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Inflammation and Neurotransmission in Mesial Temporal Lobe Epilepsy

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INFLAMMATION AND NEUROTRANSMISSION IN MESIAL TEMPORAL LOBE EPILEPSY

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Serum expression of inflammation-associated microRNAs in Mesial Temporal Lobe Epilepsy patients. Bárbara Leal, Cláudia Carvalho, Ricardo Ferreira, João Chaves, Andreia Bettencourt, Joel Freitas, Daniela Boleixa, João Lopes, João Ramalheira, Berta Martins da Silva, António Martins da Silva, Paulo P Costa **(submitted)**

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Inverse relationship between serum levels of miR-22 and hippocampal P2X7 receptor overexpression in patients with Mesial Temporal Lobe Epilepsy. Bárbara Leal, Cláudia Carvalho, João Chaves, Andreia Bettencourt, Ricardo Ferreira, Rui Rangel, Agostinho Santos, Daniela Boleixa, Joel Freitas, João Lopes, João Ramalheira, Berta M.Silva, Paulo P Costa, António Martins da Silva & Paulo Correia-de-Sá **(in preparation)**

No cumprimento do disposto no referido Decreto-Lei, o autor desta tese declara que interveio na concepção e na execução do trabalho experimental, na interpretação dos resultados e na redação dos manuscritos supracitados, sob o nome de **Bárbara Leal**.

“Maybe the shortest distance between two mind points is not a straight line”

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"Quem caminha sozinho pode até chegar mais rápido, mas aquele que caminha acompanhado, com certeza, chega mais longe" (Clarice Lispector)

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Resumo

A Epilepsia do Lobo Temporal Mesial com Esclerose do Hipocampo (MTLE-HS) é a epilepsia parcial mais frequente nos adultos. Estes doentes são geralmente refractários, com mais de 80% a não responderem à terapia medicamentosa. Nestes casos, a cirurgia ablativa do hipocampo e da amígdala constitui o tratamento mais eficaz. A amigdala-hipocampectomia é uma das cirurgias mais bem-sucedidas no tratamento da epilepsia tendo, no entanto, cerca de 38% de recidivas vários anos após a cirurgia. Assim, para os doentes com MTLE-HS a remissão das crises constitui uma necessidade médica ainda por atingir. Sendo a compreensão do processo epiletogénico crucial para o desenvolvimento de novas terapias, o maior entreve a sua concretização é o desconhecimento dos mecanismos que originam a MTLE-HS.

Vários estudos têm revelado que a neuroinflamação poderá ter um papel fundamental no desenvolvimento da MTLE-HS. O dano neuronal, as convulsões febris ou a actividade neuronal excessiva podem levar à activação da resposta imune inata com consequente expressão de citocinas pro-inflamatórias. Estas moléculas interferem com a actividade de canais iónicos e de receptores de neurotransmissores, alterando a estabilidade sináptica e diminuindo o limiar para o desenvolvimento de crises epilépticas. Através da activação de receptores ionotrópicos P2X7, o ATP libertado durante a actividade neuronal excessiva possui uma acção pleiotrópica no controlo da interacção entre neurónios e glia, na neuroinflamação e na defesa do organismo a agentes estranhos. Após a sua libertação, o ATP é rapidamente metabolizado extracelularmente em adenosina. A adenosina é um potente anticonvulsivante endógeno uma vez que inibe a libertação de neurotransmissores e induz hiperpolarização das membranas pós-sinápticas. O papel da adenosina na epilepsia está, ainda, envolto em alguma controvérsia, já que a sua acção depende do balanço entre a activação de receptores inibitórios ou facilitatórios, respectivamente dos subtipos A_1 e A_{2A}, que são os mais abundantes no sistema nervoso central

Estudos recentes mostram que tanto a neuroinflamação como a sinalização purinérgica podem ser moduladas por mecanismos epigenéticos, nomeadamente por acção de microRNAs. Estes são pequenas moléculas de RNA não codificante que funcionam como reguladores pós-transcripcionais da expressão génica. A observação de que os níveis circulatórios destas moléculas são muito estáveis e habitualmente reflectem a sua produção tecidular, nomeadamente no cérebro, sugere que estas moléculas podem constituir bons biomarcadores do processo epileptogénico.

O principal objectivo deste trabalho foi o de tentar elucidar os mecanismos envolvidos no processo epileptogénico da MTLE-HS, dando uma ênfase particular à inflamação

mediada pelas purinas e aos seus mecanismos reguladores. Para tal, realizaram-se estudos genéticos, epigenéticos e de expressão génica num grupo de 196 doentes com MTLE-HS, 24 dos quais sujeitos a amigdalo-hipocampectomia, e de 342 indivíduos controlo.

Os doentes MTLE-HS apresentavam, quer no hipocampo quer na região cortical anterior, um processo inflamatório caracterizado pela presença de células de microglia HLA-DR +, e sobre-expressão de TLR4 e IL-1β. Verificou-se, ainda, que a citocina anti-inflamatória, IL-10, se encontrava aumentada nos doentes com MTLE-HS. Estes doentes apresentavam, também, níveis séricos aumentados de miR-146a e miR-132, que são microRNAs frequentemente associados à inflamação. Verificou-se ainda que o genótipo rs16944TT do gene que codifica para a IL-1β parece ser um factor de susceptibilidade para o desenvolvimento da MTLE-HS.

Paralelamente ao fenótipo pró-inflamatório encontrado no sangue periférico e no cérebro dos doentes com MTLE-HS verificou-se a existência de uma desregulação na via de sinalização mediada pela adenosina nestes doentes. Esta consiste numa sobre-expressão hipocampal e cortical do receptor A_1 e da enzima intracelular ADK, que força a captação de adenosina pelas células, na região cortical; O receptor A_{2A} também se encontrava sobre-expresso na região cortical.

A expressão do receptor P2X7 também se encontrava aumentada, tanto no hipocampo como na região cortical adjacente dos doentes MTLE-HS. Paralelamente verificou-se uma diminuição significativa dos níveis séricos do miR-22, particularmente evidente em doentes refractários à terapêutica. Sabe-se que o miR-22 restringe a expressão do receptor P2X7 no cérebro de roedores. Foi ainda demonstrado que o processo epileptogénico pode ser antecipado por antecedentes de convulsões febris ou pela presença de isoforma ApoE ε4.

Em conclusão, os resultados obtidos mostram que o desenvolvimento da MTLE-HS humana parece estar associado a um fenótipo pró-inflamatório e a uma alteração significativa das vias de sinalização mediadas por purinas, tanto ATP como adenosina. Comprovou-se, ainda, que estas alterações não se limitam à região hipocampal mas expandem-se para o córtex temporal anterior facilitando a propagação das crises. Sabendo que os mecanismos que regulam a complexa interacção entre o sistema purinérgico, a neuroinflamação e a neurotransmissão necessitam ser aprofundados, os resultados sugerem que esta relação tripartida poderá impor-se como uma nova via a atingir na terapêutica da epileptogénese no contexto da MTLE-HS resistente aos fármacos.

Abstract

Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) is the most frequent focal epilepsy in adulthood. It is usually refractory with over 80% of the patients presenting a poor response to conventional anti-epileptic drugs (AEDs). Refractory patients are often subjected to surgical resection of the hippocampus and amygdala in order to control seizures. This is one of the most successful epilepsy surgeries. Nevertheless, it is reported a seizure recurrence of 38% several years after surgery. For MTLE-HS patients the efficient resolution of seizures is still an unmet clinical need. Understanding the epileptogenic process is fundamental to the development of new AEDs, but the pathophysiological mechanisms leading to MTLE-HS remain largely unknown.

Mounting evidence suggests that neuroinflammation is paramount in epileptogenesis. Activation of innate immune mechanisms and inflammatory responses induced in the brain by febrile seizures, injury, or cell death has been associated with acute symptomatic seizures and with a high risk of developing epilepsy. Inflammatory molecules may interfere with normal neurotransmission and synaptic plasticity phenomena, thus contributing to lower seizures threshold. Excessive neuronal firing leads to the release of huge amounts of ATP to the synaptic cleft and neighbouring cell interfaces. ATP-gated low affinity slow desensitizing ionotropic P2X7 receptors have a pleiotropic role modulating neuron-glia interaction, neuroinflammation and host defence. Once in the extracellular medium ATP is rapidly catabolised to adenosine by ecto-nucleotidases. Adenosine is a well-known endogenous antiepileptic molecule, since it attenuates neuronal activity by pre-synaptically inhibiting neurotransmitter release and by controlling neurotransmitter responsiveness at post-synaptic sites. Controversy still exists concerning adenosine's role on epileptic hippocampi perhaps because adenosine neuromodulation in this region might result from a balance between inhibitory A_1 and facilitatory A_{2A} receptors, which are the most abundant receptor subtypes in the brain. Recent studies suggest that neuroinflammation and purinergic signalling mechanisms may be modulated by epigenetic mechanisms through the action of microRNAs (miRNAs), which are non-coding RNA molecules that regulate gene expression at post-transcriptional level. Taking into consideration that miRNA levels are very stable in biological fluids and normally reflect tissue production, it was suggested that these molecules could be good biomarkers for epilepsy.

The main aim of this study was to approach MTLE-HS pathogenesis focusing in particular the mechanisms underlying neural inflammation and purinergic signalling unbalance in order to unravel new molecular targets for therapeutic intervention in drug-resistant

epilepsy. To this end, genetic, epigenetic and gene expression studies were performed in a cohort of 196 MTLE-HS patients, 24 of which underwent amigdalo-hippocampectomy, and 342 controls.

Inflammation characterized by the presence of HLA-DR$^+$ microglia and overexpression of TLR4 and IL-1β was evident both in the hippocampus and anterior temporal cortex of MTLE-HS patients. We also observed that IL-10 is overexpressed in MTLE-HS patients; interestingly, this anti-inflammatory cytokine is primarily produced by circulating monocytes, which are the precursors of brain microglia. Likewise, serum levels of inflammation-associated miRNAs, like miR-146a and miR-132, were also upregulated in patients compared to control individuals. Moreover, MTLE-HS patients had a higher frequency of the rs16944TT genotype coding for the pro-inflammatory cytokine, IL-1β, than control individuals.

In parallel to the pro-inflammatory phenotype, MTLE-HS patients also exhibit marked changes in the adenosine signalling system. This was evidenced by significant increases in the expression of the A_1R and adenosine kinase (ADK) both in the hippocampus and adjacent neocortex. It is worth to note that ADK overexpression is linked to increased inactivation of the nucleoside being the most relevant driving force for the reuptake of adenosine in the brain. Concerning the A_{2A} receptor, we observed that it was upregulated in the anterior cortical region, but not in the hippocampus.

The P2X7R expression was also higher in the hippocampus and anterior temporal lobe of MTLE-HS patients compared to control individuals. We detected a reduction in the miR-22 serum levels, particularly in drug-refractory patients; this may be clinically relevant since miR-22 is a known P2X7R suppressor in the brain of rodents. Our findings show that patients expressing ApoE ε4 as well as patients with febrile seizures (FS) antecedents had an earlier MTLE-HS onset.

In conclusion, data suggest that the pathogenesis of human MTLE-HS is associated with a pro-inflammatory profile underlying significant changes in the purinergic signalling pathway, involving both ATP and adenosine. Additionally, we showed that these alterations are not limited to the hippocampal formation, but are also evident in the anterior temporal lobe which might facilitate seizures propagation. Knowing that the mechanisms that modulate the complex interplay between the purinergic signalling cascade, neuroinflammation and neurotransmission require further investigations, the results presented here prompted us to hypothesize that targeting these pathways may constitute a valuable novel approach for the treatment of refractory MTLE-HS.

Table of contents

Abbreviation list

5'NT	-	5' Nucleotidase
A1R	-	Adenosine A_1 Receptor
A2AR	-	Adenosine subtype A_{2A} receptor
AchE	-	Acetylcholinesterase
ADA	-	Adenosibe deaminase
ADK	-	Adenosine Kinase
ADP	-	Adenosine Phosphate
AEDs	-	Anti-Epileptic Drugs
AMPA	-	α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic acid
ApoE	-	Apolipoprotein E
Argo	-	Argonaute
ATP	-	Adenosine Triphosphate
BBB	-	Blood-Brain Barrier
BDNF	-	Brain-derived Neurotrophic Factor
CA	-	Cornus Ammonis
cAMP	-	cyclic AMP
CNT	-	Concentrative Nucleoside Transporters
DAB	-	3'3 -Diaminobenzidine tetrahydrochloride
DAMP	-	Damage-Associated Molecular Pattern
DNA	-	Desoxiribonucleic Acid
EctoN	-	Ectonucleotidases
EEG	-	Electroencephalography
ENT	-	Equilibrative nucleoside transporters
FS	-	Febrile Seizures
GABA	-	Gamma Aminonutyric Acid
GWAS	-	Genome Wide Association Studies
HLA	-	Human Leukocyte Antigen
HMGB1	-	High Mobility Group Box 1
ILAE	-	International League Against Epilepsy
IRAK	-	IL-1 Receptor-Associated Kinase
LTP	-	Long Term Potentiation
miRNA	-	microRNA
MRI	-	Magnetic Resonance Imaging
mRNA	-	messenger Ribonucleic Acid
MTLE-HS	-	Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis
MyD88	-	Myeloid Differentiation primary response gene 88
NF-kB	-	Nuclear Factor - Kappa B
NGF	-	Nerve Growth Factor
NMDA	-	N-methly- D-aspartate
OR	-	Odds Ratio
PAMP	-	Pathogen-Associated Molecular Pattern
PCR	-	Polymerase Chain Reaction
RFLP	-	Restriction Fragment Lenght Polymorphism
RNA	-	Ribonucleic Acid

SAH	-	S-adenosylhomocysteine
SAM	-	S-adenosylmethionine
SNP	-	Single Nucleotide Polymorphism
SPECT	-	Single-photon emission computed tomography
SPSS	-	Statistics for Social Package
SSP	-	Sequence Specific Primer
TLR	-	Toll-like Receptor
TNF	-	Tumour Necrosis factor
TRAF	-	Tumour Necrosis factor receptor-associated factors
UBC	-	Ubiquitin C
WHO	-	World Health Organization

Scope of the thesis

Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS), the most common focal epilepsy in adults, is highly refractory to pharmacological treatment with more than 80% of patients having a poor response to anti-epileptic drugs (AEDs). In these cases, surgery for removal of affected area is the most effective treatment for seizure remission, but can have detrimental effects on patients' life and a high cost for health national systems. So, it urges to identify new therapeutic targets what can only be accomplished with a better knowledge of epileptogenic processes and their regulatory mechanisms.

The aim of this work is to contribute to fill the gaps in our knowledge regarding the pathogenesis of epilepsy, focusing on the role of inflammation as an epileptogenic mechanism and on the importance of the purinergic signalling pathway in epilepsy. Specifically, objectives were as follows:

1. To characterize MTLE-HS pathogenic mechanisms based on patients' clinical history specifically looking for Febrile Seizure antecedents.

2. To study the importance of inflammation in epilepsy development.

3. To analyse the contribution of purines to the regulation of neurotransmission and neuroinflammation

4. To evaluate the contribution of regulatory epigenetic mechanisms in MTLE-HS development.

Studies were performed in a cohort of 196 MTLE-HS patients, 24 of which were submitted to amygdalo-hippocampectomy. The study comprised genetic, gene expression and epigenetic analyses.

CHAPTER I

General Introduction

1. Epilepsy history: from the sacred to the unknown

Epilepsy is a chronic neurologic disease that affects approximately 50 million people worldwide, 80% of them living in low or middle-income countries. In Europe it is estimated that more than 6 million inhabitants suffer from this pathology, according to data from International League Against Epilepsy (ILAE) and World Health Organization (WHO). In Portugal, according to an estimate made by LPCE (*Liga Portuguesa Contra a Epilepsia*), 4 – 7 /1000 inhabitants are affected by this condition. Accordingly to world health organization, 2.4 million people / year are diagnosed with epilepsy.

Epilepsy is one of the oldest identified conditions with records dating back to 4000 B.C. The term Epilepsy derives from the ancient Greek "ἐπιλαμβάνειν = *epilambanein*" that means "to seize, possess, or afflict". The definition is based on the belief that a seizing person was possessed by demons. Nowadays, remarkably, in some parts of the world this is still believed! In 400 B.C., Hippocrates hypothesized that phenomena that characterize a seizure have origin in the brain. This was proven only in the 19th century by Fritsch and Hitzig, and John Hughlings Jackson, who demonstrated that a seizure is a manifestation of a disturbed neuronal electrical activity. In 1930, the EEG's development, allowed the observation of the abnormal neuronal activity, demonstrating definitely that a seizure is a dysregulation in the normal neuronal transmission. Although major advances on the study of epilepsy have been made, a major gap arises concerning the etiopathogenic mechanisms and treatment.

A brief introduction to the basic principles of neuronal functioning will be necessary to understand the main aim of this work: to contribute to the advancement of knowledge on the pathogenesis of epilepsy.

1.1. Brain function and control of synaptic transmission

The brain is the most complex organ of vertebrates. It receives, processes, and analyses messages from the surroundings generating a response. Are those responses that allow us to think, breath, and move, speak, show emotion, generate memories and regulate all our other bodily functions.

The functional unit of the brain is the neuron, as identified by Ramón y Cajal in 1891. The brain contains an overwhelming one hundred billion of neurons that communicate with each other through chemical and electrical signals in a process known as synaptic transmission. The information is generated and propagated within the neuron through an electrical signal, the action potential. This is a crucial and transient event in which the

membrane potential changes in milliseconds (Figure 2). Neurons, as all animal cells, are electrically polarized, maintaining a voltage difference across the plasma membrane. This electrical polarization is the result of a complex interplay between ion channels and ion pumps present in the cell membrane. In neuronal membranes different types of ion channels exist (Figure 1).

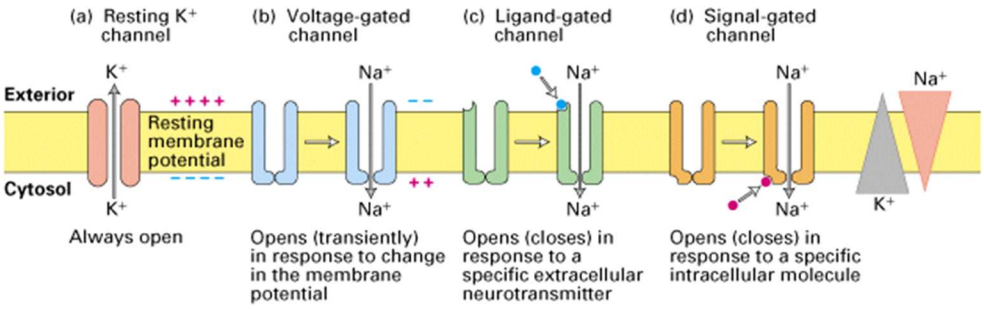

Figure 1 – Ionic channels in neurons membrane a) Resting K^+ channels the resting potential across the membrane. (b) Voltage- gated channels respond to differences in potential membrane. c) Ligand-gated channels open or close in response to the binding of a neurotransmitter (ex AMPA, NMDA); d) Signal-gated channel is couple to a neurotransmitter receptor (not depicted) and respond to intracellular signals (Ca^{2+}, cGMP, cAMP, Gα subunits) generated by the neurotransmitter – receptor interaction. (AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP = cyclic Adenosine Monophosphate; cGMP = Cyclic Guanosine Monophosphate NMDA =N-methyl - D aspartate) From Lodish 4th Edition.

In the resting state, extracellular sodium (Na^+) and chloride (Cl^-) concentrations are higher than their intracellular concentrations whilst potassium (k^+) ions are more concentrated in the intracellular medium. In this situation intracellular medium is negative making the resting potential with the value of ≈ -70mV. When a signal is received by receptors in the pre-synaptic membrane, Na^+ channels open and the cell interior becomes more positive. In response to this voltage change, or depolarization, voltage-gated Na^+ channels open and Na^+ ions continue to enter in the cell rendering the cell more positive (≈ +30mV). At this point, sodium channels close and potassium channels open and the system reach neutrality. With the K^+ efflux, the membrane begins to repolarize towards its rest potential. In addition to voltage-gated, other k^+ channels open in response to calcium (Ca^{2+}) influx (Ca^{2+} - activated K^+ channels). In this way, the resting potential is overreached and membrane potential reaches the potassium equilibrium voltage ≈ -90mV. This hyperpolarization or afterhyperpolarization inhibits the neuron from starting another action potential, preventing it from becoming overactivated and assuring that signal transmission is unidirectional. The Na^+/K^+ pump and Inward Rectifier K^+ channels (Kir) drive the K^+ influx and the resting potential is finally reached (Figure 2). Contrarily to what happens with neurotransmitter receptors, the action potential functions accordingly to the principle

of all-or-none, meaning that the stimulus induces or not the action potential which strength is not determine by stimulus intensity.

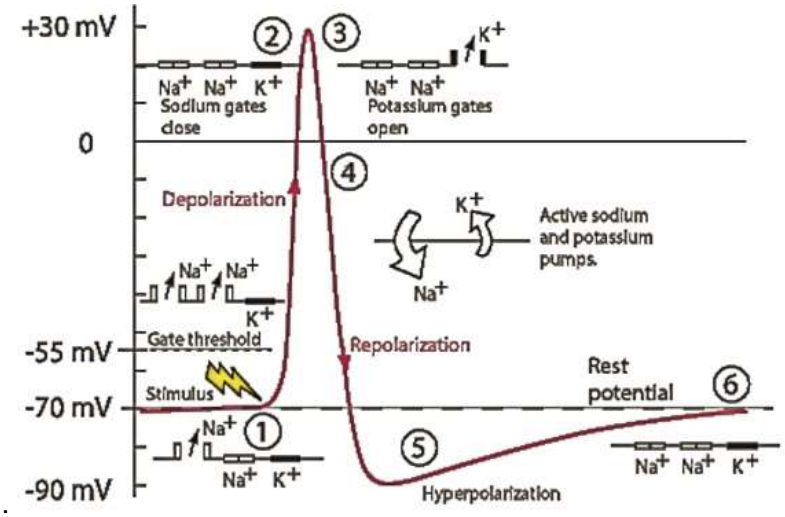

Figure 2 – The Action potential. (1) A stimulus leads to the opening of sodium channel which activates voltage-gated Na^+ channels with continuous influx of Na. (2) Sodium channels close and (3) potassium channels open in order to revert the cell polarity and membrane begins to repolarize. Other potassium channels are activated in response to calcium influx and the resting potential is overreached towards the potassium equilibrium voltage ≈ -90mV. The Na^+/K^+ pump and Inward Rectifier K^+ channels (Kir) drive the K^+ influx and the resting potential of ≈ -90mV is finally reached. From http://hyperphysics.phy-astr.gsu.edu

The action potential is then transmitted throughout the neuron till the axons where it induces the release of a neurotransmitter. In the synaptic cleft the neurotransmitter binds to a receptor in the post-synaptic membrane. This may have an excitatory – allow the generation of an action potential – or inhibitory action – stops the formation of an action potential. This communication is done in a fast way and allows neurons to communicate with other neurons in the neighbourhood or in the vicinity.

A tight regulation of ionic balance is fundamental for a succesfull neurotransmission. Other levels of regulation, with a myriad of molecular mechanisms, function for a fine-tuning synaptic transmission[1].

In 1990's several authors suggested that astrocytes may be an important player in this regulation. It was observed that synaptic-derived glutamate, was able to activate the neighbouring astrocytes leading to Ca^{2+} astrocytic influx [2]. Additionally, the Ca^{2+} influx lead to the release of glutamate from astrocytes which modulated synaptic activity. The observations of a cross-talk between astrocytes and neurons lead to the creation of the 'tripartite synapse' concept[2] (Figure 3). It is now known that ≈ 40% hippocampal synapses

are surrounded by astrocytes that sense and respond to neuronal activity [3]. The key player in astrocytic excitability is the Ca^{2+} intracellular concentration which can be controlled by intrinsic oscillations or by synaptic modulation of vesicle release[4]. It has been observed that Ca^{2+} levels are nonlinearly modulated by simultaneous neurotransmitters and synaptic pathways (for review see e.g. Perea et al, 2009[5]). Experimental studies showed that the Ca^{2+} signal generated in one astrocyte can propagate as a calcium wave to dozens of neighbouring astrocytes. Nowadays it is known that the calcium wave stimulates not only the release glutamate but also other gliotransmitters exerting excitatory (e.g. ATP, serine) and inhibitory (e.g. GABA, adenosine) effects.

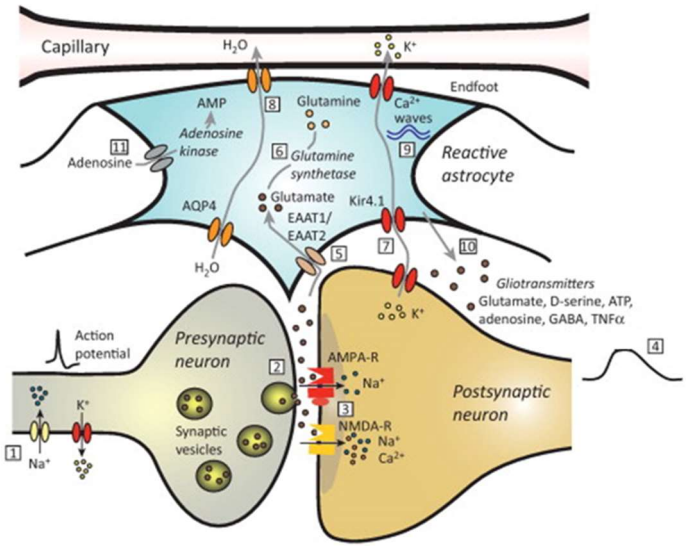

Figure 3 – Schematic model of a Tripartite Synapse showing interactions between astrocytes and excitatory neurons. (1)Voltage-gated Na^+ and K^+ channels generate action potentials in the presynaptic neuron, leading to glutamate release in the synaptic cleft (2). Glutamate activates its receptors (AMPA and NMDA) (3) in the postsynaptic membrane, leading to the Na and Ca^{2+} influx initiating an action potential in the postsynaptic neuron (4). (5) Astrocytes uptake glutamate through specific transporters EAAT1 and EAAT2 converting it to glutamine by the action of glutamine synthetase (6). 7) K^+ released from neurons through voltage-gated (outwardly rectifying) K^+ channels, enters the astrocyte via Kir4.1channels and distributed into capillaries. Water balance modulation is performed by AQP4 at astrocytic endfoot processes (8). Ca^{2+} waves (9) generated within the astrocyte leads to the release of gliotransmitters (10). The enzyme adenosine Kinase (ADK) in astrocyte membrane removes adenosine from synaptic cleft. (AMP = adenosine monophosphate; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AQP4 = Aquaporin-4; ATP = adenosine triphosphate; EAAT = **excitatory amino acid transporter**, GABA = Gamma Aminonutyric Acid; Kir = inwardly rectifying K^+; NMDA = N- Methyl D-aspartate; TNF α= Tumour Necrosis Factor alpha) (Adapted from Devinsky et al, 2013[6])

Besides their importance in synaptic transmission, astrocytes also play an important in the maintenance of Blood-Brain Barrier (BBB) integrity releasing molecules that form and sustain tight gap junctions. These cells are important for cellular homeostasis as well, modulating the ionic and water balance allowing exchanges with capillary vessels[5,6]

(Figure 3). In this context, movement of potassium ions is important for the regulation of cerebral blood flow in response to variations in neuronal activity[7].
Unlike neurotransmission that occurs within milliseconds and targets precisely the post-synaptic neuron, astrocytic-born effects may last for several seconds and mediates volume transmission throughout cells in the vicinity. This allows astrocytes to control synaptic tuning/strength and contributes to long term synaptic plasticity (Long Term Potentiation, LTP), which are important phenomena involved in learning and memory.

Synaptic transmission is a tightly regulated process and its dysregulation is associated with the development of several neurological pathologies, such as epilepsy.

1.2 Epilepsy: dysregulation in action

The term epilepsy encompasses a wide range of different syndromes[8] characterized by an "abnormally increased predisposition for epileptic seizures and the neurobiological, cognitive, psychological and social consequences"[9]. Seizures are a manifestation of an "abnormal excessive or synchronous" neuronal activity resulting in electric discharges[9]. The discharges may occur in both hemispheres in generalized seizures or may be limited to a group of neurons in a defined localization in one hemisphere in focal epilepsies. Seizures occur due to a dysregulation of normal neurotransmission that results in excessive excitability or diminished inhibition.
Dysregulation of neurotransmission may occur due to unbalanced ionic gradients, disequilibrium between excitatory and inhibitory neurotransmitters levels, or malfunctioning of neurotransmitters receptors. Several factors can contribute to these unbalances. The aetiological classification of seizures has evolved through the years and recently ILAE have proposed a new classification: structural, metabolic, immune, infectious, genetic, or unknown. In structural epilepsies a distinct structural lesion underlies the occurrence of seizures. A great number of metabolic diseases are associated with epilepsy. In these cases, seizures are associated with a well-defined metabolic defect with biochemical changes throughout the body. Immune epileptic seizures develop due to autoimmune-mediated inflammation of the CNS. Infectious epilepsies are the most common aetiological subtype. The term does not refer to seizures occurring in the context of a CNS infection such as meningitis but rather to seizures occurring in the context of an infection with an agent such as HIV, tuberculosis, or neurocysticercosis. Genetic epilepsies result from a, known or presumed, genetic defect. In epilepsies of unknown aetiology is not possible to know the causal factor and diagnosis relies only in specific electroclinical semiology.

One aetiology does not discard other intervenient although can help to define the therapeutical options. For example, genetic factors can underlie structural or metabolic abnormalities. In fact, it has been reported that 70-88% of epilepsy susceptibility is attributable to genetic factors being even suggested the existence of a genetic basis transversal to all epileptic syndromes. Recently, a meta-analysis of Genome Wide Association Studies (GWAS) suggested that mutations in the sodium voltage-gated channel alpha subunit 1 (SCN1A) gene may be a transversal susceptibility factor for generalized and focal epilepsies[10]. The majority of epilepsies have complex multifactorial aetiologies with the interaction of multiple acquired and genetic factors in an unknown ethiopathogenic mechanism[11]. The diverse epileptic syndromes have different clinical manifestations, prognosis, and treatment options.

Epilepsy diagnosis is based on clinical, imaging and electroclinical data. The occurrence of an epileptic seizure does not determine epilepsy. In fact, more than 10% of population worldwide may have a seizure during their life time. These are mainly due to drugs, alcohol, or sleep privation. The diagnosis of epilepsy requires the occurrence of at least 2 unprovoked (reflex) seizures 24h apart[12] or "one unprovoked seizure when the risk for another is known to be high (>60%)" (ILAE, 2014).

1.2.1 Refractory Epilepsy: much more than seizures

Although great advances have been made, to date no cure for epilepsy exists. The available therapies are mostly anti-seizure in detriment of anti-epileptogenic approaches. This means that they only inhibit excessive excitability preventing the symptoms (seizures), but do not change the underlying disease process (epileptogenesis). Currently, there are more than 25 available Anti-Epileptic Drugs (AEDs) targeting different molecules, resulting mainly in enhanced GABAergic neurotransmission or inhibition of Na^+ influx (Figure 4). It is described that 30% of epileptic patients are refractory to treatment having a poor response to AEDs and continue to have seizures[13]. Patients are considered refractory if they do not respond to maximal doses of two or more conventional AEDs used for more than 2 years. Recurrent seizures contribute to the progression of epilepsy as well as to its physical and social impairments. The burden of disease may have a greater impact than the seizures itself. Epileptic patients often suffer from discrimination, social stigma and prejudice. Daily life domains such as employment, education, cognition or interpersonal skills may become affected[14]. Refractoriness can have a serious impact in quality of life with several associated risks such as injuries, increased morbidity and mortality[15-18]. Refractory epilepsy is also associated with major socioeconomic costs with more than 5 million euros of expenses per year only in Europe. More than 70% of this

budget are claimed to be for indirect expenses with unemployment from the patient and their familiar caregiver [19].

Multiple mechanisms are associated to refractoriness. Some patients are refractory from the start due to individual factors, such as variants of genes encoding for certain proteins such as P-glycoprotein, which is important for pharmacokinetics (e.g. blood-brain barrier permeability) and pharmacodynamics of AEDs. Other patients have an initial positive response but seizure-induced changes, both in drug targets or brain structures, or even seizure severity lead to the development of refractoriness.

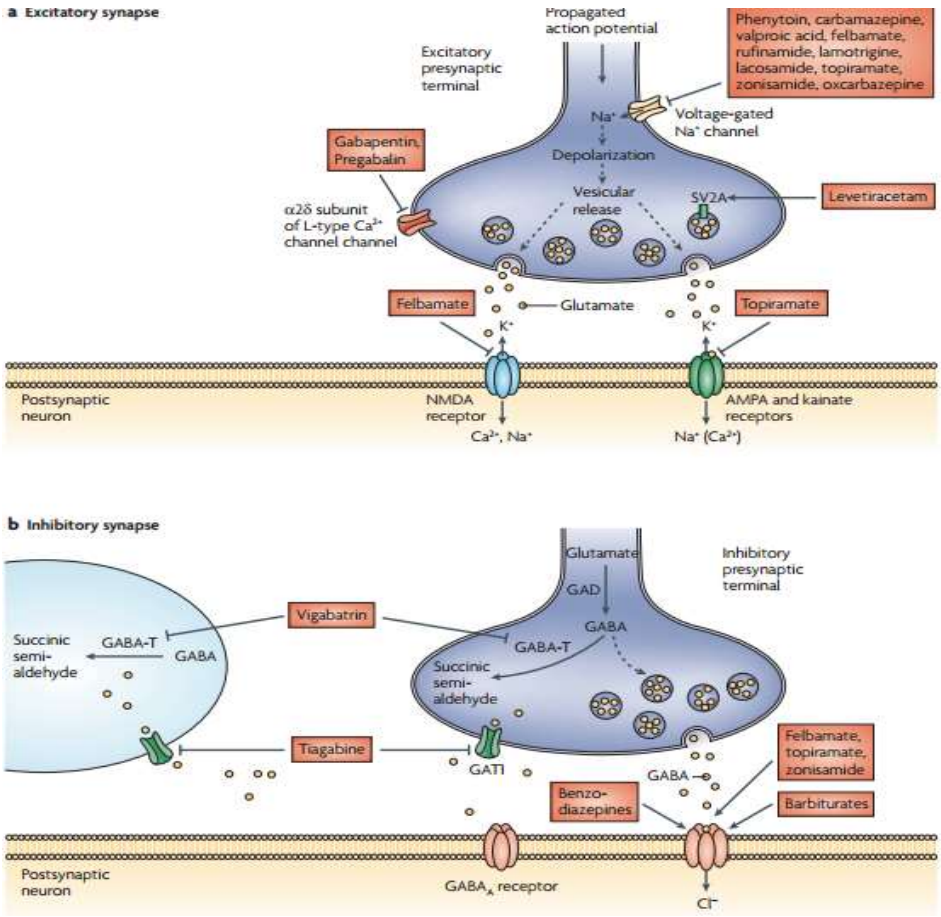

Figure 4 – Action mechanisms of currently available AEDS. AEDS can act both in a) excitatory and b) inhibitory synapses. AEDS target several molecules such as voltage-gated channels modifying essentially Na^+ and Ca^{2+} currents and NMDA and AMPA receptors. GABA transport, metabolism and receptors are also target by AEDs. Sodium channels are target by different AEDs and the same AED molecule may have different action mechanisms and the net effect is the downmodulation of excitability in order to prevent seizures. GABA= γ aminobutyric acid; GABA T = GABA transaminase; GAD = glutamic acid decarboxylase; GAT-1 = GABA transporter 1; NMDA = N-Methyl-D-aspartate; SV2A = synaptic vesicle glycoprotein 2A From: Bialer and White, 2010[20]

In refractory cases, non-phamacological tratments may be used with moderated efficacy. Among these are included dietary strategies, such as ketogenic diet, neurostimulation, or even focal cooling of the brain. The most widely used in focal epilepsies is the surgical removal of seizure foci. Patients must have a complete pre-surgical assessment comprising brain MRI (minimum 1.5 tesla), prolonged video-EEG recording, ictal and interictal SPECT, neuropsychological assessment and functional brain MRI. After this evaluation not all patients are suitable for the procedure.

The most refractory epileptic syndrome is Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS).

2. MTLE-HS: an unsolved puzzle

Temporal Lobe Epilepsy (TLE) is the most common focal epilepsy accounting for 60% of all epileptic patients. Two types of TLE are considered depending on the region of temporal lobe that is being affected. In Neocortical or Lateral TLE, seizures involve the outer region of temporal lobe and are frequently associated with tumours. The most common type is Mesial Temporal Lobe Epilepsy (MTLE) in which seizures have origin in internal structures of the temporal lobe such as hippocampus and other structures of limbic system. It usually begins in the late childhood or early adolescence[21]. A prototypical MTLE seizure usually lasts 1 minute or less and consists of an aura followed by automatisms and consciousness alterations with probable amnesia [21]. Auras are frequently epigastric or a déjà-vu sensation, but fear, anxiety, or an unknown sensation, have also been described. Limb automatisms, dystonic posturing and oroalimentary automatisms are also frequently reported [22].
The most common neuropathological finding in MTLE is Hippocampal Sclerosis (HS) characterized by selective neuronal loss, gliosis and neuronal reorganization in the CA1 (cornus ammonis), CA3 and CA4 subregions [23-27]. CA4 is the region most vulnerable to damage whilst CA2, dentate gyrus and subiculum are more preserved[21,28,29].

Neuronal loss can affect both excitatory and inhibitory (interneurons) pathways and is accompanied by mossy fibres (axons of granule cells) sprouting within the molecular layer of dentate gyrus[30]. These mossy fibres can establish excitatory synapses with dendrites from the inner molecular layer originating a recurrent excitatory circuit[31]. It has been observed that this synaptic reorganization may extend to the contralateral hemisphere[28] supporting the hypothesis that recurrent seizures contribute to anatomical and functional abnormalities[32]. Gliosis is an HS hallmark with dense astrocyte proliferation (astrogliosis) in regions such as dentate gyrus, CA1 and CA3 subfields where neuronal loss is observed[22]. It has been reported that astrocytes within epileptic focus present distinct

morphologies and functional phenotypes. These abnormalities may comprise, among others, changes in K^+ channels, in glutamate transporters or in glutamate-metabolizing enzymes, and may contribute to sustained excitability. Microglial activated cells are also present in these regions[33]. Different patterns of abnormalities may be found in patients[34] and currently, accordingly to Blumcke *et al.* four major HS types are considered[35] (table 1).

Table 1 – Histopathological features of HS subtypes

HS type	Main Histopathological Characteristics
1a (classic hippocampal sclerosis)	Severe cell loss in CA1 and moderate loss in other subfields, excluding CA2
1b (severe hippocampal sclerosis)	Severe cell loss in all hippocampal subfields
2 ("atypical" hippocampal sclerosis)	Severe neuronal loss restricted to CA1
3 ("atypical" hippocampal sclerosis)	Severe neuronal loss restricted to the hilar region

Dispersion of the dentate gyrus' granule cell layer has also been observed[36]. It is claimed that cytoarchitectural changes of granule cell layer are associated with increased abnormal neurogenesis as well as with modifications in molecular mechanisms that govern cell migration[36].

It has also been described that network reorganization and structural abnormalities extend far beyond the hippocampus and can also be observed in white matter with the presence of heterotopic neurons in the subcortical region[37]. Impaired white matter integrity has been associated with an early seizure onset[38] and may contribute to seizure propagation[39]. Neuropathlogical abnormalities may also be found in other constituents of the limbic system, such as amygdala, or even in other brain regions.

The casual relation between HS and MTLE is not yet fully understood[40]. In one hand prolonged seizures can cause cell death and HS is a consequence of epilepsy. Accordingly, it has been observed that one-third of asymptomatic first-degree relatives of MTLE patients have MRI features of HS[41] and HS has also been observed in an elderly population without seizures[42]. These data suggest that the lesion is not sufficient to develop MTLE and that when observed in MTLE patients is rather a consequence of seizures. On the other hand, as discussed above, changes seen in HS may contribute to seizure development or progression. The observation supports the idea that HS is a cause of seizures is the observation that surgical removal of sclerotic hippocampus is effective in seizure control in 50-60% patients[43,44].

Retrospective studies show that the majority of HS patients have a history of an initial precipitating injury such as central nervous system infection, head or birth trauma, hypoxia

peripartum or febrile seizures (FS)[45]. Among these factors FS is the most common: it is claimed that up to 80% of MTLE-HS patients have a previous history of FS[46-49]. Febrile Seizures, the most common neurological event in children, consist of seizures occurring in the context a febrile illness without evidence of a CNS infection[50]. Epidemiological studies show that 5% of children under the 5 years age have at least one FS[51].

It has been hypothesized that after the initial insult there is a latency period with an abnormal cascade of damage repair, leading to atrophy and sclerosis of hippocampus[25]. Supporting this association, imaging studies have shown that prolonged and lateralized FS can produce acute hippocampal injury with oedema that resolves within 5 days[52]. The follow-up of these children showed changes in hippocampal symmetry consistent with injury and neuronal loss[52]. So, it is believed that FS initiate the abnormal network reorganization that will lead to the development of an epileptogenic zone. Accordingly, it has been observed that FS are associated with modifications in ionic channels and neurotransmitters' receptors contributing in this way to neuronal excitability[53-61]. Alternatively, the asymmetry could represent a return (post-acute oedema) to a pre-existing hippocampal abnormality similar to that identified in family members of MTLE patients with history of FS[62]. Conversely, a recent study, observed that complex FS do cause white matter abnormalities that are seen 6 months later. But the follow-up revealed that the abnormalities disappear in the majority of children 1 year after the FS episode [63].

Although, the relation between FS and MTLE-HS is recognized it still remains controversial [64]. Epidemiological studies have showed that 2 to 10% of children who had at least one FS will develop afebrile seizures subsequently[65-67]. Later, Vestergaard *et al.*[68], evaluated the association between FS and epilepsy in a population-based cohort of 1.54 million persons (49 857 FS and 16 481 epilepsy) and found that 7% of children with FS developed epilepsy during the 23 years of follow-up[68]. Similar results were found in a study in the English population[69]. Some authors consider that FS occur primarily in predisposed (genetic factors, development dysplasia or pre/post acquire condition such as trauma) individuals being a marker of individuals that are predetermined to develop MTLE-HS. Others believe that FS may occur in uncompromised brain but lead to MTLE-HS development only in predisposed individuals. In some studies it is considered that FS by itself lead to epilepsy development in individuals who otherwise might not develop MTLE-HS[68].

The study of the role of FS or other initial precipitant factor in MTLE-HS development has some limitations inherent to the fact that the observations are, in the majority of cases, retrospective. Exhaustive medical records may not always be available, incidental precipitating factors could be listed by mistake or testimonial from older relatives may not

be obtainable[22]. This scenario is further complicated since antecedents are not always present in MTLE-HS. It will be important to understand this relation since history of initial precipitating factors as well as age at onset of epileptic seizures and duration of the latency period may affect the clinical presentation and the prognosis of MTLE-HS. Over 80% of the patients have a poor response to conventional AEDs. In such cases, surgical ablation of the hippocampus and amygdala is the last resource to control epileptic seizures. Notwithstanding this, 47% and 38% of the patients report seizure recurrence 10 and 18 years after surgery, respectively[43,44]. Thus, these patients remain with unmet clinical needs. Understanding the epileptogenic process is fundamental to the development of new AEDs, but the mechanism leading to MTLE-HS remains largely unknown.

3. Uncovering MTLE-HS pathophysiological mechanisms

The majority of data on molecular mechanisms that govern seizure in MTLE-HS comes from animal models. From the different seizure-induced models existent the ones that most resembles MTLE-HS are *Status Epilepticus* (SE) models. SE can be induced by different protocols such as kindling, kainic acid or pilocarpine injection, hyperthermia among others. The most used to study MTLE-HS are kainic acid or pilocarpine injection since are the ones less labor-intensive and mirror clinic-pathological features such as the latency period, the development of spontaneous motor seizures and a spectrum of anatomical abnormalities that resemble hippocampal sclerosis. Nevertheless, all data obtained in the study of animal models should be carefully extrapolated since these animals develop very rapidly much more severe seizures than human MTLE-HS.

The fact that MTLE-HS is, in a great proportion of cases, submitted to resection surgery, allow us the unique opportunity to study what happen in the epileptic focus.
The combination of animal models studies with observations in human tissue has already shown that the epileptogenic proces is associated with a profound change in gene expression. These changes are dynamic and affect all epileptogenic phases. These studies also allowed us to understand that epileptogenesis is not limited to alteration in neurotransmitters' pathways or ionic channels, but other mechanisms are involved as well.

The interplay between ATP, adenosine, neurotransmission and neuroinflammation seems to be important in MTLE-HS development. Accordingly, studies, both in animal models and MTLE-HS have demonstrated that the inflammatory response is among the biological processes most upregulated in epileptogenesis as well as in chronic epilepsy phase [70-72].

3.1. Inflammation in the Central Nervous system: repair and neuroprotection

The presence of the Blood-Brain barrier (BBB), the absence of a conventional lymphatic system, and the low monocytes and lymphocytes traffic with the periphery may lead one to think that Central Nervous System (CNS) is an immune-privileged organ. Nevertheless, it is more accurate to consider the CNS as an immunological specialized organ as inflammatory reactions do occur in CNS. These inflammatory reactions can be originated within the CNS or be imported from the periphery [32,33].
Nowadays, cytokines are claimed to be important in neuronal development controlling neurite outgrowth, neurogenesis and cell survival [73]. A role in modulation of synaptic pruning, transmission and plasticity in adult brain, has been described as well [74].

In normal physiological conditions pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, and their receptors are constitutively expressed at low levels in different brain regions by astrocytes, microglia cells, neurons and endothelial cells[73]. Pro-inflammatory cytokines can modulate voltage-gated and receptor-coupled ionic channels [75-77] as well as neurotransmitter's receptors[78,79] (Table 2). In fact, IL-1β and IL-6 are described to have a general inhibitory action on CNS voltage-gated channels inhibiting Ca^{2+}, Na^{+} and K^{+} currents (for review see Vezzani *et al.*, 2015[73]). Concerning receptor-gated ion channels, IL-1β promotes NMDA-mediated Ca^{2+} influx[80-82]. This happens since in the hippocampus IL-1R1 is co-expressed with NMDA receptors[80,83] and is able to interact with its NR2B subunit[83]. Through the activation of a complex signalling cascade of proteins kinases, cytokines promote the phosphorylation of NR2B[80,81,84], and of AMPA[85] and GABAA receptor subunits[86,87]. In this way, pro-inflammatory cytokines influence ionic and neurotransmitter - induced currents, and consequently the strength of synaptic transmission (for review see Vezzani et al, 2015[73]). Additionally, IL-1β can favour the release of several neurotransmitters such as glutamate, GABA or adenosine[88,89]. The net effect of the cytokine action will depend on the presence of other cytokines / signalling molecules, cytokine concentration, time of exposure, receptors that are being activated and brain region affected. In accordance, several studies demonstrated that a fine-tuned cytokine production is necessary for learning and cognition[90,91] and that a dysregulation may lead to excitotoxicity.

3.1.1. Inflammation and epilepsy: a vicious cycle

Glial cells – astrocytes and microglial cells – are crucial for brain homeostasis and damage repair. When these cells are stimulated by infectious agents or injured they become activated, proliferate and produce inflammatory mediators that will then resolve injury and repair the damage. The inflammatory reaction stimulates anti-inflammatory molecules and growth factors resolving the inflammatory response.

When this process is deregulated, due to failure in normal feedback mechanisms or to a genetic predisposition, high levels of inflammatory molecules may cause neurodegeneration and neurotransmission imbalance with the development of several pathologies.

Table 2 – Effects of cytokines on voltage-gated channels (VGC) and ligand gated channels (LGC) (adapted from Vezzani et al, 2015[73])

Cytokine	VGC	Effect	LGC	Effect
IL-1β	VGSCs - Nav	Increased Na^+ currents Hyperalgesia	NMDAR-NR2B	Increased Ca^{2+} influx Hyperexcitability/Excitotoxicity Increased seizure susceptibility
	α1subunit	Reduced Na^+ currents Neuroprotection		
	VGCCs – Cav L- and N-type	Reduced Ca^{2+} influx Reduced Ca^{2+} currents Neuroprotection	GABA-A R β2/β3 subunits α5 subunit	Increased GABA current (Xenopus laevis oocytes) Increased tonic GABA current
	VGKC – Kv	Reduced K^+ currents Neuroprotection Increased excitability Hyperalgesia		
TNFR1 (p55)	5VGSCs – Nav 1.3 Nav1.7; Nav1.8	Enhanced TTX-R and TTX-S Na^+ currents Pain facilitation	AMPA R – GLUR2	Increased Ca^{2+} influx Hyperexcitability/Excitotoxicity
	VGCCs – Cav	Decreased Ca^{2+} currents	5AMPAR-GLUR1 5NMDAR-NR1	Increased seizure susceptibility
			GABA-A R β2/β3 subunits	Decreased inhibitory synaptic strenght
TNFR2 (p75)	5VGSCs – Nav Nav1.7; Nav1.8	Enhanced TTX-R and TTX-S Na^+ currents Pain facilitation	AMPAR-GLUR2 GLUR3	Decreased response to glutamate
			KA-GLUR6/7 5NMDAR-NR2	Decreased seizures
	VGCCs – Cav Cav3.2	Increased Cav3.2 expression		
IL-6	VGSCs – Nav Nav1.7	Increased number of spikes; Decreased latency to first AP Hyperexcitability	mGLUR2/3	Alterations in presynaptic glutamate release and changes in synaptic network activity
	α1 subunit	Reduced Na^+ currents Neuroprotection	AMPAR-GLUR2	Reduced Ca^{2+} influx
	VGCCs – Cav L-Type	Reduced Ca^{2+} currents Neuroprotection	NMDAR-NR1 GABA-A R	Decreased GABA current

Active inflammation has been documented not only in traditionally assumed inflammatory epilepsies but also in patients with pharmacoresistant epilepsies of diverse causes, especially MTLE-HS[92]. This inflammation is translated in the activation and proliferation of microglial cells and upregulation of pro-inflammatory cytokines. Until recently, inflammatory reactions were considered an epiphenomenon of seizure-induced damage. Nowadays, several studies have demonstrated that inflammatory molecules may indeed contribute to seizure propagation and exacerbation[76]. In fact, neuroinflammation appears to be an important component in epileptogenesis, reflecting complex cross-talks between microglia, astrocytes and neurons[6,93].

Microglial cells, the brain resident macrophages, are central players in brain immune responses being the mediators of both innate and adaptive immune mechanisms. The outcome of its activation is context dependent and determined by type and duration of the inflammatory stimulus and by the molecules produced and its receptors[94]. Animal and human studies have demonstrated that microglial cells are important to seizure-induced inflammation. Its excessive activation can lead to cellular dysfunction and death being correlated with seizure frequency and duration[95,96]. It has also been observed that microglia have a prompt cytokine release in response to seizures and that it can stay activated several hours after seizures[33]. Microglia activation may be modulated by astrocytes that in vitro were able to inhibit microglial phagocytosis as well as the production molecules and reactive oxygen species[97]. Animal models have also demonstrated that seizures alone can activate microglia and astrocytes even in the absence of neuronal damage[98,99].

Activated astrocytes and neurons release molecules, such as Il-1β and high-mobility group box 1 (HMGB1), which act in autocrine or paracrine ways activating complex signalling cascades culminating in change of gene expression. Whilst IL-1β activates its receptor in astrocytes, neurons and microglial cells, HMGB1 is recognized by the Toll-like Receptor 4 (TLR4) expressed in glial cells and neurons[100,101]. The engagement of TLR4 leads to the activation of the innate immune response. Through the recruitment of MyD88, the transcription factor Nuclear Factor kappa B (NF-kB), is activated as well as it is the production of pro-inflammatory mediators. Due to its functions in neurotransmission, cytokine upregulation will lead to hyperexcitability causing the occurrence of individual seizures. Several inflammatory cytokines (such as IL-1β, TNFα and IL-6) as well as TLRs (for example TLR4 and TLR9) and NF-kB are rapidly induced by seizures, or by brain injury, in activated astrocytes and microglia[99,102-107] (Figure 5). The continuous and exacerbated pro-inflammatory cytokine expression will promote long-term changes in molecular and cellular processes involved in epileptogenesis and lowering seizure

threshold. In this way, it is suggested that inflammatory factors participate in glial scar formation contributing to seizure-related hippocampal pathology, such as neuronal death, reactive gliosis and mossy fibre sprouting. Curiously, the abnormal astrocyte organization is not observed in other non-epileptic gliosis models[108].

Inflammation-induced seizures cause more damage perpetuating brain inflammation and initiating a vicious cycle of inflammation and excitability.

Evidencing the role of inflammatory molecules in epilepsy propagation, the injection of IL-1R antagonist (IL-1Ra)[99] as well as the genetic or pharmacological blockade of IL-1β affords seizure reduction[109,110].

Moreover, it has been observed that some conventional AEDs have an anti-inflammatory action [111] whilst the administration of anti-inflammatory drugs can also have anti-convulsant effects with seizure reduction[112].

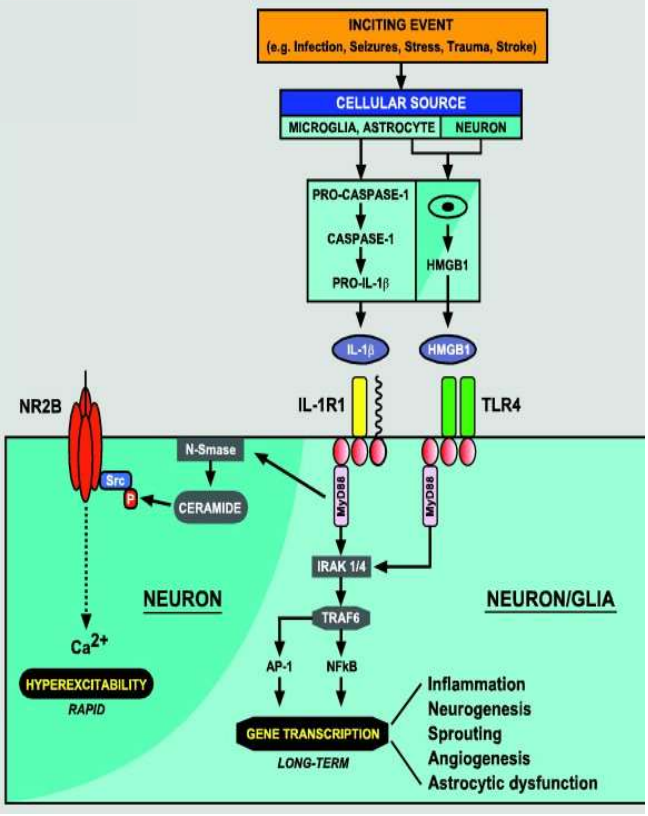

Figure 5 – Schematic model of IL1R/TLR4 signalling in epilepsy. An initial insult leads to the activation of microglial cells, astrocytes and neurons. Pro-inflammatory cytokines such as IL-1β and danger signals such as HMGB1 activate its respective receptors and elicit the inflammatory events cascade with a complex signalling pathway involving several kinases. A result is the phosphorylation of NR2B of NMDA receptor with consequent increase in Ca^{2+} influx and a rapid induction of hyperexcitability. The continuous stimulation of these signalling pathways leads to long-term changes in molecular and cellular pathways involved in epileptogenesis and the vicious cycle inflammation – hyperexcitability. IRAK = Interleukin-1 receptor-associated kinase ; MyD88 = Myeloid differentiation primary response gene 88; TLR = Toll-like Receptors, TRAF = Tumour necrosis factor receptor-associated factor (Adapted from Vezzani et al, 2012[113])

Other components of the innate immune response, besides TLR, such as complement proteins have also been demonstrated to play an important role in epilepsy development[72,114].

Inflammatory reactions and seizure activity may induce the breakdown of the blood-brain barrier (BBB). This will lead not only to the intrusion of peripheral immune cells, enhancing neuroinflammation, but also to accumulation in brain of serum molecules such as albumin that impair neurotransmission[115-117].

Inflammatory reactions, BBB integrity, and neuronal repair may be regulated by different mechanisms including apolipoproteins. Apolipoprotein E (ApoE), a constituent of many types of lipoproteins, plays a key role in the CNS where it is released by astrocytes and microglia[118]. Under diverse physiological and pathological conditions CNS neurons also express this protein albeit at lower levels than astrocytes[119]. Due to its function in cholesterol and phospholipid transport, ApoE plays an important role in the maintenance and repair of myelin and neuronal membranes' integrity[120]. It has also been demonstrated that ApoE enhances the effect of growth factors promoting neuron survival and sprouting[121]. It is claimed that ApoE is also important in neurotransmission, since it has a regulatory role in calcium homeostasis, modulating indirectly the function of various ion-dependent receptors[118,122]. In view of this, ApoE plays and important role in structural plasticity important for higher brain functions such as memory and learning[119].

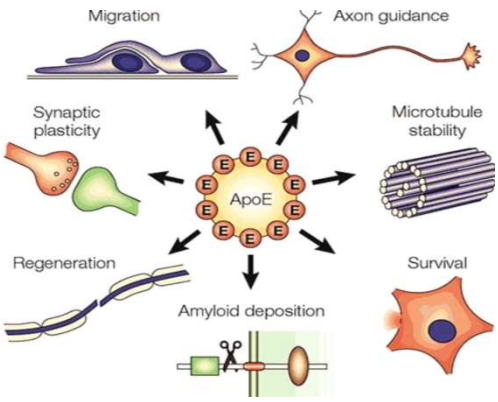

Figure 6 - ApoE roles in Central Nervous System. ApoE modulates axonal outgrowth and sprouting, cell migration, neuronal survival and repair, and microtubule stability. It can also influence synaptic transmission and plasticity and deposition of amyloid plaques. Herz *et al.*, 2000[123]

Astrocyte dysfunction, with consequent decrease in ApoE expression leads to a less efficient neuroprotective response with failure of repair and remodelling mechanisms leading to the progression of a variety of CNS disorders[119] (Figure 6). The human ApoE gene, on chromosome 19q13.2, codes for 3 isoforms: ApoE ε2, ε3 and ε4. These

isoforms have different efficiencies in cholesterol and phospholipid transport being the ApoE ε4 the less efficient. This isoform is a well-established major risk factor for Alzheimer´s disease and is associated with faster progression of disability in multiple sclerosis[124,125].

Inflammatory reaction may also be modulated by purinoreceptors and its ligands, ATP and adenosine, upon releasing from cells in response to stressful conditions.

3.2. ATP and purinoreceptors: a signal alert

ATP (adenosine triphosphate) is the cell primary source of chemical energy having important roles in cell development and functioning. ATP modulates a variety of functions such as cellular survival[126], cell proliferation and differentiation[126], axonal growth and maturation[127], excitability[128] and glia activation and cytokine release[129]. In humans ATP is primarily obtained by mitochondrial oxidative phosphorylation. ATP functions are operated through the activation of P2 purinoreceptors that have been shown to be one of the first functionally active receptors in the developing membrane. The activation of these receptors in synergy with growth factors, cytokines and chemokines drive cell differentiation[130] and also have an important role in neuronal network mapping and neurogenesis modulation[131] during embryonic cortical development. ATP is also important in Ca^{2+} wave's generation[131] in adult progenitor cells.

P2 receptors are expressed in all cell types including neurons, astrocytes, microglia, oligodendrocytes and endothelial cells distributed throughout the CNS[132]. Based on molecular cloning, mechanism of action and pharmacological studies these receptors can be divided in 2 subfamilies: the ligand-gated ionotropic P2X receptors (P2XR) and the metabotropic G protein-couple P2Y receptors (P2YR)[132] (Figure 7). Both subtypes are variably expressed by all cell types in different combinations[132]. Seven subtypes of ionotropic receptors[133] - P2X1 – 7- and 8 subtypes of metabotropic receptors[134] – $P2Y_{1, 2, 4, 6, 11,12, 13, 14}$ - have been identified so far. Some receptors have a well-defined function and expression profile while for others controversy still exists.

P2XRs are ligand-gated ion channels which have a restrictive agonist selectivity, responding only to ATP. Each receptor subunit possesses 2 hydrophobic transmembrane domains, a large extracellular loop where ligand binds and intracellular N and C terminus[135]. P2XRs form hetero or homomultimers that allow the rapid and non-selective flux of mono and divalent cations (Na^+, K^+ and $Ca2^+$)[135]. These receptors have functions in several vital cell functions such as survival, differentiation, proliferation as well as

astrocyte and microglia activation[136]. P2XRs are expressed pre and postsynaptically and have been demonstrated to contribute to synaptic plasticity and to synaptic transmission, particularly to fast synaptic activity[137,138].

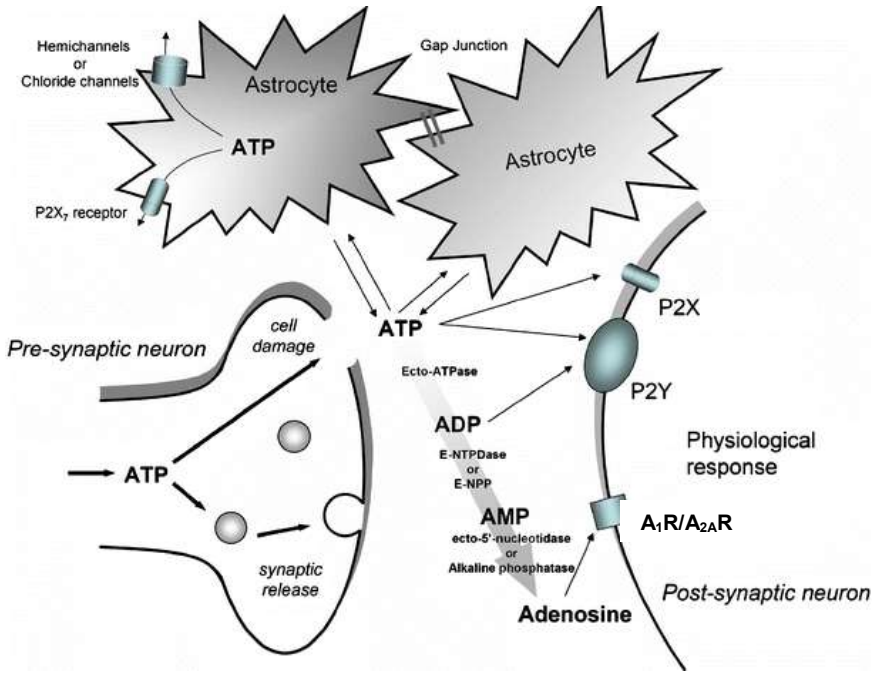

Figure 7 - Schematic nucleotide metabolism in brain cells. ATP is released from astrocytes and neurons to the extracellular space where it activates the purinergic receptors P2X and P2Y. In the extracellular space ectoenzymes rapidly degrade ATP to AMP and adenosine. These activate distinct receptors in neurons and glial cells inducing different physiological responses. ADP = Adenosine MonoPhosphate; AMP = Adenosine MonoPhosphate; ATP = Adenosine Triphosphate Adapted from Majumber *et al.*; 2007[139]

In normal brain, P2XRs currents are small and not uniformly detectable[135,140]. During high neuronal activity, P2XR may lead to Ca^{2+} influx resulting in neurotransmitter exocytosis, especially of glutamate[141,142]. Several studies have conflicting results with both excitatory and inhibitory actions of P2XR activation being reported. It was observed that treatment of hippocampal slices with a P2XR agonist had excitatory effects[143] whilst inhibition of post-synaptic receptors facilitated long term potentiation[144]. It was then observed that excitatory or inhibitory effects may also depend on the P2XR subtype that is being activated. Whereas, activation of pre-synaptic P2X2R enhanced the release of excitatory neurotransmitter onto the CA1 interneurons[145], pre-synaptic P2X7R stimulation reduced excitatory field potentials in the hippocampal CA3 region[146]. Furthermore, P2X7R activation has been demonstrated to lead to the release of both GABA and glutamate in hippocampus [147,148], nerve terminals[149,150] and astrocytes[151] supporting a context-specific role of P2XR in neurotransmission .

The metabotropic P2YRs have a broad range of agonists' selectivity, responding to extracellular uridine (UTP, UDP and UDP-glucose) or adenine (ATP, ADP) nucleotides with different pharmacokinetics depending on the receptor subtype. P2YR are involved in slower acting pre-synaptic functions regulating cell growth and survival, neurotransmission, and inflammation[134]. Whereas P2X have been associated mainly with facilitatory effects on neurotransmission, P2YRs have been suggested to play an overall inhibitory role[152]. This is accomplished by different action mechanisms depending on the coupled G protein: while $P2Y_{1,\ 2,\ 4,\ 6,\ 11}$ subtypes couple to Gq proteins $P2Y_{12,\ 13,\ 14}$ receptors couple to Gi proteins inhibiting adenyl cyclase[153]. These receptors are highly expressed on astrocytes where they have been shown to have a role in the propagation of astrocytic calcium waves induced by astrocyte-mediated ATP release[154,155].

In normal physiological conditions, P2YRs inhibit voltage-dependent Ca^{2+} influx[156] thereby limiting vesicular exocytosis of neurotransmitters[157]. Accordingly, the $P2Y_{1,2,4}$ receptor subtypes have been shown to inhibit glutamatergic neurotransmission in the hippocampus[158]. It has also been demonstrated that higher astrocytic Ca^{2+} levels, subsequent to P2YR activation, enable GABAergic inhibitory postsynaptic currents onto interneurons resulting in increased synaptic inhibition[156]. Conversely, the $P2Y_4$ receptor activation blocked GABA release[159].

The low ATP extracellular levels rapidly increase in pathological conditions such as inflammation, cell death, hypoxia or intense neuronal activity[128,137]. This is due to the ATP release from neurons astrocytes or microglial cells. ATP is released directly from damage cells, by exocytosis or transported in vesicles stored with other neurotransmitters (GABA or Glutamate) or ATP-only vesicles[160]. Thus released ATP can act either as a neurotransmitter or a co-neurotransmitter signalling to glial cells information about neuronal activity. ATP can also be released non-vesicularly through plasma membrane carriers, including the P2X7 receptor itself and hemichannels containing connexins and pannexins (see Figure 7).

3.2.1. ATP and P2X7: response to damage

Neuronal stimulation leads to a Ca^{2+}-dependent, glutamate-independent ATP release[161] and decreased ectonucleotidase activity has been claimed to be the responsible for the extracellular ATP accumulation[162] observed during seizures. Additionally, administration of ATP analogues leads to the generation of motor seizures[163] and to an exacerbation in seizure activity[164,165]. The role of purinoreceptors in epileptogenic process has been demonstrated in animal models with the use of pharmacological and genetic tools. These

studies have demonstrated that targeting different P2 receptors may have a beneficial effect on seizure susceptibility suggesting a potential new therapeutic option. Nevertheless, a study suggests only a limited P2 action in modulating seizure activity[166].
One of the most studied purinoreceptor is P2X7. It has been demonstrated that the P2X7R, which are expressed in neurons[146,165,167,168], astrocytes[167] and microglia[167,169], have pleiotropic effects modulating neuron-glia interaction, host defence and neuroinflammation[170].

P2X7 receptors are low affinity ATP-gated ion channels, which activation is only possible under stressful conditions, like during brain damage, hypoxia, or excessive neuronal activity detected during the course of seizures, which favour high extracellular ATP accumulation to the millimolar concentration range[170,171]. Yet it may also be functionally relevant under physiological conditions, such as during synaptic plasticity phenomena triggered by high frequency stimuli inherent to learning and memory processes[172].
Once activated, P2X7R behaves as a non-desensitizing cation channel involved in the long-lasting influx of Na^+ and Ca^{2+} and also in the K^+ efflux depending on ionic concentration gradients[171,173]. A unique P2X7R characteristic is that with prolonged activation, the P2X7R may form a reversible plasma membrane pore that is permeable to hydrophilic molecules up to 900Da[174]. Molecular models argue that pore formation occurs by successive acquisition of subunits to the existing oligomeric structures, while others suggest that subtle structural changes may also contribute to pore formation. Recent studies have described that pannexin-1 is required in this process[175]. The pore formation activates a series of signalling pathways (Map kinase, Nf-kB)[176] important for cell function and survival.
The P2X7R is crucial for microglial cells, where it has a trophic function modulating their activation and proliferation[177], with expression of pro-inflammatory cytokines, IL-1β and TNF-α, and reactive oxygen species[178-180]. Through this action P2X7R may modulate neuronal cell death. The presence of the P2X7R in pre-synaptic nerve terminals and astrocytes justifies its role on GABA and glutamate release[150].

Up-regulation of the P2X7 receptor expression has been observed in the hippocampus and cortex of animal models of *status epilepticus* or TLE[165,181-185], as well as in the neocortex of human patients with TLE[181,182,186]. This upregulation is evident not only in microglial cells but have also been described in neurons[165,169,182,186], especially in glutamatergic nerve terminals, granular layer of the DG and cortical layer II-III[165,182]. The observed P2X7R overexpression in neocortical nerve terminal of drug-resistant epileptic patients is claimed to down-modulate GABA and glutamate uptake, which endures GABA

signalling, increases GABAergic rundown, and, thereby, unbalances glutamatergic excitation[186].

In accordance with the pro-convulsive P2X7 role, Jimenez-Pacheco *et al.*, showed that P2X7R antagonism may reduce the number of spontaneous seizures and that this effect is maintained after treatment cessation[181,182]. The reduction in seizure severity is accompanied by a reduced hippocampal and cortical cell death and consequent gliosis[165,182]. The association between P2X7R antagonist and seizure severity reduction was confirmed by other research groups[187,188]. Genetic or immunological P2X7R blockade afford seizure reduction[165]. In particular, P2X7R blockade reduce hippocampal microglia proliferation and the release of IL-1β[165,182,188]. Conversely, it was observed that pre-treatment with P2X7R agonists is associated with increased induced seizures.

The role of the remaining P2XR in seizure development is still controversial with upmodulation[183,189], downmodulation or no changes in expression being reported[169] (for review see *Engel et al.,* 2016[190])

For metabotropic receptors the studies are scarce probably due to the lack of efficient antagonists that have low affinity, high cross-reactivity with other P2 receptors, and low BBB permeability[153]. The $P2Y_{12}$ receptor has been observed to be up-regulated after seizure induction[183]. This receptor seems to be crucial for the formation of seizure-induced microglial extensions[191]. It has also been observed that seizure-induced microglia activation increase astrocytic $P2Y_1$ receptor – mediated spontaneous excitatory postsynaptic currents[192]. In astrocytes, the $P2Y_1$ receptor activation by ADP promotes reactive astrogliosis and Ca^{2+} waves generation[192].

The use of $P2Y_1$ and $P2Y_{12}$ receptor antagonists in the therapy of pathologies, such as thrombosis, may anticipate their use in epilepsy treatment in the near future. To accomplish that, it is necessary to understand the purinergic signalling cascade in the CNS, which also depends on adenosine formation, a breakdown product of the ATP catabolism.

3.3. Adenosine signalling pathway: endogenous neuromodulation

Adenosine, a purine ribonucleoside, is a ubiquitous homeostatic molecule that participates in fundamental processes for cell viability and adaptability namely energy state (ATP and ADP), redox reactions, nucleic acid maintenance (DNA and RNA), signalling pathway (cAMP) and epigenetic control [1].

Adenosine is present in all vertebrate's cells. with an important role in immune response, cardiovascular system, gastrointestinal tract and nervous system [193], [194] where it acts as

an “endogenous modulator” [195-199]. In fact, adenosine is claimed to have important functions in neuronal development, learning and memory processes, and in regulation of the circadian rhythm [200]. Adenosine has neuroprotective actions acting on pre and post-synaptic neurons and on glial cells as well. In this way it modulates synaptic transmission and synaptic plasticity [1].

Adenosine can be produced intra or extracellularly. Within the cell, 5’ adenosine monophosphate (5′-AMP) is hydrolysed by 5′-nucleotidase to adenosine that then can follow different metabolic pathways (Figure 8). Adenosine can be metabolized either by adenosine deaminase (ADA) forming inosine and hypoxanthine or by xanthine oxidase originating uric acid [201].

Neurons and glia can release directly adenosine via adenosine bi-directional transporters or release ATP that is then converted to adenosine by the action of ectoenzymes in the extracellular space (Figure 8) [202]. In normal physiological conditions, both process are infrequent and adenosine extracellular levels are low [193,203,204].

Adenosine transporters can be equilibrative nucleoside transporters (ENTs) or concentrative nucleoside transporters (CNTs)[205] . ENTs (ENT1-4) are passive bidirectional transporters widely expressed in CNS[206] (Figure 8). On the other hand CNTs perform an active, Na^+-dependent adenosine transport[207,208]. These transporters have been localized in microglia, liver, and choroid plexus among others[209].

Adenosine‘s functions are mainly operated by the activation of four distinct G-protein-coupled receptor subtypes (P1 = A_1, A_{2A}, A_{2B} and A_3) [210-212] (Figure 9). These receptors are more abundant in the brain tissue than in other organ confirming the important role of adenosine in brain functioning. Adenosine receptors have distinct pharmacological profiles, tissue distribution, adenosine affinities, and different G-couple proteins (Figure 9). A_1 and A_{2A} receptors have a high affinity to adenosine, being activated even in physiological levels whilst A_{2B} and A_3 are low affinity receptors being activated only with high adenosine levels that happen in a pathological state[213].

All subtypes have a similar molecular architecture with a seven-transmembrane α-helice domain with an extracellular N-terminus and an intracellular C-terminus. The N-terminus has glycosylation sites important for trafficking to the membrane whilst the C-terminus has serine and threonine residues which can be phosphorylated leading to receptor desensitization. The C-terminus and the third intracellular loop allow the coupling to G proteins[214,215] (Figure 9).

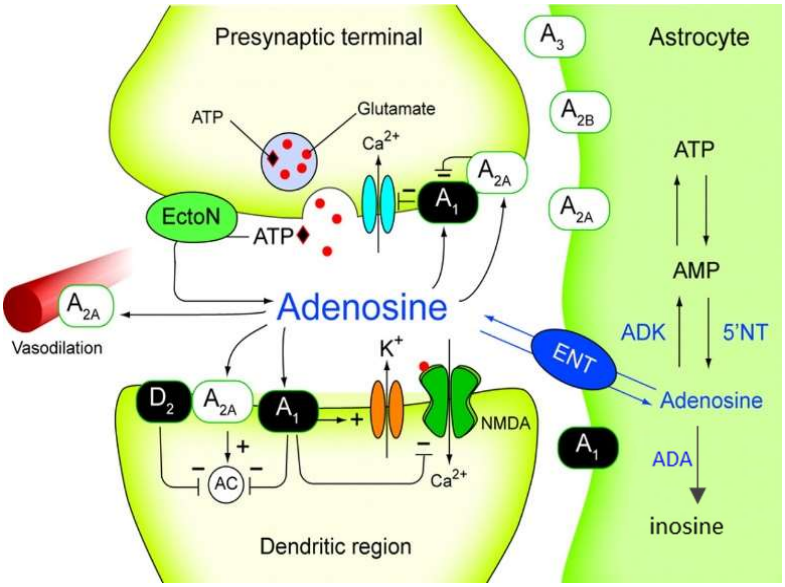

Figure 8 – Schematic model of adenosinergic transmission. Adenosine is released directly from the cells via ENTs or CNTs or is synthesized from cell-derived ATP by Ectonucleotidases. Adenosine has a neuromodulator role signalling mainly via inhibitory A_1R or facilitatory $A_{2A}R$. $A_{2A}R$ may form heterodimers with A_1R, blocking its action or with subunits of other receptors Intracellularly Adenosine is metabolized by ADK or ADA. ADA= Adenosine deaminase; ADK= adenosine Kinase; AMP= Adenosine Monophosphate, ATP= Adenosine Triphosphate; ENT= equilibrative nucleoside transporters; EctoN = Ectonucleotidases Adapted from Benarroch 2008[213]

The different receptors have affinity to different G proteins what influences its activity. Whilst A_1R and A_3AR are coupled to $G_{i/o}$ proteins inhibiting cAMP production, which decreases PKA activity and CREB phosphorylation, $A_{2A}R$ and $A_{2B}R$ are coupled to $G_{s/olf}$ proteins with opposite signalling effects[210,216,217]. Other signalling pathways have been also been described such as the activation of mitogen-activated protein kinase (MAPK) pathway by A_1R[218].

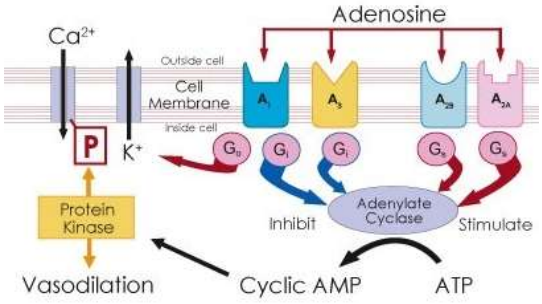

Figure 9 - Adenosine Receptors. The four subtypes have a structure of a seven-transmembrane α-helice domain with an extracellular N-terminus and an intracellular C-terminus. A_1R and A_3 are couple to Gi proteins whilst A_{2A} and A_{2B} are coupled to Gs protein having opposite effects. From Ham et al, 2012[219] and www.quora.com/What-roles-does-Adenosine-play-at-a-cellular-and-systems-level

In the brain, adenosine subtypes A_1 and A_{2A} seem to be the most important [197]. Adenosine A_1 receptors (A_1R) are distributed throughout the brain region being the second most abundant metabotropic receptor. This receptor is particularly abundant in the hippocampus, cerebral cortex, cerebellum and dorsal horn of spinal cord[220-222] being expressed by pre and post-synaptic neurons[223] as well as by astrocytes[224], microglia[225], and oligodendrocytes[226]. A_1R activation has a predominantly inhibitory action blocking pre-synaptic glutamate release by inhibition of Ca^{2+} influx[216,227-230] and control post-synaptical neurotransmitter responsiveness via potassium channels activation causing hyperpolarization[231-236 216,237]. Activation of K^+ channels by A_1R may also decrease GABA-A currents[238]. Using animal models it was observed that A_1R activation may have a lasting effect on synaptic inhibition by causing AMPAR internalization[239]. Astrocytic A_1R activation stimulates the release of growth factors (e.g. NGF – nerve growth factor) and of proteins (e.g. S100B) that stimulate neuronal survival and neurite outgrowth among others[240].

The A_{2A} receptors are present especially in basal ganglia and in the hippocampus, where it presents a low density[241-243]. A predominant location on hippocampal glutamatergic terminals has been described[244,245] and recent studies have evidence it's astrocytic and microglia expression[246-251]. It has been claimed that $A_{2A}R$ activation can afford both neurodegeneration and neuroprotection depending on the involvement of different cell types at different stages of the brain damage[252-254]. It has been observed that astrocytic and neuronal[255] $A_{2A}R$ activation may have opposing effects, which also depend on its pre- or post-synaptic[256] receptor actions.

Synaptic $A_{2A}R$ activation has facilitatory effect in the hippocampus being associated with high extracellular glutamate levels. In fact, $A_{2A}R$ activation stimulates glutamate release[248,257,258] and also prevents its uptake[257], by intracellular pathways that interfere with Ca^{2+} and Na^+ levels respectively. In this way, A_{2A} receptor may lead neuronal excitation and/or excitotoxicity. This excitotoxicity may be further aggravated since $A_{2A}R$ activation also upregulates astrocytic GABA uptake [259]. Interestingly, increased neuronal activity leads to enhanced synaptic $A_{2A}R$ activation[248,258,260]. Additionally, A_{2A} receptors can also attenuate the neuroprotective action of A_1R [261,262]. It has been observed that in the hippocampus, A_1R and $A_{2A}R$ are colocalized and coexpressed indicating that can interfere functionally with one another [241,261]. A further degree of complexity is added by the fact that $A_{2A}R$ could form heterodimers with other neurotransmitter's receptor subunits such as dopamine D2 and D3, glutamate mGluR5 among others. Nevertheless, it is suggested that this receptor has important physiological functions such as in memory formation.

Microglial $A_{2A}R$ activation is involved in microglia proliferation and activation with the production of trophic factors such as BDNF, or pro-inflammatory mediators[249-251,263,264].

Contrarily to what has been described in the periphery, in CNS, $A_{2A}R$ activation is claimed to have a pro-inflammatory role, especially in conditions of high glutamate levels[249 263].

3.3.1 Adenosine receptors in epilepsy

Adenosine has been considered the main endogenous antiepileptic molecule. Its extracellular levels have been described to rise immediately after seizures[265] as a consequence of high neuronal activity. This is suggested to be a mechanism to counteract the increased neuronal activity and prevent excitotoxicity and damage. In line with this, several studies have demonstrated that administration of inhibitors of adenosine transporters or of adenosine metabolism, that raise adenosine extracellular levels, attenuate seizure activity in diverse animal models [266]. Nevertheless, besides the fact that these compounds may have serious side-effects, adenosine role depends on the balance of inhibitory A_1R and facilitatory $A_{2A}R$.

Accordingly to its neuroprotective functions, the A_1R is upregulated in epileptic patients[267,268]. In line with this it was observed that the injection of A_1R agonists limits seizure activity in a wide number of epileptic animal models[269,270] and in human cortical slices[271] controlling seizures spreading. Equally, genetic or pharmacological blockade of these receptors worsen seizures in different animal models leading to generalization of focal seizures, development of spontaneous seizures and enhancing duration and severity of seizures[272-274]. Conversely, the $A_{2A}R$ activation has been associated with higher seizure activity and lower seizure threshold in diverse epileptic syndromes[275-278]. It has also been observed that genetic or pharmacological blockade of $A_{2A}R$ affords a robust neuroprotection with seizure reduction, in different animal models[276,277]. Although scarce, a recent study showed that $A_{2A}R$ may be upregulated in MTLE-HS patients[246].

3.3.2. Adenosine kinase hypothesis of epileptogenesis

From the enzymes that catabolise adenosine (Figure 7), adenosine kinase (ADK), synthetized by astrocytes, has the highest adenosine affinity[279].
Thus, under baseline conditions, is most likely to affect the rate of intracellular adenosine catabolism, being the key regulator of extracellular adenosine levels in the brain, by forcing adenosine cellular uptake by equilibrative nucleoside transporters[280]. Experimental studies support a role for ADK in brain injury associated with astrogliosis.

The ADK hypothesis of epileptogenesis, developed by Detlev Boison, states that after a brain insult (e.g.: seizure, hypoxia) extracellular adenosine levels increase as a consequence of ATP degradation[266]. Under these conditions adenosine A_1 receptors are

down-regulated whereas A_{2A} receptors are up-regulated, promoting astrocytic proliferation[266]. This astrogliosis leads to up-regulation of ADK, decreasing extracellular adenosine, and consequently may cause seizures. In fact, immunohistochemistry studies and western blot analysis have demonstrated an overexpression of astrocytic ADK in the hippocampus and temporal cortex both in animal models and in MTLE-HS patients[281,282]. The up-regulation of ADK leads to a decreased in adenosine extracellular levels with consequent inhibition of neuroprotective signalling that affects both neurotransmission and neuroinflammation among other factors.

4. Regulation of gene expression and MTLE-HS

Epileptic seizures are associated with a profound change in gene expression affecting neurotransmission, ionic channels, and neuroinflammation among others. Studies demonstrate that the gene expression is dynamically regulated and the expression patterns of acute and latent epileptogenic phases may significantly differ from the chronic phase. Gene expression may be modulated by genetic factors. Recently, it has been reported that epigenetic mechanisms also play a role in epileptogenic process and can be influenced by seizure-induced cellular changes.

4.1. Genetics and MTLE-HS: is it in our genes?

Although the origin, causal relation and interplay between FS and MTLE-HS are not yet fully understood, genetic determinants are thought to be involved[283,284]. These conditions are expected to have a complex inheritance for which several genetic as well as environmental factors contribute[285,286]. Some studies have demonstrated that FS and MTLE-HS[287] share genetic susceptibility factors, strengthening evidence for a causal relation between the two entities.

The importance of genetic and environmental factors in FS has been demonstrated in studies showing that FS may be familial (genetic contribution) or sporadic (environmental contribution). It was shown in animal models that fever can induce seizures in almost all strains suggesting that genetic factors are not determinant for FS development[288-290]. In fact, the raise in body temperature, *per se*, can cause seizures since several ionic channels are temperature-sensitive[291,292]. Hyperthermia also leads to hyperventilation and alkalosis which are associated with increased excitability[293]. Nevertheless, the observation that different strains have different temperature thresholds for seizure induction suggests a role for the genetic background[294,295]. A familial aggregation of FS has been demonstrated. Concordance rate in monozygotic twins has been estimated to be 19 - 68% and 6-14% in dizygotic twins[296-299]. Several studies have shown that first-degree relatives of FS patients have a higher risk to develop FS compared with general population[284,300].

The FS hereditability rate has been claimed to be around 75% and several genes have been associated with FS susceptibility. Many of these susceptibility genes code to sodium channels – SCN1A and SCN2A – to GABAA receptor subunits (GABRG2) as well as to the IL-1β gene. A single nucleotide polymorphism (SNP) in the P2X7R gene, rs208294, conferring a gain-of-function effect has also been associated with susceptibility to FS[301].

Different epidemiological studies have demonstrated that genetic factors are important in MTLE-HS as well[302-304]. Although familial MTLE-HS forms are reported[285], the majority of MTLE-HS are sporadic cases in which MTLE-HS presents a complex multifactorial aetiology and inheritance pattern[286]. Genome-wide association studies (GWAS) and candidate genes have revealed associations with some common genetic variants that influence expression levels or function of neurotransmitter receptors, ionic channels, cytokines or regulatory proteins. Some of these variants confer susceptibility for MTLE-HS development while others influence its clinical characteristics, such as age at onset[305] or drug response[306]. Remarkably, the most consistent associations found are with variants described as susceptibility factors for FS strengthening the casual relation between these two entities. The polymorphism rs16944 in the IL-1β gene, which leads to high cytokine expression, has been described as a susceptibility factor for both FS[307] and MTLE-HS[308,309]. It is thought that the association with MTLE-HS rs16944 reflects the high prevalence of FS in this population. Recently, a European consortium study, with several European cohorts including ours, demonstrated an association between variants in the SCN1A gene, especially the rs7587026 polymorphism, and MTLE-HS development but only in individuals with FS antecedents[287]. This raises the question if patients with and without FS (or other initial precipitant injury) have different susceptibilities and even disease courses and mechanisms. In fact, it is known that an early disease onset, FS antecedents (particularly complex ones), and disease duration may influence MTLE-HS clinical presentation.

4.2. Epigenetics and epilepsy: “DNA is not our destiny”

The definition of epigenetics has evolved during the years, and is now understood as mitotically heritable changes in gene expression that are not due to modifications in the DNA sequence. These epigenetic phenomena are highly influenced by internal, external and environmental factors allowing the cells to respond to the different stimuli induced, for example, by diverse physiological states (aging, pregnancy, sports, disease, emotional condition, *etc.*). The combination of the genetic and epigenetic profile determines the individual response to the presented stimuli, allowing the organism to adapt and evolve in response to environmental challenges. The diverse epigenetic mechanisms - DNA

methylation, histone modification, non-coding RNAs (Figure 10) - modify genome reading, producing a different gene expression profile.

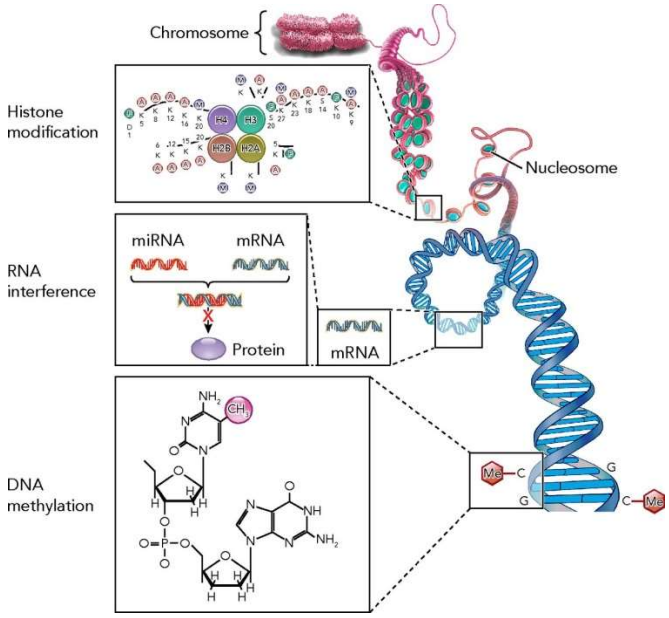

Figure 10 – Epigenetic mechanisms. Gene expression may be modulated ate several levels since post-transcriptional histone modifications that affect chromatin stability, DNA methylation that inhibits gene expression, or RNA interference molecules that are post transcriptional modulators of gene expression From Hagood, 2014[310]

In the brain, epigenetic mechanisms are important in neurodevelopment and damage repair modulating physical parameters and function of newly formed cells. Epigenetic mechanisms are also important in the modulation of synaptic transmissions regulating higher brain functions such as memory, cognition and language.

Methylation of cytosines in CpG motifs of DNA sequence leads to silencing of gene transcription. It is a dynamic and persistent molecular process that is seen in normal organism functions such as Parent-of-origin imprinting, X chromosome inactivation in females, aging, lineage commitment, for example, during neurogenesis and neural plasticity. Several studies have demonstrated and association between DNA methylation and epilepsy development. Changes in DNA methylation profile have been reported in MTLE-HS patients and may be associated with epileptogenic phenotype such as refractoriness and cognitive impairment. DNA methylation changes are subtle and accumulate over time what may contribute to epilepsy chronicity. DNA methylation may be influenced by other intervenients in the epileptogenic mechanism such as adenosine. The primary methyl group donor is S-adenosylmethionine (SAM) that is converted to S-adenosylhomocysteine (SAH) which is then further hydrolysed to adenosine and

homocysteine. The constant adenosine removal, for example in situations of ADK overexpression, stimulates the continuous SAH hydrolysis promoting DNA methylation and gene inactivation[311]. DNA methylation may also influence other epigenetic markers.

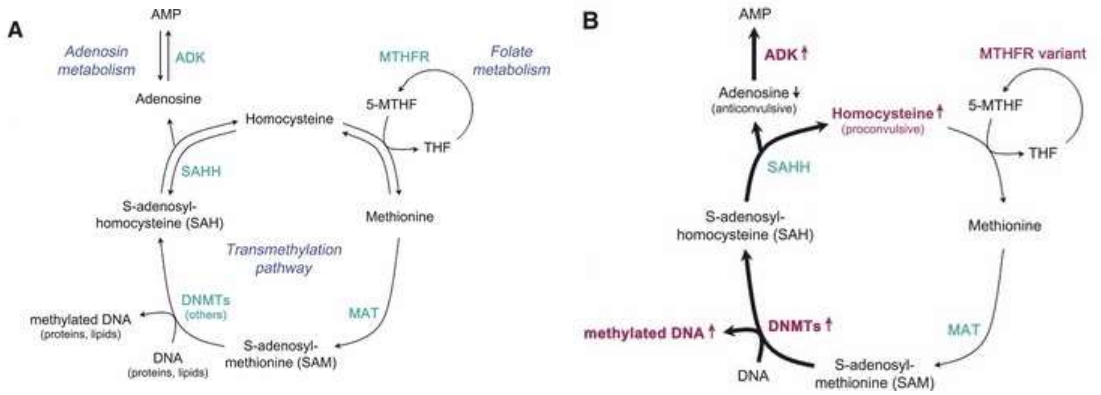

Figure 11 – DNA methylation and adenosine Kinase hypothesis of epileptogenesis. a) in abnormal situation SAM is the methyl group donor in all methylation reactions being converted to SAH that is then further hydrolysed to adenosine and homocysteine. b) ADK overexpression as observed in epilepsy, with consequent adenosine removal, shift the reaction equilibrium towards a constant hydrolysis of SAH promoting DNA methylation which may be associated with chronicity of epilepsy From Kobow et al., 2012[311]

Histones can undergo post-translational modifications that affect histone – DNA and histone – histone interaction promoting or inhibiting gene expression. Histone modification studies in epilepsy are scarce but it has been observed that chronic seizures cause histone-specific changes in the hippocampus. These modifications were found highly correlated with changes in gene expression of signalling molecules or growth factor such as BDNF.

4.2.1 MicroRNAs and epilepsy: another level of regulation

Only 1.5% of the human genome is responsible for protein codification. It is estimated that about 80% of the human genetic information is transcribed as non-coding RNA, known to regulate every aspect of cellular function, development, embryogenesis, differentiation and organogenesis, cell growth and programed cell death [312]. MicroRNAs (miRNAs) are short non-coding RNA molecules (19-25 nucleotide length) that function as post-transcriptional regulators of gene expression.

MicroRNAs are transcribed from exons, introns or intergenic regions of protein-coding genes by RNA polymerase II (Figure 12). One transcript may origin a single miRNA chain or several ones. This molecule is then processed in the nucleus by the Drosha/DGCR8 heterodimer, forming a precursor molecule, the pre-miRNA, approximately 70-kb-long.

Pre-miRNA is then transported to the cytoplasm, where it is further processed by a second RNAse III (Dicer) in an approximately 22-nucleotide long mature miRNA duplex (miRNA/miRNA*). This complex is then separated and the leading strand (miRNA) binds to Argonaute 2 (Ago2) protein which is part of the RNA-induced silencing complex (RISC) along with transactivation-responsive RNA-binding protein (TRBP)[313-315]. Within this complex, the 5' region of the miRNA, binds to the 3' region of the target mRNA and the complementarity of this binding determines the mechanism of regulation. When there is full complementarity, the RISC complex degrades the target mRNA due to its endonuclease activity. A partial pairing induces de-adenylation of the target RNA, causing structural disruption and translation repression[316-318]. The net effect of miRNAS is the downregulation of protein synthesis, modulating the homeostasis of several biological processes such as immune response and neurotransmission. An interesting feature is the ability of one miRNA to modulate diverse genes from the same or different pathways. Additionally, one mRNA molecule can have several binding sites for different miRNAs.

In CNS, microRNAs are present in all cells but their expression profile is described to differ significantly between neurons of different brain regions and even between neurons in the same region. MiRNAs regulate differentiation, function, migration, synaptic transmission and plasticity, not only in neurodevelopmental stages but also in adulthood. These molecules are also claimed to have a role in neuroprotection. It has been observed that endogenous seizure-tolerance mechanisms elicited by precondioning mechanisms (exposure to brief non-harmful seizures) is associated with a change in miRNAs profile expression with up and downmodulation of several miRNAs[319,320].

Lawrie et al. were the first to suggest that miRNAs could be used as noninvasive biomarkers for disease diagnostics[321]. These authors detected the presence of several miRNAs in serum of cancer patients, not describing, nonetheless, if these molecules derived from tumour cells or were originated from the response to the tumour[321]. Another group detected both tumour and normal-derived cell-free miRNAs in plasma and serum of cancer patients in an extraordinarily stable form[322]. Later, Chen and collaborators confirmed the presence of stable miRNAs molecules in sera and demonstrated that its expression levels were consistent and reproducible among individuals from the same species[323]. In this study was also demonstrated that different pathologies produce different serum miRNAS expression profiles[323]. The presence of miRNAs in other body fluids has also been widely reported[324-326] with variable concentration and relative composition among them[326].

In plasma and serum, circulating miRNAs are claimed to be mostly microvesicle-free and associated with the Ago protein[327] (figure 12). In other fluids cell-free miRNAs are

contained in microvesicules such as apoptotic bodies, shedding microvesicles, High Density Lipoprotein (HDL) particles or exosomes[328-331].

The existence of cell-free circulating miRNAs has raised some controversy. Whilst some authors defend that circulating miRNAs are merely by-products of the normal cell metabolism others support that these molecules are selectively exported having an important function in intercellular communication.

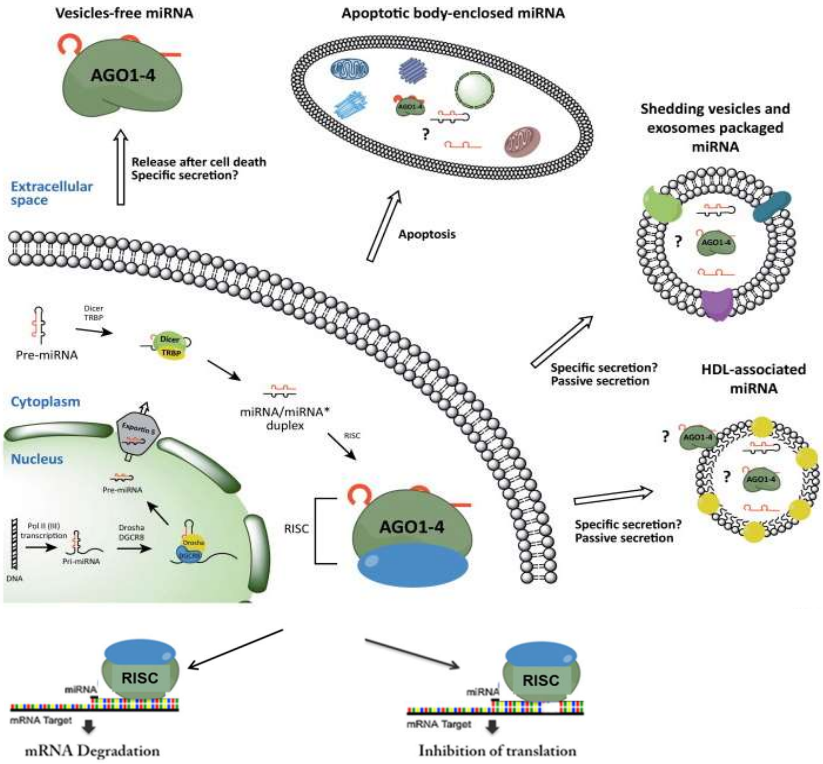

Figure 12 – Schematic model of miRNAs biogenesis and extracellular transport. Pri-miRNAs molecules are synthetized in the nucleus from exons, introns or intergenic regions by RNA polymerase II. Within the nucleus these molecules are processed by the endonuclease complex Drosha-DGCR8 given origin to pre-miRNA molecules which are actively transported to the cytoplasm by exportin-5. Here pre-miRNA molecules are further processed by the endonuclease Dicer into ~22-nucleotide miRNA/miRNA* duplexes. The leading strand, miRNA, is incorporated in an Argonaute protein (in humans is Argo 2), a component of RISC complex. Within this complex miRNA binds to its target mRNA thus interfering with processing or leading to degradation. MiRNAs are exported to circulation either in vesicles or in vesicle-free form connected to Argo2 Protein. Vesicles that can transport miRNA molecules are exossomes and apoptotic bodies. The presence of pre-miRNA or Argo-free miRNA in these vesicles has not been confirmed. The presence of miRNAs in HDL is also controversial. Ago = Argonaute; HDL = high-density lipoprotein miRNA = microRNA: mRNA= messenger RNA; RISC = RNA-induced silencing complex (Adapted from Turchinovich et al, 2012[327])

Despite the controversy on its transport and biological meaning, cell-free circulating miRNAs are consistently described as good biomarkers for several pathologies and good indicators of the physiological state of the organism[324-326].

Several miRNAs have been shown to be up or down-regulated after an epileptic seizure. In recent years, targeted and genome-wide studies have reported more than 100 miRNAs with a different expression profile in epilepsy [42,332-344]. Interestingly, changes in expression of inflammation-associated microRNAs are frequently reported [339,345-347]. Dysregulation of microRNAs that modulate purinergic signalling has also been recently described[346,348]. AEDs, such as sodium valproate, were observed to influence miRNAs expression profile[349,350] as well as other epigenetic markers such as DNA methylation[351].

Experimental and clinical studies highlight the complex cross-talk between inflammation, neurotransmission and the purinergic signalling cascade. A dysregulation, genetic or epigenetic, in one system may influence the entire network contributing to seizures and MTLE-HS development. In this context, it is important to clarify the interactions and the level of dysregulation that may be seen in MTLE-HS patients.

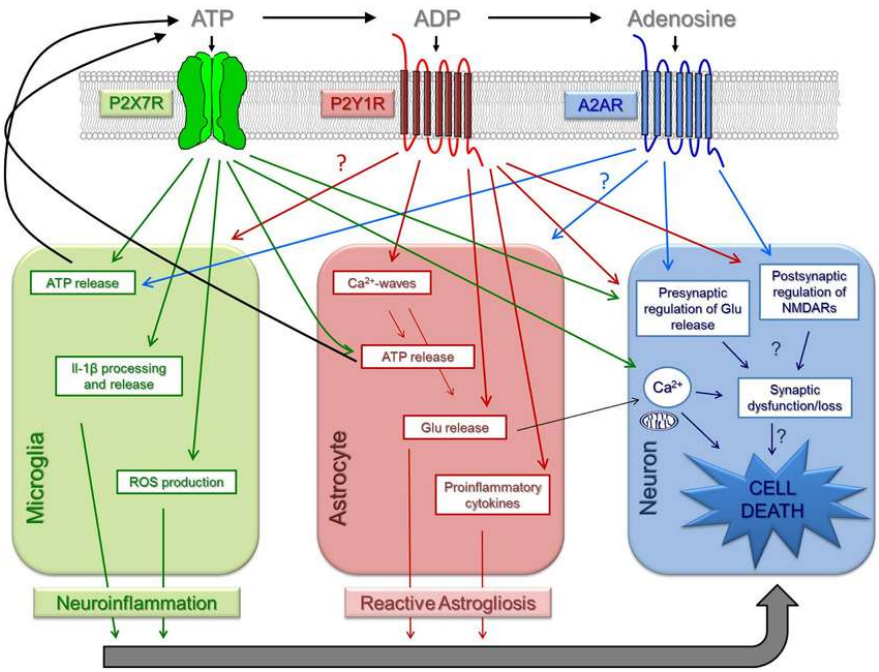

Figure 13 – Interplay between purinergic system, neurotransmission and inflammation. Purinergic receptors exist in all brain cell types. Released ATP binds to P2X7R in microglia promoting its activation and pro-inflammatory cytokines production. ATP may also be metabolized in extracellular medium and the resultant metabolites may binding to purinoreceptors in astrocytes or neurons promoting astrocytosis and interfering with Glutamate and GABA transmission and ionic balance. Excess excitability and pro-inflammatory reactions contribute to neuronal death. ADP = adenosine Diphosphate; ATP = adenosine Triphosphate; NMDAR = N-Methyl-D- Aspartate receptor; ROS = Reactive Oxygen Species From Rodigues et al, 2015[352]

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CHAPTER II

Results and discussion

Manuscript 1

Age of onset of Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis: the effect of apolipoprotein E and Febrile Seizures

Age of onset of Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis: the effect of apolipoprotein E and Febrile Seizures

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Abstract

Purpose: Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) is the most frequent pharmaco-resistant epilepsy. It has been associated with febrile seizures (FS) in childhood. Its aetiology remains unclear but genetic factors are involved. Apolipoprotein E (ApoE) is the main lipoprotein secreted in brain. It has a critical immunomodulatory function, influences neurotransmission and it is involved in repairing damaged neurons. ApoE ε4 is an isoform of ApoE with altered protein function, previously associated with refractoriness and early onset epilepsy. This study was undertaken to determine if ApoE isoforms are risk factors for MTLE-HS and influence clinical characteristics.

Methods: A group of 188 MTLE-HS patients (101 F, 87 M, mean age =44.7 ± 11.6 years, 100 with FS antecedents) was studied and compared with a group of 342 healthy individuals in a case-control genetic association study. Data was analysed with Pearson Chi-squared Test or Student's t test, as appropriated.

Results: No differences in ApoE ε4 allelic frequencies between MTLE-HS patients and controls or between MTLE-HS subgroups were observed. Nevertheless, ApoE ε4 carriers had an earlier MTLE-HS onset (11.0 ± 7.9 years in ApoE ε4 carriers *vs*. 14.4 ± 11.2 years in ApoE ε4 non-carriers $p<0.05$). Additionally, we observed that MTLE-HS patients with FS antecedents had a statistical significant early disease onset (11.5 ± 8.7 years in FS^+ *vs*. 16.0 ± 12.1 years in FS^-, $p<0.01$).

Conclusions: Our data shows that ApoE ε4 and FS may not participate directly in ethiopatogenic mechanisms of MTLE-HS but could hasten the disease development in predisposed individuals.

Introduction

Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) is the most frequent pharmaco-resistant epilepsy. Retrospective studies show that the majority of HS patients have a history of initial precipitating injury such as central nervous system infection, head or birth trauma or febrile seizures (FS). Among these factors FS is the most common: some authors claim that up to 50% - 80 % of MTLE-HS patients have a previous history of complicated FS.

Apolipoprotein E (ApoE), a constituent of many types of lipoproteins, plays a key role in the Central Nervous System (CNS) where it is released by astrocytes and microglia [1]. Under diverse physiological and pathological conditions CNS neurons also express this protein albeit at lower levels than astrocytes [2]. ApoE, due to its function in cholesterol and phospholipid transport, is involved in different functions like memory, learning, neuronal repair, structural plasticity, and in the maintenance of myelin and neuronal membranes' integrity during development and aging. ApoE is also important in neurotransmission, since it has a regulatory role in calcium homeostasis, modulating indirectly the function of various ion-dependent receptors [1, 3]. Several studies have demonstrated that ApoE enhances the effect of growth factors promoting neuron survival and sprouting [4]. Dysfunction of astrocytes, with consequent decrease in ApoE expression leads to a less efficient neuroprotective response with failure of repair and remodelling mechanisms leading to the progression of a variety of CNS disorders [2].

The human ApoE gene, on chromosome 19q13.2, encodes for 3 isoforms: ApoE ε2, ε3 and ε4. The ApoE ε3 isoform is the most common and provides higher protection against oxidative stress compared to the other isoforms. It was observed that mice expressing human ApoE ε3 had less neurodegeneration subsequent to oxidative stress than mice expressing ApoE ε4 or ε2 [1]. The ApoE ε 4 isoform is a well-established major risk factor for Alzheimer´s disease and is associated with faster progression of disability in multiple sclerosis [5, 6].

The role of ApoE in epilepsy development is still controversial. The ApoE ε4 isoform was associated with an increased risk for medically refractory epilepsy [7, 8], for late post-traumatic seizures [9] and for nonlesional MTLE [10] but other studies failed to prove this relation in nonlesional TLE patients [11] and in MTLE-HS patients [12]. An association between this isoform and age of onset of temporal lobe epilepsy was found in several studies [13, 14, 15]. The ApoE ε4 allele has also been associated with cognitive impairment in epileptic patients [16]. With this study we aim to clarify the importance of FS and the role of ApoE in MTLE-HS development. For that we compared ApoE's allelic and

genotypic frequencies in a cohort of MTLE-HS patients and in healthy individuals. We also tried to correlate genetic data with clinical parameters such as Febrile Seizures antecedents and age of onset. We hypothesised that if ApoE ε4 is a key player in epileptogenic mechanism it will be a susceptibility factor for MTLE-HS or will influence its development predisposing for instance for an early onset.

Methods

Subjects

In this case-control study we included 188 consecutive MTLE-HS adult patients [101 F, 87 M, 44.7 ±11.6 years, age at onset= 13.6 ± 10.7 years, disease mean duration = 31.1 ± 13.0 years] followed up at a tertiary epilepsy centre from North of Portugal. All patients had MTLE-HS diagnosis based on clinical and electrophysiological studies (EEG and/or video-EEG monitoring) and on brain MRI (minimum 1.5T) features as defined by Wieser [17]. Definition of HS was based on brain MRI findings criteria which comprised atrophy, T2 hyperintensity signal and altered internal structure on one or both hippocampi associated or not with other imaging criteria like ipsilateral fornix atrophy, ipsilateral mamillary bodies' atrophy or ipsilateral entorhinal abnormalities. EEG studies (scalp or depth) showed focal temporal or fronto-temporal inter- ictal or/and ictal activity in 70% of the patients. We excluded other MTLE-HS aetiologies like HS due to dual pathology). Patient may have visual and/or verbal memory impairment but patients with other abnormalities in neurological examination were excluded. We enrolled patients with bilateral MTLE-HS criteria. At the time of the study all, but one, patients were under pharmacological treatment, 38 patients in mono and 149 patients in polytherapy and 148 patients were refractory to pharmacological treatment (table 1). Carbamazepine was the most used anti-epileptic drug in both patients in mono (45%) or polytherapy (56%). Levetiracetam was the most used adjunct in polytherapy (22%). Data concerning FS' antecedents was collected from patient medical records and 100 patients had a history of FS (table 1). Considering a complex FS as one that had focal features, prolonged (>10 minutes) or recurrent within a 24-hour period, 38 MTLE-HS patients had antecedents of complex FS, 47 had antecedents of simple FS and for 15 patients we did not have sufficient data to FS classification. The control population comprised 342 healthy individuals (212 F, 130 M, 37.7±11.6 years (17 – 67)) voluntarily recruited among blood donors, ethnically matched, from the same geographical area. This population was inquired regarding multiple diseases including neurological pathologies. Individuals with neurological diseases (epilepsy, Alzheimer's disease, febrile seizures among others) or a positive familial history of these pathologies were not included in the study. The study was

approved by the Hospital Ethical Committee and all individuals gave written informed consent in accordance with Declaration of Helsinki.

Table 1 – Demographic and clinical data from MTLE-HS population studied

Clinical /demographic data	Patients (n total =188)
F/M	101 / 87
Age ± SD, years (range)	44.7 ± 11.6 (13 - 76)
Age of onset ± SD, years (range)	13.6 ± 10.7 (0 - 65)
Disease mean duration ± SD , years (range)	31.1 ± 13.0 (1 - 69)
Hippocampal Sclerosis (Left /Right / Bilateral)	104 / 72 / 12
Febrile seizures antecedents (Yes / No)	100 / 88
AED (0 / 1 / 2 / ≥ 3)	1 / 38 / 59 / 90
Refractory to treatment	148

s.d. =standard deviation

ApoE genotyping

Peripheral blood samples (10ml) were collected in EDTA. Genomic DNA was obtained from Proteinase-K treated peripheral blood leukocytes using a Salting-Out procedure. Genotyping of the ApoE polymorphisms was performed using a Polymerase Chain Reaction Restriction Fragment–Length Polymorphism (PCR-RFLP) assay as described previously [18].

Statistical Analysis

ApoE phenotypic frequencies were estimated by direct counting. Comparisons of ApoE isoform frequencies between patients and controls were performed using the Pearson Chi-squared Test or the Fisher's Exact Test when n≤5. Normal distribution of ages of onset between different groups was analysed with Kolmogorov – Smirnov test. Student's t test was used to compare distributions of age of onset in ApoE ε4 carriers and non-carriers. Data was analysed with SPSS v.21 software and results were considered significant at $p \leq 0.05$ for all statistical tests. Hardy–Weinberg equilibrium was tested.

Results

The ApoE ε4 isoform was present in 40 MTLE-HS and 54 control individuals (1 with ApoE ε4 allele in homozygoty) whilst 148 patients and 288 control individuals did not have this isoform. Thus, the allelic frequency of ApoE ε4 was similar between MTLE-HS patients and controls (10.6% in MTLE-HS vs. 8.0% in controls, p=n.s., Odds Ratio (OR) = 1.36, 95% Confidence Interval (CI) = 0.887 - 2.09) (table 2). The genotype E2/E2 was absent in the population studied. The frequencies of other genotypes and isoforms were similar in

MTLE-HS patients and controls (table 2). We observed that the ApoE ε4 carriers had a lower age at onset of MTLE-HS (11.0 ± 7.9 years in ApoE ε4 carriers vs. 14.4 ± 11.2 years in ApoE ε4 non-carriers p=0.032, Fig. 1a).

Table 2 – Frequency of ApoE isoforms and genotypes in the MTLE-HS patients and healthy controls

	Control population		MTLE-HS				
apoE	n	f	n	f	OR	95% CI	p Value
allele (n)		684		376			
Ɛ2	43	6.3%	18	4.8%	0.750	0.426 - 1.32	n.s.
Ɛ3	586	85.7%	318	84.6%	0.917	0.645 - 1.30	n.s.
Ɛ4	55	8.0%	40	10.6%	1.36	0.887 - 2.09	n.s.
Genotype (n)		342		188			
E2/E2	0	0.0%	0	0.0%	-	-	-
E2/E3	40	11.7%	15	8.0%	0.655	0.351 – 1.22	n.s.
E2/E4	3	0.8%	3	1.6%	-	-	-
E3/E3	248	72.6%	133	70.4%	0.917	0.618 - 1.36	n.s.
E3/E4	50	14.6%	37	19.7%	1.43	0.896 - 2.29	n.s.
E4/E4	1	0.3%	0	0.0%	-	-	-

apoE = apolipoprotein E; CI = confidence interval; n.s. = no significance OR = odds ratio

According to Febrile Seizures (FS) antecedents, 2 MTLE-HS sub-groups were formed. The subgroup “FS positive” was constituted by 100 patients and the subgroup “FS negative” was comprised by 88 patients. No differences in ApoE allelic or genotypic frequencies were found between these 2 subgroups (table 3). Additionally, we observed that patients with FS antecedents had an early MTLE-HS onset than patients without previous history of FS (11.5 ± 8.7 years in FS+ vs. 16.0 ± 12.1 years in FS-, p=0.005, Fig. 1b).

Discussion

The population studied in this work is a homogeneous population with all patients presenting Hippocampal Sclerosis. As has been described, 53% of MTLE-HS patients had a history of Febrile Seizures. Although, the relation between FS and MTLE-HS is recognized it still remains controversial. Vestergaard et al. [19], evaluated the association between FS and epilepsy in a population-based cohort of 1, 54 million persons (49, 857 FS and 16, 481 epilepsy) and found that 7% of children with FS developed epilepsy during the 23 years of follow-up.

Table 3 – Frequency of ApoE isoforms in MTLE-HS patients with and without Febrile Seizures antecedents

	FS negative		FS positive				
apoE	n	f	n	f	OR	95% CI	p Value
allele (n)		176		200			
Ɛ2	8	4.5%	10	5.0%	1.11	0.426 - 2.87	n.s.
Ɛ3	154	87.5%	164	82.0%	0.651	0.366 - 1.16	n.s.
Ɛ4	14	8.0%	26	13.0%	1.73	0.872 - 3.43	n.s.
Genotype (n)		88		100			
E2/E2	0	0.0%	0	0.0%	-	-	-
E2/E3	8	9.1%	7	7.0%	0.753	0.261 - 2.17	n.s.
E2/E4	0	0.0%	3	3.0%	-	-	-
E3/E3	66	75.0%	67	67.0%	0.677	0.358 - 1.28	n.s.
E3/E4	14	15.9%	23	23.0%	1.58	0.756 - 3.30	n.s.
E4/E4	0	0.0%	0	0.0%	-	-	-

apoE = apolipoprotein E; CI = confidence interval; n.s. = no significance OR = odds ratio

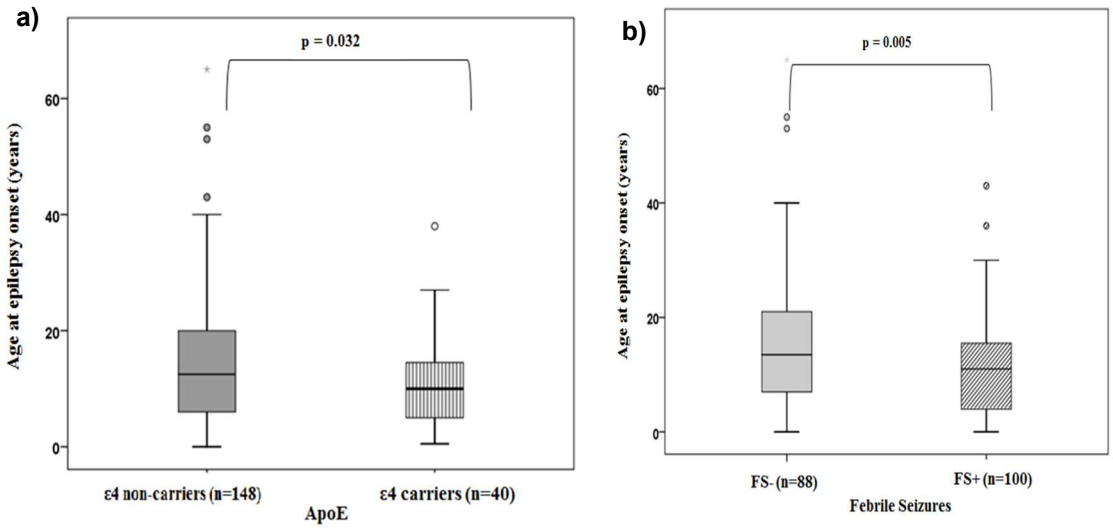

Figure 1. Age at seizure onset in MTLE-HS subgroups. a) ApoE ε4 carriers vs. non carriers; b) FS positive vs. FS negative

Some authors consider that FS occur primarily in predisposed (genetic factors, development dysplasia or pre/post acquire condition such as trauma) individuals being a marker of individuals that are predetermined to develop MTLE-HS. Others believe that FS may occur in uncompromised brain but lead to MTLE-HS development only in predisposed individuals. In some studies it is considered that FS by itself lead to epilepsy development in individuals who otherwise might not develop MTLE-HS [19]. Our results

show that, at least, FS predispose to an early onset of MTLE-HS. This is in accordance with previous studies [20]. It may be hypothesized that the occurrence of a febrile seizure haste the abnormal network reorganization that will lead to the development of the epileptogenic zone in predispose individuals [20]. Supporting this association, imaging studies have shown that prolonged and lateralized FS can produce acute hippocampal injury with oedema that recovers within 5 days [21, 22]. The follow-up of these children showed changes in hippocampal symmetry consistent with injury and neuronal loss associated with prolonged FS [21].

Our results also support the hypothesis that ApoE ε4 predisposes to an early development of MTLE-HS, in accordance with a recent meta-analysis study [14]. ApoE plays an important role in the maintenance and repair of neurons, by distributing lipids necessary for proliferation, synaptogenesis and axons' myelinisation [23]. As ApoE ε4 allele is associated with impaired neuronal cholesterol and phospholipid metabolism [24], we hypothesized that patients with this allele are more prone to impaired neuronal recovery after an insult. ApoE ε4 is associated with CNS network instability and with lower protection against oxidative and inflammatory cascade. These factors could influence neuronal growth and recovery, leading to a chronic vicious cycle of damage and neuronal loss and consequently to hippocampal atrophy contributing to an earlier onset of the disease as observed in our population. Accordingly, it has been described in animal models that ApoE ε4 seems to increase microglia activation and astrogliosis leading to a more pronounced hippocampal injury [25]. So, we propose that apolipoprotein could not participate in etiopathogenic process of MTLE-HS but the presence of ApoE ε4 allele may hasten the disease development in predisposed individuals. It has been claimed that an early disease onset may influence MTLE-HS prognosis. In MTLE-HS network reorganization and structural abnormalities extend far beyond the hippocampus and can also be observed in white matter. Impaired white matter integrity has been associated with an early seizure onset [26] and may have an important role in seizure propagation in MTLE-HS [27].

The finding of this study contrasts with some studies in the literature. Discrepant results are very common in genetic studies of complex diseases that arise from the interaction of several genes with additional environmental factors. Beside the fact that genes may have population differences in allele prevalence other factors can contribute to controversial results [13, 14]. For instance, the discrepancies may be due to differences in phenotype definition with inclusion of different epilepsy types [15], due to disease heterogeneity [11, 13, 28] or due to a limited sample size, with consequent lack of statistical power to detect small genetic effects as usual in complex diseases [12, 29].

Uncovering the contribution of FS and ApoE to MTLE-HS could be important for the development of potential preventive therapeutically measures. For that, replication studies with larger cohorts and refinement of sample homogeneity (as achieved in our population) are necessary.

Acknowledgments

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Manuscript 2

Immunogenetic Predisposing Factors for Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis

Immunogenetic Predisposing Factors for Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis

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(submitted)

Abstract

Purpose: Neuroinflammation appears as an important epileptogenic mechanism. Experimental and clinical studies have demonstrated an upregulation of pro-inflammatory cytokines such as IL-1β and TNF-α, in Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS). Expression of these cytokines can be modulated by polymorphisms such as rs16944 and rs1800629, respectively, both of which have been associated with Febrile Seizures (FS) and MTLE-HS development. The Human Leukocyte Antigen (HLA) system has also been implicated in diverse epileptic entities suggesting a variable role of this system in epilepsy. Our aim was to analyse the association between immunogenetic factors and MTLE-HS development. For that rs16944 (-511 T>C, IL-1β), rs1800629 (-308 G>A, TNF-α) polymorphisms and HLA-DRB1 locus were genotyped in a Portuguese Population.

Methods: We studied 196 MTLE-HS patients (108 females, 88 males, 44.7 ±12.0 years, age of onset= 13.6 ± 10.3 years, 104 with FS antecedents) and 282 healthy controls in a case control study.

Results: The frequency of rs16944 TT genotype was higher in MTLE-HS patients compared to controls (14.9% in MTLE-HS vs 7.7% in controls, p=0.021, OR [95%CI] = 2.20 [1.13 - 4.30]). This association was independent of FS antecedents. No association was observed between rs1800629 genotypes or HLA-DRB1 alleles and MTLE-HS susceptibility. Also, no correlation was observed between the studied polymorphisms and disease age of onset.

Conclusion: The rs16944 TT genotype is associated with MTLE-HS development what may be explained by the higher IL-1β levels produced by this genotype. High IL-1β levels may have neurotoxic effects or imbalance neurotransmission leading to seizures.

Keywords: epilepsy; HLA-DRB1; cytokines; hippocampus; immunogenetics,

Introduction

Temporal lobe epilepsy (TLE) is the most frequent focal epilepsy in adults and is characterized by complex partial seizures originated, in the majority of cases, in mesial structures, particularly the hippocampus [1, 2]. Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) is the most common drug-resistant epilepsy. Retrospective studies show that patients with HS often have a history of initial precipitating injury such as febrile seizures (FS), central nervous system infection and head or birth trauma [1]. Among these factors FS is the most common: some authors claim that up to 50% - 80 % of MTLE-HS patients have a history of complex FS [1]. It has been hypothesized that after the initial insult there is an abnormal cascade of damage repair, with the maintenance of chronic inflammation, leading to atrophy and sclerosis of hippocampus [1]. Accumulating evidence strongly supports the importance of neuroinflammation in the development of HS [3, 4]. Clinical data suggest that epilepsy development is associated with changes in the immunological profile [5]. In fact, it has been demonstrated that several inflammatory cytokines, such as IL-1β, TNF-α and IL-6, are rapidly induced by seizures, or by brain injury, in activated astrocytes and microglia [6, 7, 8]. Also, microarray studies in TLE animal models showed that inflammatory response is among the biological process most upregulated during epileptogenesis [9].These results are corroborated by studies in the human brain from drug-resistant epileptic patients in which the upregulation of inflammatory mediators, such as TNF-α and IL-1β was observed [6, 10]. Expression of these cytokines can be modulated by polymorphisms in the promoter region of its genes. Higher TNF-α production has been associated with the rs1800629 A allele. The rs16944 T allele, that produces higher IL-1β levels, has been associated with MTLE-HS development in several populations [11, 12, 13]. This allele has also been described as a susceptibility factor for FS [14, 15]. Nevertheless, both associations are still controversial since posterior studies did not replicate these results.

It has also been observed that HS patients have higher numbers of CD4 and CD8 T cells in brain tissue than controls [16]. Beach et al demonstrated that HS patients have an increase in HLA-DR-immunoreactive microglia and in HLA-DR-immunoreactive perivascular cells comparing to controls [17]. The Human Leukocyte Antigen (HLA) system has been implicated in diverse epileptic entities, such as Juvenile Myoclonic Epilepsy (JME) [18, 19, 20, 21] and Lennox-Gastaut syndrome [22, 23] suggesting a variable role of this system in epilepsy. Nevertheless, the information about HLA in focal epilepsies is scarce. Ozkara et al described an association of HLA-DR4, -DR7, and HLA-DQ2 alleles and DR4-DQ2, DR7-DQ2 haplotypes with MTLE-HS development in a Turkish population [24]. Recently, in a Brazilian population, a higher frequency of HLA-

DRB1*1302 allele in MTLE-HS patients comparing to controls was described, although without statistical significance [25].

In order to clarify the relationship between inflammation and MTLE-HS development, we proposed to analyse the association between rs16944 (-511 T>C, IL-1β), rs1800629 (-308 G>A, TNF-α) and HLA-DRB1 system and the development and clinical features of MTLE-HS in a Portuguese population.

Material and Methods

Subjects

In this case-control study we included 196 consecutive MTLE-HS adult patients [108 F, 88 M, 44.7 ±12.0 years, age at onset= 13.6 ± 10.3 years, disease mean duration = 31.1 ± 13.2 years] followed up at a tertiary epilepsy centre from North of Portugal. All patients had MTLE-HS diagnosis based on clinical and electrophysiological studies (EEG and/or video-EEG monitoring) and on brain MRI (minimum 1.5T) features as defined by Wieser [26]. Definition of HS was based on brain MRI findings criteria which comprised atrophy, T2 hyperintensity signal and altered internal structure on one or both hippocampi associated or not with other imaging criteria like ipsilateral fornix atrophy, ipsilateral mamillary bodies' atrophy or ipsilateral entorhinal abnormalities. EEG studies (scalp or depth) showed focal temporal or fronto-temporal inter- ictal or/and ictal activity in 70% of the patients. We excluded other MTLE-HS aetiologies like HS due to dual pathology. Patient may have visual and/or verbal memory impairment but patients with other abnormalities in neurological examination were excluded. We enrolled patients with bilateral MTLE-HS criteria. At the time of the study all, but one, patients were under pharmacological treatment, 36 patients in mono and 159 patients in polytherapy with 155 patients refractory to pharmacological treatment (table 1). Carbamazepine was the most used anti-epileptic drug in both patients in mono (45%) or polytherapy (56%). Levetiracetam was the most used adjunct in polytherapy (22%). Data concerning FS' antecedents was collected from patient medical records and 104 patients had a history of FS (table 1). Considering a complex FS as one that had focal features, prolonged (>10 minutes) or recurrent within a 24-hour period, 41 MTLE-HS patients had antecedents of complex FS, 49 had antecedents of simple FS and for 14 patients we did not have sufficient data to FS classification. The control population comprised 282 healthy individuals (174 F, 108M, 37.7±11.6 years (17 – 67)) voluntarily recruited among blood donors, ethnically matched, from the same geographical area. This population was inquired regarding multiple diseases including neurological pathologies. Individuals with neurological diseases (epilepsy, Alzheimer's disease, febrile seizures among others) or a

positive familial history of these pathologies were not included in the study. The study was approved by the Hospital Ethical Committee and all individuals gave written informed consent in accordance with Declaration of Helsinki.

Table 1 – Demographic and clinical data from MTLE-HS patients

Clinical /demographic data	Patients (n total =196)
F/M	108/88
Age, years, mean ± SD (range)	44.7 ± 12.0 (13 - 78)
Age of onset, years, mean ± SD (range)	13.6 ± 10.3 (0 - 55)
Disease duration, years, mean ± SD (range)	31.1 ± 13.2 (3 - 69)
HS (Left / Right / Bilateral)	109 / 75 / 12
Febrile Seizures (Complex / Simple / Undet)	41 / 49 / 14
AEDs (0/ 1 / 2 / ≥ 3)	1/36/67/92
Refractory to treatment	155

AED = Anti-epileptic Drugs; s.d= standard deviation

Genotyping

Peripheral blood samples (10ml) were collected in 5% EDTA tubes. Genomic DNA was obtained from Proteinase-K treated peripheral blood leukocytes using a Salting-Out procedure [27].

The rs16944 polymorphism was genotyped using a pre-designed TaqMan allelic discrimination assay (C___1839943_10, Applied Biosystems Foster City, CA, USA) in a Rotor Gene 6000 Real-Time PCR machine (Corbett Life Science). Genotyping of rs1800629 was assayed by a PCR-RFLP methodology. Briefly, the region flanking the polymorphism was amplified with a forward (5'-AGG CAA TAG GTT TTG AGG GCC AT-3') and a reverse (5'-CAG CGG AAA ACT TCC TTG GT-3') primer, followed by an enzymatic digestion with NcoI enzyme (Nzytech). PCR products of 264bp, 244bp and 20bp were analysed by electrophoresis in a 4% agarose gel and visualised using UV fluorescence after staining with ethidium bromide.

HLA-DRB1 genotyping was carried-out with sequence-specific primers (PCR-SSP) based on primer sequences previously described [28]. PCR products were visualized under ultraviolet light after electrophoretic separation on 1.5% agarose gel containing ethidium bromide. Genotypes were deduced from the amplification patterns.

Statistical Analysis

Frequencies of the rs16944, rs1800629 genotypes and HLA-DRB1 phenotype were determined by direct counting. HLA frequencies in patients and controls were compared using the Pearson chi-squared test or the Fisher's exact test as appropriate. Mean values were compared using the t-test. For rs16944 and rs1800629 logistic regression model was used to estimate ORs and 95% CIs. The major homozygote genotype was used as the reference group. Data was analysed with SPSS v.23 software and significant levels were set at $p \leq 0.05$.

Results

The population studied was in Hardy-Weinberg equilibrium. The frequency of rs16944 TT genotype was significantly higher in MTLE-HS patients comparing to controls (14.9% in MTLE-HS vs. 7.7% in controls, p=0.021, OR [95%CI] = 2.20 [1.13 - 4.30], table 2). No differences in allelic frequencies were observed.

The rs1800629 allelic and genotypic frequencies were similar in MTLE-HS patients and controls (table 3). The phenotypic frequencies of each HLA-DRB1 allele are shown in Table 4. There were no statistically significant differences in HLA-DRB1 phenotype frequencies between MTLE-HS patients and control population.

We constituted 2 MTLE-HS subgroups, considering FS antecedents. We observed that allelic and genotypic frequencies of rs16944 (table 2) and rs1800629 (table3) were similar between these 2 subgroups. Also, no association was found between rs16944, rs1800629 or HLA-DRB1 alleles and MTLE-HS age of onset.

Discussion

Our results support a role for rs16944 TT genotype in MTLE-HS development independently of FS antecedents. Kanemoto et al. were the first to describe an association between rs16944 T allele and MTLE development [11, 12] but later studies, in different populations, did not replicate these results suggesting that this is a specific association of the Japanese population [29, 30, 31, 32, 33]. Later on, rs16944 T allele was ascribed to indicate susceptibility for localization related epilepsy such as TLE [13]. This allele has also been associated with a poorer anti-epileptic drug response [13]. A meta-analysis study from Kauffman et al. pooled all these results observing that rs16944 T allele had a moderate effect on TLE-HS susceptibility [34], which is in agreement with our findings. It has been demonstrated that the rs16944 T allele is an enhancer of IL-1β production [35]. Accordingly, it has been observed, in both animal models and clinical

studies, that an overexpression of this cytokine is associated with more frequent and severe epileptic activity in HS [6]. IL-1β overexpression is more pronounced in microglial cells and astrocytes of the cortex and hippocampus that are brain regions most affected by neuronal damage in this pathology. Upregulation of IL-1β may lead to exacerbation of inflammatory responses resulting in increased neuronal damage. Nonetheless, several experimental evidences suggest that the IL-1β epileptogenic potential may be attributed to other mechanisms besides its direct neurotoxic effect. IL-1β not only leads to an increase in nitric oxide levels but it interferes with N-methyl-D-aspartate (NMDA) receptors and ion channels activities increasing neuronal excitability, thus contributing to induction and propagation of seizures [6, 36]. In view of this, it is important to clarify the importance of rs16944 in MTLE-HS since it may be a good biomarker for epilepsy susceptibility and ultimately lead in new directions in epilepsy treatment [37], particularly in patients refractory to the currently available AEDs.

It has been observed in animal models that seizures also induce the expression of TNF-α, yet the importance of this cytokine in epileptogenesis remains elusive [6]. Several studies have shown that TNF may have a dual role in epilepsy, contributing either to exacerbation or prevention of seizures [38]. This duality of function depends on the activation of different signalling receptors that may be influenced by the TNF-α extracellular concentration. It has been described that rs1800629 A is a high TNF-α producer and an association between TNF-α expression levels and the occurrence of FS has been described [38]. Few studies have addressed the importance of rs1800629 in epilepsy development and no association has been observed either with susceptibility or with response to AEDs [33]. Our data do not support a role for this polymorphism in pathophysiological mechanism of MTLE-HS. However, it would be interesting to study other polymorphisms in the promoter region of TNF-α gene.

Concerning HLA Class II, our results are in accordance with a recent study in a Brazilian population in which no statistically significant differences in HLA-DRB1 frequencies were observed between MTLE-HS patients and controls. Nevertheless, a possible role for the HLA system in MTLE-HS development cannot be excluded. In fact, several studies have suggested an association between HLA Class I alleles and epilepsy development. Eeg-Oloffson et al. described that TLE patients had a lower frequency of HLA-A1 and B8 alleles [39]. Interestingly, the HLA-B8 allele is part of an extended haplotype that encompasses the rs1800629A allele, HLA-A1-B8-DR3-DQ2-rs1800629A. Also, it has been observed that HS patients have increased CD8 / CD4 T cells ratio compared with controls [16]. A recent proteomic study has also described that proteins associated with

MHC class I processing pathway are one of the most upregulated during the chronic phase of epileptogenesis [40].

MTLE-HS is a complex polygenic disease arising from the interaction of multiple genes with environmental factors in which each gene has only a small contribution to disease susceptibility. In spite the fact that our immunogenetic study has the largest MTLE-HS cohort published so far, it may still be underpowered to detect the smaller effect of TNF-α gene and HLA-DRB1 locus in MTLE-HS susceptibility.

In the literature, contradictory results on the role of immunogenetic factors in MTLE-HS development can be found. Differences in allelic prevalences, as it is the case for rs16944, can lead to discrepancies when several populations are studied. Additionally, discrepancies between studies can also be due to differences in phenotype definition such as inclusion of cohorts with variable FS antecedents. Thus, to improve knowledge on the relationship between immunogenetic factors and MTLE-HS larger cohort studies with sample homogeneity (as achieved in our study) are necessary. It would also be important to study other HLA loci and immune system-related genes, such as IL-10 and IL-6.

Conclusion:

In our Portuguese population we observed that rs16944TT genotype allele may be a susceptibility factor for MTLE-HS. This association is independent of FS antecedents and may be a reflex of IL-1β interference in neuronal excitability and, therefore, in disease severity and progression. More studies are needed to clarify the role of other immunogenetic factors in MTLE-HS development. This could be especially relevant because a better understanding of the pathophysiological role of these immunogenetic factors in MTLE-HS development could shed light on the relationship between inflammation and epilepsy, with beneficial consequences for epilepsy treatment in a group of patients refractory to the currently available AEDs.

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Table 2: rs16944 genotypic and allelic frequencies in controls, MTLE-HS patients and subgroups of patients accordingly to FS antecedents

rs16944 genotypes	Controls (n=221) n (%)	MTLE-HS (n=194) n (%)	OR [95%CI]	p	FS No (n=90) n (%)	FS Yes (n=104) n (%)	OR [95%CI]	p
				0.064				**0.263**
CC	98 (44.3)	76 (39.2)	1		30 (33.3)	46 (44.2)	1	
CT	106 (48.0)	89 (45.9)	1.08 [0.72 - 1.63]	0.705	44 (48.9)	45 (43.3)	1.50 [0.81 - 2.79	0.200
TT	17 (7.7)	29 (14.9)	2.20 [1.13 - 4.30]	**0.021**	16 (17.8)	13 (12.5)	1.87 [0.80- 4.48]	0.150
Alleles	**%**	**%**			**%**	**%**		
C	68.3	62.1	1		66.7	65.9	1	
T	31.7	37.9	1.32 [1.00 - 1.75]	0.060	42.2	34.3	0.71 [0.47 - 1.07]	0.101

CI = confidence interval; FS= Febrile Seizures; MTLE – HS = Mesial Temporal Lobe Epilepsy – Hippocampal Sclerosis; n.s. = no significance; OR = Odds ratio

Table 3: rs1800629 genotypic and allelic frequencies in controls, MTLE-HS patients and subgroups of patients accordingly to FS antecedents

rs1800629 Genotypes	Controls (n=217) n (%)	MTLE-HS (n=182) n (%)	OR [95%CI]	p	FS No (n=83) n (%)	FS Yes (99) n (%)	OR [95%CI]	p
				0.217				**0.347**
GG	154 (71.0)	143 (78.6)	1		63 (75.9)	80 (80.8)	1	
GA	56 (25.8)	34 (18.7)	0.65 [0.40 - 1.06]	0.085	16 (19.3)	18 (18.2)	1.13 [0.53 - 2.39]	0.752
AA	7 (3.2)	5 (2.7)	0.77 [0.24 - 2.48]	0.660	4 (4.8)	1 (1.0)	5.08 [0.55 - 46.58]	0.151
Alleles	**%**	**%**			**%**	**%**		
G	85.2	88.0	1		88.1	90.4	1	
A	16.1	12.0	0.72 [0.48 - 1.07]	0.104	14.3	10.1	0.67 [0.35 - 1.25]	0.204

CI = confidence interval; FS= Febrile Seizures; MTLE – HS = Mesial Temporal Lobe Epilepsy – Hippocampal Sclerosis; n.s. = no significance; OR = odds ratio

Table 4: Phenotype frequency of HLA-DRB1* alleles in controls and MTLE-HS patients

HLA - DRB1 allele	Control Pop (n= 282)		MTLE-HS (n= 196)		OR	95% CI	p value
	n	f	n	f			
DRB1*01	66	23.4%	54	27.6	1.253	0.82 - 1.89	0.289
DRB1*03	44	15.6%	30	15.4	0.983	0.59 - 1.62	0.948
DRB1*04	69	24.47%	53	27.04	1.144	0.76 - 1.73	0.526
DRB1*07	72	25.53%	57	29.2	1.205	0.80 - 1.80	0.371
DRB1*08	24	8.51%	13	6.67	0.768	0.38 - 1.54	0.459
DRB1*09	14	4.96%	5	2.56	0.504	0.18 - 1.42	0.188
DRB1*10	11	3.9%	4	2.05	0.516	0.16 - 1.64	0.255
DRB1*11	55	19.5%	29	14.9	0.721	0.44 - 1.17	0.192
DRB1*12	9	3.19%	13	6.67	2.167	0.90 - 5.15	0.075
DRB1*13	84	29.79%	50	25.6	0.813	0.54 - 1.22	0.322
DRB1*14	17	6.03%	9	4.62	0.754	0.33 - 1.72	0.504
DRB1*15	56	19.86%	35	18.0	0.883	0.55 - 1.40	0.602
DRB1*16	13	4.61%	12	6.15	1.357	0.60 - 3.02	0.457

CI = confidence interval; HLA = Human Leukocyte Antigen; MTLE – HS = Mesial Temporal Lobe Epilepsy – Hippocampal Sclerosis; n.s. = no significance; OR = odds ratio

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Manuscript 3

Brain Expression of inflammatory mediators in Mesial Temporal Lobe Epilepsy patients

Brain Expression of inflammatory mediators in Mesial Temporal Lobe Epilepsy patients

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(submitted)

Abstract

Neuroinflammation may be central in epileptogenesis. In this we analysed inflammatory reaction markers in brain tissue of Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) patients. TLR4, IL-1β and IL-10 gene expression as well as the presence of activated HLA-DR+ microglia was evaluated in 23 patients and 10 cadaveric controls. Inflammation characterized by the presence of HLA-DR^{+} microglia and TLR4, IL-1β overexpression was evident in hippocampus and anterior temporal cortex of MTLE-HS patients. Anti-inflammatory IL-10 was also overexpressed in MTLE-HS patients. Our results show that hippocampal neuroinflammation extends beyond lesional limits, as far as the anterior temporal cortex.

Hippocampus, cytokines, inflammation, epilepsy, activated microglia

Highlights

- Activated microglia is present in hippocampus and anterior cortex of MTLE-HS
- IL-1β and TLR4 are upregulated in hippocampus and cortex of MTLE-HS patients
- Cortical and hippocampal upregulation of the anti-inflammatory cytokine IL-10 was observed in MTLE-HS patients
- Inflammatory response associated with seizure propagation

1. Introduction

Active inflammation has been documented not only in traditionally assumed inflammatory epilepsies but also in patients with pharmacoresistant epilepsy of diverse causes (Vezzani et al., 2011a, Vezzani and Ruegg, 2011). MTLE-HS is the most frequent focal epilepsy in adulthood. It is usually refractory with over 80% patients presenting a poor response to conventional anti-epileptic drugs (AEDs). Refractory patients are often subjected to surgical resection of the hippocampus and amygdala in order to control seizures. This is one of the most successful epilepsy surgeries. Nevertheless, it is reported a seizure recurrence of 38% at 18 years of follow-up after surgery (Hemb et al. , 2013). For MTLE-HS patients the efficient resolution of seizures is still an unmet clinical need. Understanding the epileptogenic process is fundamental for the development of new AEDs, but the mechanisms leading to MTLE-HS remain largely unknown. Retrospective studies show that MTLE-HS patients often have a history of initial precipitating injury such as febrile seizures (FS), central nervous system infection and head trauma or hypoxia peripartum (Fisher et al. , 1998). Among these factors FS is the most common with up to 80 % of MTLE-HS patients reporting a history of complex FS (Fisher, Sperber, 1998). It has been hypothesized that after the initial insult there is an abnormal cascade of damage repair, with the maintenance of chronic inflammation, leading to atrophy and sclerosis of hippocampus (Fisher, Sperber, 1998). In fact, imaging studies have shown that prolonged and lateralized FS can produce acute hippocampal injury with oedema that resolves within 5 days (Scott et al., 2003). The follow-up of these children showed changes in hippocampal symmetry consistent with injury and neuronal loss (Scott, King, 2003). So, it is believed that FS initiate the abnormal network reorganization that will lead to the development of an epileptogenic structure. Alternatively, the asymmetry could represent a return (post-acute oedema) to a pre-existing hippocampal abnormality (Fernandez et al. , 1998).

In normal physiological conditions pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, and their receptors are constitutively expressed at low levels in different brain regions by astrocytes, microglia cells, neurons and endothelial cells (Vezzani and Viviani, 2015). These proteins are claimed to be important in neuronal development, controlling neurite outgrowth, neurogenesis and cell survival (Vezzani and Viviani, 2015). These pro-inflammatory cytokines can also modulate voltage-gated and receptor-coupled ionic channels (Kulkarni and Dhir, 2009, Vezzani et al. , 2013, Viviani et al. , 2007) as well as neurotransmitter's receptors (Balosso et al. , 2009, Stellwagen et al. , 2005) controlling synaptic pruning, transmission and plasticity in the adult brain (Marin and Kipnis, 2013). In fact, IL-1β and IL-6 seem to have a general inhibitory action on CNS voltage- or ligand -

gated channels inhibiting Ca^{2+}, Na^{+} and K^{+} currents (for review see (Vezzani and Viviani, 2015)). In ligand-gated ion channels, IL-1β is observed to be mainly excitatory increasing NMDA-mediated Ca^{2+} influx (Viviani et al. , 2003). Additionally, IL-1β can promote excitability through the downmodulation of the astrocytic glutamate transporter (GLUT-1) (Viviani, Bartesaghi, 2003) while promoting the release of excitatory neurotransmitters such as Glutamate or ATP (Devinsky et al. , 2013). In fact, neuroinflammation appears to be an important component in epileptogenesis, reflecting complex cross-talks between microglia, astrocytes and neurons (Aronica et al. , 2012, Devinsky, Vezzani, 2013).
Cytokines can also influence the strength of synaptic transmission as they can modulate NMDA, AMPA and GABAA receptor expression and their sub-unit composition. In accordance, several studies demonstrated that a fine-tuned cytokine production is necessary for learning and cognition and that a dysregulation may lead to excitotoxicity (McAfoose and Baune, 2009, Yirmiya and Goshen, 2011).
Seizure-induced cell death leads to the release of endogenous molecules (DAMPs) such as HMGB1 that are recognized by TLRs expressed in glial cells and neurons (Bianchi, 2007, Mazarati et al. , 2011). The engagement of TLRs leads to the activation of innate immunity with the production of pro-inflammatory mediators. In fact, it has been demonstrated in animal models that several inflammatory cytokines (such as IL-1β, TNF-α and IL-6), as well as TLRs (e.g. TLR4 and TLR9), are rapidly induced by seizures, or by brain injury, in activated astrocytes and microglia (De Simoni et al., 2000, Jankowsky and Patterson, 2001, Maroso et al., 2010, Ravizza et al., 2005, Tan et al., 2015, Vezzani et al. , 1999, Vezzani et al. , 2000). Also, microarray studies in rodent models of TLE showed that inflammation is one of the most upregulated biological processes during epileptogenesis (Gorter et al. , 2006). Inflammatory molecules contribute to decrease seizure threshold by direct effects on neuronal excitability (Vezzani and Baram, 2007) and may also activate transcription of genes involved in neurogenesis, cell death, and synaptic plasticity (Vezzani et al., 2008b, Widera et al., 2006). In this way, inflammatory molecules can also participate in glial scar formation contributing to seizure-related hippocampal changes, such as neuronal loss, reactive gliosis and mossy fibre sprouting.

These experimental results are corroborated by studies in human brain tissue from drug-resistant epilepsies. In these patients, activation of hippocampal microglia with concomitant over-expression of HLA-DR (Beach et al. , 1995) and upregulation of inflammatory mediators has been evidenced (Aronica et al., 2007, Choi et al., 2009, Crespel et al., 2002, Jamali et al., 2006, Kan et al., 2012, Omran et al., 2012, Ravizza et al., 2008, Vezzani et al., 2011b). Upregulation of several inflammatory players has also been observed in the cerebrospinal fluid and serum of epileptic patients (de Vries et al.,

2016). Moreover, it is known that some conventional AEDs have an anti-inflammatory action (Matoth et al., 2000) and that administration of anti-inflammatory drugs can also have anti-convulsant effects with reduction of seizures (Hancock et al., 2013, Radu et al., 2017). On the other hand, it has been observed that anti-inflammatory cytokines, such as IL-10, may protect against seizures.

Studies using surgically-removed anterior cortical region are scarce. This region is thought to contribute to seizure propagation in MTLE-HS patients (Bartolomei et al., 2008). Thus, the aim of this study was to characterize the expression of inflammatory mediators, namely IL-1β, TLR4, and IL-10, both in the hippocampus and anterior temporal cortex.

2. Material and methods

2.1 Population

Resected fresh human tissue obtained from 23 MTLE-HS patients (13F, 10M, see Table 1) who underwent epilepsy surgical treatment (selective amygdalohippocampectomy or anterior temporal lobectomy) at Neurosurgery Department of Hospital Santo António – Centro Hospitalar e Universitário do Porto (HSA – CHUP) has been analysed. The decision for surgery was taken by HSA multidisciplinary epilepsy team incorporating neurologists, neurosurgeons, neuroradiologists, neurophysiologists and neuropsychologists. All patients were resistant to maximal doses of two or more conventional AEDs used during for more than 2 years. Pre-surgical assessment was discussed by the team analysing the results of brain MRI, prolonged video-EEG monitoring, ictal and interictal SPECT, neuropsychological assessment and functional brain MRI, in order to precise the epileptogenic zone and to determine the suitability of the patient for surgical intervention. Surgical specimens of the hippocampus and of the anterior temporal lobe were collected. A complete coronal slice of 0.5 cm thick was removed 3 cm posterior to the tip of the temporal pole. Samples were recovered in ice-cold synthetic CSF (10mM glucose, 124mM NaCl, 3mM KCl, 1mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26mM $NaHCO_3$, 2mM $CaCl_2$, pH=7.40) and immediately cryopreserved in liquid nitrogen. The amount of tissue removed did not differ from the strictly necessary for successful surgical practice. All patients gave written informed consent as stated in Declaration of Helsinki. As for the controls the same temporal lobe region from 10 human autopsies (8M, 2F; 67.0 ± 10.9 years) were analysed. Tissue was collected by a similar procedure from cadavers, with no known previous history of neurological disease, examined at the Forensics Institute of Porto within a short post-mortem delay (of 4 to 7 hours). This work was approved by the ethics committee of the participant institutions.

Table 1 – Clinical and demographic data from surgery MTLE-HS patients

Clinical /demographic data	MTLE-HS (n total =23)
F/M	13 /10
Age at surgery ± SD, years (range)	39.6 ± 9.8 (24 - 60)
Age of onset ± SD, years (range)	10.3 ± 6.8 (1 - 28)
Disease mean duration ± SD , years (range)	29.3 ± 9.0 (10 - 49)
Post-surgery time ± SD , years (range)	6.9 ± 1.3 (5 - 10)
Hippocampal Sclerosis (Left /Right)	15 / 8
Febrile seizures antecedents (Yes / No)	15 / 8
Engel classification (I / II / III / IV)	16 / 2 / 4 / 1

Sd = Standard deviation

2.2 Immunohistochemistry

Immunohistochemical staining was performed in 2 µm-thick sections with the mouse monoclonal anti-human HLA-DR alpha-chain clone TAL. 1B5 antibody (Dako, Agilent Technologies, Denmark) and the Novolink Polymer Detection kit procedures (Leica, Biosystems, Cambridge, UK). Heat-mediated antigen target retrieval was performed with 10 mM sodium citrate pH 6. Antibody optimum dilution was determined in a tissue positive control to be 1:400. Slides with replacement of the primary antibody with an antibody of the same immunoglobulin isotype were integrated in each experiment as negative labeling controls.

2.3 RNA extraction and gene expression quantification

RNA was isolated from the fresh brain tissue, using commercially available extraction kit RNeasy® blood and Tissue kit (Qiagen) following manufacturer´s instructions. cDNA was synthesized with an available commercial kit (Nzy First-Strand cDNA Synthesis Kit) in a Biometra thermocycler. IL-1β (hs01555410_m1), IL-10 (hs00961622_m1), TLR4 (hs00152939_m1) and the reference gene Ubiquitin C (UBC) (hs00824723_m1) expression was quantified by Real Time PCR with specific primers and probes (Taqman® Kits, Applied Biosystems, USA) and a NzySpeedy qPCR mastermix (Nzytech, Portugal) in Corbett Rotor Gene 600 Real Time Thermocycler machine (Corbett Research, UK). UBC gene was chosen as the reference gene since its expression has relatively low variability in the regions studied (Trabzuni et al. , 2011). Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4 Statistical analysis

Differences in ΔCt (Ct target gene – Ct UBC) were evaluated using two-tailed Student's t-test or Mann´s-Whitney test when appropriated. Normal distribution was evaluated with Kolmogorov – Smirnov test. Spearman's correlation coefficients were used to test interactions between age and expression levels. Logistic regression was used to test dependence of expression levels and age at onset, disease duration, FS antecedents and Engel classification. Analyses were done with SPSS v.23 software Package (IBM SPSS Statistics, USA) and significant levels were set at $p<0.05$.

3. Results

In order to assess inflammatory response in MTLE-HS patients, we analysed the presence of activated HLA-DR$^+$ microglia and quantified the expression of inflammatory markers – TLR4, IL-1β and IL-10 - in the hippocampus and anterior temporal cortex of MTLE-HS patients and cadaveric controls.

The presence of HLA-DR$^+$ cells was prominent in the hippocampus (Fig 1a) and also in the anterior cortical region (Fig 1c) of MTLE-HS patients. The presence of HLA-DR+ cells was not detected in the hippocampus and anterior temporal cortex of control individuals (Fig 1b and 1d, respectively).

MTLE-HS patients had significantly higher levels of expression of TLR4, IL-1β, and IL-10 in the anterior temporal cortex than control individuals (Table 2, Figure 2). The same was observed in the hippocampus where the expression of TLR4, IL-1β, and IL-10 was also higher in MTLE-HS patients than in controls (Table 2, Figure 3).

Table 2 – Relative expression of inflammatory markers in brain of MTLE-HS comparing to controls

cytokine	Hippocampus (fold change)	p	Anterior Cortex (fold change)	p
TLR4	2.3	0.028	5	0.002
IL-1β	13	0.000019	4	0.002
IL-10	2	0.027	2	0.027

Since control individuals were (on average) older than MTLE-HS patients, we performed a correlation analysis to verify if this difference could bias our results. No correlation was observed between age and the expression levels of the inflammatory mediators, TLR4, IL-1β or IL-10, in the two cerebral regions obtained from control individuals and MTLE-HS patients. A univariated linear model analysis was used to assess possible confounding

effects of gender, age of onset, febrile seizures past history, or Engel classification. No significant correlations between these factors and gene expression were detected

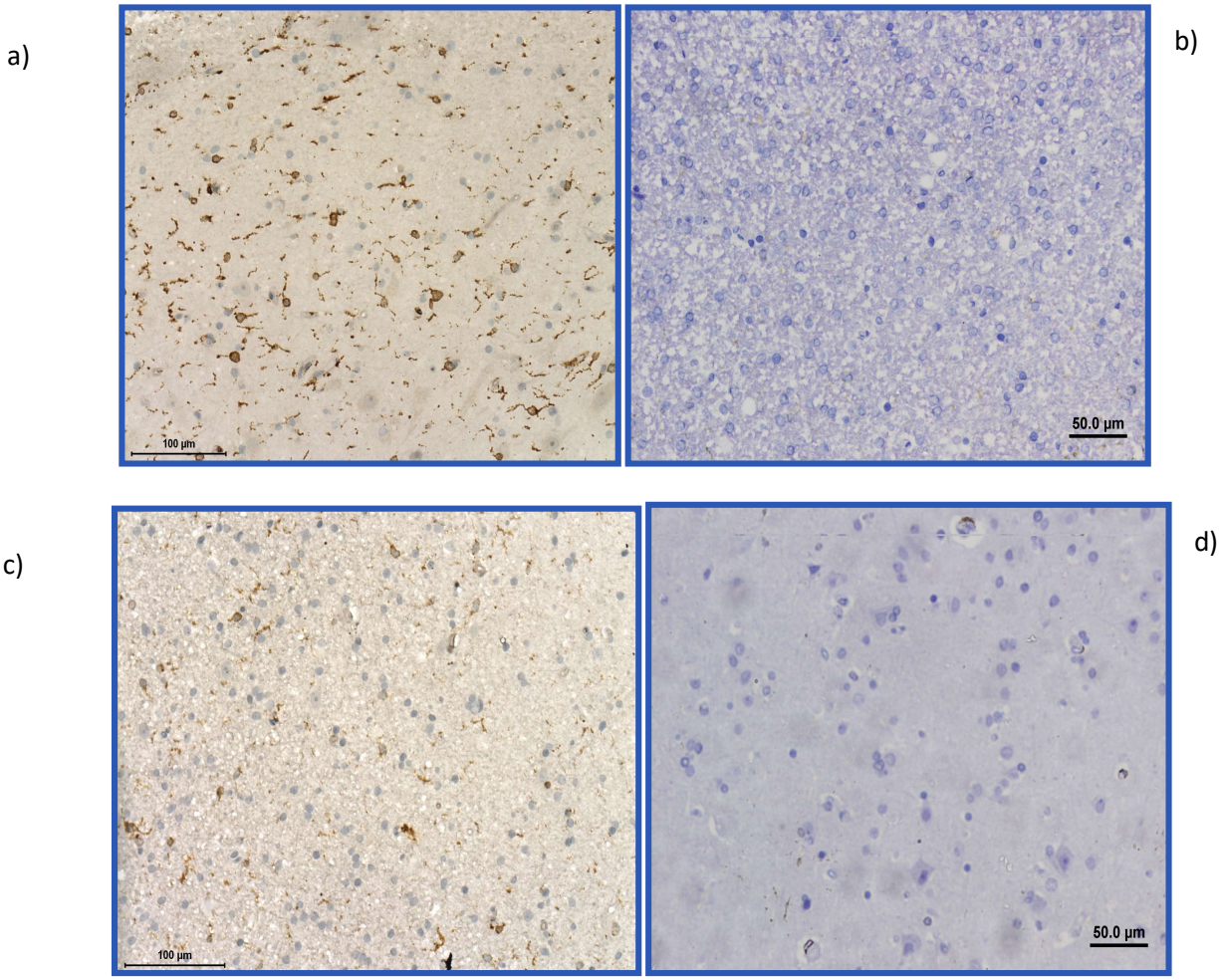
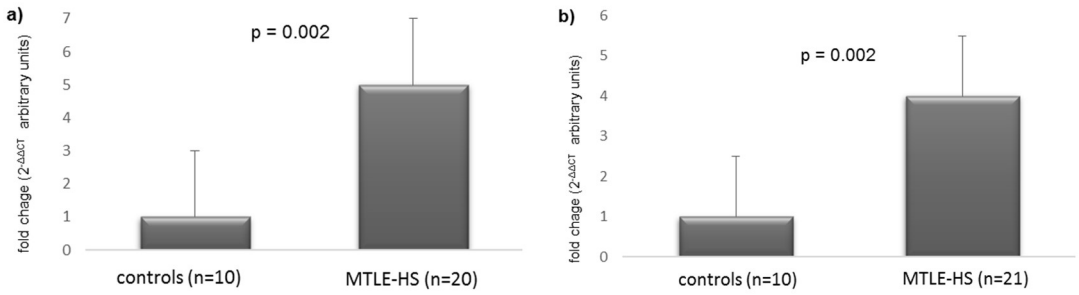


Figure 1 - HLA labeling showing immunoreactivity in the hippocampus (a and b) and anterior cortex (c and d) of MTLE-HS patients (a and c) and controls (b and d). Shown images are representative of all individuals studied**.** HLA-DR+ cells were observed in the hippocampus and anterior temporal cortex of MTLE-HS patients (n=10), but not in control individuals (n = 8). Scale bars: 50 or 100 µm, as indicated.



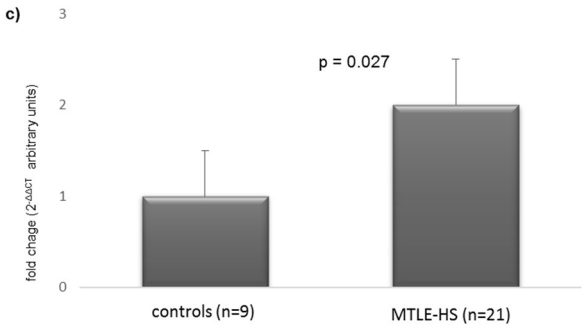


Figure 2 - Cortical expression of inflammatory mediators in MTLE-HS patients and in control individuals. a)TLR4; b) IL-1β: c) IL-10. MTLE-HS patients had a higher expression of inflammatory mediators than control individuals. Values are presented as mean ± SEM (standard error mean)

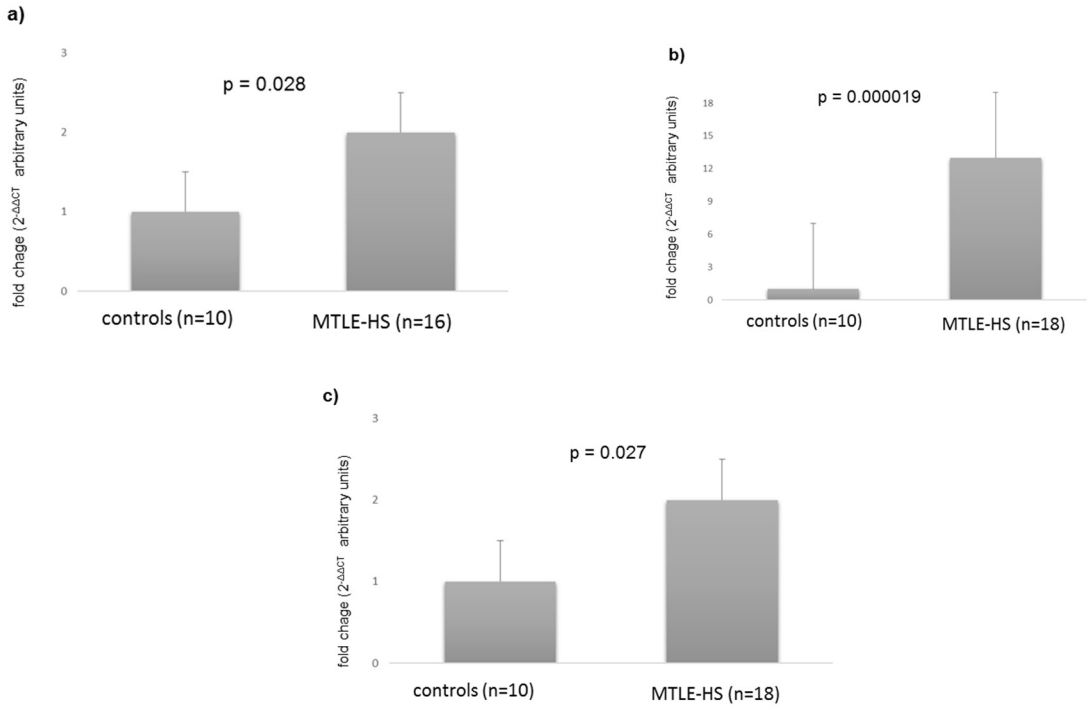


Figure 3 - Hippocampal expression of inflammatory mediators in MTLE-HS patients and control individuals. a)TLR4; b) IL-1β: c) IL-10 MTLE-HS patients had a higher expression of inflammatory mediators than controls. Values presented as mean ± SEM (standard error mean)

4. Discussion

The main goal of this study was to evaluate the inflammatory response in brain tissue of MTLE-HS patients. We observed the presence of activated HLA-DR+ microglial cells in the hippocampus and anterior temporal cortex of MTLE-HS patients. Similar results have

already been observed both in animal models (Avignone et al., 2008) and in the hippocampus of MTLE-HS patients (Beach et al. 1995).

Concomitant with microglia activation we have observed the overexpression of the pro-inflammatory cytokine, IL-1β, and of the immune receptor, TLR4, in the hippocampus and anterior temporal cortex of MTLE-HS patients. A large number of studies, both in animal models and epileptic patients corroborate our results (Bianchi, 2007, Crespel, Coubes, 2002, Jankowsky and Patterson, 2001, Kauffman et al., 2008, Vezzani et al., 2008a, Vezzani et al., 2011b, Vezzani et al., 2000). Although a dysregulation in inflammatory molecules has been widely described, it is yet not known if the inflammatory process is secondary to recurrent seizures or if it is a consequence of persistent neuronal loss and/or reactive gliosis.

The presence of activated microglia in the anterior cortical region of epileptic patients demonstrates the existence of a persistent inflammatory reaction extending beyond the limits of the initial epileptic focus, as far as the anterior temporal cortex, thus suggesting that the epileptogenic process is more diffuse than it is generally acknowledged.

Increased neuronal activity leads to the release of molecules such as the chromatin-derived HMGB1 that is recognized by the TLR4. Activation of this receptor culminates in the production of pro-inflammatory cytokines and molecules involved in neuronal survival and repair. Upregulation of HMGB1 and astrocytic TLR4 has been widely described in animal models and epileptic patients (Maroso et al. 2010) but the driving-forces causing this effect remain elusive. We have detected TLR4 transcripts in the brain of control individuals. Chakravarty and Herkenham (2005) have also demonstrated the presence of TLR4 transcripts, without showing the protein, in CNS-resident cells and that activation of these receptors sustaining neuroinflammation was independent of systemic cytokine effects. These results suggest that TLR4 expression is under a tight control of post-translational mechanisms. It has been shown that seizures modify cell microenvironment and metabolism which may be associated with post-translational changes in gene expression (Jankowsky and Patterson, 2001, Vezzani, 2005). In line with this and taking into consideration that we have observed a more pronounced TLR4 upregulation in the anterior cortex, one may hypothesize that TLR4 expression is driven by seizures. Accordingly, it has been observed that a higher seizure frequency is associated with higher TLR4 expression (Pernhorst et al., 2013).

Cell damage or intense neuronal firing leads not only to HMGB1 release, but also to the accumulation of extracellular ATP. Whilst HMGB1 activates TLR4, ATP binds to purinoceptors, namely to P2X7R, which most possibly cooperate to increase IL-1β

production. Thus, seizure-induced IL-1β production may lead to dysregulation of neurotransmission initiating a vicious cycle of inflammation and neuronal excitation. In fact, it has been described that persistent and chronic activation of innate immune response, mainly the TLR4 – IL-1β axis, may be crucial for seizure development (Aronica and Gorter, 2007; De Simoni et al., 2000; Devinsky et al., 2013; Gorter et al., 2006; Iori et al., 2016; Omran et al., 2012; Ravizza et al., 2008; Sheng et al., 1994; Vezzani et al., 2011b; Vezzani et al., 2000). In line with this, we have observed that IL1β is overexpressed both in the hippocampus and anterior cortex region, which is more notorious in the hippocampus coinciding with the major cell loss observed in this region compared to the anterior temporal cortex. On their own, cytokines can contribute to cell death via promotion of nitric oxide release and reactive oxygen species production. Accordingly, it was observed that HMG1B release is stimulated by IL-1β (Ravizza and Vezzani, 2006, Vezzani et al., 2008a). In spite of this, the epileptogenic potential of this cytokine has been mainly attributed to a dysregulation of neurotransmission, since IL-1β overexpression is associated to extracellular glutamate accumulation, ionic imbalance and higher NMDA currents, while GABA currents are diminished (for review see (Vezzani and Viviani, 2015)). In animal models, a prompt IL-1β, TNF-α, and IL-6 upregulation has been observed after seizure induction (Ravizza and Vezzani, 2006). However, the increase in TNF-α and IL-6 was only transient, whilst IL-1β levels remained higher long after seizure cessation (Ravizza and Vezzani, 2006, Vezzani et al., 2008a). Additionally, the injection of the IL-1R antagonist (IL-1Ra) (Vezzani, Moneta, 2000), as well as genetic or pharmacological blockade of IL-1β, affords seizure reduction (Maroso et al. , 2011, Noe et al. , 2013). These observations suggest that IL-1β may be an important player in epileptogenesis. Accordingly, a polymorphism in the IL-1B promotor region that induces high cytokine expression has been described as a susceptibility factor for MTLE-HS in different populations (Kauffman, Moron, 2008), including in the cohort studied (Leal et al., unpublished data; see Chapter II – Manuscript 3).

Exacerbated inflammatory responses are restrained by the production of anti-inflammatory cytokines, such as IL-10. This cytokine has a broad anti-inflammatory action decreasing pro-inflammatory protein expression, down-modulating HLA-DR and inhibiting the activation of macrophages / microglial cells (Murray, 2006). IL-10 can also mediate neuronal survival an adult neurogenesis (Pereira et al., 2015, Perez-Asensio et al. , 2013). So, the IL-10 upregulation observed in this study may be a brain response to seizure activity in order to control inflammation and consequent excitotoxicity, and to prevent and repair seizure-induced neuronal cells damage. Few studies have addressed the role of this cytokine in MTLE-HS. Nevertheless, in has been demonstrated that IL-10 levels

increase in the brain and in the serum of patients with different epileptic syndromes (Kan et al., 2012). Interestingly, polymorphisms resulting in high IL-10 expression may be protective factors for FS (Ichiyama et al. , 2008).

5. Conclusion

The acute or long-term exposure to inflammatory cytokines can determine modifications in neurotransmission with opposite effects. The net overall effect will depend on cytokine balance and target cell. Our study gives further support to previous data showing a persistent activation of the innate immune response in epilepsy. The upregulation of the IL-1β – TLR4 axis in epilepsy was particularly evident in the anterior cortical area, thus suggests that it might contribute to seizure propagation. The observed IL-10 upregulation may be a compensatory mechanism to overcome the exacerbated inflammatory response. A better understanding of the dynamics between pro- and anti-inflammatory molecules in the context of seizure mechanisms may provide insights useful for the identification of new therapeutic targets.

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Manuscript 4

Serum expression of inflammation-associated microRNAs in Mesial Temporal Lobe Epilepsy patients

Serum expression of inflammation-associated microRNAs in Mesial Temporal Lobe Epilepsy patients

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(submitted)

Abstract

Background: Neuroinflammation is an important epileptogenic mechanism. MicroRNAs (miRNA) are small non-coding RNA molecules that function as post-transcriptional regulators of gene expression. In this way, microRNAs control different biological processes including immune system homeostasis and function. Several evidences, both in patients and animal studies, have demonstrated an abnormal brain expression of miR-146a, miR-132 and miR-155 in Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS). Knowing that miRNAs expression is very stable in biological fluids such as plasma or serum our aim was to characterize the expression of these miRNAs in serum of MTLE patients and try to correlate expression levels with clinical characteristics.

Methods: Serum expression levels of miR-146a, miR-132 and miR-155 were quantified by Real-Time PCR in the serum of 73 MTLE-HS patients (39F, 34M, 42.4 ± 11.6 years) and 80 healthy individuals (52F, 28M, 42.1 ± 10.5 years). Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results: Serum levels of miR-146a and miR-132 was 2 fold higher in MTLE-HS patients comparing to controls ($p=0.009$ and $p=0.021$, respectively). MTLE-HS patients and control individuals had similar serum miR-155 expression levels. No other associations were observed.

Conclusion: In accordance with other studies this work supports a role of inflammation and respective regulatory mechanisms in epileptogenic process. Our results show that the inflammation-associated miR-146a and miR-132 may influence MTLE-HS development. The serum quantification of miR-146a and miR-132 may be a reliable analysis method and miRNAs may be suitable biomarkers for epileptogenesis. Understanding the role of these molecules in epilepsy's pathogenesis may also provide the development of novel therapeutic targets for the treatment of drug-refractory epilepsy.

1. Introduction

Epileptic seizures are associated with a profound change in gene expression. Microarray studies have demonstrated that inflammation is one of the most upregulated processes during epileptogenesis, in both clinical and experimental studies (Gorter et al., 2006). Overexpression of pro-inflammatory cytokines during epileptogenesis has been demonstrated, particularly if seizures are associated with cellular death (Vezzani and Baram, 2007). The epileptogenic potential of pro-inflammatory cytokines is due to much more than a direct neurotoxic effect, but they can also interfere with neurotransmitter receptors and ionic balances disrupting the depolarization threshold (Balosso et al., 2008).

Gene expression is modulated not only by genetic mechanisms, but epigenetic factors play an important role, as well. Epigenetic mechanisms are diverse such as DNA methylation, histone modification or non-coding RNAs. MicroRNAs (miRNAs) are short non-coding RNA molecules (19-25 nucleotide length) that function as post-transcriptional repressors of gene expression. This is accomplished through mRNA degradation or inhibition of its translation (Du and Zamore, 2005). The net effect is the downregulation of protein synthesis, modulating several biological processes, such as immune responses and neurotransmission. One interesting feature is the ability of a single microRNA to modulate diverse genes from the same or different pathways. Additionally, one mRNA molecule can have several binding sites for different miRNAs.

MicroRNAs have been considered potential biomarkers for several pathological conditions, like drug resistant colorectal cancer, endothelial dysfunction, hypertension and glioblastoma. In the last years, a role for these molecules on epilepsy development and progression has been described, both in animal models or epileptic patients (Henshall et al., 2016). The most studied epileptic syndrome, in this context, has been Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS), the most common focal epilepsy in adults. MTLE-HS development is frequently associated with antecedents such as traumatic brain injury, foetal hypoxia and most frequently Febrile Seizures (Wieser, 2004). In fact, 50-80% of MTLE-HS patients report a history of febrile seizures (FS) that have been described as a predisposing factor for early disease development (Leal et al., 2016). It is known that the majority of MTLE-HS patients (up to 90%) are refractory to currently available anti-epileptic drugs (AEDs) (Wieser, 2004). In these cases, surgery for removal of affected area is the most effective treatment for seizure remission, but it can have detrimental effects on patients' life and, therefore, constitutes a tremendous burden for health care systems.

Several miRNAs have been shown to be up or downregulated after an epileptic seizure. In recent years, targeted and genome-wide studies have reported more than 100 miRNAs with a different expression profile in epilepsy (Alsharafi et al., 2015; Ashhab et al., 2013; Gorter et al., 2014; Henshall et al., 2016; Kan et al., 2012; Omran et al., 2013; Peng et al., 2013; Roncon et al., 2015; Sun et al., 2016; Surges et al., 2016; Wang et al., 2015a; Wang et al., 2015b; Yan et al., 2017; Zucchini et al., 2014). These differences can be in the expression levels or in cellular location. Interestingly, microRNAs that regulate the immune system, particularly pro-inflammatory responses, are the ones most frequently reported alterations (Bot et al., 2013).

The first study of miRNA in epilepsy has demonstrated an increase of hippocampal miR-146a levels in TLE-HS patients and in animal models (Aronica et al., 2010). MiR-146a is considered a dominant negative regulator of neuroinflammation acting on a feedback loop (Saba et al., 2014). It is particularly important in astrocytes and glial cells where it is responsible for fine-tuning the response of these cells to cytokines, such as IL-1β (Iyer et al., 2012). In the recent years, several other studies have contributed to strengthen the linkage between brain miR-146a upregulation and epilepsy (Bot et al., 2013; Gorter et al., 2014; He et al., 2016; Hu et al., 2012; Omran et al., 2012; Roncon et al., 2015; Song et al., 2011).

Another important miRNA for immune response control is the miR-132, a very abundant miRNA in neurons (Peng et al., 2013). The miR-132 participates in the regulation of several neurological processes, such as synaptic plasticity, neuronal excitability, cellular death, neuronal differentiation and neurite outgrowth, among others (Edbauer et al., 2010; Hwang et al., 2014; Luikart et al., 2011; Magill et al., 2010; Remenyi et al., 2013; Wanet et al., 2012). It has been argued that miR-132 might play a role in neuroprotection (Jimenez-Mateos et al., 2011; Yuan et al., 2016), since it downmodulates inflammatory response after TLR-4-induced astrocyte-related inflammation (Kong et al., 2015). Accordingly, it has been described that miR-132 is overexpressed in the brain tissue of both animal models and TLE patients (Bot et al., 2013; Peng et al., 2013; Ren et al., 2016; Yoshimura et al., 2016; Yuan et al., 2016).

MiR-155 overexpression has been associated to inflammation as well (Ashhab et al., 2013). This microRNA is important for modulation of several CNS functions, such as the blood-brain barrier permeability, altering the phenotype and neurovascular function of the cerebral endothelium specifically by the downregulation of different components that participate in cell-to-cell and cell-to-matrix adhesion (Lopez-Ramirez et al., 2014). The overexpression of miR-155 has been described to negatively regulate neurogenesis

(Woodbury et al., 2015). In the immune system, it acts mainly as a pro-inflammatory molecule (Cardoso et al., 2012); miR-155 promotes monocyte differentiation to the pro-inflammatory phenotype M1 (Cardoso et al., 2012) and regulates T cells activation, differentiation and proliferation (Murugaiyan et al., 2011; Rodriguez et al., 2007). It has been observed that high levels of the miR-155 is associated with higher levels of the pro-inflammatory cytokine, TNF-α (Ashhab et al., 2013), while it inhibits the expression of anti-inflammatory cytokines (Cardoso et al., 2012).

Moreover, different studies demonstrated that the use of antagomirs (miRNA inhibitors) for different miRNAs reduce seizure-induced cellular damage, EEG profiling (Henshall et al., 2016; Ren et al., 2016) and may protect against the development of neurodegenerative disorders (Butovsky et al., 2015). All these data indicate that inflammation-associated miRNAs may be relevant in epilepsy development. It is known, that miRNAs are quite stable in biological fluids such as plasma or serum and normally reflect tissue production (Turchinovich et al., 2012). Therefore, they could prove to be useful biomarkers for diagnosis, prognosis or optimization of treatment in epilepsy. Notwithstanding this, the majority of the studies were performed in brain tissue, not in plasma or serum, prompting for the need to investigate whether their levels in more easily accessible biological fluids reflect disease conditions of the CNS, like epilepsy.

The aim of this study was to evaluate the expression levels of circulating inflammation-associated miRNAs, miR-146a, miR-155 and miR-132, in MTLE-HS patients compared to a control population. We also wanted to analyse the existence of correlations between miRNAs serum level and clinical characteristics, such as FS antecedents, disease duration, age at onset or AEDs response.

2. Materials and Methods:

2.1. Population:

In this study 73 MTLE-HS patients (39F, 34M, 42.4 ± 11.6 years) (Table 1) followed up at a tertiary epilepsy centre from North of Portugal were included. All patients had MTLE-HS diagnosis based on clinical and electrophysiological studies (EEG and/or video-EEG monitoring) and on brain MRI (minimum 1.5T) features, as defined by Wieser (Wieser, 2004). Definition of HS was based on brain MRI findings criteria, which comprised atrophy, T2 hyperintensity signal and altered internal structure on one or both hippocampi, associated or not with other imaging criteria like ipsilateral fornix atrophy, ipsilateral mammillary bodies atrophy or ipsilateral entorhinal abnormalities. We excluded patients with other MTLE-HS aetiologies like HS due to dual pathology. Patients may have visual

and/or verbal memory impairment but patients with other abnormalities in neurological examination were excluded. At the time of the study 21% of patients were in monotherapy and 79% in polytherapy, and 57 patients were refractory to pharmacological treatment (table 1). Carbamazepine was the most used anti-epileptic drug in both patients in mono (45%) as well as in polytherapy (56%). Levetiracetam was the most used adjunct in polytherapy (22%). Data concerning Febrile Seizures antecedents was collected from patient medical records. Thirty-nine patients had a history of FS (Table 1). The control population comprised 80 healthy controls (52F, 28M, 42.1 ± 10.5 years) voluntarily recruited among blood donors, ethnically matched, from the same geographical area. This control population was inquired regarding multiple diseases. Individuals with neurological diseases (epilepsy, Alzheimer's disease, febrile seizures among others) or a positive familial history of these pathologies were not included. The study was approved by the Hospital Ethical Committee and all individuals gave written informed consent in accordance with Declaration of Helsinki**.**

Table 1 – Clinical and demographic data from MTLE-HS patients

Clinical /demographic data	MTLE-HS (n total =73)
F/M	39 /34
Age ± SD, years (range)	42.4 ± 11.6 (13 - 68)
Age of onset ± SD, years (range)	13.6 ± 10.6 (1 - 55)
Disease mean duration ± SD , years (range)	28.8 ± 12.8 (5 - 58)
Hippocampal Sclerosis (Left /Right/Bilateral)	40 / 30 / 3
Febrile seizures antecedents (Yes / No)	39 / 34
Type FS (Complex / Simple / Undetermined)	19 / 14 / 6
AED (0 / 1 / 2 / ≥ 3)	1 / 15 / 27 / 30
Refractory to treatment	57

2.2. MiRNAs quantification

Peripheral blood was collected in dry glass tubes (Vacuette, GBO, Germany), centrifuged at 490g for 20 minutes and serum aliquots were stored at -20ºC. RNA was extracted using the miRNeasy® Serum/Plasma Kit (Qiagen, Germany), according to the manufacturer's protocol. Synthesis of cDNA was performed with the Taqman®MicroRNA reverse Transcription Kit (Applied Biosystems, USA) and microRNA assay (Taqman® MicroRNA Assays – Applied Biosystems, USA). The reaction was performed in a Biometra Alfagene thermocycler accordingly to the manufactures' instructions. The quantitative RT-PCR amplification was run with a NzySpeedy qPCR mastermix (Nzytech, Portugal) in a Corbett Rotor Gene 600 Real Time Thermocycler (Corbett Research, UK). Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative

expression was calculated using the 2-ΔΔCT method. MiRNAs levels were evaluated in serum, a cell-free body fluid that has no known RNA species, at constant levels that could be used for normalisation. To overcome this problem, equal serum volumes were used for each subject and the same threshold was used for each target miRNAs so that expression levels could be compared between samples. MicroRNAs levels are expressed as arbitrary units calculated 50-Ct (Wang et al., 2010).

2.3. Statistical analysis

Differences in ΔCt were evaluated using a two-tailed Student's t-test or Mann´s-Whitney test as appropriated. Normal distribution was tested using the Kolmogorov – Smirnov method. Spearman's correlation coefficients were used to evaluate interactions between disease duration and expression levels. Analyses were done using the SPSS v.23 software and significant levels were set at $p<0.05$ for all statistical analysis.

3. Results:

Serum expression levels of miR-146a (Figure 1) and miR-132 (Figure 2) were higher in MTLE-HS comparing to controls ($p = 0.009$ and $p = 0.021$, respectively). Circulating miR-155 expression levels were similar between MTLE-HS patients and controls (Figure 3).

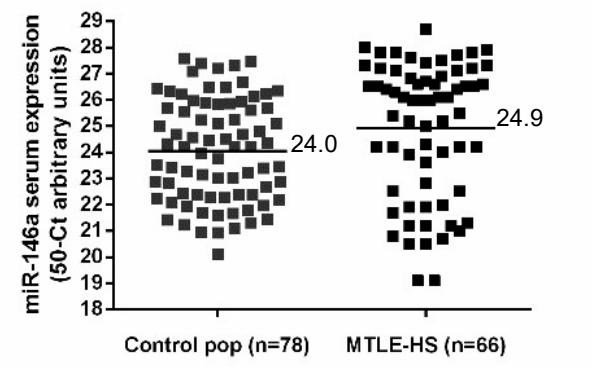

Fig. 1 - Circulating miR-146a expression levels in control population and MTLE-HS patients. Fold change calculated as $2^{-\Delta\Delta CT}$

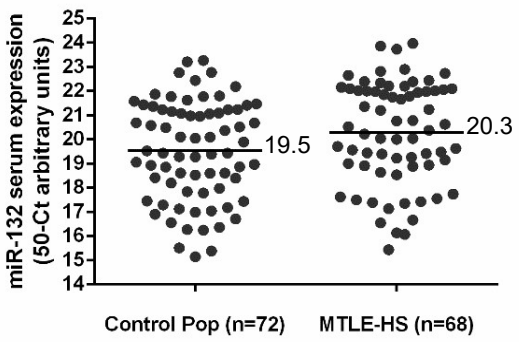

Fig. 2 - Circulating miR-132 expression levels in control population and MTLE-HS patients. Fold change calculated as $2^{-\Delta\Delta CT}$

No association between miRNAs expression levels and refractoriness for treatment was observed (data not shown). No correlation was found between miRNAs expression levels and age at onset, disease duration or number of AEDs (data not shown).

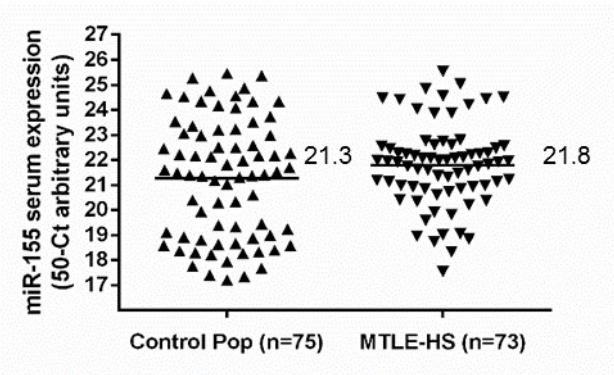

Fig. 3 - Circulating miR-155 expression levels in control population and MTLE-HS patients.

Neuroinflammation is emerging as an important process in epileptogenesis and its regulatory mechanisms may be of particular interest in the development of new AEDs. Our studies, in accordance with previous reports, support a role for inflammation-associated miR-146a and miR-132 in epilepsy development. Although, our results show that these miRNAs are unlikely to be sufficiently sensitive or specific to be suitable biomarkers of epilepsy its widely observed upregulation may give important clues for a better understanding of the epileptogenic process.

MiR-146a and miR-132 upregulation may be a reflex of a disturbance in the regulatory network of neuroinflammation, contributing for development and progression of MTLE-HS. Our results as well as other previous studies, support this hypothesis. After an initial insult (for example a seizure) an upregulation of the inflammatory response with higher pro-inflammatory cytokine levels, is observed (Omran et al., 2012; Ravizza et al., 2008). In this inflammatory milieu, there is an upregulation of regulatory microRNAs, namely miR-146a and miR-132 (among others) in order to overcome the exacerbation of the inflammatory response (Gorter et al., 2014). Persistent seizures can lead to a chronic inflammatory state with sustained higher microRNAs levels.

Although miR-146a upregulation in epilepsy has been widely described its exact role in epileptogenic process is not yet clear (An et al., 2016; Aronica et al., 2010; Bot et al., 2013; Gorter et al., 2014; Hu et al., 2012; Omran et al., 2012; Song et al., 2011; Wang et al., 2015b). In vitro studies have shown that the stimulation of an astrocytic inflammatory response stimulates miR-146a synthesis. This miRNA will then act in a negative feedback loop inhibiting IL-1β-mediated inflammatory response downstream effectors, such as IRAK-1 (Interleukine-1 receptor-associated kinase), IRAK-2 and TRAF-6 (TNF Receptor Associated Factor-6) (Iyer et al., 2012). Studies in animal models and MTLE patients demonstrate brain miR-146a upregulation in both latent and chronic epileptogenic phases (Aronica et al., 2010; Omran et al., 2012). It has also been observed that the miR-146a upregulation in the latent phase is driven by IL-1β overexpression after an initial seizure and that it downmodulates cytokine levels (Omran et al., 2012). Therefore, it is argued

that in this phase miR-146a upregulation may be a compensatory mechanism to overcome the exacerbated inflammatory response caused by seizure-induced damage. In line with this, Iori et al, have recently demonstrated that injection of a miR-146a mimic has an anti-seizure effect downmodulating IL-1 signalling pathway (Iori et al., 2017). In the chronic phase, miR-146a upregulation is concomitant with IL-1β overexpression (Omran et al., 2012). Some studies have shown that miR-146a downregulates the complement factor H, an inhibitor of the inflammatory cascade, leading in this way to an exacerbation of inflammation (He et al., 2016).

MiR-132 besides neuroinflammation also regulates synaptic transmission (Yoshimura et al., 2016). It has been demonstrated that miR-132 has an anti-inflammatory activity targeting IRAK4 and, consequently, the expression of IL-1β and IL-6 (Kong et al., 2015). It can also suppress inflammation downregulating acetylcholinesterase (AChE) (Shaked et al., 2009). Our results, in accordance with previous studies in both epileptic patients and animal models, demonstrate that miR-132 is associated with epilepsy development (Bot et al., 2013; Hwang et al., 2014; Remenyi et al., 2013; Yoshimura et al., 2016). MTLE-HS is associated with neuronal damage, astrocytosis and mossy fibre sprouting. On one hand, mir-132 targets HBRGF (Heparin-binding EGF-like growth factor), and is essential for the transition from inflammation to cell proliferation in the wound healing process (Li et al., 2015) participating in neuroprotection mechanisms important for brain damage repair. On the other hand, it has been described that miR-132 upregulation leads to enhancement in Ca^{2+} and k^{+} channels currents and disruption of depolarizing threshold (Xiang et al., 2015). In this way, there is impairment in synaptic transmission and neuronal excitability with consequent exacerbation of epileptic seizures (Xiang et al., 2015). Accordingly, it has been observed that miR-132 silencing supresses neuronal apoptosis and mossy fibber sprouting decreasing the occurrence of spontaneous seizures (Huang et al., 2014; Jimenez-Mateos et al., 2011; Yuan et al., 2016). Tight regulation of a gene is the result of the cooperation of several miRNAs and its action may depend on the presence of other molecules. Accordingly, miR-132 can cooperate with miR-146a to counteract seizure-induced inflammation.

TNF-α is an important cytokine in the epileptogenic process, playing a role in neurodegeneration or neuroprotection, depending on the receptor that is being activated. It has been reported that expression levels of this cytokine are positively correlated with miR-155 levels, a miRNA described as a pro-inflammatory regulator (Ashhab et al., 2013). Ashhab et al have demonstrated that miR-155 is upregulated in in brain tissue of animal models and children with MTLE-HS (Ashhab et al., 2013). These authors observed the upregulation of both TNF-α and miR-155 in all phases of the epileptogenic process, particularly in the acute phase, and postulate that the inflammatory process is important

for the exacerbation and progression of epileptic seizures. This is supported by evidence that miR-155 antagonists protect against pilocarpine-induced seizures (Cai et al., 2016). We do not observe an association between miR-155 serum expression levels and MTLE-HS development. Whilst we included adult patients in a chronic state of disease with 28.8 ± 12.8 years of disease duration Ashhab et al quantified miR-155 in children and in an immature mouse model. It may be hypothesized that miR-155 may have a role restricted to epileptogenesis in the immature developing brain and that in later developmental stages and in chronic epilepsy stage its action on epileptogenic process is not fundamental. Accordingly, it has been observed a continuous changing in miRNAs expression pattern in chronic epilepsy (Roncon et al., 2015) demonstrating the dynamics of epileptogenic process.

Conclusions

This study supports a role for inflammation-associated miRNAs, miR-146a and miR-132, in MTLE-HS development and progression. These results also validate the quantification of circulating miRNAs as a reliable analysis method suggesting that it may be useful to monitor epileptic patients, as has been recently suggested (Sun et al., 2016). Since, miRNAs are pleiotropic molecules, it is important to clarify all the epileptogenic pathways that are being modulated by these miRNAs. This is important for a better understanding of the molecular mechanisms that drive seizure development and progression and could help in the identification of new therapeutically targets.

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Manuscript 5

Adenosine Receptors and Adenosine Kinase Upregulation in Mesial Temporal Lobe Epilepsy Patients

Adenosine Receptors and Adenosine Kinase Upregulation in Mesial Temporal Lobe Epilepsy Patients

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(in preparation)

Abstract

Adenosine is considered a most important endogenous anti-epileptic agent, since it fine-tuning modulates synaptic transmission and neuroinflammation. The endogenous adenosine levels are tightly modulated by adenosine kinase (ADK) activity, the highest affinity metabolizing enzyme for the nucleoside. Disequilibrium between adenosine receptors activation and nucleoside controlling enzymes has been reported in epilepsy. Astrogliosis, a hallmark of Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS), is the main responsible for the observed upregulation of ADK and, consequently, the low extracellular levels of adenosine in epileptics. This finding prompt to the hypothesis that augmentation of endogenous adenosine levels may effectively control seizures. Complexity to this idea, however, arises from the fact that adenosine acts through high-affinity inhibitory A_1 and facilitatory A_{2A} receptors in the brain, which activation balance might be affected during the epileptogenic process. This study was designed to investigate the mRNA expression of ADK, A_1R and $A_{2A}R$ in the hippocampus and anterior temporal cortex of a cohort of 23 MTLE-HS patients compared to 9 cadaveric controls. Patients with MTLE-HS exhibit higher expression of A_1R ($p = 0.007$) and ADK (p = n.s.) in the hippocampus compared to control individuals. These differences were accentuated in the anterior temporal cortex ($p = 0.001$ for A_1R and ADK). While the expression of $A_{2A}R$ was higher in the cortex of MTLE-HS patients, no differences were observed comparing hippocampal samples from epileptic patients and controls. Results are in accordance with the hypothesis that overexpression of the adenosine metabolizing enzyme, ADK, in the hippocampus and anterior temporal cortex of MTLE-HS patients may contribute to decrease the threshold and contribute to seizures propagation, as it is usually associated with low endogenous levels of adenosine. Decreases in the extracellular concentration of adenosine may drive a compensatory increase in the expression of high affinity A_1R and $A_{2A}R$ in the brain of MTLE-HS patients, which magnitude may differ between the hippocampus and anterior temporal cortex. The pathophysiological meaning of this disequilibrium deserves further investigations in order to test its repercussions at the receptor protein level and its impact on neuronal cells function.

Introduction

Adenosine is a ubiquitous homeostatic molecule that participates in fundamental processes concerning cell viability and adaptation, namely energy state, redox reactions, nucleic acid maintenance and epigenetic control [1]. Extracellular adenosine levels are a paracrine danger signal for dysregulation in cellular activity. Adenosine‘s functions are operated by the activation of four distinct G-protein-coupled receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) [2]. The high affinity, A_1 and A_{2A},, receptors are more abundant in the brain tissue than in other organ [6], suggesting an important role of adenosine in brain activity [3] where it acts as an “endogenous neuromodulator” [4-8]. The A_1R is distributed throughout the brain being the second most abundant metabotropic receptor in the central nervous system. Activation of the A1R has a predominantly inhibitory action controlling excitatory glutamatergic synapses leading to reduced presynaptic neurotransmitter release and to postsynaptic hyperpolarization [9-14]. The A_{2A}R is present predominantly in basal ganglia, being also found at a lower density in the hippocampus [15-17]. In the hippocampus, it is predominantly located on glutamatergic nerve terminals [18,19], but recent studies have demonstrated its presence in astrocytes and microglia [20-25]. Synaptic A_{2A}R activation facilitates the release of glutamate from hippocampal nerve terminals [22,26] and attenuates the A_1R-mediated neuroprotection [27,28]. In microglia, activation of the A_{2A}R is associated with cell proliferation and to the production of cytokines and growth factors [23-25,29,30].

Unbalanced adenosine metabolism has been implicated in pathological conditions, such as epilepsy. From the enzymes that metabolize adenosine, adenosine kinase (ADK), synthetized by astrocytes, has the highest affinity for adenosine [31]. Thus, under baseline conditions, ADK is the most likely enzyme affecting intracellular adenosine levels in the brain; active phosphorylation of adenosine into AMP by ADK continuously decreases the intracellular concentration of the nucleoside favouring its uptake from the extracellular space [32]. Previous experimental studies support a role for ADK in brain injuries associated with astrogliosis, a morphological hallmark of Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) that is the most common partial epileptic syndrome in adulthood. Interestingly, MTLE-HS is highly refractory to pharmacological treatment, with more than 80% of patients having a poor response to anti-epileptic drugs (AEDs). In these cases, surgery for removal of affected area is the most effective treatment for seizure remission, but can have detrimental effects on the patient´s life and a high cost for health systems. Therefore, identification of novel therapeutic targets to control drug-refractory epilepsy is deeply needed. Recent evidences have highlighted the role of the adenosine signalling pathway in epilepsy development and progression.

The ADK hypothesis of epileptogenesis states that after a brain insult (e.g. seizure, hypoxia, etc.) extracellular adenosine levels increase as a consequence of ATP degradation [33]. Under these conditions, the A_1R is downregulated whereas the $A_{2A}R$ is upregulated, promoting astrocytic proliferation [33]. Astrogliosis leads to an upregulation of ADK resulting in the consequent decrease in extracellular adenosine and seizures threshold. In fact, immunohistochemistry studies and Western blot analysis have demonstrated an overexpression of astrocytic ADK in the hippocampus and temporal cortex both in animal models and MTLE-HS patients [34,35]. Studies in animal models, showing that selective A_1R agonists [36,37] or $A_{2A}R$ antagonists [35,38] might protect against diverse epileptic syndromes, suggest that manipulation of adenosine signalling pathways maybe a useful therapeutical target to control epilepsy. Nevertheless, the role of adenosine in the epileptic hippocampus is controversial since adenosine neuromodulation in this region might result from a balance between inhibitory A_1R and facilitatory $A_{2A}R$ responses [39].

This study was designed (1) to investigate the mRNA expression of ADK, A_1R and $A_{2A}R$ in the hippocampus and anterior temporal cortex of MTLE-HS patients compared to control individuals, and (2) to test if there is any correlation between expression changes and clinical features. To the best of our knowledge, there are no studies investigating simultaneously the expression of these proteins in epileptic human patients.

Material and methods

Population

Resected fresh human tissue obtained from 23 MTLE-HS patients (13F, 10M, see Table 1) who underwent epilepsy surgical treatment (selective amygdalohippocampectomy or anterior temporal lobectomy) at Neurosurgery Department of Hospital Santo António – Centro Hospitalar e Universitário do Porto (HSA – CHUP) has been analysed. The decision for surgery was taken by HSA multidisciplinary epilepsy team incorporating neurologists, neurosurgeons, neuroradiologists, neurophysiologists and neuropsychologists. All patients were resistant to maximal doses of two or more conventional AEDs used during for more than 2 years. Pre-surgical assessment was discussed by the team analysing the results of brain MRI, prolonged video-EEG recording, ictal and interictal SPECT, neuropsychological assessment and functional brain MRI, in order to determine the suitability of the patient for surgical intervention. Surgical specimens of the hippocampus and of the anterior temporal lobe were collected. A complete coronal slice of 0.5 cm thick was removed 3 cm posterior to the tip of the temporal pole. Samples were recovered in ice-cold synthetic CSF (10mM glucose,

124mM NaCl, 3mM KCl, 1mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26mM $NaHCO_3$, 2mM $CaCl_2$, pH=7.40) and immediately cryopreserved in liquid nitrogen. The amount of tissue removed did not differ from the strictly necessary for successful surgical practice. All patients gave written informed consent as stated in Declaration of Helsinki. As controls the same temporal lobes region from 9 human autopsies (8M, 1F; 70.0 ± 7.8) were analysed. Tissue was collected by a similar procedure from cadavers, with no known previous history of neurological disease, examined at the Forensics Institute of Porto within a short post-mortem delay (of 4 to 7 hours). The collection of autopsy brain material was made accordingly to Decree-Law 274/99, of July 22, published in Diário da República–1st SERIE A, No. 169, of 22-07-1999, Page 4522, on the regulation on the ethical use of human cadaveric tissue for research. This work was approved by the ethics committee of the participant institutions.

Table 1 **Clinical and demographic data from surgery MTLE-HS patients**

Clinical /demographic data	MTLE-HS (n total =24)
F/M	14 /10
Age at surgery ± SD, years (range)	39.8 ± 9.6 (24 - 60)
Age of onset ± SD, years (range)	11.0 ± 7.6 (1 - 28)
Disease mean duration ± SD , years (range)	28.8 ± 9.3 (10 - 49)
Post-surgery time ± SD , years (range)	7.0 ± 1.3 (5 - 10)
Hippocampal Sclerosis (Left /Right)	15 / 9
Febrile seizures antecedents (Yes / No)	16 / 8
Engel classification (I / II / III / IV)	17 / 2 / 4 / 1

Quantification of ADK, A_1R and $A_{2A}R$ expression in the human brain

RNA was isolated from the fresh brain tissue, using the commercially available extraction kit RNeasy® blood and Tissue kit Qiagen, Germany) according to manufacturer´s instructions. cDNA was synthesised with an available commercial kit (Nzy First-Strand cDNA Synthesis Kit, Nzytech, Portugal) in a Biometra thermocycler, accordingly to manufacturer´s instructions. A_1R (hs00417073_m1), $A_{2A}R$ (hs00181231_m1), ADK (hs00169123_m1) and the reference gene Ubiquitin C (UBC, hs00824723_m1) expression was quantified by Real Time PCR with specific primers and probes (Taqman® Kits, Applied Biosystems, USA) and a NzySpeedy qPCR mastermix (Nzytech, Portugal) in Corbett Rotor Gene 600 Real Time Thermocycler machine (Corbett Research, UK). UBC gene was chosen as the reference gene since its expression showed relatively low variability in expression levels in the regions studied [40]. Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Differences in ΔCt were evaluated using two-tailed Student's t-test or Mann´s-Whitney test when appropriated. Normal distribution was evaluated with Kolmogorov – Smirnov test. Spearman's correlation coefficients were used to test interactions between disease duration or age and expression levels. Analyses were done with SPSS v.23 software and significant levels were set at $p<0.05$

Results

In the anterior temporal cortex, the expression of ADK was 83-fold higher in MTLE-HS patients compared to cadaveric controls (p=0.001) (Figure 1c). The expression of the A_1R in MTLE-HS patients was 28-fold higher than in controls (p=0.001) (Figure 1a), whereas an increase of only 1.7-fold (p>0.05) was observed concerning the expression of the $A_{2A}R$ in MTLE-HS versus controls (Figure 1b).

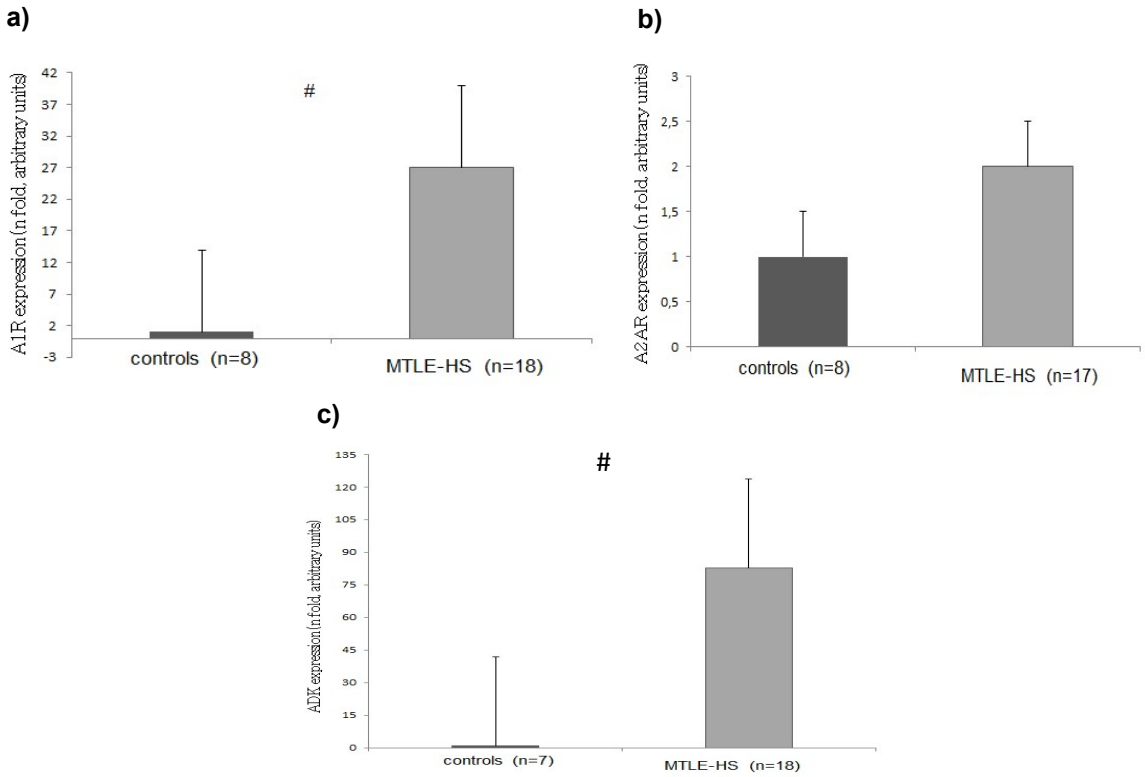

Figure 1 - MTLE-HS patients have a higher cortical A_1R, $A_{2A}R$ and ADK expression. a) A_1R expression, b) $A_{2A}R$ expression and c) ADK expression. Expression in controls is defined as 1 and relative expression in MTLE-HS patients is calculated with the $2^{-\Delta\Delta Ct}$ method. Values presented as mean ± SEM (standard error mean) # p =0.001

In hippocampal foci, ADK expression was 28-fold higher in MTLE-HS patients (p = n.s.) than in control individuals (Figure 2c). The expression of the A_1R was 17-fold higher in

MTLE-HS patients than in controls (p = 0.007) (Figure 1a), whereas no difference was observed between the two groups regarding the $A_{2A}R$ expression levels (Figure 1b).

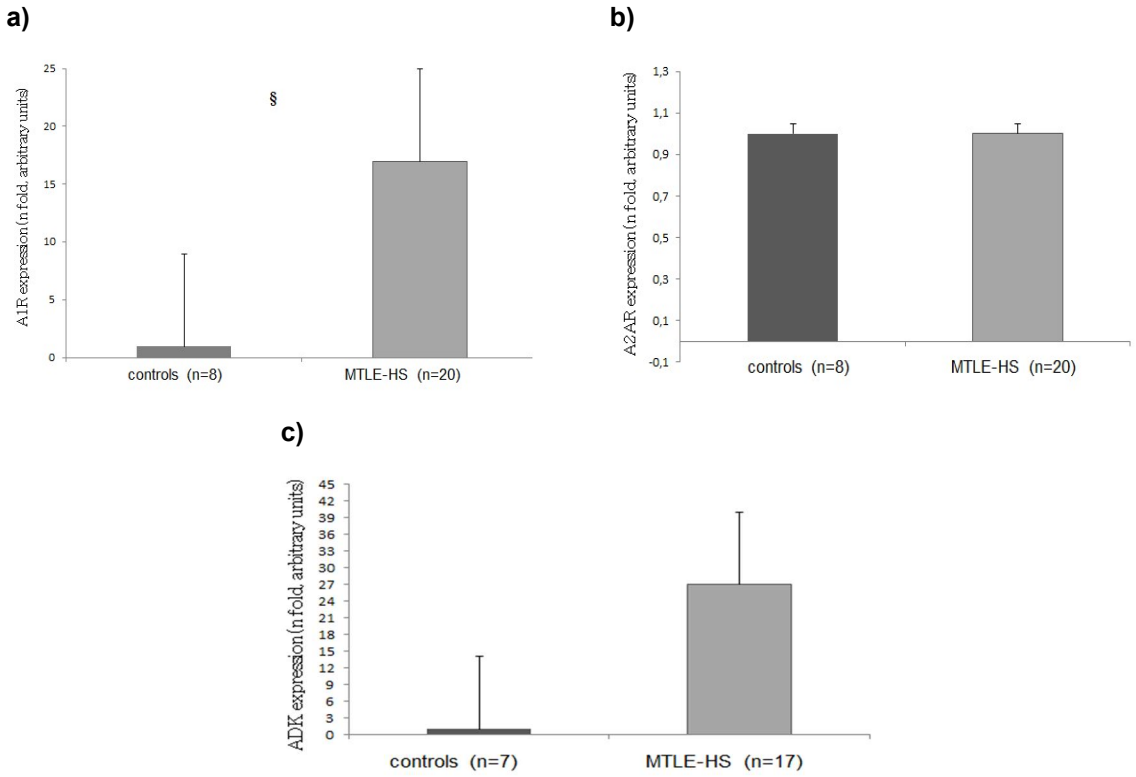

Figure 2 - MTLE-HS patients have a higher hippocampal A_1R and ADK expression. a) A_1R expression, b) $A_{2A}R$ expression and c) ADK expression. Expression in controls is defined as 1 and relative expression in MTLE-HS patients is calculated with the $2^{-\Delta\Delta Ct}$ method. Values presented as mean ± SEM (standard error mean) § p =0.007

Taking into consideration that MTLE-HS patients were (on average) younger than the control group, we performed a correlation analysis to verify if this difference was affecting the results obtained. No correlation was observed between age and the expression of A_1R, $A_{2A}R$ or ADK both in the hippocampus and anterior temporal cortex isolated from control and MTLE-HS patients. A univariate linear model analysis was used to assess possible confounding effects, such as age, gender, age-of-onset, febrile seizure antecedents, and Engel's classification (Table 2 and Table 3). It was observed that males had lower hippocampal A_1R and ADK expression levels (p = 0.02 and p = 0.002, respectively, see Table 2) compared to females. Despite this difference, comparing only male individuals, MTLE-HS patients still had a higher A_1R and ADK expression than controls (data not shown). No correlations between age-at-onset, disease duration, febrile seizure antecedents or Engel's classification and hippocampal or cortical A_1R, $A_{2A}R$, and ADK expression were observed (see Table 2 and Table 3). Interestingly, in the anterior temporal cortex ADK expression has a positive influence in A_1R expression (table 4).

Table 2 - Multivariated analysis for A_1R, $A_{2A}R$ and ADK expression in the hippocampus

Clinical parameters	A_1R (ΔCt)			$A_{2A}R$ (ΔCt)			ADK (ΔCt)		
	Unstandardized Coefficients		p	Unstandardized Coefficients		p	Unstandardized Coefficients		p
	B	Std. Error		B	Std. Error		B	Std. Error	
(constant)	13.00	5.98	**0.05**	0.22	3.97	0.96	30.98	12.51	**0.03**
Gender	-4.17	1.58	**0.02**	-0.69	1.05	0.52	-10.77	2.73	**0.002**
Age at onset	0.18	0.10	0.11	0.11	0.07	0.14	0.07	0.28	0.81
Disease Duration	-0,13	0.09	0.16	0.06	0.06	0.30	-0.21	0.16	0.21
FS antecedents	0.30	1.58	0.85	1.14	1.05	0.29	-3.23	4.00	0.44
Engel Classification	-1.65	0.78	**0.05**	-0.04	0.51	0.93	-2.20	1.75	0.23

B = beta coefficients

Table 3 - Multivariated analysis for A_1R, $A_{2A}R$ and ADK expression in the anterior temporal cortex

Clinical parameters	A_1R (ΔCt)			$A_{2A}R$ (ΔCt)			ADK (ΔCt)		
	Unstandardized Coefficients		p	Unstandardized Coefficients		p	Unstandardized Coefficients		p
	B	Std. Error		B	Std. Error		B	Std. Error	
(constant)	14.3	7.8	0.1	4.4	3.7	0.3	23.4	14.7	0.1
Gender	-2.28	2.26	0.33	-0.20	1.09	0.86	-4.22	4.28	0.34
Age at onset	0.11	0.14	0.47	-0.01	0.07	0.94	0.27	0.27	0.34
Disease Duration	0.01	0.10	0.89	0.02	0.05	0.73	0.02	0.19	0.91
FS antecedents	-2.61	2.08	0.23	0.19	1.03	0.86	-5.42	3.93	0.19
Engel Classification	-0.62	1.30	0.64	0.16	0.62	0.80	-1.36	2.47	0.59

B = beta coefficients

Table 4 – Linear regression as ADK cortical expression as constant variable

Variable	Unstandardized Coefficients		Sig.
	B	Std. Error	
(Constant)	-3.583	2.889	0.229
A_1Rrcortex	1.642	0.165	$2.71x10^{-9}$
A_{2A}Rcortex	0.116	0.441	0.795

B = beta coefficients

Discussion

The results presented here are in accordance with the hypothesis that overexpression of the adenosine metabolizing enzyme, ADK, in the hippocampus and anterior temporal cortex of MTLE-HS patients may contribute to decrease the threshold and contribute to seizure propagation, as it is usually associated with low endogenous adenosine levels. Data also prompted us to suggest that decreases in the extracellular concentration of adenosine may drive a compensatory increase in the expression of high affinity A_1R and $A_{2A}R$ in the brain of MTLE-HS patients, which magnitude may differ between the hippocampus and anterior temporal cortex.

Adenosine has been considered the main endogenous antiepileptic molecule. Raises in the extracellular levels of endogenous adenosine immediately after seizures have been demonstrated to result from excessive neuronal firing [41]. This feature may have beneficial effects to counteract increased neuronal activity and to prevent excitotoxicity and cellular damage. In line with this, it has been demonstrated that administration of inhibitors of adenosine transporters and/or metabolizing enzymes attenuate seizure activity in diverse animal models by increasing adenosine accumulation in the extracellular milieu [33]. In this context, the beneficial effects of adenosine are exerted mainly through the activation of inhibitory A_1R [36]. This receptor pre-synaptically blocks the release of glutamate by inhibiting the Ca^{2+} influx and controls neurotransmitter responsiveness through the activation of potassium channels causing hyperpolarization of the post-synaptic membrane [42,39]. Accordingly, the injection of A_1R agonists limits seizure activity in a wide number of epileptic animal models [36,37] and in human cortical slices [43], thus controlling seizures spreading. Likewise, genetic ablation or pharmacological blockade of these receptors worsen seizure activity in different animal models leading to generalization of focal seizures, development of spontaneous seizures and enhancing the duration and severity of the seizures [44-46]. Notwithstanding this, controversial findings concerning up- or down-regulation of A_1R expression in the context of epilepsy have been revealed. While in animal models dowregulation of the A_1R expression is more often reported [47], studies using brain samples from human patients [48,49] point towards an upregulation of the A_1R, in accordance with our work. Here, we demonstrate that the A_1R expression is up-regulated both in hippocampus, the lesioned region, and in the anterior temporal cortex. The coincidental increase in the expression of ADK and A_1R, led us to hypothesize that overexpression of the A_1R may be a consequence of the reduced levels of extracellular adenosine (associated with excessive ADK activity) that are required to activate this receptor in order to overcome neuronal excitability in epileptic patients. Notably, these changes were much more evident in the

anterior temporal cortex than in the sclerotic hippocampus of MTLE patients. One, thus, may argue that the more accentuated changes in the expression of ADK and compensatory upregulation of the A_1R occurring at the anterior temporal cortex may be due to failure of the sclerotic hippocampus to respond adequately to untreatable chronic seizures, such as those occurring in MTLE-HS patients.

It is also worth note that upregulation of the A_1R may not be directly translated into a higher functional efficacy of the receptor, as this depends on the amount of endogenous (or exogenous) ligand and on the efficacy of second messenger intracellular cascades. Moreover, it has been demonstrated that A_1R activation is time-dependent [50]. Pharmacological studies using experimental animals have demonstrated that A_1R agonists only have an effect if injected shortly after the insult losing their efficacy if administrated long after [50]. This may be due to desensitization caused by prolonged exposure to adenosine [51], which levels dramatically increased after an epileptic insult. Interestingly, desensitization of presynaptic A_1R may be accomplished by co- activation of $A_{2A}R$ [27,28]. As a matter of fact, it has been shown that A_1R and $A_{2A}R$ are colocalized on nerve terminals of the hippocampus allowing a functional interplay between these two adenosine receptors [27,15], even if a disequilibrium between their expression levels is verified, as we detected here between the hippocampus and anterior temporal cortex.

The $A_{2A}R$ has been implicated in the development of several neurological conditions, including epilepsy [20,29,52,53], and this may not be exclusively due to counteraction of the A_1R neuroprotective role. The $A_{2A}R$ may have a direct action on neurotransmission, being associated with increased extracellular glutamate accumulation. In fact, activation of the $A_{2A}R$ stimulates the release of glutamate [22,26,54] and prevents its reuptake [54] by coupling to mechanisms that interfere with intracellular Ca^{2+} and Na^{+} levels respectively. Thus, the $A_{2A}R$ most frequently favours neuronal excitation and/or excitotoxicity, which may be further aggravated since $A_{2A}R$ activation upregulates astrocytic GABA uptake [55] and neuronal firing leads to enhanced synaptic $A_{2A}R$ activation [56]. Accordingly, we observed that the $A_{2A}R$ is upregulated in the brain MTLE-HS patients in agreement with previous reports [20,57,53]. As mentioned above, overexpression of the $A_{2A}R$ was more evident in the anterior temporal cortex compared to the sclerotic hippocampus of MTLE-HS patients, which may be associated to facilitation of seizures propagation and severity [58]. Likewise, using animal models of epilepsy it has been shown that activation of the $A_{2A}R$ is associated with increases in seizure activity and lower seizure thresholds [59,60,38,61]. Genetic ablation or pharmacological blockade of the $A_{2A}R$ affords a robust neuroprotection with seizure reduction, in different epileptic animal models [60,38]. The mechanisms by which this neuroprotection is accomplished are unknown, but it is thought

that they are not limited to interference on neurotransmitter levels. The $A_{2A}R$ is not only expressed by neurons and astrocytes, but they are also expressed by microglial cells. Microglial $A_{2A}R$ activation is involved in cell proliferation and activation leading to the production of trophic factors, such as brain derived neurotrophic factor (BDNF) and diverse cytokines (e.g. IL-6, IL-1β) [23-25,29,30]. It has been observed that $A_{2A}R$ blockade curbs microglia activation in animal models of Parkinson's disease [62,63] or traumatic brain injury [29]. However, these observations are in contrast with studies in peripheral inflammation and central pathological conditions showing that it is the $A_{2A}R$ activation, rather than its blockage, that produces a neuroprotective effect [64,65]. Conflicting results suggest that the role of the $A_{2A}R$ in neuroinflammation depends on multiple other factors, including cells microenvironment and cell-type specific localization of these receptors. When inflammation is driven by microglia cells, the $A_{2A}R$ blockade exerts a neuroprotective effect preventing cell damage, which would otherwise contribute to seizures propagation [23]. In addition, it has been proven that the effect of the $A_{2A}R$ controlling neuroinflammation depends on the extracellular levels of glutamate; $A_{2A}R$ activation plays a pro-inflammatory role when the extracellular levels of glutamate are high, as occurring during and after epileptic seizures [29]. This may be of particular importance in patients with MTLE-HS where neuroinflammation is seen as an important factor for seizure development and propagation [66].

Unlike the results of the present study, members of this research team showed recently that hippocampal astrogliosis observed in MTLE-HS patients was accompanied by a proportionate increase in the $A_{2A}R$ immunoreactivity, evaluated both by Western blot analysis and by immunofluorescence confocal microscopy [20]. Up-regulated hippocampal $A_{2A}R$ were localized predominantly in the membrane of GFAP-positive astrocytes in close proximity to ecto-5′-nucleotidase/CD73, an enzyme that converts extracellular ATP into adenosine [20]. Here, we show that the mRNA levels coding the $A_{2A}R$ were similar in hippocampal homogenates of control and MTLE-HS patients, whereas a small (1.7-fold) increase in the receptor expression was found in the adjacent neocortex of MTLE-HS compared to control individuals. The difference between the two studies may be justified by experimental evidences suggesting that upregulation the $A_{2A}R$ might not be associated to augmentation of gene expression, but is rather due to post-translational modifications in receptor trafficking to the plasma membrane [20,67], which turned to be more effective in astroglial cells of drug-refractory epileptic patients.

Activation of the astrocytic $A_{2A}R$ promotes astrogliosis and the subsequent increase in the expression levels of ADK; this was observed in our study and is in agreement with the findings from other experimental and clinical studies [34,68,69,33]. The fact that we have

observed higher levels of both ADK and $A_{2A}R$ mRNAs in the anterior temporal cortex than in the sclerotic hippocampus is in accordance with a recent proteomic study, showing that in early and latency periods hippocampal region is submitted to more dramatic changes whilst in the chronic epilepsy phase these changes move towards seizure propagation pathways, including the adjacent neocortex [70].

In accordance with the ADK hypothesis of epileptogenesis [33], upregulation of this enzyme may be a reflex of $A_{2A}R$-induced astrogliosis that accompanies neuronal cell loss. After a first epileptic seizure, intracellular ATP catabolism drives the release of adenosine into the extracellular milieu through equilibrative nucleoside transporters. Under these circumstances, activation of upregulated inhibitory A_1R tend to curtail the increased neuronal excitation. This is observed since during first epileptic seizures adenosine activates preferentially overexpressed A_1R in detriment of down-modulated $A_{2A}R$. With continuous seizure occurrence, huge amounts of ATP are released from stimulated and/or damaged neuronal cells. Once in the extracellular space, ATP is rapidly converted into adenosine by a cascade of ecto-nucleotidases. The rate limiting enzyme of the ecto-nucleotidase cascade is the ecto-5'-nucleotidase/CD73, which dephosphorylates AMP into adenosine. Interestingly, our group demonstrated that the ecto-5'-nucleotidase/CD73 is highly expressed in hippocampal astroglial cells of MTLE-HS patients in close proximity to the $A_{2A}R$ [20]. This finding strengthens the hypothesis that adenosine derived from the extracellular catabolism of ATP activates preferentially the $A_{2A}R$ subtype, which was demonstrated many years ago using the rat as animal model [71,72]. The preferential activation of the $A_{2A}R$ drives astrocyte cells proliferation with the subsequent increase in ADK levels in the astrogliotic region. ADK upregulation inverts the adenosine transport equilibrium from the efflux to the reuptake, which results in a significant decrease in the extracellular adenosine levels leading to failure in the activation of the A_1R, in spite of its compensatory upregulation. Thus, during recurrent seizures a new purinergic equilibrium is established, which favours neuronal excitotoxicity and extensive astrogliosis. It appears that ADK upreglation is more than an epi-phenomenon of seizures, as data from several studies show that ADK upegulation is sufficient *per se* to cause seizures even in the absence of astrogliosis [73,68,74]. Conversely, it has been demonstrated that ADK knockout animals are more resistant to develop seizures [74].

In light of the above theory, it seems paradoxical that both ADK and the A_1R are upregulated in epileptic patients. Even more, if one considers that A_1R upregulation is often associated with higher ADK expression levels [75,76]. As mentioned before, the intracellular phosphorylation of adenosine into AMP by ADK forces the reuptake of the nucleoside, thus reducing its extracellular accumulation to levels that are unable to

activate the inhibitory A_1R. As a consequence, neuronal cells tend to increase the expression levels of the A_1R, which under the circumstances may not be enough to compensate its lack of activation due to insufficient endogenous ligand. Also, the overexpressed $A_{2A}R$ may counteract A_1R-mediated inhibition of neuronal activity. It is important to notice that the $A_{2A}R$ expression is claimed to be very stable in situations of brain damage. In contrast to the A_1R receptor-mediated effects, it was observed that $A_{2A}R$ antagonists are able to revert brain damage even if administrated long after the insult, suggesting that under such conditions the $A_{2A}R$ is continuously activated not suffering desensitization [77-79]. Besides astrogliosis, continuous activation of the $A_{2A}R$ leads to impairment in neurotransmission and neuroinflammatory response with increased expression of IL-6, IL-1β and BDNF, which were described to be upregulated in epilepsy. These microglial-originated proteins are claimed to be in a complex interplay with A_1R, $A_{2A}R$ and ADK influencing their expression [80-83].

To the best of our knowledge this is the first study to investigate the expression of adenosine receptors, both A_1 and A_{2A}, and ADK in the hippocampus and anterior temporal cortex of MTLE-HS patients. The observed A_1R, $A_{2A}R$, and ADK upregulation must be analysed in an integrated manner since endogenous adenosine may influence the epileptogenic processes in different mutually-influencing ways, as for instance neurotransmission and neuroinflammation. Understanding these complexities inherent to the purinergic system may prompt for new targets for therapeutic intervention in drug-resistant epilepsy.

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Manuscript 6

Inverse relationship between serum levels of miR-22 and hippocampal P2X7 receptor overexpression in patients with Mesial Temporal Lobe Epilepsy

Inverse relationship between serum levels of miR-22 and hippocampal P2X7 receptor overexpression in patients with Mesial Temporal Lobe Epilepsy

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(in preparation)

Abstract

Objective: Mounting evidences implicate the participation of ATP-gated ionotropic P2X7 receptors (P2X7R) in epilepsy and other neurological disorders. Increased expression of P2X7R has been demonstrated in the brain of patients with Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS). The P2X7R modulates glial activation, cytokines production and neurotransmitter levels after brain injury. Recent studies indicate that the P2X7R expression may be downmodulated by miR-22 in a mouse model of status epilepticus. MicroRNAs (miRNAs) are non-coding RNA molecules that regulate gene expression at post-transcriptional level. Taking into consideration that miRNA levels are very stable in biological fluids and normally reflect tissue production, here we compared the expression of P2X7R and miR-22 respectively in the brain and serum of MTLE-HS patients.

Methods: The P2X7R expression was quantified in brain samples of 23 patients with MTLE-HS and 10 cadaveric controls. MiR-22 expression levels were evaluated in the serum of 40 MTLE-HS patients and 48 healthy individuals.

Results: The P2X7R expression was higher in the hippocampus and anterior temporal lobe ($p=0.012$) of MTLE-HS patients compared to control individuals. The opposite was observed in the serum levels of miR-22 ($p=0.029$) when comparing the two groups. The difference in the serum levels of miR-22 between MTLE.HS patients and control individuals was accentuated when only MTLE-HS patients refractory to medications were considered ($p=0.015$).

Conclusion: Our results show for the first time that downmodulation of miR-22 production is associated with over expression of P2X7R in the hippocampus and anterior temporal lobe of human MTLE-HS patients. The putative implication of miR-22 de-repression of P2X7R in epileptogenesis and seizure propagation linked to exacerbation of inflammatory responses and excitatory over inhibitory neurotransmission unbalance requires further elucidation. Nevertheless, our hypothesis is that targeting the miR-22 – P2X7R axis may be a novel strategy to develop new antiepileptic drugs.

Introduction

Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS) is the most frequent focal epilepsy in adults. Patients often present a history of Febrile Seizures (FS) in childhood and more than 80% are refractory to available anti-epileptic drugs (AEDs). In such cases, surgical ablation of the hippocampus and amygdala is the last resource to control epileptic seizures, yet this procedure may have devastating effects on patients' quality of life and constitutes a significant burden for national health care systems. Notwithstanding this, seizure recurrence may occur 10 and 18 years after surgery in 47% and 38% of the patients, respectively[1; 2], and these patients remain with unmet clinical needs. Understanding the epileptogenic process is paramount to the development of new AEDs, but the mechanism leading to MTLE-HS remains largely unknown.

Recent accumulating evidence suggests a role for microRNAs (miRNAs) in epileptogenic mechanisms[3]. These small non-coding RNA molecules function as post-transcriptional regulators of gene expression controlling biological processes, such as immune responses and neurotransmission. Different miRNA expression profiles have been described in epilepsy with several miRNAs being up or downregulated after epileptic seizures, both in human patients and animal models[3; 4]. Interestingly, miRNAs are quite stable in biological fluids such as plasma or serum and normally reflect remote tissue production[5] so that circulating miRNAs have been proposed as promising novel biomarkers for diagnosis, prognosis and/or optimization of the anti-epileptic treatment[3; 4]. In animal models, targeting specific miRNA molecules is associated with seizure reduction[6]. Recently, Jimenez-Mateo et al. (2015) demonstrated that circulating miR-22 is downregulated in an animal model of epilepsy[7]. Interestingly, miR-22 displays a neuroprotective role regulating neuronal death and apoptosis in a model of traumatic brain injury[8]. It is also involved in the regulation of neuronal excitability and neuroinflammation, which are mechanisms tightly linked to the activation of P2X7 purinoceptors (P2X7R) [7] as one among many other targets identified in the central nervous system.

P2X7 receptors are low affinity ATP-gated ion channels, which activation is only possible under stressful conditions, like during brain damage, hypoxia, or excessive neuronal activity detected on the course of seizures, which favour high extracellular ATP accumulation to the millimolar concentration range[9; 10]. Yet it may also be functionally relevant under physiological conditions, such as during synaptic plasticity phenomena triggered by high frequency stimuli inherent to learning and memory processes[11]. Once activated, the P2X7R behaves as a non-desensitizing cation channel involved in the long-lasting influx of Na^+ and Ca^{2+} but also in the efflux of K^+, depending on the ionic concentration gradients[10;12]. Prolonging its activation, the P2X7R may form a reversible

plasma membrane pore that is permeable to hydrophilic molecules up to 900Da[13]. Using brain samples from both human and animals, it has been demonstrated that the P2X7R is expressed in neurons, astrocytes and microglia having pleiotropic effects modulating neuron-glia interaction, host defence and neuroinflammation[9]. In microglial cells, the P2X7R has a trophic function modulating their activation and proliferation[14], which leads to the expression of pro-inflammatory cytokines, like Il-1β and TNF-α, and of reactive oxygen species[15-17]. Through this action the P2X7R may modulate neuronal cell death. The presence of the P2X7R in pre-synaptic nerve terminals and astrocytes justifies its role on GABA and glutamate release[18].

Recently, our group showed that the P2X7R is over expressed in neocortical nerve terminals of drug-resistant epileptic patients; once activated, this receptor leads to down-modulation of GABA and glutamate uptake, which endures GABA signalling, increases GABAergic rundown, and, thereby, unbalances glutamatergic neuroexcitation[19]. Upregulation of the P2X7 receptor expression has also been verified in the hippocampus and cortex of animal models of status epilepticus or TLE[20-22], as well as in the neocortex of human patients with TLE[19; 21; 22]. These authors showed that P2X7R antagonism may reduce the number and duration of spontaneous seizures and gliosis and that this effect is maintained after treatment cessation[21; 22]. The association between P2X7R antagonist and seizure severity reduction was confirmed by other research groups[23;24]. Taking into consideration that there is an inverse relationship between miR-22 and hippocampal P2X7 receptor overexpression in a mouse model of status epilepticus[7;25] this study was designed to investigate whether the same occurs in human patients with MTLE-HS.

Material and Methods

Quantification of the P2X7 receptor expression in the human brain

Resected fresh human tissue obtained from 23 MTLE-HS patients (13F, 10M, see Table 1) who underwent epilepsy surgical treatment (selective amygdalohippocampectomy or anterior temporal lobectomy) at Neurosurgery Department of Hospital Santo António – Centro Hospitalar e Universitário do Porto (HSA – CHUP) has been analysed. The decision for surgery was taken by HSA multidisciplinary epilepsy team incorporating neurologists, neurosurgeons, neuroradiologists, neurophysiologists and neuropsychologists. All patients were resistant to maximal doses of two or more conventional AEDs used during for more than 2 years. Pre-surgical assessment was discussed by the team analysing the results of brain MRI, prolonged video-EEG recording, ictal and interictal SPECT, neuropsychological assessment and functional brain MRI, in order to determine the suitability of the patient for surgical intervention. Surgical

specimens of the hippocampus and of the anterior temporal lobe were collected. A complete coronal slice of 0.5 cm thick was removed 3 cm posterior to the tip of the temporal pole. Samples were recovered in ice-cold synthetic CSF (10mM glucose, 124mM NaCl, 3mM KCl, 1mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26mM $NaHCO_3$, 2mM $CaCl_2$, pH=7.40) and immediately cryopreserved in liquid nitrogen. The amount of tissue removed did not differ from the strictly necessary for successful surgical practice. All patients gave written informed consent as stated in Declaration of Helsinki. As controls the same temporal lobes region from 10 human autopsies (8M, 2F; 67.0 ± 10.9) were analysed. Tissue was collected by a similar procedure from cadavers, with no known previous history of neurological disease, examined at the Forensics Institute of Porto within a short post-mortem delay (of 4 to 7 hours). This work was approved by the ethics committee of the participant institutions.

RNA was isolated from the fresh brain tissue, using commercially available extraction kit RNeasy® blood and Tissue kit Qiagen) according to manufacturer´s instructions. cDNA was synthesised with an available commercial kit (Nzy First-Strand cDNA Synthesis Kit) in a Biometra thermocycler, accordingly to manufacturer´s instructions. P2X7R (hs0017521_m1) and the reference gene Ubiquitin C (UBC) (hs00824723_m1) expression were quantified by Real Time PCR with specific primers and probes (Taqman® Kits, Applied Biosystems, USA) and a NzySpeedy qPCR mastermix (Nzytech, Portugal) in Corbett Rotor Gene 600 Real Time Thermocycler machine (Corbett Research, UK). UBC gene was chosen as the reference gene since its expression showed relatively low variability in expression levels in the regions studied[26]. Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Quantification of the miR-22 expression in the human serum

Serum samples were obtained from peripheral blood of 40 MTLE-HS patients (23F, 17M, 43.0 ± 12.2, 30 refractory to treatment, Table 2) and 48 age-matched controls (28F, 20M, 42.0 ± 10.8) without known neurological disease. Patients were followed up at the Epilepsy Outpatient Clinic of the HSA – CHP. All patients had MTLE-HS diagnosis based on clinical and electrophysiological studies (EEG and/or video-EEG monitoring) and on brain MRI (minimum 1.5T) features. Definition of HS was based on brain MRI findings criteria which comprised atrophy, T2 hyperintensity signal and altered internal structure on one or both hippocampi associated or not with other imaging criteria like ipsilateral fornix atrophy, ipsilateral mammillary bodies' atrophy or ipsilateral entorhinal abnormalities. We

excluded other MTLE-HS aetiologies like HS due to dual pathology. At the time of the study 10 patients were not refractory to pharmacological treatment (Table 2).

Table 1 – Clinical and demographic data from surgery MTLE-HS patients

Clinical /demographic data	MTLE-HS (n total =23)
F/M	13 /10
Age at surgery ± SD, years (range)	39.6 ± 9.8 (24 - 60)
Age of onset ± SD, years (range)	10.3 ± 6.8 (1 - 28)
Disease mean duration ± SD , years (range)	29.3 ± 9.0 (10 - 49)
Post-surgery time ± SD , years (range)	6.9 ± 1.3 (5 - 10)
Hippocampal Sclerosis (Left /Right)	15 / 8
Febrile seizures antecedents (Yes / No)	15 / 8
Engel classification (I / II / III / IV)	16 / 2 / 4 / 1

Data concerning FS' antecedents was collected from patient medical records and 21 patients had a history of FS (Fable 2). Control individuals were voluntarily recruited among blood donors, ethnically matched, from the same geographic region. Peripheral blood was collected in tubes without anticoagulant (Vacuette, GBO, Germany), centrifuged at 490g and serum aliquots were stored at -20ºC. RNA was extracted using the miRNeasy® Serum/Plasma Kit (Qiagen, Germany), according to the manufacturer's protocol. The synthesis of cDNA was performed with the Taqman®MicroRNA reverse Transcription-Applied Biosystems Kit (Applied Biosystems, USA) and specific primer for miR-22 (Taqman® MicroRNA Assays – Applied Biosystems, USA). The reaction was performed in a Biometra Alfagene thermocycler accordingly to manufacturer's instructions. The quantitative RT-PCR amplification was run with specific primers and probes for miR-22 (Taqman® MicroRNA Assays – Applied Biosystems, USA) and NzySpeedy qPCR mastermix (Nzytech, Portugal) in a Corbett Rotor Gene 600 Real Time Thermocycler (Corbett Research, UK). Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. MiR-22 level was evaluated in serum, a cell-free body fluid that is not known to have constant levels of a particular RNA species, hindering expression normalization by an endogenous control or housekeeping gene. To overcome this problem, the same serum volume was used for each subject and the same threshold was used so that expression levels could be comparable between samples. Therefore, microRNA levels are expressed as 50-Ct [27].

Statistical analysis

Differences in ΔCt were evaluated using two-tailed Student's t-test or Mann´s-Whitney test when appropriated. Normal distribution was evaluated with Kolmogorov – Smirnov test.

Spearman's correlation coefficients were used to test interactions between disease duration and expression levels. Analyses were done with SPSS v.23 software and significant levels were set at $p<0.05$

Table 2 – Clinical and demographic data from MTLE-HS with miR-22 quantification

Clinical /demographic data	MTLE-HS (n total = 40)	
	Refractory (n = 30)	Non refractory (n=10)
F/M	23 / 17	7 / 3
Age ± SD, years (range)	43.0 ± 12.2 (24 – 68)	42.4 ± 12.3 (20 – 60)
Age of onset ± SD, years (range)	12.4 ± 9.8 (1 - 32)	12.8 ± 11.2 (1 - 51)
Disease mean duration ± SD , years (range)	30.7 ± 12.6 (6 - 56)	29.6 ± 13.9 (8 - 58)
Hippocampal Sclerosis (Left /Right / Bilateral)	18 / 11 / 1	7 / 3 / 0
Febrile seizures antecedents (Yes / No)	15 / 15	6 / 4
AED (0 / 1 / 2 / ≥ 3)	0 / 6 / 8 / 16	1 / 2 / 6 / 1

Results

The P2X7R expression was higher both in the hippocampus (Figure 1a) and in the anterior temporal lobe (Figure 1b) of MTLE-HS patients compared to control individuals. This difference was significantly higher in samples from the anterior temporal cortex ($p=0.012$, Figure. 1b).

The multivariate analysis did not reveal any association between hippocampal P2X7R expression and age at onset, disease duration, gender, Engel's classification or Febrile Seizures (FS) antecedents (Table 3). We also failed to detect any association between the P2X7R expression in the anterior temporal cortex and the duration of the disease, gender and FS antecedents (Table 3). Conversely, an association with Engel's Classification was observed, with Class III patients presenting a P2X7R expression in the anterior temporal cortex similar to controls (Table 3). We also detected an association between P2X7R expression in the temporal lobe and age-at-onset, with patients showing higher P2X7R expression presenting a later disease onset (Table 3).

To confirm that differences in the P2X7R expression were not due to the age discrepancy among MTLE-HS patients and the control group, we performed a Spearman´s correlation analysis. No correlation was observed between the expression levels of the P2X7R in the anterior temporal lobe (Figure. 1c and 1d) and the hippocampus (Figure 1e and 1f) and age of surgery or death in MTLE-HS or controls, respectively.

In 9 out of the 23 MTLE-HS patients submitted to surgery we were able to access the serum levels of miR-22, in addition to the quantification of the P2X7R expression in the

two brain regions of interest. Comparison of the results obtained in the serum of these patients with the control population (48 individuals) is shown in Figure 2a. The serum levels of MiR-22 were lower in MTLE-HS patients when compared to the control population (Figure 2a). This difference was maintained when a larger cohort including 40 surgical and non-surgical MTLE-HS patients was compared to an age-matched control population (p=0.029, Figure 2b).

An inverse relationship between the serum levels of miR-22 and the P2X7R expression in the hippocampus (Figure 2c) and anterior temporal cortex (Figure 2d) from operated MTLE-HS patients.

Table 3 – Multivariated analysis for P2X7R expression

Clinical parameters	Hippocampal P2X7R (ΔCt)			Cortical P2X7R (ΔCt)		
	Unstandardized Coefficients		P	Unstandardized Coefficients		p
	B	Std. Error		B	Std. Error	
(constant)	3.024	2.923	0.321	4.443	1.442	**0.008**
Disease Duration	0.010	0.044	0.826	-0.009	0.017	0.615
Gender	-0.172	0.718	0.815	0.222	0.344	0.528
FS antecedents	-0.017	0.992	0.987	-0.164	0.425	0.704
Engel Classification	0.229	0.484	0.645	0.630	0.205	**0.008**
Age at onset	0.033	0.071	0.648	-0.077	0.034	**0.039**

B = beta coefficients

Table 4 – Multivariated analysis for miR-22 serum expression

Clinical parameters	miR-22 (ΔCt)		
	Unstandardized Coefficients		p
	B	Std. Error	
(constant)	15.327	3.486	0.000
Disease Duration	1.592	3.206	0.623
Gender	1.487	0.937	0.122
FS	0.411	0.973	0.675
Age	-1.493	3.220	0.646
Age at onset	1.599	3.222	0.623
Number AEDs	-0.552	0.525	0.301

B = beta coefficients

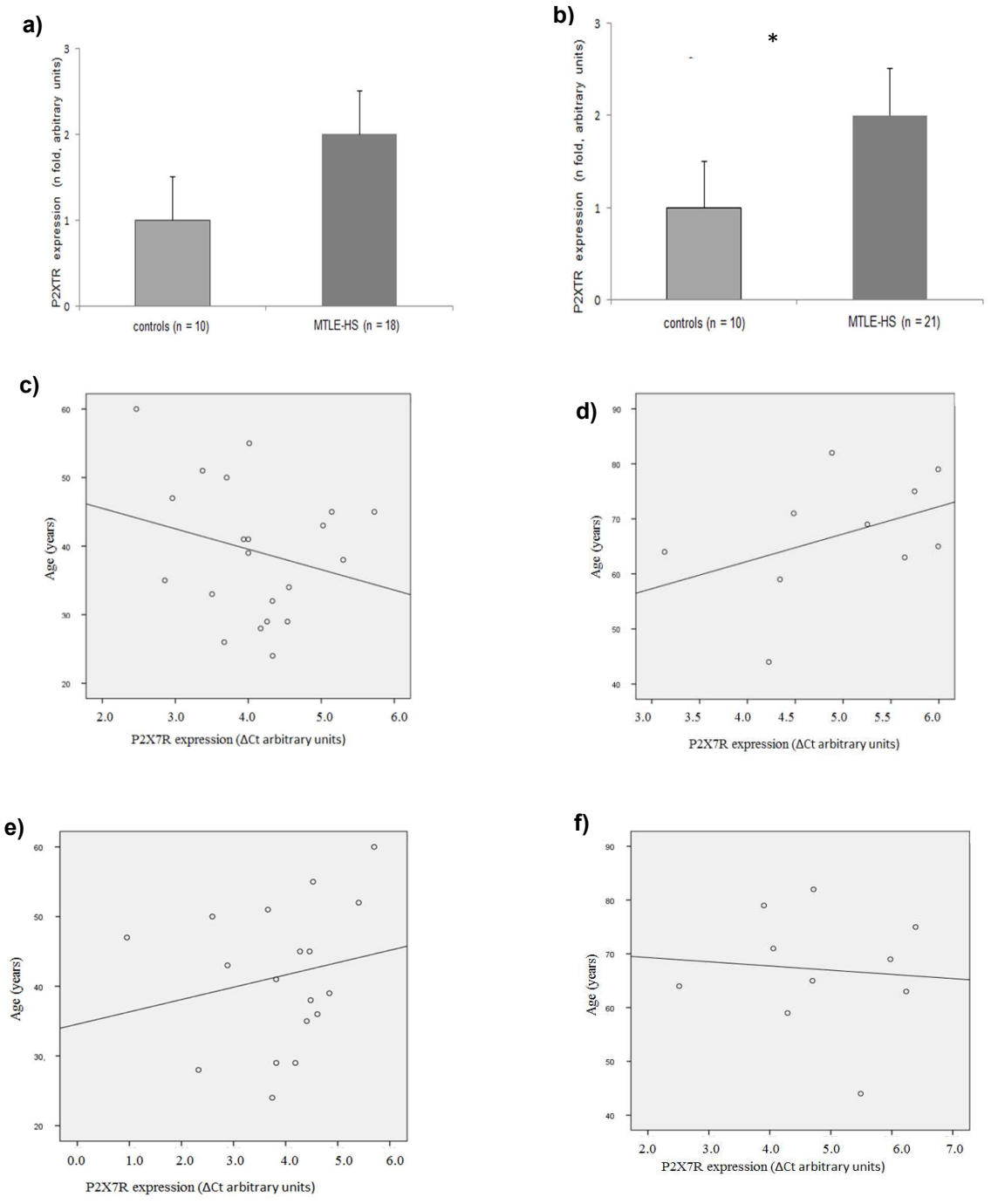

Figure 1 – P2X7R expression in the hippocampus (a) and anterior temporal cortex (b) of controls and MTLE-HS patients. P2X7R expression levels in the hippocampus and anterior temporal cortex were 2-fold higher in MTLE-HS patients compared to the control population. The expression of the P2X7R was not correlated with age, both in the anterior temporal cortex (c and d) and in the hippocampus (e and f), of MTLE-HS patients (c and e) and control individuals (d and f). Bar graphs are mean ± SEM (standard error mean) *p = 0.012

Interestingly, upon dividing the MTLE-HS patients group accordingly to their response to AEDs, we observed that patients with a good response to medication had a miR-22

serum levels similar to the control population whereas patients that were refractory to multiple AEDs have reduced levels of miR-22 in the serum (p = 0.015. Figure 2e). A multivariate regression analysis was performed and no association between miR-22 serum levels and age, gender, age-at-onset, disease duration, FS antecedents or the number of AEDs, was observed (Table 4).

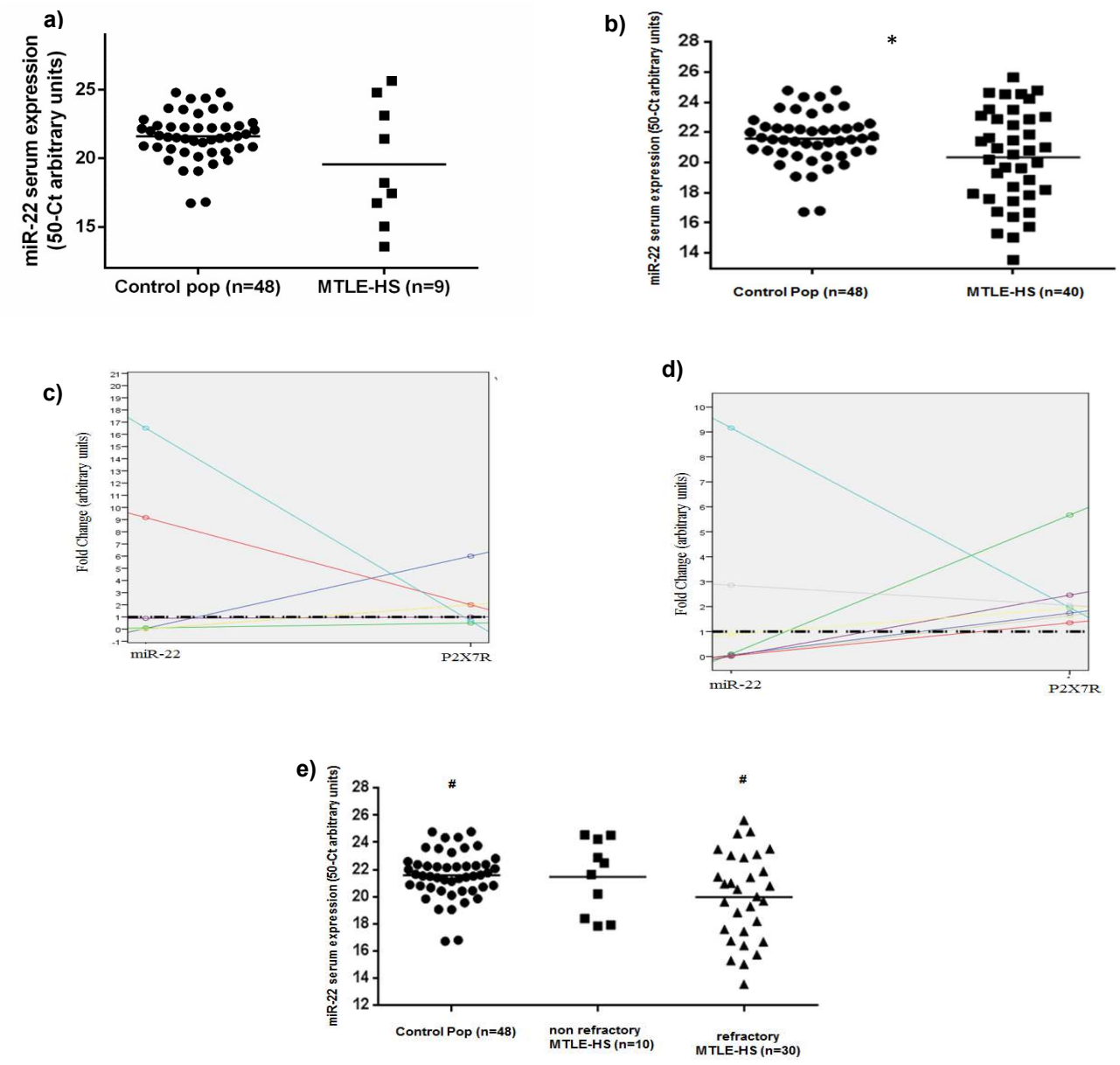

Figure 2 – Serum levels of miR-22 in MTLE-HS patients proposed for surgery and control individuals (a). This comparison was also made using a larger cohort of MTLE-HS patients (b). We observed an inverse relationship between the serum levels of miR-22 and the P2X7R expression in the hippocampus (c) and anterior temporal cortex (d) of operated MTLE-HS. The difference between the miR-22 expression levels to the control population was accentuated in drug-refractory MTLE-HS patients compared to non-refractory (e). Black dashed line in c) and d) represent the control population. *p = 0.029; #p = 0.015

Discussion

Our results show for the first time, that downmodulation of miR-22 in the serum is inversely related with the P2X7R overexpression in the hippocampus and anterior temporal cortex of human MTLE-HS patients, in accordance with previous observations in animal models[7].

Situations of brain damage and other stressful conditions, such as ischemia, trauma, or excessive neuronal activity, lead to a rapid upregulation in transcription and translation of P2X7R[28]. This typically coincides with an increase in extracellular ATP levels that favours activation of low-affinity non-desensitizing P2X7 receptors. Signalling through these receptors may affect both neuroinflammation and neurotransmission contributing to exacerbation and propagation of seizures throughout the brain. Our results showing an upregulation of the P2X7R subtype in MTLE-HS patients which is particularly evident in the anterior temporal cortex a region that contributes to seizure propagation, are in agreement with the overall pro-epileptic role attributed to this receptor by several authors. The observation that cortical P2X7R expression is higher in patients with a later disease onset may favour the importance of this receptor in the development of MTLE-HS indicating that its expression is important in disease establishment and progression.

Some controversy, however, exists on the dominant cell localization of P2X7R in the brain. It has been suggested that P2X7R are localized predominantly on glial cells, including microglia, oligodendrocytes and astrocytes, although neuronal expression has also been reported[19; 29]. It appears that the differential cellular expression of the P2X7R is highly dependent on the model and/or stage of the epileptic process under evaluation. For instance, in TLE or status epilepticus animal models [21; 30; 31] the P2X7R has been localized predominantly in microglial cells of the CA3 hippocampal region coinciding with major neuronal cell loss in this area[19-22]; however, this pattern significantly changes in the CA1 and dentate gyrus regions where the P2X7R was found to be more abundant in neuronal cell populations[7; 20; 30]. One may argue that this may reflect different roles of the P2X7R according to distinct phases of epileptogenesis. *Ab initio*, P2X7R upregulation may be relevant in order to negatively compensate neuronal overexcitation[32] and to overcome cellular damage that arises from excessive neuronal activity. As a consequence of this phenomenon there is a raise in extracellular ATP with consequent surplus P2X7R activation promoting proliferation and activation of microglial cells[14]. This results in the concomitant production and release of pro-inflammatory cytokines[15-17] that are required for damaged cells repair. In vitro studies suggest the existence of a positive feedback loop between IL1-1β and P2X7R[33]. In this situation of altered excitability, inflammatory milieu

and activated microglia, the extracellular levels of ATP continue to escalate. The long-term P2X7R activation may lead to sustained and uncontrolled inflammatory reactions, cell death and hyperexcitability[9]. Prolonged P2X7R activation rends cells more susceptible to ATP-induced death due to the formation of a plasma membrane pore resulting in intracellular Ca^{2+} overload and to the efflux of potassium and other relevant metabolites in a manner that is independent of inflammatory actions[6;12;34]. This scenario triggers synaptic neurotransmission deficiencies by interfering with glutamate homeostasis and ATP/adenosine metabolism. In addition, inflammatory cytokines may also interfere with neurotransmitters signalling, namely via NMDA receptors, leading to neuronal hyperexcitability[34]. All of these events aggravate neuronal network instability and dysregulation characterizing epilepsy, thus contributing to seizures prolongation and propagation to other brain regions.

Studies using epilepsy animal models and brain samples from MTLE patients indicate that P2X7R are also present on cortical nerve terminals[19-22; 32; 35; 36]. Mounting evidence suggest that neuronal P2X7R activation regulates the extracellular levels of both GABA and glutamate, indirectly interfering with neuronal excitabilitability. While, in one hand, it has been shown that the P2X7R activation interferes with the release of neurotransmitters[37], due to massive dysregulation of cytoplasmic ion homeostasis and consequent alteration of depolarization thresholds[32; 38]. On the other hand, it was observed that P2X7R activation downmodulates Na^{+}-dependent GABA and glutamate uptake in synaptic nerve terminals isolated from epileptic patients, thus contributing to the extracellular accumulation of these neurotransmitters[19]. The raise in extracellular GABA may not be protective as one may initially predict and may even contribute to neuronal hyperexcitability due to paradoxical GABaergic “rundown” in the epileptic human brain[19]. In line with these observations, P2X7R antagonists exhibit potent anticonvulsant effects in animal models of status epilepticus. Some authors claim that there is a reduction in seizure severity associated with reduced neuronal damage[22;24] while others argue that only the number and frequency of seizures is reduced[21]. An interesting result is that the effect is maintained, and sometimes even amplified, when treatment with P2X7 antagonists is discontinued[21]. Notwithstanding this, contradictory results regarding the P2X7R effect in seizures development have been reported using different animal models. For instance, while P2X7R inhibition leads to exacerbation of pilocarpine-induced seizures, a situation that is commonly associated with high neuronal death and reduced astrocytic cells damage in the CA3 hippocampal area, blockade of P2X7R activation had no effect in seizures outcome in kainic acid-induced status epilepticus[39]. As mentioned before, these idiosyncrasies may be justified taking into consideration the type of cells

predominantly affected in each given epilepsy model, as well as the mechanism and phase of the epileptogenic process that is being considered. In our study, we observed an overall increase in the P2X7R expression in homogenates of the hippocampus and anterior temporal cortex isolated from human patients with MTLE-HS without paying much attention to any particular cell type. Notwithstanding this, using Western blot analysis and immunofluorescence confocal microscopy we showed that the P2X7R is present in isolated nerve terminals of the human neocortex and that its expression is increased in VAMP-1 positive nerve terminals of the neocortex of MTLE patients compared to control individuals, despite intense astrogliosis was documented by GFAP immunostaining in the epileptic brain[19].

Mounting evidence suggests that acute and chronic P2X7R expression is tightly controlled in the CNS and that dysregulation of these mechanisms during epileptogenesis may affect disease severity and progression. Recently, Jimenez-Mateo et al. (2015) showed that Kainic acid unilateral injection induces P2X7R overexpression in the ipsilateral epileptogenic focus, but not in the contralateral hippocampus[7]. This has been ascribed to post-transcriptional repression of the P2X7R in the contralateral hippocampus by a microRNA molecule, the miR-22[7]. These authors also showed that miRNA targeting of the P2X7 purinoreceptor opposes seizure development[7]. Coincidently or not, both P2X7R and miR-22 are regulated by the same transcription factor which action is dependent on intracellular Ca^{2+} levels[25]. The specificity protein 1 (Sp1) has been shown to induce P2X7R transcription in vitro and Sp1 occupancy of the miR-22 promoter region is blocked under conditions of high Ca^{2+} influx into the cells, such as those occurring during excessive P2X7R activation driven by ATP released from cells during seizures. This calcium-sensitive feed-forward loop regulating the expression of the ATP-gated P2X7R is accompanied by a pro-convulsive lack of miR-22-mediated post-translational repression of the P2X7R protein leading to its overexpression at that plasma membrane[25]. Our results are in accordance with this theory, since we observed that MTLE-HS patients refractory to AEDs possess higher than control levels of P2X7R in the hippocampus and adjacent neocortex whilst mirR-22 is downregulated in the serum, which normally reflects the rate of microRNAs production in remote tissues, like the brain[5]. It is also worth noting that our results are in keeping with those observed in the brain of epileptic animals used to validate serum microRNA quantification. A similar relationship has also been observed in patients with amyotrophic lateral sclerosis[40].

It is particularly interesting to observe that non-refractory MTLE-HS patients have similar miR-22 expression as controls whilst refractory patients to medication have a lower expression of this microRNA in the serum. In an animal model of traumatic brain injury low

miR-22 expression is associated with higher neuronal cell damage[8]. Patients that do not respond to multiple AED regimens present more severe and recurrent seizures. It is tempting to assume that, in these cases, sustained extracellular ATP accumulation together with P2X7R overexpression results in uncontrolled neuroinflammatory reaction and synaptic transmission dysregulation, with cell damage and seizure progression acting as a positive feed-forward loop. Since the P2X7R activation is essential for physiological higher brain functions like memory and cognition, this dysregulation may occur only in predisposed individuals.

The mechanisms underlying the lack of miR-22-induced post-transcriptional repression associated with P2X7R overexpression may constitute novel molecular targets for the treatment and follow-up of drug-refractory MTLE-HS.

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CHAPTER III

General Conclusions and Future Perspectives

Mesial temporal lobe epilepsy with Hippocampal Sclerosis (MTLE-HS), the most frequent focal epilepsy in adulthood, is also the most refractory to medical treatment with over 80% of patients presenting a poor response to conventional anti-epileptic drugs (AEDs). Most often, currently available AEDs only inhibit excessive neuronal discharges or promote synaptic transmission inhibition, thus preventing symptoms (seizures) but do not change the course of the underlying disease process (epileptogenesis). In drug-refractory cases, surgical resection of the hippocampus and the amygdala is the last resource to control recurrent epileptic seizures. However, this procedure may have a detrimental impact on patients' quality of life and constitutes a significant burden for national health care systems. Nearly half of the patients report seizure recurrence many years after surgery[43,44]. Thus, efficient resolution of epilepsy is still an unmet medical need. Understanding the epileptogenic process is paramount to the development of newer and eventually more effective therapeutic strategies, but due to the fact that it is frequently an indolent process the mechanism(s) leading to MTLE-HS development remains largely unknown.

This study aimed at contributing to fill some gaps in our knowledge regarding the pathogenesis of MTLE-HS in the human brain, focusing on the role of neuroinflammation as a critical epileptogenic mechanism and, consequently, on one of its most relevant players, the purinergic system. More specifically, our findings contributed (1) to elucidate further the linkage between Febrile Seizure antecedents and Apolipoprotein E isoforms (ε4) on early disease onset of drug-refractory MTLE-HS in human patients; (2) to characterize the inflammatory profile of these patients analysing cerebral tissue expression of pro-inflammatory cytokines, such IL-1β, and cell biomarkers, like TLR4 and HLA-DR; (3) to show that this pro-inflammatory profile may be dramatically altered to increase MTLE-HS susceptibility in patients exhibiting single nucleotide polymorphisms of the IL-1β gene (e.g. rs16944) and in those expressing microRNAs able to epigenetically regulate immune responses (e.g. miR-146a, miR-132); (4) to demonstrate in the human brain (i) that upregulation of purinoreceptor subtypes, like A_1, A_{2A} and P2X7, as well as the adenosine metabolizing enzyme, ADK, is positively associated with increased susceptibility to develop drug-resistant MTLE-HS, and (ii) that low levels of miR-22 in the serum may adequately predict overexpression of the P2X7 receptor in the hippocampus and adjacent neocortex of MTLE-HS patients. A summary of the results obtained in this thesis is presented in Table 1.

Table 1 – Summary of the results obtained in this thesis

Manuscript	Type of study	Biological material	Observation
Manuscript 1	Genetic	DNA	ApoE ε4 and early disease onset FS and early disease onset
Manuscript 2	Genetic	DNA	rs16944TT in IL-1B gene is a susceptibility factor for MTLE-HS
Manuscript 3	Expression	Brain tissue	Hippocampus: ↑ activated microglia, IL-1β, TLR4, IL-10 Cortex: ↑ activated microglia, IL-1β, TLR4, IL-10
Manuscript 4	Expression	Serum	↑ miR-146a, miR-132
Manuscript 5	Expression	Brain Tissue	Hippocampus: ↑ A_1R, ADK Cortex: ↑ A_1R, A_{2A}, ADK
Manuscript 6	Expression	Brain tissue Serum	Hippocampus: ↑ P2X7R Cortex: ↑ P2X7R Serum: ↓ miR-22, specially in refractory patients

Cell damage or intense neuronal activity leads to the release of danger molecules, such as ATP and HMGB1. The HMGB1 activates TLR4 whilst ATP binds to P2X7R. The two receptors may cooperate to increase the production of pro-inflammatory cytokines in the brain, without depending on the circulating immune response. Once in the extracellular medium, ATP is rapidly catabolised by ecto-nucleotidases into adenosine. Ecto-5'-nucleotidase/CD73 is the rate limiting enzyme of this reaction. This enzyme has a close proximity with the $A_{2A}R$ that favours the receptor activation, thus promoting astrocytes proliferation (scar formation) and microglia activation resulting in the production of neurotrophic factors, such as BDNF (Brain-Derived Neurotrophic Factor). Limitation of ATP catabolism to the intracellular milieu during increased neuronal activity increases the intracellular adenosine levels, which triggers a partial leakage of the nucleoside to the extracellular fluid through equilibrative nucleoside transporters. Adenosine outflow contributes to the downmodulation of synaptic transmission via the activation of both pre- and post-synaptic A_1R. The concerted action of all these players curbs excitatory synaptic transmission and leads to brain repair and stimulation of cell survival. Under normal conditions, the pro-inflammatory action stimulates predominantly regulatory mechanisms, namely the production of anti-inflammatory cytokines such as IL-10, or the transcription of microRNAs, such as miR-22, miR-146a or miR-132, which contribute to restrain the inflammatory reaction and prevent the excessive cellular damage.

Interestingly, results from this study show that genes coding for P2X7R **(manuscript 6)** and TLR4 **(manuscript 3)** were upregulated in the hippocampus and adjacent neocortex of MTLE-HS patients. One may, thus, hypothesize that this may positively link to exacerbation of inflammatory reactions with the consequent upregulation of the production of pro-inflammatory cytokines, such as IL-1β **(manuscript 3)** (see also Figure 1). The exacerbated inflammatory response (mediated by the P2X7 receptor) leads not only to an excessive neuronal cell loss but also to the imbalance of synaptic neurotransmission affecting ionic currents favouring the release of neurotransmitters and impairing the uptake of GABA and glutamate, in a way that favours neuroexcitability / neuroexcitotoxicity. Neuronal excitation is further enhanced by the activation of adenosine $A_{2A}R$, which is overexpressed at gene and protein levels in the anterior temporal lobe **(manuscript 5)** and hippocampus[246] of MTLE-HS patients (Figure 1). Thus, activation of $A_{2A}R$ besides favouring neuroinflammation (see above), it is also involved in the upregulation of GABA uptake while promoting the release of glutamate and glutamate-induced excitotoxicity.

High neuronal excitability contributes to seizure recurrence with sustained high ATP extracellular levels. Extracellular ATP accumulation together with overexpression of the non-desensitizing P2X7R results in an uncontrolled neuroinflammatory reaction and synaptic transmission dysregulation, thus leading to a vicious cycle of cell damage and seizure progression. As the P2X7R activation is essential for physiological brain functions, like memory and cognition, this dysregulation may occur only in predisposed individuals. Part of this predisposition may be given, among other yet unidentified susceptibility factors, by polymorphisms (e.g. rs16944TT) in the IL-1β gene, which frequency we found to be increased in the MTLE-HS patients population **(manuscript 2)**. The rs16944TT genotype is associated with higher IL-1β gene expression and, in view of this, may contribute to failure in the regulatory mechanisms with consequent exacerbation of inflammatory responses. Considering the rs16944TT genotype as a susceptibility factor may allow the identification of individuals that are at higher risk of developing MTLE-HS after a precipitating insult (e.g. febrile seizures, brain infection, head trauma).

In normal conditions, the P2X7R expression is negatively modulated by miR-22; this epigenetic control of the P2X7R expression may contribute to downmodulate the inflammatory input (Figure 1). In epileptic-stressed cells, high intracellular Ca^{2+} levels, generated by the P2X7R activation or by NMDA receptors under the influence of IL-1β, impairs miR-22 gene expression with the consequent lack of miR-22-mediated post-translational repression of the P2X7R. Interestingly, we found that the miR-22 serum levels were significantly decreased in MTLE-HS patients, especially in those refractory to

AEDs **(manuscript 6)**. Moreover, we also demonstrated an inverse relationship between serum levels of miR-22 and P2X7R overexpression in the hippocampus and anterior temporal cortex of MTLE-HS patients.

In our opinion, all these issues integrate a positive feedback loop that leads to the perpetuation of the inflammatory response in a vicious cycle of inflammation-excitability.

Retrospective studies show that MTLE-HS patients often have a history of initial precipitating injury, such as febrile seizures (FS), central nervous system infection, head trauma or peripartum injuries[3]. Among these factors FS is the most common with up to 50% - 80 % of MTLE-HS patients reporting a history of complex FS[25]. A causal relation between FS and MTLE-HS development still remains controversial. We have shown here that FS are associated with an early MTLE-HS disease onset, probably because it hastens the abnormal network reorganization favouring the development of an epileptogenic zone in predisposed individuals[353] **(manuscript 1)**. Imaging studies have shown that prolonged and lateralized FS can produce acute hippocampal injury with oedema that usually recovers within 5 days[46,118]. The follow-up of these children has shown changes in hippocampal symmetry consistent with injury and neuronal loss[5]. It will be important to understand this association since history of initial precipitating factors as well as age at onset of epileptic seizures and duration of the latency period may affect the clinical presentation and prognosis of MTLE-HS. Another interesting question is if patients with and without FS antecedents have differences in the severity and prognosis of MTLE-HS.

Data from this study revealed that the presence of the ApoE ε4 isoform also hastens the disease development **(manuscript 1)**. Apolipoprotein E has a crucial role in cholesterol and phospholipid transport, being important for neuronal repair, structural plasticity, and in the maintenance of myelin and neuronal membranes' integrity during development and aging. ApoE participates also in neurotransmission, since it has a regulatory role in calcium homeostasis, modulating indirectly the function of various ion-dependent receptors[8,9]. ApoE ε4 is associated with impaired neuronal cholesterol and phospholipid metabolism and seems to increase microglia activation and astrogliosis leading to a more pronounced hippocampal injury[10]. In this way, it may be hypothesized that patients with this isoform are prone to exhibit impaired neuronal recovery after a precipitating insult, leading to an earlier disease onset which may negatively influence MTLE-HS prognosis. Impairment of neuronal cholesterol and phospholipid metabolism associated with ApoE ε4 may have tremendous implications on network disorganization and structural abnormalities extending far beyond the hippocampus, also including the white matter.

These changes have been associated with an early disease onset[11] and may have an important role in seizure propagation in MTLE-HS[12]. Since seizures have also been associated with permanent neuronal network reorganization, structural abnormalities of the epileptic brain may contribute to neurological and psychiatric comorbidities[13].

Hippocampal Sclerosis (HS) is characterized by neuronal cell loss, abnormal mossy fibre sprouting and astrogliosis. Astrocyte proliferation may be driven by seizures. Our hypothesis is that ATP-derived adenosine, via the $A_{2A}R$ activation, plays a critical role in astrogliosis leading to astrocytic ADK overexpression **(manuscript 5)**. According to the ADK hypothesis of epileptogenesis[14] upregulation of this adenosine metabolizing enzyme exerts a dual effect. At first, the expression levels of ADK remain low and ATP-derived adenosine rapidly increases. With continuous recurrence of seizures, sustained high adenosine levels contribute to inhibitory A_1R desensitization while activating more resistant $A_{2A}R$, which drive astrocytes proliferation with subsequent ADK upregulation. Excessive intracellular conversion of adenosine into AMP by upregulated ADK modifies the transmembranar gradient of the nucleoside facilitating its cellular uptake by equilibrative nucleoside transporters, resulting in decreases in the extracellular adenosine content. Eventually, this mechanism designed to restrain excitotoxicity and astrogliosis, will lose its power and became unable to compensate excessive ATP-derived adenosine production, with consequent continuous excitatory $A_{2A}R$ activation. ADK overexpression may also be associated with dynamic changes in gene expression seen in MTLE-HS. ADK upregulation favours DNA methylation, by constantly removing adenosine resultant from S-adenosylhomocysteine (SAH) hydrolysis, which leads to the release of methyl groups from its precursor, S-adenosylmethionine (SAM). Thus, ADK modulates the epigenetic control of the different genes involved in epilepsy. Characterization of the DNA methylation profile in our cohort of MTLE-HS patients would, therefore, be of interest to go more in depth on how these changes may affect the course and severity of this disease.

Remarkably, we have observed that endogenous retaliatory mechanisms seem to attempt to compensate for the exacerbated inflammatory response and neurotransmission imbalance. In particular, we found that the A_1R is overexpressed in the hippocampus and anterior cortical temporal lobe of MTLE-HS patients **(manuscript 5)**. The A_1R is the main responsible for the inhibitory neuromodulatory effects of adenosine in the brain. Its activation blocks the release of glutamate from nerve terminals by reducing Ca^{2+}-mediated exocytosis. The A_1R also controls neurotransmitter responsiveness by hyperpolarizing the post-synaptic membrane through the activation of potassium channels. In this sense, A_1R overexpression triggered by low adenosine in the extracellular milieu may transiently

compensate neuroexcitotoxicity produced by excessive glutamate release during surplus epileptic neuronal firing.

The anti-inflammatory cytokine, IL-10, was also found to be upregulated in the brain of MTLE-HS patients **(manuscript 3)**, further supporting a role for compensatory mechanisms in the context of epilepsy.

In addition, we also observed an upregulation of serum miR-146a and miR-132 in MTLE-HS patients compared to the control population **(manuscript 4)**. Interestingly, the expression of miR-146a is driven by activation of the TLR signalling cascade and it acts on a negative feedback loop resulting in inhibition of IL-1β expression. Likewise, mir-132 also inhibits the expression of pro-inflammatory cytokines. One may, therefore, hypothesize that reinforcing the expression of these epigenetic regulators may have a putative role in the control of exacerbated inflammatory reactions in the epileptic brain. MicroRNAs are pleiotropic molecules that modulate different genes and, thereby, may affect other processes involved in epileptogenesis. In this regard, miR-132 is also able to modulate the expression of Ca^{2+} and K^{+} channels, which may contribute to synaptic neurotransmission dysregulation. Functional studies are, therefore, required in order to elucidate which mechanisms are predominantly affected by miRNAs in epileptogenesis.

It should be emphasized that seizures potently modify cells microenvironment and metabolism, which may impact on the mechanisms regulating cellular homeostasis at the post-translational gene expression level. The loss of efficient extra- and intra-cellular regulatory mechanisms may explain changes in cells phenotype towards a more pro-inflammatory, less adequate for neuronal transmission or even more prone to neuronal cell elimination, which is compatible with the pathological findings detected in the brain of MTLE-HS patients.

One of the most relevant findings of this study is the clear demonstration that changes observed in the sclerotic hippocampus, the epicentre of this syndrome, expand to the adjacent anterior temporal cortical region, where more pronounced differences were found for the majority of genetic markers analysed in this study. This observation is supported by a recent proteomic study showing that seizures induce dynamic changes in gene expression in these regions. In early and latency periods, the changes in the hippocampal region are more pronounced whilst in the chronic phase they affect more evidently the anterior temporal cortex[15]. These differences may be associated with the role of the adjacent neocortex in seizure propagation. This observation also supports the theory that inflammation is not a mere seizure epi-phenomenon, but indeed it may have an important role in propagation and development of MTLE-HS as well.

We must be aware that we are studying chronic epileptic patients and some of the observed changes may not be related to epileptogenesis, but may be a reflex of recurrent episodes of sustained high cellular activity, changes in intracellular medium, or could even be caused by AEDs. Caution is, thus, warranted and further studies to clarify the role of these factors must be undertaken.

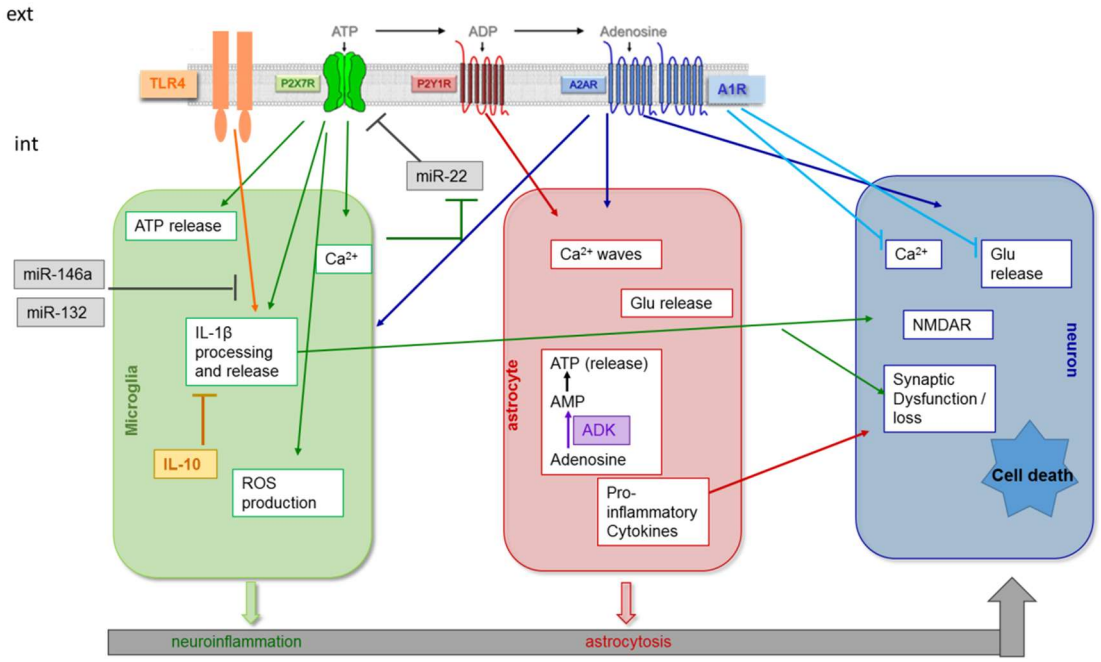

Figure 1 – Neuronal and glial cells communication is mediated through a complex interplay: focus on the purinergic system crosstalk with the inflammatory response. ATP released by neurons, astrocytes or microglia is a danger signal. ATP binds to microglial P2X7R stimulating activation and proliferation of microglial cells. P2X7R activation, in addition to the activation of TLR4 by HMGB1 released by injured cells, leads to the expression of pro-inflammatory cytokines. Pro-inflammatory signals continue to promote microglia activation and impair synaptic neurotransmission. ATP continues to be release due to high neuronal activity. Once in the extracellular space, ATP is catabolised to adenosine by ecto-nucleotidases, which preferentially activates the $A_{2A}R$ in detriment of the A_1R. Whilst the fast desensitizing A_1R have an inhibitory effect, long lasting activation of the $A_{2A}R$ stimulates glutamatergic neurotransmission and GABA uptake. Activation of the $A_{2A}R$ also promotes microglia activation, in particular when high levels of glutamate are available in the extracellular milieu, as seen during seizures. A self-propagating cycle of neuroinflammation and neurodegeneration is then established. While a series of anti-inflammatory cytokines and microRNAs attempt to constrain the exacerbated neuroinflammatory response, the system reaches a state of unbalanced cell environment where failure of common regulatory mechanisms decrease seizures threshold and facilitate propagation to dissemination regions. Adapted from Rodigues et al, 2015[16]

A complex interplay between neuroinflammation and purinergic signalling with a positive feedback loop between them has been demonstrated. This suggests that targeting the inflammation – purinergic axis is worth to pursue in order to design novel strategies for the treatment of drug-resistant MTLE-HS. Notwithstanding this, several questions regarding

the interplay between these two mutually - influenceable major systems still remain unanswered. Although it is out of the scope of this work it would be interesting to characterize the genetic / epigenetic profile affecting inflammation and purinergic signalling pathways in MTLE-HS patients showing seizure recurrence after resective surgery.

Hipocrates, in 400 B.C., said *"It seems to me that the disease (epilepsy) is no more divine than any other. Men think it is divine merely because they don't understand it"*. Remarkably, in 2017 we still don't! Nonetheless, "the truth is out there" and with the results of this work we are closer to unravel it. The notion that inflammation, and the interplay between inflammatory reactions – purinergic system - neurotransmission, may contribute to seizure perpetuation in MTLE-HS may lead towards new directions in the epilepsy treatment in the near future.

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