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PHARMACOLOGICAL APPROACHES TO STUDY AND COUNTERACT EPILEPTOGENESIS IN ACQUIRED EPILEPSY:
FOCUS ON THE ROLE OF MICROGLIA

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Degree of Doctor of Philosophy

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ABSTRACT

For decades, research on the mechanisms underlying the development of epilepsy has focused mainly on neurons, and more recently research activities have addressed the role of non-neuronal cells, in particular microglia and astrocytes. Microgliosis is a common phenomenon occurring in brain tissue exposed to epileptogenic insults as well as during recurrent seizures in animal models. Microgliosis is also described in brain specimens from human drug-resistant epilepsy foci. This evidence raised the hypothesis that microglia may contribute to pathologic outcomes in epilepsy, such as seizures, neuronal cell loss and neurological comorbidities.

We focused our studies on understanding the temporal pattern of microglia reactivity and proliferation in a murine model of acquired epilepsy, in order to explore whether microglia play a role in epilepsy outcomes. To this aim, CSF1R inhibitors (CSF1Ri), namely PLX3397 and GW2580, were administered to mice undergoing epilepsy development to (1) deplete microglia or (2) to block microglia proliferation, respectively. The results showed that CSF1Ri did not modify synaptic transmission or neuronal excitability, neither prevented epilepsy development. However, CSF1Ri prevented salient neuropathological features of epilepsy, such as structural abnormalities (cortical thinning), neuronal cell loss and cognitive impairment. Notably, blockade of microglial proliferation in mice with already established chronic epilepsy reduced seizures frequency.

Our findings highlight that microglia play distinct roles in distinct disease stages and suggest that timely interference with microglia may attenuate neuronal cell loss and seizures.

Our set of evidence separates cell loss from seizure development in epilepsy and highlights microglia as a cellular target for early neuroprotective intervention. Moreover, since microglial proliferation during the chronic disease stage contributes to seizures, pharmacological interference with this microglia function may offer a potential target for improving seizures control.
Acknowledgements

First, I would like to thank my supervisor, Dr. Annamaria Vezzani for giving me the opportunity to attend the Open University PhD program. I am grateful to her for the scientific guidance and her support that undoubtedly accompanied me throughout these four years. Thank you, Annamaria!

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I am grateful to Dr Teresa Ravizza for her supervision in the project and for sharing her experience that definitely helped me grow up professionally.

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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AQP4</td>
<td>Acquaporin 4</td>
</tr>
<tr>
<td>ASDs</td>
<td>Anti-seizures drugs</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>C1q</td>
<td>Complement component 1q</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine-ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD11b</td>
<td>Integrin alpha M subunit</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Tyrosine recombinase enzyme</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CSF1Ri</td>
<td>Colony stimulating factor 1 receptor inhibitor</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Chemokine (C-X3-C motif) ligand 1</td>
</tr>
<tr>
<td>DAM</td>
<td>Disease-associated microglia</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DTA</td>
<td>Difteria Toxin A</td>
</tr>
<tr>
<td>DTR</td>
<td>Difteria toxin A receptor</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EEC</td>
<td>ENIGMA-Epilepsy Consortium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EEG</td>
<td>Electroencephalographic</td>
</tr>
<tr>
<td>ENIGMA</td>
<td>Enhancing Neuro Imaging Genetics by Meta-Analysis</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAT1</td>
<td>GABA transporter type 1</td>
</tr>
<tr>
<td>GC</td>
<td>Granule cells</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HSVTK</td>
<td>Herpes simplex virus thymidine kinase</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>Ih</td>
<td>HCN-mediated current</td>
</tr>
<tr>
<td>ILs</td>
<td>Interleukins</td>
</tr>
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<td>ILAE</td>
<td>International league against epilepsy</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid or kainate</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccaride</td>
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<tr>
<td>MDTs</td>
<td>Multidrug transporters</td>
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<tr>
<td>MES</td>
<td>Maximal electroshock</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage inhibitory factor</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTLE</td>
<td>Mesial temporal lobe epilepsy</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
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<td>NORSE</td>
<td>New onset refractory status epilepticus</td>
</tr>
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<td>NORT</td>
<td>Novel object recognition test</td>
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<td>P2X</td>
<td>Purinergic receptor</td>
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<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>P-gp</td>
<td>P glicoprotein</td>
</tr>
<tr>
<td>PPF</td>
<td>Paired pulse facilitation</td>
</tr>
<tr>
<td>PS</td>
<td>Population spike</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
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<td>S100-β</td>
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<td>SE</td>
<td>Status epilepticus</td>
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<td>Subgranular zone</td>
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<td>SV2A</td>
<td>Synaptic vesicle glycoprotein 2A</td>
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<td>SVZ</td>
<td>Subventricular zone</td>
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<td>TGF-β</td>
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<td>Temporal lobe epilepsy</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Trem2</td>
<td>Triggering receptor expressed on myeloid cells 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1: INTRODUCTION
1.1 Definition of Epilepsy

The International League against Epilepsy (ILAE) has defined epilepsy as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition” (Fisher et al., 2014). Epilepsy is defined as a disorder or a family of disorders to emphasize that it includes different diseases and clinical conditions. Since the term “disorder” is poorly understood by lay people and may minimize the burden of epilepsy, ILAE recently agreed that epilepsy should be considered a disease (Fisher et al., 2014).

The primary hallmark of epilepsy is the seizure, defined as “an episode of neurologic dysfunction during which synchronized neuronal firing leads to clinical changes in motor control, sensory perception, behaviour or autonomic function” (Stafstrom, 2006). A seizure is a transient alteration of brain activity that may be an objective sign or a subjective symptom and can be associated to loss of awareness, stiffening, jerking as well other emotional and cognitive sequelae. The clinical manifestations of seizures depend on the involved brain area(s) (Devinsky et al., 2018).

The epileptic seizures are caused by hyperexcitability of clusters of neurons and by the hypersynchrony of neuronal networks in the brain. Hyperexcitability refers to a reduced physiological threshold of neuronal excitability, which contributes to the generation of a seizure event. Hypersynchrony refers to a population of neurons that fire at the same time at a similar rate (Stafstrom, 2006).

1.1.1 Classification of Epilepsy

The ILAE classification framework, which was revised in 2017, is a guide for the diagnosis of individuals presenting with seizures. Epilepsy classification begins with the determination of whether seizure initiation, referred to as onset, is focal or generalized (Fisher, 2017). Seizures are focal when they arise in one or more localized brain regions or hemisphere, generalized when they begin with a widespread distribution over both
Seizures have unknown onset when the onset is not identified but other manifestations are known (Fisher et al., 2014; Devinsky et al., 2018). A schematic classification of seizures is reported in Figure 1.1: seizures are determined on clinical grounds, according to behavioural manifestations, imaging and electroencephalographic (EEG) findings.

![Figure 1.1](image)

**Figure 1.1.1 - Seizures classification by ILAE 2017.** a) Basic seizures classification. b) Expanded seizures classification according to the presence of motor features; taken by Devinsky *et al.*, 2018.

After determining the seizures type, the clinical diagnosis of epilepsy is defined by any of the following conditions: (i) at least two unprovoked seizures occurring >24 h apart, (ii) one unprovoked seizure and an increased probability of further seizures like the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the next 10 years (Fisher *et al.*, 2014). Evidence for increased probability of having additional seizures
includes patterns of epileptiform activity on EEG and abnormalities on brain imaging (Falco-Walter et al., 2018).

Clinically, the epilepsy type is diagnosed and classified as generalized epilepsy (i.e., absence, myoclonic, atonic, tonic, tonic-clonic seizures) and focal epilepsy (i.e., the level of awareness can be impaired or not, motor or non-motor seizures). A “combined generalized and focal epilepsy” and an “unknown” category have been included in the classification since many epilepsies include multiple types of seizures (Scheffer et al., 2017) as depicted in Figure 1.1.2 (Devinsky et al., 2018). Seizures type, EEG and imaging features that tend to occur together are incorporated into a cluster of features and diagnosed as an epilepsy syndrome (Scheffer et al., 2017; Falco-Walter et al., 2018).

![Figure 1.1.2 - Framework for the classification of the epilepsies](image)

**Figure 1.1.2 - Framework for the classification of the epilepsies.** Classification bases on the diagnosis of an epileptic seizure type, which is determinant for the diagnosis of an epilepsy type and potentially an epilepsy syndrome. If possible, aetiology and associated comorbidities should be identified and considered. * Seizure onset. Taken by Devinsky et al., 2018.

Epilepsies are often classified according to their causes. The ILAE has defined six etiologic categories: (1) *structural*, when structural abnormalities visible on neuroimaging are likely the cause of the patients’ epilepsy. Abnormalities may be acquired after an acute injury or due to malformations of cortical development; (2) *genetic*, from a known or presumed pathogenic variant (mutation) of significant effect in causing the individual’s epilepsy; (3) *infectious*, the commonest aetiology worldwide when seizures are a core symptom of the disorder or epilepsy is developed after the acute infection, (4) *metabolic*, in
case of a well delineated metabolic defect with seizures depending on biochemical changes throughout the body (such as porphyria, uremia etc); in many cases, metabolic disorders will have a genetic defect; (5) Immune, when seizures are symptoms of an immune disorder (e.g, autoimmune-mediated); (6) unknown aetiologic category, when a specific diagnosis is not possible (Scheffer et al., 2017). During the diagnosis workflow, neurological comorbidities such as cognitive impairment and psychiatric disorders should be considered (Devinsky et al., 2018).

1.1.2 Temporal Lobe Epilepsy

Among acquired human epilepsies is temporal lobe epilepsy (TLE), which represents a common form of epilepsy characterized by drug-resistant focal seizures originating from temporal lobe structures (Engel et al., 2012). The ILAE does not recognize TLE as unique syndrome, indeed many forms of TLE exist (Berg and Cross, 2010). Mesial (M) TLE is one of the most prevalent form of TLE. The common pathological correlate of MTLE is hippocampal sclerosis, but it remains undetermined whether it is the initial cause of MTLE or its consequence (Engel et al., 2012). Hippocampal sclerosis is discussed in paragraph 1.6.

Potential causes of MTLE include mesial temporal sclerosis, perinatal injury, prolonged febrile seizures and glial tumours (Blair, 2012; Engel et al., 2012). One potential cause of TLE and other acquired epilepsies, most often mimicked in animal models, is status epilepticus (SE), "a clinical situation in which a seizure is manifested continuously for more than 5 minutes, or in which multiple seizures are repeated at very short intervals representing a continuous condition of epileptic activity". It is a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms which lead to abnormally prolonged seizures, or both. This clinical condition often results in long-term neurological consequences, including neuronal death and maladaptive alterations of neuronal networks, which may contribute to spontaneous seizures and neurological deficits (Trinka et al., 2015).
1.1.3 Unmet clinical needs

Epilepsy currently affects around 65 million people worldwide and is considered the third leading contributor to the global burden of disease for neurological diseases (Devinsky et al., 2018; Moshe et al., 2015).

People affected by epilepsy suffer the consequences of living with a chronic unpredictable disease that implicates the decline of their autonomy in the daily activities (Moshe et al., 2015). Epileptic seizures reduce the quality of life of patients by determining strong socioeconomic and educational disadvantages and by increasing the risk of death and injury (Ventola, 2014). Studies report a higher risk of premature mortality in individuals with epilepsy (Devinsky et al., 2018). Mortality is due either to direct effects of seizures (for example, sudden unexpected death) or indirect effects (for example, adverse effects of anti-seizure drugs (ASDs) or other drugs, accidents; Devinsky et al., 2018).

The first-line treatments for epilepsy are ASDs that in most cases are successful but mainly symptomatic treatments. However, despite the availability of various ASDs, approximately one-third of newly diagnosed patients fails to achieve seizure control. This condition, defined as drug-resistance, is referred to as the failure of at least two ASDs that are appropriately chosen, adequately dosed, and used for an appropriate period (Kwan et al., 2010). Clinically, drug-resistance can occur with distinct patterns: de novo, when patient never experience a seizure-free time window from the onset of the epilepsy; delayed resistance, when seizures freedom is initially achieved but then seizures become uncontrollable; waxing-and-waning pattern, with fluctuations between seizures control and drug-resistance; or forms of drug resistant epilepsy which becomes drug responsive (Schmidt and Löscher, 2005). For drug-resistant patients, surgery has the highest probability of attaining seizure freedom by removing the epileptic focus. However, patients may not be eligible for surgery. Neuro-stimulation devices, dietary therapies or clinical trials with new ASDs are the remaining options (Moshe et al 2015; Devinsky et al., 2018).
Another major issue in epilepsy management is the lack of therapeutic options for preventing epilepsy in those patients at high-risk of developing epileptic seizures after an acute brain injury such as neurotrauma, stroke and SE (White and Löscher, 2014). In addition, current pharmacotherapy does not arrest epileptogenesis or resolve the epilepsy-associated co-morbidities. Thus, the need for more effective therapies remains urgent.

1.2 Neuro-centric pharmacotherapy of epilepsy: towards non-neuronal targets

Historically, search for treatments of seizures in epilepsy has focused on drugs targeting neuronal mechanisms of hyperexcitability and hypersynchrony, thus providing a variety of cellular targets for modifying the intrinsic excitability properties of neurons and modulating classical neurotransmission (Rogawski et al., 2016). As reported in Table 1.2.1, ASDs can be classified into broader categories (1) the modulators of voltage-gated ion channels, including sodium, calcium, and potassium channels. (2) The enhancers of GABA-mediated inhibition by acting on GABA<sub>A</sub> receptors, the GABA transporter (GAT1), the GABA-synthesizing enzyme glutamic acid decarboxylase or the GABA-metabolizing enzyme GABA transaminase. (3) The direct modulators of synaptic release by targeting the release machinery, including synaptic vesicle protein 2A (SV2A) and the α2δ subunit of voltage-gated calcium channels. (4) The inhibitors of synaptic excitation mediated by ionotropic glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors (Rogawski et al., 2016).
In order to overcome the seizure burden in patients with epilepsy, ASDs have been administered either in monotherapy or in various combinations (Devinsky et al., 2018; Löscher et al., 2020). However, when epilepsy displays drug-resistance, the chance of therapeutic success with further drug combinations becomes progressively less likely (Chen et al., 2018). This also suggested that the development of new ASDs based on the neuro-centric principles may not provide substantial improvement in the pharmacotherapy of epilepsy. Thus, the search for novel therapeutic targets outside classical ion channels and chemical neurotransmission is currently an area of intensive investigation for developing novel strategies for medically refractory epilepsy (Boison, 2010; Löscher et al., 2020).

In the last two decades, research has focused on the mechanisms underlying epileptogenesis (the development of epilepsy), far beyond the mere processes underlying

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**Table 1.2.1 - Mechanisms of action of ASDs (adapted from Rogawski et al., 2016)**

<table>
<thead>
<tr>
<th>Molecular target</th>
<th>ASDs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Voltage-gated ion channels</strong></td>
<td></td>
</tr>
<tr>
<td>Voltage-gated sodium channels</td>
<td>Phenytoin, fosphenytoin, carbamazepine</td>
</tr>
<tr>
<td>Voltage-gated calcium channels</td>
<td>Ethosuximide</td>
</tr>
<tr>
<td>Voltage-gated potassium channels</td>
<td>Ezogabine</td>
</tr>
<tr>
<td><strong>GABA inhibition</strong></td>
<td></td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptors</td>
<td>Phenobarbital, primidone, benzodiazepines,</td>
</tr>
<tr>
<td>GABA transporter</td>
<td>including diazepam, lorazepam, and clonazepam;</td>
</tr>
<tr>
<td>GABA transaminase</td>
<td>Tiagabine</td>
</tr>
<tr>
<td><strong>Synaptic release machinery</strong></td>
<td></td>
</tr>
<tr>
<td>SV2A α2δ subunit Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
<td>Levetiracetam and its analogues</td>
</tr>
<tr>
<td><strong>Ionotropic glutamate receptors</strong></td>
<td></td>
</tr>
<tr>
<td>AMPA receptor</td>
<td>Gabapentin, pregabalin</td>
</tr>
<tr>
<td>Mixed/unknown</td>
<td>Perampanel</td>
</tr>
<tr>
<td></td>
<td>Valproate, felbamate, topiramate, zonisamide, rufinamide, adrenocorticotrophin</td>
</tr>
</tbody>
</table>
ictogenesis (the transition from interictal state to a seizure), thus leading to a new concept for epilepsy therapy and for biomarkers discovery.

In particular, non-neuronal cells have been studied to address their potential role in the pathogenesis of seizures and comorbidities, and as putative new targets to counteract the cascade of events that lead to epileptic seizures generation and recurrence (i.e., epileptogenesis).

1.3 Epileptogenesis

Epileptogenesis is a complex and dynamic multifactorial process converting a healthy brain network into a hyperexcitable one, thus having an enhanced probability to generate spontaneous recurrent seizures (Pitkanen et al., 2015). It is characterized by the development and extension of tissue capable of generating spontaneous seizures, thus resulting in the development of an epilepsy condition and its progression after the condition is established (Pitkanen and Engel, 2014). Both acquired injuries, such as neurotrauma, stroke, infections, SE, as well as gene mutations trigger epileptogenesis, as observed in patients and in animal models (Pitkanen et al., 2015). Epileptogenesis differentiates from the term ictogenesis that is the propensity to generate epileptic seizures, including initiation and evolution of the epileptic seizures, otherwise referred to as the transition from an interictal to a seizure state (Blauwblomme et al., 2014; Pitkanen and Engel, 2014).

The epileptogenic cascade of events is envisaged to occur after an acute event or a gene mutation, or both, that disturb brain homeostasis, followed by a clinically silent period and then by the occurrence of spontaneous seizures (Thom, 2014). The term epileptogenesis was initially referred to the period between the epileptogenic insult and diagnosis of epilepsy (Figure 1.3.1 A). As revised by ILAE, it is now considered a continuum process (Figure 1.3.1 B) that starts at the time of the brain injury and extends beyond the first spontaneous seizure (Dudek and Staley, 2012; Pitkanen et al., 2015). This concept is therapeutically relevant since it suggests that epileptogenesis can be targeted also after the onset of the
disease. Therefore, the anti-epileptogenic interventions could be designed either for preventing epilepsy onset or for providing disease modification (i.e. less frequent seizures or seizures with shorter duration or reduced generalization of seizures, change from drug-resistant to drug-responsive seizures) and for improving the related neuropathology and comorbidities. Indeed, it is well-known that comorbidities such as anxiety, depression and cognitive decline often accompany epileptogenesis (Kanner et al., 2014). Thus, it is clinically relevant to study cellular and molecular modifications occurring during epileptogenesis to find new therapeutic strategies. These mechanistic insights may also help to find prognostic biomarkers in individuals at high-risk of developing epilepsy after an initial insult. Indeed, the study of epileptogenesis has important implications for both therapy and biomarkers discovery (Pitkanen and Engel, 2014; Thom, 2014).

![Figure 1.3.1 - Definitions of epileptogenesis](image.png)

**Figure 1.3.1 - Definitions of epileptogenesis.** (A) Epileptogenesis was previously considered to occur during the latent period between the brain insult and the first unprovoked seizure. (B) Currently, epileptogenesis is considered to extend beyond the latent period and the first clinical seizure (*taken by* Pitkanen et al., 2015).

1.3.1 *Experimental models of epilepsy and epileptogenesis*

The pathogenesis of seizures and the potential targets for therapy as well as biomarker discovery can be studied in animal models that mimic the clinical condition, and subsequently targets and biomarkers should be validated in eligible patient populations. Spontaneous seizures in rodents are induced by different brain injuries (i.e., structural,
infectious, SE) or by genetic interventions (Devinski et al., 2018). Also, non-rodent species represent useful model organisms to study seizures and epilepsy, such as cats and dogs, or non-mammalian species, such as zebrafish (Devinsky et al., 2018). Table 1.3.1 (taken by Raol and Brooks-Kayal, 2012) summarizes a few examples of commonly used models of seizures and epilepsy that mirror the human condition.

Table 1.3.1 - Commonly used models of seizures and epilepsies

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Human condition</th>
<th>Common use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilocarpine</td>
<td>Focal (temporal lobe)</td>
<td>Mechanism of epileptogenesis, cognitive deficits and epilepsy, therapy development</td>
<td>(Curia et al., 2008)</td>
</tr>
<tr>
<td>Kainic acid</td>
<td>Focal (temporal lobe)</td>
<td>Mechanism of epileptogenesis, cognitive deficits and epilepsy, therapy development</td>
<td>(Williams et al., 2009)</td>
</tr>
<tr>
<td>Pentylene tetrazol</td>
<td>Generalized seizures</td>
<td>Seizure mechanism, drug screening</td>
<td>(Lösgcher, 2009)</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Multiple acute seizures, childhood epilepsies</td>
<td>Seizure-induced cognitive deficits</td>
<td>(Velísek et al., 1995)</td>
</tr>
<tr>
<td>Focal application of penicillin, picrotoxin</td>
<td>Focal (neocortical) seizures</td>
<td>Mechanism of seizure generation and spread</td>
<td>(Fisher, 1989)</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>Focal seizures</td>
<td>Mechanism of seizure generation and spread, mechanism of epileptogenesis</td>
<td>(Fisher, 1989)</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>Infantile spasms</td>
<td>Mechanism of long-term consequences of seizures, therapy development</td>
<td>(Stafstrom, 2009)</td>
</tr>
<tr>
<td>Doxorubicin + lipopolysaccharides + p-chlorophenylamine</td>
<td>Infantile spasms</td>
<td>Mechanism of long-term consequences of seizures, therapies development</td>
<td>(Chudomelova et al., 2010)</td>
</tr>
<tr>
<td>Electrical and chemical kindling</td>
<td>Focal (temporal lobe) epilepsy</td>
<td>Mechanism of epileptogenesis, anticonvulsant and antiepileptogenic drug screening, cognitive deficits</td>
<td>(Morimoto et al., 2004)</td>
</tr>
<tr>
<td>Maximal electroshock (MES)</td>
<td>Generalized seizures</td>
<td>Drug screening</td>
<td>(Lösgcher, 2011)</td>
</tr>
<tr>
<td>Trauma</td>
<td>Focal epilepsy</td>
<td>Mechanism of epileptogenesis, cognitive deficits and cell death, therapy development</td>
<td>(Pitkänen et al., 2009)</td>
</tr>
<tr>
<td>Hypoxia/ischemia</td>
<td>Hypoxic–ischemic encephalopathy</td>
<td>Mechanism of epileptogenesis, cognitive deficits and cell death, therapy development</td>
<td>(Jensen and Baram, 2000)</td>
</tr>
<tr>
<td>Temperature</td>
<td>Febrile seizures</td>
<td>Mechanism of long-term consequences of seizures, therapy development</td>
<td>(Bender et al., 2004)</td>
</tr>
<tr>
<td>Genetic models</td>
<td>Mutations-linked epilepsies</td>
<td>Mechanism of seizures and epilepsy</td>
<td>(Avanzini et al., 2007)</td>
</tr>
<tr>
<td>\textit{In vitro} models</td>
<td>Seizures</td>
<td>Mechanism of seizures, preliminary drug screening</td>
<td>(Wahab et al., 2010)</td>
</tr>
</tbody>
</table>
The choice of the appropriate rodent model, including the sex, age and strain, provides an opportunity to investigate epilepsy development, since the epileptogenic process including the onset time of spontaneous seizures, their frequency and severity and the associated comorbidities, can be monitored prospectively in individual animals (Devinsky et al., 2018; Pitkänen et al., 2017).

A wide range of experimental models of seizures and epilepsy are available. Models of acute seizures are helpful to investigate ictogenesis and can be used to screen anti-seizures drugs. These models are characterized by self-remitting seizures induced by single or repetitive injections of chemo-convulsive agents (kainic acid, bicuculline, pentylentetrazol), by electrical stimulation or short hyperthermia. Acute seizures models are not apt to study epileptogenesis therefore they provide limited information on the potential therapeutic effects of antiepileptogenic drugs (Simonato et al., 2014).

To best address epileptogenesis, chronic epilepsy models should be used to monitor spontaneous seizures onset and recurrence in longitudinal studies. The use of animal models allow to study the molecular, cellular and structural changes occurring in the brain during epileptogenesis, and in particular before seizure onset, since human tissue is generally unavailable to study early stages of disease development (Becker, 2018).

In vivo models of acquired epilepsy are induced by various types of lesions, including SE, traumatic brain injury, hyperthermia, and viral infection mimicry (Becker, 2018; Loscher, 2002). SE causes up to 10% of all acquired human epilepsies and can be induced experimentally by electrical stimulation of specific seizure-prone brain areas or by chemoconvulsants (e.g., paraoxon, kainic acid and pilocarpine), which administration can be systemic, intraventricular or intracerebral (e.g., intra-cortical, intra-hippocampus or intra-amygdala). Both electrical and chemical SE are followed by a latent period that lasts few days to weeks depending on the model, then spontaneous seizures occur, and chronic epilepsy develops. After SE, several hallmarks of epileptogenesis have been described
including reactive gliosis, neuronal cell loss and synaptic reorganization thus replicating salient features of the neuropathology described in patients with MTLE (Iori et al., 2013).

1.3.2 Temporal lobe epilepsy: the role of the limbic structures

Seizures in TLE mainly involve the limbic structures of the temporal lobe, accordingly surgical resection of this region often provides seizures control (Clusmann and Schramm, 2012). TLE seizures may originate in the hippocampus, in the amygdala or in the entorhinal cortex as assessed by electroencephalogram recordings and shown by electrophysiological characterizations (Bartolomei et al., 2005; Levesque et al., 2015; Turski et al., 1989).

One of the brain areas mainly involved in neuronal hyperexcitability underlying seizures in acquired epilepsies, as TLE or post-traumatic epilepsy, is the hippocampus. This brain area is part of the temporal lobe and includes the dentate gyrus (DG), the hippocampal formation or Cornu Ammonis (CA), subdivided into CA1, CA2, CA3 and CA4 sectors, and the subiculum (Anand and Dhalbiv, 2012). Three layers characterize each region: the molecular (I), the cellular (II) and the polymorphic (III) layers. The pyramidal neurons are the principal excitatory cells of CA1-CA4 sectors, while granule cells (GC) form the main cellular layer of the DG. These cells are interconnected forming an axonal circuitry defined trisynaptic circuitry, as depicted in Figure 1.3.2. The GCs are the principal excitatory cell type in the DG (granule cell layer) and their axons, named the mossy fibres, project to the pyramidal cells of the CA3 region. GCs include the hilar region. A number of diverse interneurons population occupies the hilar region, named the polymorphic layer (Navidhamidi et al., 2017). Hilar interneurons comprise the glutamatergic mossy cells with excitatory input to the GCs, (Buckmaster et al., 1996; Jackson and Scharfman, 1996; Wenzel et al., 2000) and the GABAergic inhibitory interneurons (Acsády et al., 1998; Forti and Michelson, 1998; Wenzel et al., 1997), such as pyramidal basket cells and diverse neuronal subtypes, mostly immune-reactive for GABA-ergic and co-localizing with other neuroactive
compounds such as parvalbumin, calretinin, somatostatin and neuropeptide Y (Ribak, 1992; Sloviter, 1994).

One of the pathological features of hippocampal sclerosis in TLE, is neuronal cell loss in the polymorphic layer of the dentate hilus (Jinde et al., 2013). Studies support that selective loss of hilar interneurons contributes to the development of epileptogenesis in animal models (Heinemann et al., 1992; Hsu, 2007; Huusko et al., 2015). For example, the loss of GABA-ergic interneuron subtypes in human epileptic hippocampi, such as parvalbumin-positive neurons, was proposed as a mechanism that promotes epileptogenesis by reducing the inhibition on the excitatory GCs in the DG and pyramidal cells in the hippocampus, thus promoting a hyperexcitable state (Arellano et al., 2004; Drexel et al., 2017). Similarly, the loss of hilar glutamatergic mossy cells has been proposed as a mechanisms that promotes hyperexcitability since inhibitory interneurons do not receive their excitatory input and in turn fail to inhibit GCs (Jinde et al., 2013; Sloviter, 1991). Moreover, the loss of mossy cells was described to trigger mossy fibres sprouting, which generates aberrant innervation of post-synaptic targets such as granule cells (Sutula et al., 1989) thus promoting hyperexcitability (Jiao and Nadler, 2007; Jinde et al., 2013; Nadler, 2003). Therefore, there is selective loss of mossy cells and GABAergic interneurons subtypes in the DG in TLE, whereas GCs are less affected (Jinde et al., 2013). GCs can undergo cell dispersion, i.e widening of GC layer (Houser, 1990; Thom et al., 2005). However, the association between the vulnerability of neurons subclasses and the risk of spontaneous seizures is not clear (Arellano et al., 2004; Buckmaster and Dudek, 1997), even though an aetiology-specific link cannot be excluded (Huusko et al., 2015).

Anatomically, the hippocampus receives inputs from other structures of the temporal lobe, such as perirhinal, parahippocampal and entorhinal cortices. A major input to the DG, namely the perforant path, originates from the entorhinal cortex (Amaral et al., 2007). The flux of information between the DG of the hippocampus and the entorhinal cortex (EC) is coordinated by the trisynaptic circuitry, as depicted by Figure 1.3.2 (Deng et al., 2010). The
EC represents an example of a *cortico-cortical loop* that can amplify and propagate seizure discharges (Vismer et al., 2015). In TLE, the hippocampal-EC communication is critical for generation and propagation of epileptic activity (Vismer et al., 2015). Moreover, the hippocampus and the EC are both involved in cognitive functions that are often impaired in TLE (Acsády and Káli, 2007; Andersen et al., 1971).

The EC afferent fibres project to the dendrites of the GCs in the molecular layer of the DG (Amaral et al., 2007). The DG is a major route of information to the hippocampal formation and due to its unique anatomical organization, the DG processes the cortical sensory information that underlies episodic memories (Amaral et al., 2007) and modulates the cortical excitation inputs into the hippocampal formation.

*Figure 1.3.2 - The hippocampal circuitry.* (a) Drawing of the hippocampal trisynaptic circuitry; (b) the excitatory trisynaptic pathway EC–dentate gyrus–CA3–CA1–EC illustrated by solid arrows.
Evidence from animal models and TLE patients indicate that the DG acts as a filter that limits the excitatory input from the entorhinal cortex into the hippocampus, and disrupting this function may contribute to the epileptogenic process underlying TLE (Heinemann et al., 1992; Hsu, 2007; Navidhamidi et al., 2017).

The typical hippocampal sclerosis in TLE is characterized by extensive loss of CA1, CA3 and CA4 pyramidal neurons (Blümcke et al., 1999), and hilar interneurons, including inhibitory interneurons and excitatory hilar mossy cells (Sloviter, 1987, 1991), whereas most GCs and CA2 neurons are preserved (Blümcke et al., 2012).

The neuronal cell loss in the DG and the GCs dispersion (Aronica and Crino, 2011; Blumcke, 2009) may contribute to the hyperexcitability of granule cells (Sloviter, 1987, 1991; Zappone and Sloviter, 2004), and together with molecular, synaptic and cellular changes, including glia reactivity, may contribute to epilepsy development in experimental models (Pitkanen and Lukasiuk, 2011).

1.3.3 Mechanisms underlying epileptogenesis

After an epileptogenic injury, a complex network of changes has been described in the brain areas of seizure generation, best in the hippocampus although the epileptogenic process involves also other limbic and extralimbic areas (Jutila et al., 2002).
Figure 1.3.3 summarizes the modifications that occur during epileptogenesis, including neurodegeneration, aberrant neurogenesis, axonal sprouting, blood-brain barrier (BBB) dysfunction, reactive gliosis with neuroinflammation, recruitment of inflammatory cells into brain tissue, reorganisation of the extracellular matrix and of the molecular signature of individual neuronal and non-neuronal cells (Pitkanen and Lukasiuk, 2009, 2011).

Neurodegeneration occurs at a very early stage after SE and it does not appear to be associated with epilepsy development, although it may play a role in cognitive dysfunctions. Indeed, even though neuronal cell death has been reported after prolonged seizures, it is not yet understood whether neuronal cell death is one of the causes or a mere consequence of epileptic activity (Dingledine et al., 2014). For example, seizure-induced neuronal death is reported as the results of the excessive excitatory neurotransmission that provokes the entry of Na⁺ and Ca²⁺ into the cell, thus leading to osmolytic stress, swelling and excitotoxicity (Fujikawa, 2005). However, when a neuroprotective strategy is applied before or shortly
after SE, it may ameliorate the behavioural deficits and the functional decline, but epilepsy development is not prevented (Brandt et al., 2006; Pascente et al., 2016).

In addition to neuronal cell death, both axonal injury and axonal sprouting have been described during epileptogenesis (Pitkänen et al., 2000). In particular, mossy fibres sprouting has been proposed as one mechanism of increased network excitability, but this is still controversial if its prevention arrests epilepsy development (Buckmaster, 2012; Pitkänen et al., 2000). In particular, aberrant mossy fibres establish excitatory feedback circuitries with both normal and ectopic GCs in the hippocampus, thus potentially increasing excitability, but they also innervate inhibitory basket cells, thus reducing neuronal excitability (Buckmaster, 2012; Devinsky et al., 2018; Sloviter et al., 2006).

Neurogenesis is affected by seizures (Gray and Sundstrom, 1998). An epileptogenic event like SE transiently triggers adult neurogenesis, which then returns to baseline during epilepsy development, and is reduced in chronic epilepsy (Chen et al., 2020). The aberrant hippocampal neurogenesis triggered by seizures includes increased neural progenitors’ proliferation, impaired migration of newly born neurons leading to ectopic location of GCs in the hilus, thus determining aberrant connectivity and enhanced excitability (Jiruska et al., 2013; Parent et al., 1997; Pun et al., 2012; Scharfman, 2002; Scharfman and Gray, 2007; Shtaya et al., 2018). There is evidence that altered neurogenesis also affects learning and memory (Barkas et al., 2012; Westacott et al., 2021) and that strategies reducing this phenomenon, such as genetic ablation of newly born neurons, attenuate both seizures development and cognitive deficits in a murine pilocarpine model of chronic epilepsy (Cho et al., 2015; Danzer, 2019).

Glial cells react and proliferate during the epileptogenic process, but their contribution to epilepsy development is still poorly explored. A more detailed description of this phenomenon referred to as reactive gliosis is given next (paragraphs 1.3.4 and 1.4-1.6).

After an epileptogenic insult, also BBB permeability functions are altered and result in leakage of serum albumin into the brain parenchyma and its accumulation in astrocytes.
and neurons. It is reported that albumin activates TGFβ signalling in astrocytes by inducing a reduced expression of inwardly rectifying potassium channels and AQP4 water channels, and increased synthesis of inflammatory mediators. Gap junctions among astrocytes are also altered as well as glutamine synthase cellular location and glutamate reuptake (Ivens et al., 2007; Kim et al., 2012). Serum albumin also triggers aberrant neurogenesis and excitatory synaptogenesis acting as a potent pro-synaptogenic signalling molecule, suggesting that protection of BBB integrity may prevent these effects (Weissberg et al., 2015). In addition, clinical and experimental evidence showed that peripheral immune cells including monocytes, neutrophils and lymphocytes may enter the CNS in association with BBB damage and may exert effects on neuronal cell survival which are still under assessment (Prinz and Priller, 2017; Varvel et al., 2016; Zattoni et al., 2011). BBB impairment has also potential consequences for drug distribution into the brain (Löscher and Friedman, 2020). BBB opening has been also associated with induction of multidrug transporters (MDTs) such as P-glicoprotein (P-gp) that increases the efflux of ASDs from the brain into blood, and therefore has been proposed as one of the causes of drug resistance (Löscher et al., 2020; Schinkel and Jonker, 2003; Sun et al., 2003). Accordingly, P-gp blockers have been successfully applied to overcome drug-resistance in animal models of epilepsy (Brandt et al., 2006; van Vliet et al., 2006).

Demyelination was recently reported to be a critical mechanism in the pathogenesis of epilepsy, since findings have shown that CNS myelin sheaths are affected in epilepsy patients (Concha et al., 2009; de Curtis et al., 2021; Nilsson et al., 2008; Scanlon et al., 2013). Moreover, epileptic seizures also occur in patients affected by other demyelinating diseases, such as multiple sclerosis (Anderson and Rodriguez, 2011). The presence of demyelination associated with epileptogenesis was confirmed in animal models, with myelin damage accompanied by mature oligodendrocytes loss in the hippocampus of epileptic rats, in a lithium-pilocarpine model of epilepsy (Luo et al., 2015; Ye et al., 2013). The myelin sheath is formed by mature oligodendrocytes that surround axons to regulate electrical
impulse conduction and maintain neuronal communication. Despite only limited data on the mechanisms of myelin dynamics are available, data report that after demyelination and oligodendrocytes loss in the hippocampus of epileptic rats, transient changes in the reservoir of oligodendrocytes precursors occurred to repair myelin damage, thus implicating a role of myelin, oligodendrocytes and their precursors in epileptogenesis (Luo et al., 2015). 

Gene expression studies in experimental models have revealed altered gene transcription and changes in epigenetic mechanisms during epileptogenesis. Alterations in expression of voltage-gated and receptor-operated ion channels, neuropeptides, neurotrophins, and immune molecules are the result of these genomic and non-genomic changes (Dingledine et al., 2017; Pitkanen et al., 2015). The epigenetic mechanisms described in epileptogenesis include DNA methylation, histone modification and changes microRNA biosynthesis (Henshall and Kobow, 2015; Henshall et al., 2016). The available data show altered levels of microRNAs in the hippocampus of patients with TLE and in animal models of epilepsy; in particular, up to nine novel miRNAs appear to influence seizures or hippocampal pathology (Henshall et al., 2016). For example, silencing of brain-specific miR-134 exerted potent anti-seizure effects in experimental models (Henshall et al., 2016) and transient application of a synthetic mimic of miRNA 146a reduced spontaneous seizures in epileptic mice and prevented disease progression (Iori et al., 2017).

Severe brain insults such as SE may alter the molecular composition and the function of both voltage-gated and receptor-gated ion channels, thus resulting in acquired channelopathies in dendritic, somatic and axonal channels. Importantly, acquired channelopathies after the brain injury may contribute to lower seizure threshold (Bernard et al., 2004; Chen et al., 2001; Su et al., 2002). For example, Hyperpolarization-activated Cyclic Nucleotide-gated channels (HCN-) are voltage-gated ion channels, with the HCN1 subtype highly expressed in cortex and hippocampus, where it modulates the excitability of pyramidal neurons (Jung et al., 2011). Dendritic HCN1 loss is described in several studies as an acquired channelopathy in epilepsy (Jung et al., 2011; Marcelin et al., 2009; Shin et
al., 2008). In particular, both *in vitro* and *in vivo* models of acquired epilepsy have shown decreased HCN1 expression and function in CA1 pyramidal neurons and in the entorhinal cortex neurons, in early epileptogenesis and in chronic epilepsy, due to channel internalization and transcriptional downregulation (Jung et al., 2011). In a rat models of kainic acid-induced SE, HCN1 changes are reflected by decreased currents (I_h) 24 h after SE, in association with an enhanced excitability of EC pyramidal neurons (Shah et al., 2004).

Also T-type Ca^{2+} channels have a long association with epilepsy, due to their ability to promote neuronal bursts (Poolos and Johnston, 2012). During epileptogenesis CA1 pyramidal neurons displayed bursting behaviour associated with an increased currents mediated by T-type Ca^{2+} channels (Su et al., 2002; Yaari et al., 2007). Channels mediating T-type currents were also found to be upregulated in a pilocarpine model before the onset of spontaneous seizures (Becker et al., 2008; Poolos and Johnston, 2012).

**1.3.4 Neuron-astroglia interaction during epileptogenesis**

Glial cells cooperate to maintain brain homeostasis and experimental and human epileptic foci are characterized by changes in astroglia and microglia morphology, induced proliferation and changes in their molecular phenotype (Devinsky et al., 2018; Wetherington et al., 2008). Reactive gliosis is an important hallmark of epileptogenesis which attracts considerable interest for its potential role in epilepsy, and as a potential therapeutic target.

Although neurons remain the cardinal cells in the brain involved in the neurological manifestations of epilepsy, in the last decade research has been focused on the role of non-neuronal cells, namely astrocytes and microglia, in hyperexcitability phenomena (Wong, 2019).

Astrocytes and neurons communicate in a bidirectional way at various levels (Devinsky et al., 2013; Patel et al., 2019; Wong, 2019) being astrocytes the third element of the tripartite synapse. For example, *astrocytes* are involved in neuronal cell migration during brain development and contribute to neurotransmission and synaptic plasticity by regulating
the extracellular ions and water and by the release of gliotransmitters with neuromodulatory functions. Astrocytes contribute to maintain BBB permeability functions (Friedman et al., 2009; de Lanerolle et al., 2010). They are essential for regulating brain homeostasis and neuronal function (Dong and Benveniste, 2001). After acute brain injuries, astrocytic homeostatic functions such as regulation of extracellular K+ and water and glutamate re-uptake are disturbed resulting in alterations of neurotransmission, neuronal hyperexcitability, and promoting seizures activity (Devinsky et al., 2013; Robel et al., 2015).

Failure of both glutamate uptake and reduced glutamine synthesis have been reported in astrocytes in epilepsy models (Verhoog et al., 2020). In fact, glial glutamate transporters are downregulated (Ueda et al., 2001) and glutamine synthetase levels are decreased (Eid et al., 2019; Verhoog et al., 2020). Neuronal glutamate release determines the activation of the metabotropic glutamate receptors (mGluR5 and mGluR3) in astrocytes, which release gliotransmitters and cytokines (Aronica et al., 2005a, 2005b). Specifically, increase in intracellular Ca^{2+} oscillations resulting from altered neurotransmission, induces astrocytes to release gliotransmitters such as glutamate, D-Serine, ATP, adenosine and GABA (Navarrete et al., 2013). The release of glutamate by astrocytes leads to the activation of (extra-synaptic) NMDA receptors that evoke slow inward currents in neurons (Navarrete et al., 2013) that drive neuronal network synchronization, thus providing an example of the modulatory activity of astrocytes on synapses that may promote excitotoxicity.

D-serine is a relevant gliotransmitter in epilepsy binding to the glycine site of the NMDA receptor thereby promoting excitatory neurotransmission (Mothet et al., 2000). Moreover, the inflammatory mediators released by reactive astrocytes activate their cognate receptors on endothelial cells of the BBB, thus provoking tight junctions disruption that result in the influx of albumin, normally absent in the brain parenchyma (Devinsky et al., 2013; Heinemann et al., 2012). As demonstrated by studies on TLE patients and epilepsy models (Seifert and Steinhäuser, 2013), astrocytes are implicated in the pathophysiology of
epilepsy by influencing the synchronization of neuronal firing causing hyperexcitability, and contributing to seizures and neurotoxicity.
1.4 Microglia in the adult healthy brain

Microglia are the brain resident immune cells of the CNS and they provide homeostatic and protective functions to neurons during CNS development and in adult life under physiological conditions (Hammond et al., 2018). Microglia represent around 10% of adult CNS cells, with variable cell density across distinct CNS regions (Lawson et al., 1990). Recent fate-mapping studies have shown that microglia originate from early erythromyeloid progenitors in the extraembryonic yolk sac (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Kierdorf et al., 2013; Schulz et al., 2012). Early embryonic microglia differentiate into mature microglia (Matcovitch-Natan et al., 2016) but the process by which they migrate from the yolk sac to the brain and achieve their unique cell identity is poorly understood (Ginhoux et al., 2010; Hammond et al., 2018). Although microglia has been deeply studied in the last few decades, their origin during development and in the adult brain has been often debated (Ginhoux and Garel, 2018). In fact, due to the similarities with other myeloid cells, such as macrophages, adult microglia was initially believed both to self-renew upon local expansion and to derive from recruited peripheral blood monocytes (Ginhoux et al., 2013). Previous evidence has shown that circulating monocytes recruited into the brain parenchyma could differentiate into a microglial-like population (Ginhoux et al., 2013; Lawson et al., 1992), but recent pioneer studies using parabiosis have provided new evidence of microglial turnover, excluding the recruitment of blood monocytes to maintain microglia pool in the brain (Ajami et al., 2007; Ginhoux et al., 2010). Indeed, it is now well documented that bone-marrow-derived monocytes do not appear to generate brain microglia (Bruttger et al., 2015) and that microglial cells are maintained in brain through a self-renewing turnover, throughout development, adulthood and aging (Askew et al., 2017; Tay et al., 2017). In particular, the whole microglia population has a low-rate turnover and cells are self-renewed several times throughout life in mice with 1% of microglia dying by apoptosis each day (Askew et al., 2017; Tay et al., 2017). Despite species-specific differences in microglial
biology exist, findings in humans have confirmed that more than 96% of microglia can be slowly renew throughout life (Réu et al., 2017).

As depicted in Figure 1.4.1, microglia are highly adaptable cells of the CNS that play a role of surveillance of the brain microenvironment. They can physically interact with neurons, regulate neurogenesis and promote the phagocytosis of damaged neurons. They also contribute to the synapse formation, dendritic shaping and the engulfment of unnecessary synapses (Eyo et al., 2017; Hiragi et al., 2018). Microglia are key players for maintaining CNS health and promote tissue repair, and alterations of their homeostatic functions have been implicated in CNS diseases and pathological neuroinflammation (Morris et al., 2013; Nayak et al., 2014; Ransohoff and Perry, 2009; Tremblay et al., 2011). A more detailed insight of microglial functions is reported in the following paragraphs.

Figure 1.4.1 - Functional states of microglia in the healthy brain. (taken from Gomez-Nicola and Perry, 2015).
1.4.1 Microglial cells heterogeneity and their functional features

Historically, microglia have been phenotypically characterized based on their morphology, density and the presence of a panel of immune membrane molecules (De Biase et al., 2017; Lawson et al., 1990; Schmid et al., 2002). It is well-known that microglia morphology and function are tightly coupled (Gomez-Nicola and Perry, 2015), although microglia reactivity is a highly dynamic process which cannot be entirely described by their morphology (Davis et al., 2017; Streit et al., 1999). Cutting-edge technologies such as single-cell RNA sequencing and single-cell mass spectrometry allowed to describe the spatial and temporal diversity of microglia and to study the complex cell diversity based on different surface markers. Therefore, the spatial, temporal, and functional heterogeneity of microglia during CNS development, homeostasis, aging and disease have been described, as depicted in Figure 1.4.2 (Masuda et al., 2020), showing that these cells are heterogeneous in CNS regions and during the entire brain lifespan both in physiological and pathological conditions (Davis et al., 2017; Masuda et al., 2020).

The heterogeneity of microglia is a direct reflection of the local microenvironment that can induce reprogramming of microglia towards different functional states (Hammond et al., 2019; Masuda et al., 2019; Prinz et al., 2019; Wright-Jin and Gutmann, 2019). For example, in the injured adult brain, the neuronal expression or secretion of “find me” signals such as fractalkine (Sokolowski et al., 2014) and “eat me” signals such as calreticulin (Fricker et al., 2012), drive microglia towards a phagocytic phenotype for removing dying or damaged cells.

Heterogeneity of microglia phenotypes during brain development and in adult brain have been characterized (Masuda et al., 2019; Matcovitch-Natan et al., 2016), whereas evidence for spatial heterogeneity still remains scarce (Silvin and Ginhoux, 2018). Earliest evidence of microglia heterogeneity reported brain-region specific cell density which was higher in the hippocampus and thalamus and lower in the cerebellum (Lawson et al., 1990), and these findings were confirmed by advanced morphology analysis that highlighted
clusters of microglia subpopulations showing specific morphologies and functions after a neuroinflammatory challenge such as lipopolysaccharide (LPS) (Verdonk et al., 2016). Furthermore, diversity in microglial cell density has been reported also between male and female mice: cells were more abundant in cortex, hippocampus and amygdala in male vs female mice (Benusa et al., 2020). In addition, evidence exists that male and female microglia are functionally distinct and respond differently to noxious stimuli (Posillico et al., 2015; Villa et al., 2018).

As the main brain resident immune cells, the heterogeneity of microglia influences the brain milieu, since these cells continuously probe the brain microenvironment for detecting signals of damage or cell distress. Upon disturbance of CNS homeostasis, microglia reactively migrate to the sites of injury/infection/neurodegeneration and begin to proliferate (Davis et al., 2017). Microglia reactivity is best described as “a complex multistage activation process, which broadly encompasses their transformation from a ramified to an amoeboid morphology by first retracting the microglia processes and then extending dynamic protrusions, followed by cellular locomotion” (Davis et al., 2017).

Indeed, CNS injury triggers microglia that react by producing various inflammatory mediators and nitric oxide and become phagocytic (Hanisch and Kettenmann, 2007). For example, by releasing pro-inflammatory cytokines such as interleukins, tumour necrosis factor (TNF) and complement component 1q (C1q), microglia induce astrocytes to display a reactive phenotype, thus amplifying their response and contributing to a potentially harmful neuroinflammatory action in the brain parenchyma (van Rossum and Hanisch, 2004). Indeed, administration of LPS as a pro-inflammatory challenge to mice genetically ablated of microglia have shown that LPS alone does not induce astrocytes inflammatory phenotype, which is indeed microglia dependent (Liddelow et al., 2017). Also, microglial cytokines such as TNF can induce astrocytes to disrupt tight junctions, thus contributing to the BBB leakage (Wachtel et al., 2001). According to these studies, reactive microglia in
part coordinate and direct CNS neuroinflammation, also by attracting surrounding microglial cells to the site of activation (Garden and Möller, 2006).

As key players of synaptic connectivity in development, microglia also regulate synaptic pruning to eliminate supernumerary synapses in the adult brain (Hong and Stevens, 2016) by phagocytosis, induced by the astrocytic-mediated activation of the classical complement cascade (Schafer et al., 2012; Stevens et al., 2007). Synaptic stripping in the adult healthy and diseased brain was described as a mechanism that disrupt synapses through the engulfment of pre- or post-synaptic elements (Tremblay, 2011). Among the pathways that have been proposed to regulate this mechanism, a possible candidate is the microglia fractalkine receptor (CX3CR1) that plays an important role in the synapses maturation, elimination and functional connectivity, even though the involvement of this signalling in synaptic engulfment is not yet clear (Paolicelli et al., 2011; Zhan et al., 2014). ATP and purinoceptors are other molecular candidates that attract microglial processes towards the synapses (Kettenmann et al., 2013).

Microglia also regulate neurotransmission by expressing receptors for glutamate, GABA, monoamines and cannabinoids (Kettenmann et al., 2011). In response to a neurotransmitter stimulation in fact, microglia display a remarkable degree of phenotypic plasticity, in terms of morphology and release of molecules, and may exhibit either neurotoxic or neuroprotective functions (Davis et al., 2017). For example, it is reported that activation of NMDA receptor subunits on amoeboid reactive microglia enhances the release of inflammatory mediators such as TNF, IL-1β and nitric oxide (Murugan et al., 2011), which all exert profound effects on neuron functions and survival. Also the stimulation of metabotropic glutamatergic receptor II (mGluR2) triggers TNF-induced neurotoxicity associated with Fas death receptor ligand, thus promoting neuronal death by activating Caspase 3 in neurons (Taylor et al., 2005). However, the activation of mGluR3 in microglia have been shown to prevent glutamate release by microglial cells, to reduce microglial reactivity after LPS stimulation, thus preventing microglial toxicity towards neurons.
(McMullan et al., 2012). Also, GABA receptors are expressed by microglia and evidence suggest that upon their activation they are able to modulate cytokines release from microglia. For example, microglial expression of GABA_B receptors increases in response to brain injuries and the receptor activation attenuates LPS-induced release of cytokines such as IL-6 (Kuhn et al., 2004). Both the inhibitory nature of GABA neurotransmitter and the compensatory increase of its receptors in microglia after an injury, support the evidence that GABA receptors promote a neuroprotective microglial phenotype, as also confirmed by other studies (Mead et al., 2012). It is noteworthy that, in general, the modulation of neurotransmitter receptors activity on microglia provides either neurotoxic or neuroprotective effects, thus highlighting the importance of dissecting microglial functions and phenotypes to evaluate the therapeutic potential of these cells in neurological disorders (Liu et al., 2016).

Figure 1.4.2 - Microglial heterogeneity during development, aging, and disease. Microglia are heterogeneous during embryonic life in mice, heterogeneity gradually decreases during development and is limited during adulthood. Heterogeneity increases again during aging. In contrast, regional heterogeneity is limited at early postnatal time points in the white matter and less is known during
aging. During disease microglia display more heterogeneous sub-states in a context-dependent manner. Highly diverse transcriptional microglial clusters are indicated by differentially coloured cells (taken by Masuda et al., 2020).

1.4.2 Spectrum of microglia reactivity and phenotypes

Initial attempts to characterize microglial “states of activity” was based on limited molecular expression profiles, and microglia were categorized as either “activated” or “resting” but these terms do not properly define the broad range of existing microglial phenotypes (Benusa et al., 2020). Indeed, as previously described in this chapter, microglia are constantly extending and retracting their processes to survey their surroundings, thus displaying high activity levels (Nimmerjahn et al., 2005; Sierra et al., 2014). According to the current knowledge, the term “surveying”, is more appropriate than “resting”. Similarly, a more appropriate term for “activated” is “reactive” (Benusa et al., 2020).

In an oversimplified way, reactive microglia have been further divided into two extremely polarized functional states, namely “M1-like” and “M2-like” as illustrated in Figure 1.4.3, referring to the pro- and anti-inflammatory phenotypes based on expression profiles applied to macrophages (Martínez and Gordon, 2014). However, evidence show that reactive microglia, display a variety of changes in their inflammatory profiles (Crotti and Ransohoff, 2016).

After injuries or acute conditions, simultaneous triggering of Toll-Like Receptors and Interferon-γ signalling pathways induces a pro-inflammatory and neurotoxic state of microglia, which produce cytokines and chemokines (TNF, IL-6, IL-1β, IL-12, and CCL2) and upregulates the nicotinamide adenine dinucleotide phosphate (NADPH) and the inducible nitric oxidase. These enzymes produce reactive oxygen species (ROS) and nitric oxide, which alter the function of Ca²⁺-permeable ion channels and potentiate NMDA receptor–mediated excitotoxicity. This chain of events leads to neuronal injury and hyperexcitability (Benusa et al., 2020; Chen et al., 2016; Colonna and Butovsky, 2017; Dong et al., 2009).
Besides a pro-inflammatory response, microglia display phenotypes to promote clearance of debris, extracellular matrix deposition and angiogenesis in order to resolve brain inflammation. Anti-inflammatory microglia can release cytokines such as IL-10 and TGF-β, growth factors (i.e. CSF1), and neurotrophic factors (such as NGF, BDNF and GDNF) (Colonna and Butovsky, 2017). Furthermore, microglia can be activated by specific interleukins (IL-4, IL-13, IL-10, IL-1Ra) or by the activation of nuclear receptors (i.e. estrogen receptors, ERs and Peroxisome Proliferator-Activated Receptors, PPARs) that act as transcriptional factors and regulate the expression of genes that promote the restoration of tissue homeostasis (Saijo et al., 2013). One of the best characterized markers of M2-like cells is the enzyme arginase 1 (Arg1) that downregulates the production of nitric oxide, thus promoting mechanisms underlying wound healing and matrix deposition (Cherry et al., 2014; Munder, 2009; Munder et al., 1999).

However, it is now well-known that a wide range of “in-between” activity states exists and a simple categorization into one or the other functional state is not sufficiently informative, as microglia rarely display a net bias toward either the M1-like or M2-like phenotype. Transcriptome studies showed that microglia activation vary in a context-dependent manner, and in CNS disorders the multiple phenotypes of microglia can be associated with different neuropathological conditions and disease stages (Colonna and Butovsky, 2017). For example, based on a specific pattern of surface markers (e.g Iba1, cd11c, downregulated Cx3Cr1 and Tmem119; upregulated Trem2), Keren-Shaul and colleagues described a subclass of reactive microglia, defined as Disease Associated Microglia, that was identified in models of chronic neurodegeneration such as Alzheimer’s disease, amyotrophic lateral sclerosis, and tauopathy, as well as in multiple sclerosis (Benusa et al., 2020; Keren-Shaul et al., 2017).
1.4.3 - Schematic diagram of microglial inflammatory response. After brain injury, surveying microglia become “reactive” and adopt a wide spectrum of intermediate phenotypes with two extreme poles: M1-like microglia with a pro-inflammatory profile that consists of decreased expression of neurotrophic factors and increased levels of pro-inflammatory chemokines and cytokines and reactive oxygen species, or M2-like microglia with anti-inflammatory phenotype including the resolution of the inflammatory profile, neurogenesis and the clearance of debris (amoeboid shape). Polarization between the two extreme phenotypes is represented by the spectrum colour bar and double-pointed arrows. Image modified from Victor and Tsirka, 2020, using Biorender.com

1.5 Clinical and experimental evidence of microgliosis in epilepsy

Extensive microglia reactivity, referred to as microgliosis, occurs in human and experimental epilepsy foci (Beach et al., 1995; Borges et al., 2003; Drage et al., 2002; Morin-Brureau et al., 2018). Interestingly, microglia reactivity and the extent of their activation are strictly associated with seizure occurrence in terms of time after last seizure, duration of seizures, brain region of seizure onset and generalization (Mirrione and Tsirka 2011). Both in MTLE patients and in animal models, the hippocampal areas with neuronal death, such as CA1 and CA3 sectors, or regions with cortical malformations in patients with cortical
dysplasia, exhibit microgliosis (Beach et al., 1995; Borges et al., 2003; Taniwaki et al., 1996; Tooyama et al., 2002). Moreover, in the sclerotic hippocampus of MTLE patients, microglia exhibit reactive phenotype and an amoeboid shape (Morin-Brureau et al., 2018) and increased expression of pro-inflammatory molecules (Crespel et al., 2002; Leal et al., 2017; Ravizza et al., 2008).

Increased microglia density was observed also in rodent hippocampus within 48 h after kainic acid-induced SE (Andersson et al., 1991) or pilocarpine-induced seizures (Borges et al., 2003; Shapiro et al., 2008). A recent study in transgenic mice expressing GFP in microglia reported that microglia exhibit increase in cell body size with shortening of their processes within 24 h after seizures induced by systemic kainic acid administration (Avignone et al., 2008). These studies describe microglia reactivity as secondary, namely occurring after the neuronal injury following the initial seizures (Eyo et al., 2017). On the other hand, data also suggest a rapid microglia reactivity within 3 and 8 hours following kainic acid administration, even before neuronal death has occurred (Eyo et al., 2014; Rappold et al., 2006; Taniwaki et al., 1996). Less is known about the phenotype and the molecules expressed on their surface in their reactive state, during and after seizures, except for induction of Iba1, CD68 or CD11b.

Overall, the available evidence shows that microglia reactivity is associated with seizures and neuronal stress/injury (Eyo et al., 2017).

1.6 Microglia in epileptogenesis: their role in neuroinflammation

Neuroinflammation is a common process in epileptogenesis as shown in several experimental models of epilepsy such as SE, stroke, trauma as well as in structural human epilepsies (Vezzani et al., 2011a). Neuroinflammation is defined as a homeostatic phenomenon induced by various tissue injuries and consists of the rapid production of molecules with pro-inflammatory or anti-inflammatory properties by cells of the innate immune system.
Typically, this process is triggered to defend tissues against pathogen invasion during infections, but it can be also activated by endogenous “danger signals” released by threatened cells (Bianchi, 2007). Neuroinflammation occurs as a result of the reactivity of brain resident parenchymal cells, such as microglia and astrocytes, but also neurons. As a consequence, leukocytes may penetrate the brain through the BBB (including both components of peripheral innate immunity such as macrophages and granulocytes as well as lymphocytes as part of adaptive immunity).

Despite it is not known whether and how microglia contribute to seizures and/or are involved in epileptogenesis, evidence exists that these cells release pro-inflammatory mediators after epileptogenic insults or during seizures that, directly or indirectly, enhance neuronal excitability thus contributing to seizures (Hiragi et al., 2018; Vezzani et al., 2019). For example, the release of IL-1β that acts on its receptor IL-1R1 promotes seizures in NMDAR-dependent manner, and the pharmacological blockade, or genetic strategies, for the inactivation of this signalling provide anti-seizures effects (Devinsky et al., 2013; Vezzani et al., 2011b).

Moreover, microglia express a variety of immune receptors on their cell membranes, such as chemokines receptors, purinergic receptors, Toll-like receptors (TLRs) and danger signals that allow microglia to communicate with the surrounding cells and to initiate the immune response, for example, in response to danger signals released after cell injury or stressful events (Garden and Möller, 2006; Younger et al., 2019). For example, TLRs are a family of receptors that recognize either pathogen- or damage-associated molecular patterns (PAMPs and DAMPs or danger signals) to elicit an immune response (Vezzani, 2006), as in the case of TLR4 that can be activated either by LPS or by endogenous ligands (i.e high mobility group box 1, HMGB1) resulting in pro-inflammatory effects that contribute to epileptogenesis (Iori et al., 2013; Maroso et al., 2010).

The process of neuroinflammation also relies on neuron-microglia interaction and an example of relevant mediator of neuron-microglia communication is fractalkine, CX3CL1
Fractalkine is expressed predominantly by neurons and binds to its receptor, CX3CR1, which is selectively expressed by microglia (Ransohoff and Perry, 2009). The activities of fractalkine signalling may vary in different CNS regions and in different disease conditions (Cardona et al., 2006). Interestingly, increased levels of both fractalkine and CX3CR1 have been observed in resected hippocampal tissue from epileptic patients and in animal models of MTLE (Roseti et al., 2013; Xu et al., 2012; Yeo et al., 2011), and fractalkine increased levels have also been reported in CSF of epileptic patients (Ali et al., 2015; Cardona et al., 2006; Kettenmann et al., 2011). Recently, as assessed in human MTLE tissue microtransplanted into Xenopus oocytes, fractalkine was reported to modulate GABA_A mediated current by promoting its stability (Roseti et al., 2013). Moreover, experimental data report that neuronal fractalkine provides an inhibitory effect on the activation of CX3CR1-expressing microglia and the genetic deletion of this receptor increases microglia-mediated neurotoxicity (Cardona et al., 2006; Kettenman et al., 2011). Blockade of the receptor signalling by an antibody anti-CX3CR1, in a rat model of electrically induced SE, resulted in reduced microglia reactivity and neurodegeneration, while infusion of fractalkine only provided minor effects, suggesting a complex role of this chemokine and its receptors in microglia responses (Ali et al., 2015).

Purinergic signalling is also upregulated in microglia following kainic acid administration in rodent models of epilepsy (Avignone et al., 2008; Ulmann et al., 2013). Purinergic receptors such as P2X7 sense ATP concentrations released by cells after brain injury (Garden and Möller, 2006; Kettenmann et al., 2011) and the activation of these receptors by ATP promotes the release of reactive oxygen species and IL-1 β, thus leading to neurotoxicity (Parvathenani et al., 2003). Studies report that seizures also upregulate the microglia expression of purinergic receptors, such as P2X4, P2Y6 and P2Y12 in the hippocampus (Avignone et al. 2008; Ulmann et al. 2013). Interestingly, a lack of P2Y12 receptors in transgenic P2Y12-KO mice resulted in the exacerbation of seizures after SE,
thus suggesting that microglial P2Y12 receptors provide an anti-epileptogenic effect (Eyo et al., 2014).

Purinergic receptors are also expressed by astrocytes, therefore they may play an important role in the communication between these cell populations during the inflammatory response (Matejuk and Ransohoff, 2020). For example, ATP or other soluble nucleotides released from astrocytes acts on the purinergic receptors on microglia, thus promoting microglial motility and phagocytosis, after systemic kainic acid in rats (Inoue et al., 2007; Koizumi et al., 2007).

In support of the crosstalk between microglia and astrocytes in neuroinflammation, recent data support a causal relationship of reactive microglia and astrogliosis during epileptogenesis. Specifically, in a mouse model of pilocarpine induced-SE, microglia release of proinflammatory cytokines was shown to induce reactive astrocytes, which subsequently caused increased excitability in a CA$^{2+}$-dependent manner, increasing seizures susceptibility (Sano et al., 2021). Evidence that reactive microglial cells play a role in the induction of a reactive phenotype in astrocytes was demonstrated also in mice systemically injected with LPS (Liddelow et al., 2017).

A deeper knowledge is needed to uncover the wide range of soluble mediators released by microglia and their receptors in epileptogenic brain tissue. Therefore, microglia inflammatory profile during epileptogenesis and during ictal and interictal phases needs to be investigated in more-depth (Eyo et al., 2017).

1.6.1 The role of microglia in seizures

Although microglia contribute to neuroinflammation, which is a phenomenon involved in seizures, the direct role of these cells in neuronal hyperexcitability and ensuing seizures is still controversial and poorly understood (Victor and Tsirka, 2020). After SE, surveillant microglia sense the damage and migrate to the region of insult, where they remain reactive for several weeks post-seizures (Borges et al., 2003). Depending on the model of
epilepsy, microglia reactivity can vary in extent and duration (Alyu and Dikmen, 2017; Benson et al., 2015).

Cytokines have been reported to be upregulated after seizures as measured by mRNA and protein expression of IL-1β, IL-6, TNF, TGF-β, and vascular endothelial growth factor (VEGF), among others. In particular, IL-1β is an ictogenic molecule since it promotes seizures by enhancing NMDA receptor activity on post-synaptic cells (Viviani et al., 2003), reduces HCN1-mediated $I_h$ currents (Frigerio et al., 2018a) and decrease GABA-mediated neurotransmission (Roseti et al., 2015). Thus, IL-1β may be a crucial target for controlling seizures both in animal models and in human drug-resistant conditions, such as New-Onset Refractory Status Epilepticus (NORSE) (Koh et al., 2021; Vezzani et al., 2019).

TNF is also involved in neuronal excitability and is released by microglia and astrocytes to maintain neuronal excitation at physiological levels (Stellwagen and Malenka, 2006). In particular, TNF promotes release of glutamate and increases GABA receptor endocytosis, thus reducing GABA inhibitory action and increasing neuronal excitability (Stellwagen et al., 2005; Victor and Tsirka, 2020). A dual role of TNF in susceptibility to seizures was reported depending on the receptor subtype activated. A pro-ictogenic role of TNF is mediated by the TNF receptor type 1, while an anti-ictogenic effect is mediated by the activation of TNF receptor 2 (Balosso et al., 2013).

According to these results, since reactive microglia express ictogenic inflammatory molecules during epileptogenesis (Ravizza et al., 2008), it was hypothesized it may contribute to seizure generation. However, the genetic ablation of microglia before pilocarpine injection in rodent models of epilepsy exacerbated seizure severity, thus suggesting that microglia may protect the CNS from exaggerated neuronal activity (Mirrione et al., 2010; Victor and Tsirka, 2020). Additional studies reported that the administration of minocycline, a non-selective microglia inhibitor, administered post-SE decreased the ensuing spontaneous seizures, suggesting that microglia are involved in the generation of seizures (Barker-Haliski et al., 2016; Wang et al., 2015; Wolf et al., 2020). By contrast, other
studies showed that minocycline has partial or no efficacy on seizures in rodent models of epileptogenesis (Dupuis et al., 2016; Russmann et al., 2016; Wang et al., 2012).

Thus, there is controversy on the role of microglia in seizures due to conflicting results, thus highlighting the need of further investigations.

1.6.2 The role of microglia in neuronal death

Microglial cells are known to take part in developmental processes and in the adult brain for their essential role on synaptic pruning and clearance of debris, but the engagement of such functions in a pathological context may lead to an excessive synapses or neuronal cell loss that also provokes cognitive decline (Song and Colonna, 2018). Evidence suggests a strong link between microglia and neurodegeneration; indeed, microglia have been linked to several neurodegenerative and neurological conditions such as Alzheimer’s, Parkinson’s disease, dementia, prion diseases, multiple sclerosis, amyotrophic lateral sclerosis and also TLE (Block and Hong, 2007; Block et al., 2007; Hanisch and Kettenmann, 2007; Marín-Teva et al., 2011) but how microglia influence the neurodegenerative process during CNS diseases has been debated for decades.

In particular, findings support that microglia is involved in neurodegenerative phenomena associated with epilepsy (Beach et al., 1995). Studies in mice exposed to SE have shown that reactive microglia persist in brain regions of neuronal cell loss, and its reactivity may precede evidence of neurodegeneration (Borges et al., 2003; Somera-Molina et al., 2007, 2009). In accord, minocycline reduced neuronal death associated with seizures in rodents exposed to kainic acid (Heo et al., 2006). Minocycline was also found to reduce SE-induced neuronal cell loss in lithium-pilocarpine injected rats, an effect associated with inhibition of microglia reactivity and reduced IL-1β and TNF expression in the hippocampus, while astrocytic reactivity was not affected (Wang et al., 2015). Also macrophage inhibitory factor (MIF) reduced microglia reactivity and neurodegeneration in an intrahippocampal kainic acid mouse model (Rogove and Tsirka, 1998), suggesting that
microglia was involved in neuronal death subsequent to seizures. Moreover, the inhibition of the microglia-specific fractalkine receptor using selective antibodies reduced seizure-induced neurodegeneration in the hippocampus (Ali et al., 2015).

Despite the evidence that microglial cells are involved in neurodegeneration and that inhibiting microglial functions may promote neuron survival, the mechanisms that regulate the overall phenomenon are still under consideration. As reviewed by Block and colleagues, microglia express pattern-recognition receptors (PRRs) that in the presence of neurotoxic stimuli induce microglia to produce free reactive oxygen species, in a NADPH-oxidase dependent manner, that may cause neuronal damage as well as amplify the pro-inflammatory cascade in microglia (Block and Hong, 2007). For example, TLRs family expressed in microglia are essential in inducing neurotoxic factors release from microglial cells (Olson and Miller, 2004), thus representing an interesting target for studying microglial-dependent neurotoxicity.

1.6.3 The role of microglia in aberrant neurogenesis

Neurogenesis is a physiological lifelong phenomenon and studies in mice reported that sustained adult neurogenesis reduces anxiety and depression-like behaviours (Hill et al., 2015), improves the ability of discrimination between similar contexts (Sahay et al., 2011) and regulates mood and cognitive functions (Christian et al., 2014; Scharfman and Gray, 2007). Neurogenesis primarily occurs in the subventricular zone (SVZ) of the lateral ventricles and in the hippocampus within the subgranular zone (SGZ) of the dentate gyrus (DG) (Eriksson et al., 1998; Kempermann et al., 2004).

Evidence shows that upon epileptogenic stimuli, adult neurogenesis is aberrantly triggered and abnormal connections originate among newly-born and mature neurons, thus participating in hyperexcitability phenomena and behavioural impairments (Cho et al., 2015; Gray and Sundstrom, 1998; Jessberger and Parent, 2015; Parent et al., 2006; Scharfman and Gray, 2007). Indeed, pilocarpine-induced SE provokes aberrant hippocampal neurogenesis
and preventive ablation of adult neurogenesis results in reduction in chronic seizures and 
rescue of epilepsy-associated cognitive deficits (Cho et al., 2015).

Microglia regulate adult hippocampal neurogenesis by modulating cell survival and 
migration and the proper incorporation of newly born granule cells into the hippocampal 
circuitry (Eyo et al., 2017; Victor and Tsirka, 2020). Microglia also regulate the proliferation 
and differentiation of newly generated cells into mature neurons both in physiological and 
epileptic conditions (Luo et al., 2016a). For example, depletion of microglia in hippocampal 
cultures reduced both survival and proliferation of neural stem/progenitor cells, thus 
providing evidence of the trophic and proliferative role of microglia in the neurogenic 
process (Nunan et al., 2014).

After SE, neurogenesis transiently increases and microglia may phagocyte newly 
formed granule cells, thus regulating their number in the DG (Jessberger et al., 2005; Luo et 
al., 2016b; Parent et al., 1997). By contrast, reactive microglia have been proposed to 
facilitate aberrant migration of newly-born neurons in the hippocampal DG, thus 
contributing to the generation of ectopic neuronal cells that promote a dysfunctional 
excitatory circuitry (Yang et al., 2010). This latter study reports that stereotaxic injection of 
LPS into the DG, to prime microglia reactivity, promotes the development of ectopic hilar 
basal dendrites in the hippocampus. This phenomenon was prevented by minocycline (Yang 
et al., 2010). In addition, fractalkine receptor knockout mice injected with kainic acid 
showed a delay in the maturation of newly born neurons (Xiao et al., 2015), therefore 
supporting the involvement of this specific microglia signalling in aberrant neurogenesis 
associated with seizures (Eyo et al., 2017).

1.7 Strategies and tools to interfere with microglia

Recently, a variety of methods have been developed to interfere with microglia to 
provide new insights into the role of these cells in pathophysiological conditions. These are 
toxins-based methods or pharmacological and gene-based approaches designed to
manipulate microglia in healthy and diseased contexts by *depleting the whole cell population* or by *targeting a specific cellular function* (Jäkel and Dimou, 2017).

1.7.1 Toxin-based models of microglial depletion

Early studies have applied *toxin-based tools* such as clodronate-containing liposomes, which are selectively engulfed by phagocytes such as monocytes/macrophages and microglia. Once phagocytosed, liposomes release clodronate that depletes the cells by causing apoptosis. Since liposomes do not readily cross the BBB, their intracerebral application is necessary to deplete microglia and macrophages in the CNS (Han et al., 2019; Van Rooijen and Sanders, 1994). Moreover, this procedure allows rapid but short-lasting depletion and induces cytokine release, astrocytic reactivity and endothelial damage, therefore it is not considered the best tool to deplete microglia (Han et al., 2019; Van Rooijen and Sanders, 1994).

Another toxin-based approach to physically remove microglia is to generate *Cd11B-HSVTK* transgenic mice (Gowing et al., 2006; Green et al., 2020). In this model, the *Cd11b* promoter which is selectively expressed in myeloid cells including microglia (Heppner et al., 2005) controls the expression of the HSVTK gene (herpes simplex virus-derived thymidine kinase). This gene is constitutively expressed in myeloid cells and when ganciclovir is administrated to mice, it activates HSVTK thus initiating the cascade of biochemical reactions that convert ganciclovir into its active form, which induces apoptosis in Cd11b-positive cells. Complete depletion of Cd11b+ cells results in defects of normal hematopoiesis, thus leading to severe anemia in mice (Heppner et al., 2005), therefore this method has been optimized by transferring wild-type bone marrow into treated mice or by an intracerebroventricular administration of ganciclovir to more selectively kill microglia in the CNS (Heppner et al., 2005; Green et al., 2020). Other toxin-based models to eliminate myeloid cells exploit the Diphteria Toxin (DT) that inhibits protein synthesis in the target cells, thus inducing programmed cell death (Waisman et al., 2015). Specifically this
approach requires the selective expression of the DTA (Diphteria Subunit A) or its receptor (DTR) under selective microglial promoters, such as Cx3cr1 or Tmem119, in CreLoxP mouse lines with DTR/DTA expression inducible by tamoxifen administration (Bruttger et al., 2015; De Luca et al., 2019; Kaiser and Feng, 2019). This DT-based model has important advantages and has been optimized to obtain an inducible and more selective microglia depletion, but it is not devoid of criticisms. In fact, this system only provides a short-lived ablation of microglia, with variable efficiency and toxicity depending on the Cre recombinase used and induces cognitive impairment (Green et al., 2020; Waisman et al., 2015).

1.7.2 CSF1R and genetic models of microglial depletion

Research has identified the Colony Stimulating Factor 1 Receptor (CSF1R) as a major regulator of the survival, proliferation and differentiation of myeloid cells, including microglia. CSF1R is also expressed in hematopoietic stem cells, neural progenitor cells, renal and colonic epithelial cells, oocytes and embryos thus playing critical roles in development and physiology, immunity and inflammation as reviewed by Stanley and Chitu (Stanley and Chitu, 2014).

The CSF1R is a tyrosine kinase receptor with three distinct domains (Figure 1.7.1): the intracellular domain containing a juxtamembrane domain, eight tyrosine residues that become phosphorylated during receptor activation and a kinase domain, a transmembrane domain and an extracellular domain that binds to its specific ligands, CSF1 and IL-34 (Stanley and Chitu, 2014).
Figure 1.7.1 - Signalling pathways regulated by CSF1R in myeloid cells. Ligand (CSF1) binding stabilizes CSF-1R in its dimeric form and leads to activation of the CSF-1R kinase that promotes multiple downstream cascades specific to each biological function (light yellow boxes): proliferation, survival, differentiation, cell adhesion, motility and phagocytosis as reviewed by Pixley and Stanley (Pixley and Stanley, 2004).

Both ligands are secreted by neurons, while CSF1 is also released by glial cells. The inactive conformation of CSF1R shifts from the monomeric to its dimeric active state. Upon binding to IL-34 or CSF1, CSF1R dimers stabilize thus promoting the autophosphorylation of specific tyrosine residues and distinct intracellular signals according to the biological function to be accomplished: survival, proliferation, differentiation, as shown in Figure 1.7.1 (Stanley and Chitu, 2014).

The CSF1R signalling has been extensively studied to design genetic models of microglial depletion (Green et al., 2020). Generating CSF1R knock out mice provided complete microglial depletion from CNS and reduced monocytes/macrophages populations in the periphery (Elmore et al., 2014). As review by Green and colleagues, conditional knockout mice, with Cre recombinase expressed under a specific microglial promoter and a loxP-flanked Csf1r, provided spatial and temporal control of microglia resulting in transient
elimination of microglia with tamoxifen (Buttgereit et al., 2016; Green et al., 2020). This approach also allows the myeloid cells transplantation into the brain. However, notable disadvantages need to be taken into account: mice show a shortened lifespan and neurodevelopmental abnormalities (Cronk et al., 2018; Dai et al., 2002; Erblich et al., 2011). Genetic defects in CSF1 and IL-34 ligands cause only a partial reduction of microglial cell numbers (Colonna and Butovsky, 2017; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990) and developmental defects are reported (Dai et al., 2002; Michaelson et al., 1996).

1.7.3 Pharmacological interventions to target microglia: CSF1R inhibitors

Small molecule CSF1R inhibitors are a class of orally bioavailable chemical compounds that are selective for receptors with tyrosine kinase activity, penetrate the BBB and provide sustained microglial blockade until their withdrawal. CSF1R inhibitors (CSF1Ri) can be formulated in the rodent chow, thus affording a non-invasive strategy to interfere with CSF1R signalling and transiently target microglial population.

The first identified CSF1Ri is Pexidartinib, with two screened compounds PLX3397 and PLX647 (Elmore et al., 2014). Pexidartinib has been approved by US FDA for the clinical treatment of tenosynovial giant cell tumours, strongly encouraging its application to other disorders involving myeloid dysfunctions (Tap et al., 2015). PLX3397 binds to CSF1R in its juxtamembrane domain thus trapping the kinase in the autoinhibited state (Tap et al., 2015). In a dose-dependent manner, these inhibitors provide robust reduction of microglial numbers in brain parenchyma, thus reinforcing the evidence that microglia survival rely on CSF1R signalling in adult mice (Elmore et al., 2014). In particular, PLX3397 at 290 ppm provides a 50% depletion of microglia from brain parenchyma during 3 days of administration and a 99% of depletion if the treatment is continuously administered for 3 weeks (Elmore et al., 2014). Upon drug withdrawal microglia repopulate the brain parenchyma within 3 days with no contribution from peripheral myeloid cells (Elmore et al., 2014).
Using a CSF1Ri-based approach to eliminate microglia allows to modulate the extent of microglial depletion using different concentration of inhibitor and duration of treatment, thus providing a range of 20-99% depletion (Dagher et al., 2015; Elmore et al., 2014; Najafi et al., 2018; Spangenberg et al., 2019). Indeed, several studies reported that using CSF1Ri such a NJ-40346527 and GW2580 specific functions such as cell proliferation and self-renewal can be targeted, without inducing cell loss (Gómez-Nicola et al., 2013; Mancuso et al., 2019; Olmos-Alonso et al., 2016). In particular, GW2580 is a potent selective compound that binds to CSF1R kinase by competing with ATP, thus inhibiting an ATP-dependent tyrosine kinase-mediated transduction signal that ultimately directs myeloid cells proliferation (Conway et al., 2005). These inhibitors attenuated neurodegeneration, neuroinflammation, disease progression, cognitive and behavioural impairment in several mouse models of CNS disorders as reviewed by Green et al., 2020.

CSF1Ri represent a valid tool to interfere with microglia not requiring invasive surgery or genetic manipulation, and fast microglia repopulation occurs after inhibitor discontinuation (Green et al., 2020).
CHAPTER 2: AIMS OF THE THESIS
For decades, research on the mechanisms underlying the development of epilepsy has focused mainly on neurons with the aim of finding means to counteract hyperexcitability underlying the epileptic seizure. However, despite the availability of at least twenty anti-seizures drugs, about one third of patients has drug refractory seizures, thus the identification of new therapeutic targets is an urgent clinical need.

Based on this consideration, research activities have addressed the role of non-neuronal cells, such as microglia and astrocytes, in the mechanisms underlying epilepsy development. In particular, reactive microglia have been described after epileptogenic insults in animal models and in human drug-resistant brain specimens, begging for the question whether these cells play a role in seizures, cell loss and neurological comorbidities in epilepsy.

The scope of this research project was to characterize the role of microglia in epileptogenesis using CSF1Ri inhibitors, as follows:

1. To study the pattern of microglia reactivity and proliferation in one representative murine model of acquired epilepsy
2. To study the involvement of microglia in epileptogenesis, in particular in epilepsy-associated structural brain modifications such as cortical thinning and neuropathology by depleting microglia with the CSF1R inhibitor PLX3397
3. To study the role of microglia proliferation in seizures, cell loss and cognitive deficits using GW2580, a CSF1R inhibitor specifically blocking microglia proliferation.

These pharmacological approaches were applied to the animal model in order to counteract microgliosis during specific disease phases. The interference with microglia was measured on parameters of clinical relevance such as spontaneous recurrent seizures and epilepsy-associated cognitive deficits and neuropathology.
CHAPTER 3: GENERAL MATERIALS AND METHODS
3.1 Animals

C57BL6N male mice (8 weeks-old, 25-30 g; Charles River, Calco, Italy) were housed (5/cage) in the SPF facility at a constant room temperature (23°C) and relative humidity (60 ± 5%) with free access to food and water, and with a fixed 12 h light/dark cycle. After surgery for EEG set up implantation, or sham surgery, mice were individually housed in the presence of environmental enrichment (e.g., toilet paper, straw, nesting material) (Hutchinson et al., 2005). All experimental procedures were conducted in conformity with institutional guidelines, in compliance with national (D.L. n.26, G.U. March 4, 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Procedures involving animals were conducted at the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, which adheres to the principles set out in the above-mentioned laws, regulations, and policies governing the care and use of laboratory animals.

3.2 Murine model of acquired epilepsy

We used a well-established mouse model of epilepsy induced by convulsive status epilepticus (SE) provoked by intra-amygdala injection of kainic acid (Frigerio et al., 2018b; Iori et al., 2017; Mouri et al., 2008).

Surgery. Briefly, mice were implanted under 1.5% isoflurane anaesthesia with a 23-gauge cannula unilaterally positioned on top of the dura mater for the intra-amygdala injection of kainic acid (from bregma, mm: nose bar 0; AP -1.0, L -3.1) (Franklin and Paxinos, 2008). A bipolar Teflon-insulated stainless-steel depth electrode (60 µm O.D.) was implanted in the dorsal hippocampus contralateral to the injected amygdala (from bregma, mm: nose bar 0; AP –1.8; L +1.5; -2.0 below dura mater). Additionally, a cortical surface electrode was implanted onto the ipsilateral somatosensory cortex (from bregma, mm: nose bar 0; AP –1.8; L -1.5). Finally, two screw electrodes were positioned over the nasal sinus and the cerebellum, as ground and reference electrodes, respectively. Electrodes were
connected to a multipin socket and secured to the skull by acrylic dental cement (Iori et al., 2017; Frigerio et al., 2018b). At the end of stereotaxic surgery, perioperative analgesia with buprenorphine (Bupaq, 0.1 mg/kg, s.c; Alcyon Italia S.p.a., Cherasco, CN, Italy) was carried out by treating mice once-a-day for 3 consecutive days (Di Sapia et al., 2021).

*Status epilepticus.* One week after surgery, freely moving mice were connected to the EEG set up the day before beginning the experiment to record baseline EEG for about 24 hours. In order to evoke SE, kainic acid (KA, 0.3 μg in 0.2 μl; Sigma-Aldrich, S. Louis, MO, USA, #K0250) was dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and injected unilaterally into the basolateral amygdala using a needle protruding 4.0 mm below the implanted cannula (Iori et al., 2017; Frigerio et al., 2018b). SE developed after around 10 min from injection and SE was defined by continuous spiking activity with a frequency >1Hz, intermixed with high amplitude and frequency discharges lasting for at least 5 seconds with a frequency of >8Hz. The spikes were defined as sharp waves with amplitude at least 2.5-fold higher than the standard deviation of baseline signal and duration <100 ms, or as a spike-and-wave with duration <200 ms (Iori et al., 2017; Frigerio et al., 2018b). The total number of spikes was measured during 24 h after KA administration (Clampfit 9.0, Axon Instruments, Union City, CA, USA). Mice were injected with diazepam (10 mg/kg, intraperitoneally, i.p.) 40 min after KA injection to inhibit motor seizures and improve survival, although EEG-monitored SE was not interrupted. **Figure 3.2.1** shows examples of EEG tracings that illustrate SE generalization to both brain hemisphere.
Figure 3.2.1 - Representative EEG tracings of baseline and SE. The examples report two traces that represent the left (HP=hippocampus) and the right (S1=somatosensory cortex) hemispheres, respectively. Image taken from Di Nunzio et al., 2021.

Epilepsy. Mice exposed to SE lasting for at least 3 h develop spontaneous seizures (90% of mice) that recur for more than 2 months (Iori et al., 2017). Spontaneous seizures consist of EEG paroxysmal events lasting 30-60 sec simultaneously occurring in amygdala, hippocampus and somatosensory cortex, bilaterally, and are accompanied by generalized motor convulsions (Iori et al., 2017). Epilepsy onset is defined by the appearance of spontaneous seizures after at least 48 h from the end of SE (to exclude acute symptomatic seizures). EEG activity was recorded using the Twin EEG Recording System (version 4.5.3.23) connected with a Comet AS-40 32/8 Amplifier (sampling rate 400 Hz, high-pass filter 0.3 Hz, low-pass filter 70 Hz, sensitivity 2000 µV p-p; Grass-Telefactor, West Warwick, R.I., USA). We reckoned the total number of EEG seizures during the recording period; seizure frequency was estimated by dividing the total number of seizures by the number of recording days. Digitized EEG data were processed using the Twin record and review software. EEG analysis of seizures was done by two independent investigators blinded to the treatment, who reviewed all the EEG tracings in the electronic files of each mouse. Deviation of ≤5% from concordance was considered acceptable; otherwise, EEG tracing was additionally analysed by a third person to look for consensus. EEG was monitored (24/7) during different time windows representative of acute, subacute and chronic disease development (see the Experimental protocols for details in the Results).
Sham mice were implanted with electrodes and guide cannula but were not exposed to SE and they served as controls for behavioural and post-mortem analyses.

### 3.3 Novel object recognition test

We have used a well-established behavioural test, the novel object recognition test (NORT), which allows to evaluate non-spatial memory, i.e recognition memory in mice (Denninger et al., 2018; d’Isa et al., 2014). The NORT relies on the natural tendency of rodents to spontaneously approach and explore novel objects over familiar ones and it is considered a non-stressful tool for the study of memory in animals, since punishments or starvation/water restriction regimens are not employed (d’Isa et al., 2014).

**Procedure.** Before performing the test, all mice were temporarily disconnected from EEG set up and habituated to their home cage for 48 h. The test was performed in the open-square grey arena (40×40 cm) surrounded by 30-cm high wall. Mouse behaviour was remotely monitored via video camera. All experiments were started between 9:00 and 10:00 am. Before starting the test, mice were acclimated to the testing room for 1 h in individual home cages. As illustrated in Figure 3.3.1, NORT is performed during three days. On the first day, mice were allowed to habituate in the arena for 5 min (habituation phase). After 24 h, namely the familiarization phase, mice were placed into the open field for 10 min in the presence of two identical objects positioned in internal non-adjacent squares. Mice belonging to different experimental groups were randomized to assign an object to each mouse, thus avoiding selection bias. The following objects were used: black plastic cylinders (4×5 cm); transparent scintillation vials with white cups (3×6 cm); metal cubes (3×5 cm). Cumulative exploration time of both objects and of each object separately was recorded. Exploration was defined as sniffing, touching, and stretching the head toward the object at a distance of not more than 2 cm. After 24 h, during the recognition phase, mice were placed for 10 min in the open field which contained one object presented during the familiarization phase (familiar object, F) and a novel object (N). In this phase mice were tested for memory
(d'Isa et al., 2014) and the following parameters were recorded: time spent exploring N vs F, as well as cumulative exploration time (novel+familiar, N+F). Novel object recognition was quantified using the discrimination index (N-F/N+F): the difference between the time spent exploring the novel and the familiar objects (N-F) divided by the sum of total exploration time (N+F). Mice displaying total exploration time <8 sec during the familiarization phase were excluded from the test (Di Nunzio et al., 2021; Frigerio et al, 2018b).

![Figure 3.3.1 - Novel object recognition test.](image)

**Figure 3.3.1 - Novel object recognition test.** The procedure starts with mice habituation to an empty arena. After 24 h, mice freely explore two identical objects for 10 min (familiarization). After 24 h, mice are exposed to a novel object and the familiar one to be tested in the non-spatial memory task (10 min, test).

### 3.4 Barnes Maze test

The Barnes maze test is one of the available tools to measure spatial memory and learning in mice and is based on the principle that the exposition to an adverisive environment, i.e an enlightened platform, stimulate mice to learn and remember the location of an escape hole (Gawel et al. 2019).

**Procedure.** Before performing the test, mice were temporarily disconnected from EEG set up and habituated to their home cage for at least 48 h. The test was performed as previously described (Gawel et al., 2019; Van Den Herrewegen et al., 2019). Briefly, before starting the experiment, mice were acclimatized to the testing room for 1 h in individual home cage. The maze (Figure 3.4.1) was placed in the centre of an enlightened room. Mice belonging to different experimental groups were randomized to assign an escape hole to each mouse, thus avoiding selection bias. Behavioural testing included three phases: 1.
Habituation phase, in which mice were introduced to the environment for 2 minutes and gently accompanied to the escaped hole; 2. Learning phase lasting three consecutive days, in which the animals learn to find the location of the escape hole (day 1-3; 3 trials/day; 2 minutes/trial); 3. One probe trial (day 4) carried out using a closed escape hole. During the training phase, total latency time to enter the escape box was recorded. On the probe day, the escape hole was closed, and each mouse was given 2 minutes to explore the maze, therefore the primary latency time to identify the target hole was measured, then the mouse was returned to its home cage. Mice were excluded from the probe trial if they remained immobile in their home cage or did not perform properly due to immobility for >2 minutes during the learning days or during the probe trial (Di Nunzio et al., 2021).

Figure 3.4.1 - Barnes Maze. Left, the platform maze. Right, on the first day mice were placed in the maze and gently pushed towards the escape hole. After 24 h and for three subsequent days, mice start the learning sessions and freely explore to identify the escape hole (day 1-3; 3 trials/day; 2 minutes/trial). After 24 h, the escape hole is closed, and mice are tested for spatial memory (Probe-day 4).

3.5 Post-mortem brain MRI and image analysis

At the end of EEG recordings, mice were sacrificed for post-mortem MRI analysis and subsequent histological analysis. Mice were deeply anaesthetized by injecting ketamine (75 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.), then perfused via ascending aorta with 50 mM ice-cold PBS, pH 7.4 for 3 minutes followed by ice-cold 4% paraformaldehyde (Merck, Darmstadt, Germany, #104005) in PBS for 5 minutes. After perfusion, the electrodes were carefully removed from the brain, mice were decapitated and the head of the
mouse was inserted in a plastic tube, fixed on the mouse-compatible MRI holder with tape, then immediately scanned.

Neuroimaging was performed on a 7T small bore (12 cm wide) animal scanner (Bruker Biospec, Ettlingen, Germany). Two actively decoupled radio frequency coils were used: a volume coil of 7.2 cm diameter used as the transmitter and a surface coil as the receiver. A 3D rapid acquisition with relaxation enhancement (RARE) T2-weighted sequence was performed to assess anatomic changes. The morphologic images were obtained with a voxel size of 100 x 100 x 100 µm (matrix size 300 x 100 x 120 and field of view 3 x 1 x 1.2 cm); repetition time [TR] = 2500 ms, effective echo time [TE] = 50 ms, and a RARE factor of 16, for 1 average (Acquisition time: 30 min). At the end of MRI procedures, brains were post-fixed for 45 minutes at 4° C, then transferred to 20% sucrose in PBS for 24 hours at 4° C. Then, the brains were immersed in -45°C isopentane for 3 minutes and stored at -80°C until immunohistochemistry was performed.

MRI images were analysed to measure the volume of selected brain regions and the thickness of cortices involved affected by epilepsy. Measurements were undertaken in the hemisphere ipsilateral to the injected amygdala.

Brain region volume was quantified using the multi-atlas segmentation approach embedded in the ANTs software library (Advance Neuromaging Tools)(Avants et al., 2011; Wang et al., 2013).

Specifically, five reference brains had 39 brain regions manually identified on every MRI section co-registered on the mouse brain histology atlas (Bai et al., 2012) by Franklin and Paxinos (http://www.bioeng.nus.edu.sg/cfa/mouse_atlas.html#_ftnref1).

The thickness of the temporal cortices was assessed by measuring the distance between the external capsule and the brain surface using atlas-based co-registered images covering the full antero-posterior extension of the specific cortical region. In each mouse brain, values obtained from each MRI image (n= 45 images/area/mouse) were averaged, providing a single value for each area, and this value was used for statistical analysis.
3.6 Immunohistochemistry and histological evaluation of neuronal cell loss

3.6.1 Brain tissue preparation for histological analysis

Mice were deeply anesthetized (10% ketamine+10% medetomidine+80% saline; 10 ml/kg, i.p.) and perfused via ascending aorta with ice-cold phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PAF) in PBS. Brains were removed from the skull and post-fixed for 90 min in 4% PAF in PBS at 4°C, transferred in 20% sucrose in PBS for 24 h at 4°C, then frozen in n-pentane for 3 min at −50°C, and stored at −80°C until assay.

Serial coronal (-1.34 to -1.58 mm from bregma, 40 µm) and horizontal slices (-5.04 to -6.84 mm from bregma, 40 µm; Franklin and Paxinos, 2008) were cut on a cryostat throughout the dorsal extension of the hippocampus and the entorhinal cortex, respectively. For each anterio-posteriority level, 1 slice every 4 (or 8, see 3.6.3 Nissl staining) was collected onto a gelatine-coated slide. The adjacent slices were collected in 1X PBS for immunohistochemistry (IHC, in free-floating; see 3.6.2). Sampling strategy of slices was designed to obtain at one slice for each level of antero-posteriority for quantitative analysis of each marker/staining to provide an average value representative of the whole area of interest. Quantitative analyses were performed in the dorsal hippocampus and in the entorhinal cortex ipsilateral to the injected amygdala since the histopathology is mainly restricted to the injected hemisphere (as described in Chapter 4 and by Mouri et al, 2008). Slices at the same anteroposterior levels were matched for each control and experimental mouse.

3.6.2 Immunohistochemistry

Markers of microglia (Iba1), astrocytes (S100β), and leukocytes (CD45) were analysed using 3 coronal hippocampal slices/marker. Slices were incubated overnight at 4°C with a primary anti-S100β antibody (1:3000, Abcam) in 3% fetal bovine serum (FBS) in 0.1% Triton X-100 in Tris buffered saline (TBS), or with a primary anti-Iba1 antibody (1:1000, Wako) in 1% Normal Goat Serum (NGS) in 0.1% Triton X-100 in PBS, or with a
primary anti-CD45 biotinylated antibody (1:500, BD Pharmingen) in 3% FBS in 0.1% Triton X-100 in PBS. Immuno-reactivity was tested by avidin–biotin–peroxidase (Vector Labs, USA). Sections were stained using diaminobenzidine. At the end of immunohistochemical procedures, slices were mounted onto gelatine-coated slides, dehydrated in graded alcohols, and cover slipped.

Images of the whole dorsal hippocampus were captured at 20X magnification using a Virtual Slide scanning microscopy system (Olympus, Germany) and digitized. Iba1, S100β and CD45 were quantified either by counting in each slice the number of positive cells, and by measuring the cell body size (Iba1 and S100β only). The number of cells was expressed either as total number/section or as cell density (number of cells/area µm²). An algorithm was created to segment and analyse stained cells using Fiji software, as shown in Figure 3.6.1 as well as previously described (De Blasio et al., 2017; Zanier et al., 2015). Data obtained in each of the 3 brain sections for each marker from each mouse were averaged, thus providing a single mean value for each marker/mouse, and this value was used for the statistical analysis.

**Figure 3.6.1 - Quantification of glial cells.** Representative microphotographs of the entorhinal cortex of one representative sham control mouse. The original image (left panel) was scaled to microns, binarized and segmented using Fiji (middle panel). Cells with >15 µm² area were selected and counted (profiles of selected cells for analysis in the right panel). Scale bar = 50 µm. Image taken from Altmann et al., 2021.

### 3.6.3 Nissl staining

One slice every 4 coronal (dorsal hippocampus) or 8 horizontal (entorhinal cortex) sections were stained with cresyl-violet. Cell loss was quantified as previously described using 3 slices for each area of interest (Frigerio et al, 2018b). Briefly, images of the dorsal
Hippocampus and of the entorhinal cortex were captured at 20X magnification using a Virtual Slide scanning microscopy system (Olympus, Germany) and images were digitized. As far as the hippocampus is concern, neuronal cell loss was quantified by manually reckoning the number of Nissl-stained neurons in CA1 and CA3 pyramidal cell layers, and the hilar interneurons. For the entorhinal cortex, the image processing was done using Fiji software (Schindelin et al., 2012). An algorithm was created to segment and analyse every Nissl-stained cell over the entire manually selected region of interest (ROI), as previously described (Pischiutta et al., 2018). Briefly, images were scaled into microns, and the background subtracted. Then an optimised threshold selected in a pilot study was applied across all the experimental groups to identify the Nissl-stained positive area, and the images were binarized. In order to unequivocally distinguish Nissl-stained neurons from glial cells and to count neuronal cells only, we excluded from the counting those cells with an area below the cut off value of 25 µm² by using Fiji software, as previously described (Pischiutta et al., 2018; Zanier et al., 2015). Figure 3.6.2 illustrates a representative microphotograph of the segmentation method. Once segmented, all cells positive for Nissl were automatically quantified. The number of cells was expressed either as total number/section or as cell density (number of cells/area µm²). Data obtained in each of the 3 slices from each area were averaged, thus providing a single mean value for each brain area/mouse, and this value was used for statistical analysis.
Figure 3.6.2 - Quantification of neuronal cell loss. Representative microphotograph showing Nissl staining in the entorhinal cortex of a sham control mouse. To distinguish neurons from glia, we segmented every cell over the entire entorhinal cortex slice by excluding those cells with an area below the cut-off value of 25 µm², by using Fiji software. Using this procedure, only neurons were counted (black profile), while glial cells (red arrows) were excluded from the quantitative analysis. Image taken from Altmann et al., 2021.

3.7 Evaluation of hippocampal excitability and basal neurotransmission

3.7.1 Brain tissue preparation for acute brain slices

Naïve mice were sacrificed by cervical dislocation, the brains were quickly removed and cut in ice-cold modified artificial cerebrospinal fluid (aCSF) containing the following (in mM): 87 NaCl, 2.5 KCl, 1 NaH2PO4, 75 sucrose, 7 MgCl2, 24 NaHCO3, 11 D-glucose, and 0.5 CaCl2. Coronal brain slices (350 um thick) at the level of the dorsal region (septal pole) of the hippocampus were cut with vibrating-blade microtome VT1000s (Leica Microsystems GmbH). Then, slices were transferred into an incubating chamber and submerged in aCSF containing (in mM): 130 NaCl, 3.5 KCl, 1.2 NaH2PO4, 1.3 MgCl2, 25 NaHCO3, 11 D-glucose, 2 CaCl2 and constantly bubbled with 95% O2 and 5% CO2 at room temperature. Slices were incubated for at least 1 h, then transferred in a submerged recording
chamber and perfused with oxygenated aCSF at a rate of 2 ml/min and a constant temperature of 28-30°C (Altmann et al., 2021).

3.7.2 Extracellular recordings

Extracellular recordings of population spikes (PS) were obtained in CA1 pyramidal layer using glass micropipettes filled with 3 M NaCl. Stimulation of Schaffer collaterals was delivered by a Constant Voltage Isolated Stimulator (Digitimer Ltd., Welwyn Garden City, UK) through bipolar twisted Ni/Cr stimulating electrode. Population spike (PS) was recorded in pyramidal layer, while field excitatory postsynaptic potentials (fEPSP) were recorded in stratum radiatum. PS amplitude was measured as the amplitude of the first negative deflection overriding the field EPSP waveform. The input-output curves were plotted as the relationship of PS amplitude or fEPSP slope versus stimulus intensity (2V steps). Five consecutive tracings were averaged for each stimulus intensity. To examine the Paired-pulse facilitation the stimulation was set to approximately the half maximal fEPSP response and the pairs of stimuli were delivered with 25, 50, 150, 200 and 250 ms inter pulse interval (IPI). The first pulse and second pulse elicited by the first and second stimulus are referred to as fEPSP1 and fEPSP2, respectively. The PPF value was calculated as the fEPSP2/fEPSP1 ratio for any given IPI. Data were amplified and filtered (from 10 Hz to 3 kHz) by a DAM 80 AC Differential Amplifier (World Precision Instruments, Sarasota, FL, USA), and digitized at 10 kHz by a Digidata 1322 (Molecular Devices, Foster City, CA, USA).

3.8 Flow cytometry

Mice were anesthetized, briefly perfused with saline for blood displacement and brain tissue removed. Hippocampi ipsilateral to injected amygdala (in SE-exposed groups) or from both hemispheres (in sham mice) were rapidly dissected out on ice and mechanically dissociated, then incubated with 0.4 mg/ml collagenase type IV (Sigma-Aldrich) for 30 min at 37°C. Then, the homogeneous cell suspension was loaded on a isotonic 90% Percoll
gradient and centrifuged for 30 min at 4°C at 10,800 rpm to enrich for inflammatory cells and discard myelin debris, as previously described (De Feo et al., 2017). All harvested cells were then immediately stained and thereafter acquired on the Canto II (Becton Dickinson/BD Biosciences) flow cytometer and analysed with FlowJo software (Tree Star). The following antibodies were employed: CD11b-PE-Cy7 (1:300, BD, clone M1/70), CD45-PB (1:300, Biolegend, clone 30-F11), Ly6C-FITC (1:200, BD, clone AL-21), Ly6G–PerCP-Cy5.5 (1:200, BD, clone 1A8). Live viability was assessed by the Zombie NIR™ Fixable Viability Kit (1:500, Biolegend). Prior to detailed analysis, cells were always gated on single and live cells, by excluding dead cells with the live/dead Zombie NIR™ Fixable Viability Kit (Biolegend). Immune cells were identified as live microglial cells (CD45loCD11b+Ly6G-Ly6C-), monocytes (CD45hiCD11b+Ly6G-Ly6Chi), neutrophils (CD45+CD11b+Ly6G+) and lymphocytes (CD45+CD11b-) as detailed in the gating strategy in each figure legend (Altmann et al., 2021; Di Nunzio et al., 2021).

3.9 Statistical analysis of data

All efforts were made to minimize the number of animals used and their suffering according to the principles of the 3 Rs (Replacement, Reduction and Refinement; https://www.nc3rs.org.uk/the-3rs). Quantification of each experiment was done blindly. The sample size was a priori determined based on previous experience with this animal model, and statistical hypotheses, endpoints and statistical tests were prospectively defined.

Statistical analysis was performed by GraphPad Prism 8 (GraphPad Software, USA) for Windows using absolute values. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum (n = number of individual mice or samples) or as mean ± standard error mean (SEM). Choice between parametric and non-parametric tests depended on passing D’Agostino and Pearson normality test.

The temporal distribution of spikes during SE, the treatment effect on number of seizures and duration, the learning rate (measured as total latency during the daily trials of
the Barnes Maze) were analysed by two-way ANOVA or mixed models (Goonewardene, 2004). Mann-Whitney test for two independent groups or one-way ANOVA followed by Tukey’s post-hoc test for more than two independent groups were used for statistical analysis of SE onset and duration, MRI data, microglial and neuronal cell count, for discrimination index in the NORT and probe in the Barnes Maze. Statistical significance was considered with p<0.05.

The statistical analysis performed were accurately revised by a biostatistician, Dr L. Porcu.
CHAPTER 4: THE EXPERIMENTAL MODEL OF ACQUIRED EPILEPSY

Part of the results presented in this chapter have been published in:

4.1 Description of the experimental model of epilepsy

The experimental mouse model of acquired epilepsy we used has been extensively described previously (Frigerio et al., 2018b; Iori et al., 2017; Jimenez-Mateos et al., 2011; Mouri et al., 2008). It is based on the induction of a primary brain injury, *status epilepticus (SE)* provoked by the injection of KA in the basolateral nucleus of the amygdala. KA is an agonist of ionotropic glutamate AMPA/Kainate receptor subtype and local or systemic injection of convulsive doses of KA induce seizures associated with neuronal cell loss in forebrain (Ben-Ari and Cossart, 2000).

After 10 minutes from KA injection, SE starts and is characterized by continuous spiking activity evaluated with EEG analysis and by associated motor convulsions (Iori et al., 2017). After 40 minutes from KA, an i.p injection of diazepam is administered in order to reduce mortality by attenuating SE motor component but without altering EEG SE (Jimenez-Mateos et al., 2011; Iori et al., 2017; Frigerio et al., 2018b). In this model, SE has an average duration of 12.1±1.4 hours (*Mean ± SEM*; Iori et al., 2017).

SE triggers epileptogenesis and spontaneous seizures develop after the acute insult (*Mean ± SEM*, onset, 6.2±0.5 days), and recur for months (Di Nunzio et al., 2021; Frigerio et al., 2018b; Iori et al., 2017). Spontaneous convulsive seizures originate and spread in the limbic system, and also involve the neocortex (Jimenez-Mateos et al., 2011; Iori et al., 2017; Frigerio et al., 2018b). Mice develop cognitive deficits after SE (Di Nunzio et al., 2021; Frigerio et al., 2018b; Liu et al., 2013).

As extensively described in Chapter 1 of this thesis, clinical and experimental evidence showed that *microgliosis* is a major feature of the epileptogenic brain. Thus, in this study we have characterized the temporal pattern of microglia reactivity and proliferation in our murine model of acquired epilepsy by combining immunohistochemistry and flow cytometry.

This model mimics features of temporal lobe epilepsy with hippocampal sclerosis. In particular, neuronal cell loss is observed in the hippocampus ipsilateral to the injected
amygdala (Mouri et al., 2008; Iori et al., 2017) and in extrahippocampal areas ipsilateral to the injected hemisphere (Jimenez-Mateos et al., 2011; Iori et al., 2017). Less extensive cell loss was reported in the contralateral hemisphere in our previous studies. We evaluated therefore the extent of the neurodegeneration by quantifying the neuronal cell loss in crucial epileptogenic areas in this model, such as the hippocampus and entorhinal cortex (Iori et al, 2017; Frigerio et al, 2018b) in both hemispheres.

4.2 Specific materials and methods

Experimental plan. In order to characterize the pattern of microgliosis during epileptogenesis, a detailed time-course experiment was designed as follows: mice were exposed to stereotaxic surgery as described in General Materials and Methods and after one week of recovery in their home cage, they were connected to the EEG set up and exposed to SE. Microglia was assessed 2 h, 24 h, 5 days, 7 days, 14 days and 3 months post-SE that represent the crucial phases of disease development (n=5-6 mice/time-point). Iba1-positive cells were analysed by quantitative immunohistochemistry in the hippocampus ipsilateral to the KA-injected amygdala, a crucial epileptogenic area in this model (Iori et al, 2017; Frigerio et al, 2018b).

4.3 Results

4.3.1 Microglia reactivity and proliferation

Iba1 immunoreactivity showed reactive microglia from 24 h to 3 months after SE: Iba1 positive cells displayed hypertrophy with retraction of distal ramifications (Figure 4.3.1 C, D) as compared to cells with small cell bodies and extensive ramifications in sham hippocampi (Figure 4.3.1 A). Quantitative analysis showed a significant increase of cell body size in SE-exposed mice at all the time points analysed starting from 24 h post-SE after SE (Figure 4.3.1, left), thus confirming the morphological changes of cell activation (Figure 4.3.1). Also, the number of Iba1 positive cells was significantly increased in SE-exposed vs sham mice after 5 days until 3 months post-SE (Figure 4.3.1, right).
Figure 4.3.1 - Analysis of microglia in the hippocampus of mice exposed to status epilepticus.

Panels A-D: Representative photomicrographs (2x; scale bar: 50 µm) showing Iba1-positive cells in CA1 subfield in sham mice (A) and status epilepticus (SE)-exposed mice sacrificed 2 h (B), 24 h (C) and 3 months (D) after SE onset. Inserts show a magnified microglial cell (60x; scale bar: 20 µm).

The graphs below depict the quantitative analysis (averaged from at least three sections): the average cell body size/section (left) and number of Iba1-positive cells/section (right) in sham mice and SE-exposed mice sacrificed at the various time points. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values (sham n=5; SE n=5-6 mice/group). *p<0.05, **p<0.01 vs sham by Kruskall-Wallis test followed by Dunn’s multiple comparison test.
Consistent with immunohistochemistry, a significant increase of microglial cells was measured 7 days post-SE vs shams in dissociated hippocampal tissue (Figure 4.3.2, upper left). A significant increase in the number of macrophages was also measured within the same hippocampi (Figure 4.3.2, upper right).

Figure 4.3.2 - Flow cytometry quantification of microglia and macrophages. Above, absolute cell counts of microglia (left) and macrophages (right) in the hippocampus of sham and SE-exposed mice sacrificed 7 days after SE onset (n=4; each value results the total number of cells from pools of hippocampi from 3 different mice). Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. *p<0.05, Mann Whitney U test. Below, representative plots depicting gating strategy in flow cytometry analysis for separation of phenotypically identified microglia and macrophages in sham and SE-exposed mice.

4.3.2 Evaluation of neuronal cell loss in epileptic mice

We have first evaluated neuronal cell loss in the hippocampus and entorhinal cortex of chronic epileptic mice compared with sham control mice by Nissl-staining. The analysis confirmed a significant reduction of neurons in the dorsal ipsilateral hippocampus of
epileptic mice compared with sham mice in CA1, CA3 and hilar region of DG (**Figure 4.3.3 A**). Differently, a significant reduction of CA1 neurons and CA3 but not in the hilar region was observed in the contralateral hippocampus (**Figure 4.3.3 A**). In the ipsilateral entorhinal cortex, a significant reduction of neurons was measured compared with sham mice, whereas no neuronal damage was observed in the contralateral entorhinal cortex (**Figure 4.3.3 B**).

Next, we evaluated if the neuronal cell loss was present in the ipsilateral ventral hippocampus. The results of the neuronal counts have shown no differences in the epileptic mice compared with sham mice (**Figure 4.3.3 C**).
Figure 4.3.3 - Quantification of neuronal cell loss by Nissl-staining. A) Representative photomicrographs of dorsal hippocampus (CA1, CA3 and hilus) in the ipsilateral and contralateral hemispheres to the injected amygdala (n=8-9 respectively), compared with sham control mice (N=7-14) and the relative quantification (number of neurons/section averaged from at least three sections). B) Representative photomicrographs of horizontal sections of the entorhinal cortex in the ipsilateral and contralateral hemispheres to the injected amygdala (n=21-15 respectively), compared with sham control mice (n=20) and the relative quantification (number of neurons/area µm², averaged from at least three sections). C) Representative photomicrographs of horizontal sections of ventral ipsilateral hippocampus (CA1, CA3 and hilus) in epileptic vs sham mice (n=8/group) and the relative quantification (number of neurons/section averaged from at least three sections). Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. *p<0.05, **p<0.01, vs sham; °p<0.05, °°p<0.01 vs ipsi, ANOVA followed by Tukey’s test. Ctx=cortex; I-V= neuronal cell layers.

4.4 Discussion

It was previously reported that microglia display reactive morphology and proliferates after SE in rodents exposed to systemic KA (Avignone et al., 2008). Our results support previous findings by showing microglia reactivity and proliferation in our intra-
amygdala KA model of acquired epilepsy. Data showed that microglia react and proliferate during epileptogenesis and this effect persist in chronic epilepsy, specifically within the first week and at 3 months after SE.

We also characterized the neurodegeneration in this model of acquired epilepsy (Frigerio et al., 2018b). Previous studies described a progressive pattern of neuronal death following electrically induced SE (Pitkanen and Sutula, 2002), and that pilocarpine-induced SE causes progressive neuronal damage across brain regions (Fujikawa, 1996).

Our results showed that neuronal cell loss is prevalent in the dorsal ipsilateral hippocampus and it does not extend to the ventral hippocampus. Neuronal cell loss was observed in the entorhinal cortex but only ipsilaterally to the injected amygdala, since the contralateral side was preserved. Note that post-mortem analyses in the following chapters will mainly refer to the ipsilateral hemisphere, unless otherwise indicated, where damage was prevalent.

The following chapters describe the pharmacological strategies used to interfere with microglia and uncover its role in epileptogenesis: two different CSF1R inhibitors were applied to mice, namely PLX3397 to deplete microglial cells and GW2580 to block their proliferation.
CHAPTER 5: MICROGLIA DEPLETION PREVENTS CORTICAL THINNING IN A MURINE MODEL OF ACQUIRED EPILEPSY

The results reported in this study have been published in:

Andre Altmann*, Mina Ryten*, Martina Di Nunzio* et al.

*First shared co-authorship
5.1 Introduction

The ENIGMA-EPILEPSY consortium (EEC), who is running the largest international neuroimaging study in epilepsy, has recently identified patterns of shared grey matter reduction in cortical regions (cortical thinning) across epilepsy syndromes, including idiopathic generalized epilepsies, TLE with/wo hippocampal sclerosis, other types of focal epilepsies. These structural brain abnormalities represent a common feature across epilepsies as shown by MRI brain scans of 2149 individuals with diverse epilepsy etiologies and syndromes compared to 1727 matched healthy controls (Whelan et al., 2018). However, mechanisms underlying these structural changes have not been described yet, thus it is important to identify possible causes of cortical thinning in human epilepsies.

To identify the molecular basis of this regional vulnerability in the broad spectrum of epilepsies, and the potential clinical consequences, the brain regions showing cortical thinning have been aligned to a region-specific gene expression map available from the Allen Institute for Brain Science: Healthy Human Brain Gene Expression Atlas. More than 2500 differently expressed genes have been identified by this analysis revealing an enrichment for microglial genes in cortical thinning vulnerable regions vs non vulnerable regions, in particular, for microglial reactive state. Indeed, it is well known that microglia display a reactive morphology in epileptic foci and in the areas where seizures generalize both in surgical specimens from epilepsy patients and in experimental models (Ravizza et al., 2008). This evidence therefore has identified microglia as a cell population potentially involved in the cortical thinning observed in the epilepsies.

The aim of this study was to interrogate microglia involvement in epilepsy development, cortical thinning and neuropathology, and in potential functional consequences such as cognitive decline, using an established murine model of TLE. We targeted reactive and proliferating microglia by reversibly depleting this cell population from the brain parenchyma with PLX3397, a blocker of the CSF1R involved in proliferation, differentiation and survival of microglia. As previously described by Elmore and colleagues,
PLX3397 in food pellet depletes microglia by ~95% after 3 weeks of treatment. After mice are returned to a non-medicated diet, devoid of PLX3397, microglia re-populate the brain parenchyma within one week (Elmore et al., 2014).

5.2 Specific Materials and Methods

5.2.1 Justification of the treatment schedule and experimental design

An orally available inhibitor of CSF1R (Tap et al., 2015), PLX3397 was formulated 290 mg/kg in a standard chow diet (AIN-76A, Research Diets, Inc New Brunswick, NJ, USA), corresponding to a daily dosing of ≈ 52 mg/kg in mice and a placebo diet was used as control (standard chow diet AIN-76A). Both research diets were stored at 4°C until use.

Microglia depletion in mice fed with a PLX3397-supplemented diet has been extensively described before (Buttgereit et al., 2016; Elmore et al., 2014; Huang et al., 2018; Waltl et al., 2018). Studies report that a treatment schedule of 21 days allows depletion of 99% of microglia from brain parenchyma and after drug withdrawal microglia re-populates the brain by 50% within 3 days, and at completion by one week (Elmore et al., 2014).

In order to characterize an appropriate protocol of drug administration during epileptogenesis, naïve mice were fed with PLX3397-supplemented diet for 21 days. Daily food intake and body weight were measured to ensure mice were healthy and food intake was not affected by the medicated diet. At the end of the treatment (21 days), mice were killed and brains were processed for immunostaining of the microglial marker Iba1, flow cytometry analysis (Figure 5.3.1) or for electrophysiological experiments (Figure 5.3.5).

Based on the results in naïve mice, we designed the experiment with mice to be exposed to SE that were randomized as depicted in Figure 5.2.1.

Cross-sectional study. As depicted in Figure 5.2.1 A, mice were kept for two weeks under either placebo or PLX3397-diet, then they were implanted with electrodes and one injection cannula for kainate administration (see Materials and Methods in Chapter 3). One week after surgery mice under the same placebo or PLX3397-diet regimen were exposed to
SE. The PLX3397 diet was continued after SE until mice were sacrificed 14 days (n=6/group) post-SE, in order to verify that microglia depletion was maintained after SE induction (Figure 5.2.1 A). Sham mice were run in parallel serving as control (n=7).

Longitudinal study. As depicted in Figure 5.2.1 B, a group of mice was prepared for a longitudinal study, in order to study the involvement of microglia in the development of epilepsy and the related structural brain modifications, as follows. Mice were fed with either PLX3397-supplemented diet or placebo diet (n=14/group) for 2 weeks, then mice were implanted with electrodes and injection cannula, and after one week from surgery they were exposed to SE (time 0) while keeping them under the same placebo or PLX3397-diet regimen during the whole procedures (n=3 mice/group died after SE and n=1 mouse in the placebo group died in the chronic phase; they were excluded from any subsequent analysis). The PLX3397 diet was continued for one week after the onset of epilepsy. At this time, mice under the PLX3397 diet were switched to the placebo diet until the sacrifice. Control mice exposed to SE received a placebo diet throughout the whole experiment.

Mice were monitored (24/7) from SE induction until 3 weeks thereafter to encompass the acute phase of epilepsy development. Animals were again EEG monitored in the chronic disease phase, as indicated in Figure 5.2.1 B. At the end of the EEG recordings, mice were behaviourally tested in NORT. Then, mice were sacrificed and their brains were exposed to post-mortem MRI, then processed for histology (see Material and Methods for details). As described in paragraph 5.3.4, for behavioural analysis we included additional sham mice (n=13-16) and placebo-diet fed SE mice (n=8) that were run in parallel with PLX3397-fed or placebo-diet fed mice of Figure 5.2.1 B. These placebo-diet fed mice (n=8) were EEG monitored only during the chronic disease phase (55-90) and displayed seizures frequency (1.65 ± 0.75, n=10; p=0.41 by Mann-Whitney test) similar to longitudinally monitored placebo-diet fed mice of Figure 5.2.1 B. The same mice were also included in post-mortem MRI and histopathological brain analyses.
In addition to the longitudinal study, a group of mice that had experienced SE (n=10), was monitored for 2 weeks during chronic epilepsy (55-69 days), then switched to PLX3397-supplemented diet (70-84 days): EEG recordings were performed for 2 weeks to assess the number of seizures of each mouse (baseline) and for 2 weeks during PLX3397 treatment (2 out of 10 mice were excluded because displaying no seizures at baseline). This treatment schedule is depicted in Figure 5.2.1 C. These mice were tested during chronic epilepsy to study the involvement of microglia in the generation of chronic seizures.

**Figure 5.2.1 - Schematic representation of the experimental design.** Mice were randomized in a cross-sectional (A) and a longitudinal (B) experiment, where the time of onset of spontaneous seizure (box onset) occurred approximately one week (7 d) after SE (mean ± SEM: 6.2±0.5 days; n=21). The PLX3397-supplemented diet was given to mice for three weeks (21 days) before the induction of status epilepticus (SE, time 0). One week before the induction of SE, mice underwent stereotactic surgery for electrode implantation to allow EEG recordings (1 w before SE, black arrow). EEG was monitored (24/7) at the time intervals indicated by dashed lines. After SE, the medicated diet was protracted until the onset of spontaneous seizures and continued for 7 additional days (14 d) after onset. Overall, the treatment schedule was continued for approximately 5 weeks. Panel A depicts the protocol of the cross-sectional experiment where mice were sacrificed respectively 14 days for immunohistochemistry (IHC). Mice in Panel B were switched to the placebo diet 7 days after the onset of SRS, which means approximately 14 days after SE (14 d) and monitored for one additional week (21 d), then removed from the EEG and returned to their home cage. Dashed black arrows...
indicate mice that were excluded from the study because of mortality after SE or in seizures. Mice were monitored longitudinally, and EEG recordings were performed again during chronic epilepsy (55-90 d). At the end of the EEG recordings, mice were exposed to a behavioural test (NORT), then sacrificed. Brains were exposed to post-mortem MRI, then processed for histology (see Material and Methods for details). An additional group of mice exposed to SE received a placebo diet throughout the whole experiment, serving as control mice. Panel C depicts the chronic treatment protocol, where PLX3397-supplemented diet was administered during chronic epilepsy (n=10). Mice were monitored longitudinally, and EEG recordings were performed during two subsequent recording windows: 55-69 days to assess seizures baseline (2 mice were seizure-free at the baseline, thus they were excluded) and 70-84 days to evaluate the effect of PLX3397 on seizures number (as indicated in green brackets).

5.3 Results

5.3.1 Effects of microglia depletion in naive mice

The mice fed with the PLX3397 diet grew in weight similarly to placebo diet fed mice during three weeks of treatment (Mean ± SEM; g; Placebo, day 1, 25.1±0.2; day 21, 28.2±0.5; PLX3397, day 1, 24.7±0.5; day 21, 26.7±0.4; n=5-8). Also, daily food intake was comparable in the two experimental groups (Mean ± SEM; g; Placebo: 3.7 ± 0.1; PLX3397: 3.3 ± 0.1; n=5-8). In accordance with what previously observed by Elmore and colleagues, PLX3397-supplemented chow (290 mg/kg diet) provided >90% microglia depletion in hippocampus and entorhinal cortex of naive mice vs mice fed with placebo diet (n=6-8), as assessed by Iba1 quantitative immunohistochemistry (Figure 5.3.1 A). Accordingly, flow cytometry analysis of microglia from forebrain of PLX3397-supplemented diet fed mice showed a cell number reduction of ~94% compared to placebo diet fed mice (Figure 5.3.1 B). No effect of PLX3397 was found on the number of S100β positive astrocytes (Mean ± SEM; average number of cells in three adjacent hippocampal slices; Placebo: 1343 ± 29.4; PLX3397: 1357 ± 13.9; n=5-6), thus confirming the selectivity of PLX3397 for microglia.
**Figure 5.3.1** - **PLX3397 depletes microglia in the brain of naive mice.** Panel A: Representative photomicrographs of Iba1 immunoreactivity in the hippocampus and entorhinal cortex of mice fed with placebo non-medicated diet (n=6-8) or PLX3397-supplemented diet (n=5) for 21 days. Box-and-whisker plots show the quantification of the number of Iba1 positive cells/section averaged from the cell count in three adjacent hippocampal slices, as depicted by median, minimum, maximum and single values in the hippocampus and entorhinal cortex. **p<0.01 vs placebo by Mann Whitney test.** Scale bars: 200 µm. Ctx = cortex; GC = granule cell layers; CA1 = CA1 pyramidal cell layer; II-V cortical neuronal layers. Panel B: Flow cytometry analysis on brain immune cells after 21 days treatment with PLX3397-supplemented diet. The total cell number of live microglial cells (CD45<sup>lo</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>-</sup>) in the hippocampus (pooled hippocampi from the two hemispheres/mouse; n=2-3 mice) is quantified in the graph and shown in representative plots. Data are shown as Mean ± SEM, **p<0.01 vs placebo by Mann Whitney test.

Similarly, PLX3397 displayed no effects on neuron number in the hippocampus and in the entorhinal cortex, as depicted in **Figure 5.3.2.**
Figure 5.3.2 – Lack of effects of PLX3397 on neurons in naïve mice. Panel A. Photomicrographs of Nissl-stained coronal hippocampal slices (CA1, CA3 and hilus subfields) from brains of naïve mice fed with placebo diet (n=4) and PLX3397-supplemented diet (n=5) for 21 days. Relative quantifications of the average number of Nissl-stained neurons/section in each hippocampal subfield are reported in the graph (right). Panel B. Photomicrographs of Nissl-stained horizontal entorhinal cortex slices in naïve mice fed with placebo diet (n=4) and PLX3397-supplemented diet (n=4) for 21 days. II-V cortical neuronal layers. Relative quantification of neuronal density is shown in the graph (right).
5.3.2 Microglia depletion in mice exposed to SE and developing epilepsy

As shown in Figure 5.2.1 A, mice fed for 21 days with the PLX3397-supplemented diet in order to achieve microglial depletion (>95%), or placebo diet, were exposed to SE.

Then, mice were sacrificed 14 days post-SE during the same diet regimen for immunohistochemistry analyses to verify the efficacy of PLX3397 in providing microglial depletion after SE. As described previously (Chapter 4), SE induces glial cells reactivity and proliferation within 24 h after KA injection and this effect persists until chronic epilepsy, as previously shown in Figure 4.3.1 (Chapter 4). Figure 5.3.3 shows that the number of microglia and their cell body size were increased after SE in placebo-fed mice compared to sham (**p<0.01 vs sham by one-way ANOVA followed by Tukey’s multiple comparisons). PLX3397-fed mice showed microglia depletion after SE, as shown by the quantification of microglial cell number (**p<0.01 vs placebo by one-way ANOVA followed by Tukey’s multiple comparisons) (Figure 5.3.3 A). The microglia cell surviving PLX3397-induced depletion after SE appeared morphological activated as shown by increased cell body size (Figure 5.3.3 B). Astrocytes number and body size were increased similarly in SE group under placebo or medicated diets compared with sham (**p<0.01 by one-way ANOVA followed by Tukey’s multiple comparisons) (Figure 5.3.3 C, D), confirming PLX3397 selectivity for microglia.
Figure 5.3.3 – PLX3397 depletes microglia without affecting astrocytes. Total cell number/section and average cell body size/section of Iba1- (A, B) and S100β-positive cells (C, D) were averaged from three slices of different anteroposteriority level, representative of the whole dorsal hippocampus, ipsilateral to the injected amygdala. Sham and SE-exposed mice: placebo and PLX3397-diet fed mice were sacrificed 14 days after SE (experimental protocol in Figure 5.2.1). Box-and-whisker plots depicting median, minimum, maximum and single values. ** or °° p<0.01 vs sham or placebo by ANOVA followed by Tukey’s post-hoc test.

5.3.3 Microglia depletion effects on SE and spontaneous seizures

SE developed similarly in PLX3397-supplemented and placebo diet fed mice: microglial depletion did not modify the onset, duration and severity of SE as shown in Figure 5.3.4. In agreement with these results, hippocampal neuronal excitability in naïve mice fed with PLX3397 for 21 days was not modified by microglia depletion, as assessed in acute hippocampal slices (Figure 5.3.5, n=5-6 mice/group; see Chapter 3, General Materials and Methods for details).
Figure 5.3.4 – Lack of effects of PLX3397 on acute SE outcomes. Panel A: box-and-whisker plots depicting median, minimum, maximum, and single values of status epilepticus (SE) onset and total duration in mice fed with placebo non-medicated diet (n=10) or PLX3397-supplemented diet (n=11) for 3 weeks before SE induction. Panel B, the temporal spike distribution during SE: each data point represents the mean cumulative number of spikes during progressive 1-hour intervals, for each experimental group. The dotted line represents the threshold number of spikes/hour (3.600) below which SE ends (inter-spike intervals longer than 1 second).
Figure 5.3.5 – Lack of effects of PLX3397 on basal neuronal excitability and neurotransmission. Extracellular recordings of population spikes (PS) evoked in CA1 pyramidal neurons by electrical stimulation of the Schaffer collaterals. The relationship between stimulus intensity, i.e. current input, and PS peak amplitude, i.e. neuronal output, is considered as an index of neuronal excitability. Slices from microglia-depleted mice showed an input-output curve similar to slices from control mice fed with placebo diet indicating no changes in neuronal excitability. PS amplitudes are shown as percentage of maximal response (mean ± SEM; n=6 slices from 5-6 mice/each group). Representative traces of PS obtained in response to 24 V stimulation are shown.

Moreover, the onset (Mean ± SEM; days after SE; Placebo: 5.0±0.4; PLX3397: 6.8±0.8), frequency and duration of spontaneous seizures were not altered in mice transiently fed with PLX3397-supplemented diet as compared to placebo-diet fed mice (Figure 5.3.6). The mice fed with PLX3397, or placebo diet grew in weight similarly during the treatment (Mean ± SEM; Placebo before SE: 24.0±1.2 g; after SE: 25.7±1.4 g; PLX3397 before SE: 23.3±1.7 g; after SE: 26.2±1.1 g (Figure 5.2.1 B).
Figure 5.3.6 – Lack of effects of PLX3397 on epilepsy development. The PLX3397-diet was initiated 21 days before exposing mice to SE and continued until each mouse experienced the first spontaneous seizure (indicated as day 1 in this Figure) and for one additional week thereafter (as described in the experimental design in Figure 5.2.1 B). Then, mice were switched to the placebo diet until end of the experiments. Panel A shows box-and-whisker plots depicting median, minimum, maximum and single values of the number of spontaneous seizures/day and their average duration during days 1–7, 8–15 and 55–90 from epilepsy onset (day 1) in the placebo (n=10) and PLX3397-supplemented diet (n=11) groups. Outliers were identified for the parameter Number of seizures/day in the Placebo group (n=1 in days 1–7) and in the PLX3397 group (n=2 in days 8–15 and n=2 in days 55–90), their omission did not change the results of the primary statistical analysis (Mixed-effects Model) therefore they were not removed from the corresponding data set. Panels B, C report the daily seizure number (Mean ± SEM) occurring in mice fed with placebo diet (n=10) or with PLX3397-supplemented diet (n=11).
5.3.4 Microglia depletion effect on cognitive impairment associated with epilepsy

Considering the natural tendency of mice to explore novel objects for which they have an innate preference over a familiar one, their ability to recognize a set of novel objects in an otherwise familiar environment is considered a measure of recognition memory (d’Isa et al., 2014). This cognitive parameter is impaired during epilepsy development and in chronic epileptic mice (Iori et al., 2017; Frigerio et al., 2018b). Therefore, at the end of the EEG monitoring, memory was tested in epileptic mice transiently fed with placebo or PLX3397-supplemented diet, and sham control mice in the NORT (Figure 5.3.7) to assess the effects of microglia depletion on the development of the memory deficit. Previous data showed that a prolonged PLX3397 treatment did not affect behaviour in naïve mice tested in the elevated plus maze, in the open field and in the Barnes maze (Elmore et al., 2014), therefore we did not test the effect of PLX3397 in sham mice. In this experiment, some mice (sham, n=3; placebo-diet, n=5; PLX3397-diet, n=3) were excluded from the behavioural test due to scarce object exploration during the familiarization phase (a total time of exploration < 8 seconds; see Materials and Methods for details). Therefore, additional control mice (sham and placebo epileptic mice, depicted in grey) were used. These mice were run in parallel with placebo- or PLX3397-treated mice (longitudinal study described in Figure 5.2.1) and they were EEG monitored in the terminal disease phase (day 55-90). Their seizure frequency (0.91±0.26, n=8) was similar to mice depicted in Figure 5.3.6 (1.65±0.75, n=10; p=0.41 by Mann Whitney test).

During the test, the time of exploration of both objects, novel and familiar, was similar in all the experimental groups (Figure 5.3.7 A).

As expected, sham mice explored preferentially the novel object, with a discrimination index (DI) of 0.29 ± 0.04 (mean ± SEM; n=13; Figure 5.3.7 B), whereas chronic epileptic mice fed with placebo diet displayed similar exploration of the familiar and the novel object resulting in reduced DI (-0.11 ± 0.09; n=13; p<0.01 vs sham by one-way ANOVA followed by Tukey’s multiple comparisons), which indicates memory impairment.
Chronic epileptic mice that were depleted of microglia at the time of SE induction and during the initial phases of disease development (Figure 5.2.1 B), displayed an increased DI vs placebo diet SE-mice (DI, 0.1 ± 0.07, n=8; p<0.05 by Mann-Whitney test), approaching DI value of sham mice. Thus, microglia depletion in the early disease phase prevented the deficit in NORT. Mice total travelled distance (cm) and speed (cm/s) during the test were also evaluated to assess mice general motility and no differences were detected for both parameters among the three groups (Figure 5.3.7 C, D).

Figure 5.3.7 - Microglia depletion with PLX3397 attenuates cognitive deficit in the NORT. The novel object recognition test (NORT) was performed in epileptic mice fed with placebo- (n=13) or PLX3397-supplemented diet (n=8), and sham controls (n=13) during early disease development. Mice showing a total exploration time <6 sec during the familiarization phase were excluded from the subsequent analyses (sham n=3; placebo n=5; PLX3397 n=3). Total time of exploration of the two objects (A), travelled distance (C) and speed (D) were evaluated to assess mouse natural explorative behaviour and motility. Memory (B) was evaluated by measuring the discrimination index (DI), which was calculated as time spent (s) exploring the familiar (F) and the novel (N) object as follows: (N - F)/(N + F). Data are shown by box-and-whisker plots depicting median, minimum, maximum and single values, differences significant at **p<0.01 vs sham by ANOVA followed by Tukey's test; °p<0.05 vs placebo by Mann-Whitney test.
5.3.5 Effects of microglia depletion on cortical thinning and neuron loss

First, the thickness and volume of selected cortical brain regions were studied in chronic epileptic mice (n=18) compared to sham control mice (n=13-16), and we confirmed the structural changes as predicted by the ENIGMA-Epilepsy findings in humans (Whelan et al., 2018). The volume of the lateral ventricles was enlarged by 2-fold in epileptic mice fed with placebo diet (n=10) vs sham mice (not exposed to SE, n=13-16; **p<0.01 by ANOVA followed by Tukey's test; Figure 5.3.8 A). In accordance with the clinical evidence, we measured a significant reduction in the volume of the entorhinal and perirhinal cortices (*p<0.05 by ANOVA followed by Tukey's test; Figure 5.3.8 E, F). Notably, a significant reduction in the thickness of entorhinal and perirhinal cortices was also measured in the same mice (respectively: **p<0.01; *<0.05 by ANOVA followed by Tukey's test; Figure 5.3.9 A, B). No significant changes were observed in other brain areas such as the hippocampus, caudato-putamen, and the thalamus, although their average volumes trended lower than the corresponding values in sham mice (Figure 5.3.8 B-D).

Microglial depletion during the initial phases of epilepsy development prevented the decrease in thickness of the entorhinal cortex (°p<0.05 vs placebo by ANOVA followed by Tukey's test; Figure 5.3.9 A). Thus, microglia during the early disease phases may play a role in the cortical thinning observed in the chronic disease phase. However, microglia depletion did not affect the thinning of the perirhinal cortex (Figure 5.3.9 B) or the ventricle volume changes occurring in epileptic mice (**p<0.01 vs sham by ANOVA followed by Tukey’s multiple comparisons).
Figure 5.3.8 – Lack of effects of PLX3397 on brain volumes in epileptic mice. Quantification of the volumes of lateral ventricle (panel A, three sham mice values were discarded because of unusual ventricle size), hippocampus (panel B), caudato-putamen (panel C), thalamus (panel D), entorhinal (panel E) and perirhinal (panel F) cortices, as assessed by quantitative post-mortem MRI performed at the end of the longitudinal experiment (placebo: n=18; PLX3397: n=7), and in sham mice (n=13-16). Grey symbols represent additional sham (n=5-8) and epileptic mice fed with placebo diet (n=8).
run in parallel with experimental mice (black) of the longitudinal experiment described in Figure 5.2.1 (and see p. 94 for details). Four mice in PLX3397 group did not undergo MRI analysis therefore they were not included in the subsequent histological analyses. Each panel depicts also MRI images showing representative ROIs traced to quantify the volumes of the various brain regions. Box-and-whisker plots depicting median, minimum, maximum and single values. Lateral ventricle, entorhinal cortex **p<0.01; perirhinal cortex *p<0.05 vs sham by ANOVA followed by Tukey’s post-hoc test. Scale bars: 1 cm.

Figure 5.3.9 - Microglia depletion with PLX3397 attenuates the thinning of the entorhinal cortex. Quantitative post-mortem MRI analysis of entorhinal (panel A) and perirhinal (panel B) cortices in epileptic mice at the end of EEG monitoring. Grey symbols represent sham and epileptic mice fed with placebo diet (n=8/group) run in parallel with experimental mice (black) of the longitudinal experiment described in Figure 5.2.1 (and see p.95 for details). Four mice in PLX3397 group did not undergo MRI analysis therefore they were not included in the subsequent histological analyses. Data are reported in box-and-whisker plots depicting median, minimum, maximum and single values, (placebo, n=18; PLX3397, n=7), and in sham mice (not exposed to status epilepticus; n=16). *p<0.05 and **p<0.01 vs sham; °p<0.05 vs placebo by ANOVA followed by Tukey's test. MRI images depict representative levels showing the ROIs used to quantify the cortical thickness. The white line within the ROI was manually drawn to measure the cortical thickness. Scale bar: 1 cm.
No correlation was found between seizure frequency and cortical thickness in chronic epileptic mice (Spearman r: 0.3, p=0.3, in either placebo- or PLX3397-fed mice), in accordance to what previously described by ENIGMA epilepsy study (Whelan et al., 2018).

*Nissl-staining.* Neuronal cell loss was evaluated in the hippocampus and in the entorhinal cortex of chronic epileptic mice. Mice receiving placebo diet showed a significant decrease in neuronal cell density (**p<0.01 vs sham by ANOVA followed by Tukey's test; **Figure 5.3.10**) and in their average cell body size in the entorhinal cortex (*p<0.05 vs sham by ANOVA followed by Tukey's test; **Figure 5.3.10**). The reduction in cell density, but not of the average cell body size, in epileptic mice was prevented by microglia depletion in the early disease phase in this brain area (°°p<0.01 vs placebo diet by ANOVA followed by Tukey's test; **Figure 5.3.10**). A different outcome was observed when the hippocampal neurodegeneration was quantified as depicted in **Figure 5.3.11**. In fact, chronic epileptic mice displayed a reduced number of neurons in CA1, CA3 and hilus compared to sham mice (**p<0.01 vs sham by ANOVA followed by Tukey's test; **Figure 5.3.11**). A similar neuronal cell loss was measured in mice that received PLX3397-supplemented diet during the early phases of epilepsy development (*p<0.05 or **p<0.01 vs sham by ANOVA followed by Tukey's test; **Figure 5.3.11**).
Figure 5.3.10 - Microglia depletion with PLX3397 attenuates the neuronal cell loss in the entorhinal cortex. Representative Nissl-stained sections (top row) of the entorhinal cortex in the experimental groups (sham, n=14; placebo, n=15; PLX3397, n=7), and the relative quantification of the number and the average size of Nissl-stained neurons (bottom row). Each parameter results from the average value of the quantification of at least three slices per group, sampled at different horizontal levels to represent the entorhinal cortex. Grey symbols represent additional sham (n=7) and epileptic mice fed with placebo diet (n=7) run in parallel with experimental mice (black) of the longitudinal experiment described in Figure 5.2.1 (and see p. 95 for details). Two sham and three placebo mice were excluded from the analysis due to poor quality of Nissl staining. Data are shown by box-and-whisker plots depicting median, minimum, maximum and single values. **p<0.01 vs sham; °°p<0.01 vs placebo diet by ANOVA followed by Tukey’s test. Scale bar: 250 µm.
Lack of effects of PLX3397 on the neuronal cell loss in the hippocampus.

Representative photomicrographs and relative quantifications of Nissl-stained neurons/section (averaged from at least three sections from different anteroposterior level representative of the dorsal hippocampal area) in the CA1 (above line), CA3 (middle line) and hilus (bottom line) of the hippocampus in chronic epileptic mice fed with placebo or with PLX3397-supplemented diet 21 days before SE and for one week after SRS onset (protocol in Figure 5.2.1 A), and in sham mice. Data are shown by box-and-whisker plots depicting median, minimum, maximum and single values. **p<0.01 vs sham by ANOVA followed by Tukey's test. Scale bar: 250 µm.

5.3.6 Microglia density in forebrain areas of naïve mice and in epilepsy

To shed light on the different behaviour of microglia in temporal cortex vs hippocampus for neuroprotection outcomes, as well as differences in microglia role in cortical thinning in enthorinal vs perirhinal cortices, we performed immunohistochemical analysis of Iba1 positive cells to measure microglia density in the different brain areas. As shown in naïve mice (Figure 5.3.12), microglia density was significantly higher in the entorhinal and perirhinal cortices compared to the hippocampus (*p<0.05, **p<0.01 by ANOVA followed by Tukey’s post-hoc test). In epileptic mice, microglia density was significantly increased in the entorhinal cortex but not in perirhinal cortex compared with sham mice (***p<0.05 vs respective sham by Mann-Whitney test).
Figure 5.3.12 - Entorhinal cortex is enriched in microglial cell numbers compared to other brain regions in sham and epileptic mice. Box-and-whisker plots depicting median, minimum, maximum and single values in the hippocampus, entorhinal and perirhinal cortices in sham control (panel A) and placebo diet-fed epileptic (panel B). Only 4 mice of the experimental group (see Figure 5.2.1 B) were analyzed for Iba1 staining based on slices availability. *p<0.05, **p<0.01 vs sham hippocampus by ANOVA followed by Tukey’s post-hoc test; **p<0.01 vs respective sham (B) by Mann Whitney test. Ent Ctx = entorhinal cortex; PRhin Ctx = perirhinal cortex.

We confirmed that after PLX3397 withdrawal (Figure 5.2.1 B), microglia repopulated the brain parenchyma as described by previous evidence (Elmore et al., 2014). Microglial cell number was significantly increased in the hippocampus of chronic epileptic mice fed with placebo or with PLX3397 during early disease development, compared with sham mice (Mean ± SEM; cells/μm²; Sham: 627.6 ± 13.1; Placebo: 953.6 ± 39.7; PLX3397: 1032 ± 50.4; p<0.01 by ANOVA followed by Tukey’s post-hoc test). Similarly, microglia body size was significantly enlarged in both conditions compared with sham (Mean ± SEM, μm²; Sham: 27.9 ± 0.4; Placebo: 32.3 ± 0.6; PLX3397: 32.17 ± 1.1; p<0.01 by ANOVA followed by Tukey’s post-hoc test). In the entorhinal cortex, microglia density was significantly increased in epileptic mice vs sham, as shown in Figure 5.3.12 B. Similarly, microglia density in epileptic mice fed with PLX3397 during early disease was increased compared with sham (Mean ± SEM, n=4-7; cells/μm²; PLX3397: 58 ± 2.7 vs sham: 40 ± 0.9; p<0.01 by Kruskal-Wallis test followed by Dunn’s multiple comparisons). Also, the average cell body size was significantly increased in epileptic mice ± PLX3397-treated vs
sham mice (Mean ± SEM, n=4-7; µm², Sham: 28.5 ± 0.8; Placebo: 33.9 ± 1.0; PLX3397: 35.5 ± 0.9).

5.3.7 Microglia depletion in chronic epileptic mice

Similarly to what observed in the longitudinal study (Figure 5.2.1 B and Figure 5.3.6), when microglia was depleted in chronic epileptic mice for two weeks (as depicted in Figure 5.2.1 C), no effects were observed on seizures frequency and duration (Mean ± SEM; Baseline 55-69 days: number of seizures/day, 1.4 ± 0.5; seizures duration (s), 46.2 ± 3.9; 70-84 days, during PLX3397-supplemented diet: number of seizures/day, 2.1 ± 0.9; seizures duration (s), 41.5 ± 2.4; p=0.672 by Wilcoxon matched-pairs signed rank test).

Microglia depletion in PLX3397-treated mice was confirmed in the hippocampus by immunohistochemical analysis of Iba1-positive cells compared with epileptic mice under placebo diet (n. of cells, mean ± SEM; PLX3397-epileptic mice: 46.1 ± 17.8 vs placebo-epileptic mice: 1163 ± 48.8; p<0.01 by Mann-Whitney U test, n=6-8). The surviving microglial cells displayed reactive cell body size, similar to epileptic mice (hippocampus, average cell size µm², PLX3397-epileptic mice: 34.7 ± 0.6 vs placebo-epileptic mice: 33.8 ± 0.9; n=6-8, p<0.01 vs by Mann-Whitney U test).

5.4 Discussion

The ENIGMA-Epilepsy Consortium has identified brain structural abnormalities, consisting of shared patterns of grey matter reduction, across several epilepsy syndromes. These abnormalities, referred to as cortical thinning, correlated with epilepsy duration but not with the frequency of seizures (Whelan et al., 2018). Indeed, progressive cortical thinning distinct from normal aging was detected in patients with focal epilepsy (Galovic et al., 2019). The ENIGMA-Epilepsy Consortium has further investigated this phenomenon in human specimens and found out that it spatially correlated with gene expression-predicted microglia density and microglia reactive states. In particularly, microglia reactivity was
identified as a potential contributor to the underlying cause of cortical thinning (Altmann et al., 2021). Therefore, in our study, PLX3397 was used as a pharmacological tool to explore the involvement of microglia in cortical thinning in the murine model of TLE (Iori et al., 2017).

We have confirmed that >90% depletion of microglia was achieved with 21 days of treatment with PLX3397 formulated in animal chow, without affecting survival or morphological features of astrocytes and neurons (Elmore et al., 2014; Liu et al., 2020). This method of microglia depletion was shown not to induce inflammation, or BBB damage. Notably, the behavioural studies performed in naïve mice or non-human primates depleted of microglia have shown that cognitive and motor functions were unaffected by microglia depletion, thus suggesting that microglia are not required for cognition and neuronal survival in physiological conditions (Elmore et al., 2014; Green et al., 2020). Accordingly, we showed that microglial depletion did not affect the basal neuronal excitability in naïve mice, as assessed by extracellular recordings in hippocampal slices. Moreover, our study has shown that microglia do not contribute to seizures, since both the initial epileptogenic insult (SE), the epilepsy onset and the chronic seizures were not modified by cell depletion. A previous study showed that microglia depletion for 21 days with PLX3397 slightly but significantly increased seizure generalization during the early phase of SE (first 2 hours) in mice intraperitoneally injected with pilocarpine. In this study, SE severity was evaluated using the Racine’s scale to assign mice a behavioural seizure score (Liu et al., 2020). In the same study, an increase in seizure generalization was reported in microglia depleted mice during 30 min after systemic pentylenetetrazol (Liu et al., 2020). Since seizure recording was very short in both instances it is difficult to predict the net effect of microglia depletion in the two models (for ex., SE after pilocarpine lasts for about 18-24 h). Moreover, the induction of SE, using a systemic route, results in systemic inflammation, which in condition of myeloid cells depletion may be altered, thus contributing to the observed effects. In fact, pilocarpine-induced peripheral activation of proinflammatory mechanisms contributes to SE
by altering BBB permeability and allowing convulsive concentrations of pilocarpine to reach the brain (Marchi et al., 2007).

Another study applied toxin-based strategies of microglial ablation in rodents intracerebroventricularly injected with kainic acid, then reporting exacerbation of SE based on a Racine’s scale (Wu et al., 2020). Notably, the depletion strategy per se may affect disease outcomes (Green et al., 2020). Indeed, toxin-based models provide short-lived microglial depletion and induce a storm pro-inflammatory cytokines such as TNF and IL-1β (Bruttger et al., 2015) that may contribute to ictogenesis, thus worsening epileptic seizures (Vezzani, 2014). Moreover, these toxin-based strategies of microglial ablation require repetitive administration of active biological compounds, such as tamoxifen, which plays pleiotropic effects on cell types expressing its target, namely the oestrogens receptor (Green et al., 2020).

In our study, the lack of effects of microglia depletion on both SE and on the onset and generation of epileptic seizures reinforced the evidence that microglia unlikely contribute to hyperexcitability and seizures during epileptogenesis. No effects on seizures were observed even after PLX3397 withdrawal when microglia repopulated the brain. Compensatory mechanisms mediated by repopulated microglia might have resulted in a null effect on seizures. Since we did not characterize the repopulated microglia in our experimental setting we cannot exclude this hypothesis. However, evidence from mRNA gene expression in healthy adult mice demonstrated that after PLX3397 withdrawal, newly repopulated microglia exhibit similar phenotype and reactivity to LPS as the original microglia (Elmore et al., 2015; Huang et al., 2018), thus suggesting that repopulating microglia do not substantially differ from resident microglia. In line with these results, a recent study showed that repopulated microglia do not modify kainic acid induced SE in mice (Wu et al., 2020).

Furthermore, our study has also addressed the role of microglia in seizures during chronic epilepsy, when microgliosis is still abundant, as depicted in Chapter 4, Figure 4.3.1.
We did not observe any effect of microglial depletion on seizures frequency and duration in mice with an already established disease. Lower doses of PLX3397, that do not deplete microglia but modulate their activation state, attenuated chronic seizures in pilocarpine and kainic acid models of epilepsy (Srivastava et al., 2018), suggesting that reduction of microglia reactivity without depleting the cells may attenuate seizures.

Despite no effect on seizures, microglial depletion in mice during early disease development has prevented cortical thinning in the entorhinal cortex. This supports the involvement of microglia in the structural changes occurring in brain areas involved in the seizure circuitry. Notably, microglia expansion in epileptic mice occurs in the entorhinal but not in perirhinal cortex, where no involvement of microglia in cortical thinning is observed.

Data showed that cortical thinning is due to reduced neuronal cell density and reduction of neuronal size. While reduction of neuronal density was prevented by microglial depletion, neuronal shrinkage persisted, which may explain why the entorhinal cortex volume was still reduced in microglia-depleted mice. However, neurodegeneration was not prevented in the hippocampus, thus supporting the heterogeneity of microglial functions across distinct brain regions (Hammond et al., 2019; Masuda et al., 2019). Our data support that microglia reactivity, and possibly the consequent production of pro-inflammatory cytokines, are involved in neurodegeneration in epilepsy (Hiragi et al., 2018). Accordingly, neuroprotective effects have been reported in animal models of epilepsy where non-selective microglial inhibitors, such as minocycline, MIF or anti-fractalkine antibody, were applied (Hiragi et al., 2018). Our data also dissociate neurodegeneration in the entorhinal cortex from spontaneous seizures generation since seizures persist despite neuroprotection.

Notably, the neuroprotective effect of microglial depletion in the entorhinal cortex was associated with improvement of cognitive deficit measured by one memory test that depends on the entorhinal cortex integrity (Wilson et al., 2013). Since a limitation was encountered during the test, it became necessary to pool additional mice to the original
longitudinal ones (i.e sham and placebo), as described in the *experimental design* and results of this Chapter. During the design, the *extra* mice were randomized and run in a parallel cohort that displayed similar epilepsy severity to the original mice, with the intent to avoid dishomogeneity and to control the risk of introducing a bias in the result. Indeed, we promptly considered that adding extra mice could possibly introduce variability, whether the groups of mice to be pooled do not show homogeneity: for example, the extra mice could perform different from the original group, thus leading to extreme variability in the placebo group and to uninterpretable results; also the extra mice could be much more compromised vs the original longitudinal ones, thus increasing the difference with the treatment group and leading to a biased effect of PLX3397. However, despite the additional placebo mice showed a slightly better performance vs the original mice, the *merged* placebo group displayed a significatively higher cognitive impairment compared to the treatment group. Therefore, the the risk of dishomogeneity and misinterpretation was controlled and our results provide evidence of the effect of microglia depletion on this cognitive parameter.

Altogether, our results highlight that microglia are heterogeneous and play different functions during epilepsy development. Indeed, there is evidence for transcriptomic heterogeneity of microglia in different cortical and subcortical regions (Masuda et al., 2019, 2020). Moreover, our data show that epileptic seizures and cortical thinning are independent processes and that seizures are not the only contributor to morbidity in people with epilepsy (Bell et al., 2016). Early intervention on microglia may prevent disease-related cortical thinning, neuronal cell loss and cognitive deficits, opening up new areas for treatment in common human epilepsies.
CHAPTER 6: MICROGLIA PROLIFERATION PLAYS DISTINCT ROLES IN ACQUIRED EPILEPSY DEPENDING ON THE DISEASE STAGE

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6.1 Introduction

Clinical evidence from drug-resistant epilepsy patients and experimental models of acquired epilepsy highlighted that microgliosis is one of the processes that commonly occur during epileptogenesis and in drug-resistant human epilepsy (Borges et al., 2003; Morin-Brureau et al., 2018; Ravizza et al., 2008). SE and other brain insults such as trauma, infection or febrile seizures, induce reactive microglia in injured brain areas, as assessed by morphological, functional and molecular analyses (Avignone et al., 2008; Hiragi et al., 2018; Wyatt-Johnson and Brewster, 2020). Reactive microglial functions have been implicated in epileptogenesis in animal models of acquired epilepsy (Eyo et al., 2017; Morin-Brureau et al., 2018). In particular, reactive microglia release potential pathogenic molecules such as pro-inflammatory mediators and reactive oxygen species (Devinsky et al., 2013), and activation of CX3CR1 (Yeo et al., 2011) and purinergic receptors P2X7 and P2X4 (Jimenez-Pacheco et al., 2013; Ulmann et al., 2013) signalling pathways contribute to neuronal cell death and seizures in animal models of epilepsy. However, there are studies suggesting that microglia may also display neuroprotective functions, since genetic deletions of purinergic receptors P2Y12 resulted in seizures exacerbation (Yeo et al., 2011), and genetic ablation of microglia increased the severity of SE and chronic seizures in mice (Mirrione et al., 2010; Wu et al., 2020). Therefore, the heterogeneity of microglia roles supports evidence of the wide spectrum of phenotypes that these cells acquire during epileptogenesis, as determined by gene expression studies (Benson et al., 2015).

The contribution of microglia in disease development has been addressed by limited studies in rodents, where minocycline administration during epileptogenesis may either attenuate spontaneous seizures (Wang et al., 2015), or display no effects (Zhang et al., 2016). These controversial results are likely due to lack of specificity of minocycline for microglia and it is uncertain which phenotypes were inhibited by the drug.

Recently, a class of small molecule inhibitors of CSF1R has been developed to address the signalling that regulates proliferation, differentiation and survival of microglia
(Chitu et al., 2016). Specifically, GW2580 is an orally available highly selective inhibitor of the CSF1R, which blocks CSF1R tyrosine kinase activity, thus preventing microglia/monocyte proliferation (Conway et al., 2005; Uitdehaag et al., 2011). As previously described, microglia proliferate during epileptogenesis and an increased cell density is reported in epileptic foci in human epilepsy (Eyo et al., 2017; Gershen et al., 2015; Scott et al., 2017). Clinical relevance of microgliosis for human epilepsies is supported by the evidence that microglial cells number is associated with seizure frequency in human epilepsy (Boer et al., 2006) and that reactive microglia coexist with reduced neuronal density in forebrain regions (Morin-Brureau et al., 2018). Interestingly, GW2580 has been successfully applied to improve disease progression in several pathological conditions displaying microgliosis, such as in models of multiple sclerosis, prion disease, Alzheimer's disease and amyotrophic lateral sclerosis (Crespo et al., 2011; Gómez-Nicola et al., 2013; Martínez-Muriana et al., 2016; Olmos-Alonso et al., 2016).

As previously described in Chapter 4, microglia react and proliferate shortly after SE and the increase in cell body size and number is maintained during chronic epilepsy. Thus, as described in Part I of this chapter, pharmacological interference with microglia proliferation was set out during the early phase of epilepsy development, to study whether it affects seizures, neuronal cell loss and cognitive deficits, in a murine model of acquired epilepsy. Additionally, microglial proliferation was targeted with GW2580 during chronic disease to assess its role in established chronic seizures, as described in Part II.

6.2 Specific Materials and Methods

6.2.1 Justification of the treatment schedule with GW2580

GW2580 (LC Labs, Woburn, MA, USA) was formulated by TestDiet (London, UK, Europe) in food pellet (LabDiet® 5V75 containing 1000ppm GW2580, corresponding to a daily dose of 166 mg/kg body weight), and regular food pellet diet (LabDiet® 5V75) was used as placebo diet. Both diets were stored under vacuum at 4°C until use. Previously
published work reported that in-diet formulation of GW2580 provides efficacious blockade of microglia proliferation in mice (Gerber et al., 2018; Martínez-Muriana et al., 2016; Olmos-Alonso et al., 2016). CSF1R blockade results in reduced microglial cell number, according to flow cytometry and immunofluorescence analysis, then confirmed by Iba1 staining (Gomez-Nicola et al., 2013; Olmos-Alonso et al., 2016; Martínez-Muriana et al., 2016; Gerber et al., 2018). According to pharmacokinetics studies, a dose of 80 mg/kg of GW2580 by oral gavage allowed to reach the blood therapeutic concentrations (0.8-1 µM) for 12 hours and the administration of 160 mg/kg/day of GW2580 ensured the blockade of CSF1 signal activation in vivo (Conway et al., 2005).

Treatment schedule was decided based on protocols previously established in models of experimental autoimmune encephalomyelitis (EAE), where three days of pre-treatment allowed to attain the steady-state drug concentration in the brain (Gomez-Nicola et al., 2013). To design the optimal protocol of drug administration to effectively prevent microglia proliferation during epileptogenesis, mice were fed with GW2580-supplemented diet for three days before inducing SE. Daily food intake and body weight were measured to ensure mice were healthy and food intake was not modified by the medicated diet. This would assure that mice receive the daily dose of 166 mg/kg GW2580 that blocks microglial proliferation within two days (Neal et al., 2020).

6.2.2 Experimental design

The experimental protocols are shown in Figure 6.2.1. GW2580 was administered to mice during the early phase of epilepsy development or to chronic epileptic mice.

As depicted in Figure 6.2.1 A, mice were randomized into two experimental conditions: a group of mice was assigned to a GW2580-supplemented diet regimen and another group of mice was fed with a placebo diet. After three days under the assigned diet regimen, all mice were exposed to SE. In SE-exposed mice fed with placebo diet, 2 mice were lost at follow-up: 1 mouse died during SE (excluded from Figure 6.3.2) and 1 mouse...
died a few days post-SE (excluded from the subsequent studies). In the GW2580 group, 3 mice died a few days post-SE (excluded from the subsequent studies) and 1 mouse was omitted from post-SE investigations because of developing infection around the EEG implant. The remaining SE-exposed mice (n=13 placebo; n=16 GW2580) were randomized into two experimental groups as follows:

1) Mice underwent a cross-sectional design (Figure 6.2.1 A, red arrow and pink box) where treatment was carried out for 7 days. Then, mice were sacrificed and brain harvested to determine the effects of GW2580 on glial cells by immunohistochemistry (n=5 placebo; n=7 GW2580). The results of this protocol are reported in Part I of this chapter.

2) Mice underwent a longitudinal experiment (Figure 6.2.1 A) to determine the effects of GW2580 on epilepsy development. Mice were treated for 21 days after SE, then they were switched to a placebo diet for the rest of the experiment (n=8 placebo; n=9 GW2580). Primary outcomes were evaluated to assess epilepsy development: seizures onset, duration and frequency of seizures were determined by EEG analysis; NORT and Barnes maze were performed to test cognitive deficits; at day 91 post-SE, mice were sacrificed and brain harvested for histological analysis. The results of this treatment schedule are described in Part I of this chapter.

3) As depicted in Figure 6.2.1 B, 10 mice were exposed to SE and monitored until chronic epilepsy: After a spontaneous seizure baseline was monitored during 14-days EEG recording under placebo diet (from day 58 to day 71 post-SE), mice were fed with GW2580-supplemented diet (from day 72 until day 85) in order to assess the effects of GW2580 on spontaneous seizures (Figure 6.2.1 B). A group of 9 mice exposed to SE under placebo diet was run in parallel and similarly monitored to serve as a control group (Figure 6.2.1 C). The results of this experiment are reported in Part II of this chapter. An overall discussion of the results is reported at the end of Chapter 6.
Figure 6.2.1 - Schematic representation of the experimental design. Panel A depicts the experimental protocol related to treatment with GW2580 (n=20) starting 3 days before SE (day 1). A control group of mice fed with placebo diet and exposed to SE was run in parallel (n=15). In the cross-sectional experiment, mice were sacrificed 7 days post-SE and brain processed for immunohistochemistry (IHC; red arrow and pink box). In the longitudinal experiment, SE-exposed mice were fed with GW2580-supplemented or placebo diet for 21 days, then switched to placebo diet. NORT was performed during days 17-21 and Barnes maze during days 86-90 (in green). EEG recording epochs are shown by dotted lines. On day 91 post-SE, mice were sacrificed and brains collected. Dashed black arrows indicate mice that were excluded from the study because of mortality during/after SE and seizures or infection around EEG implant. Panel B depicts the experimental protocol applied in chronic epileptic mice (n=10) fed with placebo diet until day 71, then switched to the GW2580-diet (day 72). EEG was recorded in two 14-day epochs (24/7) from day 58 to day 71 during placebo and from day 72 until day 85 during the treatment, then mice were sacrificed. Panel C depicts the experimental protocol in epileptic mice fed with placebo diet and used as controls of GW2580-treated mice. EEG was recorded during a time window overlapping with monitoring of GW2580-treated mice.
Part I: Effects of GW2580 on SE-induced glia reactivity and epilepsy development

6.3 Results of the cross-sectional study

As depicted in Figure 6.2.1 A, mice were fed with GW2580- or placebo-supplemented diet starting 3 days before SE and for 7 days thereafter. Then, mice were sacrificed and brain harvested to determine the effects of GW2580 on glial cells by immunohistochemistry (n=5 placebo; n=7 GW2580).

Food intake was similar in mice fed with placebo diet and GW2580-supplemented diet for three days before SE induction (Mean ± SEM; Placebo, n=15: 5.6 ± 0.3 g; GW2580, n=20: 5.1 ± 0.2 g), thus providing the expected dose of 166 mg/kg GW2580 in mice under medicated diet.

6.3.1 Blockade of SE-induced microglial proliferation

To assess the effects of GW2580 on microglia, the drug’s cell target, SE-exposed mice were fed with placebo or GW2580-diet for three days (n=5-7; protocol in Figure 6.2.1 A), then sacrificed 7 days after SE. SE induced an increase of Iba1 immunoreactivity in mice fed with placebo diet vs sham mice (not exposed to SE; n=6; Figure 6.3.1 B vs A) which was prevented by GW2580 (Figure 6.3.1 C vs B; two GW2580-treated mice were excluded due to poor staining quality, n=5). The number of Iba1-positive cells was increased in SE-exposed mice under placebo diet vs sham mice, and GW2580 treatment prevented this increase (Figure 6.3.1 D). Cell body was also enlarged in SE-mice fed with placebo, thus indicating microglia reactivity, but this parameter was not affected by GW2580 (Figure 6.3.1 E). In naïve mice, GW2580 did not modify Iba1-positive cell number (mean ± SEM; Placebo: 531 ± 12, n=5; GW2580: 549 ± 16, n=6) or their body size (µm², Placebo: 25.7 ± 0.76; GW2580: 25.9 ± 0.7).

6.3.2 SE-induced gliosis and leukocytes extravasation

Astrocytes. To confirm the target specificity of GW2580 for microglia, the number and cell body size of S100β-positive astrocytes were measured in hippocampal slices
adjacent to the brain slices processed for Iba1. SE induced the increase of cell body size in both placebo- or GW2580-treated mice vs sham mice (Figure 6.3.1 F-H and J). GW2580 did not modify cell number or the increase in cell body size in mice exposed to SE (Figure 6.3.1 F-J).

Leukocytes. Peripheral immune cells such as monocytes/macrophages expressing CSF1R are known to migrate from the blood into the brain after SE (Ravizza et al, 2008; Varvel et al., 2015). As described in Chapter 4, flow cytometry analysis detected macrophages in the hippocampi within 7 days after SE. Immunoreactivity of CD45, a pan leukocyte antigen, in the hippocampus of SE-exposed mice showed an increased cell number in placebo diet-fed vs sham mice (Figure 6.3.1 L vs K; panel N), which was reduced in GW2580-treated SE mice (Figure 6.3.1 M vs L; panel N).
Figure 6.3.1 - Effect of GW2580 on SE-induced gliosis and leukocyte extravasation in the hippocampus. Panels A-C: representative photomicrographs (20x; scale bar: 50 µm) showing Iba1-positive cells in CA1 subfield of sham (A), SE mice fed with placebo (B) or with GW2580 diet (C). Inserts in each panel show a magnified microglial cell (60x; Scale bar: 20 µm). Quantitative analysis of number of Iba1-positive cells (D) and their average cell body size (E). Panels F-H: representative photomicrographs (20x; scale bar: 50 µm) showing S100β-positive astrocytes in CA1 subfield of sham (F), SE mice fed with placebo (G) or with GW2580 diet (H). Inserts in each panel show a magnified astrocytic cell (60x; Scale bar: 20 µm). Quantitative analysis of the number of S100β-positive cells (I) and their average cell body size (J). Data (D, E, I, J) are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. Sham n=6; SE n=5 mice/group. *p<0.05, **p<0.01 vs sham; ^p<0.05 vs Placebo by Kruskall-Wallis test followed by Dunn’s multiple comparison test. Panels K-M: representative photomicrographs (20x; scale bar: 50 µm) showing CD45-positive cells in CA1 subfield of sham (K), SE mice fed with placebo (L) or with GW2580 diet (M). Quantitative analysis of the number of cells in the various experimental groups is reported in N. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values *p<0.05 vs Placebo by Mann-Whitney U test. n.d, not detectable.

6.3.3 Lack of effects of GW2580 on status epilepticus

First, we have studied the development of SE in GW2580-fed mice vs placebo mice. SE parameters were not significantly modified by the treatment as shown by measuring the time to the onset of SE, the duration and the severity of SE, and the number of spikes/h during EEG recording (Figure 6.3.2).
In agreement with these results, hippocampal excitatory neurotransmission and neuronal excitability in naïve mice were not modified by GW2580 administered with a similar schedule as in SE-exposed mice. In order to match with the treatment schedule before SE induction, naïve mice were fed with GW2580 or placebo for three days then they were sacrificed and extracellular recordings were performed in the CA1 region of acute hippocampal slices (see General Materials and Methods for details, in Chapter 3). GW2580 did not alter synaptic transmission as assessed by measuring input-output curve of fEPSPs (field excitatory postsynaptic potential) recorded in stratum radiatum, (Figure 6.3.3 A) and
the amplitude of action potentials (PS: population spikes; Figure 6.3.3 B). Also, presynaptic short-term plasticity was evaluated by measuring paired-pulse facilitation of fEPSPs, which was similar in GW2580- vs placebo-fed mice (Figure 6.3.3 C).

Figure 6.3.3 – Lack of effects of GW2580 on hippocampal basal transmission and excitability in naïve mice. Extracellular recordings in acute hippocampal slices of naïve mice fed with placebo diet or with GW2580-supplemented diet (n=5-6 mice/group). A) input-output curve of field excitatory postsynaptic potentials (fEPSP) evoked in CA1 pyramidal neurons by electrical stimulation of the Schaffer collaterals (number of slices: Placebo n=13, GW2580 n=11). B) CA1 pyramidal neurons population spike (PS) amplitude in response to increasing stimulation intensities of Schaffer collaterals (number of slices: Placebo n=15, GW2580 n=19). C) Paired Pulse Facilitation (PPF) at distinct inter-pulse intervals (i.e., 25, 50, 100, 150, 200, 250 ms). PPF is expressed as the ratio of the slope of responses to second and first stimuli (fEPSP2/fEPSP1). Data are presented as mean ± SEM (number of slices: Placebo n=9, GW2580 n=10). Respective representative traces are shown in insets.

As described in Figure 6.2.1 A, mice exposed to SE were randomized into two different studies: a cross-sectional and a longitudinal study. As reported, the cross-sectional study has shown that the protocol used blocked microglial proliferation induced by SE,
without reducing morphological reactivity of microglia. Moreover, SE was not modified by GW2580, suggesting that any effects on epileptogenesis could be reliably attributed to the blockade of microglial proliferation rather than to modification of the epileptogenic insult.

Next, the results of the longitudinal study are reported. The effects of GW2580 were assessed on primary epilepsy outcomes: seizures onset, duration, and frequency, and on cognitive deficits and neurodegeneration.

6.4 Results of the longitudinal study: Effects of GW2580 on epilepsy outcomes

As described in the experimental design (Figure 6.2.1 A), mice were fed for 3 days with GW2580- supplemented or placebo diet, then exposed to SE, and the diet regimen was protracted for 21 days after SE to include the initial phase of epileptogenesis, then mice were switched to the placebo diet until the end of the experiment.

Mice were EEG monitored (24/7) for three months after SE (until day 91) to determine the time to onset of the first spontaneous seizure, and frequency and duration of spontaneous seizures at predetermined post-SE epochs: 1-17 days (GW2580 treatment period, no EEG monitoring was done during NORT at days 18-21); 22-38 days (GW2580 wash-out period after switching to placebo diet); 60-81 days (chronic epilepsy phase). At the completion of the EEG analysis, mice were tested in the Barnes Maze (days 86-90), then sacrificed and brain collected for post-mortem histopathology.

Animal’s weight was similar in the two experimental groups (mean ± SEM, before SE, placebo: 27.1 ± 0.7 g; GW2580: 26.4 ± 0.6 g; 24 h post-SE, placebo: 26.0 ± 0.6 g; GW2580: 24.4 ± 0.5 g; 90 days-post SE, placebo: 29.8 ± 0.9 g; GW2580: 29.8 ± 0.5 g), suggesting that there are no adverse effects due to the medicated diet.

6.4.1 Lack of effect of GW2580 on epilepsy

The onset of spontaneous seizures in mice fed with placebo diet occurred 5.7 ± 0.9 days after SE (mean ± SEM, n=8), in accordance with previous evidence in this model of
epilepsy (Iori et al, 2017; Frigerio et al, 2018b). No differences in this parameter was measured in GW2580 treated mice ($mean \pm SEM$: 5.0 ± 0.8 days, n=9).

EEG recordings were done in each mouse during three epochs: 1-17 days during placebo or GW2580-diet administration; 22-38 days after drug withdrawal referred to as drug wash out; 60-81 days during chronic epilepsy (Figure 6.2.1 A). Spontaneous seizures were detected in each mouse and seizure frequency (Figure 6.4.1) was calculated as the average number of seizures per day in each mouse during each recording epoch. Daily seizure frequency distribution in placebo and GW2580 experimental groups is shown in Figure 6.4.1 B. GW2580 did not modify seizure frequency (A, B) and their average duration (B) compared to placebo during the entire the recording periods (Figure 6.4.1, one mouse in the GW2580 group died at day 55 therefore seizures were reckoned only until day 38). Representative traces of seizures for each experimental group are reported in Figure 6.4.1 C and the arrows indicate the average duration of each seizure. Motor components of spontaneous seizures were not systematically analysed with video-monitoring, but previous evidence showed that EEG seizures were associated with motor generalized seizures (Iori et al., 2017). Mice experiencing generalized convulsions during EEG seizures were observed in this study by the investigator in both groups.
Figure 6.4.1 – Lack of effects of GW2580 on epilepsy development. A) The number of daily seizures (top) and their average duration (bottom) in 3 subsequent phases of disease development: days 1-17 (GW2580 treatment period); days 22-38 (GW2580 wash-out period after switching to
placebo diet); days 60-81 (chronic epilepsy phase). Experimental protocol in Figure 6.2.1 A. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. Mixed effects models’ statistics showed no significant differences in seizures frequency and duration in the two groups during the disease development. Placebo n=8; GW2580=9. One mouse in the GW2580 group died at day 55 therefore seizures were reckoned only until day 38. Colour code identifies each mouse during the different recording periods. B) Average daily number of seizures throughout the experiment both in placebo (above) and GW2580-treated mice (below), same mice of panel A. Data are presented as mean ± SEM. C) Two representative EEG tracings of spontaneous seizures in chronic epileptic mice from placebo or GW2580 group. Left hippocampus, HP; right ipsilateral somatosensory cortex, S1). Black arrows delimit the duration of each seizure event.

6.4.2 Effect of GW2580 on cognitive deficits

Literature data showed that a prolonged GW2580 treatment did not affect behaviour in naïve mice tested in the open field and in the T maze (Olmos-Alonso et al., 2016), therefore we did not test the effect of GW2580 in sham mice. As described in the experimental protocol (Figure 6.2.1 A), during days 18-21 post-SE EEG recordings were interrupted in mice to test animals in NORT (GW2580 (n=9), placebo (n=8) mice; sham mice (n=10). NORT is a test for non-spatial memory involving both hippocampus and entorhinal cortex (Wilson et al., 2013; Denninger et al., 2018). The novel object exploration in sham mice was 70% of the total exploration time with a discrimination index (DI) of 0.3 ± 0.05 (mean ± SEM; n=10; Figure 6.4.2 B). By contrast, mice that had experienced SE fed with placebo diet displayed equal exploration time of the familiar and the novel object resulting in reduced DI (0.11 ± 0.06; n=8; p<0.01 vs sham), which indicates memory impairment. Mice that had experienced SE fed with GW2580 diet were similarly impaired with a reduced DI comparable to placebo diet SE-mice (DI, 0.14 ± 0.03, n=9).

Results of total exploration time travelled distance and speed of mice in the open field were not modified by SE or GW2580 treatment as shown in Figure 6.4.2.
Figure 6.4.2 - Effects of GW2580 on cognitive deficits during epilepsy development. A) Total time of exploration of the objects during the day of the test. B) Discrimination index (DI) in the Novel Object Recognition Test (NORT). C) Travelled distance (cm) and D) speed (cm/s) of mice in the arena during the NORT, day 3. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. Sham, n=10; Placebo, n=8; GW2580, n=9. *p<0.05, **p<0.01 vs sham by Kruskal-Wallis test followed by Dunn’s multiple comparison test. Results of the Barnes maze test are shown in E, F. The total latency (E) and primary latency (F) to find the escape hole in the Barnes Maze during the training trials (E, days 1-3) and during the probe trial (F, day 4) in chronic epileptic mice. Data in panel E are mean ± SEM of average values reckoned for each mouse from 3 daily trials. *p<0.05 Placebo or GW2580 vs sham at the same day of training; #p<0.05 vs sham at day 1 by 2-way ANOVA for repeated measures (p=0.003) followed by Tukey’s multiple comparison test. Data in panel F are presented as box-and-whisker
plots depicting median, interquartile interval, minimum and maximum, and single values. *p<0.05 vs sham by Kruskall-Wallis test followed by Dunn’s multiple comparison test. Sham n=10; Placebo n=5; GW2580=7: same mice tested in NORT during epileptogenesis. Three placebo diet mice and one GW2580 mouse did not perform the test because of either preceding death or displaying immobility in their home cage.

During chronic epilepsy, after completion of EEG recordings, mice were tested in the Barnes maze (protocol in Figure 6.2.1 A; see Materials and Methods for details) to assess hippocampal-dependent spatial memory (Van Den Herrewegen et al., 2019). Mice showing immobility in their home cage (three placebo diet mice and one GW2580-treated mouse) were not considered eligible for the test. The remaining mice (n=10 sham; n=5 placebo; n=7 GW2580) were behaviourally tested and none of them showed motor seizures during the entire test. As shown in Figure 6.4.2 E, the total latency to find the escape hole during the daily training trials was significantly shorter at days 2 and 3 vs day 1 (p<0.05) in sham mice (n=10), showing that mice were learning the task. By contrast, mice that had experienced SE of the placebo (n=5) or GW2580 group (n=7) appeared to be impaired in learning the task, since the total latency did not change over the daily trials (Figure 6.4.2 E) (p<0.05 vs sham).

Beside learning, the Barnes maze test allows to measure the memory retention of a learnt task (Sadeghian et al., 2019). The concept of memory retention can be described as the ability or not to consolidate spatial learning in a long-term memory as previously assessed (Barkas et al., 2012; Sadeghian et al., 2019; Pascente et al., 2016) and evidence suggests that spatial learning and memory retention can be uncoupled mechanisms (Barkas et al., 2012).

During the probe trial (day 4; Figure 6.4.2 F), primary latency in mice that had experienced SE of the placebo group was significantly increased compared to sham mice therefore showing memory retention impairment. The primary latency measured in mice that had experienced SE treated with GW2580 indicates a better performance than SE placebo mice, suggesting a partial recovery of behavioural deficit, however these mice were not statistically different from either placebo or sham mice (Figure 6.4.2 F). Therefore, to
further assess the memory retention, per each group an index was calculated (Figure 6.4.3) as follows: the latency time to find the target hole during the last trial of the training (day 3, Figure 6.4.2 E) vs the probe trial (day 4, Figure 6.4.2 F). No differences were assessed in placebo SE mice (mean ± SEM, latency day 3 vs day 4; 95.4 ± 7.3 vs 80.4 ± 20.2 s) denoting memory impairment, while a significant reduction of 40% was found in GW2580-treated mice (95.6 ± 13.8 vs 58.3 ± 15.4 s, p = 0.03 by Wilcoxon matched-pairs signed rank test) indicating improved memory retention. Sham mice displayed 46% reduction of the latency time during the probe trial (35.3 ± 6.7 vs 19.9 ± 6.2 s, p < 0.001).

Figure 6.4.3 - GW2580 ameliorate performance in memory retention. Memory retention index was calculated by comparing for each mouse in each experimental group: the latency time to find the target hole during the last trial of the Training (day 3, Figure 6.4.2 E) vs latency time to find the target hole during the Probe (day 4, Figure 6.4.2 E). Data are reported in box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. * or ** vs respective trial (day 3) by Wilcoxon matched pairs signed rank test performed to assess the differences in each experimental group: sham/placebo/GW2580 trial (day 3) vs sham/placebo/GW2580 probe (day 4).

6.4.3 Effect of GW2580 on neurodegeneration and reactive gliosis

After completion of behavioural tests, epileptic and sham mice were sacrificed (91 days-post SE; protocol in Figure 6.2.1 A) for assessing neuropathology in the hippocampus and the entorhinal cortex, two limbic areas pivotally involved in the epilepsy circuitry
As previously reported (Terrone et al., 2018), placebo mice that have experienced SE (n=7, one mouse died at day 82 before perfusion) showed neurodegeneration in CA1, CA3 pyramidal layers and loss of interneurons in the hilus of the dentate gyrus in the hippocampus ipsilateral to the injected hemisphere as compared to sham mice (n=8; 2 mice were discarded because of poor staining quality) (Figure 6.4.4 B, E, H vs A, D, C). Treatment with GW2580 during SE and the initial phases of epileptogenesis (until day 21 after SE) provided neuroprotective effects by preventing the loss of CA1 pyramidal neurons (Figure 6.4.4 C vs A, B) and hilar interneurons (Figure 6.4.4 I vs G, H; n=8 mice). As reported in Figure 6.4.4 J, neuronal cell counts confirmed CA1 and hilar interneurons protection in GW2580 treated mice, while CA3 pyramidal cell loss (Figure 6.4.4 E vs D) was not prevented by GW2580 (Figure 6.4.4 F vs E, bargram in J). A significant reduction of neurons in layers II-III of the entorhinal cortex was observed in epileptic mice, either placebo or GW2580 treated mice, compared to sham mice, showing that GW2580 had no neuroprotective effects in this cortical area (Figure 6.4.4 K; quantification of cell number in box and whiskers plot).
Figure 6.4.4 - Effect of GW2580 on neurodegeneration in the hippocampus and entorhinal cortex. Panels A-I depict representative photomicrographs of Nissl-stained CA1, CA3 pyramidal neurons and hilar interneurons (h) in chronic epileptic mice fed with placebo diet (B, E, H) or with GW2580-supplemented diet (G, F, I) during epileptogenesis and in sham mice (A, D, G). Scale bar: 50µm. Panel J shows quantitative analysis of neurodegeneration. Sham n=8; Placebo n=7; GW2580=8: 2 sham mice were discarded because of poor staining; 1 Placebo mouse died at day 82 before perfusion. Panel K depicts representative photomicrographs of Nissl-stained neurons in the entorhinal cortex in chronic epileptic mice compared to sham mice; scale bar: 50 µm. Quantitative
analysis of neuronal density (neurons/µm²) is reported in plots. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values. *p<0.05, **p<0.01 vs sham; †p<0.05 vs Placebo by Kruskall-Wallis test followed by Dunn’s multiple comparison test.

Glial cells number and reactivity were assessed by Iba1 and S100β staining: the number of cells and their average body size were similarly increased in placebo- and GW2580-treated mice compared to sham mice (Figure 6.4.5). This result is compatible with the treatment schedule since at the time of sacrifice mice were under placebo diet for several weeks after treatment interruption (protocol in Figure 6.2.1 A).

**Figure 6.4.5 - Effect of GW2580-treatment during epileptogenesis on chronic glial reactivity.** Quantitative analysis of the number of Iba1- (A) and S100β-positive cells (C) and their average cell body size (B, D) in chronic epileptic mice fed with placebo diet or with GW2580-supplemented diet during epileptogenesis. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum, and single values (sham n=6; SE n=6-8 mice/group). *p<0.05, **p<0.01 vs sham by Kruskall-Wallis test followed by Dunn’s multiple comparison test.
The longitudinal study has shown that microglial proliferation did not contribute to seizures onset and recurrence but plays a role in neurodegeneration in the hippocampus. Mild improvement in memory retention was also observed. The lack of effects of GW2580 on spontaneous seizures onset, duration and frequency assessed by EEG monitoring, are in agreement with lack of effects on SE and on hippocampal excitability in naïve mice.

In Part II of this Chapter, the results related to the role of microglia proliferation in chronic epilepsy are reported. The effects of GW2580 on cognitive deficits and neurodegeneration were not assessed since these events already develop during the initial phase of epileptogenesis and were therefore studied with the previous intervention protocol.
Part II: Effect of GW2580 on established seizures in chronic epileptic mice

Experimental design. To assess the effects of blockade of microglial proliferation with GW2580 on established spontaneous seizures in chronic epileptic mice, 10 epileptic mice under placebo diet were exposed to SE (protocol in Figure 6.2.1) and EEG monitored at day 58 post-SE for two weeks (14-days; 24/7) until day 71 post-SE, to establish the baseline of spontaneous seizures in each mouse. Then, at day 72 mice were fed with GW2580-supplemented diet for 2 weeks to determine the treatment effect in each mouse as percent variation of seizure number compared to corresponding pre-injection baseline (Figure 6.5.1). Based on food intake in GW2580-treated mice (mean ± SEM: 5.3 ± 0.2 g), animals received the average daily dose of 166 mg/kg, which blocked microglia proliferation in vivo within 2 days (Neal et al., 2020). In parallel, one group of 9 epileptic mice was fed with the placebo diet and was similarly monitored between day 58 and day 85 (24/7) post-SE (protocol in Figure 6.2.1 C). The EEG recording period was divided in two subsequent 14-day epochs, in order to reckon seizure baseline variation during a time window overlapping with the time of EEG monitoring in GW2580-treated mice. Post-mortem brain processing revealed the presence of cortical damage in 2 out of 10 GW2580-treated mice, due to previous stereotaxic surgery; therefore, these mice were excluded from seizure evaluation.

6.5 Results

The results of seizure evaluation are reported in Figure 6.5.1. GW2580 reduced seizures in 6 out of 8 mice: 50% of mice (n=4) were seizure-free during GW2580 treatment and 25% of mice (n=2) displayed 60% and 67% seizure reduction, respectively. Two mice (25%) did not experience seizure reduction, one of which showed increased seizures (Figure 6.5.1 B).

Mice in the placebo control group displayed seizure variation between the two subsequent recording epochs (14 days each) intrinsic to the model: over the whole recording
time an average 36% seizure reduction occurred in 3 out of 9 mice, whereas 6 mice showed increased seizure number (Figure 6.5.1 A).

GW2580 treatment resulted in significant seizure reduction compared to placebo, as the % variation of seizure number vs baseline during GW2580 treatment was significantly different from the corresponding variation in the placebo group (Figure 6.5.1 B vs A; p=0.014 by exact two-tailed Wilcoxon rank-sum test). Moreover, the rate of responders, calculated by setting the clinical criteria of 50% seizure reduction (Perucca, 2018) was significantly higher in GW2580-treated mice (6 out of 8 mice) than in placebo mice (1 mouse out of 9 mice) (p=0.0152 by Fisher's exact two-tailed test), reinforcing the evidence of an effect of the treatment on established spontaneous seizures.
Figure 6.5.1 - Effect of GW2580 on spontaneous seizures in epileptic mice. A) Waterfall plot shows the percent variation in total number of seizures in each epileptic mouse (n=9; mice are identified by progressive numbers) under placebo diet during two subsequent epochs (day 72-85 vs day 58-71) of (24/7) EEG monitoring. B) Waterfall plot shows the percent variation in total number of seizures in each epileptic mouse (n=8; mice are identified by progressive numbers) during
GW2580 treatment (day 72-85) vs baseline (placebo diet, day 58-71). Number of seizures during the corresponding recording periods for each individual mouse are shown in the inset tables (A, B). Data in the two experimental groups were compared by taking into account the variability in the number of seizures during the entire monitoring period (day 58-85) intrinsic to the natural history of the disease. A-priori determined summary statistics was chosen for each mouse to take into account that both treatment and natural history of the disease covariate. Summary statistics is the % variation of seizure number during treatment or corresponding placebo (days 72 to 85) vs respective baseline (days 58 to 71) calculated as follows: number of seizures in days 72 to 85 minus number of seizures in days 58 to 71 (baseline) divided by number of seizures in days 58 to 71 (baseline) x 100. For each mouse the summary statistic is shown in the waterfall plot of panels A and B. Summary statistics in the two experimental groups were compared using the two-tailed Wilcoxon rank-sum exact test (p=0.014). No statistical difference was measured when comparing the seizure number during GW2580 treatment vs respective baseline (p=0.266 Wilcoxon signed-rank test) or when comparing seizure number during day 72-85 in GW2580-treated mice vs seizure number in corresponding monitoring days in placebo (day 72-85) (p=0.063 by Mann-Whitney U test).

Histological assessment of microglia in post-mortem brain specimens showed a significant increase in the number (mean ± SEM: Sham, 641 ± 27, n=6; Placebo-epileptic, 1141 ± 67, n=6; p<0.01) and cell body size (µm², mean ± SEM: Sham, 27.7 ± 0.5; Placebo-epileptic, 30.8 ± 0.8; p<0.05) of Iba1-positive cells in chronic epileptic mice under placebo diet compared to sham mice, therefore showing microglial proliferation in the chronic disease phase (as previously described in Chapter 4, Figure 4.3.1). Epileptic mice under GW2580-supplemented diet showed both number (mean ± SEM: 701 ± 16, n=8; p<0.01, Kruskall Wallis test) and cell body size (µm², mean ± SEM: 27.4 ± 0.9; p<0.05, Kruskall Wallis test) of Iba1-positive cells similar to sham mice, thus denoting that GW2580 blocked microglial proliferation and reactivity.

The number of seizures did not correlate with the number of Iba1-positive cells in GW2580-treated mice (p=0.4, Spearman correlation test) and the therapeutic effect of GW2580 on seizures, expressed as % variation vs baseline (Figure 6.5.1 B), was not significantly correlated with the reduction of Iba1 cell body size, (r=0.731; p=0.061, Spearman correlation test). Similarly, the total number of seizures in each mouse during
GW2580 treatment was not significantly correlated with the body size of Iba1-positive cells ($r=0.704; p=0.077$), as summarized in Table 6.5.1.

Table 6.5.1 - Correlation between chronic epileptic seizures and cell body size

<table>
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<tr>
<th># Mouse</th>
<th>Seizure (% change vs baseline)</th>
<th>Iba1 cell body size (difference with sham)</th>
<th>Total number of seizures</th>
<th>Iba1 cell body size ($\mu m^2$)</th>
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<td>17</td>
<td>-100.0</td>
<td>-0.53</td>
<td>0</td>
<td>27.15</td>
</tr>
</tbody>
</table>

Also, adjacent hippocampal slices were processed for immunostaining of astrocytes and macrophages. GW2580 treatment did not modify the number of S100β-positive astrocytes ($mean \pm SEM$, Sham, 1131 ± 24.1; Placebo, 1519 ± 59.8**; GW2580, 1581 ± 69.4**; **p<0.01 vs sham by Kruskall-Wallis followed by Dunn’s multiple comparison test) or their average body size ($\mu m^2$, $mean \pm SEM$: Sham, 30.2 ± 0.7; Placebo, 34.2 ± 1.2*; GW2580, 34.6 ± 1.1*; *p<0.05 vs sham), nor the number of macrophages ($mean \pm SEM$: Sham, not detectable; Placebo, 16.3 ± 2.9; GW2580, 19.6 ± 4.2; n.s. by Mann-Whitney U test).

These results implicate microglial proliferation in chronic seizures reinforcing the hypothesis that pharmacological intervention targeting microglia can exert diverse effects in different disease stages.
6.6 Discussion

Chapter 6 reports the results of a study that explored the role of microglia proliferation during disease development and in chronic epilepsy, using an established murine model of acquired epilepsy (Iori et al., 2017; Frigerio et al., 2018b). Specifically, microglia proliferation was pharmacologically blocked with the CSF1R inhibitor GW2580 to shed light on the pathophysiological role of this cellular function. This study provided novel evidence for the different role of microglia proliferation in two distinct disease phases.

As shown in Part I of this Chapter, both SE and epilepsy development were not affected by blockade of microglia proliferation while interference with this cell function mediated neuroprotection in the hippocampus. In accordance, previous studies reported that blockade of microglia proliferation in mice mediates neuroprotection, without inducing SE modifications (Feng et al., 2019). Moreover, in murine models of chronic neurodegeneration, blockade of microglia proliferation in the initial disease phase, reduced the release of neurotoxic molecules implicated in neuronal cell loss, like inflammatory mediators (Gómez-Nicola et al., 2013; Mancuso et al., 2019; Olmos-Alonso et al., 2016).

Our results reinforce the link between microglia and neurodegeneration, previously suggested by studies that applied less selective interventions such as minocycline, MIF, or the inhibition of fractalkine signalling in SE models (Eyo et al., 2017; Ali et al., 2015). This link between microglia and neurodegeneration is mainly restricted to the proliferative activity of microglia during the acute disease phases, rather than to newly proliferating microglia after GW2580 withdrawal. In fact, in our epilepsy model neurodegeneration develops at completion within 7 days post-SE (Frigerio et al., 2018b), thus when mice are still under GW2580 treatment.

Macrophages also contribute to neuronal damage after SE since preventing the infiltration of these peripheral immune cells into the CNS after SE (Feng et al., 2019) reduces neuronal cell loss (Varvel et al., 2021). We found that GW2580 reduced leukocyte extravasation into the hippocampus, a phenomenon that may contribute to neuroprotection.
GW2580 also reduces monocytes proliferation (Conway et al., 2005) but a recent study demonstrated that only resident microglia proliferate in the hippocampus after SE, whereas the infiltrating monocytes do not (Feng et al., 2019). In particular, monocyte/macrophage cells account for the majority of infiltrating cells in our model, as shown by flow cytometry and immunohistochemistry (not shown). Previous results showed that treatment with GW2580 reduced monocytes infiltration in murine models of SE (Feng et al., 2019) and multiple sclerosis (Crespo et al., 2011; Martinez-Muriana et al., 2016), without affecting the number of circulating monocytes (Leblond et al., 2015; Priceman et al., 2010), thus suggesting that GW2580 reduced extravasation of tissue macrophages in our SE-exposed mice.

We also assessed whether neuroprotection observed when microglial proliferation was prevented was associated with rescue of cognitive deficits. However, mice that had experienced SE under either placebo or GW2580 diet were similarly impaired in both recognition and spatial memory compared with sham mice, although there was an improvement in memory retention of GW2580-treated mice in Barnes Maze. The persistence of cognitive deficits in GW2580-exposed mice may be due to the incomplete neuroprotection in the hippocampus and to the damage in the entorhinal cortex, two areas specifically involved in NORT. Our data also showed that the interference with microglia proliferation has diverse effects in the hippocampus (partial neuroprotection) vs the entorhinal cortex (lack of neuroprotection), thus supporting the brain region heterogeneity of microglia and different sensitivity of brain regions to microglia dysregulation (Grabert et al., 2016; Hammond et al., 2019; Masuda et al., 2020; Prinz et al., 2019).

GW2580 did not modify SE onset, severity and duration or the development of spontaneous seizures during epileptogenesis. Accordingly, basal excitatory neurotransmission and neuronal excitability were not modified by GW2580. These results indicate that microglia proliferation during the early post-injury phase is not crucial for the induction of neuronal network hyperexcitability underlying seizure generation. Specifically,
the lack of effects on the spontaneous seizures is unlikely to be due to newly proliferating microglia after GW2580 withdrawal since chronic spontaneous seizures were also not modified during GW2580 treatment. Interestingly, as shown in the cross-sectional study in Chapter 6, despite blockade of cell proliferation, microglia may retain reactive morphology during epileptogenesis, in accordance with previous observation in a model of prion disease (Gomez-Nicola et al., 2013). It is therefore possible that reactive microglia promote seizures even in the absence of cell proliferation by promoting cellular processes mobility and contacts onto neuronal soma (Merlini et al., 2021).

In Part II, the effect of GW2580 was evaluated also on established spontaneous seizures in chronic epileptic mice, through an unbiased summary statistic that took into account seizure number variation intrinsic to the disease itself. Blockade of microglial proliferation during chronic epilepsy has provided a significant reduction in spontaneous seizures number compared with a parallel group of epileptic mice fed with the placebo diet. Indeed, previous evidence has shown that targeting CSF1R by PLX3397 with a treatment protocol affecting only a set of microglia genes reduced seizures in epileptic mice (Srivastava et al., 2018). Moreover, in organotypic hippocampal cultures spontaneous seizure-like activity was reduced by GW2580 (Liu et al., 2019).

Altogether the results suggest that microglia function and involvement in epilepsy depend on the disease phase. Therefore further studies are necessary to characterize the molecular signatures of microglia during epilepsy development, and after the disease is established (Bosco et al., 2018).

Interestingly, our results show that GW2580 did not provide seizures reduction in two out of eight epileptic mice, despite reduction of microglia proliferation was achieved similarly in all mice. Notably, microglia cell body size was increased over sham mice only in non-responder mice, while it was reduced by GW2580 in responder mice, thus suggesting that the therapeutic effect of GW2580 on seizures is associated with both blockade of cell
proliferation and reduction of microglia reactivity. In support, the cross-sectional study (Part I) showed that GW2580 treatment did not affect seizures in the early disease phase when cell body size was not reduced, despite microglial proliferation was prevented. However, microglia cell body size may be simply affected by seizure number rather than contributing to seizures.

This study highlighted different roles of microglia proliferation in the early disease phase compared with chronic epilepsy. While during early disease development microglia proliferation, possibly in concert with extravasated macrophages, contributes to neuronal cell loss in the hippocampus, at a late disease stage it affects chronic seizures. Thus, timely pharmacological interference with microglia proliferation may offer a potential target for improving disease outcomes. Furthermore, both our results and previous evidence suggest that seizures and neurodegeneration in epilepsy are two distinct phenomena. Molecular analysis of microglia phenotype at different disease phases, and after specific cell functions are occluded, may shed light on novel druggable targets for disease modification.
CHAPTER 7: CONCLUSION
The main goal of this PhD project was to provide insights into the role of microglia during epileptogenesis by interfering with its survival, reactivity, and proliferation during critical times of disease development, in a well-established mouse model of acquired epilepsy. Previous studies reported that shortly after kainic acid-provoked SE, a pro-inflammatory microglia profile dominates the milieu (Benson et al., 2015), and others demonstrated that also non-inflammatory changes in microglia may exert pro-epileptic effects (Zhao et al., 2018). Thus, at first, the identification of a proper time window of intervention may be challenging, since microglial phenotypes are very dynamic (Benson et al., 2015). Then, the next step will be to study the phenotype of microglia in those critical time points with high-throughput technologies, by RNA sequencing, an endeavour that goes beyond the aim of this thesis but was already initiated in our laboratory.

As a first important achievement, we have identified the time points at which microglia react and proliferate after SE, thus highlighting two time-windows of intervention: during early epileptogenesis and during the chronic disease phase.

When applying CSF1R inhibitors during epileptogenesis, we found that microglial depletion or inhibition of cell proliferation both prevented neuropathological outcomes in the entorhinal cortex or in the hippocampus with improvement of cognitive deficits, without modifying seizures. Our data provide new evidence in support of microglia heterogeneity in CNS (Masuda et al., 2020; Prinz et al., 2019) by detecting an enrichment of microglia density in the entorhinal cortex compared with the hippocampus in healthy conditions. The heterogeneity across brain regions may explain why depletion of microglia prevented neurodegeneration in the entorhinal cortex, where cell density is higher, but not in the hippocampus. Also, human evidence reports an enrichment for genes associated with microglial reactive states in the entorhinal cortex (Altmann et al., 2021) that may explain why blockade of microglia proliferation, but retention of activated morphologic features, was not sufficient to prevent the neuronal cell loss in this brain region. Our findings also
show that microglial depletion did not prevent the neurodegeneration in the hippocampus, whereas blockade of microglial proliferation has induced neuroprotective effects in this area.

Also, leukocytes extravasation occurs after SE and monocyte/macrophage cells account for most infiltrating cells. Previous studies have demonstrated that preventing the infiltration of these peripheral immune cells reduces neuronal cell loss, thus providing the evidence that extravasated monocytes/macrophages also contribute to neurodegeneration (Feng et al., 2019; Varvel et al., 2021). Moreover, when applying CSF1R inhibitors, also monocytes and macrophages are affected, since this receptor also regulate the survival and proliferation of all myeloid cells other than microglia. In line with this, previous evidence and our own findings have shown that PLX3397 depletes also circulating monocytes and macrophages, thus reducing the infiltration of other immune cells into the brain (data not shown; Elmore et al., 2014), and GW2580 reduces extravasation of monocytes/macrophages. For GW2580, the mechanisms underlying the reduced extravasation are not yet clear, since this drug affects proliferating cells and as previously reported, only microglia proliferate after SE while macrophages do not (Feng et al., 2019). A possible explanation of this reduction could be that blockade of proliferation reduced the chemoattractant power of microglia in recruiting monocytes/macrophages, but experimental evidence cannot be provided yet. Our data suggest that CSF1R inhibitors, may promote neuroprotection by suppressing microgliosis and limiting monocytes/macrophages extravasation, for example GW2580 could be used to explore this hypothesis further.

When applying CSF1R inhibitors, also neuronal progenitors deserve consideration, since it was shown that they also express CSF1R (Stanley and Chitu, 2014). Moreover, microglia play important roles in neurogenesis, as described in Paragraph 1.6.3 of this thesis and previously (Eyo et al., 2017; Victor and Tsirka, 2020), by guiding migration of newly born neurons and by providing trophic support to neuronal progenitors. Therefore, the application of CSF1R inhibitors may affect neurogenesis either directly, by the interference
with neuronal progenitors, or indirectly, by the interference with neuron-microglia interactions. Notably, aberrant neurogenesis is one of the mechanisms triggered by an epileptogenic insult and may contribute to epileptogenesis itself, by the integration of newly born neurons that form ectopic and abnormal neuronal connections, thus increasing hyperexcitability phenomena and behavioural impairments (Cho et al., 2015; Gray and Sundstrom, 1998; Jessberger and Parent, 2015; Parent et al., 2006; Scharfman and Gray, 2007). Indeed, evidence exists that selective ablation of adult neurogenesis reduces chronic seizures and epilepsy-associated cognitive deficits (Cho et al., 2015).

However, all these aspects go far beyond the aims of this thesis project and were not investigated here, therefore the possible implications of these inhibitors in neurogenesis remain unexplored and their future investigation should be considered to add a piece in the puzzle.

Our study highlighted that targeting microglia during epileptogenesis may exert neuroprotective effects in different brain regions, despite does not prevent epilepsy development. However, blockade of cell proliferation in the chronic disease phase was sufficient to reduce seizures. Therefore, microglia play different roles in different disease phases and our data suggest that timely targeting of microgliosis may exert therapeutic effects, for example by ameliorating neurological symptoms and providing seizures control. These effects may either depend directly on modification of microglia functions or be indirectly mediated by compensatory changes in parenchymal cells such as neurons and astrocytes.

Altogether these findings may open new areas of studies and therapeutic interventions that could reduce comorbidities in epilepsy by ameliorating the quality of life of patients, and attenuate seizures. For example, CSF1R inhibitors could be tested as coadjuvant therapy with already approved ASDs to provide disease-modification in drug-resistant patients. Despite appropriate protocols of intervention cannot be directly deduced by our results, the clinical translation of these findings can be potentially explored, since
CSF1R inhibitors are well tolerated in humans and are currently tested in clinical trials. For example, PLX3397 has been approved for the treatment of the tenosynovial giant cell tumour (Monestime and Lazaridis, 2020) and is under investigation in monotherapy or as coadjuvant for several solid tumours such as glioblastoma (see at ClinicalTrials.gov - Study Identifier: NCT01790503; full link to clinical studies with PLX3397, accession date: 17.11.2021, https://clinicaltrials.gov/ct2/results?cond=&term=PLX3397&cntry=&state=&city=&dist=)

Near perspectives. Further investigations are needed to better understand the mechanisms underlying the reduction of chronic seizures when microglial proliferation was inhibited. For example, blockade of cell proliferation may switch microglia towards an anti-inflammatory phenotype thus reducing the release of ictogenic cytokines such as IL-1β, TNF, IL-8.

An additional aspect that should be further explored is the consequence of microglia depletion or proliferation blockade, on the transcriptional and translational phenotype of neurons and astrocytes. This analysis may reveal compensatory changes in these cells therefore highlighting potential targets for therapeutic interventions. In line with that, a deeper study of monocytes and macrophages contribution to epilepsy pathogenesis should be further investigated in the presence of CSF1R inhibitors and complementary approaches, to dissect the roles of these cells from microglia. Moreover, the effect of CSF1R inhibitors on neurogenesis should be addressed to avoid misinterpretations.

Finally, our data should prompt additional investigations in other models of acquired epilepsy such as post-traumatic epilepsy, a condition also associated with microgliosis.

In conclusion, a deeper understanding of microglia functions in epilepsy remains instrumental for envisaging clinical translation of experimental findings.
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