MARCELO ANANIAS TEOCCHI

"EXPRESSÃO HIPOCAMPAL DE GENES ENVOLVIDOS EM VIAS DE APOPTOSE EM PACIENTES COM EPILEPSIA DO LOBO TEMPORAL"

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MARCELO ANANIAS TEOCCHI

"EXPRESSÃO HIPOCAMPAL DE GENES ENVOLVIDOS EM VIAS DE APOPTOSE EM PACIENTES COM EPILEPSIA DO LOBO TEMPORAL"

Tese de **Doutorado** apresentada à Pós-Graduação em Saúde da Criança e do Adolescente da Faculdade de Ciências Médicas da Universidade Estadual de Campinas - UNICAMP, para a obtenção do título de **Doutor** em **Ciências**, área de concentração **Saúde da Criança e do Adolescente.**

Orientadora: Profa. Dra. Lília Freire Rodrigues de Souza Li

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A tarefa da ciência não é simplesmente identificar a mudança do padrão estrutural em todas as coisas, mas *considerar essa mudança uma coisa simples*. A ciência começa com a hipótese, que está sempre presente, embora possa ser inconsciente, esquecida ou, às vezes, até mesmo negada: *Existe uma ordem simples na natureza; é possível uma forma simples de revelar a experiência; a tarefa da ciência é descobri-la.*

L. L. Whyte, *Accent on Form*

Entre todos os elementos, o Sábio tomaria a água como seu preceptor. A água é submissa, mas conquista tudo. A água extingue o fogo ou, vendo que pode ser derrotada, escapa como vapor e toma nova forma. A água carrega a terra macia ou, quando desafiada pelas rochas, procura um novo caminho em torno... Satura a atmosfera de modo que o vento morre. A água cede passagem para os obstáculos com uma humildade enganadora, pois nenhum poder pode impedi-la de seguir o seu caminho traçado rumo ao mar. A água conquista submetendo-se, nunca ataca, mas sempre ganha a última batalha.

De *The Wheel of Life*, de John Bloteld, p. 78

Look deep into nature, and then you will understand everything better.

Albert Einstein

Resumo

A epilepsia do lobo temporal associada à esclerose hipocampal [ELT(EH)] é o tipo mais comum de epilepsia focal que causa crises refratárias. A morte neuronal na EH pode ser desencadeada por danos excitotóxicos e citocinas específicas. Pesquisas em modelos experimentais de crises convulsivas ressaltaram a citocina pleiotrópica fator de necrose tumoral (TNF) como um importante efetor/mediador de neuroinflamação e morte celular. Além disso, esses modelos sugeriram que o TNF possa ter uma ação dicotômica por meio de seus dois receptores: ativação da morte celular programada (via TNFRSF1A) ou atuação na sobrevivência celular (via TNFRSF1B), através do fator nuclear kappa B (NFkB). Klotho (KL), originalmente identificada como uma proteína antienvelhecimento, tem se destacado como um importante hormônio regulador de cálcio e fósforo. Sua função cerebral é desconhecida; porém, camundongos *knockout* para *Kl* apresentam características que remetem ao envelhecimento humano, com neurodegeneração e redução de sinapses no hipocampo. Em modelos de doença renal crônica e colite, foi comprovado que o TNF inibe KL através do NFkB. Nosso objetivo é identificar alvos críticos na epileptogênese e na fisiopatologia molecular da ELT(EH). Avaliamos a expressão relativa do RNAm de cinco genes-alvo: *TNF*, *TNFRSF1A*, *TNFRSF1B*, *NFKB1* e *KL*. A expressão gênica foi avaliada em amostras de tecido hipocampal de 14 pacientes com ELT(EH) e comparadas com cinco amostras de controles *post mortem*. Além disso, ambos os receptores do TNF foram analisados nas amostras hipocampais por imuno-histoquímica. Todos os cinco genes avaliados apresentaram expressão significantemente alterada nos

pacientes com ELT(EH) ($P<0,05$). A expressão de ambos os receptores foi constatada nos tecidos dos pacientes. Este é o primeiro estudo a relacionar KL e epilepsia. Nossos dados reforçam o componente inflamatório da EH e sugerem que o TNF possa inibir a expressão de KL no hipocampo dos pacientes. A repressão de KL abre novas frentes de pesquisa que podem contribuir para a compreensão da complexa fisiopatologia da ELT(EH). Além disso, uma vez que TNF, TNFRSF1A e NFKB1 são protagonistas na via extrínseca da apoptose, concluímos que a sinalização do TNF participe criticamente da neurodegeneração hipocampal associada à ELT. Existem controvérsias sobre o papel do TNFRSF1B. Sua ativação pode estar relacionada a mecanismos de sobrevivência, hipótese corroborada pela concomitante hiperexpressão de NFKB1; todavia, já foi demonstrado que o TNFRSF1B pode reforçar a ação do TNFRSF1A. Apresentamos evidências de que KL e a sinalização do TNF constituem um importante eixo para estudos farmacológicos, especialmente em relação aos benefícios de uma terapia anti-inflamatória nesses pacientes.

Abstract

Temporal lobe epilepsy associated with hippocampal sclerosis [TLE(HS)] is the most common form of focal epilepsy that causes refractory seizures. Neuronal death in HS can be triggered by excitotoxic damage and specific cytokines. Previous research in seizure models indicates that the pleiotropic cytokine tumor necrosis factor (TNF) as an important effector/mediator of neuroinflammation and cell death. Through its two receptors, TNF can play a dichotomous role in animal seizures: programmed cell death activation (via TNFRSF1A) or cell survival actuation (via TNFRSF1B), through the nuclear factor kappa B (NFkB) activation. Klotho (KL), originally identified as an antiaging protein, is emerging as an important calciophosphoregulatory hormone. Its cerebral function is unclear; however, the *Kl* knockout mouse exhibits a phenotype resembling human aging presenting neural degeneration and a reduction of synapses in the hippocampus. Studies have demonstrated that TNF downregulates KL through NFkB in animal models of chronic kidney disease and colitis. Our aim is to identify critical targets in epileptogenesis to clarify the molecular pathophysiology in TLE(HS). We evaluated the relative mRNA expression of five target genes: *TNF*, *TNFRSF1A*, *TNFRSF1B, NFKB1* and *KL*. Gene expression was performed in resected hippocampal tissue samples from 14 TLE(HS) patients and compared to five *post mortem* controls. Moreover, an immunohistochemistry assay was done to verify the activation of both TNF receptors in patient and control tissues. We found that all target genes were differentially regulated in the TLE(HS) patients ($P<0.05$). Both TNF receptors were clearly activated in patient's tissues. This is the first study relating KL to

epilepsy. Our data corroborates the prominent role of inflammation in HS and suggests that TNF might affect KL expression in hippocampus. As a multifunctional protein, KL downregulation in TLE(HS) patients opens several possible avenues of research that will help us to understand the complex pathophysiology in HS. Furthermore, since TNF, TNFRSF1A and NFKB1 are key factors in the death receptor signaling canonical pathway, we conclude that TNF signaling plays a crucial role in TLE hippocampal neurodegeneration. There is still some controversy on TNFRSF1B role. Its augmentation could be related to a survival mechanism because the concomitant NFKB1 upregulation; however, it has already been demonstrated that TNFRSF1B may reinforce TNFRSF1A action. Our evidence reveals KL and the TNF pathway as an important axis for pharmacological studies regarding the benefits of an anti-inflammatory therapy in these patients.

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Lista de Abreviaturas e Siglas

ACE: *angiotensin I converting enzyme (peptidyl-dipeptidase A) 1*

AP1: proteína ativadora 1 ou *activator protein 1*

APAF1: *apoptotic peptidase activating factor 1*

BAX: *BCL2-associated X protein*

BID: *BH3 interacting domain death agonist*

cAMP: monofosfato de adenosina cíclico ou *cyclic adenosine monophosphate* ou *3'-5'-cyclic adenosine monophosphate*

CASP10: *caspase 10, apoptosis-related cysteine peptidase*

CASP2: *caspase 2, apoptosis-related cysteine peptidase*

CASP3: *caspase 3, apoptosis-related cysteine peptidase*

CASP6: *caspase 6, apoptosis-related cysteine peptidase*

CASP7: *caspase 7, apoptosis-related cysteine peptidase*

CASP8: *caspase 8, apoptosis-related cysteine peptidase*

CASP9: *caspase 9, apoptosis-related cysteine peptidase*

CDKN1A: *cyclin-dependent kinase inhibitor 1A (p21, Cip1)*

COX: ciclo-oxigenase ou *cyclooxygenase*

DAXX: *death-domain associated protein*

DD: domino de morte ou *death domain*

DIABLO: *diablo, IAP-binding mitochondrial protein*

DISCs: *death-inducing signaling complexes*

DR: receptores de morte ou *death receptors*

EH: esclerose hipocampal

ELT: epilepsia do lobo temporal

ELT(EH): epilepsia do lobo temporal associada à esclerose hipocampal

ELTM: epilepsia do lobo temporal medial

FADD: *FAS-Associated Death Domain*

FAS: *Fas (TNF receptor superfamily, member 6)*

FGF23: *fibroblast growth factor 23*

FLIP: *FLICE-inhibitory protein*

GABA: ácido gama-aminobutírico ou *gamma-aminobutyric acid*

HGNC: *HUGO Gene Nomenclature Committee*

HMGB1: *high-mobility group box 1*

HTRA2: *HtrA serine peptidase 2*

HUVECs: *human umbilical vein endothelial cells*

IAP: proteínas inibidoras da apoptose ou *inhibitors of apoptosis proteins*

IGF1: *insulin-like growth factor 1 (somatomedin C)*

ILAE: Liga Internacional Contra a Epilepsia ou *International League Against Epilepsy*

JNK: *c-Jun N-terminal kinase*

KL: *klotho*

MAP3K5: *mitogen-activated protein kinase kinase kinase 5*

NFkB: *nuclear fator kappa B*

PI3K: fosfoinositídeo 3-quinase ou *phosphoinositide 3-kinase*

PKA: proteína quinase A ou *protein kinase A*

PLA2: fosfolipases A2 ou *phospholipases A2*

RIPK1: *receptor (TNFRSF)-interacting serine-threonine kinase 1*

SNC: sistema nervoso central

tBID: BID truncado ou *truncated BID*

TGFB: fator de transformação do crescimento beta ou *transforming growth factor beta*

TNF: fator de necrose tumoral ou *tumor necrosis factor*

TNFRSF10A : *tumor necrosis factor receptor superfamily, member 10a*

TNFRSF10B : *tumor necrosis factor receptor superfamily, member 10b*

TNFRSF1A: *tumor necrosis factor receptor superfamily, member 1a*

TNFRSF1B: *tumor necrosis factor receptor superfamily, member 1b*

TNFRSF21: *tumor necrosis factor receptor superfamily, member 21*

TNFRSF25: *tumor necrosis factor receptor superfamily, member 25*

TP53: *tumor protein p53*

TRADD: *TNFRSF1A-associated via death domain*

TRAF2: *TNF receptor-associated factor 2*

TRPV5: *transient receptor potential cation channel, subfamily V, member 5*

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1. Introdução

1.1. Epilepsia

Epilepsia é uma desordem neurológica caracterizada por crises recorrentes e não provocadas (1). Supõe-se que essas crises sejam o resultado de uma descarga elétrica transitória, excessiva e irregular proveniente de um grupo de neurônios (2, 3). Na maioria dos casos, os pacientes apresentam epilepsia idiopática, ou seja, de causa indeterminada. Contudo, diversos fatores podem desencadear crises e epilepsias, tais como traumatismos cranianos, certas drogas ou tóxicos, interrupção do fluxo sanguíneo cerebral em virtude de um acidente vascular ou de problemas cardiovasculares, doenças infecciosas e tumores (4). Em alguns casos, a causa da epilepsia é de caráter genético (4).

As manifestações clínicas mais comuns da epilepsia podem incluir alterações de consciência, motoras, sensoriais, cognitivas, autonômicas ou eventos psíquicos (3, 5-7). As epilepsias formam, então, uma combinação de desordens neurológicas crônicas provenientes de funções cerebrais alteradas, que podem ocorrer concomitantemente a outras doenças neurológicas ou isoladamente, e em muitos casos se tornam progressivas com relação aos distúrbios cognitivos, frequência e gravidade das crises. A enorme diversidade de sintomas, etiologias, gravidade e prognóstico sugerem que sejam consideradas como um conjunto de doenças ou síndromes, o que difere de uma enfermidade clínica uniforme (1).

Considerada um problema de saúde pública pela Organização Mundial de Saúde, a epilepsia acomete cerca de 50 milhões de pessoas no mundo (8). Sua prevalência varia de acordo com a idade, sexo, etnia e tipo de síndrome epilética e diverge entre países desenvolvidos e em desenvolvimento – um reflexo de condições socioeconômicas antagônicas (8, 9). Aproximadamente 80% dos casos de epilepsia concentram-se em regiões em desenvolvimento, pois nessas áreas os pacientes estão mais expostos a fatores ou condições que podem levar a danos cerebrais permanentes (8). Em países desenvolvidos, os novos casos anuais estão entre 40 a 70 por 100 mil pessoas na população em geral; naqueles em desenvolvimento, esse índice frequentemente corresponde ao dobro (10). Além disso, diferentemente dos países desenvolvidos, nos países em desenvolvimento o pico de incidência ocorre em adultos jovens, fato que reflete provavelmente diferenças etiológicas. Infelizmente, há poucos estudos epidemiológicos sobre epilepsia no Brasil (4, 9).

1.2. Epilepsia do lobo temporal medial

Em 1989, a epilepsia do lobo temporal (ELT) foi incluída na classificação da Liga Internacional Contra a Epilepsia [ILAE (*International League Against Epilepsy*)] (5). A classificação publicada pela ILAE em 2010 trouxe um avanço significativo, pois reconhece a ELT medial (ELTM) associada à esclerose hipocampal (EH) (11). Todos os pacientes integrantes deste estudo apresentaram esta condição.

A ELT caracteriza-se como a mais frequente das epilepsias parciais ou focais, representando aproximadamente 50% dos casos em adultos e tem

como manifestação típica, a crise parcial complexa (12). Acomete principalmente as estruturas mediais do lobo temporal, sendo a ELTM a forma mais comum de ELT (3).

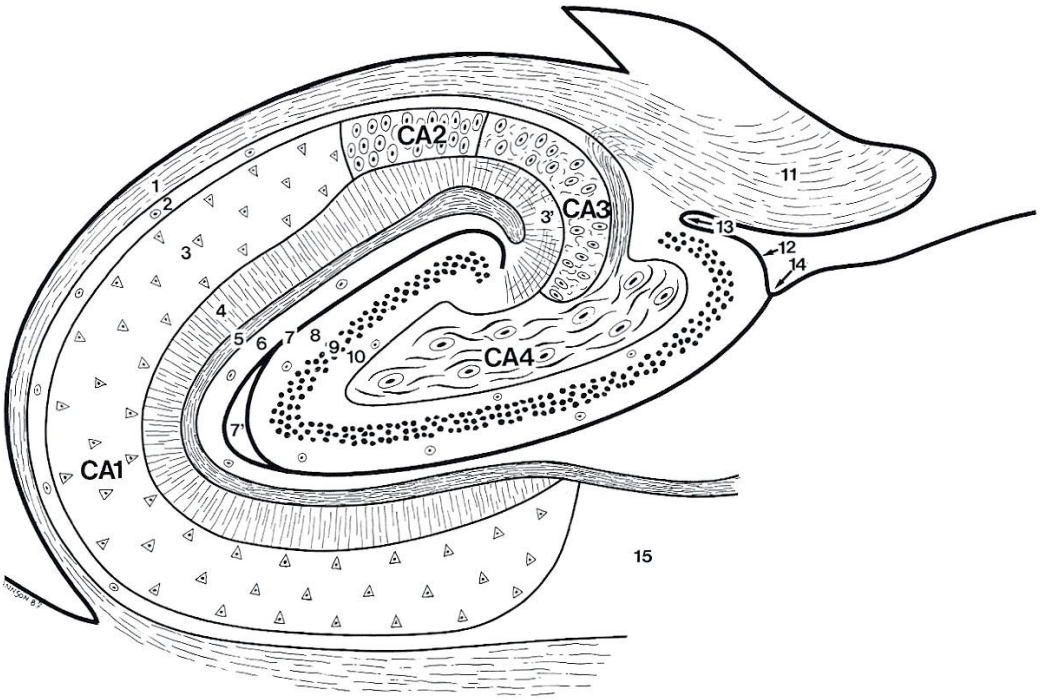

Figura 1. Diagrama do corte transversal do corpo hipocampal [Extraído de Duvernoy (13)].
Cornu ammonis:
1 *Alveus*; 2 *Stratum oriens*; 3 *Stratum pyramidale*; 3' *Stratum lucidum*; 4 *Stratum radiatum*; 5 *Stratum lacunosum*; 6 *Stratum moleculare*; 7 Sulco hipocampal (notar uma cavidade residual, 7')
CA1, CA2, CA3, CA4: camadas do *cornu ammonis*
Giro denteado:
8 *Stratum moleculare*; 9 *Stratum granulosum*; 10 Camada polimórfica; 11 Fímbria; 12 *Margo denticulatus*; 13 Sulco fimbriodenteado, 14 Sulco hipocampal; 15 *Subiculum*

As estruturas neurais que constituem o lobo temporal medial são o giro para-hipocampal, úncus, hipocampo, fímbria, giro dentado ou denteado, e tonsila ou amígdala (14) (Figura 1). As crises na ELTM apresentam um perfil clínico bem definido e se originam nesta porção medial do lobo temporal, especialmente no hipocampo, giro para-hipocampal e tonsila (3, 15). A primeira

crise, frequentemente, acontece no final da infância e início da adolescência e, depois de um período sem manifestações, o paciente passa a apresentar crises habituais, geralmente do tipo parcial complexa, seguidas de automatismos manuais e oromandibulares (3, 12). Manifestações clínicas como relatos de auras e de sensação epigástrica ascendente, além de medo súbito, *déjà vu*, *jamais vu* e crises autonômicas - palpitação e piloereção - são comuns (16). Depressão e alterações de humor podem ser encontradas nesses pacientes (6). Podem ocorrer distúrbios de memória verbal ou não verbal de acordo com o lado do hipocampo comprometido (7).

Sob tratamento medicamentoso (drogas anticonvulsivas), crises tônico-clônicas (generalização secundária) são pouco frequentes; todavia, comumente, com o passar dos anos, as crises tornam-se refratárias ao tratamento medicamentoso (3, 17).

Um importante estudo realizado por Semah et al. (17) relatou que apenas 11% dos pacientes com epilepsia focal associada à EH tornaram-se livre das crises convulsivas com o uso de medicamentos, o que faz da EH um importante fator prognóstico. A EH será abordada com mais detalhes na seção subsequente (item 1.2.1). A associação entre EH e ELTM é bastante comum (2, 11, 12, 15, 17-19). A resistência à medicação aliada à alta incidência denota a grande importância dessa síndrome. Na ineficácia do tratamento medicamentoso, a cirurgia de retirada do foco epilético é empregada. Nos pacientes com ELTM refratária, o processo cirúrgico mais comum é a amigdalo-hipocampectomia, que tem se mostrado bastante eficiente (3).

Rotineiramente, os exames eletroencefalográficos (EEG) mostram atividades epileptiformes unilaterais ou bilaterais nas regiões temporais médio-

basais. No entanto, o diagnóstico de ELTM não é excluído em casos de pacientes com EEG normal (3).

1.2.1. Esclerose hipocampal

A EH é o principal achado histopatológico encontrado em pacientes com ELT (20) (Figura 2). Morte neuronal seletiva, astrogliose e atrofia hipocampal são características da EH (12, 18). Os mecanismos envolvidos na extensa perda neuronal e no típico padrão da EH ainda não foram completamente elucidados. Os neurônios hipocampais são destruídos por necrose e apoptose (morte celular programada), com consequente astrogliose: proliferação anormal dos astrócitos adjuntos devido à morte neuronal (21). Além disso, o típico perfil de perda neuronal da EH associada à ELT difere daqueles encontrados em outras patologias neurológicas (2, 20, 22-24).

A perda neuronal seletiva ocorre intensamente nas áreas CA1, CA3 e hilus do hipocampo, acompanhada de astrogliose em graus variados. Já a região CA2 tem se mostrado mais resistente a esse tipo de insulto, com menor perda neuronal em comparação às outras regiões (12, 22, 25). A dispersão das células granulares é outra importante característica da EH (19, 22, 23).

Evidências crescentes indicam que outro importante componente da esclerose hipocampal seja a inflamação. Crises convulsivas, por si só, são capazes de gerar inflamação (26). A associação entre inflamação e morte neuronal na epilepsia ganhou destaque nos últimos anos, em virtude de diversos estudos que corroboram com essa evidência [revisado por Vezzani et al. (26)]. É plausível que a EH seja resultado de um processo inflamatório crônico, em que citocinas ajam como catalizadores da morte celular neuronal,

com consequente astrogliose. O componente inflamatório na epilepsia será abordado com mais detalhes no item 1.4.

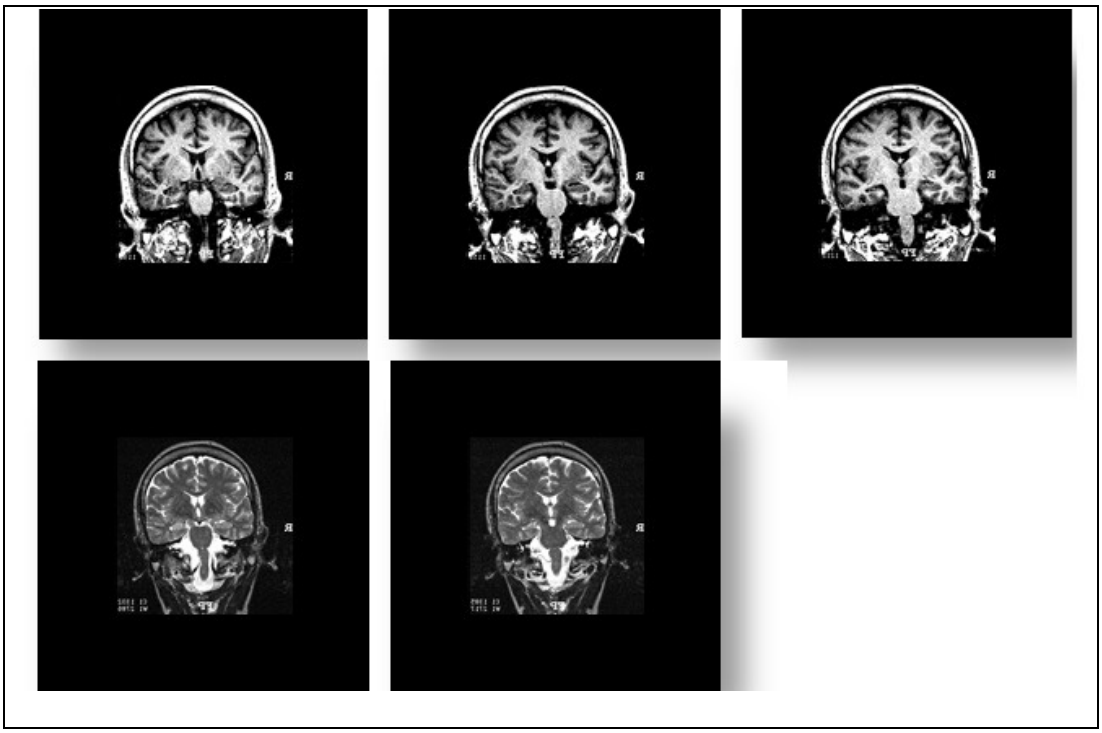

Figura 2. Imagens de ressonância magnética (MRIs) em paciente com ELT associada à EH. O paciente de 51 anos teve a primeira crise com um ano de idade. MRIs coronais IR e T2 mostram redução do volume hipocampal esquerdo com hipossinal em T1 e hipersinal em T2 (esclerose hipocampal).

Um fenômeno que tem sido bem caracterizado em pacientes com ELT associada à EH [ELT(EH)] é a reorganização axonal das células granulares. Acredita-se que após um insulto precoce, com consequente perda celular na região hilar do hipocampo, as células granulares formem novas sinapses. Isto tem levado à hipótese de que essas reorganizações axonais anormais formem um circuito local de retroalimentação excitatório sobre as células granulares, que contribui para a geração de crises crônicas (2, 15, 24).

1.3. Apoptose

A morte celular programada ou apoptose é um processo ativo de morte celular programada em que um estímulo inicial desencadeia uma cascata de eventos moleculares mediados por um grupo de peptidases denominadas caspases. De modo geral, as caspases são classificadas em iniciadoras (CASP2, CASP8, CASP9 e CASP10) e executoras (CASP3, CASP6 e CASP7) (27).

A apoptose é dividida em duas vias de sinalização específicas: a intrínseca e a extrínseca. Apesar de cada uma delas requerer iniciadores específicos, ambas convergem na ativação da CASP3, que leva à via de morte ou execução. A ativação dessa via resulta em características citomorfológicas distintas, como irregularidades (bolhas) na membrana celular, encolhimento celular, degeneração nuclear, condensação da cromatina e fragmentação do DNA (27).

Diferentemente das vias de sinalização intrínsecas que iniciam a apoptose, as vias extrínsecas requerem interações transmembranas mediadas por receptores. Estes incluem os receptores de morte (DR) que são membros da superfamília de receptores do fator de necrose tumoral (TNF) (27). A via de morte pode ser iniciada por esses receptores através da CASP8 ou da CASP10. Interessantemente, a ativação dessas caspases pode ativar as proteínas pró-apoptóticas BID (*BH3 interacting domain death agonist*) e/ou BAX (*BCL2-associated X protein*), via TP53 (*tumor protein p53*), que consequentemente pode resultar numa “comunicação cruzada” com a via intrínseca ou mitocondrial da apoptose (27).

1.3.1. Receptores de morte

Diversos membros pertencentes à família dos DR já foram descritos. A nomenclatura desses receptores é bastante complexa em virtude da existência de dezenas de pseudônimos (*aliases*). Apresentamos a sigla aprovada pelo *HUGO Gene Nomenclature Committee* (*HGNC*) (www.genenames.org), e ressaltamos que a nomenclatura proteica é idêntica ao símbolo do gene correspondente, mas nunca grafada em itálico e sempre com todos os caracteres em maiúsculo quando referida à espécie humana.

Os membros que destacamos da família dos DR são: TNFRSF1A (*tumor necrosis factor receptor superfamily, member 1a*), FAS [*Fas (TNF receptor superfamily, member 6*)], TNFRSF25 (*tumor necrosis factor receptor superfamily, member 25*), TNFRSF10A (*tumor necrosis factor receptor superfamily, member 10a*), TNFRSF10B (*tumor necrosis factor receptor superfamily, member 10b*) e TNFRSF21 (*tumor necrosis factor receptor superfamily, member 21*). Dois tipos de complexo de sinalização dos DR podem ser distinguidos. O primeiro grupo compreende os complexos de sinalização indutora de morte ou DISCs (*death-inducing signaling complexes*), que são formados no receptor FAS, TNFRSF10A ou TNFRSF10B (28). Todos os três receptores recrutam DISCs com composições similares. A formação do DISC resulta na ativação da CASP8, que é primariamente responsável pela transdução do sinal apoptótico. O segundo grupo compreende o TNFRSF1A, TNFRSF25 e TNFRSF21. Estes recrutam um grupo diferente de moléculas, responsáveis pela transdução tanto de sinais apoptóticos quanto de sobrevivência (29).

Os DISCs associados com o FAS são constituídos de receptores oligomerizados, FADD (*FAS-Associated Death Domain*), CASP8 ou CASP10 e FLIP (FLICE-*inhibitory protein*) (30). A ativação da CASP8 mediada via FAS resulta na clivagem da proteína BID da família do BCL2 para gerar o tBID (BID truncado). Este medeia a liberação do citocromo c da mitocôndria. O citocromo c se liga e ativa o APAF1 (*apoptotic peptidase activating factor 1*), assim como a pré-CASP9, formando um apoptosoma que leva à ativação da CASP9. DIABLO (*diablo, IAP-binding mitochondrial protein*) e HTRA2 (*HtrA serine peptidase 2*) promovem a apoptose pela inibição das proteínas inibidoras da apoptose [IAP (*inhibitors of apoptosis proteins*)]. A ativação da pré-CASP9 gera um efeito cascata que cliva as caspases efetoras ou executoras. Uma segunda via da apoptose mediada pelo FAS é a via da proteína adaptadora DAXX (*death-domain associated protein*), que ativa a MAP3K5 (*mitogen-activated protein kinase kinase kinase 5*). Em cascata, MAP3K5 ativa a transdução de sinal pela JNK (*c-Jun N-terminal kinase*). A ativação da JNK pode antagonizar a ação antiapoptótica de BCL2 (29, 30).

A sinalização do TNFRSF1A difere da do FAS ou da apoptose induzida pelo TNFRSF10A ou pelo TNFRSF10B. A apoptose induzida pelo TNFRSF1A envolve dois complexos de sinalização sequenciais. O complexo inicial da membrana plasmática (complexo I) é composto pelo TNFRSF1A, TRADD (*TNFRSF1A-associated via death domain*), RIPK1 (*receptor (TNFRSF)-interacting serine-threonine kinase 1*) e TRAF2 (*TNF receptor-associated factor 2*), e rapidamente sinaliza para a ativação do complexo NFkB (*nuclear fator kappa B*). Numa segunda etapa, TRADD e RIPK1 associam-se com FADD e CASP8, formando um complexo citoplasmático (complexo II).

Quando o NFkB é ativado pelo complexo I, o complexo II se associa com o FLIP (inibidor da CASP8) e a célula sobrevive. Desse modo, a transdução de sinal mediada pelo TNFRSF1A resulta em morte celular (via complexo II) quando, por exemplo, o sinal inicial do complexo I falha na ativação do NFkB. Neste caso, a CASP8 no complexo II pode levar à ativação direta da CASP3, que em cascata leva a apoptose (31).

1.4. Inflamação e epileptogênese

A última década nos revelou inúmeros estudos que relataram o papel crucial da inflamação na epileptogenese [revisado por Ravizza et al. (32)]. A conexão entre neurodegeneração e inflamação no cérebro epileptogênico emergiu como uma importante faceta para a compreensão dos mecanismos patológicos envolvidos na morte neuronal devido a crises convulsivas. Evidências crescentes também conectam astrogliose a neuroinflamação (33).

Acredita-se que a recorrência de convulsões na epilepsia seja capaz não só de induzir a morte neuronal como também de desencadear o processo inflamatório [revisado por Vezzani et al. (34)]. Todavia, a morte neuronal não é um pré-requisito para a ocorrência da inflamação; mas sim, a liberação de citocinas pró-inflamatórias é que pode resultar em morte celular e dano tecidual (35) que, consequentemente, pode perpetuar a inflamação (34). A Figura 3, publicada em outra revisão de Vezzani et al. (26), fornece importantes esclarecimentos sobre uma série de eventos fisiopatológicos gerados pelo componente inflamatório na epilepsia.

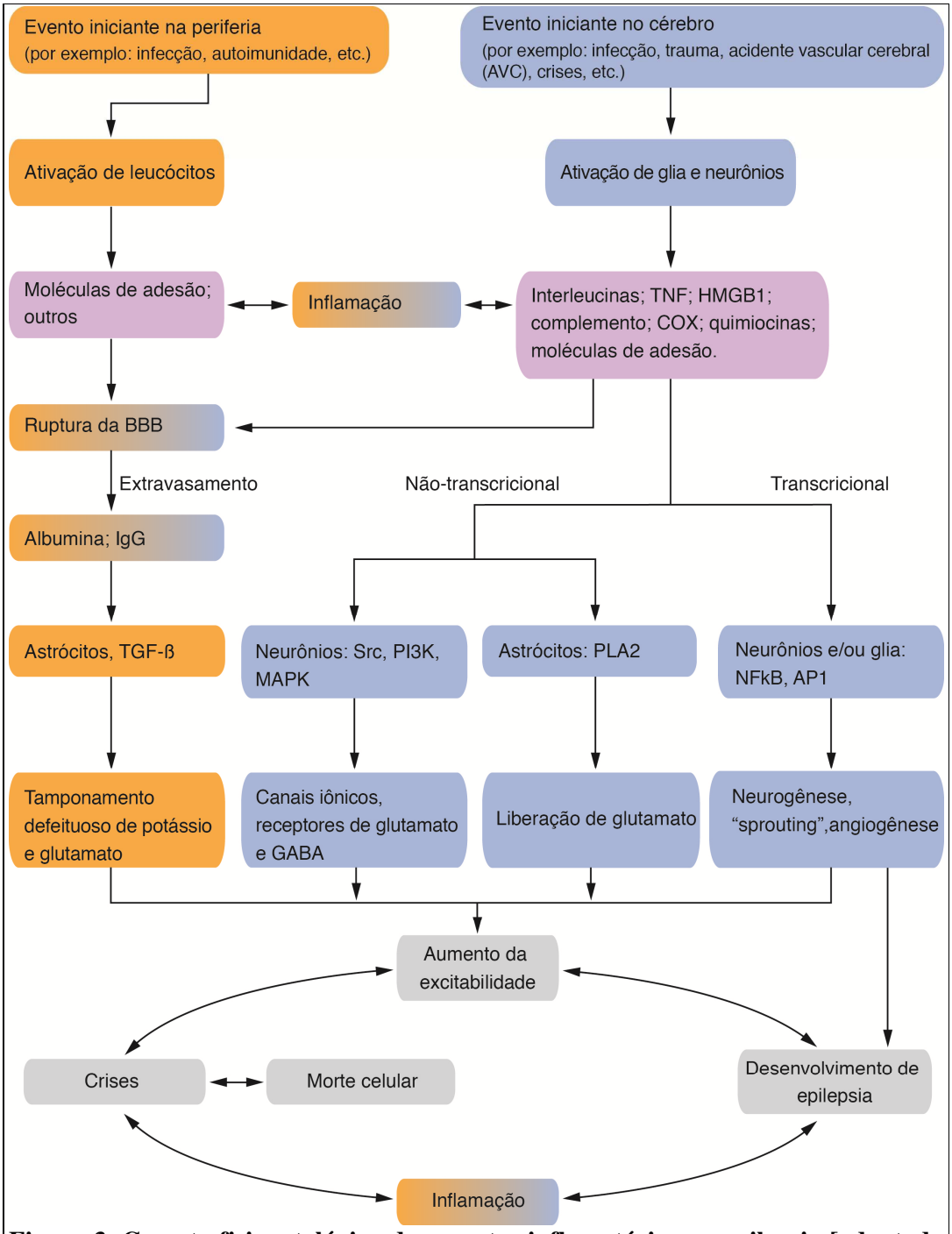

Figura 3. Cascata fisiopatológica dos eventos inflamatórios na epilepsia [adaptado de Vezzani et al. (26)]. Os eventos patológicos iniciados no sistema nervoso central (SNC) por danos locais, ou perifericamente devido a infecções ou como resultado de uma doença autoimune, podem levar a ativação de células cerebrais ou leucócitos, respectivamente. Estas células liberam mediadores inflamatórios no cérebro ou sangue, produzindo assim uma cascata de eventos inflamatórios que causam inúmeras consequências fisiopatológicas. Os efeitos da inflamação cerebral contribuem na geração de crises convulsivas individuais e morte celular, que, em sequência, aumentam a inflamação, gerando um círculo vicioso de eventos que contribuem para o surgimento

da epilepsia. A via periférica é mostrada em cor laranja, a via do SNC é evidenciada em azul, e as moléculas inflamatórias são apresentadas em roxo. A mistura de cores indica a contribuição de cada via na inflamação e no dano à barreira hematoencefálica [BBB (*brain blood barrier*)]. Abreviações: AP1, proteína ativadora 1 (*activator protein 1*); COX, ciclo-oxigenase (*cyclooxygenase*); GABA, ácido gama-aminobutírico (*gamma-aminobutyric acid*); HMGB1, *high-mobility group box 1*; PI3K, fosfoinositídeo 3-quinase (*phosphoinositide 3-kinase*); PLA2, fosfolipases A2 (*phospholipases A2*); TGFB, fator de transformação do crescimento beta (*transforming growth factor beta*).

Segundo Vezzani e Friedman (36), o componente inflamatório da epilepsia não é um fenômeno secundário ou complicação da patologia. Muito provavelmente está envolvido nos mecanismos que sustentam a hiperexcitabilidade neuronal, no surgimento e recorrência das crises convulsivas, e na progressão e gravidade da epilepsia. Dessa forma, compreender quais são os mediadores solúveis e os mecanismos moleculares críticos envolvidos na morte neuronal associada à inflamação é fundamental no esclarecimento de como as crises convulsivas contribuem com a fisiopatologia da ELTM.

1.5. Genes de interesse

1.5.1. Klotho

Klotho (KL), originalmente identificado como uma proteína antienvelhecimento, está envolvido em uma gama de funções em diversos sistemas (37), além de agir como um importante hormônio regular de cálcio e fósforo (38). O RNAm de *KL* é expresso somente em alguns órgãos, como cérebro, rins, gônadas, glândula pituitária e glândulas paratireoides (39, 40). Sua função cerebral é desconhecida; porém, camundongos *knockout* para *Kl*

(*Kl*$^{-/-}$) exibem características fenotípicas que se assemelham ao envelhecimento humano, dentre elas degeneração neural e redução de sinapses no hipocampo (39). Além disso, em humanos, KL atua na expectativa de vida celular pela repressão da proteína pró-apoptótica TP53, que regula diversos genes relacionados a apoptose (41). O Quadro 1 expõe algumas das principais atuações de KL em diferentes vias de sinalização intracelular.

Uma das mais intrigantes características de KL relaciona-se com seu efeito anti-inflamatório (42, 43). A demonstração recente de que o TNF inibe KL através do NFkB em modelos de doença crônica renal e colite (44, 45), aliado ao importante componente inflamatório da epilepsia, motivou-nos a incluir a expressão deste gene em nossas análises. Este é o primeiro estudo a relacionar KL com epilepsia.

Quadro 1. Atuações de KL em diferentes vias de sinalização intracelular

Função ou atuação de KL em vias de sinalização intracelular	Referências
Regulação da sinalização da insulina e do IGF1 [*insulin-like growth factor 1 (somatomedin C)*] e supressão de estresse oxidativo	(46-49)
Regulação da sinalização do fator de crescimento de fibroblasto 23 [FGF23 (*fibroblast growth factor 23*)]	(50, 51)
Ativação do canal iônico TRPV5 (*transient receptor potential cation channel, subfamily V, member 5*)	(52, 53)
Proteção contra disfunção endotelial e regulação da produção de óxido nítrico	(54-56)
Redução da senescência celular dependente das vias de sinalização do TP53 e CDKN1A [*cyclin-dependent kinase inhibitor 1A (p21, Cip1)*]	(41)
Influência na via de sinalização do monofosfato de adenosina cíclico (cAMP)	(56, 57)
Influência na via de sinalização da proteína quinase C (PKC)	(58)
Influência na via de sinalização Wnt	(59)

1.5.2. Fator de necrose tumoral

Diversos estudos em animais confirmam que crises convulsivas induzem a expressão de TNF no cérebro (32, 60-63). O TNF é uma importante

citocina pró-inflamatória multifuncional da superfamília do TNF. É principalmente secretada por macrófagos e sua ação depende principalmente de dois receptores: TNFRSF1A e TNFRSF1B (*tumor necrosis factor receptor superfamily, member 1B*). O TNF atua na regulação de um amplo espectro de processos biológicos que incluem proliferação celular, diferenciação, apoptose, metabolismo lipídico e coagulação (64). Inúmeras enfermidades, como doenças autoimunes, resistência à insulina, e câncer têm o envolvimento do TNF (64).

No hipocampo, o TNF regula vias de sinalização celular através de seus dois receptores (65, 66). O TNFRSF1A é expresso constitutivamente em todos os tecidos humanos e é o principal receptor de sinalização para o TNF. Um domínio de morte [DD (*death domain*)] citoplasmático, necessário para vias de sinalização apoptóticas (explanadas no item 1.3.1) e ativação do NFkB, está presente nesse importante receptor. O TNFRSF1B não contém um DD e atua em processos biológicos restritos, sendo principalmente expresso por células do sistema imunológico. Muitas evidências apoiam o envolvimento do TNFRSF1A com a ativação da apoptose, enquanto que o TNFRSF1B se relaciona com a ativação do sistema NFkB (67-69). Dessa forma, existe certa ambiguidade na ação do TNF.

Na epilepsia, alguns estudos relataram essa dualidade. Em ratos, os níveis de RNAm do *Tnf* aumentaram depois de convulsões geradas por estimulação elétrica da amígdala (61, 62), que retornou aos níveis basais em três semanas (61). Em outro estudo, um aumento similar foi observado quando convulsões límbicas foram induzidas pela injeção intra-hipocampal de ácido

caínico ou por metiodato de bicuculina ou por estimulação elétrica do hipocampo na geração de *status epilepticus* (63).

De modo diferente, o estudo de Balosso et al. (70) mostrou que a injeção intra-hipocampal de TNF recombinante murino potencialmente previniu convusões. Além disso, camundongos transgênicos para a hiperexpressão de TNF pelos astrócitos apresentaram convulsões mais curtas, enquanto que camundongos deficientes na expressão dos receptores do TNF experimentaram crises convulsivas prolongadas (70). Acredita-se que essa característica dicotômica do TNF nas convulsões esteja relacionada com sua concentração e na sinalização gerada pelos seus dois receptores (60, 70). Novamente, a grande maioria dos estudos implica que, em modelos convulsivos, a via do TNFRSF1A esteja associada com a morte celular neuronal e que a do TNFRSF1B se relacione com efeitos anticonvulsivos (60).

Apesar de intensamente estudado em animais, estudos clínicos sobre o TNF são escassos. Algumas pesquisas mostraram que após diferentes tipos de crises, os níveis séricos ou do líquido cefalorraquidiano do TNF não sofreram alterações significativas (71-74). Por outro lado, Sinha et al. (75) relataram níveis séricos elevados de TNF e outras citocinas em pacientes com diferentes síndromes epilépticas.

A ação do TNF gera um importante elo entre inflamação e morte celular, dois importantes componentes da EH associada à ELT. O entendimento do TNF nos mecanismos fisiopatológicos da ELTM é fundamental para a criação de novos tratamentos, especialmente no que diz respeito à epilepsia refratária.

2. Objetivos

O principal objetivo deste estudo é identificar, através da expressão gênica diferencial, alvos críticos que possam estar relacionados aos mecanismos fisiopatológicos da ELT(EH).

2.1. Objetivos específicos

Quantificação da expressão relativa dos genes *TNF*, *TNFRSF1A*, *TNFRSF1B*, *NFKB1* e *KL* em tecido hipocampal de pacientes com ELT(EH) e de controles *post mortem*.

Comparação estatística da expressão dos genes alvo nos dois grupos: pacientes com ELT(EH) versus controles *post mortem*.

Detecção por imuno-histoquímica dos receptores do TNF: TNFRSF1A e TNFRSF1B, no tecido hipocampal de pacientes e controles *post mortem*.

Inferência sobre a expressão de outros genes pertencentes à superfamília do TNF e associados com a via extrínseca da apoptose (receptores de morte).

3. Capítulo 1

O Capítulo 1 corresponde ao artigo intitulado “Hippocampal gene expression dysregulation of Klotho, nuclear factor kappa B and tumor necrosis factor in temporal lobe epilepsy patients”, cujo objetivo foi verificar se a expressão dos genes *KL*, *NFKB1* e *TNF* se apresentava alterada no hipocampo de pacientes com ELT(EH). Recentemente foi demonstrado que o TNF inibe KL através do NFkB em modelos de doença crônica renal e colite, que como na epilepsia, também apresentam um importante componente inflamatório.

O artigo foi submetido para o periódico *Journal of Neuroinflammation* em 1º de novembro de 2012 e aceito em 19 de março de 2013. Até a conclusão da versão final desta tese, em 29 de abril de 2013, o mesmo ainda não havia sido publicado.

Capítulo 1

“Hippocampal gene expression dysregulation of Klotho, nuclear factor kappa B and tumor necrosis factor in temporal lobe epilepsy patients”

Hippocampal gene expression dysregulation of Klotho, nuclear factor kappa B and tumor necrosis factor in temporal lobe epilepsy patients

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Abstract

Background

Previous research in animal seizure models indicates that the pleiotropic cytokine TNF is an important effector/mediator of neuroinflammation and cell death. Recently, it has been demonstrated that TNF downregulates Klotho (KL) through the nuclear factor kappa B (NFkB) system in animal models of chronic kidney disease and colitis. KL function in the brain is unclear, although Klotho knockout ($Kl^{-/-}$) mice exhibit neural degeneration and a reduction of hippocampal synapses. Our aim was to verify if the triad *KL-NFKB1-TNF* is also dysregulated in temporal lobe epilepsy associated with hippocampal sclerosis (TLE(HS)) patients.

Findings

We evaluated *TNF*, *NFKB1* and *KL* relative mRNA expression levels by reverse transcription quantitative PCR (RT-qPCR) in resected hippocampal tissue samples from 14 TLE(HS) patients and compared them to five *post mortem* controls. Four reference genes were used: *GAPDH*, *HPRT1*, *ENO2* and *TBP*. We found that *TNF* expression was dramatically upregulated in TLE(HS) patients (P <0.005). *NFKB1* expression was also increased (P <0.03) while *KL* was significantly downregulated (P <0.03) in TLE(HS) patients. Hippocampal *KL* expression had an inverse correlation with *NFKB1* and *TNF*.

Conclusions

Our data suggest that, similar to other inflammatory diseases, TNF downregulates KL through NFkB in TLE(HS) patients. The remarkable *TNF* upregulation in patients is a strong indication of hippocampal chronic inflammation. Our finding of hippocampal *KL* downregulation has wide implications not only for TLE(HS) but also for other neuronal disorders related to neurodegeneration associated with inflammation.

Keywords

Seizures, hippocampal sclerosis, neuroinflammation, TNF, KL, NFKB1, GFAP, reference genes, calcium homeostasis

Findings

Introduction

Temporal lobe epilepsy (TLE) is the most treatment resistant (refractory) partial epilepsy and only 20% of patients achieve seizure control with antiepileptic drugs (AEDs) [1]. Hippocampal sclerosis (HS) is the main pathological finding observed in excised tissue from TLE patients treated with amygdalohippocampectomy. Only 10% of TLE patients with HS, who are treated with AEDs, become seizure-free which emphasizes the importance of HS as a prognostic factor [1]. HS is characterized by an abnormal increase in the number of astrocytes associated with the destruction of nearby neurons (astrogliosis) [2]. One of the primary events in seizure-induced cell death in the hippocampus is the excessive release of glutamate with consequent overload in

intracellular calcium influx [3]. Within the neuronal cells, sophisticated homeostatic mechanisms control calcium levels. Altered Ca^{2+} sensitivity or defective Ca^{2+} regulation appear to be involved in the aging process, contributing to the progressive neurodegeneration in Alzheimer's disease and the intensified susceptibility to cell death after a seizure or stroke [3,4].

Klotho (KL), originally identified as an anti-aging protein, is involved in multiple functions in many systems and acts as an important calciophosphoregulatory hormone [5]. *KL* mRNA is expressed only in limited organs, that is the brain, kidney, reproductive organs, pituitary gland and parathyroid glands [6,7]. Its cerebral function is unclear, however Klotho knockout ($Kl^{-/-}$) mice exhibit a phenotype resembling human aging, showing neural degeneration and a reduction of synapses in the hippocampus [6]. In addition, KL regulates the cellular lifespan of human cells by repressing the pro-apoptotic tumor protein p53 (TP53) [8], which regulates a number of apoptosis-related genes.

Important components and features of medial TLE, such as hippocampal intracellular calcium imbalance, neurodegeneration, hippocampal atrophy and cognitive decline, led us to question whether KL would also be downregulated in temporal lobe epilepsy associated with hippocampal sclerosis (TLE(HS)) patients. Furthermore, several studies assert that inflammation has a crucial role in epileptogenesis [9] and an increasing body of evidence connects astrogliosis to neuroinflammation [10]. In epilepsy, the pleiotropic cytokine TNF is indicated as being an important effector/mediator of neuroinflammation and cell death [9,11-15]. Interestingly, it has been demonstrated that TNF

downregulates KL through the transcription nuclear factor kappa B (NFkB) in animal models of chronic kidney disease and colitis [16,17]. Since inflammation and neurodegeneration seem to be connected in HS, our objective was to verify if the triad *KL-NFKB1-TNF* is also dysregulated in TLE(HS).

Methods

Patients, *post mortem* controls and tissues

Electroencephalogram (EEG) video monitoring/telemetry was performed on all patients to confirm the onset of seizure in the medial temporal lobe. Dual pathologies or multifocal epilepsies were not identified. Hippocampal atrophy was detected by magnetic resonance imaging (MRI) in all patients.

Each patient signed an informed consent agreement to allow scientific use of the tissue. All procedures were carried out with the approval of the local research ethics committee, and in compliance with institutional guidelines and relevant laws.

Fourteen TLE(HS) patients had the amygdalohippocampectomy procedure performed for therapeutic reasons (Table 1). Hippocampal tissue samples from all 14 patients were immediately collected and divided into two parts. One portion was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation occurred. The second portion was fixed for histopathological analysis and HS was confirmed in all of them.

Five *post mortem* control hippocampal tissue samples (one female, four males; 28.2 ± 13.1 years; range from 19 to 50 years old) were provided by

the Instituto Médico Legal (IML) de Campinas. Despite some traumatic deaths, no neurological abnormalities were detected. Subjects passed away unexpectedly and instantly, which minimizes the occurrence and progression of neuroinflammation. The *post mortem* delay averaged 7.8 hours (range from 6 to 9 hours).

Table 1 Clinical and demographic features of TLE(HS) patients

Patients	Gender	Age (years)	Side of HS	Duration (years)	Last seizure (days before surgery)	TLE form	AED during surgery
TLE 01	F	34.6	L	28.6	nd	Familial	PHT
TLE 02	F	29.1	L	22.1	2	Sporadic	CBZ
TLE 03	F	23.8	R	22.3	7	Sporadic	CBZ
TLE 04	M	42.8	R	41.2	3	Sporadic	CBZ
TLE 05	M	41.2	L	34.2	5	Sporadic	CBZ
TLE 06	M	50.8	R	48.8	7	Sporadic	CBZ
TLE 07	M	12.7	L	9.7	3	Sporadic	CBZ, PHT
TLE 08	F	43.8	B(L)[a]	41.8	nd	Sporadic	CBZ, LTG
TLE 09	F	58.2	R	57.7	nd	Sporadic	OXC, VPA
TLE 10	F	54.9	L	50.9	nd	Sporadic	CBZ
TLE 11	F	32.1	L	31.6	nd	Familial	CBZ
TLE 12	F	38.3	L	37.0	2	Familial	OXC
TLE 13	F	54.1	L	53.3	nd	Sporadic	OXC, PHT
TLE 14	M	34.4	R	34.2	nd	Familial	OXC

[a]The hippocampal side was more affected when bilateral. When the patient's last seizure was 'nd', the seizure most likely occurred over 7 days prior to surgery. TLE(HS), temporal lobe epilepsy associated with hippocampal sclerosis; M, male; F, female; B, bilateral; R, right; L, left; nd, not determined; AED, antiepileptic drug; CBZ, carbamazepine; PHT, phenytoin; OXC, oxcarbazepine; LTG, lamotrigine; VPA, valproate.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

To extract total RNA, 1 ml of TRIzol Reagent (Life Technologies, Foster City, CA, USA) was added per 75 mg of frozen tissue samples, homogenized and then further processed according to the manufacturer's instructions. The RNA integrity number (RIN) mean in both the control and patient groups was similar: 6.68 ± 0.9441 (n = 5) and 6.155 ± 0.2484 (n = 11), respectively. Due to the fact that the RNA was unavailable, the RIN was not evaluated for three patient samples: TLE 03, TLE 11 and TLE 13. Subsequently, 1 µg of total RNA of each sample was reverse transcribed into cDNA using 200 U of Superscript III Reverse Transcriptase (Life Technologies) and 3 µg of Random Primers (Life Technologies) according to the manufacturer's instructions.

Sterilized and filtered DEPC-treated water was used in all cDNA synthesis reactions. Complementary DNA samples derived from the investigated genes were detected using an ABI PRISM 7500 Sequence Detection System (Life Technologies) and TaqMan Gene Expression Assays

(Life Technologies): 5'-FAM-labeled probes and corresponding primer pairs (Table 2). Gene names are in accordance with the approved symbol from the HUGO Gene Nomenclature Committee (HGNC) database. To select the reference genes (endogenous controls), the study of Wierschke *et al.* on human epileptogenic tissues was considered [18]. Among 12 candidate genes, the algorithm NormFinder indicated hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), enolase 2 (gamma, neuronal) (*ENO2*) and TATA box binding protein (*TBP*) as good normalization factors, since as single genes their expression levels were among the five most stable. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a very often used reference gene, showed a relatively unstable expression. However, to reinforce our results, we opted to test all four reference genes. Glial fibrillary acidic protein (*GFAP*) was used as an upregulation control [18-20]. Each qPCR was run as triplicates with 10 ng cDNA sample in 6.25 µl TaqMan Gene Expression Master Mix (Life Technologies), 0.625 µl of the respective probe/primer mix, and 0.625 µl purified and deionized H_2O.

The amplification of all samples was of the same efficiency for precise quantification of reverse transcription quantitative PCR (RT-qPCR) data. Serial fivefold dilutions, starting with 250 ng of cDNA from the RNA quantification, of a cDNA solution pooled from the control group was used. The mean C_T values, measured in triplicates, versus the log10 of the dilution was plotted. The values from linear regressions applied to these plots were also presented (not shown). The amplification efficiencies ($E = 10^{(-1/slope)}$) were close to 1.0 (100%).

Data analysis

Relative gene expression quantification data was generated and analyzed using the 7500 Software version 2.0.5 (Life Technologies). One of the *post mortem* control samples was randomly chosen as the benchmark and the quantification data from the other samples, including controls and patients, was evaluated according to this reference sample, which always had a relative quantification of 1.0. This allowed the two groups (*post mortem* controls and TLE(HS) patients) to be statistically compared.

The GraphPad Prism 5 software version 5.04 for Windows was used for the statistical analysis (San Diego, CA, USA; www.graphpad.com). The Mann-Whitney U test was used for comparison between data from the control group (n = 5) versus the patient group (n = 14). All comparison data are presented as mean and SD. Correlation among the target genes was performed by the Spearman's rank correlation (Spearman's R). The reference sample was excluded from correlation tests and differences of $P < 0.05$ were considered significant.

Results and discussion

The quality of the RNA and the reliability of reference genes to quantify gene expression in surgical tissue are crucial in interpreting epilepsy-related changes of gene expression [18,21]. The similarity in the RIN average between TLE patients and the control group (6.68 ± 0.9441 and 6.155 ± 0.2484, respectively) reinforces our results. *Post mortem* delay has always been considered a major factor when interpreting the accuracy of results from human

tissue studies; however, this may not be a factor for human brain RNA [21]. Durrenberger *et al.* concluded that *post mortem* delay caused only a minor deterioration and had no effect on RNA quality. The same results were found in previous research mentioned in their report [21]. We worked with a *post mortem* delay of between 6 to 9 hours, which would not seem to contradict the findings of the Durrenberger study.

Our results (Figure 1) showed a dramatic increase on *TNF* relative mRNA expression in hippocampal tissue of TLE(HS) patients in comparison with the *post mortem* controls (P <0.005, regardless of the reference gene). Despite the same P value for the results regarding the four reference genes used, the *TNF* expression mean in patients had an ample variation: from 29.61 ± 8.55 (*TBP*) to 110.4 ± 58.82 (*HPRT1*). The combination of *ENO2* and *TBP* as the normalization factor proved to be very stable in epileptogenic tissues [18]. Therefore, in Figure 2, we used this combination to show the relative *TNF* expression individually by subject.

Figure 1Hippocampal relative gene expression of *KL*, *NFKB1*, *TNF* and *GFAP* in the TLE(HS) patient group versus the *post mortem* control group. Different colors represent the four reference genes used: *GAPDH* (red), *HPRT1* (yellow), *ENO2* (green) and *TBP* (blue). For the three target genes (*KL*, *NFKB1* and *TNF*) and the upregulation marker (*GFAP*), four independent comparisons were performed, since each reference gene was considered as an independent variable. The y-axis represents the quantitative data of the relative mRNA expression of the target molecules in sclerotic hippocampal tissues of the TLE(HS) patient group (n = 14) compared with the *post mortem* control group (n

= 5). All data are presented as mean and SD. Mann-Whitney U tests were used for comparisons between groups. *P <0.05, **P <0.01. TLE(HS), temporal lobe epilepsy associated with hippocampal sclerosis.

Figure 2Dispersion of the relative gene expression of *KL* and *TNF* in the hippocampus of TLE(HS) patients and *post mortem* controls. The y-axis represents the quantitative data of the relative mRNA expression of *KL* and *TNF*. The x-axis corresponds to the two groups: *post mortem* controls (n = 5, circles) and TLE(HS) patients (n = 14, squares and triangles). In the control group, the unfilled circle represents the calibrator sample, whose gene expression is always 1.0. The combination of *ENO2* and *TBP* was used as the reference gene. Mann-Whitney U tests were used for comparison between groups. TLE(HS), temporal lobe epilepsy associated with hippocampal sclerosis.

Our data suggests that the marked *TNF* upregulation in patients' tissues corroborates with the chronic hippocampal inflammation in TLE(HS). Table 1 shows that several patients had their last seizure several days before the surgery, suggesting that the high *TNF* expression levels were frequent, signaling that chronic hippocampal inflammation could be intrinsic to the refractory TLE(HS) syndrome. A number of studies in animal models indicate that seizures induce *TNF* expression in the brain [9,11-14]. Our findings confirm that a similar induction also occurs in TLE patients. However, despite the intense investigation of the TNF system and its effects in seizure models, there is not unanimous agreement on its effects in TLE(HS) and clinical studies are scarce [11]. Moreover, similar to recent findings on inflammatory disease

models [16,17], our data indicates that the synthesis of KL is reduced in the sclerotic hippocampus and TNF may downregulate KL through NFkB in TLE(HS) (Figures 1 and 2).

While *KL* mRNA was significantly downregulated (*HPRT1* and *TBP*: $P < 0.001$; *GAPDH*: $P < 0.02$; *ENO2*: $P < 0.03$), the expression level of *NFKB1* was also augmented in patients (*GAPDH*, *HPRT1* and *ENO2*: $P < 0.02$; *TBP*: $P < 0.03$) (Figure 1). It remains to be elucidated whether the KL depressed expression has a role in TLE(HS) physiopathology or is only a secondary change caused by the imbalance of calcium or phosphate and/or the progression of HS in the medial TLE syndrome. Since KL is a pleiotropic protein with different functions on many systems, our findings on *KL* downregulation opens up a gamut of study possibilities in HS pathophysiology comprehension.

We initially highlighted two specific functions assigned to KL: it has a stimulating effect on the Na^+/K^+ ATPase pump activity [22] and it influences the Wnt signal pathway [23]. Considering the importance of Na^+/K^+ ATPase activity in neuronal electrical activity and excitability [24], it is important to determine if KL regulates Na^+/K^+ ATPase in the hippocampus, which could be involved with the evident synaptic reduction found in $Kl^{-/-}$ mice [6,7]. The molecular mechanisms regarding the neurodegenerative feature found in $Kl^{-/-}$ mice and analogously to HS have not yet been elucidated. By acting on the Wnt signaling, KL offers an interesting outlook as it suppresses several Wnt family members [23]. It has been demonstrated that stimulation of Wnt signaling contributes to stem and progenitor cell senescence, and persistent decreased

KL expression may affect the rate of cellular aging and have harmful impact on tissue repair mechanisms [17]. This could be a key factor in HS progression.

There is another connection between KL depression and astrogliosis. By suppressing TP53 and negatively regulating cyclin-dependent kinase inhibitor 1 (CDKN1A, also known as p21) protein levels, KL not only inhibits apoptosis but also modulates the lifespan of human cells, which may be associated with increased signaling through the insulin/insulin-like growth factor 1 (IGF-1) pathway [8]. This growth factor has a significant role in non-neuronal modulation and multiple reports have indicated that astrocytes are the main target of IGF-1 by regulating their response to tissue injury [25]. Since KL may inhibit the insulin/IGF-1 pathway [26], its downregulation could support astrogliosis by a detrimental effect on neurons and astrocyte proliferation. In fact, *Kl*$^{-/-}$ mice have neuronal cell degeneration with a drastic increase in GFAP levels [27]. As expected, *GFAP* was upregulated in patients (*GAPDH*, *HPRT1* and *ENO2*: $P < 0.001$; *TBP*: $P < 0.02$), which supports astrogliosis.

Furthermore, KL has been considered an anti-inflammatory protein and this property could be one of its most striking features. In the endothelium, KL confers protection against nitric oxide (NO)-induced dysfunction [28], reduces the expression of adhesion molecules [28] and potentially regulates T cell functions [29]. In 2007, Witkowski *et al.* reported that KL was downregulated in $CD4^+$ lymphocytes at the mRNA, protein and enzymatic (beta-glucoronidase) activity levels in healthy, older adults and especially in rheumatoid arthritis (RA) patients [29]. Regardless of the unclear link between KL activity and $CD4^+$ cell function, they proposed that KL might be involved in physiological anti-

inflammatory responses in young individuals, but these responses decreased in both healthy older adults and RA patients. Their hypothesis was supported by the fact that KL expression and activity reduction, in both older adults and RA patients' lymphocytes, occurred in concert with the downregulation of CD28, a TNF-increased dependent T cell costimulatory molecule.

The *Kl*$^{-/}$ mouse also exhibits atherosclerosis and endothelial dysfunction, which led Maekawa *et al*. [28] to test the effect of KL on vascular inflammation. KL suppressed the TNF-induced expression of adhesion molecules and NFkB activation in endothelial cells *in vitro* (human umbilical vein endothelial cells (HUVECs)) and *ex vivo* (organ culture of the rat aorta). Moreover, KL reversed the repression of the nitric oxide synthase 3 (NOS3) phosphorylation by TNF and inhibited the TNF-induced monocyte adhesion to HUVECs. These results suggest that KL may have a function in the modulation of endothelial inflammation, especially by TNF-induced NFkB inhibition. Therefore, it is plausible that KL downregulation could further exacerbate the TLE(HS) associated chronic inflammatory condition.

Moreno *et al*. related that the inflammatory cytokines tumor necrosis factor ligand superfamily member 12 (TNFSF12, also known as TWEAK) and TNF downregulate KL in renal tubular cells through an NFkB-dependent mechanism mediated by histone deacetilase 1 (HDAC1) [16]. In this regard, they observed that the HDAC inhibitors trichostatin A (TSA) or valproic acid prevented repression of KL induced by TWEAK or TNF. Among the patients studied, only TLE 09 was taking valproate during the surgery and only TLE 06 took this medication in the past. We did not observe any particular difference in

these patients compared with the others. *KL* expression was negatively correlated with *NFBK1* or *TNF* expression, while the pair *TNF* and *NFKB1* showed a positive correlation. This suggests that NFkB in patients is most likely modulated by TNF or even KL [28] and not by AEDs, although further studies are required to test the influence of AEDs in the gene expression of our targets. Gene expression data correlation was found in the three pairs tested: *KL-NFKB1* (Spearman's R = -0.3140; *P* = 0.0091), *KL-TNF* (Spearman's R = -0.3283; *P* = 0.0063), and *NFKB1-TNF* (Spearman's R = 0.4441; *P* = 0.0001).

In addition, Thurston *et al.* showed that in mouse models of inflammatory bowel disease the degree of KL inhibition was related to the severity of colitis and that attenuation of inflammation with a neutralizing anti-TNF antibody impeded this inhibition [17]. Furthermore, the neurodegenerative feature found in $Kl^{-/-}$ mice suggests that KL in the hippocampus may act as a protective autocrine hormone and its absence causes neuronal loss [30].

Our work on the mRNA level suggests that the triad *KL-NFKB1-TNF* is disrupted in the hippocampus from medically intractable TLE patients. Based on the KL function studies discussed here, we propose the first mechanistic insights into the role that this triad may play in the pathogenesis of medial TLE. It is conceivable that there is a major involvement of the KL-TNF axis in the pathogenesis of TLE(HS), particularly under chronic inflammatory conditions. Further research on the protein level will strengthen our results and the design of functional studies will be able to elucidate the role of our targets, especially KL, in the normal and pathological hippocampus. Due to the KL hormonal property [7,31] and TNF tissue diffusion as a cytokine [32,33], we believe that

the epilepsy-associated inflammation is a widespread event in the hippocampus. Finally, since KL is detectable in cerebrospinal fluid [7,31,34], it is a potential candidate as an inflammatory biomarker in epilepsy. The inflammatory component of epilepsy is not a secondary phenomenon or complication of the pathology. It is more likely involved in the mechanisms that sustain neuronal hyperexcitability, the onset and recurrence of seizures, and progression and severity of the disease [15]. The determination of a reliable biomarker of brain inflammation is urgent, in view of the fact that a number of patients would benefit from an anti-inflammatory therapy.

Abbreviations

AED: antiepileptic drug; CBZ: carbamazepine; CDKN1A: cyclin-dependent kinase inhibitor 1 (p21, Cip1); DEPC: diethylpyrocarbonate; EEG: electroencephalogram; ENO2: enolase 2 (gamma, neuronal); GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; HDAC1: histone deacetilase 1; HGNC: HUGO Gene Nomenclature Committee; HPRT1: hypoxanthine phosphoribosyltransferase 1; HS: hippocampal sclerosis; HUVEC: human umbilical vein endothelial cell; IGF-1: insulin-like growth factor 1 (somatomedin C); IML: Instituto Médico Legal; KL: Klotho; *Kl*$^{-/-}$, Klotho knockout; LTG: lamotrigine; MRI: magnetic resonance imaging; NFkB: nuclear factor kappa B; NFKB1: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NOS3: nitric oxide synthase 3 (endothelial cell); OXC: oxcarbazepine; PCR: polymerase chain reaction; PHT: phenytoin; RA: rheumatoid arthritis; RIN: RNA integrity number; RT-qPCR: reverse transcription quantitative PCR; TBP: TATA box binding protein; TLE:

temporal lobe epilepsy; TLE(HS): temporal lobe epilepsy associated with hippocampal sclerosis; TNF: tumor necrosis factor; TNFSF12: tumor necrosis factor ligand superfamily member 12 (TWEAK); TP53: tumor protein p53; TSA: trichostatin A; VPA: valproate.

Competing interest

All authors declare that they have no competing interest.

Authors' contributions

MAT designed the study, collected clinical data, performed the experiments, analyzed the data, prepared the figures and tables, and drafted the manuscript. AEDF collected clinical data and helped to perform the experiments. EPLO and HT are neurosurgeons and operated on patients. LD-L designed and coordinated the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Table 2Genes and gene expression assays analyzed in this study

Gene symbol	Name	Task	TaqMan Assay number	Amplicon (bp)	Slope	R^2	Efficiency
ENO2	Enolase 2 (gamma, neuronal)	Reference gene	Hs00157360_m1	77	-3.356	0.994	98.61
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Reference gene	4333764 F	122	-3.313	0.996	100.36
GFAP	Glial fibrillary acidic protein	Upregulation marker	Hs00909236_m1	59	-3.414	0.999	96.29
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Reference gene	4333768 F	100	-3.306	0.988	100.67
KL	Klotho	Target	Hs00183100_m1	74	-3.396	0.998	97.02
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Target	Hs00765730_m1	66	-3.388	0.997	97.30
TBP	TATA box binding protein	Reference gene	Hs99999910_m1	127	-3.407	0.993	96.57
TNF	Tumor necrosis factor	Target	Hs99999043_m1	85	-3.266	0.991	102.39

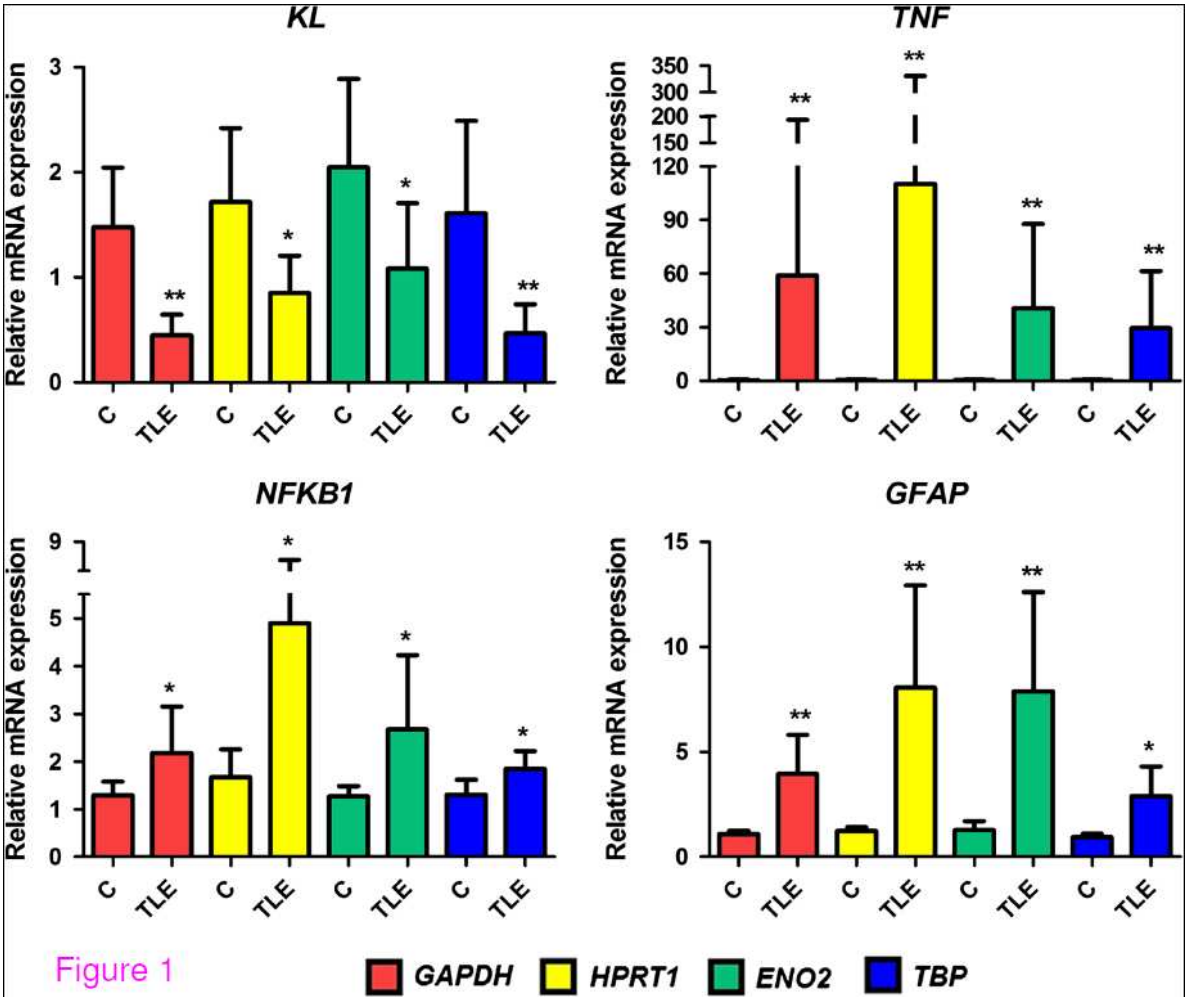
KL
TNF
NFKB1
GFAP
Relative mRNA expression
C
TLE
GAPDH
HPRT1
ENO2
TBP

Figure 1

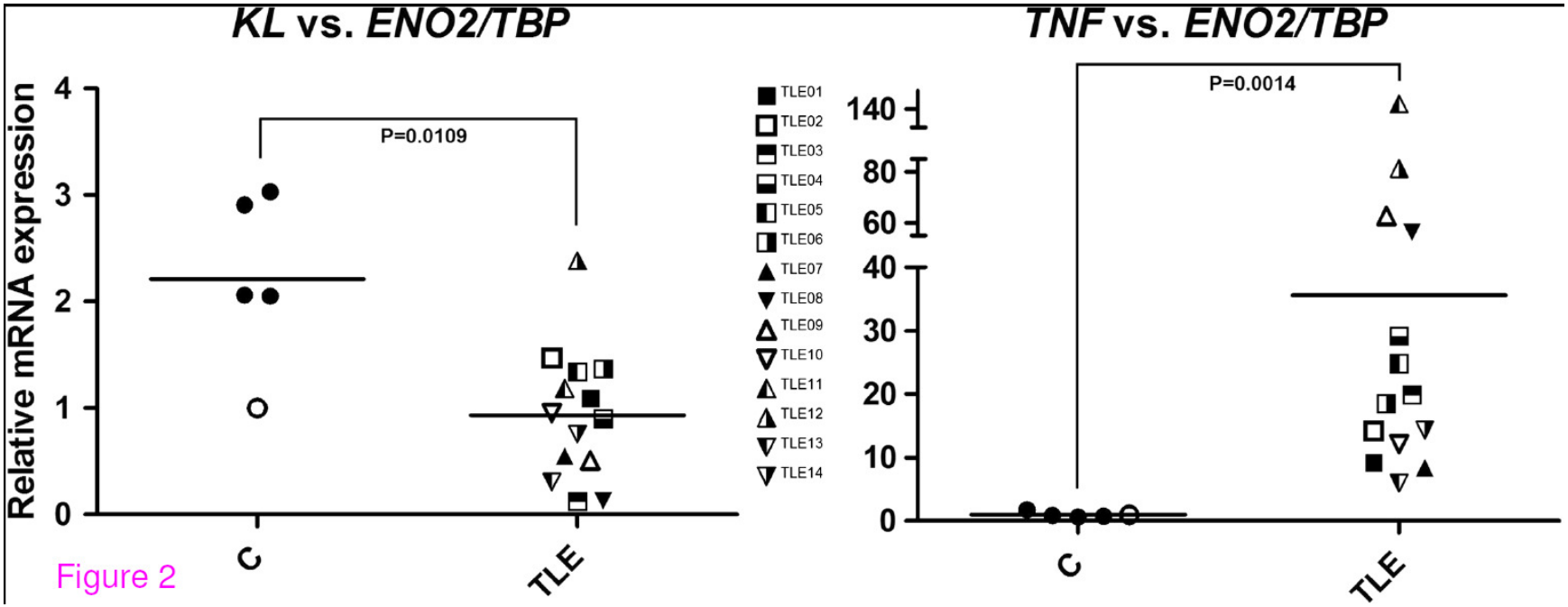
KL vs. ENO2/TBP
Relative mRNA expression
4
3
2
1
0
P=0.0109
C
TLE
TLE01
TLE02
TLE03
TLE04
TLE05
TLE06
TLE07
TLE08
TLE09
TLE10
TLE11
TLE12
TLE13
TLE14
TNF vs. ENO2/TBP
140
80
60
40
30
20
10
0
P=0.0014
C
TLE

Figure 2

4. Capítulo 2

No capítulo 2, que se refere ao artigo “Apoptosis through death receptors in temporal lobe epilepsy-associated hippocampal sclerosis”, o alvo foi a investigação da expressão hipocampal de diversos genes relacionados à sinalização da apoptose via receptores de morte nos pacientes com ELT(EH). Devido à ambiguidade da ação do TNF (apoptose versus sobrevivênvia celular), relatada nos estudos em modelos de crise convulsiva, analisamos mais detalhadamente a expressão de seus dois receptores: TNFRSF1A e TNFRSF1B.

Os periódicos *Molecular Neurodegeneration* ou *Brain* são cogitados para a submissão desse artigo.

Capítulo 2

“Apoptosis through death receptors in temporal lobe epilepsy-associated hippocampal sclerosis”

Apoptosis through death receptors in temporal lobe epilepsy-associated hippocampal sclerosis

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Abstract

Several seizure models have demonstrated that neuroinflammation and neurodegeneration are a preponderant characteristic in epilepsy. Among a number of activated cytokines, TNF emerges as a prominent effector/mediator of both events. Through its two receptors, TNF can play a dichotomous role in animal seizures: programmed cell death activation (via TNFRSF1A) or cell survival actuation (via TNFRSF1B), through the nuclear factor kappa B (NFkB) activation. Considering the lack of clinical studies, our aim is to investigate the TNF pathway in temporal lobe epilepsy associated with hippocampal sclerosis [TLE(HS)] patients. In an array expression assay, 15 upregulated genes were identified. They are involved with the TNF superfamily and are associated with the death receptor signaling and the extrinsic pathway of apoptosis. We evaluated *TNFRSF1A* and *TNFRSF1B* relative mRNA expression levels by reverse transcription quantitative PCR in resected hippocampal tissue samples from 14 TLE(HS) and compared them to four *post mortem* controls. Two normalizer factors (reference genes) were used: *HPRT1* and the geometric mean of *ENO2* and *TBP*. Moreover, double immunostaining revealed that both receptors were activated in tissue's patients. Our results showed that *TNFRSF1A* and *TNFRSF1B* were upregulated in patients ($P<0.01$ and $P<0.04$, respectively). Our data clearly suggest that the overactivation of the TNF pathway is associated with the inflammatory and neurodegenerative components of TLE(HS). Furthermore, since *TNFRSF1A* is a key factor in the death receptor signaling canonical pathway, we infer that this via plays a crucial role in TLE hippocampal neurodegeneration. There is still some controversy on TNFRSF1B role. Its augmentation could be related to a survival mechanism

because the inferred NFkB system activation; however, there is evidence that TNFRSF1B may reinforce TNFRSF1A actuation. Our evidence points the TNF pathway as an important target for pharmacological studies regarding the benefits of an anti-inflammatory therapy in these patients.

Keywords

Cell death; neurodegeneration; inflammation; astrogliosis; hippocampal sclerosis; seizures; TNF; TNFRSF1A; TNFRSF1B.

Background

In temporal lobe epilepsy associated with hippocampal sclerosis [TLE(HS)], only 10% of patients experience seizure control through the use of antiepileptic drugs (AEDs) [1]. However, the vast majority of such patients remain seizure free after undergoing a therapeutic amygdalohippocampectomy. As the main pathohistological abnormality found in resected tissue from temporal lobe epilepsy (TLE) patients, hippocampal sclerosis (HS) is characterized by astrogliosis: an irregular proliferation of astrocytes due to the destruction of nearby neurons [2].

Understanding the deleterious consequences of seizure precipitation and recurrence is fundamental to identify new therapeutic targets for the treatment and cure of pharmacoresistant epilepsy, particularly in TLE(HS) patients. The last decade showed us several studies which asserted that inflammation has a crucial role in epileptogenesis [3]. The connection between neurodegeneration and inflammation in the epileptic brain has emerged as an

important axis for the comprehension of the patho-mecanisms involved in seizure associated neuronal cell death.

Apoptosis is carried out in a regulated energy-dependent process in which an initiating stimulus triggers a cascade of events mediated by a group of cysteine proteases called caspases (CASPs). Broadly, they are classified into initiators (CASP2, CASP8, CASP9 and CASP10) and executioners (CASP3, CASP6 and CASP7) [4]. Apoptosis is divided into two main specific pathways: intrinsic and extrinsic. In spite of requesting particular triggers to start a cascade of molecular events, both pathways converge in the activation of CASP3. Then, the execution pathway is activated, resulting in distinctive cytomorphological characteristics comprising membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation [4].

Differently of the intrinsic signaling pathways that initiate apoptosis, the extrinsic signaling pathways involve transmembrane receptor-mediated interactions. These include death receptors (DR) which are members of the tumor necrosis factor (TNF) receptor gene superfamily [4]. The execution pathway can be initiated by these receptors through CASP8 or CASP10. CASP8 or CASP10 activation could trigger the pro apoptotic proteins BID (BH3 interacting domain death agonist) or BAX (BCL2-associated X protein), via TP53 (tumor protein p53), culminating in a cross talk with the mitochondrial or intrinsic pathway of apoptosis [4].

In fact, both pathways are present in TLE-associated HS. The apoptotic intrinsic signaling pathways are initiated by non-receptor-mediated intracellular signals (e.g. DNA damage, radiation) that result in changes in the

inner mitochondrial membrane. Seizures by themselves can represent an interesting example of these kinds of signals. One of the primary events in hippocampal seizure induced apoptosis is the excessive release of glutamate with consequent intracellular calcium overload, culminating in downstream swelling and rupture of intracellular organelles and activated proteolytic enzymes leading to cell death [5].

Recurrent seizures not only induce neuronal cell loss but also inflammation (reviewed in [6]). However, neuronal cell loss is not a prerequisite for inflammation to happen; rather, the liberation of proinflammatory cytokines can contribute to cell death [7], and dying cells may perpetuate inflammation [6].

Therefore, we infer that the neuronal apoptosis through death receptors pathway highlights the key molecular events involved in triggering astrogliosis and TLE-associated HS. Apoptotic or survival signals are results of the DR family activation by death ligands. Various members of this family have been described thus far, including TNFRSF1A (tumor necrosis factor receptor superfamily, member 1A), FAS [Fas (TNF receptor superfamily, member 6)], TNFRSF25 (tumor necrosis factor receptor superfamily, member 25), TNFRSF10A (tumor necrosis factor receptor superfamily, member 10a), TNFRSF10B (tumor necrosis factor receptor superfamily, member 10b) and TNFRSF21 (tumor necrosis factor receptor superfamily, member 21) [8].

In this context, understanding which are the soluble mediators and the molecular mechanisms crucially involved in the link between inflammation and neuronal cell death is instrumental to shedding light on how seizures may contribute to HS in epilepsy.

Methods

1. Subjects and tissue collection

Subject's data was reported in a recent previous study [Teocchi et al., 2013; submitted, in review]. The present study was performed with the same patient and *post mortem* control samples. Briefly, TLE and HS were detected by telemetry/video EGG and magnetic resonance image, respectively, in 14 patients. Due to the pharmacoresistance of the syndrome, they went through an amygdalohippocampectomy for treatment. All hippocampal tissue samples were immediately collected and divided into two parts. One portion was fixed for histopathological analysis and HS/astrogliosis was confirmed in all of them. The second portion was immediately snap-frozen in liquid nitrogen after surgery and stored at -80 ºC until RNA isolation.

Four *post mortem* control hippocampal tissue samples (1 female, 3 males; 22.75 ± 5.56 years ranged from 19 to 31 years old) were kindly provided by the "Instituto Médico Legal (IML) de Campinas". *Post mortem* control subjects passed away instantaneously or quickly although their deaths have been traumatic, which runs against the occurrence and progression of neuroinflammation. Neurological findings were not detected and the *post mortem* delay averaged 7.8 h (range: 6.0–9.0 h).

2. Gene Expression

All reagents were purchased from Life Technologies; Foster City, CA 94404 USA.

2.1. RNA extraction, Gene array expression and Reverse Transcription quantitative PCR (RT-qPCR)

Total RNA extraction and Reverse Transcription quantitative PCR (RT-qPCR) were done according to our previous work [Teocchi et al., 2013; submitted, in review]. TRIzol® Reagent was used for RNA extraction according to the manufacturer's instructions. The RNA integrity number (RIN) mean in control and patient groups were 7.525 ± 0.5437 and 6.155 ± 0.2484, respectively. Afterwards, 1 µg of total RNA of each sample was reverse transcribed into cDNA using 200U of Superscript® III Reverse Transcriptase and 3 µg of Random Primers. Sterilized and filtered DEPC treated water was used in all RNA procedures.

To identify potential targets associated with the apoptosis through death receptor signaling, we used the TaqMan® Array Human Apoptosis Through Death Receptors 96-well Plate (PN: 4414105). The plate contained 44 assays to genes associated with death receptor-mediated apoptosis and four assays to reference gene (endogenous control) candidates (Table 1). Gene names are in accordance with the approved symbol from the HUGO Gene Nomenclature Committee (HGNC) database. All assays were plated in duplicates: one for cDNA pooled from the TLE(HS) patients (n=12) vs. the other for cDNA pooled from the post mortem controls (n=4). Among several factors which can influence C_T (cycle threshold) and consequently qPCR results credibility, we emphasize instrument calibration and cDNA quality. The template concentration (e.g., concentration of total RNA converted to cDNA) should always be homogeneous among samples, particularly when working with pools. cDNA concentration and purity were assessed by spectrophotometry

(NanoDrop ND1000; NanoDrop Technologies, Wilmington, DE). The cDNA concentration means were 858.1 ± 30.07 and 871.9 ± 27.10 for control and patient samples, respectively. cDNA Absorbance (A260/A280) ranged from 1.84 to 1.90 considering all 16 samples. Reactions were carried out according to manufacturer's instructions. We used a total volume of 20 µL, comprising 10 µL of TaqMan® Gene Expression Master Mix (Life Technologies) and 10 µL of pooled cDNA diluted in DNase-free water. The final concentration of the pooled cDNA samples used was 10 ng in 10 µL for each 20-µL PCR reaction.

Two target genes we chosen to be tested separately by subject: TNFRSF1A and TNFRSF1B (Assay ID: Hs00153550_m1). This last was not included in the array plate. The PCR efficiency validation also was done according to our previous work [Teocchi et al., 2013; submitted, in review]. The amplification efficiencies were close to 1.0 (100%). cDNAs samples derived from the investigated genes were detected using an ABI PRISM® 7500 Sequence Detection system and TaqMan® Gene Expression Assays. For RT-qPCRs, reference genes were selected according to Wierschke et al [9]. Therefore, *HPRT1* and the geometric mean of *ENO2* and *TBP* were used as our normalization factors. Both were indicated as being the most stable reference genes in epileptogenic tissue [9]. Each qPCR was run as triplicates with 10 ng cDNA sample in 6.25 µL TaqMan® Gene Expression Master Mix, 0.625 µL of the respective probe/primer mix and 0.625 µL purified and deionized H_2O. All reactions were run as triplicates, and measurements with a difference of more than 0.3 C_T-values were excluded from analysis.

3. Immunohistochemistry

We adapted the Immunofluorescence General Protocol from Cell Signaling Technology (http://www.cellsignal.com/support/protocols/IF.html).

3.1. Tissue preparation

For the Deparaffinization/Rehydration, sections were incubated in three washes of xylene for 5 min each; in two washes of 100% ethanol for 10 min each; in two washes of 95% ethanol for 10 min each; and finally sections were rinsed twice in dH_2O for 5 min each.

For the Antigen Unmasking we tested two options: (1) Citrate: slides were brought to a boil in 10 mM sodium citrate buffer pH 6.0, then maintained at a sub-boiling temperature for 10 min. Slides were cooled on bench top for 30 min; (2) EDTA: slides were brought to a boil in 1 mM EDTA pH 8.0 followed by 15 min at a sub-boiling temperature. No cooling was necessary.

Fluorescent labeling was best visualized on confocal microscopy in the first option (citrate).

3.2. Double Immunostaining

The specimen was blocked in Blocking Buffer for 60 min. While blocking, the primary antibody was prepared by diluting as indicated on datasheet in Antibody Dilution Buffer (Human TNF RI/TNFRSF1A Antibody – Antigen Affinity-purified polyclonal Goat IgG, Catalog Number: AF225 and Human TNF RII/TNFRSF1B Antibody – monoclonal mouse IgG2A clone # 22221, Catalog Number: MAB226, R&D Systems, Inc., Minneapolis, MN 55413 USA). The blocking solution was aspirated and the diluted primary antibody applied and incubated overnight at 4 ℃.

Subsequently, the specimen was rinsed three times in PBS for 5 min each. It was incubated in fluorochrome-conjugated secondary antibody (Alexa Fluor® 546 goat anti-mouse IgG2a A-21133 and Alexa Fluor® 488 rabbit anti-goat IgG (H+L) A-11078, Life Technologies) diluted in Antibody Dilution Buffer for 1–2 hours at room temperature in dark. Later, it was rinsed in PBS as previously. Cell nuclei were counterstained with Hoechst 33258 stain (blue) according to manufacturer's instructions (Life Technologies). Again, slides were rinsed in PBS as previously. Finally, they were coversliped with Prolong® Gold Antifade Reagent (Life Technologies).

3.3. Solutions and Reagents

Solutions were prepared with Milli-Q or equivalently purified water.

Blocking Buffer: (1X PBS / 5% normal goat serum (#5425) / 0.3% Triton X-100): To prepare 25 mL, we added 2.5 mL 10X PBS, 1.25 mL normal serum from the same species as the secondary antibody (e.g., normal goat serum, normal mouse serum) and 21.25 mL dH_2O and mixed well. While stirring, we added 75 µl Triton X-100.

Antibody Dilution Buffer (1X PBS / 1% BSA / 0.3% Triton X-100): To prepare 40 mL, we added 4 mL 10X PBS and 120 µL Triton X-100 to 0.4 g BSA. We used dH_2O to bring to final. Solution was well mixed.

Antigen Unmasking: For Citrate: 10 mM Sodium Citrate Buffer: To prepare 1L we added 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) to 1L dH2O and pH was adjust to 6.0. For EDTA: 1 mM EDTA: To prepare 1L we added 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) to 1L dH_2O and pH was adjusted to 8.0.

4. Data analysis

Relative gene expression data was generated and analyzed by the 7500 Software version 2.0.5 (Life Technologies). The software GraphPad Prism 5 was used for the statistical analysis (GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The Mann-Whitney U-test was used for comparison between data from the control group (n=4) versus the patient group (n=14). For all analyzes, differences of $P<0.05$ were considered significant.

A molecular pathway related to differentially expressed genes was created by the IPA - Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com) application via the "Core Analysis" function. The array data sheet generated by the 7500 Software was uploaded into the IPA application. The differentially expressed genes were connected based on reported association among genes or proteins and its functional roles.

Results

Array results are summarized in Table 2. Fifteen (34.01%) of the studied genes were upregulated considering a cutoff of 2.0 (fold change). However, this number goes up to 31 (70.45%) if we consider a cutoff of 1.5. Choosing a precise cutoff is always a challenge in array studies and it is decisive to analyze results and select the most important targets. We preferred to adopt a more conservative cutoff: 2.0; however, considering our previous study on *NFKB1* expression in the same group of TLE(HS) patients (n=14) [Teocchi et al., 2013; submitted, in review], a 1.5 cutoff could be statistically

acceptable since the *NFKB1* expression in the array was exactly 1.5-fold and it is in accordance with that work. Curiously, only two genes had a downregulation expression: *MAPK9* and *NGF* (Table 2).

Undoubtedly, regardless the cutoff or even the reference genes, array *TNF* expression was very meaningful, particularly in comparison to the expression of the other genes. As *NFKB1*, array *TNF* expression was very similar to what we found recently [Teocchi et al., 2013; submitted, in review]. *TNFRSF1A* and *TNFRSF1B* were also upregulated in patients ($P<0.01$ and $P<0.04$, respectively) (Figure 1). Additionally, our results on hippocampal immunohistochemistry showed an evident TNFRSF1A and TNFRSF1B activation in patient's tissues (Figure 2).

Discussion

Inflammation, degeneration of neurons and HS undoubtedly are closely related [10]. Recently, we reported a marked *TNF* upregulation in TLE(HS) patients [Teocchi et al., 2013; submitted, in review], which is a indicative of chronic hippocampal inflammation. The importance of this cytokine as one of the main apoptosis "propellers" in TLE-associated HS is reinforced by our array results (Table 2). We found several differentially expressed genes associated with the extrinsic signaling pathways that initiate apoptosis, among them, members of the TNF receptor gene superfamily and other factors which play a role in cell death. This finding clearly suggests their involvement with neurodegeneration and the consequent astrogliosis in TLE(HS).

Moreover, a number of studies in animal models indicate that seizures induce the TNF expression in the brain [3, 11-14]. In hippocampus,

TNF is able to trigger its two receptors (TNFRSF1A and TNFRSF1B) to regulate cell-signaling pathways [15, 16]. TNFRSF1A is ubiquitously expressed in human tissues and is the principal signaling receptor for TNF. A cytoplasmic death domain, required for apoptotic signaling pathways and nuclear factor kappa B (NFkB) activation, is present in this major receptor. TNFRSF1B does not contain a death domain and actuates in restricted biological processes, being mainly expressed in immune cells. Through intracellular adaptors called TNF Receptor-Associated Factors (TRAFs) associated with this receptor, JNK (c-Jun N-terminal kinase) is activated during the cell survival induction. NFkB-inducing kinase (NIK) works as the downstream target of TRAF2 (TNF receptor-associated factor 2) in mediating TNF-induced NFkB activation resulting in survival. Thus, TNFRSF1A has been involved in apoptosis activation, whereas TNFRSF1B is implicated with activation of the NFkB system [17-19]. Seizure model studies imply that the TNFRSF1A pathway is associated with the deleterious effects and that the TNFRSF1B pathway is related with anticonvulsive outcomes [11].

In a recent review [11], the authors reported the doubtful TNF role in epilepsy. Rodent hippocampal *Tnf* mRNA levels exhibited an important overregulation after seizures evoked by amygdala kindling [12, 13], which returned to baseline within 3 weeks [12]. In another study [14], a similar augmentation was related to limbic seizures induced by intrahippocampal injection of kainic acid or bicuculline methiodide or by electrical stimulation of the hippocampus causing status epilepticus. The results we previously found for TLE(HS) patients only differs from those on seizure model by the fact that the *TNF* mRNA increase does not seem to be transiently enhanced. Some patients

had the last seizure several days before the surgery, implying that the high *TNF* expression levels were happening often, signalizing that chronic hippocampal inflammation could be intrinsic to refractory TLE(HS) [Teocchi et al., 2013; submitted, in review]. On the other hand, it was reported by Balosso et al. that intrahippocampal injection of murine-recombinant TNF in mice potently prevented seizures [20]. Moreover, transgenic mice overexpressing TNF by astrocytes showed shorter seizures whereas deficient mice for TNF receptors presented prolonged seizures [20]. The dichotomic effect of TNF on seizures may be related with its concentration and actuation through its two receptors [11, 20].

There are a few clinical studies on the TNF system and its effects in epilepsy, in spite of the intense investigation in animals. Some studies have reported no important TNF alterations in plasma or CSF after different kinds of seizures [21-24]. Sinha et al. [25] detected increased serum levels of TNF and other cytokines in patients with several epilepsy syndromes. The serum samples studied were prospectively collected in the immediate post-ictal phase and serial analysis during the seizure-free period revealed a reduction in TNF levels (25% to 12.5%).

Our RT-qPCR and immunohistochemistry results showed the expression of both TNF receptors in TLE(HS) patients. Both *TNFRSF1A* and *TNFRSF1B* were upregulated (Figure 1), corroborating with the TNF receptor immunolabeling performed in patient's tissues (Figure 2). The deleterious consequences of TNFRSF1A activation are well-known; however, TNFRSF1B expression is still unclear.

TNFRSF1B could be trigged as a survival mechanism to compensate the extensive neuronal cell death found in epilepsy-associated HS or its expression could potentize the harmful consequences of TNFRSF1A activation [26]. To understand the role of the TNFRSF1B in non-lymphoid cells, Tartaglia et al. investigated the cytotoxic signaling and ligand binding activities of TNFRSF1A and TNFRSF1B. They used specific agonist and antagonist antibodies for the two receptor types and demonstrated that TNFRSF1B significantly decreased the TNF concentration required for cell death without the generation of an intracellular signal. Instead, TNFRSF1B modulated the rate of TNF association with TNFRSF1A, possibly by increasing the local concentration of TNF at the cell surface through rapid ligand association and dissociation. They proposed that other cell-surface receptors, such as NGFR, may utilize an analogous "ligand passing" mechanism. Our array results showed the upregulation of *NGFR* (Table 2). Increased expression of NGFR in hippocampal neurons of TLE patients has already been described [27]. Neuronal NGFR immunoreactivity was detected in 10 of 14 cases of HS in spared neurons within the CA and hilar regions of the hippocampus, also involving glial cells, which may critically influence the neuronal survival during the epileptogenic process [27].

Several seizure model or human epileptogenic tissue studies have reported a deregulated expression of caspases [28-32]. It is well-known that sequential activation of caspases plays a central role in the execution phase of cell apoptosis. Our results emphasize the importance of CASP7 and CASP8, of which expressions were higher than 2.0 (fold change). CASP7 is an effector, responsible for cleaving important apoptotic intracellular substrates. Yakamoto

et al. found in resected tissue from TLE patients an involvement of CASP7 in endoplasmic reticulum (ER) stress [28]. Their detection of X-linked inhibitor of apoptosis (XIAP) binding CASP7 in TLE brain suggested ongoing antiapoptotic responses, which might be impeding caspases from inducing apoptosis. XIAP only can binds activated CASP7 to modulate its activity [33], and XIAP expression is regulated after experimental seizures [34].Their finding extends XIAP's implication in repressing neuronal apoptosis in seizure models [34] and reinforce the evidence for active CASP7 in TLE hippocampus. Our data on *CASP7* overexpression in HS strengthens these implications.

CASP8 is a key initiator of apoptosis via death receptor-mediated pathways, capable of inducing apoptosis by directly processing executioner caspases [31]. In a seizure model, its inhibition significantly reduced neuronal apoptosis, accompanied by decrease of truncated BID (tBID), cleaved CASP9 and cytosol cytochrome c [32]. It was shown that seizures trigger CASP8 activity in animals that received a CASP2 inhibitor [30]. However, the authors found that CASP8 activation was not a compensatory response to CASP2 inhibition because CASP8 activity was also present in vehicle-injected animals that underwent seizures and CASP8 cleavage was verified by western blotting in the absence of CASP2 manipulation [30]. This data is in agreement with our result on the upregulation of *CASP8*.

In animals, seizures also induced a significant augmentation in FAS expression within ipsilateral hippocampus from 4 to 24 h after which levels returned to baseline [31]. This same study reported an increase of FADD, while TRADD, TNFRSF25 and TNFRSF10A expressions were unaffected by seizures [31]. In contrast, it was showed by immunoblot that FADD levels were not

significantly different between control and TLE brain samples in whole cell lysates [35], which corroborates with our result on *FADD* expression (1.14-fold). FADD is the putative adaptor for FAS/CASP8 death signaling and the death receptors TNFRSF25 and TNFRSF10A are able to trigger CASP8 [36]. In our study, both - *TNFRSF25* and *TNFRSF10A* - were overregulated: 1.72 and 2.39 fold change, respectively. We did not analyze TRADD expression.

Our finding also showed an upregulation of *FAS* (1.65 fold change), but after the marked *TNF* upregulation (39.96-fold), *FASLG* got the second position: 5.46-fold. FASLG triggers apoptosis by binding to FAS [37]. Increased expression of both *Fas* mRNA and protein were evident in the adult rat brain from 4 h to 5 days after the onset of kainic acid-induced seizures [38]. Neurons with increased FAS-expression were also immunoreactive for TP53 [38]. Induced by kainite, FASLG expression increased rapidly at 6 h and returned to the basal level at 3 days in CA1 and CA3/dentate gyrus hippocampal regions [37, 39]. In addition, we found that *TNFRSF6B* was also overregulated in TLE(HS) patients (2.54 fold change). The encoded protein by *TNFRSF6B* is assumed to play a regulatory role in suppressing FASLG-mediated cell death. It acts as a decoy receptor that competes with death receptors for ligand binding [40]. We have not found any report regarding the association between TNFRSF6B expression and epilepsy.

Besides apoptosis mediated by TNF and FASLG and its receptors, TNFSF10 [also known as TRAIL (TNF-related apoptosis inducing ligand)] is another potent inducer of apoptosis [41]. Both apoptosis-inducing and nonapoptosis-inducing membrane-bound receptors have been described for TNFSF10. Only TNFRSF10A and TNFRSF10B are able to generate a death

signal [42, 43], while TNFRSF10C and TNFRSF10D are truncated and have been proposed to function as decoy receptors by binding TNFSF10 without producing a death signal, thereby inhibiting apoptosis [42, 44]. Our results on gene expression accentuate the importance of TNFSF10 and its receptors, all upregulated (Table 2). In brain tissues from TLE patients, Dörr and colleagues did not detect the expression of TNFSF10, but both apoptosis-inducing and non-apoptosis-inducing receptors were found [45]. The authors suggested that the expression and regulation of these receptors might be crucial for death or survival of an individual cell, since both apoptosis-mediating as well as apoptosis-blocking receptors were present on the different brain parenchymal cells [45]. The apoptosis-mediating TNFSF10 receptors were expressed on neurons, astrocytes, and oligodendrocytes, which indicates their possible susceptibility to TNFSF10-mediated apoptosis [45]. Moreover, considering our results and the fact that apoptosis was triggered by TNFSF10 in brain parenchymal cells including neurons, astrocytes, and oligodendrocytes from acute human brain slices [46], it is conceivable an important actuation of the TNFSF10 system in TLE(HS)-associated neuronal apoptosis.

A number of signal transduction events, initiated by inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis, converge on NFkB activation [reviewed in [47]]. This pleiotropic transcription factor, present in practically all cell types, is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL and NFKB2/p52. The heterodimeric p65-p50 complex appears to be most common. NFKBIA and NFKBIB (I-kappa-B proteins) inhibit the NFkB complex by trapping it in the cytoplasm [47]. The kinases IKBKA and

IKBKB mark I-kappa-B proteins for destruction via the ubiquitination pathway, thereby allowing activation of the NFkB complex. IKBKG is a regulatory subunit of the inhibitor of kappa B kinase (IKK) core complex which phosphorylates NFkB inhibitors, leading to the dissociation of the inhibitor/NFkB complex and ultimately the degradation of the inhibitor [47].

As previously reported, we found that *NFKB1* was upregulated in TLE(HS) patients [Teocchi et al., 2013; submitted, in review]. In the present study, our array results showed that *NFKB1* was 1.5-fold more expressed than controls. Several other genes related to the NFkB complex, such as *IKBKB*, *IKBKG*, *NFKB2*, *NFKBIA* and *RELA* also presented an increased expression (Table 2). Further studies are necessary to understand the actual role of NFkB in TLE(HS). Our finding speculates a disturbed expression of activators and repressors of the NFkB complex.

In 1994, it was related a *Tp53* overexpression associated with excitotoxicity in the adult rat brain within hours after systemic administration of the glutamate analogue kainic acid [48]. Years later and after several other reports on altered TP53 expression in seizure models, including the already mentioned study of Tan and colleagues [38], Xu et al. found TP53 positive cells in the sclerotic hippocampus from TLE patients [49]. In the same year, Engel and colleagues detected significantly higher levels of TP53 by Western blotting in hippocampal tissues from TLE patients [50]. Our result on the elevated *TP53* mRNA level extends and reinforces this data. It is still unclear if TP53 could function as a potential target for protection in seizure-induced neuronal death since TP53 regulates a large number of genes, which would need to be carefully evaluated [49].

Considering a 2.0 cutoff, we detected 15 upregulated apoptosis-related genes. In Figure 3, we propose a schematic pathway which involves all of them. Take into account a 1.5 cutoff, other 16 were revealed. Among them, *FAS*, *IKBKB*, *IKBKG*, *NFKB1*, *TNFRSF10B* and *TNFRSF25* have already been broached in this discussion. *ACTB*, *APAF1*, *BAX*, *BCL2*, *CASP9*, *CFLAR*, *DAXX*, *MAP3K5*, *PARP1* and *TRAF2* are other interesting genes of which deregulated expression seem to be due to the TLE(HS) syndrome. Lots of them have been intensively explored in experimental seizures.

In conclusion, we demonstrated that both TNF receptors are activated in the hippocampus from TLE(HS) patients. The upregulation of both genes, *TNFRSF1A* and *TNFRSF1B*, emphasize the importance of TNF in the TLE(SH) syndrome, probably as a crutial proinflammatory citokyne associated with neuroinflammation and neurodegeneration. The deregulation of several TNF superfamily genes support this proposition, indicating that apoptosis through death receptors is a preponderant component of TLE-associated HS. Based on our results, these differentially expressed molecules should be considered as important targets for treatment in refractory TLE patients.

Abbreviations

AED, antiepileptic drug; BAX, BCL2-associated X protein; BID, BH3 interacting domain death agonist; CASP, caspase; DR, death receptors; ENO2, enolase 2 (gamma, neuronal); ER, endoplasmic reticulum; FAS, Fas (TNF receptor superfamily, member 6); HPRT1, hypoxanthine phosphoribosyltransferase 1; HS, hippocampal sclerosis; JNK, c-Jun N-terminal kinase; NIK, NFkB-inducing kinase; RT-qPCR, Reverse Transcription Quantitative PCR; tBID, truncated

BID; TBP, TATA box binding protein; TLE(HS), temporal lobe epilepsy associated with hippocampal sclerosis; TLE, temporal lobe epilepsy; TNF, tumor necrosis factor; TNFRSF10A, tumor necrosis factor receptor superfamily, member 10a; TNFRSF10B, tumor necrosis factor receptor superfamily, member 10b; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1a; TNFRSF21, tumor necrosis factor receptor superfamily, member 21; TNFRSF25, tumor necrosis factor receptor superfamily, member 25; TP53, tumor protein p53; TRAF2, TNF receptor-associated factor 2; TRAFs, TNF Receptor-Associated Factors; TRAIL, TNF-related apoptosis inducing ligand; and XIAP, X-linked inhibitor of apoptosis.

Competing interests

The authors have no conflict of interest.

Authors' contributions

MAT designed the study, collected patient's clinical data, performed the experiments, analyzed the data, prepared the figures and tables, and drafted the manuscript. LD-L coordinated the study. Both authors read and approved the final manuscript.

Disclosure

The authors have no conflict of interest.

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Table 1. Array reference genes and target genes

Gene Symbol	Gene Name	Assay ID	Gene Symbol	Gene Name	Assay ID
18S	eukaryotic 18S rRNA	Hs99999901_s1	*IKBKB*	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	Hs00233287_m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	*IKBKG*	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	Hs00415849_m1
GUSB	glucuronidase, beta	Hs99999909_m1	*MAP3K5*	mitogen-activated protein kinase kinase kinase 5	Hs00178726_m1
HPRT1	hypoxanthine phosphoribosyltransferase 1	Hs99999908_m1	*MAPK8*	mitogen-activated protein kinase 8	Hs01548508_m1
ACTA1	actin, alpha 1, skeletal muscle	Hs00559403_m1	*MAPK9*	mitogen-activated protein kinase 9	Hs00177102_m1
ACTB	actin, beta	Hs99999903_m1	*NFKB1*	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Hs00765730_m1
APAF1	apoptotic peptidase activating factor 1	Hs00559441_m1	*NFKB2*	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Hs00174517_m1
BAX	BCL2-associated X protein	Hs00180269_m1	*NFKBIA*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Hs00153283_m1
BCL2	B-cell CLL/lymphoma 2	Hs99999018_m1	*NGF*	nerve growth factor (beta polypeptide)	Hs01113193_m1
BID	BH3 interacting domain death agonist	Hs00609632_m1	*NGFR*	nerve growth factor receptor	Hs00609976_m1
BIRC2	baculoviral IAP repeat containing 2	Hs01112284_m1	*PARP1*	poly (ADP-ribose) polymerase 1	Hs00242302_m1
BIRC3	baculoviral IAP repeat containing 3	Hs00154109_m1	*RELA*	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	Hs00153294_m1
CASP2	caspase 2, apoptosis-related cysteine peptidase	Hs00234982_m1	*TNF*	tumor necrosis factor	Hs00174128_m1
CASP3	caspase 3, apoptosis-related cysteine peptidase	Hs00234387_m1	*TNFRSF10A*	tumor necrosis factor receptor superfamily, member 10a	Hs00269492_m1
CASP7	caspase 7, apoptosis-related cysteine peptidase	Hs00169152_m1	*TNFRSF10B*	tumor necrosis factor receptor superfamily, member 10b	Hs00366278_m1
CASP8	caspase 8, apoptosis-related cysteine peptidase	Hs01018151_m1	*TNFRSF10C*	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	Hs00182570_m1
CASP9	caspase 9, apoptosis-related cysteine peptidase	Hs00154260_m1	*TNFRSF10D*	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	Hs00533560_m1
CFLAR	CASP8 and FADD-like apoptosis regulator	Hs01116280_m1	*TNFRSF1A*	tumor necrosis factor receptor superfamily, member 1a	Hs00205419_m1
CHUK	conserved helix-loop-helix ubiquitous kinase	Hs00175141_m1	*TNFRSF21*	tumor necrosis factor receptor superfamily, member 21	Hs00237054_m1
DAXX	death-domain associated protein	Hs00154692_m1	*TNFRSF25*	tumor necrosis factor receptor superfamily, member 25	Hs00187070_m1
DIABLO	diablo, IAP-binding mitochondrial protein	Hs00219876_m1	*TNFRSF6B*	tumor necrosis factor receptor superfamily, member 6b, decoy	Hs00234356_m1
FADD	Fas (TNFRSF6)-associated via death domain	Hs00538709_m1	*TNFSF10*	tumor necrosis factor (ligand) superfamily, member 10	Hs00174664_m1
FAS	Fas (TNF receptor superfamily, member 6)	Hs00531110_m1	*TP53*	tumor protein p53	Hs01034249_m1
FASLG	Fas ligand (TNF superfamily, member 6)	Hs00181225_m1	*TRAF2*	TNF receptor-associated factor 2	Hs00184192_m1

List of all genes investigated in the array. The target genes are involved in the apoptosis through death receptor pathway. The reference genes candidates are 18S, *GAPDH*, *GUSB* and *HPRT1*. Only *HPRT1* was effectively used as the reference gene.

Table 2. Array gene expression

Gene Symbol	Gene Expression	Gene Symbol	Gene Expression
ACTA1	1,12	*MAP3K5*	1,55
ACTB	1,74	*MAPK8*	1,05
APAF1	1,59	*MAPK9*	0,86
BAX	1,50	*NFKB1*	1,50
BCL2	1,87	*NFKB2*	3,21
BID	1,40	*NFKBIA*	2,36
BIRC2	1,31	*NGF*	0,90
BIRC3	1,27	*NGFR*	3,53
CASP2	1,38	*PARP1*	1,51
CASP3	1,44	*RELA*	2,02
CASP7	2,13	*TNF*	39,96
CASP8	2,45	*TNFRSF10A*	2,39
CASP9	1,55	*TNFRSF10B*	1,55
CFLAR	1,59	*TNFRSF10C*	4,82
CHUK	1,22	*TNFRSF10D*	3,74
DAXX	1,53	*TNFRSF1A*	3,07
DIABLO	1,45	*TNFRSF21*	1,09
FADD	1,13	*TNFRSF25*	1,72
FAS	1,65	*TNFRSF6B*	2,54
FASLG	5,46	*TNFSF10*	2,50
IKBKB	1,96	*TP53*	2,77
IKBKG	1,65	*TRAF2*	1,54

Gene expression data corresponds to the cDNA pooled from TLE(HS) patients (n=12) versus the cDNA pooled from *post mortem* controls (n=4), which was used as calibrator, e.g, gene expression equal to 1.0. The values in the table can be converted to fold change values, where the negative inverse (-1/x) is taken for values between 0 and 1 (e.g. 0.5 is converted to -2). Values greater than 1 will not be affected. A -2 value represents that the molecule is 2-fold downregulated. Only *MAPK9* and *NGF* were downregulated. *HPRT1* was used as the reference gene.

Figure 1. Hippocampal gene expression of *TNFRSF1A* and *TNFRSF1B* in TLE(HS) patients versus *post mortem* controls. *HPRT1* and *ENO2/TBP* were used as reference genes. One of the *post mortem* control samples was randomly chosen as the reference, e.g., all quantification data for the other samples, including controls and patients, was generated regarding to this reference sample, of which relative quantification was always 1.0. Samples are separated in two groups: TLE(HS) patients (n=14) and *post mortem* controls (n=4). (A) Columns are means with SD. Mann-Whitney U-tests were used for the comparison between groups. * $P<0.05$; ** $P<0.01$. (B) Marks are different geometric figures, which represent the gene expression mean of the samples. The horizontal line is the SE of the group analyzed. Circles and squares correspond to gene expression regarding *HPRT1* as the reference. The two kinds of triangles correspond to gene expression regarding *ENO2/TBP* as the reference.

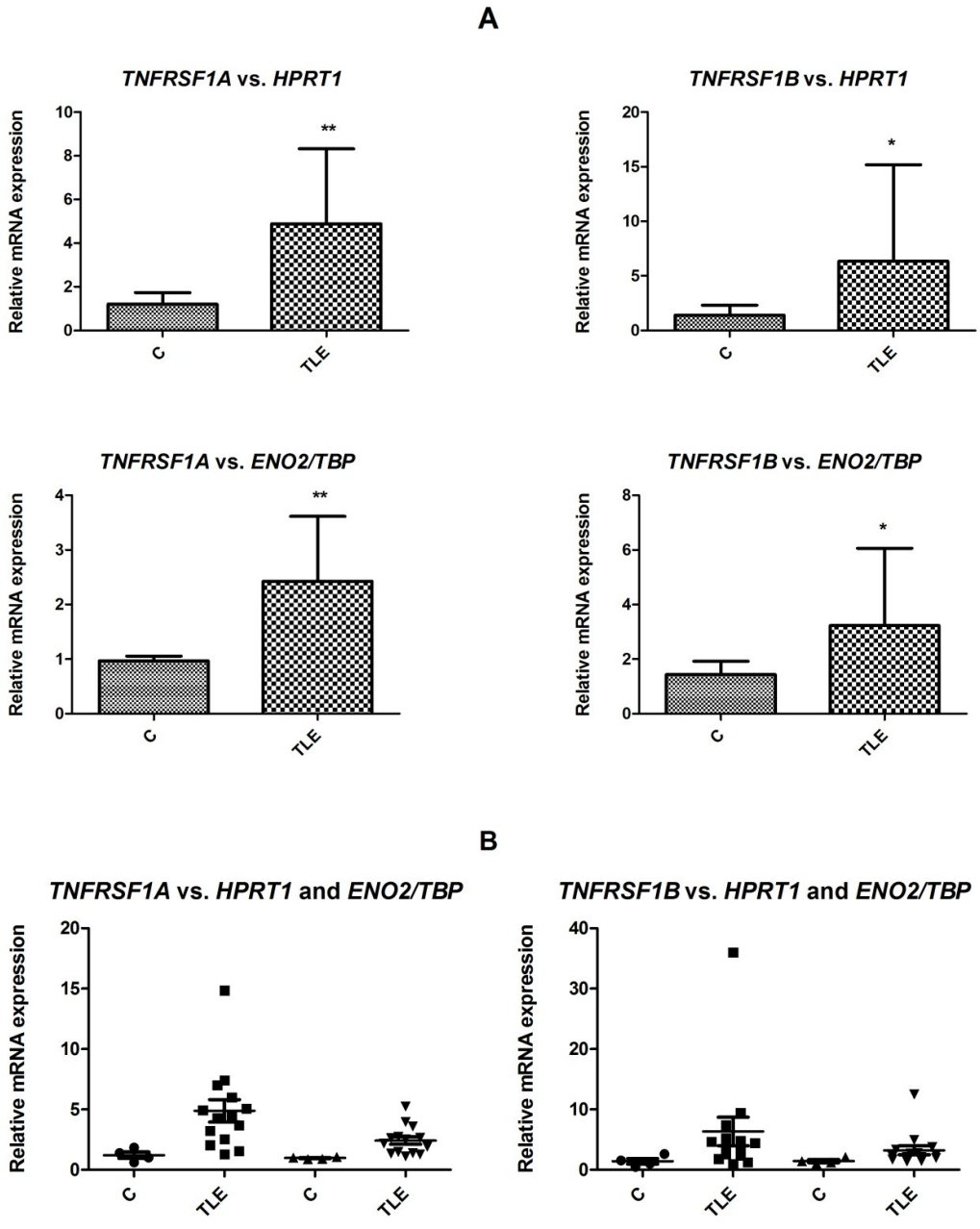

A
TNFRSF1A vs. HPRT1
Relative mRNA expression
10
8
6
4
2
0
**
C
TLE
TNFRSF1B vs. HPRT1
Relative mRNA expression
20
15
10
5
0
*
C
TLE
TNFRSF1A vs. ENO2/TBP
Relative mRNA expression
4
3
2
1
0
**
C
TLE
TNFRSF1B vs. ENO2/TBP
Relative mRNA expression
8
6
4
2
0
*
C
TLE
B
TNFRSF1A vs. HPRT1 and ENO2/TBP
Relative mRNA expression
20
15
10
5
0
C
TLE
C
TLE
TNFRSF1B vs. HPRT1 and ENO2/TBP
Relative mRNA expression
40
30
20
10
0
C
TLE
C
TLE

Figure 2. Hippocampal TNFRSF1A and TNFRSF1B double immunohistochemistry staining. This figure shows the hippocampal (CA4 region and dentate gyrus) activation of the TNF receptors in a *post mortem* control (A) and a TLE(HS) patient (B). The immunoreactivity against TNFRSF1A and TNFRSF1B is presented by cells in green and red, respectively. Nuclei are stained in blue. Note the intense immunostaining observed in the hippocampus of the TLE(HS) patient, when compared with the control tissue. The granular cell layer dispersion in the TLE(HS) patient (B) is evident.

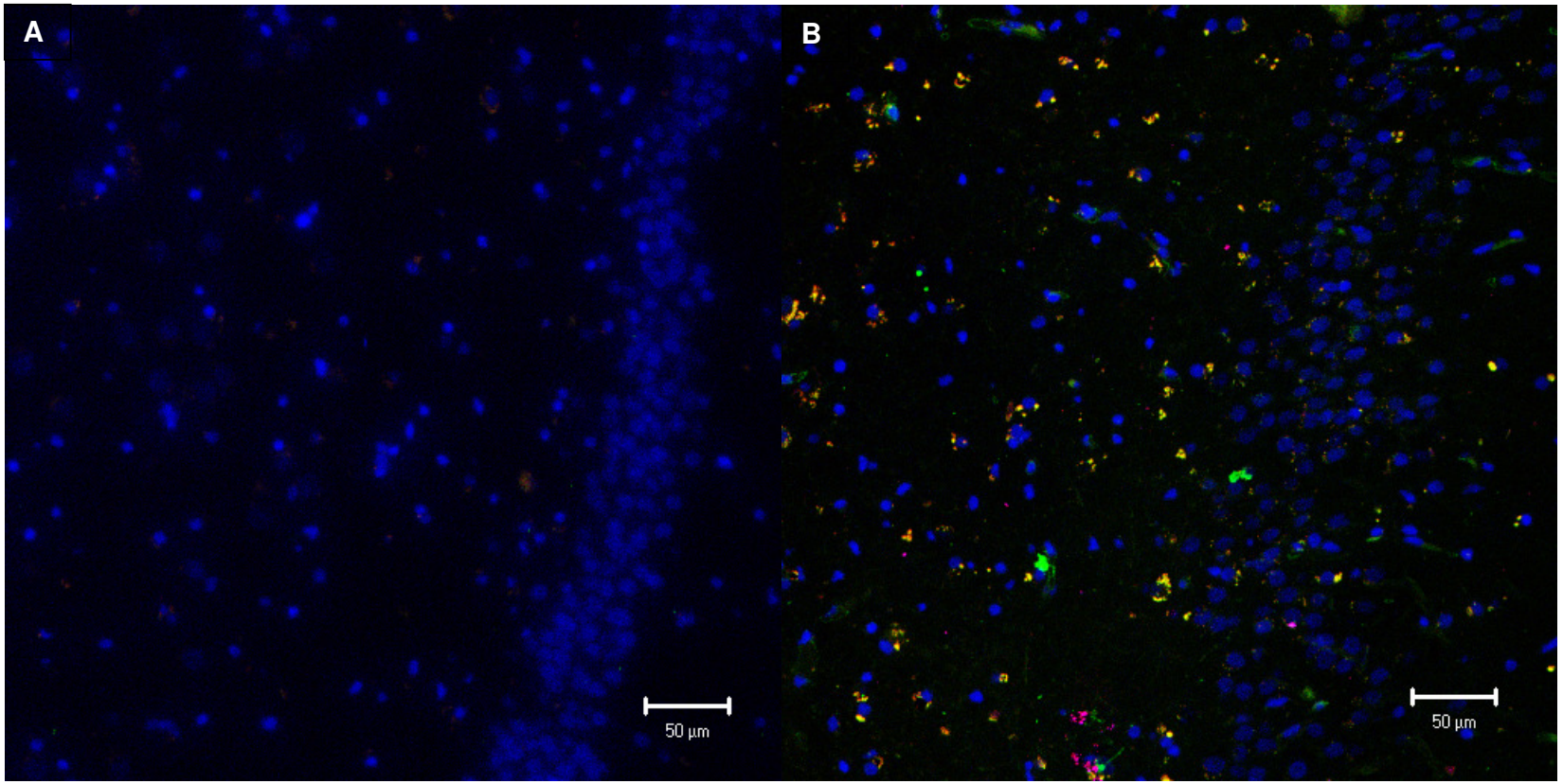
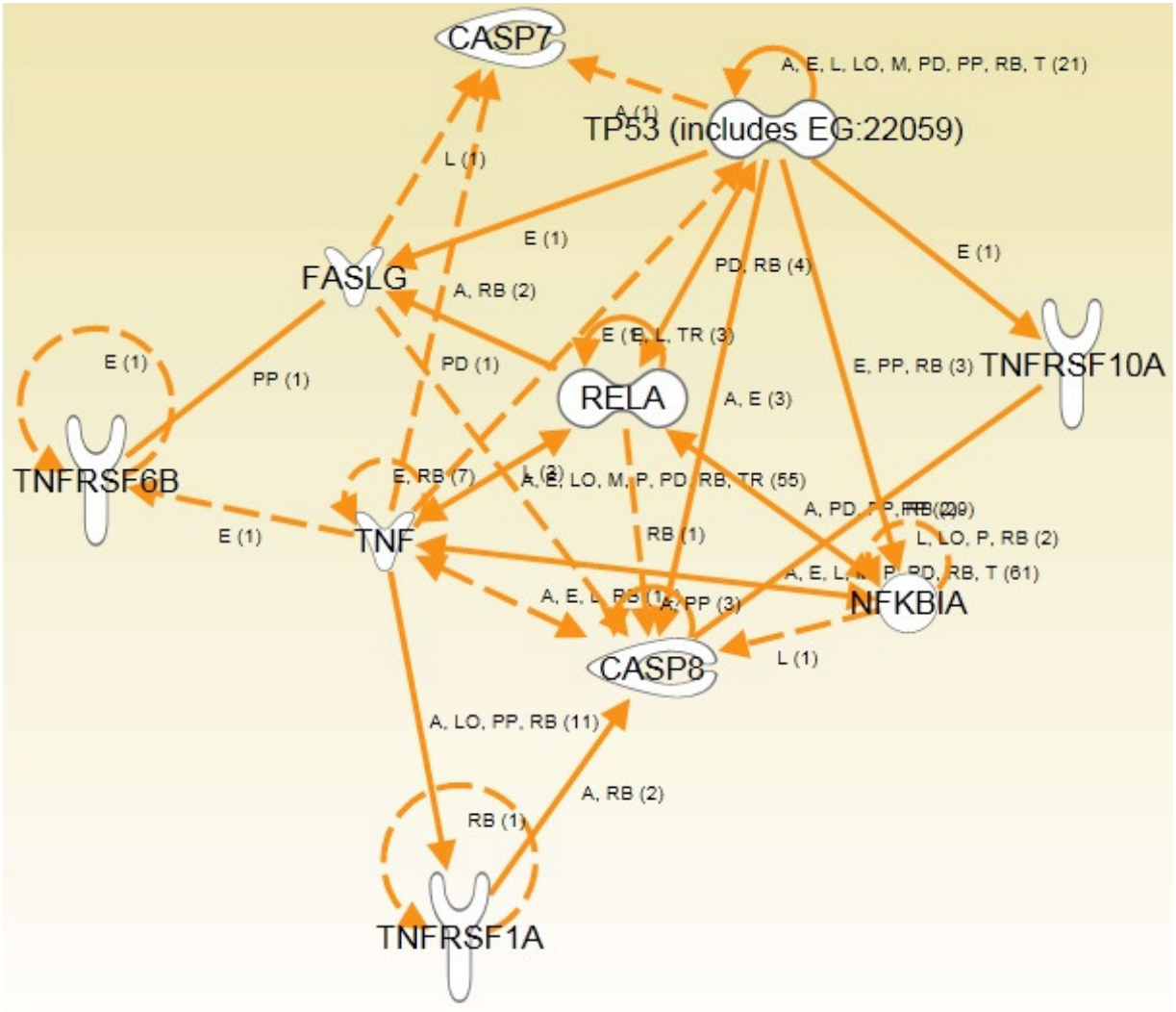


Figure 3. Apoptosis through death receptors-related genes pathway in TLE(HS). This pathway was generated by the IPA software, based on 15 genes of which expression was upregulated (cutoff 2.0-fold change) from TLE(HS) (n=12) and *post mortem* control (n=4) hippocampal tissues. The biological relationship between two genes, represented as nodes, is shown as a line. Nodes with different shapes indicate different functional class. The pathway created did not include two genes: *TNFRSF10C* and *NGFR*. The function "nervous relaxed" was used. Abbreviations: A, activation; E, expression regulation; I, inhibition; L, proteolysis; LO, localization; M, biochemical modification; P, phosphorilation; PD, protein-DNA interaction; PP, protein-protein interaction; RB, regulation of binding; T, transcription; and TR, translocation.



5. Discussão Geral

Os capítulos 1 e 2 apresentaram dados sobre a expressão alterada de genes no hipocampo de pacientes com ELT(EH). Dentre eles, destacamos a hiperexpressão bastante significativa do *TNF*, que codifica uma potente citocina pró-inflamatória; do *NFKB1*, relacionado a um importante componente do complexo NFkB, além de ter relação direta com a via de sinalização do TNF; os dois receptores do TNF: *TNFRSF1A* e *TNFRSF1B*, e finalmente detectamos a hipoexpressão de *KL* no tecido hipocampal desses pacientes.

Inúmeras funções já foram atribuídas ou inferidas à proteína KL, elegendo-a como um importante alvo na compreensão dos mecanismos fisiopatológicos da EH associada à ELT. Contudo, mais estudos são necessários para concluir se essa hipoexpressão é causa ou consequência da neuroinflamação e da neurodegeneração e se está relacionada com a progressão da EH. Em ambas as hipóteses, as implicações serão diversas. Provavelmente *KL* é inibido pelo componente inflamatório da ELT, o que ainda poderia exacerbar a inflamação.

A proteína KL estimular a atividade da bomba de Na^+/K^+ ATPase (76) e influencia a via de sinalização Wnt (59). A atividade da bomba de Na^+/K^+ tem importante impacto no funcionamento elétrico e excitabilidade dos neurônios (77); assim, é relevante determinar se KL também a regula no hipocampo, o que poderia estar envolvido com a redução sináptica que foi averiguada em camundongos *Kl*$^{-/-}$ (39, 40). Quanto à sinalização Wnt, sabe-se que KL inibe diversos dos seus membros (59). A estimulação dos membros da família Wnt

contribui com a senescência celular, sugerindo que uma redução persistente de KL poderia impactar nos mecanismos de reparo teciduais relacionados aos danos recorrentes da progressão da EH. A hiperexpressão de *GFAP*, evidenciada no Capítulo 1, reforça esse dado, além de evidenciar astrogliose.

As características anti-inflamatórias atribuídas a KL compõe uma das mais interessantes facetas dessa proteína na inferência de sua participação na fisiopatologia da ELT(EH). KL é capaz de conferir proteção contra a disfunção endotelial induzida pelo óxido nítrico (42), reduzir a expressão de moléculas de adesão (42), e potencialmente regular funções das células T (43). A conexão entre a atividade de KL e o funcionamento de células $CD4^+$ ainda é desconhecida, mas se sabe que a redução da expressão e atividade de KL nos linfócitos de pacientes com artrite reumatoide ocorreu concomitantemente como a redução de CD28, uma molécula coestimuladora das células T que é dependente do aumento de TNF (43).

Na inflamação vascular, KL foi capaz de suprimir a expressão de moléculas de adesão induzidas pelo TNF e a ativação do NFkB, tanto em cultura celular (HUVECs) quanto *ex vivo*, em cultura orgânica de aorta de rato (42). Além disso, KL foi capaz de reverter a repressão por fosforilação da enzima óxido nítrico sintetase 3 (NOS3) pelo TNF e inibiu a adesão de monócitos em células endoteliais (HUVECs) induzidas pelo TNF. É verossímil que a hipoexpressão de KL possa amplificar a inflamação na ELT(EH).

Em outras doenças com importante componente inflamatório, como nos modelos da doença renal aguda e da colite, foi demonstrado que o TNF é capaz

de reprimir KL, num mecanismo dependente da ativação de NFkB (44, 45). Na colite, três modelos murinos diferentes foram utilizados, evidenciando que a taxa de inibição de KL tinha relação direta com a gravidade da mesma. A atenuação da inflamação com um anticorpo neutralizador para o TNF impediu essa inibição (45).

A evidente hiperexpressão do *TNF* pode não só estar relacionada com a hipoexpressão de *KL*, como também com a morte celular e o importante componente neurodegenerativo da EH. De modo geral, o TNF é pró-apóptótico; enquanto KL, antiapoptótico. Também é plausível inferir que o desequilíbrio do eixo KL-TNF tenha um importante papel na ELT(EH).

Crises convulsões induzem a expressão de TNF em modelos experimentais (32, 60-63), o que corrobora com os altos valores da expressão gênica desta citocina detectados no hipocampo dos pacientes. Contudo, nem todos os pacientes tiveram crises convulsivas nos dias que antecederam à cirurgia (Tabela 1 do Capítulo 1). Nossos dados são indicativos de inflamação crônica no hipocampo desses pacientes. Além disso, encontramos evidencias de que não só o TNF se encontra hiperexpresso, como também diversos outros genes da superfamília do TNF (Tabela 2 do Capítulo 2), sendo muitos deles associados com a via extrínseca da apoptose.

Para tentarmos compreender melhor a controvérsia sobre a atuação do TNF na epilepsia, verificada pela indução de convulsões em animais, e já explicada na introdução, analisamos a expressão gênica dos receptores do TNF, além da detecção deles por imuno-histoquímica. Houve evidente marcação de ambos os receptores nos tecidos dos pacientes, reforçando o fato de também

ambos os genes estarem hiperexpressos. Dessa constatação, formulamos duas hipóteses: a hiperexpressão de *TNFRSF1B* é um mecanismo de sobrevivência celular, uma vez que este receptor não contém o DD; ou, a atividade de TNFRSF1B amplificaria a ação apoptótica de TNFRSF1A (78). A grande maioria dos estudos, oriundos de modelos experimentais, sugere que a via do TNFRSF1A esteja associada à morte neuronal; enquanto que a do TNFRSF1B, à sobrevivência (60).

Os resultados gerados pelo *array* sugeriram que a apoptose via os receptores de morte celular tem um papel crucial nos mecanismos fisiopatológicos da ELT(EH). Considerando um valor de corte correspondente a 2,0, ou seja, expressão gênica equivalente ao dobro ou a metade da do controle, obtivemos 15 genes, todos hiperexpressos. Como abordado no Capítulo 2, muitos deles já foram estudados na convulsão experimental ou até mesmo em tecidos humanos, sendo que a análise da expressão proteica por *Western blot* foi o ensaio mais comum. O presente estudo amplia o conhecimento sobre a expressão desses genes na ELTM, confirmando dados ou revelando novos intrigantes alvos, como no caso dos receptores *decoy*.

A expressão alterada das caspases já foi relatada em diversos trabalhos (79-83). Nossos resultados enfatizam a importância da CASP7 e CASP8 na morte neuronal. Outros trabalhos mostraram como a hiperexpressão de alguns dos nossos alvos pode estar envolvida com a epilepsia, como no caso do NGFR (84), FAS (82), FASLG (85), TP53 (85-87). Em tecidos cerebrais de pacientes com ELT, Dörr e colaboradores não detectaram a presença de TNFSF10, o que difere

do presente estudo, mas encontraram imunorreatividade para os quatro receptores dessa citocina: TNFRSF10A, TNFRSF10B, TNFRSF10C e TNFRSF10D (88). Também evidenciamos alteração na expressão de *NFKBIA* e *RELA*, respectivamente, genes que codificam um inibidor e um componente do complexo NFkB.

Muitos pacientes com ELTM poderiam se beneficiar de uma terapia anti-inflamatória concomitante à anticonvulsiva. Contudo, inicialmente, a inflamação pode ter um papel benéfico, antes de se tornar crônica e causar danos teciduais, como no caso da EH. A identificação de quais pacientes se beneficiariam dessa estratégia anti-inflamatória é uma questão crucial que motiva os mais recentes estudos na compreensão da fisiopatologia da ELTM refratária, determinando novos rumos para o tratamento e cura deste importante distúrbio neurológico.

6. Conclusão Geral

O presente estudo revelou com ineditismo a expressão diferencial de *KL*, *NFKB1*, *TNF*, *TNFRSF1A* e *TNFRSF1B* no hipocampo de pacientes com ELT associada à EH. Dentre estes cinco genes, apenas *KL* mostrou-se hipoexpresso.

Este foi o primeiro estudo a relacionar KL com epilepsia. Com base em algumas das muitas funções atribuídas a KL, propusemos os primeiros *insights* sobre o papel dessa proteína na patogênese da ELT medial.

A significante hiperexpressão hipocampal do *TNF* sugere que esta citocina multifuncional tenha um importante papel na fisiopatologia da ELT medial. Além disso, a mesma indica uma possível inflamação hipocampal crônica inerente a esse grupo de pacientes.

Devido à propriedade hormonal de KL e a difusão tecidual do TNF como citocina, inferimos que a inflamação na ELT seja um evento que abranja o hipocampo como um todo.

Pelo fato do hormônio KL ser detectável no líquido cefalorraquidiano, nós o indicamos como um candidato em potencial a biomarcador inflamatório na epilepsia. A importância dessa indicação consiste no fato de que ainda não é possível discriminar quais pacientes seriam beneficiados com um tratamento anti-inflamatório concomitante ao tratamento anticonvulsivo.

Constatamos por imuno-histoquímica que ambos os receptores do TNF estão evidentemente mais expressos no tecido hipocampal dos pacientes que nos controles. Essa constatação reforça a atuação do TNF nesta síndrome.

Pelo TNFRSF1A, apoiamos a hipótese de que o TNF esteja envolvido na característica perda neuronal da EH. A atuação do TNFRSF1B é incerta. Com base na literatura científica, estabelecemos duas hipóteses: mecanismo de sobrevivência e/ou estimulação da ação do TNFRSF1A.

Através de um arranjo gênico (*array*), revelamos a hiperexpressão de diversos genes da superfamília do TNF e de fatores associados à via extrínseca da apoptose (receptores de morte). Assim, acrescentamos mais evidências que corroboram com a atuação do TNF na morte neuronal.

Sugerimos que a via intrínseca da apoptose atue na EH pelos seguintes sistemas: TNF-TNFRSF1A, FASLG-FAS e TNFSF10-TNFRSF10A/B. Os estudo dessas vias e a criação de moduladores para elas podem ser estratégias de grande valia no tratamento e cura da ELT refratária.

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