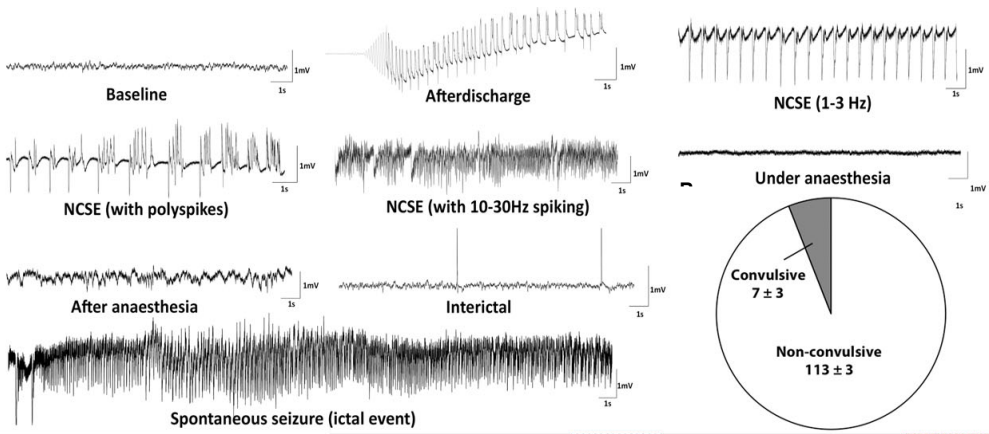

Fig 5. Intrahippocampal electroencephalogram recordings before, during and after non-convulsive status epilepticus. Background activity before stimulation (baseline), epileptiform afterdischarge during 1 hour of stimulation, epileptiform activity with varying frequency during 2 hours of self-sustained complex partial NCSE, and background activity during and after waking up from anesthesia when NCSE was terminated with pentobarbital injection, an example of a spontaneous seizure with evolving pattern, and interictal epileptiform activity. Pie chart showing the mean percentage of time exhibiting nonconvulsive or convulsive seizure semiology during 2 hours of NCSE. *Adapted and modified from Avdic et al 2018, Epilepsia.*

Electroencephalogram evaluations

Animals were continuously video-EEG–monitored (24 h/d) throughout the experimental procedure (Powerlab and Lab- chart v8.1.1; AD Instruments, Dunedin, New Zealand; sampling frequency = 1000Hz). The EEG from intrahippocampal electrodes was visually evaluated and quantified in terms of EEG patterns during NCSE, number of spontaneous and acute symptomatic seizures (Beghi et al., 2010, Beleza, 2012), and IA. Seizures (both acute symptomatic, which occur within 1 week after NCSE, and spontaneous, starting day 8 post-NCSE) were defined as epileptiform EEG activity lasting ≥10 seconds with an evolving pattern, typically consisting of initial high-frequency low-amplitude activity that over time

increases in amplitude and decreases in frequency as a spike–slow-wave pattern. Seizure frequency was quantified manually, and the total time animals exhibited seizure activity was divided into days 0-7 and 8-28 post-NCSE. IA was graded according to a 0-5 scale (0 = none, 1 = <10 spikes/h, 2 = approximately 50 spikes/ h, 3 = approximately 80 spikes/h, 4 = approximately 100 spikes/h, and 5 = >150 spikes/h).

Retroviral labeling of newborn neurons

Seven days after SE induction, rats were anaesthesized with 2% isofluorane and injected with a retroviral vector (1.0-1.1 transducing units/ml) expressing the GFP gene under the CAG promoter (Paper III) (Jackson et al., 2012). Unilateral injections in the dorsal hippocampus were performed in rats using the following coordinates: 2.0 mm caudal and 1.5 mm lateral to bregma and 1.8 mm ventral from the dura: toothbar set at -2.2mm) using a glass microcapillary.

Lipopolysaccharide injections

In Paper V, LPS, an endotoxin of gram-negative bacteria was used to experimentally induce neuroinflammation. 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 stereotaxically injected into the right dorsal hippocampus (coordinates: 4.8 mm posterior and 5.2 mm lateral from bregma; and 6.0 mm ventral from dura, tooth bar set at -3.0 mm) using a glass microcapillary. Animals received a single intracerebral injection of LPS or vehicle.

Intracerebral antibody infusion and Keppra injections

Immediately following SE, brain infusion cannulas were connected with osmotic pumps (2002 or 1007D, Alzet) carrying either mouse anti-N-cadherin Ab (1 mg/ml, Abcam, UK) (infusion rate was 0.5 µl/h for 14 days) in Paper VI or rabbit anti-CX3CR1 antibody (60 µg/ml, Abcam, UK) or recombinant rat-fractalkine (2 µg/ml, R&D systems, USA) in Paper III (infusion rate was 0.5 µl/h). Non-stimulated rats were also connected with osmotic pumps carrying vehicle (PBS, pH: 7.4) to serve as controls. The osmotic pumps were place in the subcutanoeus pocket in the dorsal region of the neck. In Paper VI, rats also received daily i.p. injections of levetiracetam (200mg/kg, or saline) (Klee et al., 2015).

Diffusion tensor imaging

Diffusion tensor imaging (DTI) is a specific MRI sequence that uses the diffusion of water molecules to generate contrast in MR images, i.e. mapping the diffusion process of water molecules in biological tissue, non-invasively (Basser and Jones, 2002, Pierpaoli et al., 1996). In Paper IV, mouse brains were imaged ex vivo in a 9.4-T magnet (Oxford Instruments, Abingdon, UK). Two regions of interest within the epileptic focus were manually marked; dorsal molecular layer of the supragranular blade of the dentate gyrus in hippocampus and the ventral CA3 region (Fig 6).

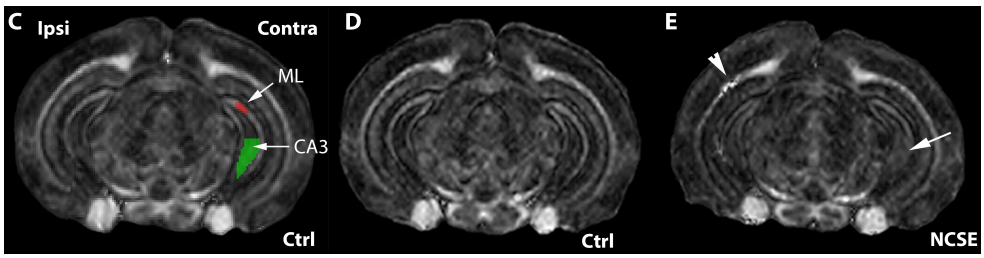

Fig 6. Representative DTI images of nonstimulated control mice (Ctrl) and 7 weeks post- NCSE. Two regions of interest were analysed, CA3 (green) and dorsal molecular layer (ML) of dentate gyrus (red), contralateral to electrode implantation. Arrow in E marks structural disruption detected in some of the animals in ventral CA3 of NCSE mice and arrowhead points at the tissue damage due to the electrode. *Adapted and modified from Avdic et al. 2018, Epilepsia*.

Behavior

Rats were subjected to a battery of behavior tests in order to evaluate memory, cognitive function and social interaction. All tests were conducted during the same time of day and under normal room lighting. Silence in the room was maintained for the duration of the tests.

Open field

The parameters in open field test spontaneous locomotor activity and anxiety-like behavior. The animals were placed individually in the center area of a square open field (80 cm x 80 cm) and divided into 16 zones, 4 central and 12 peripheral. Animals were video recorded for 10 min and during this time locomotor activity was expressed as the number of squares crossed in the center and periphery of the open field. Anxiety-like behavior was evaluated by quantifying

the time spent in each area. The open field chamber was washed with 95% ethanol solution between each rat.

Y-maze

Working memory was assessed in a Y-maze. A black Plexiglas Y-maze, elevated 100 cm from the floor, consisted of three arms with an angle of 120° between each arm. Each arm extended 50 cm with 10cm width and 15cm height. The three arms were randomly designated as A, B and C. Rats were placed individually in the center if the Y-maze and recorded for 10 min. The sequence and number of entries into each arm was quantified. The following parameters were analyzed in detail: spontaneous alteration performance (SAP), alternative arm returns (AAR) and same arm returns (SAR) (Itoh et al., 1994, Hughes, 2004, Wall et al., 2004). These paradigms are based on the tendency of rats to explore new environments. Rats will alternate between arms and explore the least visited arm by using working memory. Thus, if working memory is impaired animals will score low on the measured parameters (Holcomb et al., 1999, Wall and Messier, 2002, Hughes, 2004). The Y-maze was washed with 95% ethanol solution between each rat.

Cylinder test

A cylinder test was performed to assess fore- and/or backlimb lateralization in the rats. Rats were placed individually inside a glass cylinder (20 cm diameter x 70 cm tall). The rats were video recorded for 8 min and the number of times the rats touched the cylinder wall with the left and right forepaw respectively, was measured. The forelimb asymmetry ratio between the right and left paw was analyzed and plotted for each experimental group. The cylinder was washed with 95% ethanol solution between each rat.

Porsolt test

The porsolt swim test was performed to evaluate depressive-like behavior in rats (Porsolt et al., 1977). The animals were placed in a glass cylinder (20 cm diameter x 70 cm tall) containing 25°C water (50 cm deep to prevent the rats tail from touching the bottom). Rats were recorded for 8 min and immobility i.e. the time the rats were not active other than that required to keep the rats head above water, was measured.

Social interaction test

Social interaction assesses the preference of rodents to engage in social behavior and overall social activity (Vanderschuren et al., 1997, Lee et al., 2005). An intruder - a male, age-matched rat - was introduced into the cage of individual animals. The intruder animals were unfamiliar with the experimental animal, with which they were paired for testing. Animals were recorded for 10 min, and the time and frequency of interaction (defined as contact i.e. playing, sniffing of any part of the body of the intruder, following and chasing, initiated by the experimental rat) and passive (i.e. lack of interest when intruder initiates interaction) and active avoidance (i.e. actively avoiding when intruder initiates interaction) were quantified.

Sugar preference test

Reduced sucrose preference suggests depressive-like behavior in rats. We performed a sucrose preference test to assess anhedonia (Carvalho et al 2013). Animals were deprived of water for 6h and were afterwards presented with two bottles, one containing tap water and the other 2% sucrose water. The bottles were left in the cages throughout the experiment that lasted 72h and daily switching position of bottles (left vs right). The total intake of sucrose water and tap water was also analyzed in order to compare general fluid intake between experimental groups.

Histology

Transcardial perfusion and tissue preparation

For DTI, mice were anesthetized and transcardially perfused with 0.9% saline for 3 minutes, followed by 4% paraformaldehyde (PFA) for 15 minutes, before decapitation. Intact heads were postfixed overnight and washed with 0.9% saline for >12 hours, prior to imaging. For immunohistochemistry, rats and mice were transcardially perfused with 0.9% saline and 4% PFA for 10 min. Brains were post-fixed in PFA overnight, kept in 20% sucrose at 4°C for >24hrs, cut in 30µm coronal sections (10 series) on a freezing microtome, and stored in glycerol-based anti-freeze solution in –20°C. In Paper II, eyes were removed, enucleated and post-fixed in PFA for 4h, following perfusion. Eyes were then rinsed in PBS, incubated in 10 % sucrose (16 h) and 25 % sucrose (16 h), consecutively embedded in a Yazulla medium (30 % egg albumin, 3 % gelatin), and finally cut in 20 µm-thick sagittal cryosections (Microm HM 560, US) and stored at –20 °C.

Fluoro-Jade staining

Mounted sections were washed with KPBS, hydrated and pretreated with 0.06% potassium permanganate for 15 min, rinsed with distilled water and treated with 0.001% Fluoro-Jade (Histo-Chem, Jefferson, AR, USA) for 30 min. Following this, sections was washed with distilled water, dehydrated by treatment with ethanol and xylene and coverslipped with PERTEX mounting medium.

Immunohistochemistry

For the majority of immunostainings, the brain sections were subjected to an initial antigen retrieval step with incubation of sections in sodium citrate buffer (10nM sodium citrate, 0.05% Tween-20, pH 6.0) for 20 min at 90°C. The free-floating brain sections were incubated with the appropriate primary antibody over night at 4°C and secondary antibody for 2h at room temperature the following day. For each immunohistochemical assessment, some brain sections went through the entire protocol without primary antibody incubation, to serve as the negative control. The sections were mounted on gelatin-coated glass and coverslipped using a glycerol-based mounting medium (DABCO, Sigma).

Table 1: Primary antibodies used for immunohistochemistry

Primary antibodies			
Antigen	**Host**	**Dilution**	**Source and Catalog**
PSD-95	mouse	1:200	#ab2723, Abcam
Gephyrin	mouse	1:3000	#14711, Synaptic systems
NL-1	goat	1:750	#sc-14082, Santa Cruz Biotechnology
NL-2	goat	1:500	#sc-14089, Santa Cruz Biotechnology
Neurofascin	rabbit	1:1000	#ab31457, Abcam
N-cadherin	rabbit	1:500	#04-1126, Millipore
GFAP	mouse	1:500	#G3893, Sigma
NeuN	mouse	1:500	#2654334, Millipore
Iba1	rabbit	1:200	#01919741, Wako
ED1/CD68	mouse	1:200	#MCA1957, Biorad
CD45	rabbit	1:100	#Sc-25590, Santa Cruz Biotechnology
NG2	mouse	1:200	#05-710, Millipore
IL-6	rabbit	1:100	#ab6672, Abcam
IL-4	rabbit	1:100	#ab9811, Abcam

Microscopy and image analysis

Epifluorescence microscopy

Quantification of microglial activation in terms of cell count and morphology was performed either unilateral or bilateral with respect to the electrode/LPS/vehicle injection and viral vector injection (from 3.3 to -4.6 mm posterior to bregma) (Paper II-VI). Quantification was performed using an Olympus BX61 epifluorescence microscope (20x objective), in the granular cell layer (GCL), molecular layer (ML), CA1, CA3 and hilus region within 3-4 sections/animal. Data is expressed as mean number of cells/section. For morphological analysis of microglial phenotypes, a total number of 90-120 Iba1$^+$ cells were analyzed per animals for three different subtypes: ramified (small, round cell soma with fine processes), intermediate (elongated cell soma with shortened and thick cell processes) and round/amoeboid (swollen cell soma with few processes) (Fig 7). The relative occurrence of each subtype was expressed as the percentage of Iba1$^+$ cells/section.

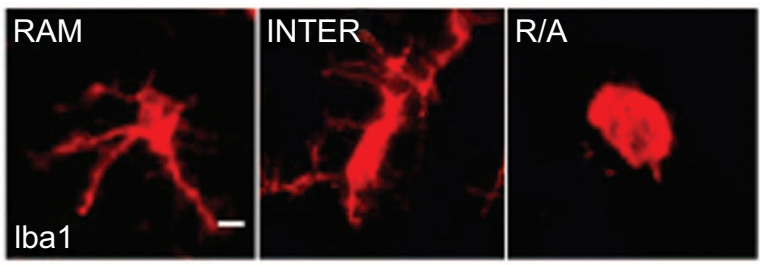

Fig 7. Images of different microglial morphological phenotypes, including ramified (RAM), intermediate (INTER) and round/amoeboid (R/A) cells.

In Paper IV and VI, number of NeuN$^+$ cells were counted in the CA1, CA3, GCL and hilus by stereological measurements using a modified optical fractionator method, set to sample all cells above the first 10µm from the surface of the section, with Olympus BH- 2 microscope, 20x objective and Visiopharm software (Visiopharm, Denmark). For all cell counts, three sections were randomly selected from one of ten parallel series, located between 3.3 and -4.4mm posterior to bregma. NeuN$^+$ cells are presented as number of cells per mm^3.

In Paper III, gross morphological analysis of retina lamination was performed in the entire retina using light microscopy, in ipsi- and contralateral eyes, respectively (Fig 8). Analysis with regard to nuclear layer morphology was performed using the ranking system 0–2 (0 = normal nuclear layer morphology and the presence of 0–10 pyknotic (shrunken) nuclei; 1 = islands of

disseminated nuclear layers without nuclei (typically the size of 1–2 cells) and 11–20 pyknotic nuclei; 2 = completely disseminated nuclear layers and >20 pyknotic nuclei).

Quantification of Iba1, ED1, CD45 and NG2- positive cells were performed in 8-12 regions of interest (ROI) within 4-6 sections/eye, approximately 500 µm from the orra serrate, using an Olympus BX61 epifluorescence microscope. Each ROI comprised the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) (Fig 8).

Semi-quantitative analysis of GFAP expression was performed in 12 representative images from four sections/eye, using Olympus BX61 epifluorescence microscope. The density of GFAP$^+$ radial Müller cell processes was graded according to a 0–5 scale in IPL (0 = none, 1 = 1–5, 2=6–10, 3=11–20, 4=21–50, 5=>50 processes) and a 0–3 scale in INL + OPL and ONL, respectively (0 = none, 1 = 1–5, 2 = 6–10, 3 = >10 processes). In addition, number of GFAP$^+$ retinal astrocytes was manually quantified in 12 representative images of the inner retina from four sections/eye. Intensity measurements of GFAP expression in the GCL, NFL, and ILM were performed in fluorescence images from 12 sections/eye.

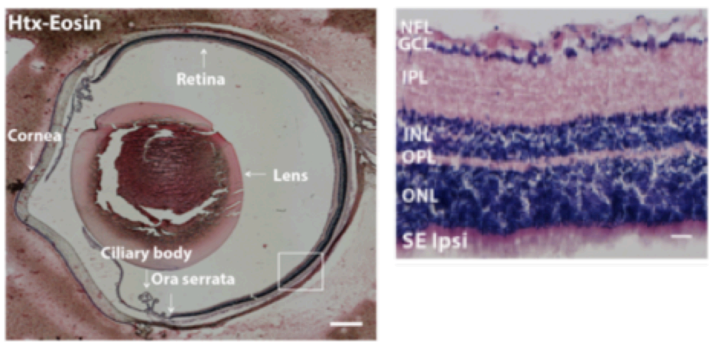

Fig 8. Photomicrographs representing the entire rodent eye and retina. Gross morphology of layers in the retina including the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

Confocal microscopy

For analyses of synaptic proteins on dendrites (Paper III), a total of 12 images were acquired per animals using a confocal laser-scanning microscope (Zeiss, Germany). Images were taken on proximal and distal parts of apical dendrites of GFP$^+$ cells in the inner and outer ML (iML and oML), respectively. Images were acquired using a z-stack at an interval of 0.2 µm. Synaptic protein analysis on cell soma was acquired from a total of 10 GFP$^+$ cells per

animal, in a z-stack at an interval of 0.5 µm. Dendrites and cell somas were selected using only the GFP channel. Synaptic proteins appeared as $Cy3^+$ spherical clusters on the GFP^+ dendrites and soma, and were analyzed in terms of cluster density (number of clusters per µm) in the iML, oML and soma, respectively.

Intensity measurements of synaptic protein expression on mature neurons in sub-regions of the hippocampus in Paper I and VI, and PSD-95 expresssion in the retinal layers, were performed with a confocal laser-scanning microscope, with a 488-nm and 561-nm excitation filter, 63x oil-immersion objective, and 5x digital zoom. Intensity measurements of cytokine expression in the retina (Paper II) were carried out with a 488-nm and 561-nm excitation filter, 40x oil-immersion objective, and 5x digital zoom. Images were taken from three representative areas from each animal. Each image was acquired in a z-stack at an interval of 0.2 µm, on average 50 slices per z-stack. The images were analyzed in ImageJ software (National Institutes of Health, Bethesda, Maaryland, USA) and the brightness and contrast corrected and noise reduced using the built-in ImageJ functions. Background intensity was measured in every image and subsequently subtracted from the mean gray value from each image in order to obtain a background-corrected mean gray value per animal.

Protein analysis

Tissue preparations

For biochemical analyses, rats were decapitated and brains were immediately removed, divided into ipsilateral and contralateral hemispheres related to electrode implantation, further dissected into hippocampus, cortex, and subcortex, frozen on dry ice, and stored at -80°C. Similarly, the spleen was removed, frozen on dry ice and stored at -80°C. Samples were homogenized on ice in lysis buffer (in mM: 50.0 Tris-HCl, 150 NaCl, 5.0 $CaCl_2$, 0.02 % NaN_3, and 1 % Triton X-100), and then centrifuged at 17,000g for 30 min at 4 °C. The supernatant was collected into a microcentrifuge tube, where the total protein concentration was determined by BCA protein assay (BCA, Pierce, Rockford, IL) as per manufacturer's instructions. Prior to decapitation, cardiac blood was collected, incubated for 2h at room temperature, centrifuged at 2500g for 30 min at 4°C, aliquoted and stored in -80°C.

Western blot

Protein samples were denatured at 99°C for 5 min in 2x Laemmli sample buffer (Biorad, Germany). Total protein of spleen (50-100 µg), brain (10-20 µg) or serum (diluted 1:50) unless otherwise mentioned was resolved on precast 4–15% mini-PROTEAN TGX (Biorad)

sodium dodecyl sulphate polyacrylamide gels and transferred using Trans-Blot Turbo mini nitrocellulose transfer packs (Biorad). Following this, the membranes were blocked for 2h at room temperature in tris-buffered saline (pH 7.4) with 0.2% (w/v) Tween 20 (TBS-T) containing 5% nonfat dried milk. Membranes were then incubated overnight at 4°C with primary antibodies diluted in TBS-T containing 0.5% bovine serum albumin (BSA) (Sigma, Germany). After washing, membranes were incubated with secondary antibody diluted in TBS-T containing 0.5% BSA for 2h at room temperature. Secondary antibodies used were either horseradish peroxidase-conjugated anti-mouse (1:5000), anti-goat (1:5000) or anti-rabbit (1:5000) (Sigma). The membranes were washed three times in TBS-T. Immunoreactive bands were subsequently visualized by enhanced chemiluminescence (Biorad), and images were acquired using Chemidoc XRS+ system (Biorad). Band intensities were quantified using ImageJ software. The relative protein expression was compared to the control levels and normalized by the expression of internal control transferrin for serum, and β- actin or GAPDH levels for spleen and brain.

Enzyme-linked immunosorbent assay (ELISA)

Levels of cytokines and chemokines (IL-1β, IL-6, IL-5, IL-4, IL-10, IL-13, tumor necrosis factor (TNF)- α, interferon (IFN)- γ, keratinocyte chemoattractant/growth related oncogene (KC/GRO)) were determined in brain, eyes, serum and spleen with multiplex ELISA kits (V-Plex Proinflammatory Panel 2 rat kit, Meso-Scale Discovery (MSD), Gaithersburg, MD, USA) according to manufacturer's instructions with minor modifications. 50 µl of protein sample was loaded per well of the MSD plate (100 µg protein). Serum was diluted 1:2. The samples were incubated overnight at 4°C with shaking. Samples were analyzed in duplicates and compared with known concentrations of protein standards. Plates were analyzed using the SECTOR Imager 2400.

Table 2: Primary antibodies used for Western blot

Primary antibodies			
Antigen	**Host**	**Dilution**	**Source and Catalog**
PSD-95	mouse	1:200	#ab2723, Abcam
Gephyrin	mouse	1:3000	#14711, Synaptic systems
NL-1	goat	1:750	#sc-14082, Santa Cruz Biotechnology
NL-2	goat	1:500	#sc-14089, Santa Cruz Biotechnology
Neurofascin	rabbit	1:1000	#ab31457, Abcam
N-cadherin	rabbit	1:500	#04-1126, Millipore
Synapsin I	rabbit	1:10000	#106103, Synaptic systems
Synapsin II	rabbit	1:2500	#106203, Synaptic systems

Table 2: Primary antibodies used for Western blot			
βactin	mouse	1:10.000	#A3853, Sigma-Aldrich
GAPDH	rabbit	1:2000	#2118, Cell Signaling Technologies
GFAP	mouse	1:500	#G3893, Sigma
NeuN	mouse	1:500	#2654334, Millipore
Iba1	rabbit	1:200	#01919741, Wako
S100β	rabbit	1:2000	#ab52642, Abcam
Parvalbumin	rabbit	1:500	#ab11427, Abcam
Neuropeptide Y	rabbit	1:500	#N9528, Sigma
CD45	rabbit	1:200	#Sc-25590, Santa Cruz Biotechnology
E-cadherin	mouse	1:200	#ab76055, Abcam
ED1/CD68	mouse	1:200	#MCA1957, Biorad
CD11b	mouse	1:200	#Sc-21744, Santa Cruz Biotechnology
Galectin-3	mouse	1:500	# ab2785, Abcam
MHCII	mouse	1:200	#MCA46G, Abd Serotec
CD8	mouse	1:400	#MCA48G, Abd Serotec
CD4	mouse	1:200	#MCA55G, Abd Serotec
CX3CR1	rabbit	1:500	#ab8021, Abcam
Transferrin	goat	1:10000	#PA3-913, Thermo Fisher

Statistical analysis

All data are presented as means ± SEM unless otherwise stated. Statistical analyses were performed with the unpaired Student's *t* test when comparing two groups, or one- or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test when comparing multiple groups. Mann-Whitney's rank sum test, applied to non-parametric data such as interictal grading, was expressed as median ± range, and Kruskal-Wallis for comparing two or more samples. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Absence of interleukin-1 receptor 1 increases excitatory and inhibitory scaffolding protein expression and microglial activation in the adult mouse hippocampus (Paper I)

The role of the pro-inflammatory cytokine IL-1 has been extensively described in both normal and pathological environments. It is upregulated in a variety of disorders and is a potent activator of the immune system. Studies have shown that IL-1 is important for neuronal modulation and regulation of synaptic processes such as plasticity. Mice lacking the receptor for IL-1β, IL-1R1, display impaired spatial memory and diminished short-term and long-term plasticity (Avital et al., 2003). Interestingly, administration of Il-1β has been shown to have negative effects on learning and memory acquisition (Yirmiya et al., 2002) and intracerebral infusion of IL-1β alters the E/I balance in the spinal cord (Kawasaki et al., 2008). Furthermore, intraventricular infusion of IL-1 has detrimental effects in the DG, suggesting that IL-1 is particularly important in the hippocampus (Yirmiya et al., 2002).

We therefore set out to investigate the changes in microglial activation and synaptic protein expression, associated to E/I balance, under physiological conditions in a mouse model lacking IL-1R1.

Increased microglial activation in IL-1R1 KO animals

Quantification of $Iba1^+$ cells in the hippocampus showed an upregulation of microglia in sub-regions of the DG, where an increased number of $Iba1^+$ cells were detected in the GCL in IL-1R1 KO animals compared to WT. In addition, we analyzed the activation states of microglia in terms of morphology and found an activated phenotype in KO animals, with an increased percentage of intermediate and decreased percentage of ramified $Iba1^+$ cells in the GCL. These results suggest that the lack of the IL-1R1/IL-1β pathway increases microglial activation and induces a pathological environment that may be detrimental to fundamental synaptic processes such as plasticity and memory formation.

Increase in excitatory and inhibitory scaffolding protein expression

In order to investigate if the lack of IL-1R1/IL-1β pathway causes changes in synaptic protein expression, we quantified a panel of synaptic proteins known to regulate the E/I

balance and found an increased expression of PSD-95 in both the GCL and inner and outer ML in mice lacking IL1-R1, compared to control animals. Interestingly, we also detected an increase in the intensity of gephyrin clusters in the inner and outer ML of the DG, whereas the perisomatic expression where gephyrin expression is concentrated was unaltered. Interestingly, further intensity measurement of PSD-95 also revealed increased expression in the amygdala, a structure that communicates extensively with the hippocampal region. No changes were detected in the expression of adhesion molecules in the DG.

In summary, our results show that the IL-1R1/IL-1β pathway changes expression of the synaptic scaffolding proteins gephyrin and PSD-95 in the hippocampus, and may thus alter synaptic transmission. These changes are associated to an activation of microglial cells in terms of both number and activation states, suggesting that neuronal synaptic activity reciprocally interacts with the inflammatory cells in the hippocampus. Further studies need to address what implications this carries in pathological contexts where synaptic transmission is disrupted, such as in epilepsy.

Immune response in the eye following epileptic seizures (Paper II)

The pathological hallmarks in the brain associated with seizures include neuronal damage, imbalance in synaptic transmission and an inflammatory response. The inflammatory response has been extensively characterized and described locally in the epileptic focus (Ekdahl et al., 2003a, Crespel et al., 2002, Ravizza et al., 2011). Cells of the innate immune system such as microglia and astrocytes change morphology and become activated, release pro- and anti-inflammatory mediators that up-regulate inflammatory factors that further orchestrate the inflammatory response (Kettenmann et al., 2011, Ekdahl et al., 2009). The seizure-induced immune response has also been reported to disrupt and permeate the BBB, which causes both acute and chronic activation and infiltration of vascular-associated blood- derived immune cells that further propagate the immune response (Legido and Katsetos, 2014). Recent evidence suggests that epilepsy is a network disturbance that manifests throughout the brain and involves subcortical nodes as well in seizure propagation (Pittau et al., 2014). Thus, the extent of the seizure activity may overlap with brain-inflammation in more remote areas of the brain. In order to address if a post-seizure immune response can be detected outside of the focus in more remote areas, we evaluated the immune response in the retina of adult rats. The retina is located at the back of the eyeball and is considered an extension of the brain but has not been shown to exhibit any seizure-induced pathology. The immune system in the retina

resembles that of the rest of the brain, with micro- and macroglia, vascular pericytes and blood-retina barrier (Pfister et al., 2013, Karlstetter et al., 2015, Liu et al., 2014a).

Lack of acute and subacute changes in immune mediators, glial activation and cell death following status epilepticus

The immune response in the epileptic focus can be detected acutely after SE (Ekdahl et al., 2003a). We therefore decided to evaluate the general cytoarchitecture and inflammatory response at 6h following SE in the retina. When performing morphological analysis of the retina it showed well-defined nuclear and synaptic layers without any changes in cytoarchitecture. Analysis of the inflammatory response did not reveal any changes in $Iba1^+$ or $Iba1^+/ED1^+$ cell number, nor was there a difference in microglial morphology. Further biochemical analysis of pro- and anti-inflammatory cytokines and chemokines did not show any changes in the eye tissue. At 1 week following SE substantial neuronal death and glial activation has been reported in the temporal lobe, including the hippocampus (Mohapel et al., 2004, Ekdahl et al., 2003b). However, at this time point cytoarchitectural analysis of the eye tissue did not reveal any changes, nor was there any glial reactivity. No changes in $Iba1^+$ cell number or microglial morphology could be detected.

Delayed glial activation and altered synaptic protein expression in the retina at 7w following status epilepticus

Prominent chronic activation of microglial cells has been observed in the epileptic focus following SE (Bonde et al., 2006). At 7 weeks post-SE we detected a prominent bilateral immune response in the retina with increased $Iba1^+$ cells, primarily located in the synaptic layers and the GCL. Microglial morphology was also significantly changed. Rats with SE showed a decreased ramified microglial phenotype and increased round/amoeboid morphology. This change was further confirmed by larger soma diameter and less extended processes of $Iba1^+$ cell in the ipsilateral retina. Moreover, intensity measurement of IL-1β showed a significant increase in the inner plexiform layer (IPL). Interestingly, the activation of microglia was not associated to neuronal damage, nor could we detect any mayor cytoarchitectural alternations. Furthermore, in addition to microglia, astrocytic activation is commonly observed in response to seizures, and in the retina Müller cells and astrocytes, together referred to as macroglia, are responsible for inducing injury-related gliosis. $GFAP^+$ Müller cell processes were significantly upregulated in the IPL in animals with SE compared to non-stimulated controls. No changes in $GFAP^+$ retinal astrocytes were found.

Moreover, we have previously reported altered expression of synaptic scaffolding proteins in response to immune changes in the brain (Chugh et al., 2013). In an attempt to analyze if the immune response was associated with changes in synaptic protein expression and even a subtle

imbalance in synaptic transmission we analyzed the expression of PSD-95 in the different layers of the retina. Intensity measurements revealed a decrease in PSD-95 clusters in the outer nuclear layer (ONL) in the retina ipsilateral to the epileptic focus.

Intraventricular CX3CR1 infusion decreases glial activation in the retina

In order to evaluate if there are any common inflammatory mechanisms between the epileptic focus and the retina, an antibody against the chemokine receptor CX3CR1 was infused intracerebroventricularly, starting at 1 week after SE. We recently reported decreased microglial activation in the temporal epileptic focus after intracerebroventricular infusion of CX3CR1 antibody during 1 week following SE (Ali et al., 2015). After 6 weeks of antibody infusion, we detected a significant decrease in the number of $Iba1^+$ cells in the contralateral retina and a bilateral change in $Iba1^+$ cell morphology with a higher percentage of ramified morphology and less intermediate and round phenotypes in rats treated with CX3CR1 antibody. In addition, Müller processes in the retina were decreased in the IPL in the CX3CR1-antibody treated SE group compared to untreated animals.

Decreased post-synaptic density-95 protein expression on dendrites of newborn neurons following CX3CR1 modulation in the epileptogenic adult rodent brain (Paper III)

New neurons are continuously produced in the subgranular zone of the hippocampus under physiological conditions and inflammation has been shown to be detrimental for adult neurogenesis in the hippocampus (Ekdahl et al., 2003a). In epilepsy, increased neuronal proliferation has been described (Parent et al., 1997, Bengzon et al., 1997), but the exact role of new neurons born in a pathological environment has not been fully elucidated in seizure propagation. Studies suggest that new neurons integrate abnormally into the network and contribute to the pathophysiology of epilepsy (Parent and Lowenstein, 2002), whereas other studies show that neurons that integrate correctly have protective effects (Jakubs et al., 2006, Jackson et al., 2012). We have previously shown that both seizure- and LPS -induced inflammatory environments may regulate the synaptic integration of newly formed neurons in the hippocampal circuitry, by altering the expression of adhesion molecules and scaffolding proteins (Chugh et al., 2013, Jackson et al., 2012), suggesting that regulation of synaptic integration of new neurons may be mediated by neuroinflammatory mechanisms. Indeed, the fractalkine/CX3CR1 pathway has been shown to regulate neurogenesis at physiological

conditions (Bachstetter et al., 2011) and we have previously reported reduced cell proliferation of immature neurons in the DG one week following SE in animals treated with CX3CR1-antibody (Ali et al., 2015).

In this study we modulated immune signaling mediated by the fractalkine/CX3CR1 pathway in an inflammatory environment following electrically-induced SE, and evaluated the expression of synaptic scaffolding proteins and adhesion molecules on newly born cells. A schematic representation of the experimental setup is shown in Fig 9.

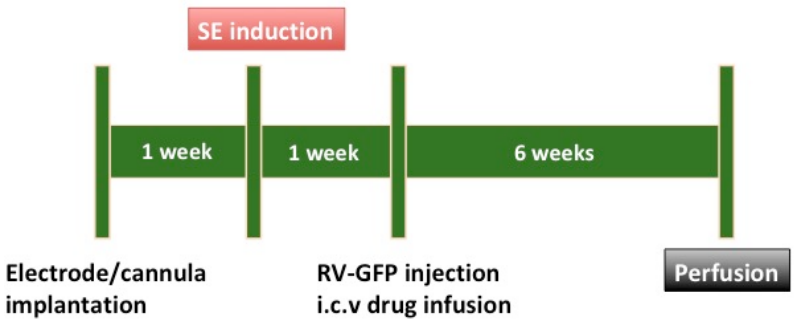

Fig 9. A schematic of the experimental setup. Animals were implanted with bipolar electrodes unilaterally and with an intracerebroventricular cannula. One week later, animals were subjected to electrically-induced SE. Seven days after SE, rats were injected with retroviral vector expressing GFP (RV-GFP) to label newly formed cells in the dentate gyrus, and immediately after RV-GFP injection, the brain infusion cannula was connected to an osmotic pump carrying either CX3CR1-antibody or vehicle (phosphate buffer saline).

We first evaluated the effects of CX3CR1 antibody infusion on microglial activation at 7 weeks post-SE. We did not detect any differences in the total number of $Iba1^+$ cells in the different sub-regions of the hippocampus. Similarly, the percentage of $Iba1^+/ED1^+$ cells did not differ. Blocking the CX3CR1 receptor did not alter the morphological profiles of microglia in the GCL or ML of the hippocampus, but when comparing the activation profiles in the dentate hilus, we found a significant increase in the number of ramified cells and reduction in intermediate morphology in CX3CR1- antibody treated rats compared to vehicle treated animals. Moreover, dendritic spines, which are the main excitatory input onto neurons, were evaluated, as well as the general morphology of the GFP^+ cells. No changes could be detected in terms of origin of dendrites, recurrent basal dendrites or immature and mature spine phenotypes.

Next, we evaluated synaptic protein expression on newly formed cells. We have previously shown altered expression of both scaffolding proteins and adhesion molecules on new neurons in the DG, in an inflammatory environment (Jackson et al., 2012, Chugh et al., 2013). Analysis of postsynaptic proteins revealed a strong reduction in the expression of scaffolding protein PSD-95 in CX3CR1-antibody treated rats compared to untreated animals.

Interestingly, these changes were not accompanied by alterations in NL-1 clusters, nor could we detect any changes in postsynaptic inhibitory proteins such as gephyrin and NL-2.

Non-convulsive status epilepticus in rats leads to brain pathology and a distinct systemic immune profile (Paper IV and V)

The majority of all SE cases typically manifest with convulsive movements. However, 20-40% of all SE cases do not present any motor features and are referred to as non-convulsive SE (NCSE). Clinical manifestations of NCSE include altered consciousness, automatism, confusion and subtle motor activity. While convulsive SE leads to neuronal damage and significant pathology, the extent of damage in NCSE is less clear. Consequently, no general consensus exists in the clinic regarding to what extent NCSE is harmful to the brain, which adds uncertainty to treatment strategies, and because of the subtle symptoms of NCSE clinical diagnosis is often delayed. New diagnostic and prognostic tools are therefore needed for earlier and more accurate stratification of the disease pathology and treatments. In this study, we sought to investigate the pathology in the brain (Paper IV) as well as characterize the systemic immune response (Paper V) following NCSE originating in the temporal lobes.

Prominent inflammation and neuronal loss in the epileptic focus following NCSE

DTI imaging of mouse brains 7 weeks following NCSE, did not reveal any disrupted integrity or changes in structure in the epileptic focus upon visual assessment, supporting the clinical difficulties of detecting robust long-term changes following NCSE in patients with standard imaging techniques. We therefore continued with biochemical analysis of common pathological markers involved in neuronal loss and inflammation in the brain. While no alterations were detected at the early time-points (6h, 24h) following NCSE, we found prominent pathophysiological changes developing over time in rats. At 1 week post-NCSE, when animals display acute symptomatic seizures (Beghi et al., 2010, Beleza, 2012), we detected a bilateral increase in both Iba1 and GFAP, markers specific for microglia and astrocytes respectively. Although studies have reported prominent neuronal loss at 1 week post-SE (do Nascimento et al., 2012, Liu et al., 2010a), we detected no changes in NeuN expression in the hippocampus at this time-point. Interestingly, at 4 weeks post-NCSE, when the majority of animals had developed spontaneous seizures, the glial response was still detected and NeuN levels were now deceased in the contralateral hippocampus.

Decreased levels of synaptic proteins in the hippocampus following NCSE

We continued to evaluate the changes in synaptic protein expression in the brain and at 1 week post-NCSE, we detected alterations in several proteins involved in excitatory and inhibitory transmission. Levels of PSD-95 and NL-1 were decreased in the contralateral hippocampus along with gephyrin and the pre-synaptic protein synII. Levels of synaptic proteins in the cortex and subcortex were unaltered, suggesting changes were specific for the epileptogenic regions. Interestingly, protein expression at 4 weeks was back to control levels and no changes were detected in terms of inhibitory or excitatory protein alterations. In an attempt to further dissect out the pathology, and study which changes are associated to epileptogenesis, we subdivided the 4 week NCSE group into two groups; rats with only acute symptomatic seizures and rats with additional spontaneous seizures during 2-4 weeks post-NCSE. Notably, the glial reaction was still prominent in both groups in the hippocampus, as well as in the cortex and subcortex in animals with NCSE and spontaneous seizures. Interestingly, neuronal death was now only present in rats that displayed spontaneous seizures. Moreover, western blot analysis of synaptic protein expression revealed a decreased level of N-cadherin in animals with spontaneous seizures in the contralateral hippocampus, compared to both controls and to rats with only acute symptomatic seizures. The change in N-cadherin levels did not correlate with seizure burden in terms of duration and frequency of seizures, suggesting a possible role for N-cadherin in the development of epilepsy, rather than a decrease due to spontaneous seizures per se.

Distinct systemic immune response in rats following NCSE

In paper IV we characterized the progression of the pathological profile in the brain. Next, we wanted to study if NCSE elicits a peripheral immune response in rats (Paper V). In rodents with severe SE, levels of circulating $CD8^+$ cytotoxic T lymphocytes have been observed, and in plasma and serum from patients with temporal lobe epilepsy increased levels of pro-inflammatory cytokines, neutrophils and cytotoxic T lymphocytes have been detected (Marchi et al., 2007, Bauer et al., 2008, Gao et al., 2017). We therefore sought to study the immune response following NCSE and compare it to serum from rats challenged with intracerebral injection of LPS, in order to evaluate the specificity of the immune reaction. ELISA analysis of common pro- and anti-inflammatory cytokines and chemokines showed an acute increase in systemic pro-inflammatory cytokines IL-6 and KC/GRO. The acute increase in KC/GRO was not specific for NCSE-induced injury but was also detected after LPS-treatment. No changes in cytokine and chemokine levels were detected at later time-points in serum.

When further evaluating a panel of proteins involved in the innate and adaptive immune response, we did not detect any major alterations at the acute stages or at 1 week post-NCSE in the serum. Western blot evaluations in the spleen however, revealed decreased levels of CD11b and E-cadherin, concomitant with an increase in CD45 and the astrocytic marker

GFAP at 6h and 24h post-NCSE, respectively. Interestingly, at 4 weeks following NCSE, when animals start displaying spontaneous seizures, several proteins were altered in the blood. Levels of MHCII, CD68 and galectin-3, markers associated with microglia, were substantially increased in serum and levels of CD4, a T helper lymphocyte marker were decreased. Interestingly, this immune profile was not detected in animals injected with LPS or in rats with only acute symptomatic seizures, suggesting a distinct pathological profile in serum that may be related to the subsequent brain pathology and development of spontaneous seizures.

Levetiracetam and N-cadherin antibody treatment counteract brain pathology without reducing early epilepsy development after non-convulsive status epilepticus (Paper VI)

Next, based on the results from Paper IV, we were interested in understanding the role of N-cadherin in epilepsy and epileptogenesis. Since N-cadherin is involved in synapse development and maturation and was found to be decreased in rats following NCSE that subsequently developed spontaneous seizures, we wanted to investigate the role of N-cadherin in the pathology and progression of epilepsy. Rats were treated with the conventional anti-epileptic drug levetiracetam in combination with intracerebral infusion of N-cadherin antibody for 4 weeks following NCSE. A schematic representation of the experimental setup is illustrated below in Fig 10.

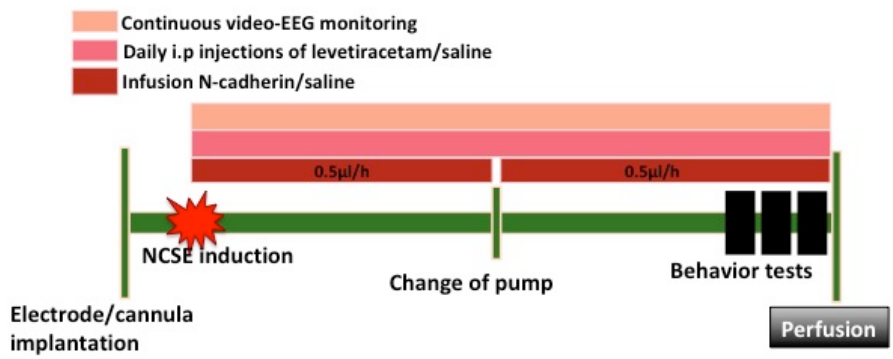

Fig 10. Schematic representation of the experimental set-up. Animals were divided into four groups; controls; NCSE-vehicle-saline; NCSE-vehicle-levetiracetam and NCSE-N-cadherin-levetiracetam. Animals were subjected to electrically-induced non-convulsive status epilepticus, infused with N-cadherin (1mg/ml) or saline, and given daily i.p. levetiracetam (200mgkg) injections for 4 weeks. Before perfusion, a set of behavior tests was conducted.

Altered brain pathology after one month of combined treatment with levetiracetam and N-cadherin antibody

We first evaluated the overall pathology in animals with NCSE and detected a significant neuronal loss in the hippocampus, with a decrease in $NeuN^+$ cells in the CA1 and hilar regions of the DG. Interestingly, the decrease in both areas was reversed by levetiracetam treatment but not with levetiracetam in combination with N-cadherin antibody. Further immunohistochemical quantification of $Iba1^+$ microglial cells in the sub-regions of the hippocampus showed prominent increase in the ML and dentate hilus following NCSE, confirming previous observations (Ekdahl et al., 2003a). Again levetiracetam treatment reduced the microglial activation, but the number of $Iba1^+$ cells was not affected by levetiracetam in combination with N-cadherin antibody. A similar patter was detected in the CA1 region, which showed increased number of $Iba1^+$ cells in animals with NCSE, and was reversed by only levetiracetam treatment. Furthermore, we evaluated the morphological profiles of activated microglia and observed a significant interaction between ramified and intermediate morphologies in untreated rats with NCSE compared to controls. Rats that were treated with levetiracetam and levetiracetam in combination with N-cadherin antibody displayed increased ramified and decreased intermediate phenotypes compared to untreated NCSE rats in the ML, while levetiracetam alone was able to reverse the microglial activation in hilus, CA3 and GCL. Moreover, immunohistochemical evaluations of synaptic protein expression following NCSE showed increased levels of PSD-95 in the iML, a region where new granule cells make synaptic connections with the surrounding network, in rats with NCSE compared controls. The increase was only reversed by levetiracetam treatment. Interestingly, in the hilus, PSD-95 expression was not altered in animals with NCSE compared to non-stimulated controls, but was despite this significantly reduced in rats with N-cadherin antibody infusion and levetiracetam, compared to untreated NCSE rats and rats treated only with levetiracetam.

No alterations in seizure burden or cognitive outcome following treatment with levetiracetam and N-cadherin antibody

When evaluating the EEG and the development of spontaneous seizures, we did not detect any changes in seizure frequency or mean duration of seizures after levetiracetam and N-cadherin antibody, despite reduced effects on the pathology in the epileptic focus. The majority of all spontaneous seizures were categorized as partial complex seizures with ambulatory behavior and occasional secondary generalization, which was not affected by the two treatments. Similarly, no changes could be detected in interictal activity in the three experimental groups post-NCSE.

Furthermore, impaired cognition and mood disorders are commonly associated with epilepsy (Kanner and Balabanov, 2002, Elger et al., 2004), and in a model of NCSE induced

by pilocarpine, similar behavior deficits have been observed (Krsek et al., 2004). We therefore evaluated cognitive and behavioral aspects in rats following NCSE, and analysis of working memory, anxiety-related behavior and social interaction did not reveal any changes in any of the experimental groups with NCSE, with or without treatment.

GENERAL DISCUSSION

The main objective in this thesis has been to characterize the immune response associated to NCSE and the development of epilepsy. We utilized an experimental rodent model that shares several similarities to clinical practice in terms of EEG patterns, semiology, acute symptomatic seizures and development of spontaneous seizures. We describe an acute release of pro- and anti-inflammatory cytokines in response to NCSE in the epileptogenic focus and blood, as well as a chronic activation of microglia and astrocytes with subsequent neuronal loss. Remarkably, the immune response was also detected in the retina after epileptic seizures and a significant glial activation was accompanied by changes in synaptic protein expression. Furthermore, a distinct systemic immune profile, specific for animals that develop spontaneous seizures after NCSE was detected, which is not present in the systemic response of animals injected with intracerebral LPS. In addition, immune activation was associated with prominent changes in excitatory and inhibitory synaptic protein levels in the hippocampus before the onset of spontaneous seizures, and we show an important link between immune mechanisms and synaptic protein expression where changes in protein levels are altered by modulating the putative fractalkine/CX3CR1 pathway. Finally, we describe decreased seizure-induced pathology in rats post- NCSE, following treatment with a common AED in combination with an intracerebral antibody against N-cadherin, without any effect on seizure development and burden.

Widespread immune responses following NCSE

Brain pathology has been extensively characterized in different animal models of convulsive SE and epilepsy, and microglial activation is known to play an important role in seizure activity and epileptogenesis (Ekdahl et al., 2003a, Ali et al., 2015, Zhao et al., 2018, Eyo et al., 2017). In clinical practice, the extent of damage associated to NCSE has been difficult to predict and few studies address the pathophysiology associated to it. We demonstrate a transient release of pro- inflammatory cytokines and chemokines in the brain acutely following NCSE, an observation made in several studies following epileptic activity (Vezzani, 2005). Increased cytokines such as IL-6, Il-1β and TNF-α are prominent features of the neuroinflammatory response in epilepsy development, known to contribute to the generation of seizures (Vezzani et al., 2011, Terrone et al., 2017). Subsequently, we detected substantial chronic glial activation in the epileptic focus at 1 and 4 weeks following NCSE, with neuronal death when animals start to develop spontaneous seizures. Further analysis of the immune response showed cortical glial activation in animals that developed epilepsy, suggesting that

larger networks are involved in epileptic activity and propagation, supporting clinical fMRI evidence (Caciagli et al., 2014, Yang et al., 2018). There is mounting evidence that the neuroinflammatory environment can persist in epilepsy and contribute to neuronal network hyperexcitability (Vezzani and Viviani, 2015). In fact, several anti-inflammatory drugs have proven to block disease progression in models of epileptogenesis (Vezzani et al., 2000, De Simoni et al., 2000), including a recent study where boosting endogenous pro-resolving mechanisms during neuroinflammation in epilepsy improved disease outcomes (Frigerio et al., 2018). Consequently, immune-modulation and anti-inflammatory therapies that address some of these mechanisms could be effective treatments for epilepsy.

Moreover, the acute cytokine response in the epileptic focus was also associated to an increase in pro-inflammatory cytokines in serum. This finding is supported by clinical reports of early and transient increase in cytokines, neutrophils, leukocytes and lymphocytes in patients with temporal epileptic seizures (Bauer et al., 2008, Gao et al., 2017). However, a similar peripheral immune response was observed in animals with LPS injections, suggesting that the immune profile was not seizure-specific at the acute phase. Interestingly, further analysis revealed a distinct peripheral immune profile in animals 4 weeks following NCSE compared to both controls and to LPS-treated animals. At this time-point, extensive gliosis and microglial phagocytic activity (Ekdahl et al., 2003a, Ali et al., 2015) is observed in the brain with subsequent changes in CD68 and MHCII expression (Shaw et al., 1994, Boer et al., 2006), and although the immune response following NCSE shares similarities to the response following intracerebral LPS injection (Herber et al., 2006, Chugh et al., 2013), serum analysis showed altered levels of MHCII, galectin-3, CD68 and CD4 specifically in animals with NCSE that subsequently developed spontaneous seizures. These changes may either be a result of the total seizure burden or act as an underlying mechanism that either propagates or counteracts epileptogenesis, but regardless of mechanisms they may offer high clinical value in patients following NCSE in terms of diagnostic and prognostic purposes. Indeed, clinical studies show elevated serum levels of galectin-3 in patients with intractable focal epilepsy and reports also suggest decreased levels of $CD4^+$ T lymphocytes in patients with TLE (Tian et al., 2016, Bauer et al., 2008).

Furthermore, we provide the first evidence that the seizure-induced immune response spreads to the retina and that a substantial immune reaction can be detected in the eyes of rats following seizure activity, including glial activation and cytokine expression. Interestingly, retinal inflammation has been observed in other neurological disorders. Clinical evidence shows pronounced retinal pathology in Alzheimer's (AD), Parkinson's (PD) and Huntington's disease, with neurodegeneration of retinal ganglion cells, micro- and macro-glia activation and microvascular abnormalities (London et al., 2013, Kaur et al., 2015, Ragauskas et al., 2014). From our studies we can conclude that the retinal pathology is a result of either the SE-insult, the accumulating seizure activity or SE-induced epileptogenesis. Whatever the mechanisms, the presence of glial pathology in the retina is highly clinically relevant in terms of diagnostic

and prognostic purposes as the eyes share many neural and vascular similarities to the brain and may offer a window to cerebral pathology. In addition, considering that it is more accessible than other parts of the brain makes it attractive for developing novel, non-invasive biomarkers. Indeed, a recent study demonstrates the use of the retina as an early biomarker of neurodegeneration in a PD model, where retinal pathology precedes the pathological manifestations in the brain (Normando et al., 2016), and in patients with AD progression, changes in the retinal nerve fiber layer have been identified as a possible diagnostic marker of early AD (Lad et al., 2018). Future studies need to correlate the extent of inflammation in the retina to seizure frequency, semiology and development, and address if these changes are linked to functional deficiencies in the retina and if patients with epilepsy present with visual disturbances.

Immune modulation

We modulated the seizure-induced pathological environment following SE and targeted the fractalkine/CX3CR1 pathway, known to mediate neuron-glia crosstalk (Arnoux and Audinat, 2015). Previous studies in our lab have shown that blocking the receptor for fractalkine by administering CX3CR1 antibody for 1 week after SE-induction significantly diminished microglial activation and neurodegeneration in the hippocampus (Ali et al., 2015). In Paper III, we extended the treatment protocol and observed reduced microglial activation in only the dentate hilus 6 weeks following CX3CR1 antibody treatment, without an effect on neurodegeneration. Notably, studies show that microglial activation subsides to a milder form in the chronic phase, compared to the strong acute activation observed in the first week following SE (Bonde et al., 2006) which may account for the minor effect on microglial activation in the hilus after long-term fractalkine/CX3CR1 modulation. Similarly, other studies have also reported decreased seizure-induced pathology following treatment with CX3CR1 antibody, where a reduced microglial number was observed 3 days following pilocarpine- induced SE (Yeo et al., 2011). However, there have been mixed reports of the effect of modulating the fractalkine/CX3CR1 pathway on microglial activation, with respect to different brain pathological environments, degrees of receptor inhibition, and timing and dose of antibody treatment (Liu et al., 2010b, Cardona et al., 2006), suggesting that the effects attained by the antibody treatment are context- and model- dependent. Nevertheless, further studies need to address the role of CX3CR1 modulation in cell-type specific changes, especially considering the heterogeneous population of microglia and microglia-released mediators.

We further evaluated the synaptic protein expression on new neurons and overall cell morphology, 6 weeks after viral injection, a critical time-point for newly formed neurons to integrate into the surrounding network. Studies have implicated the fractalkine/CX3CR1 pathway in neuronal excitability and synaptic connectivity (Yeo et al., 2011, Paolicelli et al.,

2014). Interestingly, reducing microglial activation with CX3CR1 antibody during the integration phase of newly formed neurons did not result in any major changes in neuron morphology. However, despite the lack of structural abnormalities on the newly formed neurons, such as changes in basal dendrites and spine density, as can be the case following epileptic seizures (Ribak et al., 2012, Singh et al., 2013), we did find a strong reduction in PSD-95 levels. Changes in PSD-95 levels have previously been linked to epilepsy and epileptogenesis (Ying et al., 2004, Chugh et al., 2015). Interestingly, the decrease in PSD-95 levels was not accompanied by NL-1, or by the inhibitory post-synaptic proteins gephyrin and NL-2. These results not only implicate the immune system in synaptic protein expression regulation, but also suggest that inhibiting the immune reaction locally by blocking the fractalkine/CX3CR1 pathway, a stronger inhibition of the excitatory synaptic drive can be attained, and thus prevent further epileptic activity. This speculation is supported by altered synaptic proteins on newly formed cells in a seizure-induced environment in the DG, where reduced afferent excitatory connections were reported, with higher gephyrin and lower PSD-95 expression (Jackson et al., 2012, Jakubs et al., 2006), suggesting that homeostatic mechanisms control the strength of excitatory drive and thereby counteract seizure activity. Conversely, reduced gephyrin levels and increased PSD-95 expression on newly formed neurons have recently been reported in an inflammatory environment elicited by LPS (Chugh et al., 2013), suggesting that PSD-95 expression is context-dependent and that changing the pathological environment surrounding the epileptic focus by modulating CX3CR1, results in synaptic protein alterations that may in turn affect the E/I balance. However, more studies need to confirm the functional consequences of these changes in terms of cell integration and electrophysiological properties. In addition, the effects of altering the fractalkine/CX3CR1 pathway on the development of spontaneous seizures and seizure burden also remain to be studied.

Furthermore, in Paper II we report that immune modulation with intracerebroventricular CX3CR1 antibody treatment resulted surprisingly in a delayed reduction of both micro- and macroglial activation in the retina, in animals with SE. The fractalkine/CX3CR1 pathway is involved in activation and migration of microglial cells and it is likely that the reduced immune response in the eye after CX3CR1 antibody treatment may represent an impaired migratory capacity of the immune cells, which diminishes the immune propagation from the epileptic focus (Harrison et al., 1998, Noda et al., 2011). In addition, recent evidence suggest that the fractalkine/CX3CR1 pathway is not only restricted to microglia-neuron communication but also involves indirect modulation of astrocyte activity (Catalano et al., 2013), which may account for the reduction in GFAP expression and activation of Müller cells seen in the retina. Regardless of which mechanisms are responsible for seizure-induced immune propagation to the eye, the decreased retinal glial activation following CX3CR1 antibody treatment suggests similar immune signaling mechanisms and pathways to the immune reaction in the epileptic focus and network. Thus, despite being a remote

compartment, immune modulation by way of inhibiting CX3CR1 locally diminishes seizure-induced pathological changes in the retina. These unique characteristics may offer improved clinical management and be useful for monitoring not only the disease pathology, but treatment effectiveness as well (Satue et al., 2016). The functional effects of modulating seizure-induced pathology with CX3CR1 antibody in brain and retina remain to be studied.

In addition to studying the immune modulatory properties of CX3CR1 antibody in a seizure-induced inflammatory environment, we also studied the microglial response and synaptic protein expression in an environment with a modified IL1-R1/Il-1β pathway in Paper I. The pro-inflammatory cytokine Il-1β is upregulated in a variety of diseases and immune challenges, and is a potent activator of the immune response. We demonstrate that in the absence of IL1-R1, region-specific changes in both microglial activation and PSD-95 and gephyrin expression are evident in sub-regions of the hippocampus at physiological levels. Previous studies have reported discordant results with respect to the effects of Il-1β signaling. Studies have reported that administration of Il-1β impairs learning and memory acquisition while others have demonstrated impaired spatial memory, and long- and short-term plasticity in IL-1R1 KO animals (Avital et al., 2003, Yirmiya et al., 2002). Our results suggest that lack of IL1-R1/Il-1β pathway induces a pathology-like environment in the hippocampus and that microglial activation leads to changes in synaptic protein expression that ultimately may be detrimental to fundamental processes such as memory acquisition and long-term potentiation, as has been previously observed. Notably, the changes in microglial morphology in IL1-R1 KO animals may result in significant alterations in network homeostasis maintenance and thus alter microglia-synapse interactions. Conversely, microglia is also known to respond to changes in synaptic activity and transmission (Blinzinger and Kreutzberg, 1968) and it is conceivable that the changes in the glutamatergic protein PSD-95 and inhibitory protein gephyrin in the ML potentially promote region-specific microglial activation in an area that receives high synaptic input. Moreover, an altered spine size has been previously reported in the ML in IL1-R1 KO animals (Goshen et al., 2009), suggesting that the alterations in synaptic protein expression are a compensatory mechanism and an attempt to re-establish network function. Indeed, the role of IL1-R1/Il-1β pathway has been extensively studied in synaptic activity and studies demonstrate an important role of Il-1β in regulating AMPA receptor surface expression and NMDA receptor activity, suggesting that inflammation and microglial activity can indirectly regulate and change the synaptic protein composition and signaling mechanisms important for normal neuronal function (Lai et al., 2006). Understanding the role of the IL1-R1/Il-1β pathway further, with respect to inflammation and synaptic protein alterations will be of crucial importance in several diseases where the E/I balance and neurotransmission is affected. Furthermore, future research needs to address the functional properties of changes in synaptic alterations in animals with a compromised Il-1β signaling and study its potential as a treatment strategy.

Therapy in a NCSE model

In addition to characterizing the pathology post- NCSE, we also studied the effects of levetiracetam and anti-N-cadherin antibody treatment on the neuropathological changes following NCSE. The infusion of N-cadherin antibody was based on previous findings from Paper IV, where a decrease in the excitatory adhesion molecule N-cadherin was observed specifically in animals that developed spontaneous seizures following NCSE. We therefore hypothesized that the decrease in N-cadherin was possibly related to mechanisms that are counteracting epileptogenesis and decreasing hyperexcitability. In an attempt to further reduce activity within the epileptic focus, we treated animals with intracerebral N-cadherin antibody, in combination with levetiracetam. We observed a decreased overall pathology in the epileptic focus, where neurodegeneration, microglial activation and PSD-95 expression were altered. Cell death within the dentate hilus and CA1 of the hippocampus, two regions prone to seizure-induced cell death in epilepsy (Margerison and Corsellis, 1966) was diminished after levetiracetam treatment, suggesting levetiracetam exerts a neuroprotective effect. Similar findings have previously been reported in pilocarpine-induced SE in rodents (Zheng et al., 2010, Itoh et al., 2015), as well as in stroke induced damage and TBI (Shetty, 2013), where decreased neurodegeneration was observed. Furthermore, both levetiracetam and levetiracetam in combination with N-cadherin antibody reduced PSD-95 expression in different sub-regions of the hippocampus. Studies show that both long-term blocking and ablation of N-cadherin leads to loss of PSD-95 expression at glutamatergic synapses, supporting a role for N-cadherin in PSD-95 regulation (Pielarski et al., 2013, Nikitczuk et al., 2014). These results suggest that the reduction in PSD-95 may reflect decreased hyperexcitability in the hippocampus. However, despite the effects on the pathophysiology, the treatments did not have an effect on epilepsy development, seizure load or cognitive function. This is in contrast to previous reports on delayed development of epilepsy following pilocarpine-induced SE, after treatment with levetiracetam (Margineanu et al., 2008, Glien et al., 2002). Several experimental studies have reported anti-epileptogenic effects and reduced seizure burden following levetiracetam therapy in animal models of epilepsy (Loscher et al., 1998, Stratton et al., 2003, Christensen et al., 2010). Levetiracetam is widely used in the clinic and has anti-convulsive properties in patients with epilepsy. The underlying mechanisms by which levetiracetam facilitates these mechanisms are mainly through inhibition of the synaptic vesicle protein 2A and studies have shown that it exerts disease-modifying effects in both patients and animal models (Klitgaard and Pitkanen, 2003). While our results did not display any changes in seizure frequency, there may be several reasons for this observation. The discrepancy can be attributed to either dose-dependent effects or inherently different animal models, with differences in treatment-resistant seizures. Indeed, Glien et al report a decreased seizure burden in a study where only secondary generalized seizures were recorded, while studies indicate that levetiracetam is more effective in reducing secondary generalized seizures compared to complex partial seizures (Leppik et al.,

2003). Moreover, the half-life of levetiracetam in rats is 2-3h, compared to 6-8h in humans (Loscher et al., 1998, Dooley and Plosker, 2000), suggesting that the drug is more rapidly cleared in rats compared to humans, making it difficult to reach effective and steady therapeutic levels in rats. On the other hand, it is also likely that the expected effect from levetiracetam treatment does not manifest within 4 weeks of treatment following NCSE. Consequently, future studies that evaluate doses, routes of administration and the possible disease-modifying effects of levetiracetam at later stages after electrically-induced NCSE are warranted. Similarly, while N-cadherin antibody infusion does not affect seizure frequency and duration either, we cannot exclude the possibility of effects with dose-adjusted, long-term administration.

FUTURE PERSPECTIVES

Although significant progress has been made in terms of classification, diagnosis and pharmacological management, epilepsy still remains a challenge in the clinic and many patients remain pharmacoresistant to AEDs. In addition, the ability to prevent epilepsy following a precipitating injury is a great unmet clinical need (Loscher et al., 2013) and the post-injury interval offers a time window to prevent or delay epilepsy development. Currently, all clinically available drugs are targeted at neurons, and little focus has been on the neuroinflammatory mechanisms and glial cells. However, our increased knowledge about brain inflammation in seizure potentiation supports an important role of inflammatory mechanisms in the propagation of epileptogenesis and epileptic activity, and further stratification of these pathways is needed to better understand the functional implications of inflammation in a context-dependent manner. This notion further underlines the importance of the immune response as a predictor and biomarker for SE and epilepsy development, a field that strongly relies on developing non-invasive, quantifiable tools for diagnostic, prognostic and therapeutic purposes. Furthermore, network abnormalities common to both cognitive impairment and epilepsy are far from being completely understood and future studies need to address these alterations. Understanding the network architecture and pathways underlying network reorganization in epileptic conditions and epilepsy development, including the role of newly formed neurons in the hippocampus and changes in synaptic protein expression, can help in restoring brain functions. Notably, modifying synaptic proteins that tune synaptic activity, as a possible strategy in treatment and biomarker purposes needs to be further addressed in epilepsy. Finally, utilizing relevant animal models will aid in underpinning the complex pathophysiology at multiple levels in epileptogenesis and help in validating the sensitivity and specificity of biomarkers.

Thus, identifying the complex interactions between the peripheral and local pathological changes in a network disorder that not only manifests in the brain, will help us dissect out the functional consequences of these changes in terms of epilepsy development and seizure-propagation. Future success of drug and biomarker development requires effective translation of experimental animal research to clinical settings and to more effectively bridge the clinical and preclinical areas in terms of improving the predictive value of biomarkers and ultimately offer better clinical outcomes for patients with respect to diagnosis, prognosis and treatment.

CONCLUDING REMARKS

Developing new therapeutics in epilepsy is challenging, not least because of the complexity and the heterogeneous nature of the epileptic disorders. This thesis provides evidence of a widespread and developing pathology associated to NCSE, a condition that clinically is considered not to give rise to any long-lasting changes in the brain, thus urging re-evaluation of current intervention and treatment strategies. In addition to significant inflammation in the brain, we show changes in the peripheral immune system and a distinct pathological profile in serum of rats that subsequently develop spontaneous seizures and epilepsy. Moreover, we provide the first evidence that epileptic seizures lead to an immune response in the retina, sharing similarities to the immune response in the epileptic focus. We also show that modulation of the neuron-glia cross talk, mediated by the fractalkine/CX3CR1 pathway by blocking CX3CR1, can reduce seizure-induced pathology in the hippocampus as well as in the retina, and that treatment with the commonly used levetiracetam in combination with an intracerebral antibody against N-cadherin also modulates brain pathology. Further studies need to explore the ability of CX3CR1 immune modulation to delay seizure development and epileptogenesis. Moreover, our studies provide a model that is highly relevant for future investigations aiming at elucidating the mechanisms behind the pathophysiological changes associated with NCSE and treatment-resistant epilepsy.

In conclusion, this thesis has provided substantial evidence of pathology in NCSE and demonstrates a widespread pathophysiological profile during the development of epilepsy. These crucial steps are instrumental for improving diagnostic/prognostic and therapeutic strategies and may aid in the development of novel and more easily accessible diagnostic and prognostic biomarkers with direct clinical value as a predictor of subsequent epilepsy development and disease progression.

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About the author

Una Avdic started her doctoral studies at the department of clinical sciences in 2014, in the group of 'Inflammation and stem cell therapy' at Lund University, where she has been working on understanding the pathological changes associated to epilepsy. In particular, she has been studying and characterizing the role of immune responses and synaptic protein expression before and after the development of epilepsy.

The thesis defense will take place on Tuesday, December 11 at Belfragesalen, D15 BMC, Lund University.

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