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Characterization of cell death in the *ex vivo* slice model of epileptogenesis

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RESUMO

Mais de 50 milhões de pessoas a nível mundial estão diagnosticadas com epilepsia, uma doença cerebral caracterizada por recorrentes e imprevisíveis interrupções da actividade cerebral normal, designadas por convulsões. Como qualquer doença neurológica, a epilepsia tem associado a si uma gama de consequências neurobiológicas, sociais, comportamentais e cognitivas. Atualmente, ainda não foi encontrada uma cura para esta enfermidade. Apesar dos muitos fármacos antiepilépticos, estes revelam-se ineficazes em cerca de 30% dos casos.

Nos últimos anos, a contribuição da inflamação nesta doença tem sido amplamente estudada, dado que contribui para a geração e recorrência dos ataques epilépticos, bem como para a sua progressão. Os mediadores inflamatórios têm vindo a ser descritos como sendo produzidos pela microglia activada e astrócitos, assim como por neurónios. Um mecanismo de reciprocidade também tem sido sugerido entre a inflamação e a epilepsia. A primeira pode ser tanto uma causa como uma consequência das crises epiléticas, além de ser a causa do desenvolvimento de epilepsia após o evento epilético inicial. Estudos têm demonstrado uma ligação entre os eventos inflamatórios e a morte neuronal ocorrente na epilepsia, bem como a ativação da glia e a expressão de mediadores inflamatórios. Em particular, as células da glia (astrócitos e microglia) produzem e libertam citocinas pró-inflamatórias, que têm efeitos na excitotoxicidade e na morte neuronal, sendo estes factores caracterizantes da epileptogénese. Este trabalho focou-se nas citocinas Interleucina 1β (IL-1β) e no Factor de Necrose Tumoral α (TNF-α), principalmente na sua contribuição para alguns dos processos de morte celular que acontecem nesta patologia neurológica.

Este estudo teve como objectivo principal descodificar a contribuição de cada mecanismo de morte celular envolvido num modelo de epileptogénese em fatias organotipicas de rato. Trata-se de um modelo confiável onde já foi demonstrada a ocorrência de actividade epiléptica espontânea e que permite que as células se desenvolvam de forma semelhante ao que aconteceria *in vivo*. As fatias podem ser mantidas durante mais de um mês para manipulação e avaliação a longo prazo. Este modelo de epileptogénese é considerado uma fiel reprodução do que é descrito em pacientes com epilepsia, o que o torna apto para a sua avaliação. As culturas foram preparadas a partir de ratos Sprague-Dawley com 6-7 dias de vida. Os ensaios foram realizados a 0, 3, 7, 10, 14, 17 e 21 dias *in vitro* (DIV), de modo a estudar os marcadores de morte celular e de inflamação ao longo do tempo.

O especial foco esteve nos diversos mecanismos de morte celular, monitorizando também a ativação e morfologia dos astrócitos e da microglia, e a produção das principais citocinas pró-inflamatórias envolvidas em epilepsia.

No decorrer da última década, também o inflamassoma NLRP3 tem vindo a ser referido como tendo um papel na resposta imunológica. Tendo já sido descrito no sistema nervoso central, o inflamassoma NLRP3 é produzido em altos níveis pela microglia e é o mais importante regulador da ativação da caspase1. A clivagem pela caspase1 leva à maturação de IL-1β e assim à indução de fenómenos inflamatórios. Este mecanismo é um dos que descreve a piroptose, uma das vias de morte celular estudada neste projecto. A piroptose é o maior exemplo de morte celular relacionada com a inflamação e é principalmente caracterizada pela formação do inflamassoma NLRP3. A consequente produção e excreção de IL-1β poderá influenciar as células em seu redor de forma adversa, podendo levar à rutura da barreira hematoencefálica. Outra via que leva a cascatas inflamatórias é a necrose, onde ocorre um efetivo derrame de conteúdos celulares devido ao aumento do volume dos organelos. Essa rutura liberta moléculas que iniciam cascadas inflamatórias.

Também foi realizada uma avaliação da autofagia. Normalmente com um papel protetor, a autofagia pode contribuir para a morte celular em condições de grande stress, onde se verifica a formação de estruturas – autofagossomas – que se fundem com lisossomas, libertando produtos tóxicos que se acumulam no citoplasma das células saudáveis, levando a degradação das mesmas. Por último, a apoptose também engloba um empacotamento de componentes internos, que de outra forma iniciariam uma resposta imune, para eliminação fagocitária.

A morte celular foi primariamente avaliada por western blotting. A marcação pelo NLRP3 e pelo ASC permitiu averiguar a expressão dos mecanismos de piroptose, sendo coligados à produção de IL-1β avaliada por ELISA. O marcador LC3 foi usado para averiguar os mecanismos de autofagia, mais precisamente a formação dos autofagossoma, estruturas altamente específicas deste tipo de via. Os produtos de clivagem da αII-espetrina (SBDP) forneceram mais detalhes sobre as vias de morte celular por necrose e por apoptose. A marcação por caspase3 foi também utilizada como recurso para averiguação da apoptose, bem como a avaliação da produção de TNF-α por ELISA. Os ensaios de imunofluorescência foram efetuados para avaliar as alterações morfológicas das células da glia, astrócitos e microglia, utilizando marcadores específicos, GFAP e Iba1, respetivamente. Tal procedimento é justificado pelo facto de a morfologia destas células ser indicadora de um estado de ativação, que leva astrócitos e microglia a atuar como mediadores de inflamação, produzindo e excretando citocinas inflamatórias que irão manter o ciclo de inflamação, crises e morte celular.

Devido ao severo trauma que se sabe que este modelo apresenta para as fatias de hipocampo, já era de esperar uma robusta degeneração neuronal iniciada após quatro horas do procedimento e que se poderia alastrar até 7 DIV. A necrose foi a via de morte celular mais evidente até este dia, seguindo-se uma possível contribuição da piroptose.

Os resultados obtidos mostraram-se mais preponderantes a partir de 14 DIV, aquando do aparecimento de atividade interictal (previamente estudado), revelando-se num aumento da expressão dos marcadores de morte celular responsáveis pela manutenção dos eventos inflamatórios que caracterizam a epilepsia ao longo do tempo. Os resultados combinados da expressão do NLRP3, ASC e dos níveis de IL-1β produzidos sugerem que a crónica produção de elementos inflamatórios pode levar à morte por piroptose. Também a contribuir para o ciclo da inflamação, a necrose parece ter especial relevância nas etapas finais da epileptogénese. A apoptose e a autofagia parecem não ter um papel relevante neste modelo, sendo que os seus marcadores foram observados em pouca expressão ou, comparando entre condições, não ofereciam qualquer conclusão relevante. Para conclusões mais definitivas em relação à autofagia, mais testes com outros marcadores teriam de ser efetuados. Em relação à apoptose, também não foram detetados níveis díspares de TNF-α em fatias epiléticas.

A ativação das células da glia também se alterou ao longo da epileptogénese. Os astrócitos demonstraram um fenótipo "saudável" a 7 DIV e um fenótipo ativado a 21 DIV, contrariamente à microglia que alterou de uma morfologia ativada para não-ativada ao longo do tempo. Estes resultados permitem extrapolar que os astrócitos podem ser os responsáveis pelo contexto inflamatório em estadios mais avançados da epilepsia, e muito provavelmente perpetuarem os elevados níveis de NLRP3, ASC e IL-1β.

Sumarizando, este trabalho permitiu elucidar os mecanismos de morte celular ao longo da epileptogénese, possibilitando igualmente inferir sobre a contribuição das células da glia nesta patologia.

Palavras-chave: Epilepsia, morte celular, inflamassoma NLRP3, piroptose, necrose

ABSTRACT

Approximately 50 million people worldwide have epilepsy. In the last decade, several studies demonstrated that the production of inflammatory mediators (cytokines) by activated microglia, astrocytes and neurons in epileptogenic tissue, leads to neuroinflammation. Moreover, recent studies show that NLRP3 inflammasome also drives inflammation in the CNS, particularly in this neurological disorder.

Cell death can occur due to the increase of the internal excitotoxicity. This study was performed in an ex vivo model of epileptogenesis in organotypic slices and aimed to disclosure the main pathways involved in cell death and its contribution to epileptogenesis.

An evaluation of cell death was performed through αII-spectrin cleavage assessment and by caspase3, LC3, ASC and NLRP3 protein expression. Astrogliosis and microglia activation was assessed by immunohistochemistry, with the use of specific markers, namely glial fibrillary acidic protein (GFAP) for astrocytes and ionized calcium-binding molecule 1 (Iba1) for microglia. Also, ELISA assay was carried out to quantify the production of TNF-α and IL-1β. Assays were carried out in slices with 0, 3, 7, 10, 14, 17 and 21 DIV to fully cover the course of epileptogenesis.

The initial cell trauma caused by massive deafferentation and deefferentation, which occurred during tissue slicing, resulted in enhanced SBDP145/αII-spectrin and NLRP3 levels during the first week in vitro, suggesting a strong role for necrosis and a possible role for pyroptosis during this phase. More relevant results emerged from the high production of NLRP3, ASC and IL-1β at 14 DIV, parallel with the initiation of epileptic-like events, reinforcing the idea of a strong inflammatory component in later stages of epileptogenesis and a consequent role for pyroptosis in this model. Autophagy and apoptosis do not seem to play a role in this model, since there were no significant differences for their markers, LC3 and caspase3, respectively. In what concerns apoptosis, also no change in TNF-α production was observed.

The activation state of glial cells also changed within epileptogenesis progression. Astrocytes display a “healthy” phenotype at 7 DIV and an activated one at 21 DIV, while microglia changes from an activated to a resting morphology over time. These results reinforce that astrocytes are the ultimate responsible for the inflammatory environment in late epileptogenesis, possibly being the perpetuator for the chronic levels of NLRP3, ASC and IL-1β.

In summary, this work enlightened the mechanisms of cell death along epileptogenesis, together with an assessment of glia cells contribution for this pathology.

Keywords: Epilepsy, cell death, NLRP3 inflammasome, pyroptosis, necrosis

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LIST OF ABBREVIATIONS

ACD – Autophagic Cell Death

AD – Alzheimer's Disease

AEDs – Anti-Epileptic Drugs

AIF – Apoptosis Inducing Factor

AIF2 – Apoptosis Inducing Factor 2

AIM2 – Absent in Melanoma 2

ALS – Amyotrophic Lateral Sclerosis

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASC - Apoptosis-associated Speck-like protein Containing CARD

ATP – Adenosine Tri-Phosphate

BBB – Blood Brain Barrier

BHB – β-Hidroxybutyrate

BSA – Bovine Serum Albumin

CA – Cornu Ammonis

CARD – Caspase Active Recruitment Domain

CNS – Central Nervous System

CTL – Control

DAMP – Damage-Associated Molecular Patterns

DG – Dentate Gyrus

DIV – Days *In Vitro*

DNA – Deoxyribonucleic Acid

EC – Entorhinal Cortex

EDTA – Ethykenediamine tetraacetic

EEG – Electroencephalogram

EL – Epileptic-Like

GABA - γ-Aminobutyric acid

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GBSS – Gey's Balanced Salt Solution

GFAP – Glial Fibrillary Acidic Protein

HD – Huntington's Disease

HMGB1 – High Mobility Group Box-1

HRP – Horseradish Peroxidase

HS – Hippocampal Sclerosis

Iba1 - Ionized calcium-binding molecule 1

IF – Immunofluorescence

IgG – Immunoglobulin G

IHC – Immunohistochemistry

IL-10 – Interleukin 10

IL-18 – Interleukin 18

IL-1R1 – Interleukin 1 Receptor type I

IL-1β – Interleukin 1β

IL-4 – Interleukin 4

IL-6 – Interleukin 6

IPIs – Initial Precipitating Incidents

LC3 – Microtubule associated protein 1A/1B-light chain

LRR – Leucine Rich Repeats

LTP – Long Term Potentiation

MCC 950 – N-[[(1, 2, 3, 5, 6, 7- hexahydro - sindacen - 4 - yl) amino] carbonyl] - 4 - (1- hydroxy - 1 - methylethyl) - 2 - furansulfonamide, aiarylsulfonylurea-containing

MF – Mossy Fibers

mtDNA – Mitochondrial DNA

MTLE – Mesial Temporal Lobe Epilepsy

mTNF - TNF-α transmembrane protein precursor

mTOR – Mammalian Target of Rapamycin

NBA – Neurobasal A

NF-κB – Nuclear Factor-κB

NGS – Normal Goat Serum

NLR – NOD-Like Receptors

NLRP1 – NACHT Leucine-Rich-Repeat Protein 1

NLRP3 – Nucleotide-binding Oligomerization Domain-like receptor 3

NMDA – N-Methyl-D-aspartate

NO – Nitric Oxide

NOD – Nucleotide-binding Oligomerization Domain

NP40 - Nonyl phenoxlpoylethano 40

OHSC – Organotypic Hippocampal Slice Culture

P6-7 – 6 to 7 day-old Pups

PAMPs – Pathogen Associated Molecular Patterns

PBS – Phosphate-buffer Solution

PCD – Programmed Cell Death

PD – Parkinson's Disease

PFA – Paraformaldehyde

PMSF – Phenylmethysulfonyl fluoride

PP – Perforant Path

PRR – Pattern-Recognition Receptors

PVDF – Polyvinylidene Difluoride

PYD – Pyrin

RIPA – Ristocetin Induced Platelet Agglutination

RLH – RIG-Like Helicases

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RT – Room Temperature

SBDPs – Spectrin Break Down Products

SC – Schaffer Collaterals

SDS – Sodium Dodecyl Sulphate

SDS-PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SE – *Status Epilepticus*

SEM – Standard Error of the Mean

SPECT – Single Photon Emission Computed Tomography

SRS – Spontaneous Recurrent Seizures

sTNF – TNF-α soluble form

TBS – Tris Base Solution

TBST – Tris Base Solution with Tween

TGF-β – Transforming growth factor-β

TLE – Temporal Lobe Epilepsy

TLE-HS – Temporal Lobe Epilepsy with Hippocampal Sclerosis

TLR – Toll-Like Receptors

TNFR1 – TNF Receptor type 1

TNFR2 – TNF Receptor type 2

TNF-α – Tumour Necrosis Factor-α

μ-Calpain – Calpain I

1 BACKGROUND

1.1 Central Nervous System

The central nervous system (CNS) is most commonly divided into major structural units, consisting of the major physical subdivisions, the brain and the spinal cord. The central nervous system is composed by two main classes of cells: neurons and glia (Squire, 2013).

Neurons are a highly specialized cell type and are the essential cellular elements in the CNS. They can be generated from different types of progenitor cells, as neuroepithelial cells, and are electrically excitable cells that can communicate between themselves through electrical and chemical signs (Götz & Barde, 2005; Blank & Prinz, 2013). Neurons can propagate electrical impulses and release signalling molecules, called neurotransmitters, and are considered the main targets of neurodegenerative diseases (Ransohoff, 2016). Neurotransmitters can amplify and modulate signals between neurons, as well as between neurons and other cells (Miller & Gauthier, 2007; Squire, 2013).

There are three types of glia cells in the CNS, with oligodendrocytes comprising approximately 75% of cortical glia, followed by astrocytes and microglia (Devinsky et al., 2013). The glial cells are important supportive cells but they do not conduct electrical impulses; instead they surround neurons and contribute to synaptic transmission (Miller & Gauthier, 2007). Within the healthy CNS, microglia presents itself with a distinctive morphology, with a small soma and numerous branching processes (Davalos et al., 2005). Microglia comprises about 10-15% of total brain cells and responds rapidly to pathological changes (Hubbard et al., 2013) and can contribute directly to the neuronal degeneration by producing proinflammatory cytokines and free radicals (Mildner et al., 2011; Bisht et al., 2016). Astrocytes play a variety of metabolic support roles, including furnishing energy intermediates and providing for the supplementary removal of excessive extracellular neurotransmitter secretions (Squire, 2013). Glia cells will be further characterized in following sections.

The blood–brain barrier (BBB) is an important permeability barrier to selected molecules between the bloodstream and the CNS (Marchi et al., 2014). This barrier places a dynamic interface between the brain and the circulatory system, formed by specific endothelial cells of brain capillaries, designed to defend the brain from damaging substances and pathogens (Dey et al., 2016).

1.2 The Hippocampus

The hippocampal formation is one of the most studied structures in the CNS. There is a distinction between the hippocampal formation and the hippocampus itself. According to Amaral and Witter in 1989, the hippocampal formation belongs to the limbic system and is comprised of the entorhinal cortex (EC), dentate gyrus (DG), hippocampus proper, and the subicular complex. The "hippocampus" itself refers the inclusion of the pyramidal cell fields of the proper hippocampus, Cornu Ammonis 1 and 3 (CA1 and CA3), together with the hilar and granule cells in the DG (Andersen et al., 2007).

In terms of embryonic development, the dentate gyrus and the hippocampus proper are the earliest structures in the hippocampal formation to be recognized (Swanson & Cowan, 1979). The Cornu Ammonis or Ammon's horn, referring to the CA1-CA3 pyramidal cell fields, is considered to be the central and major structural component of the hippocampal formation (Andersen et al., 2007). These layers also contain the basal dendrites of the pyramidal cells and several classes of interneurons. According to Ramón y Cajal (Ramon y Cajal, 1893), CA1 subdivision is the closest area of the EC and CA3 is in direct contact with the DG. The DG is comprised of three layers: granule cell, molecular cell

and polymorphic cell layers. The granule cell layer in comprise within the molecular layer and form a thick packed layer of cells. Together, granule cell and molecular layer for a V or U shaped structure that enclose the polymorphic cell layer (Andersen et al., 2007).

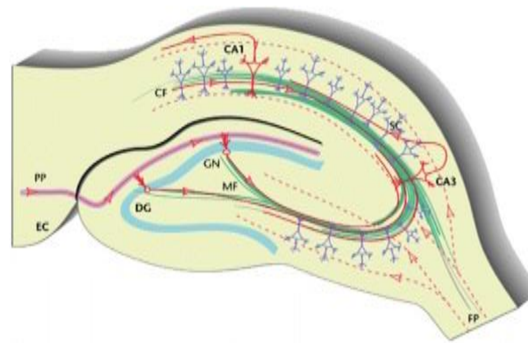


Figure 1.2 - The hippocampus structure – Entorhinal Cortex (EC), Perforant Path (PP), Commisural Fibres (CF), Cornu Ammonis (CA1-CA3), Dentate Gyrus (DG), Granule Cells (GC), Mossyfibers (MF), Fimbrial Pathway (FP) | Adapted from Phares 2006

In terms of afferents, the hippocampus (Figure 1.2) receives highly processed cortical information from the EC (Amaral & Witter, 1989) and important subcortical projections via the fimbria-fornix (Swanson & Cowan, 1979). The DG projects only to the CA3 field, via mossy fibers, and each mossy fiber (MF) expansion can make up to 37 contacts with a single CA3 dendritic cell, the dendrite of the pyramidal cells and several classes of interneurons. The granular neurons in the DG receive inputs from the EC via perforant path (PP). CA3 pyramidal neurons synapse with other CA3 neurons and with CA1 pyramidal neurons by Schaffer collaterals (SC) (Phares & Byrne, 2005). There is also a cortical connection in the direct projection from the EC that bypasses DG and terminates in the subiculum and the CA1 cell field (Andersen et al., 2007). These pathways ensure the propagation of information across the whole structure, in a unidirectional route, using the excitatory neurotransmitter L-glutamate (Storm-Mathisen, 1977). The information is propagated in a loop, called trisynaptic circuit, composed by exchange of impulses from EC to DG, DG to CA3, CA3 to CA1 and CA1 back to EC (Avoli, 2007; Stepan et al., 2015).

The hippocampus is considered the centre for learning and memory in the brain, and is associated with several chronic diseases, such as epilepsy (Andersen et al., 2007). This so happens because the hippocampus has one of the lowest epileptogenic thresholds, which makes it an exquisitely seizure-prone structure (Green, 1964; Abdelmalik et al., 2005). In fact, more than 50% of all epilepsies originate on temporal lobe structures, due to the low seizure threshold found in the limbic structures of the mesial temporal lobe (Abdelmalik et al., 2005). In an epileptic hippocampus, the DG undergoes changes, with loss of dentate hilus interneurons, newly formed granule cells and sprouting of MF. These changes suggest a remodelling of the hippocampal circuits, which is in strict correlation with epileptogenesis (Avoli, 2007). Moreover, the seizure threshold of the dorsal CA1 is the lowest in the brain. When compared with the motor cortex, it has a seizure threshold that is five to seven fold lower (Abdelmalik et al., 2005). A large proportion of the electrophysiological studies, within an epilepsy context, were performed on the hippocampus (Pare et al., 1992). It has also been proved that the surgical removal of the sclerotic hippocampus often improves the epileptic condition (McNamara, 1994).

1.3 Epilepsy

Epilepsy is a brain disorder characterized by recurrent and unpredictable interruptions of normal brain function, the so called epileptic seizures. Approximately 50 million people worldwide (with a prevalence of 3 to 5 cases per 1000 persons, depending on age and ethnic background) have epilepsy and experience neurobiological, cognitive, psychological and social consequences (Engel, 1989; Shimada et al., 2014). Epilepsy definition requires the occurrence of at least one epileptic seizure. A seizure is a transient occurrence of signs and/or symptoms due to abnormal neuronal activity in the brain (Fisher et al., 2005; Dey et al., 2016). Another crucial term for this disease is status epilepticus (SE), a medical emergency state characterized by a continuous epileptic seizure lasting over 30 minutes, or more than one seizure in a 30 minutes period, without full recovery of consciousness between seizures (Rosenow et al., 2007; Dey et al., 2016).

Epileptogenesis is the development and extension of lesions caused by the capacity of a tissue to generate a seizure and it can result in the development of an epileptic condition or its progression once epilepsy is established (Pitkänen & Lukasiuk, 2011). Epileptogenesis is characterized by several events that may be use as biomarkers for its study, such as cell loss, neurogenesis, gliosis and altered glial function, BBB damage and innate and adaptive immunity (Pitkänen & Engel, 2014). Understanding how seizures develop is critical to identify the regions where proepileptic changes may lie, designing new and more effective therapies (Bertram, 2009).

Seizure type, typical age of onset, electroencephalogram (EEG) findings and prognoses can classify different epilepsy syndromes. Predisposed seizures can be caused by genetic, environmental and/or developmental conditions. Also, transient brain insults (ischaemia, neoplasia or infection) and genetic disorders can influence neuronal metabolism or neuronal circuit assembly and thus lead to epilepsy (Planells-Cases & Jentsch, 2009).

Followed by onset age, EEG patterns and general symptoms, epilepsy syndromes can also be classified into four major categories. Idiopathic generalized epilepsy has a recurrent, but not always proved family history of epilepsy and tends to appear during childhood or adolescence. There is no nervous system abnormalities observed, other than seizures, that can be identified on either EEG or imaging studies (Berg et al., 2010; Shorvon, 2011). Symptomatic generalized epilepsy is of an acquired or genetic cause, characterized by widespread brain damage. Injury during birth is the most causable origin of this type of epilepsy and in addition to seizures, patients often have other neurological problems, as mental retardation or cerebral palsy (Berg et al., 2010; Shorvon, 2011). Cryptogenic epilepsy frequently refers to symptomatic general epilepsy that cannot be identified. They still account for 40% of adult-onset cases of epilepsy (Berg et al., 2010; Shorvon, 2011). Provoked epilepsy, in which a specific systemic or environmental factor is considered the cause of the seizures. There are no gross causative neuroanatomic or neuropathologic changes. The reflex epilepsies are included in this category, which are usually genetic (Berg et al., 2010; Shorvon, 2011; Khurana, 2014)

Temporal lobe and hippocampus are the most vulnerable brain regions to epilepsy, but several other areas can be damaged by the progress and constant injury caused by the recurrent seizures. Each affected brain region has mobility associated issues. Hippocampal epilepsy presents altered awareness, motor arrest and post-ictal confusion. Frontal lobe epilepsies exhibit altered awareness, minimal or no post-ictal confusion and frequent SE. Parietal lobe epilepsies often messes with sensory events with positive phenomenon, such as electric feeling. Occipital lobe epilepsies, on the other hand, usually have visual manifestations (Riviello, 2003; Khurana, 2014).

The impact of this disease on the health and life quality of the patient is severe. Firstly, no curative drug treatments exist for epilepsy and thus it is often acquired for life. Secondly, the current anti-epileptic drugs available, which are only symptomatic, have many side-effects and are ineffective in 30–40% of the patients (Amhaoul et al., 2014). Anti-epileptic drugs (AED) are a set of drugs that decrease the frequency and/or severity of seizures. Their goal is to maximize the quality of life by minimizing seizures, but they have three major drawbacks. The treatment is focused on symptoms and will treat neither the underlying epileptic condition nor the progress of the disorder (Bromfield et al., 2006). AEDs can have a narrow range within which seizures are controlled without toxicity. However, they can induce pharmacokinetic interactions which can cause toxicity effects, like dizziness, ataxia and nausea (Bromfield et al., 2006; Krauss & Sperling, 2011). Also, AEDs prevent seizures, but have not been shown to modify the underlying epilepsy. Studies on the mechanisms of AED action focus on neurons, ion channels and transporters, and excitatory and inhibitory neurotransmission; they rarely examine whether these drugs have effects on glia or immune function. However, anti-inflammatory effects of AEDs could be relevant to their clinical activity. The pathomechanisms that may be associated with pharmacoresistance can be classified into three general categories: disease-related, genetics and drug-related mechanisms (Krauss & Sperling, 2011). Finally, the AEDs have severe risks of cognitive complications (Binder & Steinhauser, 2006).

Among the different mechanisms that may be involved in epileptogenesis, neuroinflammation appears as one of the most critical one, as suggested in clinical reports and animal models of epilepsy (Vezzani & Rüegg, 2011; Valencia & Khurana, 2014). This is due to seizure being able to perpetuate inflammatory responses in the brain through different mechanisms, such as increased cytokine production and release. Epileptogenesis itself can set in motion a cascade of inflammatory pathways, triggering the onset of epilepsy, in a reciprocity that contributes to the comorbidities associated with epilepsy. Thus, targeting inflammation is an emerging strategy for epilepsy treatment (Vezzani & Rüegg, 2011; Valencia & Khurana, 2014).

Finally and beyond the seizure occurrence itself, neuropsychiatric comorbidities, such as depression (Kanner, 2005), autism (Jensen, 2011) and schizophrenia (Stefansson et al., 1998), are nowadays pointed out as outcomes of the epileptogenic process. Therefore, it is important to find new therapies to prevent the onset and/or progression of epileptogenesis.

1.3.1Temporal Lobe Epilepsy

The most (40%) frequent of all epilepsy syndromes is the temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) (Heuser et al., 2009). The HS histological profile includes neuronal death and astrogliosis in the hippocampal pyramidal layer, sparing only the CA2 sector, associated with Magnetic Resonance Imaging (MRI) signs of atrophy and increased hippocampal signal (Bien et al., 2007; Finegersh et al., 2011). There are few potential causes of TLE-HS, including familial cases and a presumed consequence of initial precipitating incidents (IPIs). IPIs have a latent period before the onset of seizures (or SE) and may present as non-seizure events, as hypoxia, trauma or intracranial inflammation. The remainder of TLE-HS are considered primary or idiopathic and may represent cases of pre-existing hippocampal abnormalities in the sense of a maldevelopment disorder (Bien et al., 2007).

Recent non-invasive neuroimaging techniques, like MRI and single photon emission computed tomography (SPECT), have shown that most TLE patients have pathological changes in some brain structures and network function, including cortical and subcortical structures (Coan et al., 2014; Datta

et al., 2015). Most of the neuronal cell death and active gliosis occurs in CA1 and CA3 areas (Seress et al., 2009). There is also evidence of MF sprouting in the DG region, a sign of synaptic reorganization. The MF (axons of the DG granule cells) develop collaterals that grow into the inner third of the DG molecular layers (Scharfman, 2003; Seress et al., 2009).

The severity of TLE depends on seizure frequency, epilepsy duration, and age of seizure onset (most common in adults). According to the location where seizures arise, TLE can be divided into lateral (neocortical) temporal lobe epilepsies and mesial temporal lobe epilepsies (MTLE) (Heuser et al., 2009). Clinically, MTLE is a progressive disorder where the seizures may initially be controlled with AEDs, but symptoms re-emerge in about 60 to 90% of the cases and become medically intractable (Sadler, 2006; Lee & Lee, 2013).

1.4 Neuroinflammation

The CNS has been considered an immunoprivileged site, due to the presence of the BBB. However, some studies demonstrated that immune and inflammatory reactions occur within the CNS (van Vliet et al., 2007; Cappellano et al., 2013) and inflammation is always implicated in the process of CNS pathology. Brain trauma, cerebrovascular events and cerebral infection cause a dynamic infiltration of circulating blood cells, such neutrophils, monocytes and lymphocytes (Dey et al., 2016). Those infiltrating immune cells are activated by microbial compounds of endogenous danger signals in the brain, produced by self-molecules from CNS tissue (Shichita & Yoshimura, 2016).

Neuroinflammation in the brain is a homeostatic, and often a sterile phenomenon (Ghavami et al., 2014; Shichita & Yoshimura, 2016), characterized by the presence of cytokines and established mediators of inflammation that, under physiological conditions would not, or would barely, be detected (Vezzani & Granata, 2005; Vezzani & Rüegg, 2011). These molecules are produced by cells of the immune (either adaptive or innate) system in response to infection or various kinds of pathologic threats, by brain parenchymal cells (microglia, astrocytes, and neurons) and by cells of the BBB and choroid plexus (Vezzani & Granata, 2005; Vezzani & Rüegg, 2011; Olmos & Lladó, 2014). Also, there are endogenous self-molecules that function as an alarm to warn immune cells of tissue damage are called damage-associated molecular patterns (DAMP). DAMPs are released into extracellular space from dying brain cells, but are often actively produced from abnormal or injured brain cells (Shichita & Yoshimura, 2016).

The CNS has its own innate immune cells, the microglia cells (Vezzani & Granata, 2005; Cappellano et al., 2013). Therefore, healthy CNS is defined as immunoprivileged and immunologically specialized site (Obermeier et al., 2013). Local activation of glial cells and the infiltration of circulating immune cells are important processes in neurological disorders, and have been proven to promote CNS pathologies (Shichita & Yoshimura, 2016). The activated glial cells will recognize abnormal self-molecules and induce neuroinflammation (Shichita & Yoshimura, 2016; Vezzani et al., 2016), by producing various inflammatory mediators including cytokines, such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6), chemokines, matrix metalloproteinases and reactive oxygen species (ROS) (Martinon et al., 2009; Ghavami et al., 2014). These mediators will further enhance the infiltration of cerebral immune cells, thus perpetuating the inflammation on the brain (Brown & Vilalta, 2015). Glia activation is observed during epileptogenesis in both symptomatic and genetic epilepsy models and is maintained in the chronic epilepsy phase, when the disease is already established (Pitkänen & Engel, 2014). In animal models of epilepsy there is a correlation

between the number of spontaneous seizures and the activation of both astrocytes and microglia (Ravizza et al., 2008).

Neurodegeneration refers to the progressive loss of structure or function of neurons and it can lead to devastating neurological conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Tan et al., 2015). Uncontrolled activation of microglia that follows neuroinflammation might directly be toxic to neurons (Ghavami et al., 2014). Then, even though activated microglia can phagocytose cell debris, it can also phagocytose neighbouring intact cells, thus causing neurodegeneration. Therefore, microglia can have both a protective and devastating role, as its activation and function in neurodegenerative diseases plays a significant role in mediating the diseases rather than protecting neurons (Ghavami et al., 2014).

On a first analysis, inflammation has beneficial effects, mainly due to the elimination of pathogens, recruiting other cells, clearing debris, aiding repair and providing neurotrophins. Indeed, the cytokine IL-1β promotes both the plasticity of the BBB and the production of neurotrophic factors by brain cells (Brown & Vilalta, 2015; Dey et al., 2016). On the down side, the detrimental effects are unintended side-effects of the beneficial processes, since exacerbated increase of cytokines is detrimental for BBB and other brain cells. Also, inflammation generates an anti-inflammatory activated (M2) phenotype, rather than an inflammatory (M1) one, of microglia and macrophages (Brown & Vilalta, 2015; Shichita & Yoshimura, 2016).

After its first occurrence, inflammation may become detrimental for the tissue, and thus has to be prevented before it becomes chronic or last long enough to provoke tissue damage or dysfunction. Recent studies report that seizures also can increase the BBB permeability, which can intensify and perpetuate neuroinflammation (Marchi et al., 2014; Gorter et al., 2015). Long-lasting brain inflammation has been described after ischemic stroke, traumatic brain injury, or during chronic neurodegenerative diseases, and appears to contribute to the evolution of the pathology in all the referred conditions (Vezzani & Rüegg, 2011). Also, epilepsy associated with malformations of cortical development showed a number of inflammatory mediators (cytokines) in epileptogenic tissue, produced by activated microglia and astrocytes, as well as by neurons (Levite, 2002; Bien et al., 2007). This gave rise to the hypothesis of the existence of a reciprocal link between epileptic activity and brain inflammation. So, inflammation can be both the consequence and the cause of seizures, as well as the cause of the development of epilepsy after an initial precipitating event (Vezzani et al., 2011; Vezzani & Rüegg, 2011). It now becomes clear that proinflammatory mediators, such as cyclooxygenase-2, prostaglandin E2, IL-1β, IL-6, high mobility group box 1 (HMGB1), toll-like receptor-4 (TLR4), TNF-α, transforming growth factor-β (TGF-β), and nitric oxide (NO) play important roles in seizure generation and exacerbation (Dey et al., 2016). Experimental studies demonstrated that several insults can induce a cascade of chronic inflammatory processes in the CNS, which contribute to the development of epilepsy (Nguyen et al., 2002; Walker & Sills, 2012).

The effect of seizures on neuronal death and the role of seizure-induced neuronal death in acquired epileptogenesis have been debated for decades. The prolonged and repetitive seizures that define SE typically cause brain damage, often with extensive neuronal death (Dingledine, 2014). However, the process by which seizure-evoked inflammation arises is still unknown. Although cell loss does not induce inflammation in this context, dying cells may contribute to the perpetuation of the inflammatory process (Walker & Sills, 2012; Vezzani et al., 2013).

1.5 Glial Cells

For years, epilepsy studies have focused exclusively on neurons, but in the past few years, microglial cells, resident brain macrophage-lineage cells, and astrocytes have gained attention for their ability to participate actively in pathological brain processes, thus being a new target on trying to understand the mechanisms behind epilepsy (Shimada et al., 2014). There are several features associated to this branch of cells, including the abnormal release of cytokines and the involvement of distinct intracellular messenger systems that answer to diverse brain pathogens, and have been interpreted as being either neurodegenerative or neuroprotective in nature (Lehrmann et al., 2008; Shimada et al., 2014). It is now known that glia cells play an active role in many central homeostatic processes and also during development. Tree main types of glia exist, namely astrocytes, oligodendrocytes and microglia.

Microglial cells (the innate immune cell population of the CNS) derived from progenitors that have migrated from the periphery and are from mesodermal/mesenchymal origin (Streit et al., 2004; Marín-Teva et al., 2011). They comprise 10-15% of all CNS cells and present a branched, ramified morphology. Microglia is constantly in motion, surveying their microenvironment and communicating with neurons and other glia cells via processes and protrusions. They exist in different states, including resting and activated, have phagocytosis capacity and act as critical mediators in the modulation of neurogenesis (Su et al., 2016). There is a prominent difference between the morphology of the two microglia states. Resting microglia exhibit a small, compact soma bearing long, thin and ramified processes (Yang et al., 2010). In contrast, activated microglia exhibit a macrophage-like ameboid morphology, with a larger soma and shorter, but highly ramified motile processes, that constantly monitor their immediate surroundings by extending and retracting their processes (Olah et al., 2011; Hristovska & Pascual, 2015). Morphological changes are assessable by differences in regional density, different responsiveness and differences within anatomical regions (Olah et al., 2011).

In a healthy brain, microglia are in a resting state, but upon a pathological insult, either via endogenous or exogenous stimulation, microglia can acquire an “activated state” (Blank & Prinz, 2013). This activation implies a modification in their shape to enable their phagocytic function and induce inflammatory responses through the release of multiple cytokines and inflammatory mediators, determining the fate of other neural cells around (Su et al., 2016). This activated state induces a rapid production of ATP, which in turn recruits astrocytes to amplify the ATP production and glutamate release. As a consequence, there is an increases of neuronal excitability through the metabotropic glutamate receptor 5 (Blank & Prinz, 2013). Besides, the effect of classical activation is targeted towards antigen presentation and the killing of intracellular pathogens, via an increased crosstalk with other immune cells (Cherry et al., 2014).

The activation state of microglia can be divided into two types. M1 type microglia activation is the classical activation, that leads to proinflammatory effects by producing numerous mediators, such as proteases, cytokines and ROS, that become detrimental for neurons and other glial cells (Su et al., 2016). M1 phenotype originally respond to the injury and infection, and generally act in the first line of tissue defence and promotes the destruction of invading pathogens (Tang & Le, 2016). This phenotype can be characterized by the differential expression of IL-12 and IL-10, given that its production is high for IL-12 but low for IL-10 (Cherry et al., 2014). Also, due to the fact that the production on ROS is one of the hallmarks of the microglial type, it can be characterized by the production of iNOS, which uses arginine to produce NO (Bagasra et al., 1995). This classical

activation is also associated with the production of proinflammatory cytokines such as IL-1β, TNF-α, superoxide and NO (Tang & Le, 2016).

On the other hand, M2 type microglial activation is viewed as an alternative activation, and is considered an anti-inflammatory phenotype involved in the phagocytosis of cell debris or damaged neurons and in the release of various neurotrophic factors and cytokines (Marín-Teva et al., 2011; Su et al., 2016). This phenotype is usually initiated after the onset of the classical inflammation, and it is seen as an anti-inflammatory and repairing phase, that lead to wound healing and brings back tissue homeostasis (Tang & Le, 2016). These microglia cells have thus the potential do dampen proinflammatory immune responses and promote the repair genes expression (Tang & Le, 2016). The anti-inflammatory phenotype is characterized by the production of four major anti-inflammatory cytokines including IL-4, IL-13, IL-10, and TGF-β, all known to antagonize the proinflammatory responses (Watters et al., 2005; Zhao et al., 2006). In summary, M1 microglia is associated with the classical activation and has been associated with neurotoxicity, whereas M2 microglia is considered an alternative activation pathway and is linked to neuroprotection.

Astrocytes are functionally associated with the pre- and postsynaptic nerve terminals, as the third element of a structure known as "tripartite synapse" (Lehrmann et al., 2008). The term refers to a synaptic physiology concept of a communication between astrocytes and neurons. In addition to the classic "bipartite" information flow between the pre- and postsynaptic neurons, astrocytes exchange information with the synaptic neuronal elements, thus regulating synaptic transmission (Perea et al., 2009). This role of astrocytes in synaptic transmission can be motorized by the high calcium-mediated cellular excitability of the cell (Nett et al., 2002). Neuron-to-astrocyte communication presents attributes that were classically considered to be exclusive to neuron-to-neuron communication, such as having cellular excitability, showing selective responsiveness to specific synaptic inputs, display nonlinear input-output relationships and have cell-intrinsic properties (Newman, 2005; Rieger & Deitmer, 2007; Perea et al., 2009).

Astrocytes provide a link between the vasculature and neurons, through the transport of glucose and other substances out of the bloodstream. Furthermore, their endfeet wrap around the endothelial cells contributing to the BBB maintenance (Devinsky et al., 2013). Astrocytes also play a role in the uptake of neurotransmitters, such as glutamate and gamma-aminobutyric acid, and the regulation of extracellular potassium ion concentration, crucial functions for the propagation of action potentials (Schousboe et al., 2004). In addition, astrocytes respond to all forms of CNS insults, such as infection, trauma, ischemia and neurodegenerative diseases. Moreover, these cells perform immune functions (Hull et al., 2006), synthesize and release neurotrophic factors and are involved in the formation of glial scars following injury (Silver & Miller, 2004; Sofroniew, 2009).

During development, radial glia - which differentiate into astrocytes when neuronal migration is complete - provide a supporting matrix for neuronal migration and synaptogenesis (Mori et al., 2005). Similarly to microglia, astrocytes undergo a change in morphology, molecular composition and proliferation in the epileptic foci (Devinsky et al., 2013). The morphologic change is usually addressed as "reactive astrogliosis", defined as a continuous spectrum of changes that vary with the nature and severity of diverse insults. Also, astrocytes are activated by pathogens or local non-infectious injuries, leading to the release of proinflammatory mediators (Devinsky et al., 2013).

As a consequence of severe astrogliosis, the severe astrocytic activation can lead to the occurrence of a "scar formation" (Sofroniew, 2009; Robel et al., 2015). The glial scar is characterised by the

accumulation of astrocytes in the injury site (Silver & Miller, 2004; Miller et al., 2015) and by the interaction of astrocytes with other types of cells, for instance fibromeningeal and other glial cells (Robel et al., 2015). This overreactive astrogliosis has a spectrum of changes in astrocytes that occur in response to CNS injury, and those changes vary with the nature and severity of the insult. On a normal and healthy basis, astrocytes secrete chemokines, cytokines, growth factors and extracellular matrix components that participate in wound healing (Robel et al., 2015). Reactive astrogliosis leads to a continuum of progressive alterations in molecular expression, cellular hypertrophy and, in severe cases, proliferation and scar formation, that can be translated through gain and loss of functions that impact beneficially and detrimentally on surrounding neural and non-neural cells (Sofroniew, 2009; Robel et al., 2015). Hypertrophy and upregulation of the intermediate filament glial fibrillary acidic protein (GFAP) are some hallmarks of astrogliosis (Robel et al., 2009).

Neuroglia "activation" is usually considered to be secondary to neuronal dysfunction or injury, and is believed to either participate in progressive neuron loss or in the brain's efforts to prevent further damage (Lehrmann et al., 2008, Marín-Teva et al., 2011). Both astrocytes and microglia respond to tissue insult with a complex array of factors ranging from cytotoxic mediators (cytokines, proteases, free radicals or glutamate agonists) to trophic factors, which can exert beneficial, as well as deleterious effects, upon the surrounding neurons (Marín-Teva et al., 2011, Olmos & Lladó, 2014). This response is accompanied, and often followed, by a cascade of downstream inflammatory events (Vezzani et al., 2008; Olmos & Lladó, 2014).

The activation of microglia and astrocytes can result from seizures alone, without cell loss (Dube, 2010; Vezzani, 2000). In particular, microglia has a role in two specific aspects: the initial intensity of acute seizures and the subsequent neurodegeneration that occurs (Eyo et al., 2016). Thus, microglia-neuron communication plays a critical role in acute seizures, delayed neurodegeneration and aberrant neurogenesis (Eyo et al., 2016). Activated microglia produce proinflammatory mediators within 30 minutes of seizure onset (De Simoni et al., 2000; Devinsky et al., 2013), before the actual morphological cell activation is detectable (Avignone et al., 2008). These proinflammatory mediators, produced by both microglia and astrocytes, interact with their cognate receptors overexpressed by brain microvessels, thus affecting BBB permeability at multiple levels (Devinsky et al., 2013). Activated astrocytes extend their processes outside their usual domains. The loss of astrocytic domain organization, together with dendritic sprouting and new synapse formation may contribute to the recurrent excitation in epilepsy (Oberheim et al., 2008). Astrocytes can also influence microglia through the release of ATP, affecting microglia via purinergic receptors. Like all immune effector cells, astrocytes may help limit the immune response by controlling microglial activation (Verderio & Matteoli, 2001).

Microglia activation can persist without synthesis of inflammatory cytokines. For instance, IL-1β is detected in microglia following a seizure, but its expression fades overtime and still the microglia remains morphologically activated (Ravizza et al., 2008). Moreover, microglia and astrocytes can remain activated in experimental epileptic models following the inhibition of cytokine synthesis (Ravizza et al., 2008; Maroso et al., 2011). It is also implied that microglia interact directly with neurons by means of activated astrocytes (Blank & Prinz, 2013). As a whole, the degree of activation and balance of both pro- and anti-inflammatory cytokines will determine the final contribution of the activated microglia (Battista et al., 2006). Prolonged or excessive microglial activation can cause cellular dysfunction and death (Saijo & Glass, 2011).

1.6 The NLRP3 inflammasome

Inflammasomes are multiprotein complexes, which assemble by self-oligomerizing scaffold proteins (Schroder & Tschopp, 2010). They are danger sentinels that self-oligomerize via homotypic NACHT/NOD domain interactions to form high-molecular weight complexes (probably hexamers or heptamers) that trigger caspase1 auto activation (Walsh et al., 2014).

The inflammasome belong to pattern recognition receptor (PRR) family. Two families were recently described: the NOD-like receptors (NLRs) and the RIG-like helicases (RLHs), both soluble proteins that survey the cytoplasm for danger signals from intracellular invaders (Martinon et al., 2009). Inflammasomes, in which the scaffolding and sensing proteins are members of the NLR family, have been found to be a part of innate immunity (Kugelberg, 2015; Zhou et al., 2016)

NLR proteins are intracellular leucine rich repeats (LRR)-containing proteins that resemble disease-resistance genes and form central molecular platforms. Those platforms organize signalling complexes, such as inflammasomes and NOD (Nucleotide-binding oligomerization domains) signalosomes (Martinon & Tschopp, 2005). Structurally, NLRs are multidomain proteins with a tripartite architecture containing a C-terminal region, with a series of LRRs, a central nucleotide domain termed (NACHT or NOD-domain) and an N-terminal effector domain. These NLRs are mostly expressed in the cytosol (Martinon & Tschopp, 2005).

The LRR domain is the master commander of the sensing and autoregulation of NLRs. They are formed by a structural motif of 20 to 30 amino acids with a pattern rich in the hydrophobic amino acid leucine. LRR domains are formed by tandem repeats of a β strand and an α helix (Bella et al. 2008). TLRs contain LRRs that recognize or sense the presence of intracellular danger signals, including lipoproteins, flagellin, LPS and RNA from bacteria or viruses (Adamczak et al., 2014; De Rivero Vaccari et al., 2016).

The NACHT domain, central to all NLRs, suffers oligomerization, thereby forming active, high molecular weight complexes that characterize inflammasomes and NOD signalosomes. This particular domain is believed to be crucial to NLR activation (Martinon et al., 2009)

The nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) inflammasome, also known as cryopyrin, is the best studied NLR inflammasome and most widely implicated regulator of caspase1 activation (Martinon et al., 2002). Its N-terminal protein-protein interaction domain is a pyrin (PYD), and it thus requires an apoptosis-associated speck-like protein containing a CARD (ASC) adaptor (Walsh et al., 2014). The figure 1.6.1 A shows all the NLRP3 inflammasome components. The inflammasome contains various PRRs, ASCs and caspase units, thus being a heterodimeric structure (Labbé & Saleh 2009) (Figure 1.6.1 B).

The PRRs have been shown to define the type of inflammasome to be assemble and the type of stimuli that initiates its activation (Lénárt et al., 2016). The interaction PRR/CARD is established by PYD-PYD interactions. The ASC protein is constituted by a PYD and a caspase recruitment domain (CARD), a death domain that plays a role in caspase activation (Fernández & Lamkanfi 2015; Vanaja et al., 2015). The main function of this CARD domain is to recruit the inactive procaspase1 to the inflammasome, which is followed by the cleavage and activation of procaspase1, resulting in biologically active caspase1 (Schroder & Tschopp, 2010; Gustin et al., 2015).

Caspase1 activation leads to inflammatory cascades and the eventual cleavage of pro-IL-1β and pro-IL-18 and release of active IL-1β and IL-18 (Schroder & Tschopp, 2010; Walsh et al., 2014). This is one of the mechanisms that best characterizes the pathway of cell death by pyroptosis (Fernandes-Alnemri et al., 2007; Miao et al., 2011), as IL-1β is a neuroinflammation cytokine that promotes reactive astrogliosis and increases BBB permeability reversibly (Shichita & Yoshimura, 2016) (See section 1.8.1 for more on pyroptosis).

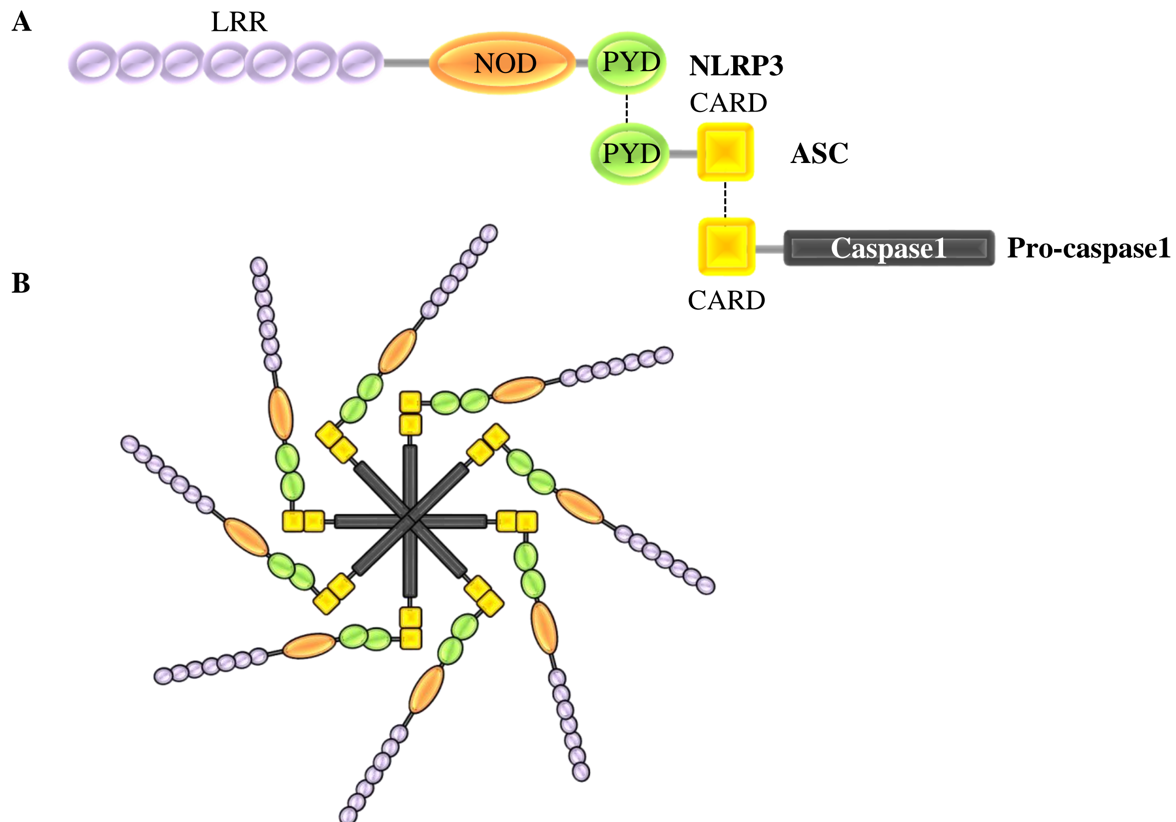


Figure 1.6.1 – The NLRP3 inflamassome components. (A) The NLRP3 (the PRR of the NLRP3 inflammasome) is constituted by LRR, NOD/NACHT and PYD domains. The ASC domain is constituted by PYD and CARD domains. NLRP3 and ASC are bond by PYD-PYD interactions. Procaspase1 is composed by an inactive caspase1 connected to a CARD domain. ASC and procaspase1 interact by CARD/CARD interactions **(B)** The heteromeric structure of the NLRP3 inflammasome

There are a few mechanisms that explain the gathering of the NLRP3 inflammasome components and its activation. The lysosomal rupture leads to cathepsin B release that binds to the NLRP3 and induces the proteolytic activation of the positive regulator of the NLRP3, resulting in the assembly of the inflammasome. This inflammasome can also be activated by the means of mitochondrial DNA (mtDNA) generation (Liu et al., 2013). The reduced activity of Na^+/K^+ ATPase decreases the gradient of ions across cell membranes, causing excitotoxic cellular response, resulting in neuronal death (Kanneganti et al., 2007; Meng et al., 2014; Singhal et al., 2014). Also, production of reactive oxygen species (ROS) from dysfunctional mitochondria and increased NF-κB signalling with aging could also potentiate the priming of NLRP3 inflammasomes in the brain resulting in an inflammatory response (Singhal et al., 2014). But one of the most important mechanisms for NLRP3 inflammasome

activation that supports a model, in which the innate immune system detects endogenous indicators of cellular danger or stress, is the activation by host-derived molecules (Schroder & Tschopp, 2010).

NLRP3 inflammasome is activated upon exposure to whole pathogens, as well as a number of structurally diverse pathogens associated molecular patterns (PAMPs), DAMPs, and environmental irritants (Schroder & Tschopp, 2010; Lamkanfi & Kanneganti, 2011). PAMPs are proteins derived from pathogens, such as bacteria or viruses. DAMPs are endogenous proteins that trigger an innate immune response when sensed by PRRs (De Rivero Vaccari et al., 2016; Shichita & Yoshimura 2016). Once these PAMPs or DAMPs are sensed, resulting in PRR activation, there is the production of inflammatory cytokines. In the case of NLRP3 inflammasome, the PRR is an NLR that form protein-protein interactions with procaspase1 and ASC. For caspase1 to be cleaved, all these proteins need to associate as a holoenzyme (Martinon et al., 2002). As mentioned before, active caspase1 has the catalytic activity necessary to process the proinflammatory cytokines pro-IL-1β and pro-IL-18 into their respective forms (De Rivero Vaccari et al., 2016).

Within the body, NLRP3 inflammasome has been shown to be expressed in lungs (Lee et al., 2015), kidney (Coll et al., 2015) and also CNS, where it is expressed at high levels by microglia (Hanamsagar et al., 2011; Liu et al., 2013). It has also been identified as a potential inflammatory target in CNS diseases, as atherosclerosis, AD, ALS, prion disease, both as key to the disease or involved in its pathogenesis (Masters, 2013) and also in major depression disorders (Alcocer-Gómez & Cordero, 2014). Astrocytes appear to lack NLRP3 expression, as they lack the ASC component (Gustin et al., 2015). However, there is no scientific consensus regarding this subject, given that astrocytes seem to incorporate the ASC component upon cell death via pyroptosis in AD (Heneka et al., 2013) and since NLRP3 was described in astrocytes in the SOD1-mouse model of ALS and in human sporadic ALS patients (Johann et al., 2015). Neurons seem to produce some inflammasome components, but in regard to NLRP3 some investigation is still needed. Nonetheless, cortical and pyramidal neurons, as well as oligodendrocytes, do exhibit some PRRs expression, mainly for NLRP1 and AIF2 inflammasome (Adamczak et al., 2014; Tan et al., 2015;).

As mentioned, inflammasome is proposed to regulate inflammation in several neurological diseases, but its role in epilepsy remains largely unknown. However, some groups have been able to disclose that IL-1β levels and NLRP3 inflammasome components levels dramatically increased at 3 hours after SE and NLRP3 or caspase1 knockdown decreased the levels of IL-1β and IL-18 at 12 hours after SE (Meng et al., 2014). This was accompanied by a significant suppression in the development and severity of spontaneous recurrent seizures (SRS) during the chronic epileptic phase. These findings suggest that NLRP3 may represent a potential therapeutic target for epileptogenesis, since its inhibition plays a neuroprotective role against neuroinflammation and neuron damages that follows SE (Meng et al., 2014; Zhou et al., 2016).

Inflammasome activity is also regulated through crosstalk between cellular stress-associated processes, such as autophagy. The induction of autophagy leads to the degradation of cellular substrates, such as protein aggregates and organelles, in autolysosomes for the recycling of metabolites (Yuk & Jo, 2013; Cadwell, 2016;). Strikingly, cells deficient in autophagy have a decreased threshold for NLRP3 inflammasome activation (Liu et al., 2013) (See section 1.8.2 for more on autophagy).

Recent studies have shown a potential role for the composite, N-[[(1, 2, 3, 5, 6, 7- hexahydro - sindacen - 4 - yl) amino] carbonyl] - 4 - (1- hydroxy - 1 - methylethyl) - 2 - furansulfonamide,

aiarylsulfonylurea-containing (MCC950) as a potential inflammasome inhibitor (Coll et al., 2015). Mostly, this compound prevents inflammasome oligomerization, an essential step for IL-1β processing (Coll et al., 2015). Hence, MCC950 is capable of inhibiting NLRP3 activation, when induced by PAMPs and DAMPs and can potentially be used in the treatment of various disorders that are bounded to that immunological response (Kugelberg, 2015; Moon et al., 2015). Moreover, MCC950 action does not seem to inhibit other inflammasomes types, such as NLRP1, NLRC4 or AIM2 (Netea & Joosten, 2015).

Similarly, it was found that ketone bodies β-hydroxybutyrate (BHB) inhibit NLRP3 inflammasome by preventing potassium efflux and reducing ASC oligomerization and speck formation (Youm et al., 2015). It is shown, in Figure 1.6.2, the molecular mechanisms of NLRP3 activation and the targets of BHB and of the pharmacologic inhibitor MCC950. As stated before, NLRP3 can be activated by ATP and lipotoxic fatty acids. BHB inhibits the potassium efflux that is essential for optimal inflammasome activation, while both BHB and MCC950 inhibit the ASC oligomerization and speck formation. In terms of mechanism, MCC950 inhibit both caspase1 and caspase11, while BHB only affects caspase1 activation (Netea & Joosten, 2015)

Taken together, MCC9950 and BHB open new approaches for the anti-inflammatory therapeutic armamentarium in which NLRP3-specific inhibition should have an important place (Netea & Joosten, 2015).

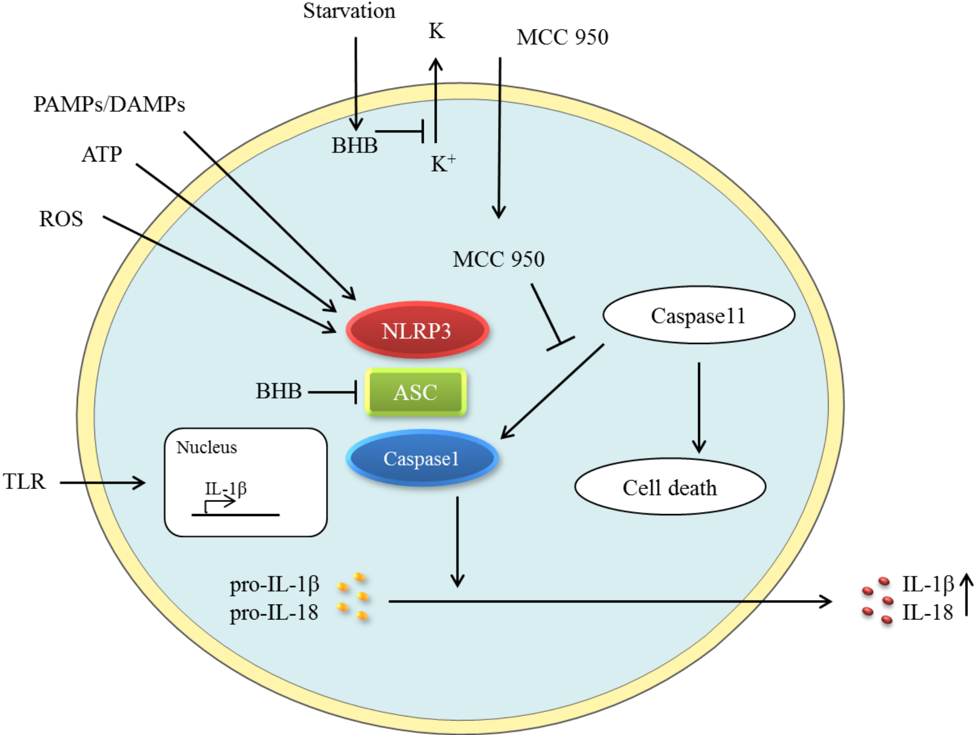


Figure 1.6.2 - NLRP3 activation and its inhibitors - MCC950 inhibit both caspase1 and caspase11, while BHB affects caspase1 activation and potassium efflux. Together, MCC950 and BHB inhibit ASC oligomerization and speck formation, essential for optimal inflammasome activation. Adapted from Netea & Joosten, 2015.

1.7 Cytokines

The role of inflammation has been widely studied in epilepsy, since there is a growing body of evidence that associates inflammation with the different types of epilepsy. This evidence correlates the inflammatory state in the epileptic brain with the increased permeability of the BBB, which leads to increased neuronal excitability (Vezzani et al., 2013; de Vries et al., 2016). Also, a clear link between inflammation, inflammasome and seizures was seen in patients with Rasmussen encephalitis (an inflammatory encephalopathy of unknown cause defined by seizures with progressive neurological disabilities), in which the inflammasome activation was associated with the increased cytokine production (Ramaswamy et al., 2013; de Vries et al., 2016)

Cytokines are generally synthesized and secreted in response to antigenic stimuli. Cytokines are soluble, potent glycoproteins involved in the regulation of growth, immune cell activation, and also inflammatory and immune responses (Medel-Matus et al., 2013). In CNS, they are secreted by glial cells (Olmos & Lladó, 2014) and have been shown to modulate neuronal activity by promoting the release of neuroactive molecules, such as NO, classical neurotransmitters and neurotrophins (Iori et al., 2016). They can be classified as proinflammatory (as IL-1β and TNF-α) and anti-inflammatory (as IL-4 and TGF-β1) (Vezzani et al., 2002; Dambach et al., 2014). Proinflammatory cytokines can activate receptor-mediated autocrine and paracrine cell signaling, resulting in different pathophysiological outcomes for different cell types. Several studies point to IL-1β, TNF-α and IL-6 as the main proinflammatory cytokines involved in seizure generation and propagation (Li et al., 2010; Pernot et al., 2011). These pathological consequences may ensue their overproduction, leading to prolonged tissue exposure to cytokines, a frequent scenery in neurodegenerative diseases and in epilepsy (Vezzani et al., 2011).

Abnormalities in the expression of cytokines and immune cells have been observed in epilepsy patients and in animal models of epilepsy. Moreover, the immune system and its associated inflammatory reactions seem to play an important role in epileptogenesis (Li et al., 2011). Indeed, data has implicated cytokines as mediators of spontaneous seizures (Vezzani et al., 2002; Vezzani et al., 2008). The presence of inflammatory mediators in the brain, which are capable of generating spontaneous seizures is a direct consequence to symptomatic pharmacoresistant epilepsies (Aronica & Crino, 2011). This happens because inflammation represents a common substrate for drug-resistant epilepsy and it can directly affect neuronal excitability (Vezzani et al., 2013).

The rapid release of cytokines activates NF-κB dependent downstream inflammatory cascades, which involves glia, neurons and the BBB, and may lead to brain extravasation of leukocytes (Vezzani et al., 2013).

1.7.1 IL-1β

The proinflammatory cytokine IL-1β is included in the IL-1 family, whose biological effects are exerted by interacting with IL-1 receptor type I (IL-1RI). Due to its three-dimensional structure, this receiver is included in the superfamily of receptors type immunoglobulins (Medel-Matus et al., 2013). IL-1 cytokines are constitutively expressed at very low levels in the human CNS (Dey et al., 2016). However, when brain injuries, like hypoxia or seizures occur, the expression of IL-1 cytokines is enhanced (Youn et al., 2013). This cytokine has been seen to induce the production of other cytokines, as well as growth factors (Rivera et al., 1994) and changes in the blood flow in the nervous system (Maher et al., 2003). IL-1β is associated with neuronal death in pathological conditions, such as

ischemia, hypoglycaemia and stroke (Medel-Matus et al., 2013), but its role in seizure-induced neuronal death is yet to be clarified.

The biological function of the IL-1 family, IL-1α and IL-1β, are mediated through the binding to its receptor (IL-1RI) in association with the receptor-accessory protein (IL-1RAcP). This complex has the ability to initiate an intracellular signal (Iori et al., 2016). Both IL-1α and IL-1β are synthesized as 31kDa precursor proteins (Boutin et al., 2003). Pro-IL-1α appears to be biologically active upon binding to IL-1R1, when it is cleaved to a 17 KDa mature form by proteases like calpain (Carruth et al., 1991). In dissimilarity, pro-IL-1β is inactive and requires enzymatic cleavage *via* caspase1 to become a 17 KDa biologically active protein. This precursor cleavage can occur in a specialized secretory lysosome or in the cytosol, giving rise to mature IL-1β able to activate signalling pathways (Chiavegato et al., 2014). IL-1β is the most studied cytokine in neuroinflammation and since it appears to have a more important role than IL-1α, this study will focus in IL-1β.

One of the most crucial signalling pathway for inflammatory response in tissue is the toll-like receptor (TLR) signalling (Iori et al., 2016). This pathway that be trigger by PAMPs, during infections, or by DAMPs, during sterile inflammation, and which functions to alert the microenvironment for imminent or on-going tissue damage (Iori et al., 2016). Both IL-1R1 and TLR4, and its ligands IL-1β and high mobility group box-1 (HMGB1) respectively, are induced in glia and neurons in experimental models of epilepsy (Vezzani et al., 2012; Vezzani et al., 2016). Interestingly, IL-1β and HMGB1 are strictly interconnected and both play a role in the biosynthesis of NLRP3 inflammasome/caspase1 and also activate NF-κB-dependent gene transcription in neurons and glia (Iori et al., 2016). Astrocytes release both IL-1β and HMGB1 (Wetherington et al., 2008; Pernot et al., 2011).

This IL-1R1/TLR4 particular signalling pathway seems to have a major role in epilepsy, since it promotes seizure onset and recurrence and its pharmacological blockade drastically reduces seizure activity (Maroso et al., 2011). This indicates that neuronal excitability is affected by both IL-1β/HMGB1. At a molecular mechanism level, the IL-1R1/TLR4 activation is capable to induce, within minutes, the Src kinase-mediated phosphorylation of the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor complex, which leads to a calcium influx (Iori et al., 2016). This pro-convulsant effect is attributed to IL-1β-mediated engagement of Src-family kinases in hippocampal neurons (Dingledine, 2014). Also, this signalling pathway may also contribute to the pathophysiology of epilepsy by increasing neuronal hyper excitability *via* blocking astrocyte-mediated re-uptake of glutamate from the synaptic space, by altering GABAergic neurotransmission and by modulating voltage-gated ion channels (Vezzani & Viviani, 2015; Dey et al., 2016)

As a proinflammatory cytokine, IL-1β can affect the BBB permeability properties, as it disrupts the tight-junction organization and the production of NO, as well as the activation of matrix metalloproteinases in endothelial cells (Vezzani et al., 2008). Also, this cytokine shows a rapid increased expression in activated glial cells during acute seizures, that do not reverse and return to basal levels after seizure decrease (Youn et al., 2013). It is also been shown that in hippocampus, the signal pathway induced by IL-1β leads to an enhancement of neuronal excitability, and a decrease in seizure threshold (Gatti et al., 2002). Hippocampal application of IL-1β can increase seizure intensity by threefold (Dingledine, 2014). Moreover, excitability is also induced by IL-1β's ability to inhibit glutamate reuptake by astrocytes and to enhance its glial release through TNF-α induction, leading to a raise in glutamate levels (Viviani et al., 2003). In addition, IL-1β prevents GABA-mediated chloride fluxes in area CA3 contributing to hyper excitability by reducing the inhibitory transmission (Wang, 2000; Dingledine, 2014). Therefore, IL-1β is classified as a pro-convulsive molecule, while inhibitors

of this cytokine are considered powerful anticonvulsants (Vezzani et al., 2010). Taken together, pharmacological treatments targeting IL-1β or its activation result in robust anticonvulsant effects, which indicate that inflammation plays an important role in epileptogenesis and it is a viable therapeutic target in this disorder (Krook-Magnuson et al., 2014).

IL-1β is of particular relevance for pyroptosis, since its maturation is a caspase1 dependent process. Caspase1 activation results not only in the production of activated inflammatory cytokines, but also in rapid cell death characterized by plasma-membrane rupture and release of proinflammatory intracellular contents (Fink & Cookson, 2005; Miao et al., 2011) (See 1.8.2 for more on pyroptosis).

1.7.2 TNF-α

The effect of IL-1β may involve other cytokines as well. It is known that IL-1β induces the synthesis of IL-6 and TNF-α in astrocytes and microglia (Vezzani et al., 2002). In particular, TNF-α is a pleiotropic cytokine that plays pivotal roles in immunity, cell proliferation, differentiation and cell death through the activation of several downstream signalling cascades (Cabal-Hierro & Lazo, 2012; Olmos & Lladó, 2014).

This cytokine is synthesized as a 26 KDa transmembrane protein precursor (mTNF) and is cleaved into a 17 KDa mature and soluble form (sTNF) (Gaur & Aggarwal 2003). TNF-α biological effect depends on the interaction with its transmembrane receptors, manly TNF receptor type I (TNFR1 or p55) and type II (TNFR2 or p75) (Li et al., 2011; Cabal-Hierro & Lazo, 2012). P55 appears to be expressed in the majority of CNS cell types and is activated by both mTNF and sTNF, whereas p75 is mainly expressed in microglia cells and it is only activated by mTNF (Dempsey et al., 2003).

In CNS, TNF-α can activate its two receptors, p55 and p75, and modulate cell-signalling pathways. The p55 receptor has been implicated in the activation of programmed cell death, and both receptors are associated with activation of the NF-κB system, a protein complex that controls transcription of DNA, cytokine production and cell survival (Li et al., 2011; Cabal-Hierro & Lazo, 2012). The p55 receptor can induce cell death signalling, since it has a functional death domain that lacks in the p75 receptor (Vezzani & Granata, 2005). This TNF-α/p55 complex is able to recruit several members of the caspase family, associated to cell death proteases, such as caspase8 and caspase10, that will cleave the effector caspase3 (Engel & Henshall, 2009). The TNF-α/p75 complex seems to signal for anti-apoptotic reactions, leading to the production of neuroprotective and anticonvulsant factors (Vezzani & Granata, 2005). In summary, p55 has been reported to mediate ictogenic effects of TNF-α, whereas p75 mediates the neuroprotective and neuromodulatory actions of this cytokine (Iori et al., 2016).

Astrocytes and neurons are able to produce TNF-α, but it is assumed that microglia is the major source of this cytokine during neuroinflammation (Olmos & Lladó, 2014). In microglia, TNF-α induces glutamate release by increasing glutaminase conversion of glutamine to glutamate. Astrocytic TNF-α stimulates release of glutamate that results in increased intracellular calcium mobilization (Iori et al., 2016).

TNF-α is a crucial proinflammatory cytokine that belongs to the TNF superfamily of ligands, that are known to be involved in the activation, differentiation, proliferation, and infiltration of immune cells into the CNS during systemic inflammation (Dempsey et al., 2003). As such, TNF-α has been shown to have a profound impact on circuit homeostasis in a manner that can promote seizures, by sponsoring the recruitment of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

receptors to postsynaptic membranes and cause endocytosis of $GABA_A$ receptors from the cellular surface, decreasing inhibitory synaptic strength (Dingledine, 2014). Also, the activation of protein kinases mediates TNF-α, as well as IL-1β, modification in the function of both receptor-gated and voltage-gated ion channels in neurons (Iori et al., 2016). Moreover, TNF-α was reported to affect seizure susceptibility in animal models, and that susceptibility can be showed by the pharmacological interventions that mimic cytokine action or block either p55/p75 receptor signalling (Iori et al., 2016). Also, this proinflammatory cytokine, is increased in hippocampal slices and is released by activated microglia inhibiting long term potentiation (LTP) (Blank & Prinz, 2013).

1.8 Cell death

Homeostasis in multicellular organisms is maintained by a balance between cell proliferation and cell death (Vanden Berghe et al., 2013). During vertebrate development, up to 50% of many types of neurons undergo cell death (Marín-Teva et al., 2011) and the fate of this dying/dead cells is mostly engulfment by phagocytes, regardless of the molecular mechanisms behind cell death (Vanden Berghe et al., 2013). The excessive or prolonged activation of receptor for excitatory amino acids that leads to neuronal death is called excitotoxicity. This excitotoxicity has been proposed to be present in the aetiology or progression of many human acute or chronic neurodegenerative diseases (Olmos & Lladó, 2014).

For a cell to be considered dead, it has to display some molecular and morphological criteria, such as loss of plasma membrane integrity, cell fragmentation and engulfment by adjacent cells (Kroemer et al., 2009). Also, it has to display certain point-of-non-return for its definition, like the massive activation of caspases, mitochondrial transmembrane and mitochondrial membrane permeabilization (Kroemer et al., 2009). Programmed cell death (PCD) is a key process in the proper development of the nervous system. The number and types of cells in the developing brain and spinal cord is regulated by PCD (Fujikawa, 2010). It also plays a key role in constructing an efficient neuronal network and, under pathological conditions, is co-responsible for the loss of neurons associated with neurodegenerative diseases (Ghavami et al., 2014).

Before enrolling in the characterization of each type of cell death, there is a need to clarify how seizure-induced neuronal injury promotes epileptogenesis. There are two possible mechanisms. First, the *recapitulation of development hypothesis*, that states that maladaptive new circuits among neurons can form to replace synapses loss during neuronal death. This mechanism involves axonal sprouting within excitatory pathways and it is amplified by the loss of inhibitory interneurons (Olmos & Lladó, 2014). The second mechanism states that molecular signals from upstream pathways and not only neuronal death *per se*, mediate the newly recognized forms of cell death that might underlie or contribute to epileptogenesis. This second hypothesis is called *neuronal* death pathway (Krook-Magnuson et al., 2014).

To characterize the cell death pathways present in the *ex vivo* model of epilepsy used in this study, the activation of the inflammasome pathways was taken into consideration, given its role as an activator of caspase1 that leads to active IL-1β and IL18. For clarity, this study classifies cell-death processes as non-inflammatory (apoptosis, autophagy, phagoptosis) and inflammatory (necrosis, necroptosis, pyroptosis) (Fink & Cookson, 2005), following the classification proposed by the Nomenclature Committee on Cell Death. From the ones mentioned below, only necrosis is not a PCD.

1.8.1 Pyroptosis

Pyroptosis is the most extreme example of inflammation-related cell death. It is a type of programmed cell death accompanied by swelling and rupture of the cells with the extrusion of cellular contents, including cytokines, into the extracellular space (Bergsbaken et al., 2010). Pyroptosis differs from apoptosis and oncosis/necrosis since it does not depend on effector caspases; is inhibited by selective caspase1 inhibitor; results in 1-2 nm pores in the plasma membrane, causing cell lysis and release of inflammatory components. The effects of pyroptotic cell death are mainly due to the maturation and release of IL-1β (Adamczak et al., 2014).

The primary distinguishing feature of pyroptosis is the formation of the inflammasome, an intracellular multimolecular complex that is required for the activation of inflammatory caspases, particularly caspase1 (Fink & Cookson, 2005).

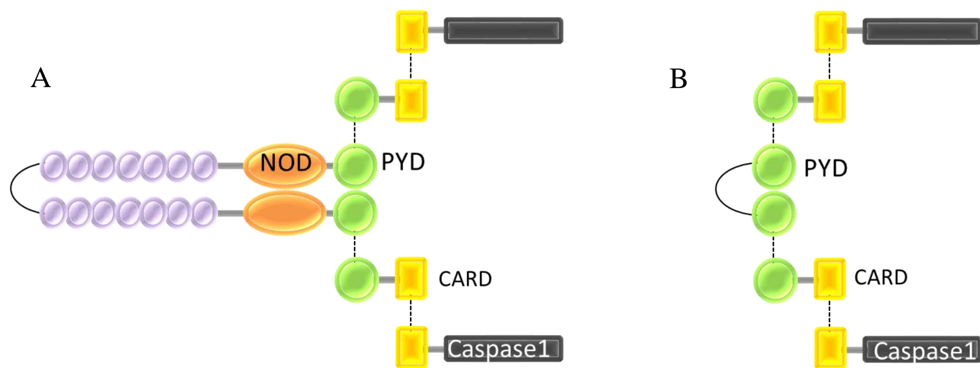


Figure 1.8.1 - Schematic representation of NLRP3 inflammasome and the pyroptosome. A) The NLRP3 inflammasome, with the NLRP3, ASC and procaspase1; **B)** Pyroptosome with ASC and procaspase1

Not only NLRP3 inflammasome activation can lead to pyroptotic cell death. The NACHT leucine-rich-repeat protein (NLRP1) inflammasome and the absent in melanoma 2 (AIM2) inflammasome can also activate this pathway, both inducing cleavage of procaspase1 and maturation of IL-1β (Denes et al., 2015; Tan et al., 2015). NLRP1 has been proposed to have a crucial role in neurological diseases and has already been proved to be activated in patients with MTLE (Tan et al., 2015). AIM2 is activated by viral, bacterial and host ectopic double stranded DNA (Denes et al., 2015).

Another multiprotein may also originate pyroptotic cell death. This complex, named pyroptosome is a supermolecular assembly of the 22 KDa protein ASC, which suffers oligomerization due to sub-physiological concentrations of potassium (Fernandes-Alnemri et al., 2007) (Figure 1.8.1). Macrophages seem to be the most affected cells, since its pyroptosis is mediated by a unique pyroptosome.

Upon activation, the NLRP3 inflammasome, and/or the pyroptosome, located in microglia and astrocytes triggers the maturation of IL-1β and IL-18 and induces pyroptotic death. The high levels of these cytokines bind to their receptor on glial cells, macrophages, neurons and endothelial cells to initiate Th-cell signalling, along with other cytokines. This initiation of Th-cell signalling triggers a complex spectrum of events that result in the exacerbation of inflammatory cascade responses within the CNS (Song et al., 2017).

The morphological features of this particular form of cell death are a combination of apoptosis and necrosis, since it exhibits condensation of the nucleus and loss of mitochondrial membrane activity, as well as plasma membrane swelling (Fernandes-Alnemri et al., 2007; Song et al., 2017). Moreover, there is also the release of proinflammatory cytokines which may aggravate inflammatory mediator-induced death, as they mediate the recruitment of other immune cells, like leucocytes, to the inflammation sites and the subsequent inflammatory response causes severe tissue damage in CNS under neuropathological conditions (Uludag et al., 2015). Also, pyroptotic cells are reported to undergo lysosomal exocytosis to release lysosomal antimicrobial peptides that facilitate the clearance of pathogens by recruiting neutrophils (Boucher et al., 2016).

Despite their similarities, there are important hallmarks that differ between apoptosis and pyroptosis and are of vital important to distinguish between both processes. Those differences can be accessed by the presence of caspase1, since it is not involved in apoptosis and pyroptosis is highly dependent on this molecule (Miao et al., 2011). Mitochondrial release of cytochrome-c, a hallmark of apoptosis, does not occur during pyroptosis, and in pyroptosis there isn't a coordinated packaging of intracellular components as observed in apoptosis. Instead, in pyroptosis occurs cellular lysis and release of inflammatory effector molecules (Krysko et al., 2008; Dingledine, 2014).

1.8.2 Autophagy

Although autophagy usually serves a protective role, as it is essential for cell survival and maintenance of homeostasis, in extreme stress conditions it can contribute to cell death (Wong, 2013). Autophagy, literally a term for "self-eating", is an essential homeostatic process, in which cytoplasmic material (macromolecules and organelles) is delivered to lysosomes for degradation as an adaptation to exogenous disruption (Su et al., 2016). There are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (Su et al., 2016). From here on, this study will address macroautophagy as a general overall autophagy.

This mechanism for regulating cell death or cell survival has acquire huge interest as a response to cell starvation and as a mechanism involved in tumorigenesis, neurodegeneration and non-caspase-linked form of death (Gimenéz-Xavier et al., 2008). Autophagic cell death (ACD) can be defined on the following criteria: i) ACD has to be a distinct mechanism, independent of apoptosis or necrosis, so situations in which autophagy triggers apoptosis or necrosis, or occurs in parallel with them, are excluded; ii) increases autophagic flux, and not just autophagic markers, in the dying cells; iii) pharmacological inhibitors or genetic approaches that suppress autophagy can rescue or prevent cell death; iv) autophagy must be responsible by itself for the final dismantling of cellular content and execute a lethal pathway (Shen et al., 2012; Ghavami et al., 2014). As biochemical features, this pathway displays beclin-1 dissociation of the Bcl-family, dependency on *atg* gene products to autophagosome formation and LC3I to LC3II conversion (Kroemer et al., 2009).

Autophagy is a procedure that features degradation of cellular components within the dying cell in autophagic vacuoles. Also, it selectively degrades catalase, leading to the accumulation of ROS and non-apoptotic death of macrophages (Ghavami et al., 2014). The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation (Fink & Cookson, 2005). The autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosome (Jaeger & Wyss-Coray, 2009). The autophagosome then fuses with intracellular lysosomes to facilitate degradation of the contents within the autophagosome by acid hydrolases. In contrast to apoptosis, caspase activation is

not required and chromatin condensation is minor (Krook-Magnuson et al., 2014). But, in similar fashion to apoptosis, autophagic pathways also progress in a series of cellular steps that involve programmed degradation of cellular components (Krook-Magnuson et al., 2014). Furthermore the resulting cell death can be either apoptotic (type I cell death) or autophagic (type II cell death), depending on the cellular setting and inducing stressor. Evidences, from mice deficient in apoptosis, place autophagy as an alternative cell death mechanism (Jaeger & Wyss-Coray, 2009).

Autophagy in the CNS can be protective under some circumstances, and lethal in others. The beneficial role is mainly associated with the maintenance of the normal balance between the formation and degradation of cellular proteins, whereas its defects have been linked to neurodegenerative diseases (Shen et al., 2012; Ghavami et al., 2014). On neurodegenerative disorders, autophagy impairment means more autophagosome formation, less efficient cargo recognition and transportation, fusion between autophagosome and lysosome that buildup on damaged organelles. The differences in the location of defects within the autophagy pathway and their molecular basis influence the pattern and pace of neuronal cell death in the various neurological disorders (Shen et al., 2012; Ghavami et al., 2014). For instance, deletion of specific autophagy-related genes in the brain may lead to axonal degeneration, meaning that this cell death pathway has a crucial role in the conservation and regulation of neuronal integrity and function (Lee et al. 2016).

In the CNS, autophagy also balances the beneficial and detrimental effect of immunity and inflammation, thereby it may protect against infectious, autoimmune and inflammatory diseases (Su et al., 2016). The interest on this mechanism in epilepsy emerged from the evidence that rapamycin, a powerful autophagy inducer, strongly modulates various seizure models and epilepsies (Giorgi et al., 2015). One of the key regulators of autophagy is the pathway controlled by the mammalian target of rapamycin (mTOR), a protein complex with a central role in different and critical cell signaling pathways. Activation of mTOR in certain cellular context results in autophagy inhibition (Romá-Mateo, 2012). This disruption of autophagy and accumulation of useless cellular components leads to cell death and it is sufficient for the generation of epilepsy (Romá-Mateo, 2012). Moreover, this mTOR pathway has been observed in different types of epilepsy and implicated in both genetic and acquired epilepsy (Gan et al., 2015).

However, it is still to be fully understood if autophagy is sufficient to execute death without help from apoptosis or necrosis, since autophagosome proliferation occurs in the context of cell death and it is executed by caspases. This may facilitate execution but does not necessarily mean cell death (Ghavami et al., 2014).

1.8.3 Necrosis

Necrosis has been characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents (Fink & Cookson, 2005). Biochemically, there are no caspases involved and, morphologically, condensation or digestion of internal cellular compartments is not observed (Krook-Magnuson et al., 2014). Necrosis deletes groups or clusters of cells and is characterized by a random breakdown of cellular constituents due to energy failure (Bengzon et al., 2002). This leads to a rapid cytoplasmic swelling (also referred as oncosis) and culminates in plasma membrane rupture and organelle breakdown (Vanden Berghe et al., 2013). Swelling and lysis of the cell is often followed by an inflammatory reaction and injury to the surrounding tissue because there is effectively a spilling of the internal contents of the dying cells into

the interstitial fluid and the release of molecules that can initiate inflammatory cascades (Dingledine, 2014).

Necrotic cell death is typically initiated by extreme physiological stress or trauma that kills cells quickly (Dingledine, 2014). This extreme physicochemical stress can be heat, osmotic shock, and mechanical stress, freeze-thawing and high concentration of hydrogen peroxide. As such, cell death occurs quickly due to the direct impact of the stress on the cell, hence this cell death process has been described as accidental and uncontrolled (Vanden Berghe et al., 2013). But this necrotic process can also occur and be induced by a set of signal transduction pathways and catabolic mechanisms, as for instance death domain receptor like TNFR1/p55 and TLR4 (Kroemer et al., 2009). This particular induction by different cellular stimuli results from extensive crosstalk between several biochemical and molecular events on various cellular levels, and it is as controlled and programmed as apoptosis, earning the name necroptosis (Galluzzi et al., 2014). This is a tricky issue if one wants to find a clear biochemical definition of necrotic cell death and positive biochemical markers that enable the unambiguously discrimination between necrosis and apoptosis (Golstein & Kroemer, 2007).

At the biochemical level, necrosis exhibits activation of calpains and cathepsins, drop of ATP levels, HMGB1 release, lysosomal membrane permeabilization, plasma membrane rupture due to rapid cytoplasmic swelling and ROS overgeneration (Krysko et al., 2008; Kroemer et al., 2009). In what epilepsy is concerned, seizure-induced neuronal death culminates in necrosis due to excitotoxic glutamatergic neurotransmission gating excessive calcium and sodium entry. This leads to osmolytic stress and cell swelling and rupture, with free radical production damaging DNA and protease activation that leads to cell and organelle membranes proteolysis (Henshall, 2007). Besides epilepsy, necrosis is also involved in other neurodegenerative diseases such as AD, PD and ALS (Fujikawa, 2010).

1.8.4 Apoptosis

The term apoptosis was proposed by Kerr and colleagues in 1972, referring to a controlled, programmed process of packaging internal components of the cell for clearance by phagocytes (Kerr et al., 1972; Bengzon et al., 2002). As such, intracellular molecules with the potential to activate immune responses are disposed of rapidly, without initiating an immune response (Krook-Magnuson et al., 2014).

Caspases have central functions in mammalian cell apoptosis (Ghavami et al., 2014). Caspases are evolutionarily conserved cysteine proteases that cleave specific substrate proteins downstream of aspartate residues (Kang et al., 2013). Caspases are classified into two different groups, namely initiator and effector caspases (Ghavami et al., 2014). Caspase8 and caspase9 are known as initiator caspases, as they are activated by external cell death triggering molecules and internal stress, as starvation and cellular dysfunction (Fink & Cookson, 2005). The initiator caspases mainly lead to mitochondrial release of cytochrome c, that triggers the formation of apoptotic bodies (Fink & Cookson, 2005).

These initiator caspases are activated through interaction of the N-terminal prodomain with an upstream receptor complex, after permeabilization of the mitochondrial outer membrane by proapoptotic members of the Bcl-family (Green, 2005; Engel & Henshall, 2009). But, for such activation to lead to cell death, the initiator caspases must cleave and activate the effector caspases

(like caspase3, caspase6 and caspase7) that lack prodomains. The trigger for the apoptotic program is thus the formation of the active effector caspases (Kang et al., 2013).

Apoptosis occurs naturally during development and serves as a means to facilitate cellular turnover in healthy tissue, and also in response to hormone withdrawal. The degraded cellular corpse is then packaged in preparation for phagocytosis by macrophages or microglia (Dingledine, 2014). This particular cell death pathway is characterized by chromatin and cytoplasmic condensation, plasma membrane leaking, formation of apoptotic bodies, as well as fragmentation of cellular compartments and DNA (Vanden Berghe et al., 2013; Dingledine, 2014;). As biochemical features, apoptosis leads to the activation of proapoptotic Bcl-2 family, consequent activation of caspases, mitochondrial transmembrane permeabilization and mitochondrial membrane permeabilization, oligonucleossomal DNA fragmentation, plasma membrane rupture, ROS over generation and single-stranded DNA accumulation (Henshall & Simon, 2005; Kroemer et al., 2009).

Besides de classical apoptosis cell death pathways, seizures also activate caspase-independent apoptosis pathways in the brain. Here, calpains are thought to be critical. Calpain is viewed as a critical coordinator of calcium-dependent signaling pathways underlying neuronal death (Vosler et al. 2008). This protein has been shown to be involved in necrotic and apoptotic cell death (Wang, 2000), since it has the ability to cleave αII-Spectrin, a structural protein of the cell membrane cytoskeleton, into a spectrin breakdown product of 145 KDa (SBDP 145) that characterizes necrosis, or into a 150 KDa fragment (SBDP 150) that can be further cleaved by caspase3 into a 120 KDa fragment (SPDP 120), that will characterize apoptosis (Czogalla & Sikorski, 2005).

Calpain inhibitors have proven to be effective neuroprotectants in models of SE (Wang et al., 2008). Recently, calpain I (μ-calpain) was shown to be responsible for triggering apoptosis inducing factor (AIF), which is a mitochondrial flavoprotein oxidoreductase, release during excitotoxicity. AIF is released from mitochondria following apoptotic signals and translocates to the nucleus, where it induces chromatin condensation (Ghavami et al., 2014). Thus, calpain and AIF may be particularly important for neuronal death in seizure models in which caspases are not activated (Wang et al., 2008; Bozzi et al., 2011).

1.9 OHSC as a model for epileptogenesis

To study epilepsy, a variety of animal models have been developed and each demonstrates different hallmarks of epilepsy, being more appropriate for some studies than for others. Epileptogenesis progression can be explored using models, such as kindling, post-SE with spontaneous recurrent seizure, traumatic brain injury (TBI)-induced epilepsy, stroke-induced epilepsy and febrile seizure models (Yin et al., 2013).

Kindling is a model of chronic seizures induced by repeated excitatory stimuli (being it electrical or chemical) that ultimately induces seizures by progressively intensifying brain excitability (Löscher, 2011). This model leads to neuronal loss in hippocampus CA1 and CA3 and is widely used in TLE since the fully kindled seizures resemble the complex partial seizures and secondarily generalized seizures (Melvin & Hardison, 2014). Post-SE model with SRS and chemically induced models, such as pilocarpine or kainite, are also often used (Löscher & Brandt, 2010). Pilocarpine and SE are known to induced neuroinflammatory responses *per se* (Vezzani & Granata, 2005; Brandt et al., 2016). The spontaneous recurrent, partial and secondarily generalized seizures, damage to hippocampus by

pilocarpine and kainate models resemble clinical characteristics of TLE, hence being representative of this disorder (Löscher, 2016). These models were mostly developed in mice and rats (Yin et al., 2013).

Organotypic hippocampal slice cultures (OHSC) became progressively more useful and advantageous for the study of several neurobiological disorders, including epilepsy and neuroinflammation (Thompson et al., 2006; Bernardino et al., 2008). Organotypic slices reproduce the intrinsic properties and the complex *in vivo* organization of cell network between neurons, astrocytes and microglia, and preserve tissue-specific cell connections, local functional circuitry and morphological architecture (Noraberg et al. 2005; Su et al., 2011). Also, cells in organotypic slices mature similarly to their *in vivo* counterparts (Gilbride, 2016). Moreover, unlike acute slices, organotypic slices can be maintained *in vitro* for weeks in an artificial growth medium (Lindroos et al., 2005) allowing to follow epileptogenesis over time.

There are two methods used to prepare slice cultures: the roller tube (Gahwiler, 1981) and the membrane interface technique (Stoppini et al., 1991). The last method is considered to be the most reliable one, with thin slices 1 to 4 cell layers thick, well preserved organization and with pyramidal neurons that resemble the organization and complexity of their dendritic processes as observed *in situ* (Gilbride, 2016). This interface method also allows the slices to stay at a boundary between the medium and the air, letting oxygen diffuse into the slice, maintaining tissue viability (Heinemann et al., 2006). Also, excitatory and inhibitory synaptic potentials can me analyzed using recording techniques (Ziobro et al., 2011).

The OHSC cultures are usually prepared from 6 to 7 day-old pups (P6-7), an age where the hippocampal cytoarchitecture is already established and can be studied after 7 to 30 days *in vitro* (DIV) (De Simoni & Yu, 2006). As the cultures are prepared from postnatal rats, they do not represent adult tissue. This so happens because, in neonatal brain tissue, dendritic trees and axons of granule cells are still under proliferation and pyramidal cells and interneurons are still in undergoing mitosis (Tau & Peterson, 2010; Masiulis et al., 2011). Additionally, GABA and glycine receptors act as excitatory, in the immature brain (Tau & Peterson, 2010; Masiulis et al., 2011). Nonetheless, in 21 DIV slices the maturation of the different cell types, receptor expression and synaptic contacts resemble the ones *in situ* (Albus et al., 2008).

The OHSC, as a model of epileptogenesis, represents a profound trauma with an effect on the survival of neurons. In slices, neuron degeneration starts after four hours of culturing and continues up to 6 DIV, being followed by a low-level of neuronal death that persists up to 28 DIV (Pozzo Miller et al., 1994). Also, there is a substantial reorganization of glia, mainly in microglial cells which respond rapidly to the cultured trauma, suffering changes in morphology, function and proliferation. The trauma caused by the massive deafferentation and deefferentation, which occurs during tissue slicing, activates M1 type of microglia as early as after two hours *in vitro*, with microglia displaying an amoeboid shape until 6 DIV. This facilitates phagocytosis of damaged cells and cellular debris (Stence et al., 2001). Between 7 and 14 DIV, the majority of microglia regain their ramified phenotype (Hailer et al., 1997; Harry & Kraft, 2009). As for astrocytes, the scar layer is formed at the top and bottom of the slices by 3 DIV and completely covers it by 7 DIV, lasting up to 21 DIV (Miller et al., 2015; Colombo & Farina, 2016). These alterations contribute to a progressively increase of excitatory activity, during culture time (Nguyen et al., 2002; Lindroos et al., 2005). Therefore, OHSC are considered a model of epileptogenesis, since the development of abnormal connectivity in the organotypic slices mimics the aberrant connectivity in epileptic brain (Pitkänen et al., 2005).

Although there are similar morphological features, spontaneous epileptiform activity without pharmacological induction has rarely been reported in OHSC. Recently, a functional characterization of spontaneous development and evolution of epileptiform activity within OHSC, which were maintained in an artificial serum-free growth medium, was described (Dyhrfjeld-Johnsen et al., 2010; Berdichevsky et al., 2012; Magalhães et al., 2017). Recordings of OSHC from 7 to 30 DIV exhibit a high incidence of spontaneous epileptiform activity and presented features related to TLE (Wong, 2011). Also, an initial peak of cell death was described to be associated with the culture trauma, while the second one, described at 7-10 DIV, coinciding with the onset of seizure-like activity, in a consequence of spontaneous, post-traumatic epileptiform discharges (Berdichevsky et al., 2012). The seizure-like activity, cell death and sprouting could be prevented by several treatments and still not lead to epileptogenesis inhibition (Berdichevsky et al., 2012). Furthermore, brain extravasation of serum due to BBB damage was seen to increase excitability leading to epileptogenesis (Devinsky et al., 2013).

These findings of spontaneous epileptiform activity in OHSC supported the idea that these slices maintained in serum-free medium could serve as an *in vitro* model of post traumatic epilepsy (Albus et al., 2013).

2 AIMS

Research directed toward the therapeutic benefits of anti-inflammatory agents is now considered crucial on the on-going search for novel antiepileptic drugs. Targeting inflammatory pathways is regarded as a novel option for the development of biomarkers and therapies for epilepsy. Thus, a full knowledge about the major types of cell death, including neuroinflammation-induced pyroptosis, which may play a role in epileptogenesis is imperative.

In this work, the organotypic hippocampal slice model of epileptogenesis will be used to assess several mechanisms of cell death throughout the course of epileptogenesis. The goal is to understand whether pyroptosis is a major contributor for this condition. Markers of pyroptosis (NLRP3 and ASC), autophagy (LC3II and LC3I), necrosis (αII-spectrin and SBDP) and apoptosis (caspase3), will be assessed. Inflammatory cytokines (mainly TNFα, IL-1β) will also be quantified. Finally, the activation of CNS immune cells (astrocytes and microglia) will be evaluated throughout culture time.

3 METHODS

3.1 Animals

Sprague-Dawley rats were obtained from Charles River (Barcelona, Spain). All the procedures were performed according to the European Union guidelines (2010/63/EY) and Portuguese law concerning the protection of animals for scientific purposes. All efforts were made to minimize animal suffering and to use the minimum number of animals.

3.2 Organotypic Hippocampal Slice Culture

In this work, OHSC were prepared from 6- to 7- days-old Sprague-Dawley rats, according to the interface culture method (Stoppini et al., 1991). Rats were decapitated, brains were removed and placed (under sterile conditions) in cold Gey's balanced salt solution (GBSS) with 25 mM glucose.

Combined entorhinal cortex-hippocampi slices were dissected out and sliced transversely at a thickness of 350μm using a McIlwain tissue chopper. Five slices were placed onto porous (0.4 μm) insert membranes (PICM 03050 Millipore, Bedford, MA) and transferred to six-well culture plates (Corning Costar, Corning, NY). Each well was filled with 1mL of medium, containing 50% Opti-MEM, 25% Hank's balanced salt solution, 25% heat inactivated horse serum, 25 mM glucose and 30 μg/mL gentamycin (Figure 3.2).

Slices were randomly divided in two groups, which undertook different culture conditions. Those designated control slices (CTL) were kept in a serum-based Opti-MEM medium, with medium renewal twice a week, and did not develop epileptiform activity. Those designated epileptic-like slices (EL), which spontaneously develop an epileptic phenotype, were changed at 3 days in vitro (DIV) to a chemically defined serum-free based medium, Neurobasal A (NBA), supplemented with 2% B27 (which contains several hormones, fatty acids and free radical scavengers), L-glutamine (1mM), 30 μg/mL gentamycin, and decreasing horse-serum concentrations (15%, 10% and 5%), until a serum-free condition was reached at 9 DIV. In EL slices, medium was renewed every second day. This protocol is different from other reports in the sense that slices are gradually deprived of serum.

Slices were maintained at 37ºC with 5% CO_2 and 95% atmospheric air for the following 3 weeks. Samples were collected at 0, 3, 7, 10, 14, 17 and 21 DIV.

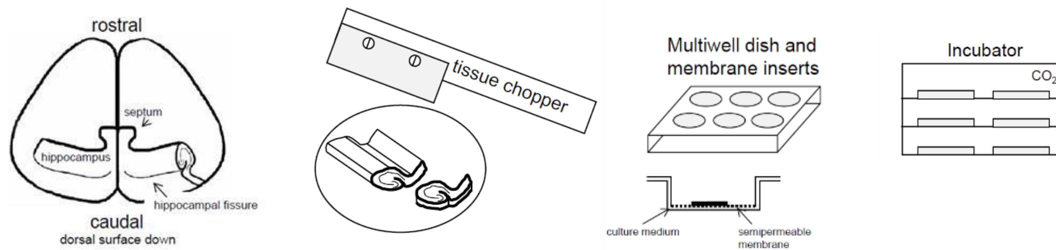


Figure 3.2 - Preparation of organotypic hippocampal slice cultures. The hippocampus and part of the entorhinal cortex are dissected out from the neonatal brain. The sections are sliced using a tissue chopper, under aseptic conditions, and slices are placed on a membrane insert in a 6-multiwell dish. Cultures are maintained at 37ºC in a CO_2 incubator (adapted from Heinemann et al., 2006).

3.3 Western Blot

Western blotting assay was performed to address protein expression changes within the course of epileptogenesis. This assay enables proteins to undergo an electrophoretic separation, after which they are immobilized in a solid support. This process makes proteins more accessible for detection and identification (Mahmood & Yang, 2012). Thus, western blotting includes the transfer of proteins from a sodium dodecyl sulfate (SDS) polyacrylamide gel to an adsorbent membrane, followed by probing with a highly specific antibody directed against the target protein (Kurien & Scofield, 2006; Ma & Shieh, 2006). Western blotting and subsequent immunodetection is a powerful tool to detect and characterize a multitude of proteins, especially the low abundant ones (Kurien & Scofield, 2006). Also, the same protein transfer can be used for multiple and successive analyses, by using specific antibodies against the proteins of interest.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing protein mixtures. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to their molecular weight, it can also be used to determine the relative molecular mass of a given protein. SDS is an anionic detergent that binds to hydrophobic regions, causing the protein to acquire a net negative charge. Thus, the protein moves in an electric field during the electrophoresis procedure, toward the positive charged electrode (Kurien & Scofield, 2006). Two gels, of different polyacrylamide content, are usually used. The stacking gel, with low polyacrylamide content, has large sized pores that allow the proteins to migrate freely and get stacked at the interface between stacking and resolving gel. The purpose of this layer is to make sure that the proteins start migrating from the same level, since they are separated based on their mass and the effect of their charge is overcome by SDS's high negativity. The sharpening of bands, which can be visually appreciated while the proteins migrate in stacking gel, is due to a phenomenon called isotachophoresis. Due to isotachophoresis the migrating proteins get sandwiched tightly between the glycinate and chloride ions that migrate along, which makes the proteins to get aligned in sharp bands (Wilson, 2010). Then, the resolving gel allows protein separation in the sample based on their molecular weight, since it has a higher polyacrylamide content, which makes the gel pores narrower.

Polyvinylidene fluoride, or polyvinylidene difluoride, (PVDF) is a highly non-reactive thermoplastic fluoropolymer produced by the polymerization of vinylidene difluoride (Mago

et al., 2008). It strongly retains proteins during protein transfer, but also reduces nonspecific protein binding that can obscure high-sensitivity detection (Jaleh et al., 2015).

Antibodies are host proteins that comprise one of the principal effectors of the adaptive immune system. They bind the antigen with a high degree of affinity and specificity. Most antigens are highly complex, as they present numerous epitopes that are recognized by a large number of lymphocytes. The lymphocyte is then activated and induced for activation and differentiation into plasma cells, resulting in a polyclonal antibody (Lipman et al., 2005). Polyclonal antibodies are produced by different cells and thus are immunochemically dissimilar, as they react with various epitopes on the antigen against which they were raised (Boenisch, 2006). The antibodies produced by a single B lymphocyte clone are called monoclonal antibodies. The monoclonal antibodies present higher homogeneity and consistency, being useful in evaluating changes in molecular conformation, protein-protein interactions, and phosphorylation states (Hollenbaugh et al., 2003; Tiller et al., 2008). In contrast, polyclonal antibodies are heterogeneous and recognize a host of antigenic epitopes, and generally are more stable (Leenaars & Hendriksen, 2005; Lipman et al., 2005).

3.3.1 Protein extraction

In all timepoints, hippocampi were dissected from five slices and kept at -80 ºC until processed. Tissue was dissociated by passing 20 times through a 25G needle (Terumo Europe, Leuven, Belgium) in 150 μL of Ristocetin Induced Platelet Agglutination buffer (RIPA, 50mM Tris pH 8.0, 1mM EDTA (Ethykenediamine Tetraacetic Acid), 150mM NaCl, 1% NP40 substitute (Nonyl phenoxlpoylethanol, Fluka Biochemika, Switzerland), 1% SDS (Sodium Dodecyl Sulfate) and 10% glycerol), containing a mixture of protease inhibitors (Sigma) and phenylmethysulfonyl fluoride (PMSF). Dissociated tissue was shaken for 15 minutes at 4°C. Tissue lysates were then centrifuged at 13000 × g for 10 min at 4ºC to remove cell debris. The supernatant was collected and total protein was quantified using the Bio-Rad DC Protein Assay Kit (Bio-Rad).

3.3.2 Western Blot assay

Samples (35μg total protein/well) and protein size marker (NZY colour protein Marker, NZYtech) were boiled at 95°C for 10 min, and electrophoresed on a 12% SDS-PAGE at a constant voltage of 80V, until the proteins reached the resolving gel, and then for 120V for 1h30. The sample buffer added to each sample contained an ionisable tracking dye, bromophenol blue, that allowed the electrophoretic run to be monitored, and glycerol, which gave density to the samples allowing them to settle to the bottom of the well when loaded.

Electroblotting assured that the proteins were transferred onto a PVDF membrane (Millipore), following the same principle that drove protein migration and separation. The membrane was placed between the gel surface and the positive electrodes in a sandwich, thus mirroring the protein migration on the gel. The apparatus containing the cassettes with the membranes was submerged in transfer buffer, an electrically conducting medium where proteins were soluble (Mahmood & Yang, 2012). Protein transfer was conducted for 90 minutes.

For protein transference effectiveness, the membranes were submerged in Ponceau S, a sodium salt of a diazo dye of a light red colour, for a rapid and reversible detection, facilitating subsequent immunological detection. Membranes were then washed and blocked for 1h with 5% powder milk in Tris base solution supplemented with Tween-20 (TBS-T) (20mM Tris base, 137mM

NaCL and 0.1% Tween-20) at room temperature (RT). Blocking is a very important step of western blotting, as it prevents antibodies from binding to the membrane non-specifically. Subsequently, membranes were probed with the primary antibodies (table 3.3), diluted in TBS-T with 3% BSA, overnight at 4°C.

Table 3.3 - Primary antibodies used in western blot

Protein	Supplier	Primary antibody	Dilution
αII-Spectrin	Sigma	Mouse monoclonal	1:500
NLRP3	Abcam	Rabbit polyclonal	1:300
Caspase3	Santa Cruz Biotechnology	Rabbit polyclonal	1:300
GFAP	Sigma	Rabbit polyclonal	1:5000
GAPDH	Cell Signalling	Mouse monoclonal	1:5000
LC3	Abcam	Rabbit polyclonal	1:500
Iba1	Abcam	Goat polyclonal	1:1000
ASC	AdipoGen	Rabbit polyclonal	1:1000

GFAP – Glial fibrillary acidic protein; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; LC3 - Microtubule-associated protein 1A/1B-light chain 3; Iba1 – Ionized calcium binding adaptor molecule; ASC – Apoptosis-associated Speck-like Protein Containing CARD

Finally, membranes were probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology, Heidelberg, Germany). Development of signal intensity was carried out using ECL Western Blotting Detection System (GE Healthcare, Buckingamshire, UK) and analysed using Chemidoc XRS$^+$ software (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. The relative expression of the proteins was calculated using computer assisted densitometry analysis with ImageJ software 1.44b and standardized for GAPDH levels. Protein levels at 0, 3, 7, 10, 14, 17 and 21 DIV were normalized to 0 DIV levels, to follow the course of protein expression since the moment at which the slices were obtained.

3.4 Immunohistochemistry (IHC)

The spatial expression of an antigen relative to an individual cell, or in the context of whole tissue, can be analysed with antibodies using immunohistochemistry (Lipman et al., 2005).

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC allows visualizing the distribution and localization of specific cellular components within cells and in the proper tissue context. While there are multiple approaches in IHC methodology, all of the steps involved are separated into two groups: sample preparation and labelling.

Fluorescence IHC, often called immunofluorescence (IF), is a powerful method for visualizing intracellular processes, conditions and structures. Indirect IF combines two different components: first, specific primary antibodies, which are used to form an immune complex with the desired protein in the cell; second, the use of fluorochrome-coupled secondary antibodies, which allow the visualization of antigen-antibody interaction through fluorescence microscopy. The secondary antibody must be directed against the species in which the primary antibody is raised. In direct IF the primary antibody is already coupled to a fluorochrome, and thus no secondary antibody is required.

In the IF procedure, the first step is the fixation, to maintain cellular and tissue architecture. Paraformaldehyde (PFA) at 4% was the chosen fixator for this study. The following step in the procedure is membrane permeabilization, in which intracellular structures become accessible for antibodies, which will be otherwise unable to pass through the lipid membranes of the cell. This was accomplished with the detergent Triton X-100. Blocking is an important step for minimizing unspecific binding of the primary antibody within the cell, normally accomplished using animal sera or bovine serum albumin (BSA). After blocking, the samples are incubated with the specific primary antibody, against the target protein, and with the fluorochrome coupled-secondary antibody.

To complete the immunoreaction, one can resort to nucleus staining with DNA dyes. This enables better orientation within cell or tissue section during microscopy and it also indicates cellular status. Fluorescent dyes that strongly bind to DNA, such as 4',6-diamidino-2-phenylindole (DAPI) or dyes from the Hoechst family, can be used in this step.

In IF final step, the samples were mounted to be suitable for microscopy. For this purpose, a mounting medium, such as Mowiol, is used to fix the sample on a microscope slide. It also prevents dehydration and increases the refractive index, which is conducive for microscopy with oil immersion objectives.

IF images were acquired by confocal microscopy, which performs a point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays. To achieve this, it uses a very high intensity excitation light, usually a laser, and a dichroic mirror that reflects this light onto two mirrors which are mounted on motors and scan the laser across the sample. The confocal microscope setup minimizes how much of the specimen is illuminated by placing a pinhole in front of the light source. Returning photons of a longer wavelength are allowed to pass through the dichroic mirror and onto a second pinhole that excludes most of the light from the specimen that is not from the microscope's focal plane. The photons that are in the focal plane are then detected and amplified by a photomultiplier tube and the final image is produced. The major advantages of confocal microscopy are the creation of an image point by point, allowing the elimination of most of the out-of-focus background, and the formation of images with better resolution than a wide field microscope. The field of view can also be made larger than that of the static objective by controlling the amplitude of the mirrors movement (Semwogerere & Weeks, 2005).

3.4.1 Preparation of slices

Slices, at each timepoint, were fixed for 1h with 4% PFA diluted in phosphate-buffer solution (PBS) at room temperature (RT), followed by an incubation in increasing concentrations of a sucrose (Sigma) solution (10% and 20% in PBS) at RT. Slices were kept in a 30% sucrose solution at 4ºC until further use.

3.4.2 Immunofluorescence assay

Slices were cut out of the insert and put in slides (2 slices per slide). Each slice was surrounded with DAKO pen (Dako, Denmark) to protect staining areas from drying out and from mixing with each other. Followed PBS, slices were incubated for 3h at RT in blocking solution containing 10% BSA, 10% horse serum and 1% Triton X-100 in PBS, which ensured simultaneous blocking and permeabilization of the tissue. Subsequently, slices were incubated with the primary antibodies (table 3.4) diluted in PBS, overnight at 4ºC. Slices were rinsed with PBS containing 0.1% Tween-20 (PBST) and the fluorochromes coupled-secondary antibodies (donkey anti-rabbit Alexa Fluor 568, donkey

anti-goat Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 488, 1:400, Life Technologies) were applied to the slices for 6h at RT. The nuclei were stained with Hoechst 33342 (1:100 dilution in PBS from a 2mg/mL stock; Invitrogen) for 40 min at RT. The slices were mounted in Mowiol (40μL per slice). Images were acquired with a frame size of 1024 x 1024 pixels on an inverted confocal laser scanning microscope (LSM 710, Zeiss, Germany) with an objective of 20x.

In this study, immunofluorescence was performed to assess astrogliosis and microglia activation throughout time.

Table 3.4 - Primary antibodies used in immunofluorescence assay

Protein	Supplier	Host	Dilution
GFAP	Millipore	Mouse monoclonal antibody	1:500
Iba1	Abcam	Goat polyclonal antibody	1:1000

GFAP – Glial fibrillary acidic protein; Iba1 – Ionized calcium binding adaptor molecule 1.

3.5 ELISA

Enzyme-linked immunosorbent assay, also known as ELISA, is one of the methods to quantify cytokines and chemokines in biological fluids and tissue culture samples. Cytokines play a role in the regulation of haematopoiesis, mediating the differentiation, migration, activation and proliferation of diverse cells. As they are pleiotropic, they possess overlapping functions and may regulate the production of other cytokines (Carson & Vignali, 1999; Remick & Granger, 2006).

The core of ELISA centres on a sandwich of the cytokine between two cytokine-specific antibodies that bind to mutually exclusive sites on that one cytokine. The first antibody, denominated capture antibody, tethers the cytokine to a solid support. The second antibody, named detection antibody is biotinylated in order to link the capture cytokine to a detection system (Remick & Granger, 2006). As such, this method presents a series of advantages, as the absence of radioactive ingredients, the robustness of the technique, relative low operating costs and little instrumentation required. As disadvantages, the sandwich immunoassay can only detect the total amount of cytokine and cannot distinguish between biological active and inactive protein (De Jager & Rijkers, 2006).

The normal goat serum, used in the reconstruction of the detection antibody, contains goat immunoglobulin-G (IgG) that binds to and blocks the proteins that the capture antibody (also IgG) would otherwise bind to. The goat serum is usually not added to the plate blocking solution but to the assay buffer or sample diluent or conjugate diluent. It is primarily used to block heterophilic reaction of antibodies in the samples to those used in the assay. Its main function is to reduce background.

Streptavidin-HRP consists of streptavidin protein that is covalently conjugated to HRP enzyme. Streptavidin binds to biotin and the conjugated HRP provides enzyme activity for detection using an appropriate substrate system, which is the measurement of biotinylated detection antibodies, in the case of ELISA.

3.5.1 ELISA assay

In this study, levels of IL-1β and TNFα were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cytokine levels from protein lysates at 0, 7, 14 and 21 DIV were normalized to 0 DIV levels, in order to follow the course of cytokine expression from the moment of slices' preparation.

3.6 Statistical analysis

All statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). The comparisons between different timepoints, within each condition control (CTL) and epileptic-like (EL) slices, were made using one-way ANOVA. All comparisons between CTL and EL slices were made using a two-way ANOVA. In both cases, a Bonferroni post-correction was carried out. The number of independent cultures or cells (N) used in each assay is indicated in the legend of each figure. In all figures data are presented as mean ± SEM. Values of $p<0.05$ were considered to account for statistically significant differences.

4 RESULTS

4.1 Assessment of cell death mechanisms

4.1.1 NLRP3-mediated pyroptosis

The increased expression of NLRP3 may represent an increased activation of the intracellular proinflammatory pathways that are involved in epilepsy (Meng et al., 2014). Also, NLRP3 activation is essential to initiate the assembly of the inflammasome. Thus, NLRP3 expression, in CTL and EL slices, was evaluated through western blot, with protein extracts obtained from the hippocampal region.

There are various isoforms that are known to exist for the NLRP3 inflammasome (Haneklaus et al., 2013). The NLRP3 band chosen in the immunoblots (Figure 4.1.1 A) stands at approximately 117-120 KDa, corresponding to the assembly of all constitutes and thus giving a notion of how much of NLRP3 constitutive domains are being produced. No activation of the inflammasome can be inferred from these results.

In CTL slices (Figure 4.1.1 B), NLRP3 immunoreactivity remained below the one obtained at 0 DIV. At 3 DIV, there is a slight increase, albeit not significant (3 DIV: 1.086±0.1762, p>0.05). This increase probably reflects cell trauma due to massive deafferentation and deefferentation, which occurred during tissue slicing. At 14, 17 and 21 DIV, NLRP3 expression significantly decreased, when compared to 3 DIV (14 DIV: 0.5135±0.09628; 17 DIV: 0.4575±0.0927; 21 DIV: 0.5069±0.1195, *p<0.05). In EL slices (Figure 4.1.1 C), NLRP3 immunoreactivity decreases up to 7 DIV, when compared to 0 DIV. As of 10 DIV, NLRP3 expression depicts a tendency for increase, although with no statistical significance (p>0.05).

When both conditions are compared (Figures 4.1.1 D-E), there are no substantial changes between CTL and EL slices until 10 DIV. From 10 DIV on, NLRP3 immunoreactivity is higher in EL slices. That increased expression is statistically significant at 14 and 17 DIV (CTL 14 DIV: 0.513±0.096 vs EL 14 DIV: 1.465±0.277, **p<0.01; CTL 17 DIV: 0.457±0.093 vs EL 17 DIV: 1.417±0.162, **p<0.01). NLRP3 expression in EL slices at 21 DIV is much higher than in the CTL ones, but with no statistical significance (CTL 21 DIV: 0.616±0.070 vs EL 21 DIV: 1.158±0.14, p>0.05).

In summary, EL slices which depict epileptic-like events from 14 DIV onwards have an increased NLRP3 expression, in relation to "non-epileptic" CTL slices. We can therefore hypothesize a role for NLRP3-mediated pyroptosis in the epileptogenesis onset. This data will be complemented with the evaluation of IL-1β levels (see section 4.2.1 for IL-1β quantification by ELISA).

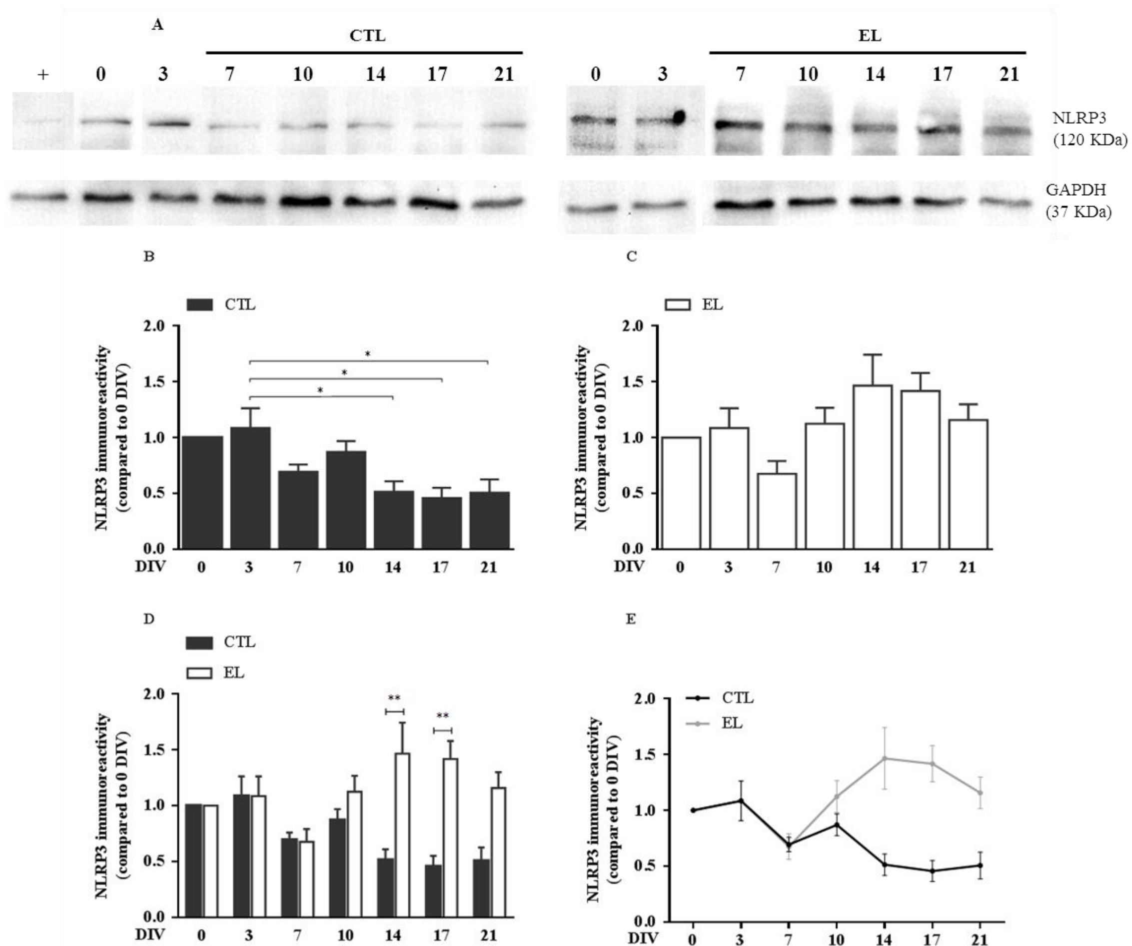


Figure 4.1.1 - Western blot analysis of NLRP3 in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of NLRP3. Lane +, slices incubated with 10µM AMPA overnight, the positive control for NLRP3 expression. GAPDH was used as the loading control. **B)** Densitometric analysis of NLRP3 for CTL slices. **C)** Densitometric analysis of NLRP3 for EL slices. **D)** Comparison of NLRP3 immunoreactivity in CTL and EL slices. **E)** Associated trend for NLRP3-mediated pyroptosis. All values are mean ± SEM. N=3-5; *p<0.05, **p<0.01. DIV, days *in vitro*.

4.1.2 ASC cleavage and inflammasome/pyroptosome formation

ASC is a 22-kDa adapter protein with an N-terminal PYD and a C-terminal CARD that exists in its free form in the cytosol. It uses its PYD to interact with the PYD of the NLRs and its CARD to interact with the CARD of procaspase1, thus linking the PYD-containing NLR family members to procaspase1. ASC is essential for caspase1 activation and generation of mature IL-1β, which suggests that this protein is a key effector of caspase1 activation. PYD-containing NLR family members such as cryopyrin (NLRP3) assemble an inflammasome complex with ASC, which in turn recruits and activates caspase1 (Fernandes-Alnemri et al., 2007). ASC expression, in CTL and EL slices, was evaluated through western blot, with protein extracts obtained from the hippocampal region.

ASC expression significantly increases after the initial trauma, an increase that lasts until 7 DIV. In CTL slices (Figure 4.1.2 B), its expression decreases, although with no statistical significance, from 7 to 10 DIV (7 DIV: 2.543±0.2850 vs 10 DIV: 1.949±0.2331, $p>0.05$) and remains at the same level from 14 DIV onwards (14 DIV: 1.841±0.018; 17 DIV: 1.534±0.0324; 21 DIV: 1.579±0.1525). In EL slices (Figure 4.1.2 C), ASC expression significantly decreases from 7 to 10 DIV (7 DIV: 2.945±0.2937 vs 10 DIV: 1.758±0.0654, $^{*}p<0.05$) and is maintained until 17 DIV (14 DIV: 1.631±0.3455; 17 DIV: 1.979±0.244). At 21 DIV, ASC expression upsurges again in comparison to 0 DIV (2.485±0.3253, $^{**}p<0.01$).

When comparing both conditions (Figures 4.1.2 D-E), no significant differences can be observed between CTL and EL slices. Nevertheless, there are specific time intervals (3-7 DIV and 17-21 DIV) where EL slices seem to have a higher ASC expression. This change in ASC expression may be related to its oligomerization and participation in the inflammasome or pyroptosome formation.

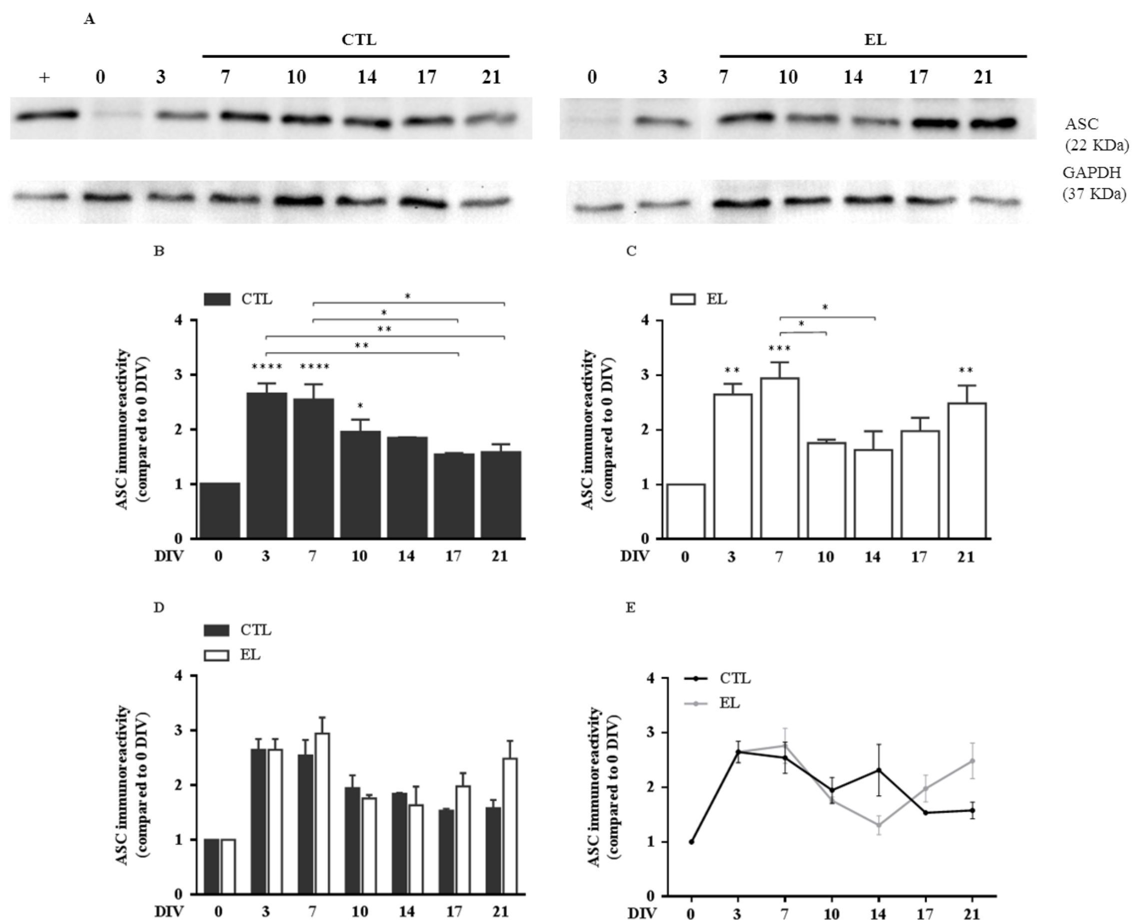


Figure 4.1.2 - Western blot analysis of ASC in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of ASC. Lane +, slices incubated with 10µM AMPA overnight, the positive control for ASC expression. GAPDH was used as the loading control. **B)** Densitometric analysis of ASC for CTL slices. **C)** Densitometric analysis of ASC for EL slices. **D)** Comparison of ASC immunoreactivity in CTL and EL slices. **E)** Associated trend for inflammasome/pyroptosome assembly. All values are mean ± SEM. N=3-4; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$. DIV, days *in vitro*.

4.1.3 LC3 cleavage and autophagy

The microtubule associated protein 1 light chain 3 (MAP-LC3 or LC3) is a protein that covalently binds phospholipids and is the main components of the autophagosome. This protein can exist in its soluble form, LC3-I (14KDa), or in a lipoprotein structure, LC3-II (17 KDa). This LC3-II form is covalently linked to a phosphatidylethanolamine that makes increases hydrophobicity and allows this protein to have higher electrophoretic mobility in SDS-PAGE. When in starvation situations, the LC3-I free form is converted to LC3-II by Atg-4 cleavage, a cys-protease, that is activated by ROS produced during starvation, leading to autophagy. This conversion is necessary for the action of Atg7 that triggers two interdependent machineries: the first leading to Atg-5/Atg-12 conjugation and linking to Atg16 and the second leading to the conjugation of LC3 to phospholipids (LC3-II). Both processes are required to generate an autophagosome *de novo*. LC3-II remains then linked to the membrane of the autophagolysosome generated after the fusion of the autophagosome with the lysosome (Gimenéz-Xavier et al., 2008).

To better correlate changes in autophagy and a more accurate measure of autophagic flux, the amount of cytosolic LC3-II and the ratio between LC3-I and LC3-II is the best option. This so happens because the LC3-I is abundant and stable in the CNS tissue, so the ratio of LC3-II to LC3-I and the amount of LC-II can be used to monitor autophagosome formation (Klionsky et al., 2008). Thus, a western blot assay, using an antibody that recognizes both forms of LC3, was carried out (Figure 4.1.3 A). LC3-II to LC3-I ratio was evaluated in CTL and EL slices.

The massive deafferentation and deefferentation that occurs during tissue slicing, causes an increase in LC3-II/LC3-I ratio at 3 DIV, corroborating autophagosome formation as of this point. In CTL slices (Figure 4.1.3 B), LC3-II/LC3-I ratio remains increased throughout culture time, in relation to 0 DIV. This increase attains levels of significance at 10 DIV (1.809±0.1512, $^{***}p<0.001$), 14 DIV (1.762±0.2173, $^{**}p<0.01$) and 17 DIV (1.584±0.1935, $^{*}p<0.05$). Similarly, autophagosome formation remains augmented in EL slices from 0 DIV (Figure 4.1.5 C), with statistical relevance at 14 DIV (1.981±0.2644, $^{**}p<0.01$) and 17 DIV (1.883±.1717, $^{*}p<0.05$).

Comparing both conditions (Figures 4.1.3 D-E), no significant changes were found in LC3-II/LC3-I ratio between CTL and EL slices, suggestive of equal autophagosome formation and no significant role for autophagy in this model of epileptogenesis.

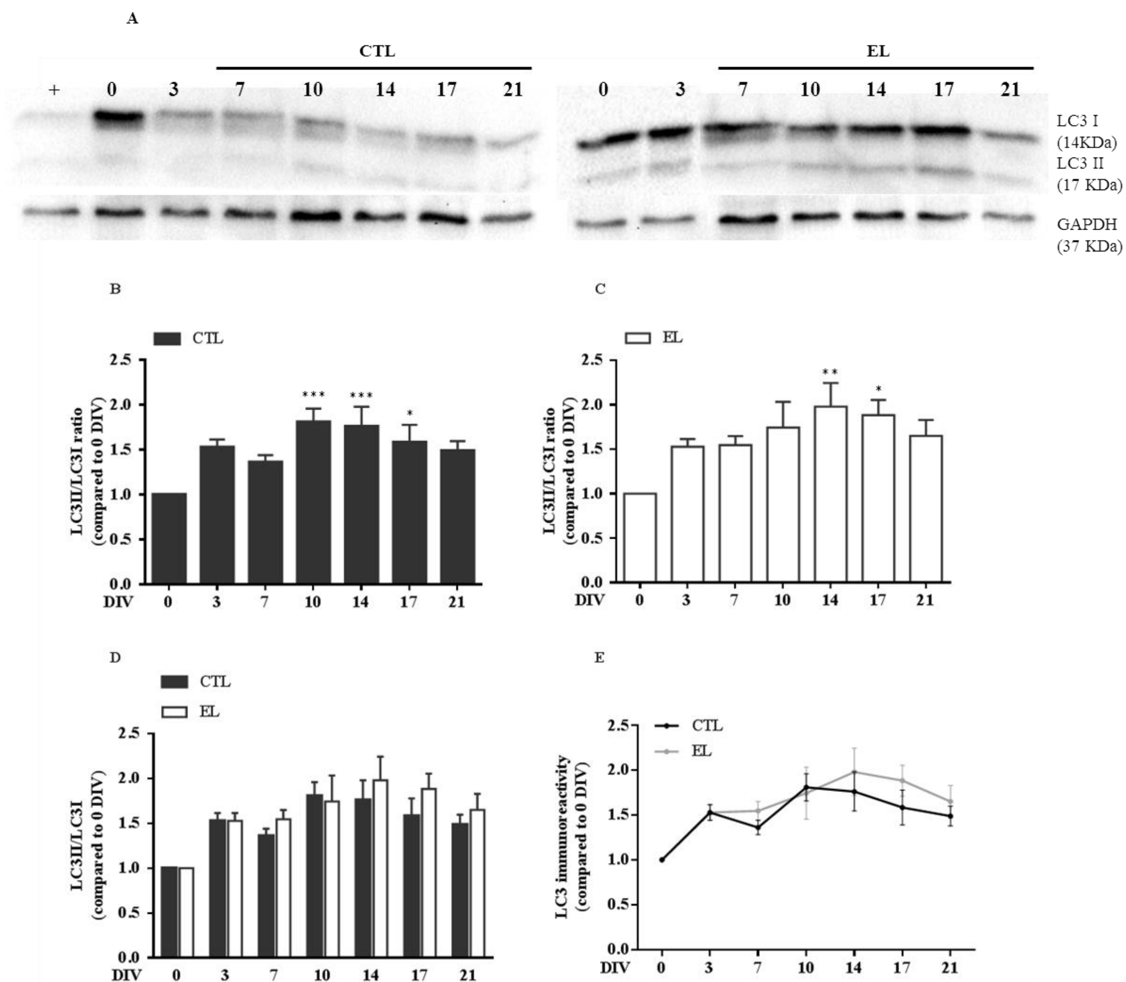


Figure 4.1.3 - Western blot analysis of LC3 in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of LC3. Lane +, slices incubated with 10µM AMPA overnight, the positive control for LC3 expression. GAPDH was used as the loading control. **B)** Densitometric analysis of LC3II/LC3I for CTL slices. **C)** Densitometric analysis of LC3II/LC3I for EL slices. **D)** Comparison of LC3II/LC3I in CTL and EL slices. **E)** Associated trend for autophagy. All values are mean ± SEM. N=3-8; *p<0.05, **p<0.01, ***p<0.001. DIV, days *in vitro*.

4.1.4 αII-Spectrin cleavage and necrosis

Spectrin is an actin binding protein that is a major component of the cytoskeletal superstructure of the erythrocyte plasma membrane. It is essential to determine the properties of the membrane, including its shape and deformability. Spectrin functions as a membrane organizer and stabilizer, composed of non-homologous α and β chains, which aggregate side-to-side in an anti-parallel fashion to form dimers, tetramers and higher polymers (Czogalla & Sikorski, 2005).

αII-Spectrin has a molecular weight of 240 KDa and is a major substrate for calpain and caspase3 proteases, with Spectrin breakdown products (SBDP) having distinct molecular sizes. Events like neuronal death, stress or injury that enhance proteolysis lead to an increase of SBDPs (Zhang et al., 2009). Degradation mediated by calpain leads to the formation of two unique and highly stable SBDPs at 150 KDa and 145 KDa (SBDP150 and SBDP145), that can be further cleaved by caspase3 yielding shorter fragments as SBDP120 (Zhang et al., 2009). The SBDP150 and SBDP145 fragments

are known to occur early in neural cell pathology and the ratio (SBDP145/Full length αII-Spectrin) indicates necrotic and excitotoxic neuronal injury and death. The 145 KDa fragment (SBDP145) is a unique calpain mediated fragment and it is used as a biochemical marker of cell death in neurodegenerative disorders (Pike et al., 2001; Pike et al., 2004). On the other hand, caspase mediated αII-Spectrin degradation yields SBDP150 formation, which is subsequently cleaved into a 120 KDa fragment, that appears to be indicative of apoptotic cell death (Yan & Jeromin, 2013). These SBDPs are highly specific for neuronal damaged, since αII-spectrin presence in glial cells is minimal (Machnicka et al., 2014).

Thus, an antibody which recognizes the full length αII-Spectrin (FL-Spectrin), as well as the calpain- and caspase3- mediated SBDPs, was used in western blot. As can be observed in Figure 4.1.4 A, this antibody identified two bands: a high molecular weight band, which corresponds to FL-Spectrin, and a lower one for SBDP145/150. Since SBDP120, which would result from SBDP150 cleavage by caspase3, did not appear in the western blot, it is plausible to assume that FL-Spectrin was only cleaved by calpain into SBDP145. Thus, SBDP145/FL-Spectrin ratio was used to assess necrotic cell death.

SBDP145/FL-Spectrin ratio peaks at 3 DIV, when compared with 0 DIV (1.543±0.1958, $^{**}p<0.01$). As mentioned before, this increase reflects cell trauma due to tissue massive deafferentation and deefferentation, which occurred during slice's preparation. However, In CTL slices (Figure 4.1.4 B), SBDP145/FL-Spectrin ratio decreased again from 3 DIV on, remaining at a similar level as 0 DIV throughout the culture time, which suggests no changes in FL-Spectrin cleavage and thus no necrotic cell death from this time on. In EL slices (Figure 4.1.4 C), SBDP145/FL-Spectrin ratio recovers to 0 DIV levels at 7 and 10 DIV. From 14 DIV on, an increase in FL-Spectrin cleavage, seen by an increased SBDP145/FL-Spectrin ratio when compared to 7 DIV, is observed (14 DIV: 1.682±0.094*$p<0.05$; 17 DIV: 1.883±0.4738, $^{*}p<0.05$; 21 DIV: 4.666±0.4498, $^{****}p<0.0001$). At 21 DIV, SBDP145/FL-Spectrin ratio is substantial increased in relation to all timepoints.

Comparing both conditions (Figures 4.1.4 D-E), there is a higher SBDP145/FL-Spectrin ratio in EL slices from 7 DIV, with that difference becoming statistically relevant from 14 DIV onwards (CTL 14 DIV: 0.365±0.118 vs EL 14 DIV: 1.682±0.094, $^{****}p<0.0001$; CTL 17 DIV: 0.330±0.070 vs EL 17 DIV: 1.883±0.474, $^{****}p<0.0001$; CTL 21 DIV: 0.353±0.038 vs EL 21 DIV: 4.666±0.4498, $^{****}p<0.0001$). Since epileptic-like events can be recorded in EL slices as of 13-14 DIV, the results suggest an increased necrotic cell death at the onset of epileptogenesis.

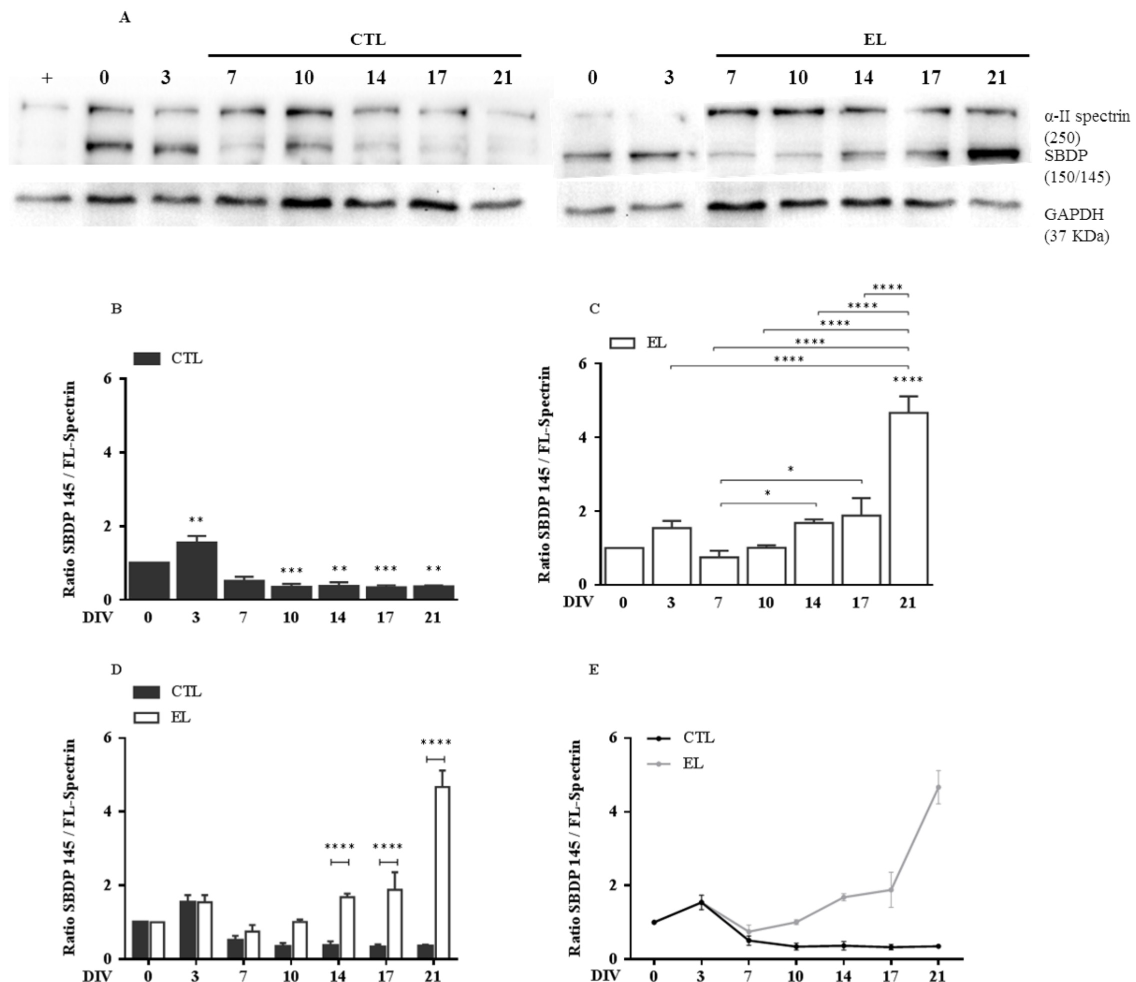


Figure 4.1.4 - Western blot analysis of alpha-II Spectrin cleavage in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of FL-Spectrin and SBDP145. Lane +, slices incubated with 10μM AMPA overnight, the positive control for FL-Spectrin cleavage. GAPDH was used as the loading control. **B)** Densitometric analysis of FL-Spectrin/SBDP 145 ratio for CTL slices. **C)** Densitometric analysis of FL-Spectrin/SBDP 145 ratio for EL slices. **D)** Comparison of FL-Spectrin/SBDP 145 ratio in CTL and EL slices. **E)** Associated trend for necrosis. All values are mean ± SEM. N=3-6; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$. DIV, days *in vitro.*

4.1.5 Caspase3 cleavage and apoptosis

Caspase3 activation can be detected in Western blot by the presence of FL-Spectrin cleavage products, as mentioned before. Following SBDPs assessment, a detection of pro (full-length, 32 KDa) and active (cleaved, 17 KDa) caspase3 can be performed (Yan & Jeromin, 2013). Caspase3-mediated cleavage by enzyme elevates their activity, providing the opportunity for positive feedback in the pathway to amplify caspase3 activation and boost transmission of the apoptotic signal (Kurokawa & Kornbluth, 2009).

SBDPs assessment did not illustrate SBDP120 occurrence, thus an antibody which detects active caspase3 was used. As can be observed in the immunoblot, Figure 4.1.5 A, the antibody identified a very light band with a molecular weight of approximately 17 KDa.

Throughout culture time, in both CTL and EL slices (Figure 4.1.5 B-C), caspase3 immunoreactivity depicted a very low expression. Indeed, always significantly lower ($^{***}p<0.001$ and $^{****}p<0.0001$) than

the one observed at 0 DIV, with no difference among the two conditions (Figure 4.1.5 D). This corroborates the findings of the previous section, where no SBDP120 was found in either condition. Figure 4.1.5 E reinforces the idea that there is little to none modulation of caspase3 activity during culture progression, and thus a not very relevant role for apoptosis in this model of epileptogenesis.

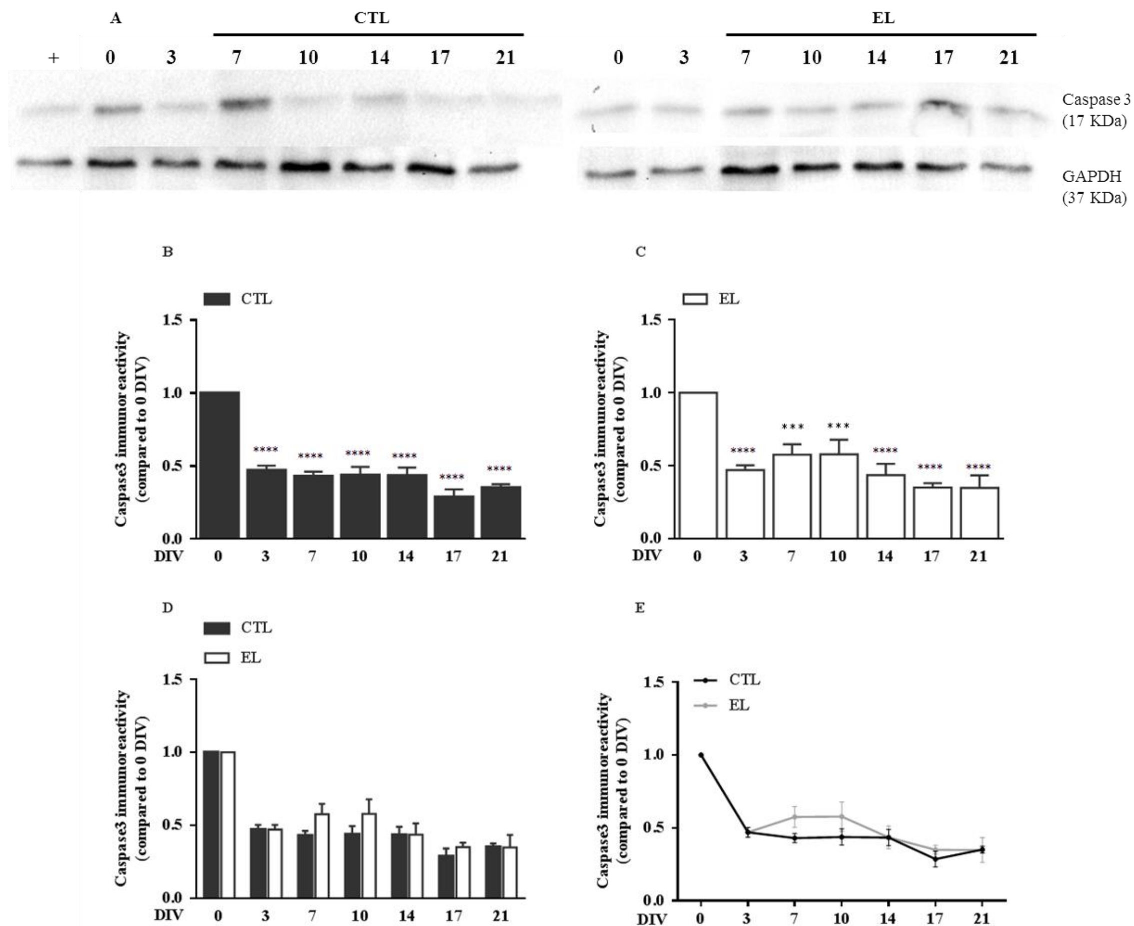


Figure 4.1.5 Western blot analysis of Caspase3 in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of Caspase3. Lane +, slices incubated with 10µM AMPA overnight, the positive control for Caspase3 expression. GAPDH was used as the loading control. **B)** Densitometric analysis of Caspase3 for CTL slices. **C)** Densitometric analysis of Caspase3 for EL slices. **D)** Comparison of Caspase3 immunoreactivity in CTL and EL slices. **E)** Associated trend for apoptosis. Performed with Image J software. All values are mean ± SEM. N=3-6; ***p<0.001, ****p<0.0001. DIV, days *in vitro.*

4.2 Cytokines quantification

Cytokines are involved in neuroimmunology, that is, in the interactions between the CNS and the peripheral immune system, as they function as neuromodulators and regulate neurodevelopment, neuroinflammation, and synaptic transmission (Ramesh et al., 2013). Although highly studied, the mechanism by which seizure activity induces cytokine production and release is still to be fully understood. For now, it is clear that cytokine can induce excitotoxicity (Bernardino, 2005; Olmos & Lladó, 2014), and being produced by glia cell during seizures, they might contribute to seizure-mediated neuronal damage (Li et al., 2011).

The model of epileptogenesis developed in organotypic slices represents a strong damage to the tissue, and might lead to the production of the principal cytokines associated with neuroinflammation in epilepsy. As such, IL-1β and TNF-α production, in CTL and EL slices, was evaluated throughout time in culture.

4.2.1 IL-1β

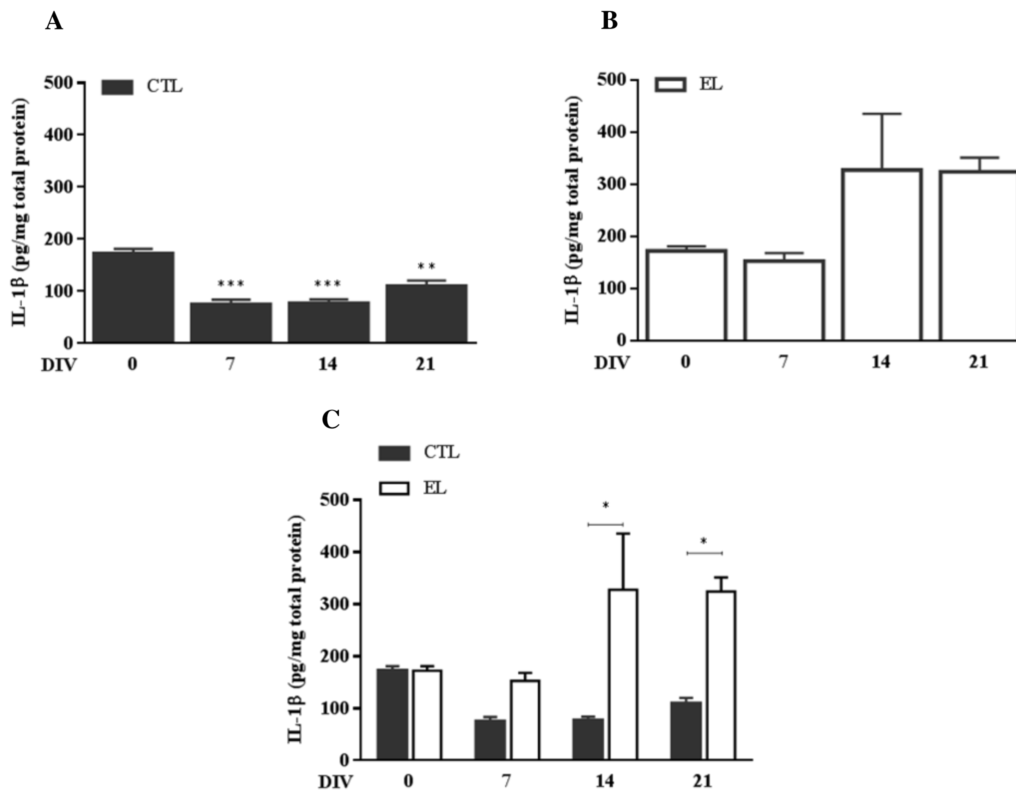


Figure 4.2.1 – Cytokine quantification through ELISA in control (CTL) and epileptic-like (EL) slices at 0, 7, 14 and 21 DIV. A) Quantification of IL-1β production levels in CTL slices. **B)** Quantification of IL-1β production levels in EL slices. **C)** Comparison of IL-1β production levels in CTL and EL slices. Wavelength correction was made by subtracting readings at 540nm from the readings at 450nm. All values are mean ± SEM. N=3-6; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. DIV, days *in vitro.*

In CTL slices (Figure 4.2.1 A), the IL-1β production significantly decreased in relation to the initial trauma (0 DIV: 172.5±8.599 pg/mg) induced by tissue's slicing (7 DIV: 74.92±8.468 pg/mg, $^{***}p<0.001$; 14 DIV: 77.08±6.842 pg/mg, $^{***}p<0.001$; 21 DIV: 109.4±10.84 pg/mg, $^{**}p<0.01$) and remained at the same level until 21 DIV. In EL slices (Figure 4.2.1 B), no decrease in IL-1β production was observed in relation to 0 DIV. Instead, IL-1β progressively increased until 21 DIV. By comparing the two conditions (Figure 4.2.1 C), it is evident the much higher production of IL-1β in EL slices from 7 DIV, with the increase becoming statistically significant at 14 DIV (CTL 14 DIV: 77.07±6.842 pg/mg vs EL 14 DIV: 327.876±107.765 pg/mg, $^{*}p<0.05$) and 21 DIV (CTL 21 DIV: 109.382±10.844 pg/mg vs EL 21 DIV: 324.555±26.974 pg/mg, $^{*}p<0.05$).

4.2.2 TNF-α

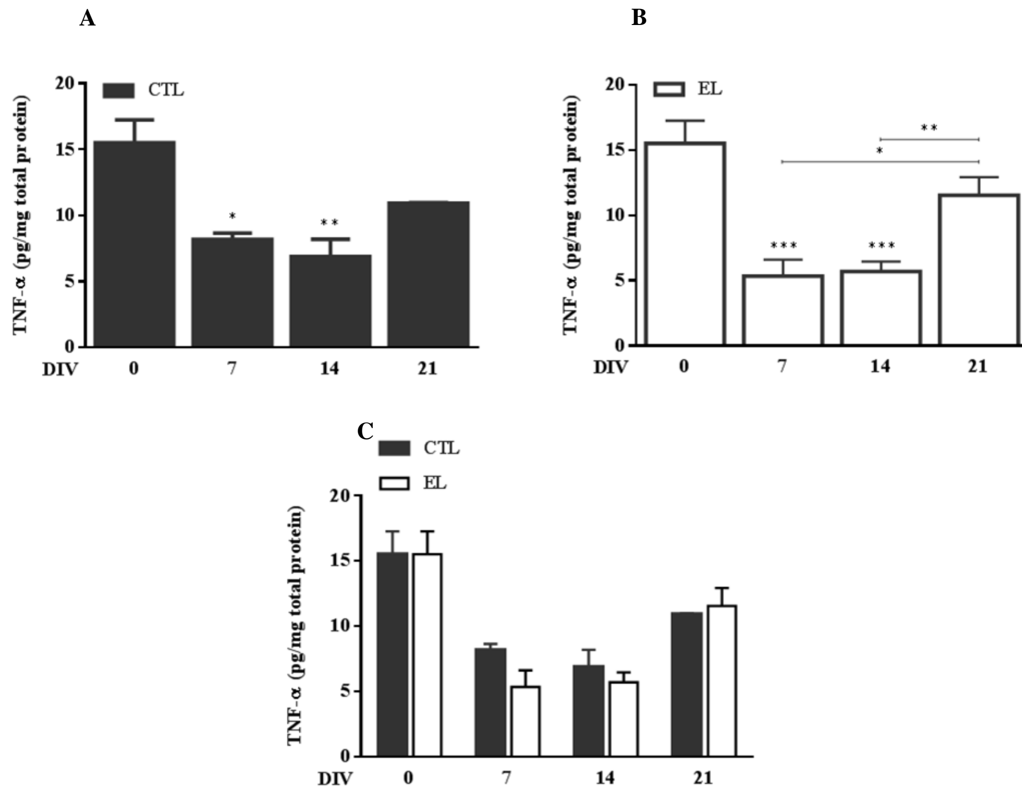


Figure 4.2.2 – Cytokine quantification through ELISA in control (CTL) and epileptic-like (EL) slices at 0, 7, 14 and 21 DIV. A) Quantification of TNF-α production levels in CTL slices. **B)** Quantification of TNF-α production levels in EL slices. **C)** Comparison of TNF-α production levels in CTL and EL slices. Wavelength correction was made by subtracting readings at 540nm from the readings at 450nm. All values are mean ± SEM. N=3-8; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. DIV, days *in vitro*.

In CTL slices (Figure 4.2.2 A), TNF-α shows the highest production at 0 DIV (15.50±1.755 pg/mg), as already referred due to the trauma induced by slices' preparation, and decreases after that (7 DIV: 8.172±0.4765 pg/mg, $^{*}p<0.05$; 14 DIV: 6.883±1.306 pg/mg, $^{*}p<0.05$). In EL slices (Figure 4.2.2 B), there was a decrease in TNF-α expression from 0 DIV, at 7 (5.342±1.259 pg/mg, $^{***}p<0.001$) and 14 DIV (5.698±0.7544 pg/mg, $^{***}p<0.001$), and an upregulation at 21 DIV (11.55±1.374 pg/mg, $^{*}p<0.05$). However, when comparing both conditions (Figure 4.2.2 C), there were no significant changes in TNF-α levels between CTL and EL slices.

4.3 Glial cells evaluation

4.3.1 GFAP and Iba1 expression

4.3.1.1 GFAP expression

GFAP is a class-II intermediate filament and the main constituent of intermediate filaments in astrocytes (Jabs et al., 2008). As such, it serves as a cell specific marker that distinguishes astrocytes from other glial cells during CNS development (Sofroniew & Vinters, 2010). An increased expression of this protein has been reported to represent the activation of astrocytes (Yang et al., 2010). Thus, GFAP immunoreactivity was assessed in CTL and EL slices (Figure 4.3.1.1 A).

Due to the initial trauma that slices undergo, an increase in GFAP expression from 0 to 3 DIV (1.931±0.1249, $^{***}p<0.001$) is observed. However, in CTL slices (Figure 4.3.1.1 B) GFAP expression significantly decreases after 3 DIV (7 DIV: 1.197±0.2112, $^{*}p<0.05$; 14 DIV: 1.155±0.1075, $^{**}p<0.01$; 17 DIV: 0.963±0.072) returning to 0 DIV levels. In EL slices (Figure 4.3.1.1 C), GFAP expression decreases to 0 DIV levels until 10 DIV (1.330±0.092, $p>0.05$) and upsurges again up to 21 DIV, with the difference becoming statistically significant at 17 DIV (14 DIV: 1.854±0.2948, $p>0.05$; 17 DIV: 2.091±0.5237, $^{*}p<0.05$; 21 DIV: 2.167±0.1098, $^{**}p<0.01$).

When comparing both conditions (Figure 4.3.1.1 D), there is evidence of a higher GFAP expression in EL slices from 14 DIV (CTL 14 DIV: 1.155±0.1075 vs EL 14 DIV: 1.854±0.2948, $p>0.05$; CTL 17 DIV: 0.964±0.073 vs EL 17 DIV: 2.091±0.524, $^{*}p<0.05$; CTL 21 DIV: 1.318±0.224 vs EL 21 DIV: 2.167±0.110, $p>0.05$). The trendline depicted in Figure 4.3.1.1 E clearly shows the increased GFAP expression in slices that display epileptic-like events, therefore corroborating an association between epileptogenesis and astrogliosis.

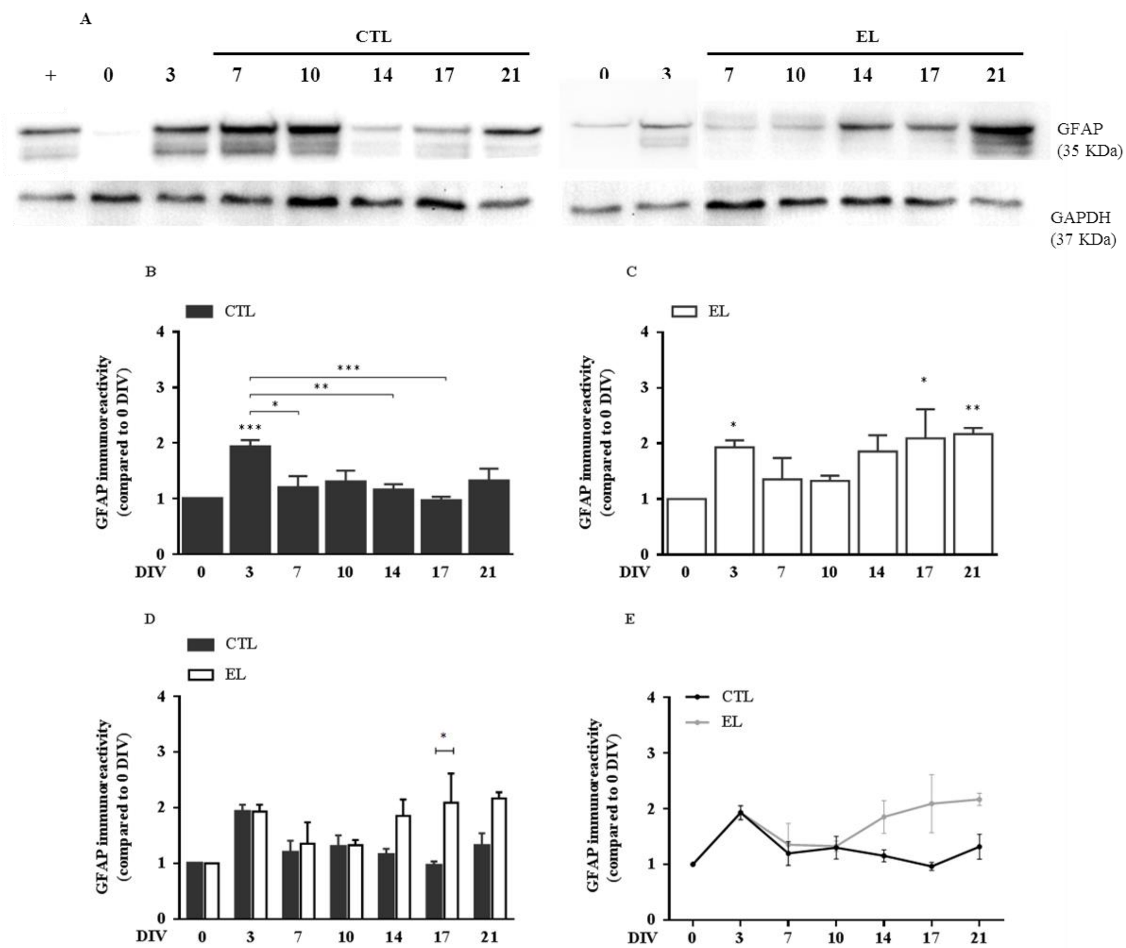


Figure 4.3.1.1 - Western blot analysis of GFAP in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of GFAP. Lane +, slices incubated with 10μM AMPA overnight, the positive control for Caspase3 expression. GAPDH was used as the loading control. **B)** Densitometric analysis of GFAP for CTL slices. **C)** Densitometric analysis of GFAP for EL slices. **D)** Comparison of GFAP immunoreactivity in CTL and EL slices. **E)** Associated trend for astrogliosis. All values are mean ± SEM. N=3-6; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. DIV, days *in vitro.*

4.3.1.2 Iba1 expression

Iba1 is a small protein with approximately 17 KDa and a localization restricted to microglia, thus being used as a microglia-specific marker (Ito et al., 1998). Iba1 is expressed in all microglia subtypes and is thus helpful for visualizing microglia morphology. Furthermore, its expression level increases upon microglia activation. Therefore, Iba1 expression was evaluated in CTL and EL slices (Figure 4.3.1.2 A).

The massive deafferentation and deefferentation occurring during tissue slicing rapidly activates microglia, which is confirmed by the increased Iba1 expression at 0 DIV. In CTL slices (Figure 4.3.1.2 B), there is a progressive and significant decrease of Iba1 expression when compared to this initial trauma (7 DIV: 0.4533±0.1304, $^{*}p<0.05$; 10 DIV: 0.4176±0.1188, $^{*}p<0.05$; 14 DIV: 0.4011±0.0899, $^{*}p<0.05$; 17 DIV: 0.224±0.009, $^{**}p<0.01$; 21 DIV: 0.1747±0.012, $^{**}p<0.01$). In EL slices (Figure 4.3.1.2 C), Iba1 expression decreases at 3 DIV, although not significantly, in comparison to the high expression observed at 0 DIV. No more changes, in relation to 3 DIV, are then observed during culture time.

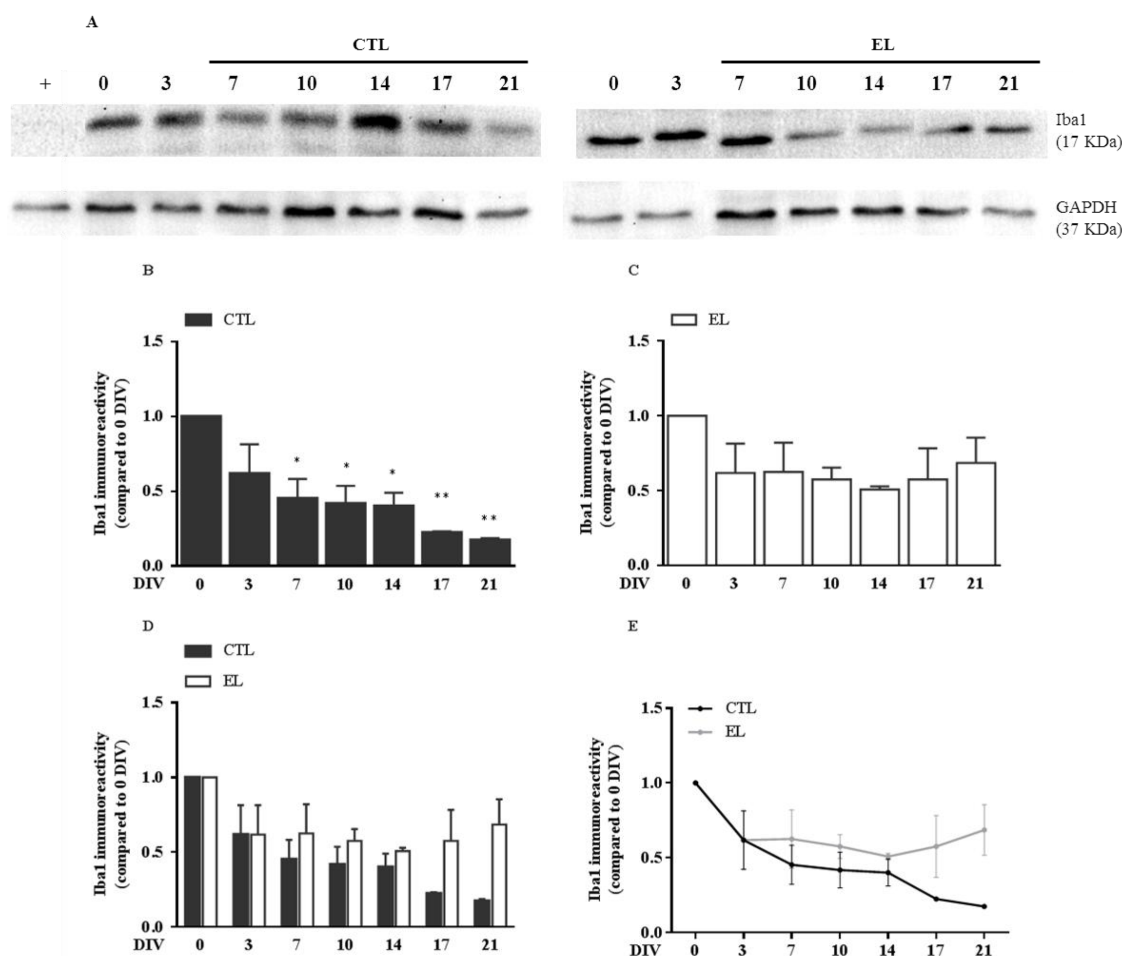


Figure 4.3.1.2 - Western blot analysis of Iba1 in control (CTL) and epileptic-like slices (EL). A) Representative immunoblot of Iba1. Lane +, slices incubated with 10μM AMPA overnight, the positive control for Iba1 expression. GAPDH was used as the loading control. **B)** Densitometric analysis of Iba1 for CTL slices. **C)** Densitometric analysis of Iba1 for EL slices. **D)** Comparison of Iba1 immunoreactivity in CTL and EL slices. **E)** Associated trend for microgliosis. All values are mean ± SEM. N=2-3; *p<0.05, **p<0.01. DIV, *in vitro.*

However, when comparing Iba1 expression in CTL and EL slices (Figures 4.1.7 D-E), a higher expression of this marker can be observed from 7 DIV on, in slices with epileptic-like behaviour (CTL 7 DIV: 0.4533±0.1304 vs EL 7 DIV: 0.6255±0.1947; CTL 10 DIV: 0.4176±0.1188 vs EL 10 DIV: 0.5751±0.0793; CTL 14 DIV: 0.4011±0.0899 vs EL 14 DIV: 0.5075±0.0217; CTL 17 DIV: 0.224±0.009 vs EL 17 DIV: 0.576±0.207; CTL 21 DIV: 0.175±0.013 vs EL 21 DIV: 0.686±0.169). These results require further experiments to achieve statistical significance and fully attest interplay between epileptogenesis and microglia activation.

4.3.2 Astrocytes and microglia morphology

Besides western blot, astrocytic and microglial activation was also assessed by immunohistochemistry. The following panels depict immunofluorescence images in CA3, CA1 and DG, obtained by confocal microscopy. Staining of the nuclear marker Hoechst, together with Iba1 (Figure 4.3.2.1 and 4.3.2.2, panels A1-4 and C1-4) and GFAP (Figure 4.3.2.1 and 4.3.2.2, panels B1-4 and D1-4) was performed at 7 and 14 DIV. The areas delimitated by dotted lines represent the amplified areas of interest from the whole hippocampus.

In CTL conditions at 7 DIV, microglia show large cell bodies with thin ramifications in every region of the hippocampus (Figure 4.3.2.1A2-4), displaying a uniform distribution and occupying distinct spatial domains with minimal overlap. In contrast, in EL slices microglia are more numerous and display larger cell bodies, with extensive and thin ramification in DG (Figure 4.3.2.1C4). In CA3 and CA1 region (Figure 4.3.2.1C2 and C3) microglia exhibit an amoeboid like morphology, with shorter and thicker primary processes. This type of morphology is thus consistent with an active microglia state.

GFAP morphology at 7 DIV is very similar between CTL and EL slices (Figure 4.3.2.1). Due to the initial insult, astrocytes show evidences of moderate reactive gliosis, with some hypertrophic cell bodies entwined with interdigited processes, but not defining an astrocytic scar (Pekny & Pekna, 2014). However, in most astrocytes, processes stay within the individual domains corroborating a non-reactive state.

In CTL slices at 14 DIV, microglia exhibits a mix of resting and amoeboid phenotype (Figure 4.3.2.2 A2-4). Both small and ramified cells, as large ones with thicker processes, can be observed, especially in the DG area (Figure 4.3.2.2 A4). Contrasting, in EL slices, the amoeboid phenotype is prevalent in the DG area (Figure 4.3.2.2 C4), whereas CA3 and CA1 exhibit a resting microglia phenotype (Figure 4.3.2.2 A2 and A3). CA3 and CA1 appear to have reversed their morphology within the time in culture. These changes in morphology have already been described in other studies (Avignone et al., 2008; Avignone et al., 2015).

As for the astrocytes, at 14 DIV, there is a higher density of cells in every region of the hippocampus displaying a mixed phenotype. Healthy astrocytes, with short processes, as well as reactive astrocytes, with large somas and long overlapping processes, can be observed, pointing to a moderate astrogliosis. However, despite the similar morphology between conditions, EL slices (Figure 4.3.2.2 D2-4) appear to have a higher GFAP expression than CTL ones, corroborating the western blot results (Figure 4.3.1.1).

At 21 DIV (Appendix B), most of the microglia cells in EL slices display a resting state, characterized by a ramified morphology, small cell bodies and very thin processes. Astrocytes clearly display a reactive phenotype, with large somas and short branches fully overlapping and creating a glial scar.

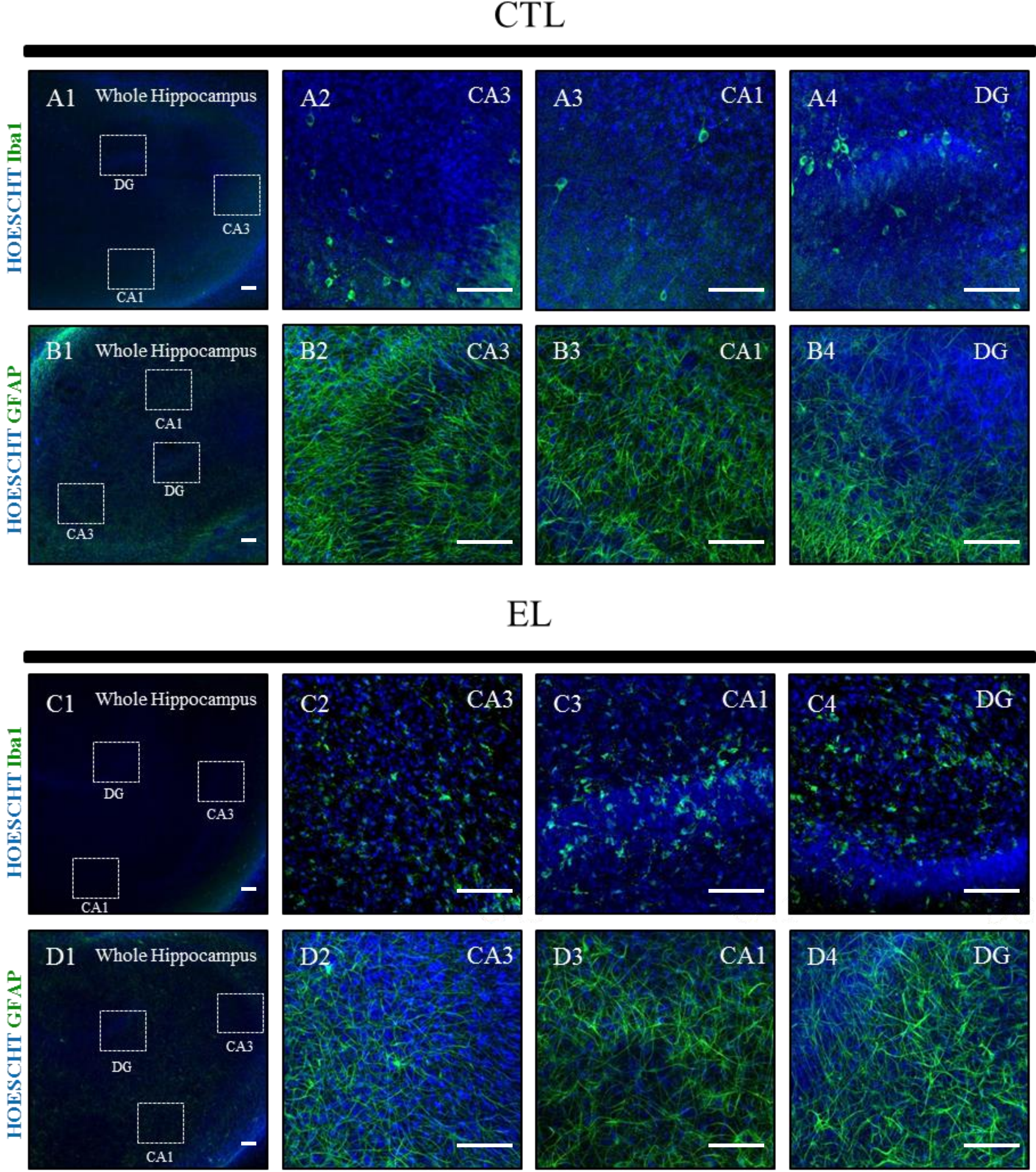
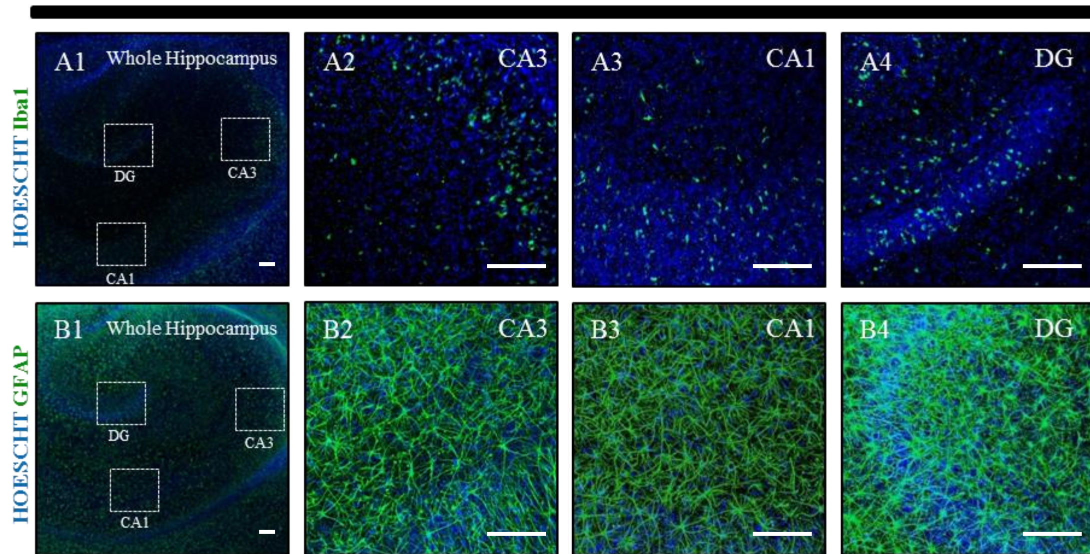


Figure 4.3.2.1 – Astrocytes and microglia activation, in CTL and EL slices, at 7 DIV. Detection of Hoechst stained nucleus (blue), together with Iba1 (green in A1-A4 and C1-C4) and GFAP (green in B1-B4 and D1-D4). Confocal images were obtained with a 5x objective (A1, B1, C1 and D1) and a 20x objective (A2-4, B2-4, C2-4, D2-4). The dotted lines delineate the magnified regions. Scale bar, 200 µm.

CTL



EL

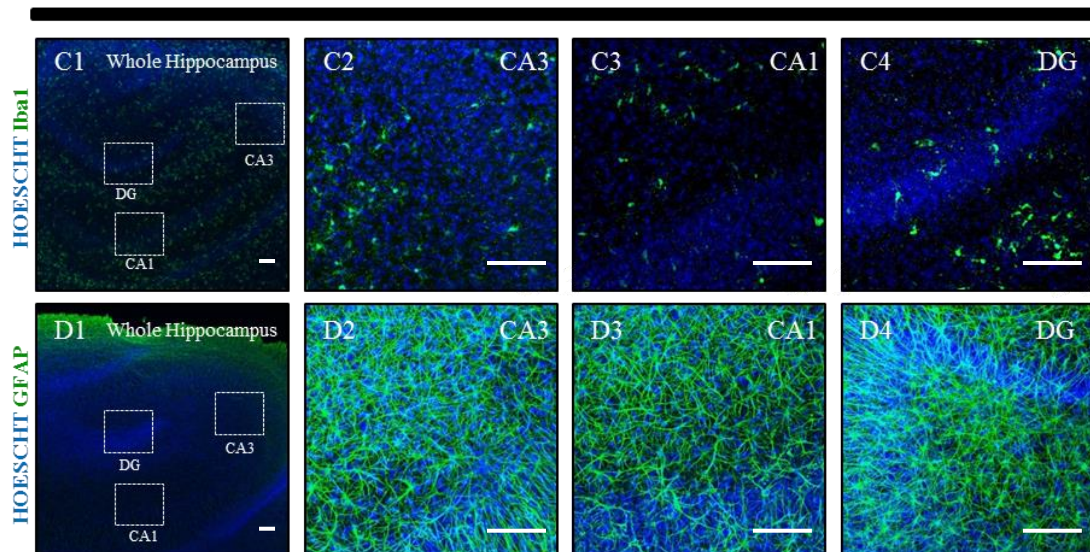


Figure 4.3.2.2 – Astrocytes and microglia activation, in CTL and EL slices, at 14 DIV. Detection of Hoechst stained nucleus (blue), together with Iba1 (green in A1-A4 and C1-C4) and GFAP (green in B1-B4 and D1-D4). Confocal images were obtained with a 5x objective (A1, B1, C1 and D1) and a 20x objective (A2-4, B2-4, C2-4, D2-4). The dotted lines delineate the magnified regions. Scale bar, 200 µm.

5 DISCUSSION

Eventually, all cells once synthesized will be confronted with phenomena that will lead to their death. This cell death cannot be completely neatly or discretely categorized, and several ways can overlap (Booth et al., 2014). In the literature, interactions among the four types of cell death approached in this study are not uncommon. Apoptosis and autophagy are closely related and mutually inhibitory (Nikoletopoulou et al., 2013), autophagy and necrosis dispute a place by the stage in which the organelles have or not regenerated effectively (Pietrocola et al., 2013), necrosis might give the impulse necessary to perpetuate pyroptosis (Nikoletopoulou et al., 2013), etc. There are several mechanisms of cell death that overlap in this epileptogenesis model and it has been proved that seizure severity and duration of SE is not a determining factor in the mechanism of cell death (Niquet et al., 2012). Due to this, this discussion aims to identify the relevance of each cell death pathway to the model and its evolution, not focusing on the crosstalk between pathways.

The timepoints for this study were chosen based on previous works with the same *ex vivo* model (Magalhães, 2015; Magalhães et al., 2017; Rosa, 2017). Electrophysiological recordings (Appendix A) revealed only physiological activity at 7 DIV, interictal activity at 14 DIV and mixed interictal and ictal activity at 21 DIV, as previously reported (Wong, 2011; Berdichevsky et al., 2012). Also, the characterization of inflammatory features in this model (Magalhães, 2015) provided some background for this study. Except for western blot, which assessed the expression of each marker continuously throughout culture, IF and ELISA were performed at 0, 7, 14 and 21 DIV, for comparison with previous results.

5.1 Contribution of pyroptosis

Studies have shown that modification to the NLRP3 gene leads to several autoinflammatory disorders. However, the underlying molecular mechanisms of these mutations are still unclear, and the NLRP3 expression may not always be a synonym for a higher NLRP3 inflammasome activation and action (Broderick et al., 2015; Freeman et al., 2017), since it takes place in the upstream side of the inflammasome assembly. So, the enhanced NLRP3 expression is not sufficient to confirm a NLRP3 inflammasome activation, as it needs to occur a priming with other initiator substances (Bauernfeind et al., 2010). Also, and admitting that a portion of the NLRP3 expression primes into NLRP3 inflammasome activation, upon activation of said inflammasome, ASC specks (pyroptosomes) form and the activated cell undergoes pyroptosis (Elliott & Sutterwala, 2015). Those pyroptosomes accumulate in the extracellular space, where they remain active and can, by themselves, recruit and activate pro caspase1 and process pro-IL-1β, available in the extracellular space and thus promote inflammatory responses (Franklin et al., 2014; Broderick et al., 2015). As the disorder progresses, the ASC specks are phagocytosed by the surrounding immune cells, leading to further activation of NLRP3 inflammasome, as they constitute a danger signal by themselves (Franklin et al., 2014; Broderick et al., 2015; Elliott & Sutterwala, 2015).

This study revealed a clear tendency for a continuous increase in the NLRP3 expression in slices that display epileptic-like activity (Figure 4.1.1 C), whereas its production remains at basal levels in slices that display only physiological activity (Figure 4.1.1 B). Also, results point to a higher production of NLRP3 in EL slices, when compared with CTL ones (Figure 4.1.1 D). As for the ASC-specks, here treated as pyroptosomes, no clear conclusion can be made, since the ASC levels did not change within each condition (Figure 4.1.2 B and Figure 4.1.2 C) and slight differences, with no statistical relevance, were found between conditions (Figure 4.1.2 D).

As mentioned before in section 1.9, the OHSC model represents a deep trauma, with fast neuronal degeneration, and consequent neuronal death, and substantial glia reorganization as early as two hours *in vitro* and up to 28 DIV (Pozzo Miller et al., 1994; Stence et al., 2001). Here, we hypothesise that the gradual serum removal undertaken by EL slices, gives rise to ROS production (Lee et al., 2010). This will activate intercellular immune mechanisms, including the NLRP3 gene, leading to the exacerbation of the inflammatory response (Singhal et al., 2014; Tozsér & Benko, 2016). Previous studies have also describe a higher NLRP3 production in EL conditions, specifically at the onset of interictal discharges (Meng et al., 2014).

The levels of NLRP3 protein expression in CTL slices confirms that there is an intrinsic transcription of the gene, that it is not exacerbated as long as there are no activators that may contribute to its priming and/or activation (Freeman & Ting, 2016). Thus, the peak of NLRP3 expression at 3 DIV is associated with culture trauma. The second peak, which begins at 10 DIV and intensifies at 14 DIV, is probably related with the onset of interictal seizure-like activity, as a consequence of spontaneous, post-traumatic epileptiform discharges (Dyhrfjeld-Johnsen et al., 2010; Berdichevsky et al., 2012) (Appendix A). As described, EL slices undergo a gradual removal of horse serum and at 9 DIV slices are placed in a serum-free medium, which triggers starvation signalling, leads to more ROS liberation by the mitochondria of damage cells and thus to more NLPR3 priming and a higher NLRP3 inflammasome activation (Zhou et al., 2016).

ASC expression, as it was analysed, may not provide clear information. NLRs exists in its free form in the cytosol (Martinon & Tschopp, 2005), where inflammasome assembly takes place and ASC only forms specks upon dimerization (Fernandes-Alnemri et al., 2007; Man & Kanneganti, 2016). ASC expression was analysed through western blot and, in the SDS-PAGE protocol, samples were denatured. This way, non-covalent and disulphide bonds were destroyed (Geddes & Lakowicz, 2007) and the oligomerization that characterizes the ASC-specks cannot be evaluated. It is only possible to state that there is a clear ASC production, in both conditions, with a slight tendency for a higher expression on EL slices at 17 and 21 DIV. That enhanced expression coincides with a higher expression of NLRP3 (Figure 4.1.2 C), IL-1β (Figure 4.2.1 A) and GFAP (Figure 4.3.1.1 C), which may lead us to conceive that, in late epileptogenesis, NLRP3 and ASC are produced and released by reactive astrocytes. Also, that turnover of the main inflammatory mediators – from microglia to astrocytes - translates into maintenance of chronic production of IL-1β, which suggests a role of pyroptosis in cell death. The production of NLRP3 by both microglia and astrocytes has been confirmed in other studies, despite some lack of consensus (Johann et al., 2015; Freeman et al., 2017). Also, recent studies have also been able to co-localize NLRP3, ASC and IL-1β in neurons, thus increased inflammasome expression may contribute to neuronal death, alongside with glial death (Debye et al., 2017).

An exacerbation on the production of IL-1β is one of the hallmarks of pyroptosis, together with the formation of the inflammasome and pyroptosome (Fink & Cookson, 2005; Tozsér & Benko, 2016). Also, this cytokine has been shown to be upregulated in response to traumatic brain injury, spinal cord damage, transient and permanent ischemia and also seizures (Acarin et al., 2000; Vezzani et al., 2016). In the current study, IL-1β production is increased in EL slices, with significant relevance at 14 and 21 DIV (Figure 4.2.1 C), whereas CTL slices exhibit steady and low levels of this cytokine (Figure 4.2.1 B). These findings support the evidences presented in other studies, in which the chronic expression of IL-1β during epileptogenesis is considered to contribute to the mechanisms underlying the onset and continuum of spontaneous seizures (Vezzani et al., 2008). Furthermore, this pro-convulsive effect of IL-1β provides evidence that inflammation is capable of driving hyperexcitability (Beamer et al., 2017). Also, and relating the peak of this cytokine expression with the GFAP peak in late

epileptogenesis, IL-1β seems to promote fibroblast and astroglial growth that potentiate the development of the glial scar (Beamer et al., 2017), as well as the regenerative responses by the induction of growth factor expression (Acarin et al., 2000).

IL-1β could be studied in association with GFAP and Iba1 in IHC studies, as to clarify its production at the different timepoints and also to clarify if there is a trade-off of the main inflammatory mediators from microglia to astrocytes (Acarin et al., 2000). NLRP3 and ASC could also be better analysed with co-immunoprecipitation and crosslinking, respectively. The co-immunoprecipitation for NLRP3 would enable subsequent antibody-based purification of its complexes and associated proteins, that could be further analysed by immunoblot or subjected to functional caspase1 activity assay (Khare et al., 2016). Also, chemical crosslinking covalently joins two or more molecules, thus capturing the oligomeric state with high sensitivity and stability, being ideal to disclose ASC specks formation, either into the inflammasome complex or into dimers that compose the pyroptosome (Khare et al., 2016).

5.2 Contribution of autophagy

In the last decade, there has been an accumulation of evidences that suggest that apoptosis, necrosis and autophagy are regulated by similar pathways, as they share initiator and effector molecules and might engage common subcellular sites and organelles. What differ between them are the cellular context and the trigger mechanism (Nikoletopoulou et al., 2013). More recently, there are also evidences that autophagy plays a role in pyroptosis (Cadwell, 2016).

However, in this model, there is little difference between CTL and EL conditions. The autophagy marker LC3 was analysed through a ratio between its two isoforms, which is described to be the most reliable way to analyse the autophagic process (Gimenéz-Xavier et al., 2008). However, this conversion of the cytosolic form of LC3I into the LC3II isoform that stays connected to the autophagosome does not necessarily result in complete autophagy (Gimenéz-Xavier et al., 2008). So, the results from Figures 4.1.3 B-E can only provide information on the autophagosome formation.

In what comes to autophagosome formation, the serum removal in epileptic slices promotes ROS release that serve as secondary messengers that converge on the mitochondria (Portt et al., 2011). That autophagosome formation is almost a paradox to the reports that state autophagy as the most impressive weapon in the cells anti-death arsenal (Wirth et al., 2013) and it is what classifies autophagy as a programmed, caspase independent cell death. The results herein presented show that the autophagosome formation is induced by the culture trauma (Figure 4.1.3 B and C) and its production stays within considerable high levels throughout all culture maintenance, in both conditions (Figure 4.1.3 D and E). Despite that, as mentioned before, this is not enough to state that there is a role for autophagy as a cell death pathway in this model.

First, there is no significant differences in autophagosome formation when we compare both conditions (Figure 4.1.3 D), while a higher autophagosome production due to the inhibition of mTOR has been reported in epileptic studies (Gan et al., 2015). Second, autophagy is specifically focused in damaged mitochondrial removal, thus inhibiting IL-1β and IL-18 production, preventing DAMPs accumulation (such as ROS and mtDNA) which indicates that this cell death mechanism inhibits or, at least, retards pyroptosis, by inhibiting the cytosolic presence of inflammasome activators (Cadwell, 2016). Furthermore, the autophagosome can incorporate the ASC subunit, leading to its inactivation (Yuk & Jo, 2013; Cadwell, 2016). This study shows that, despite the chronic production of

autophagosome, NLRP3 expression is not diminished and ASC expression is not altered as a consequence of higher LC3II/LC3I levels.

To assess the clear role of autophagy in this model, further studies are required, mainly the evaluation of inhibiting mTOR pathway (Jung et al., 2010), the formation of the autophagolysosome through the use of the auto fluorescent drug monodansylcadaverine (Shin et al., 2012), Atg4 for autophagy modulation and GFP-Atg8/LC3 for lysosomal delivery (Klionsky et al., 2008).

5.3 Contribution of necrosis

In this study, SBDP145/αII-Spectrin peaks at 3 DIV as a consequence of culture trauma, since there is the occurrence of cell membrane rupture of neural cells upon tissue slicing. Culture recovery lasts until 10 DIV. This recovery can be attributed to autophagy maintenance and clearance of excitotoxic substrates, since it was reported that autophagy is able to retard necrosis (Maiuri et al., 2007; Pietrocola et al., 2013). At the onset of interictal activity at 14 DIV (Appendix A), SBDP145/αII-Spectrin increased again in EL slices (Figure 4.1.5 C).

This increase in necrosis at 14 DIV also correlates with previous findings in the same epileptogenesis model, where overall cell death was assessed by propidium iodide (PI) uptake assays (Appendix C). PI is a polar compound, which only enters cells with damaged cell membranes and interacts with DNA emitting red fluorescence (630 nm; absorbance 493 nm). It is not toxic to cells and was proven to be a feasible marker of neuronal cell death in organotypic slice cultures (Noraberg et al., 1999). In every hippocampal region, a low PI uptake was observed at 7 DIV, when compared to 14 DIV. CA1 region was found to be the most damaged region since it displayed a higher PI uptake at 21 DIV (Magalhães, 2015, Appendix C). Thus, it's plausible to propose that the necrotic cell death peak at 14 DIV is associated to the spontaneous, post-traumatic epileptiform discharges that begins to occur at this time (Berdichevsky et al., 2012).

In EL slices, the rise on necrosis was accompanied by the increase of NLRP3, Iba1 and GFAP immunoreactivity, as well as IL-1β production. This confirms that necrotic cells release factors that evoke inflammatory response, such as HMGB1, already found to be upregulated in epileptic human brain tissue and in chronic epileptic mice (Frank et al., 2015). These factors are sensed by NLRP3, the core protein of the inflammasome (Zong & Thompson, 2006). This further leads to higher inflammasome activation and subsequent release of proinflammatory cytokines, like IL-1β (Nikoletopoulou et al., 2013). Also, a correlation between cell swelling, that precedes necrosis, and NLRP3 activation was desbribed (Compan et al., 2012). Cell volume regulation is an ancient protection mechanism for a change in extracellular osmolarity and a decrease in the extracellular osmolarity of potassium is enough to potentiate NLRP3 activation. Also, cell swelling is followed by mitochondrial damage, ROS release and effective organelle breakdown, with release of inflammatory contents, all known signals of NLRP3 inflammasome priming initiators (Liu et al., 2013; Jin & Xiao, 2015). Furthermore, the correlation between NLRP3 production, αII-Spectrin cleavage, IL-1β production and GFAP/Iba1 immunoreactivity reinforces the idea that late epileptogenesis is being sustained by astrocytes and microglia, the last one in a lower extend (Liu et al., 2013; Johann et al., 2015; Freeman et al., 2017).

5.4 Contribution of apoptosis

It was not confirmed a relevant role for apoptosis in this model. In what concerns the extrinsic pathways, TNF-α levels were found to be basal, with no significant increase in EL slices when

comparing to CTL ones (Figure 4.2.2). TNF-α would lead to an increase in the initiator caspase8, that would later provide substrates to be cleaved by the effector caspase3, leading to DNA fragmentation and death (Golstein & Kroemer, 2007). Also, an increase in caspase3 production could be sign of mitochondrial stress and damage, since it is derived from cytochrome-c release and caspase9 formation and substrate cleavage. However, caspase3 immunoreactivity also supports the theory of a not relevant apoptosis for this model, since there was no differences in EL slices (Figure 4.1.4 C) when comparing to the control conditions (Figure 4.1.4 B), either throughout time or between conditions (Figure 4.1.4 D).

Furthermore, and as it was already mentioned, apoptosis can also work in a caspase-independent way, through calpain action. Calpains acted upon calcium activation that, in case of apoptosis, is increased due to the endoplasmic reticulum stress and caspase3 is able to cleave the 145KDa SBDP into a 120 KDa fragment (Czogalla & Sikorski, 2005; Machnicka et al., 2014). That fragment would later be cleaved into procaspase3 and further to its active 17KDa form, caspase3. However, no expression of SBDP 120 (Figure 4.1.5 A) or caspase3 active form was obtained at any of the analysed timepoints. Other groups have also confirmed the caspase3 active form absence in a SE model (Fujikawa et al., 2002; Fujikawa et al., 2007).

Furthermore, if there is an actual failure in autophagy regulation that leads to cell death through a higher deposition of toxic elements into the cell cytosol instead of its elimination, this does not seem to lead to apoptosis. Instead, it rather seems to further develop into necrotic cell death exacerbation, through the deposition of toxic substrates that will promote cell swelling and later rupture. However, for more clearer declarations, the levels of ATP should be addressed, as higher levels of ATP are some of the hallmarks of apoptosis, differing from necrosis that needs lower energy levels (Meldrum, 2002). Also, to better access apoptosis and also provide further evaluation on the importance of the NLRP3 inflammasome and death by pyroptosis, the TUNEL assay can be employ (Fujikawa et al., 2002; Weise et al., 2005; Meng et al., 2014). This protocol allows the detection of DNA fragments as a result from apoptosis, labelling cells that have suffered DNA damage and that are producing cytochrome-C, allowing the researcher to evaluate neuronal loss.

5.5 Glial cells activation

Acute seizures were described significantly alter glial cells in the hippocampus (Eyo et al., 2014), which led us to investigate the morphologic differences of astrocytes and microglia in CTL and EL slices. Astrogliosis and microgliosis were evaluated via marker expression, GFAP and Iba1 respectively, through western blot and morphologic studies through IHC.

5.5.1 Astrocytic activation

Astrocytes form a significant constituent of seizure foci in the human brain (De Lanerolle et al., 2010; Devinsky et al., 2013). Astrocytes have been implicated in promoting epileptogenesis via a diversity of mechanisms, such as increased gap junction coupling, impaired glutamate transporter function, and disruption of the blood-brain barrier (Guo et al., 2017).

GFAP expression shows a peak at 3 DIV, in both CTL and EL slices. This model uses P6-7 days old rats and it has been known that, during mammalian nervous system development, neurons are first generated from neuronal precursor cells (NPCs), followed later by glia cells. This process between

neuron-to-glia formation is critical to determine how many neurons or glia are ultimately made in each brain region (Miller & Gauthier, 2007). However, upon extrinsic environmental cues, as the slicing trauma, there is a promotion of astrogenesis in NPCs that decrease the neurogenic mechanisms in order to increase astrogenic competence over developmental time (Freeman, 2010).

From 14 DIV onwards, when interictal-like discharges become evident (Appendix A), an increased GFAP expression was observed in EL slices, when compared to CTL ones (Figure 4.3.1.1 D). This rise in GFAP was corroborated by the IHC assay that showed a scar-like layer formation over the slices at 14 DIV (Figure 4.3.2.2 D2-D4). The glial scar is mainly composed of reactive astrocytes, it aids CNS axon regeneration and is essential for inflammation and tissue damage restriction (Liddelow et al., 2017). Although astrocyte hypertrophy is often thought as a hallmark of astrogliosis, some studies suggest that reactive astrocytes increase the thickness of their main cellular processes but maintain a restricted overall distribution and volume (Hubbard et al., 2013). In this model, a moderate reactivity of astrocytes was evident at 7 DIV (Figure 4.3.2.1D2-D4), with particular relevance for DG, due to the fact that the hypertrophied astrocytes in this region form a ectopic scaffold that promotes the growth of basal dendrites into the hilus, that are targeted by mossy fibres, and contribute to the recurrent excitatory circuit that facilitates seizures (Shapiro et al., 2008). The scar-like structure was clear at 14 DIV (Figure 4.3.2.2 D3), when interictal events start to take place and the inflammatory associated cell death mechanisms, as pyroptosis and necrosis, start to be relevant to hippocampal damage (Aronica & Crino, 2011). At 21 DIV (Appendix B), the astrocytic scar covers all regions of the hippocampus in EL slices (Harry & Kraft, 2009). The mentioned results lead us to hypothesize that seizure-induced astrocytic changes may directly relate to synaptic and dendritic injury following seizures (Guo et al., 2017). As such, preventive approaches that target reactive astrocytes may be effective for lessening the negative consequences of epilepsy (Guo et al., 2017).

Also, the combine results of IL-1β (Figure 4.2.1) and GFAP expression (Figure 4.3.1.1) reinforce the idea that cytokines act on astrocytic receptors. In acute and organotypic rat brain slices, some cytokines, including IL-1β, were found to inhibit glutamate reuptake and increase glial glutamate uptake, leading to hyperactivity (Robel et al., 2015; Robel & Sontheimer, 2016). Also, IL-1β is known to interfere with astrocyte gap junction communication (Dambach et al., 2014), which appears to represent a very early alteration in the process of epileptogenesis (Jabs et al., 2008; Seifert et al., 2010). This further unveils the riddle between inflammasome activation and action, IL-1β release, pyroptosis and the role of astrocytes in maintaining the inflammatory environment.

Despite the results that were obtained and the fact that most studies use GFAP immunostaining to characterize astrocyte morphology, this marker only reveals the structure of primary branches, that represent approximately 15% of the total astrocyte volume (Freeman, 2010). For a clearer and more complete differentiation of astrocytes, the same samples should be processed and labelled with markers of reactive astrocytes, such as STAT3 or vimentin (Hubbard et al., 2013; Liddelow et al., 2017).

5.5.2 Microglia activation

Iba1 expression in EL slices was higher than in CTL ones, in particular from 17 DIV (Figure 4.3.1.2 D). At 7 DIV, microglia exhibited large cell bodies with thin ramifications in every region of the hippocampus, in both conditions (Figure 4.3.2.1 A1-4 and C1-C4). Despite that, microglia appear in a higher number in EL slices, with more ramified branches and resting morphology in the DG area (Figure 4.3.2.1 D4), whereas CA1 (Figure 4.3.2.1 D3) and CA3 (Figure 4.3.2.1 D2) displayed an amoeboid, classically activated morphology. The activated morphology of these immunity guardians

remains activated in the first week after SE, as reported in literature (Shapiro et al., 2008; Kim et al., 2015), and allows microglia to act as phagocytic cells to clear damaged debris that resulted from culture trauma (Hailer et al., 1997). True enough, at 14 DIV, despite displaying a mix of activated and resting phenotype, CA1 and CA3 already display resting microglia, with smaller soma and ramified processes, appearing to have reversed their conformation. Despite that, in accordance with western blot results, EL slices exhibited slightly more Iba1 expression than CTL. These changes were already reported in literature (Avignone et al., 2008; Avignone et al., 2015; Kim et al., 2015). By the end of culture maintenance, in 21 DIV EL slices, all hippocampal region exhibits microglia in its resting form, with smaller somas and fine, ramified branches (Kim et al., 2015).

In spite of the small sample size, the results lead us to conclude that seizure activity induces changes in microglia morphology, as reported by others (Eyo et al., 2014), and that those changes may cause microglia to express a variety of cytokines, depending on the grade of neuronal affection and/or glial proximity to the degenerating area (Acarin et al., 2000). Also, microglia functions in this *ex vivo* model are essentially on regulating the regenerative responses resulted from the trauma of culture, for synaptic reorganization and repair (Harry & Kraft, 2009; Olah et al., 2011).

6 CONCLUSION AND FUTURE PERSPECTIVES

This model proved to be suitable for the study of cell death mechanisms within an epileptic context. CTL and EL slices can be considered to mimic the "healthy" and "epileptic" brain, respectively, allowing to assess changes in cell death markers, glial cell morphology and cytokine production within the course of epileptogenesis. From cell death pathways explored in this thesis, necrosis appeared as the most relevant in late epileptogenesis. Putting the results of NLRP3 and ASC expression alongside with IL-1β production, the chronic increase of inflammation also suggests a role for pyroptosis in this model. To further support our theory, 21 DIV EL slices only displayed astrogliosis, while microglia was found to be in its resting state. This allows us to state that astrocytes are the main glial cells involved in the propagation of the inflammation in this disorder, something that has been already describe in literature and in other works produced by the same group (Devinsky et al., 2013; Magalhães, 2015; Robel et al., 2015).

Depending on stimulus, severity and cell type, the hierarchy of organelle crosstalk might result in different cell death modalities. With that in mind, it is save to affirm that, in some cases, suppression of the function of a particular intracellular compartment might switch one cell death pathway to another (Zhivotovsky & Orrenius, 2010). Considering the pro-convulsant action of IL-1β, and the involvement of caspase1 in its activity, caspase1 inhibitors were seen to have anticonvulsant actions and knockout of the caspase1 gene were found to protect against seizure inducing stimuli (Edye et al., 2014). In the future, we would like to go upstream this cascade and inhibit NLRP3 in EL slices. IL-1β quantification would be considered a vital assay, since its maturation is regulated by the NLRP3 (Martinon et al., 2002).

Since there was spotted a correlation between the peaks on NLRP3 and GFAP expression and the production of IL-1β, co-localization studies of NLRP3/ASC with astrocytes and microglia would also be of interest. This was already attempted in other studies (Johann et al., 2015; Debye et al., 2017) and during this study, but no result could be drawn.

Also, an evaluation of the microglia subtype, M1 or M2, would be of interest, since changes from M1 to M2 have been described to occur (Brown & Vilalta, 2015). To accomplish this, RT-PCR for markers of each subtype, ROS for the M1 phenotype and Arg-1 for the M2 phenotype, could be used.

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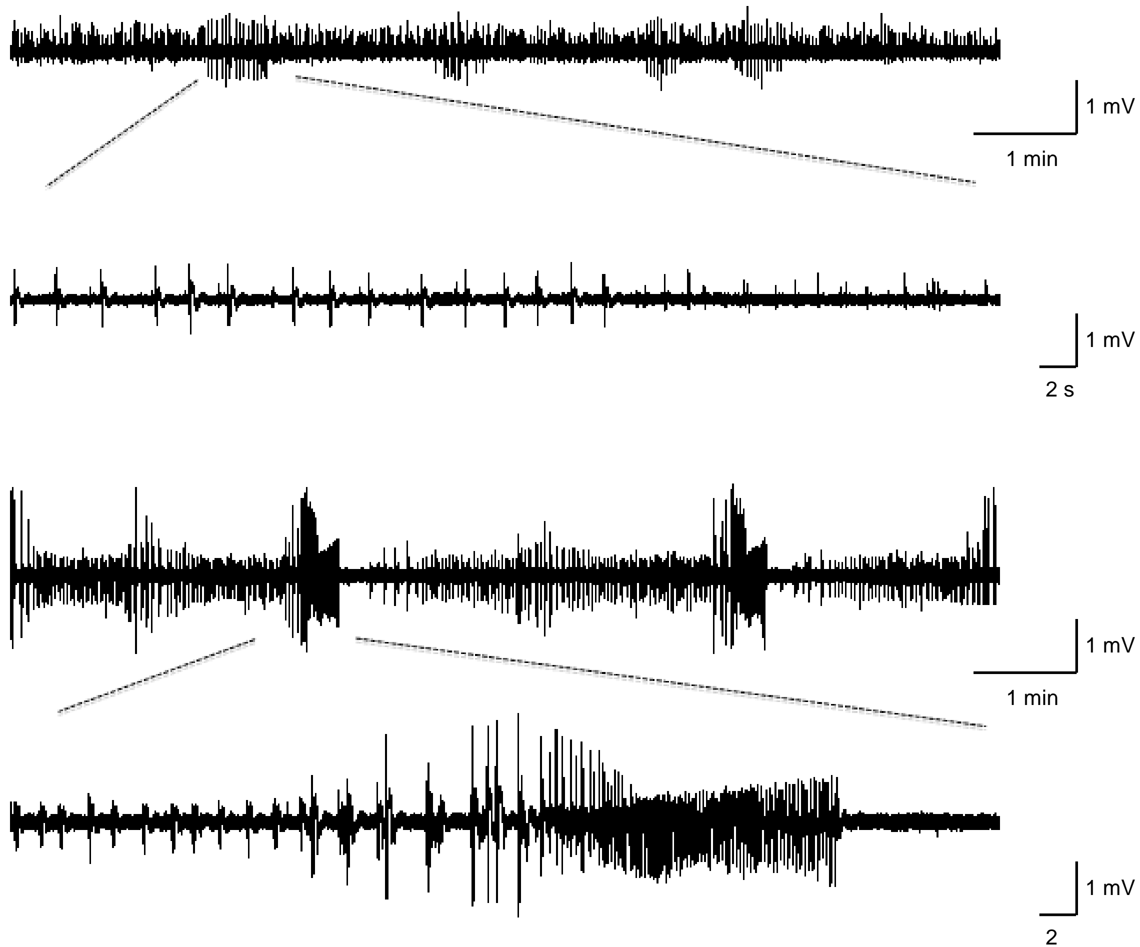
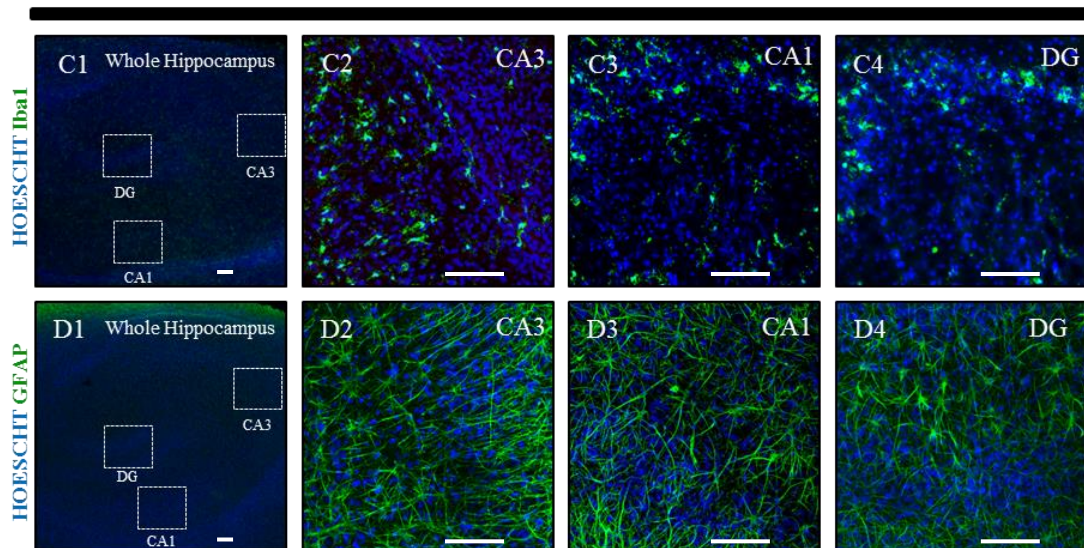
APPENDIXES

A - Electrophysiological recordings



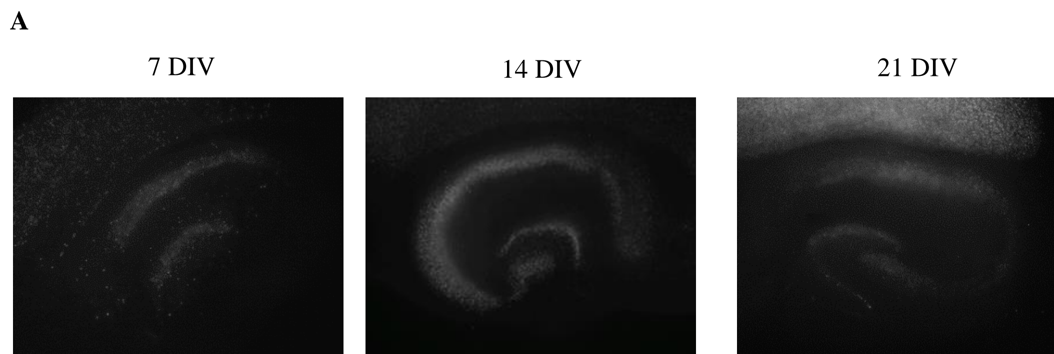
Appendix A – Representative spontaneous epileptiform activity recorded in organotypic slices. A) Interictal-like activity from an EL slice at 14 DIV; **B)** Mixed interictal and ictal.like discharges from an EL slice at 21 DIV. All vertical bars=1mV (**Magalhães et al. 2017**)

B - Astrocytes and microglia morphology at 21 DIV



Appendix B – Astrocytes and microglia activation, in organotypic slices, at 21 DIV in EL slices – Detection of Hoechst stained nucleus (blue), together with Iba1 (C1-C4 - green) and GFAP (D1-D4 – green). Confocal images were obtained with a 5x objective (C1 and D1) and a 20x objective (C2-4, D2-4). The dotted lines delineate the magnified regions. Scale bar, 200µm (**Magalhães, 2015)**

C - Propidium Iodide uptake in OHSC



Appendix C – Propidium iodide (PI) uptake in OHSC. A) Representative photomicrograph depicting PI staining show differences in cell death between DG, CA3 and CA1 regions of hippocampus throughout time in culture in EL slices. One way ANOVA followed by Bonferroni's Comparison Test. (**Magalhães, 2015**)