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PEDRO AUGUSTO TIBÚRCIO PAULINO

Efeito da inibição do miR 196b na fase aguda da epileptogênese em modelo experimental de epilepsia do lobo temporal.

Maceió
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RESUMO

A epilepsia é um distúrbio neurológico caracterizado por crises espontâneas e recorrentes. A epilepsia do lobo temporal (ELT) está associada a alta taxa (30-40%) de refratariedade ao tratamento com drogas antiepilépticas (DAE's). A busca de novas abordagens terapêuticas é uma necessidade urgente. A manipulação da expressão gênica tem sido explorada no desenvolvimento de intervenções terapêuticas em muitos processos neuropatogenéticos. Nesse sentido, os RNAs não codificadores, a exemplo dos microRNAs (miRs), são alvos preferenciais devido ao papel regulador que exercem na reorganização da expressão gênica. Recentemente, identificamos que o miR 196b-5p apresenta expressão aumentada na fase aguda da epileptogênese. Neste trabalho, realizamos a depleção dos níveis do miR-196b-5p com o objetivo de obter o conhecimento quanto ao significado funcional e ao potencial terapêutico dessa desregulação. Para isto, ratos *wistar* receberam, pela via intracerebroventricular (ICV), o inibidor do miR-196b-5p (0,4 nmol/2mL) 24h antes de serem submetidos ao *Status Epilepticus* induzido por Lítio-Pilocarpina. Observamos uma depleção significativa dos níveis hipocampais do miR-196-5p. O efeito dessa depleção foi avaliado quanto a parâmetros moleculares (expressão de marcadores da epileptogênese e do alvo SLC9A6), histoquímicos (neurônios marcados positivamente com Fluoro-Jade) e comportamentais (crises autossustentadas por 90 minutos). Como grupo controle, foram utilizados ratos *wistar* que receberam, pela via ICV 2µL de tampão salina fosfato (PBS). Os animais que receberam o inibidor do miR-196b-5p (0,4 nmol/2µL) não apresentaram alteração significativa no tempo de latência para início do SE, bem como na gravidade e frequência das crises durante 90 minutos de SE. Em relação a análise da neurodegeneração, não observamos diferença significante de células Fluoro-Jade positivas entre os grupos. Em relação a epileptogênese, não observamos diferença significante na expressão dos marcadores GFAP (astrogliose), BDNF (neuroplasticidade) e TNF-a (neuro inflamação). A análise conjunta desses dados indica que a depleção do miR-196 não apresenta potencial anticonvulsivante e antiepileptogênico. Por outro lado, observamos que os animais administrados com o inibidor do miR-196b-5p (0,4 nmol/2µL) apresentam um aumento ainda maior nos níveis de transcritos do neuropeptídeo Y (NPY), indicando um possível papel regulador do miR-196b sobre NPY. Também foi observado nos animais administrados com o inibidor do miR-196b-5p (0,4 nmol/2µL) uma diminuição nos níveis de transcritos do miR 146a-5p indicando uma possível diminuição da neuroinflamação. Em conclusão, nossos dados sugerem que o inibidor do tipo LNA é uma ferramenta útil para ensaios funcionais de inibição dos miRs, entretanto, a inibição do miR-196b-5p, nas doses e horários utilizados aqui, não alterou os processos epileptogênicos investigados.

Palavras-chave: Epilepsia; Ensaio Funcional; microRNAs; Knockdown;

ABSTRACT

Epilepsy is a neurological disorder characterized by spontaneous and recurrent seizures. Temporal lobe epilepsy (TLE) is associated with a high rate (30-40%) of refractoriness to treatment with antiepileptic drugs (AEDs). The search for new therapeutic approaches is an urgent need. Manipulation of gene expression has been explored in the development of therapeutic interventions in many neuropathogenetic processes. In this sense, non-coding RNAs, such as microRNAs (miRs), are preferred targets due to the regulatory role they play in the reorganization of gene expression. Recently, we identified that miR 196b-5p has increased expression in the acute phase of epileptogenesis. In this work, we depleted miR-196b-5p levels to gain insights into the functional significance and therapeutic potential of this dysregulation. For this purpose, Wistar rats received, via the intracerebroventricular (ICV) route, the miR-196b-5p inhibitor (0.4 nmol/2μL) 24 hours before being submitted to Status Epilepticus induced by Lithium-Pilocarpine. We observed a significant depletion of miR-196-5p hippocampal levels. The effect of this depletion was evaluated in terms of molecular (expression of epileptogenesis markers and the SLC9A6 target), histochemical (marking of Fluoro-Jade positive neurons) and behavioral parameters (assessment of self-sustained seizures through latency, severity, and frequency). As a control group, wistar rats were used that received, via ICV, 2μL of phosphate saline buffer (PBS). The animals that received the miR-196b-5p inhibitor (0.4 nmol/2μL) did not show a significant change in the latency time for the onset of SE, as well as in the severity and frequency of seizures during 90 minutes of SE. Regarding the analysis of neurodegeneration, we did not observe a significant difference in Fluoro-Jade positive cells between the groups. Regarding epileptogenesis, we did not observe a significant difference in the expression of GFAP (astrogliosis), BDNF (neuroplasticity) and TNF-a (neuroinflammation) markers. The joint analysis of these data indicates that miR-196 depletion does not have anticonvulsant and antiepileptogenic potential. On the other hand, we observed that animals administered the miR-196b-5p inhibitor (0.4 nmol/2μL) showed an even greater increase in the levels of neuropeptide Y (NPY) transcripts, indicating a possible regulatory role of miR- 196b about NPY. A decrease in the levels of miR 146a transcripts was also observed in animals administered the miR-196b-5p inhibitor (0.4 nmol/2μL), indicating a possible decrease in neuroinflammation. In conclusion, our data suggest that the LNA-type inhibitor is a useful tool for functional assays of miRs inhibition, however, the inhibition of miR-196b-5p, at the doses and times used here, did not alter the epileptogenic processes investigated.

Keywords: Epilepsy; Functional assay; microRNAs; Knockdown;

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ELT: Epilepsia do Lobo Temporal

DAEs: Drogas antiepilépticas

miR: microRNAs

LNA: Locked nucleic acid

RNA: Ácido ribonucleico

RNAm: RNA mensageiro

SE: Status epilepticus

ASO: Antisense oligonucleotide

CRE: Crises espontâneas e recorrentes

GFAP: Proteína fibrilar ácida da glia

NYP: Neuropeptídeo Y

BDNF: Fator neurotrófico derivado do cérebro

TNF-a: Fator de necrose tumoral

miRISC: Complexo de silenciamento induzido por miR

I.C.V: Injeção intracerebroventricular

OMS: Organização Mundial de Saúde

ILAE: Liga internacional contra Epilepsia.

SNC: Sistema Nervoso Central

SUDEP: Morte súbita inesperada na epilepsia

Pri-miR: MicroRNA primário.

Pré-miR: Precursor do microRNA.

AGO: Argonauta

SUMÁRIO

CAPÍTULO 1

1 INTRODUÇÃO

Epilepsia é um distúrbio neurológico caracterizado por crises espontâneas e recorrentes (CREs) que decorrem de um desequilíbrio nas vias neurais excitatórias/inibitórias (FISHER et al., 2014). Na população adulta, a Epilepsia do Lobo Temporal (ELT), a mais prevalente (MARISSAL et al., 2021), depende de determinantes ambientais que induzem mudanças estruturais e fisiológicas progressivas no cérebro dos indivíduos com baixo limiar genético de susceptibilidade. Esse processo, denominado de epileptogênese, leva a conversão de um cérebro normal em um cérebro epilético, que adquiriu uma predisposição permanente em gerar crises epiléticas espontâneas (ENGEL *et al.*, 2020). A epileptogênese inclui alguns processos biológicos bem definidos, tais como, rearranjos axonais, neurogênese aberrante, astrogliose e neuroinflamação, além de uma reorganização global na expressão gênica. De fato, genes, a exemplo de GFAP (Proteína fibrilar ácida da glia), NYP (Neuropeptídeo Y), BDNF (fator neurotrófico derivado do cérebro), e TNF-a (fator de necrose tumoral), apresentam os níveis de expressão, consistentemente, aumentados, sendo, portanto, utilizados como biomarcadores dos eventos epileptogênicos.

O tratamento clínico da epilepsia baseia-se fundamentalmente no uso de fármacos antiepilépticos (FAEs), a exemplo da fenitoína, fenobarbital, carbamazepina, benzodiazepínicos e ácido valproico (KANNER et al., 2022). Entretanto, o tratamento medicamentoso apresenta limitações, caracterizada, principalmente, pela não remissão completa das crises, em, aproximadamente, um terço dos pacientes (KWAN; BRODIE, 2000). A busca de novas abordagens terapêuticas é uma necessidade premente, e, recentemente, tem sido direcionada para abordagens de manipulação da expressão de genes regulatórios, a exemplo dos microRNAs (miRNAs). Os miRs são pequenos RNAs não codificantes responsáveis por regular pós-transcricionalmente diversos genes alvos. Para acionar a maquinaria de silenciamento, os miRs se ligam, por complementariedade de bases, aos RNAm alvos. Quando a complementariedade é perfeita, o mRNA é degradado, mas sea

complementariedade for parcial, o processo de tradução é interrompido. Ambas as situações implicam uma regulação negativa da expressão de genes codificadores (MOHR et al., 2015).

Estudo realizado por nosso grupo, mostrou que o miR 196b-5p está superexpresso na fase aguda da epileptogenese em ratos submetidos ao SE (Status Epilepticus) (ARAÚJO et al., 2016). Para entender sobre o papel funcional dessa expressão diferencial, realizamos, nesse trabalho, ensaios funcionais de perda de função do miR-196b-5p em modelo experimental. O efeito da inibição foi avaliado quanto à ensaios moleculares, histoquímicos e comportamentais. Nossa hipótese, foi de que a inibição do miR 196b-5p, durante a fase aguda da epileptogenese, afetaria o curso do SE e/ou dos principais processos biológicos que acompanham a epileptogênese, particularmente, a astrogliose, neurodegeneração, neuroinflamação e neuroplasticidade.

A dissertação está dividida em dois capítulos: o primeiro, contendo uma breve revisão de literatura, e as minhas contribuições acadêmicas obtidas durante o mestrado; e o segundo, contendo o manuscrito do trabalho experimental, a ser submetido para publicação em periódico especializado. Nosso estudo constitui-se no primeiro ensaio funcional de inibição do miR 196b, por meio da administração de um inibidor do tipo LNA pela via intracerebroventricular.

2 EMBASAMENTO TEÓRICO

2.1 Epilepsia

Epilepsia é um distúrbio neurológico caracterizado por crises espontâneas e recorrentes (CREs) resultantes de uma descarga excessiva nas vias neuronais excitatórias (FISHER et al., 2014). A Organização Mundial de Saúde (OMS) estima que 50 milhões de pessoas sejam portadoras de epilepsia, classificando essa condição como uma das mais prevalentes e custosa dentre os distúrbios neurológicos (MURRAY et al., 2012). Quanto a etiologia, a liga internacional contra epilepsia (ILAE – *Internacional League Against Epilepsy)* classifica as epilepsias em genética, infeciosa, metabólica, imunológica e estrutural. Esta última apresenta como determinantes certas alterações estruturais detectáveis ao exame de neuroimagens, tais como hemorragias, isquemia, tumores etc. (SCHEFFER et al., 2017). Na população adulta, o tipo de epilepsia estrutural mais prevalente é a Epilepsia do Lobo Temporal (ELT), que é caracterizada por crises parciais complexas originadas de alterações no sistema límbico (MARISSAL et al., 2021). Tais alterações se estabelecem no curso de um processo progressivo, denominado de epileptogênese, tendo como gatilho inicial a exposição dos indivíduos à insultos ambientais específicos, denominados de insultos epileptogênicos, dentre os quais, destacam-se o traumatismo craniano, neuro-infecções, acidente vascular encefálico, tumores e convulsão febril (LOSCHER et al., 2010).

2.2 Epileptogênese

A epileptogênese é o processo em que um cérebro normal se transforma, progressivamente, em um cérebro epilético, ou seja, àquele com predisposição permanente para gerar crises epiléticas espontâneas (ENGEL *et al.*, 2020). Esse processo cursa em três fases: inicial, latência e crônica. A fase inicial começa logo depois da exposição do indivíduo ao insulto epileptogênico, cursando com de alterações eletrofisiológicas, inflamação, morte celular e uma reorganização na expressão de genes regulatórios. O período de latência é o que se segue, e progride de forma assintomática. Esse período é acompanhado de mudanças

estruturais importantes em toda rede neural, porém o paciente não tem sinais comportamentais de crises. Dentre essas mudanças, destacam-se os eventos neuroplásticos, evidenciados por rearranjos axonais, neurogênese aberrante, e reorganização na expressão de genes secundários. Como consequência desse processo, o indivíduo começa a apresentar as CREs, caracterizando o início da fase crônica (PITKANEN et al., 2015). As CREs, por sua vez, podem agir como um novo distúrbio epileptogênico, causando alterações secundárias, que levam a um efeito progressivo das mudanças estruturais no cérebro epilético.

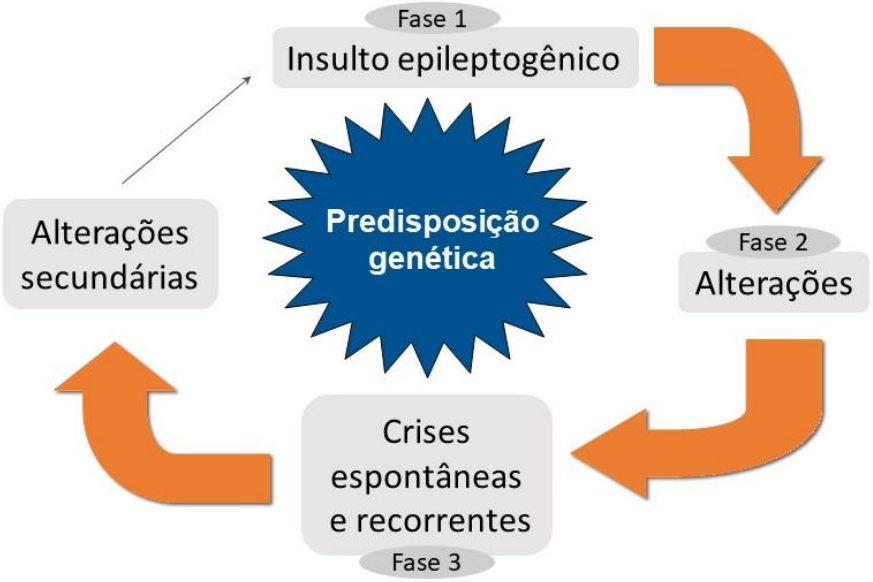

Figura 1 – Processo epileptogênico. Fonte: Elaborado pelo autor (2023).

Os genes que apresentam uma reorganização consistente da expressão em resposta à diferentes insultos epileptogênicos, são utilizados como marcadores da epileptogênese. GFAP (Proteína fibrilar ácida da glia), um filamento intermediário específico de astrócitos, apresenta seus níveis aumentados durante toda a epileptogênese. Essa superexpressão é um marcador biológico da astrogliose reativa, presente ao longo da epileptogênese (BRENNER et al., 2021). Outros genes superexpressos na epileptogênese que atuam como marcadores são: o Neuropeptídeo Y (NPY), relacionado em vários processos neurais como dor, excitabilidade e neurogênese (CATTANEO et al., 2021); fator neurotrófico derivado do cérebro (BDNF) que também está

relacionado com a excitabilidade neuronal e a neurogênese (BINDER et al., 2001); e TNF-a (fator de necrose tumoral), que é uma importante citocina pró inflamatória (KHABOUSHAN et al., 2022).

2.3 Tratamento para epilepsia

O tratamento clínico da epilepsia baseia-se fundamentalmente no uso de fármacos antiepilépticos (FAEs), como fenitoína, fenobarbital, carbamazepina, benzodiazepínicos e ácido valproico, também conhecidos como FAEs primários (KANNER et al., 2022). Porém existem desafios no uso de tratamentos medicamentosos, como a não remissão completa das crises em um terço dos pacientes (KWAN; BRODIE, 2000), devido a mecanismos de farmacorresistência a serem elucidados (WANG et al., 2015). Além do mais, os pacientes que são resistentes têm um risco, de duas a dez vezes maior, para morte súbita, conhecida como SUDEP (*Sudden Unexpected Death in Epilepsy*) (FATTORUSSO et al. 2021). Uma alternativa terapêutica para os pacientes farmacorresistentes é a ressecção cirúrgica das estruturas epileptogênicas, (AHMAD *et al.*, 2020) entretanto, essa intervenção pode levar a diversos efeitos adversos como déficit cognitivo e de memória, além de transtornos neuropsiquiátricos (SHIMIZU *et al.*, 2006; MAEHARA et al., 2013)

Assim, há uma forte necessidade de se buscar uma abordagem terapêutica eficiente em reduzir a frequência e gravidade das crises, com diminuição dos efeitos adversos e sem deixar grandes sequelas nos pacientes. Nesse sentido, esforços têm sido direcionados para abordagens de manipulação da expressão de genes regulatórios, a exemplo dos microRNAs (miRNAs).

2.4 MicroRNAs

Os microRNAs (miRs), são pequenos RNAs não codificadores, com aproximadamente 22 nucleotídeos, que conseguem regular a expressão de genes, em nível pós-transcricional. A biogênese do miRNA começa no núcleo, pela ação da polimerase II (Pol II), responsável por transcrever o miRNA primário (pri-miRNA), que consiste numa cadeia simples dobrada em si mesmo, num formato de gancho. O pri-miRNA é, então, processado pela DGCR8 (RNA

DiGeorge Syndrome Critical Region 8) e Drosha (enzima ribonuclease III), os dois componentes formam o complexo microprocessador responsável pela clivagem e formação do precursor-miRNA (pré-miRNA) com 60 pb (DENLI et al., 2004). Os pré-miRNAs são exportados para o citossol através da Exportin 5, e então, sofrem a ação catalítica da Dicer-like 1, dando origem ao miRNA duplex maduro de 22 pb (RYAN et al., 2015). O duplex se liga a uma proteína argonauta (AGO), formando um complexo de silenciamento gênico chamado de miRISC (miRNA-induced silencing complex), onde uma das fitas é ejetada, ficando apenas a fita madura (JUŰWIK et al., 2019). O complexo miRISC pode atuar por uma complementariedade perfeita ao RNAm alvo, levando assim a degradação deste mRNA; alternativamente, o complexo atua por uma complementariedade parcial de bases, levando a uma repressão traducional (MOHR et al., 2015).

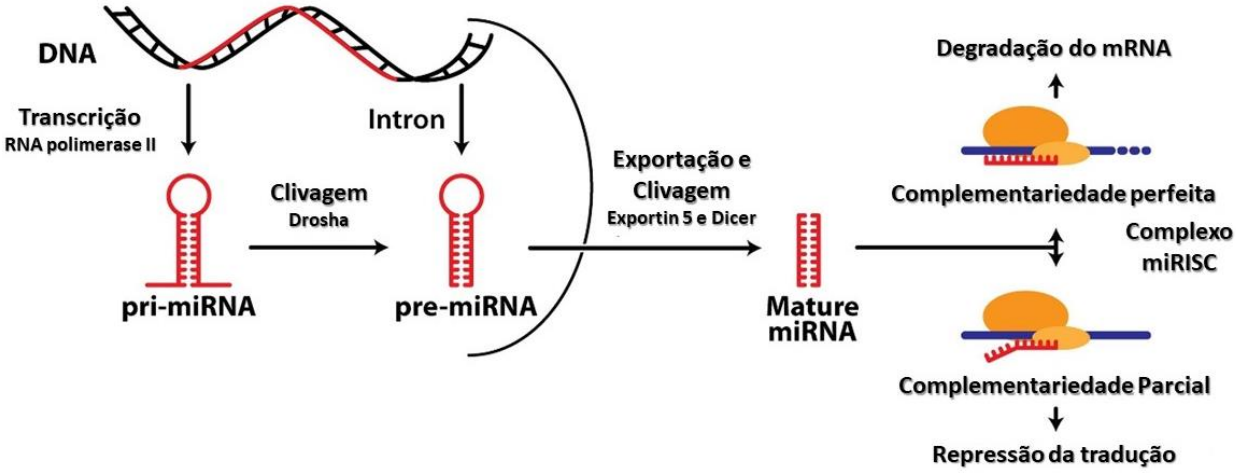

Figura 2 – Biogênese do microRNA. Adaptado de Ryan et al. (2015).

A expressão dos miRs pode servir como biomarcadores para diversos processos, como sinalização intracelular, movimento celular, inflamação, morte celular (ZHANG et al., 2012; GAGLIARDI et al., 2019). Além do mais, uma desregulação na expressão dos miRs foi associada a diversos processos patológicos, dentre os quais, a epilepsia, como relatado no estudo de Raoof et al., 2018, que demonstrou miRs como potenciais biomarcadores para diagnostico.

O nosso grupo de pesquisa identificou um conjunto de miRs expressos diferencialmente nas fases aguda e crônica da epileptogênese, por meio de hibridação em *microarray* acoplada a validação individual por RT-qPCR. Um

destes miRs identificado foi o miR 196b-5p que apresentou superexpressão na fase aguda da epileptogenese induzida por Lítio-Pilocarpina (ARAÚJO et al., 2016). O miR 196b já é bem descrito para diversos tumores, dentre eles o glioma, onde sua superexpressão está associada há um péssimo prognostico patológico, crises convulsivas e resistência a medicações (MA et al., 2012; MA, 2018; YOU et al., 2021;), porém seu envolvimento na epilepsia ainda é desconhecido.

2.5 Ensaios Funcionais de inibição dos microRNAs

Estudos de expressão gênica diferencial acoplada a ensaios funcionais tem mostrado que os miRs são essenciais na epileptogênese, tornando essas moléculas atrativas para o desenvolvimento de drogas com potencial terapêutico (LI et al., 2018; DOGINI et al, 2013, JIMENEZ-MATEOS et al, 2013, RESCHKE et al, 2015). Ao induzir a depleção ou o aumento dos níveis de transcritos de genes alvos, é possível avaliar os efeitos biológicos de ganho ou perda de função de uma molécula específica, e por conseguinte, propor significado funcional e efeitos terapêuticos.

Dentre as metodologias de indução de perda de função de um miR, o uso de moléculas chamadas de oligonuleotideo antisense (ASO) (STENVANG et al., 2012) tem ganhado destaque. ASO é um inibidor de miR, também denominado de antimiR, que irá competir com um miR específico pelo seu mRNA alvo (STENVANG; KAUPPINEN, 2007). Existem diversos tipos de ASO, e cada um deles pode conter modificações químicas para melhorar algumas de suas propriedades, tais como intensidade de inibição, eficiência de internalização celular, especificidade e distribuição (DUYGU et al., 2019). Há três gerações de ASOs, de acordo com o tipo de modificações químicas implementadas para aprimorar os processos de entrega, toxicidade e internalização destas substâncias (GHEIBI-HAYAT et al., 2020). O LNA (*Locked nucleic acid*), mostrado na figura 3, é um ASO classificado como terceira geração (mais atual) que apresenta uma modificação química na estrutura da sua ribose, onde há uma ligação entre 2'O e 4'C, a qual confere estabilidade na complementariedade, melhor velocidade no emparelhamento de bases e melhor resistência frente as

endonucleases (ROOIJ et al., 2014), resultando, portanto, numa molécula inibitória mais eficaz.

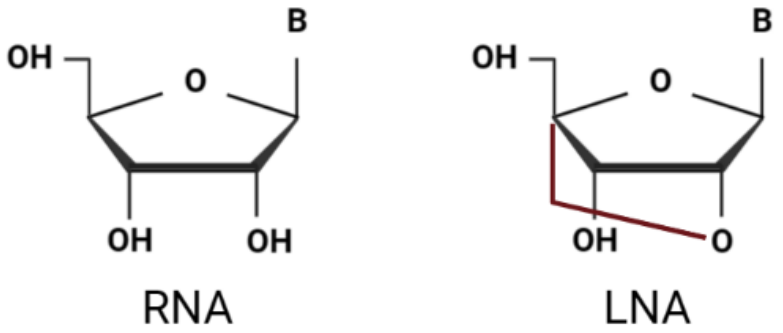

Figura 3 – Estrutura do LNA comparada a um monômero de RNA. A conformação normal de uma ribose fica “bloqueada” através uma ligação entre os átomos 2’O e 4’C. B = Base nitrogenada; O = oxigênio; H = hidrogênio. Fonte: Elaborado pelo autor (2023).

Em epilepsia, um dos estudos pioneiros com antagomir do tipo LNA mostrou que a depleção do miR-132 resulta na redução da morte neural induzida por crises (Jimenez-Mateos et al. 2011). Posteriormente, o mesmo grupo usou essa ferramenta para silenciar o miR-134, o que resultou em ação anticonvulsivante e neuroprotetora (Jimenez-Mateos *et al.* 2012). Tais estudos, portanto, apontam que a manipulação da expressão de miRs se faz necessário para elucidar novas moléculas com potenciais terapêuticos para a ELT.

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3 RELEVÂNCIA E IMPACTO DO PROJETO PARA O DESENVOLVIMENTO CIENTÍFICO, TECNOLÓGICO OU DE INOVAÇÃO

Nosso grupo de pesquisa, identificou a expressão desregulada de diversos miRs através de análise de *microarray* e validação por RT-qPCR em modelo experimental de ELT e tecido humano. Dentro os miRs desregulados, o miR-196b-5p apresentou superexpressão na fase aguda da epileptogenese. Para entender o significado dessa mudança de expressão, faz-se necessário o uso de ensaios funcionais de perda de função. Desta forma, é necessário utilizar uma das técnicas de *knockdown,* como a utilização de um inibidor do miR-196b. Em trabalhos anteriores, depletamos os níveis do miR-196b em ensaios *in vitro*. Neste trabalho, complementamos esse estudo, realizando ensaio de *knockdown in vivo* por meio da injeção intracerebroventricular do inibidor do miR-196b em modelo experimental de ELT induzido por Lítio-Pilocarpina. As contribuições desta dissertação se referem aos dados moleculares, histológicos e comportamentais referentes a caracterização dos efeitos dessa inibição em ratos induzidos ao SE.

4 OUTRAS CONTRIBUIÇÕES DURANTE O MESTRADO

4.1 Coautoria no artigo: **Functional Manipulation of microRNAs in Status Epilepticus-induced Temporal Lobe Epilepsy: Systematic Review of Pre-clinical Studies**, a ser submetido na revista ***PLOS One***. Nesta revisão, avaliamos estudos de interferência funcional de miRNAs na epileptogênese em animais induzido ao SE. Sessenta e três estudos preencheram os critérios de elegibilidade, nos quais a modulação funcional de 38 miRs foram estudadas no processo epileptogênico. Participei ativamente nesta revisão em etapas como: Seleção de artigos de acordo com os critérios de exclusão e inclusão através de títulos e/ou resumos; Atualizações através das pesquisas de palavras chaves nas plataformas de buscas; Revisão das informações retiradas dos artigos selecionados para compor as tabelas do artigo; Avaliação da qualidade dos artigos conforme critérios padronizados e estabelecidos. Foram exclusas as

publicações duplicadas, os artigos com análises experimentais sem modulação funcional de miRs ou que utilizarão modulação funcional, porém não tiveram como modelo experimental o SE. A partir dos dados desta revisão sistemática novos estudos poderão surgir com um objetivo mais claro em miRs com potenciais terapêutico, excluindo algumas de suas limitações e já conseguindo melhores resultado para avanço nas etapas antes da aplicação clínica.

4.2 Coautoria no artigo: **Differential Gene Expression in Patients with Temporal Lobe Epilepsy: Systematic Review Linked to Bioinformatics Analysis** a ser submetido na revista ***Epilepsy*.** Nesta revisão, avaliamos os estudos experimentais de pacientes com epilepsia do lobo temporal que tiveram expressão gênica diferencial validadas por métodos individuais. 210 estudos preencheram os critérios de elegibilidade, sendo selecionados 448 genes. Minha participação nesta revisão corresponde a seleção de artigos de forma independente de acordo com os critérios de exclusão e inclusão através de títulos e/ou resumos recuperados usando a plataforma Rayyan. Foram exclusos as publicações duplicadas, os estudos não relevantes e os artigos com outras doenças ou com pacientes epilépticos, porém sem validação por métodos individuais. A partir dos dados desta revisão sistemática alguns genes podem ser sugeridos como marcadores epileptogênicos por apresentarem dados em concordância com os estudos selecionados, servindo de fontes para estudos sobre as bases moleculares da epilepsia.

4.3 Coautoria no artigo: **MgAl-layered double hydroxide-based nanosystem for functional studies of microRNAs inhibition** a ser submetido na revista ***Nanotechnology*.** Neste artigo experimental, desenvolvemos uma nanopartícula capaz de internalização com eficiência e sem toxicidade um inibidor para estudos funcionais com miRs. Foram realizados ensaios *in vitro* e *in vivo* para avaliar a internalização celular da nanopartícula, eficiência na entrega do sistema e toxicidade. Minha participação neste artigo compreende desde reuniões e discussões de protocolos e medidas a serem realizadas para viabilizar melhor o projeto como também na participação, em bancada, dos experimentos realizados, como: A partir dos dados deste artigo foi possível dar

continuidade ao projeto aqui escrito, que foi analisar o papel fisiológico do miR 196b-5p em ratos *Wistar*s submetidos ao SE 24h após uma dose i.c.v do inibidor do tipo LNA.

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CAPÍTULO 2

Effect of miR 196b inhibition on the acute phase of epileptogenesis in an experimental model of temporal lobe epilepsy.

Abstract: Epilepsy is a neurological disorder characterized by spontaneous and recurrent seizures. Temporal lobe epilepsy (TLE) is associated with a high rate (30-40%) of refractoriness to treatment with antiepileptic drugs (AEDs). The search for new therapeutic approaches is an urgent need. Manipulation of gene expression has been explored in the development of therapeutic interventions in many neuropathogenetic processes. In this sense, non-coding RNAs, such as microRNAs (miRs), are preferred targets due to the regulatory role they play in the reorganization of gene expression. Recently, we identified that miR 196b-5p has increased expression in the acute phase of epileptogenesis. In this work, we depleted miR-196b-5p levels to gain insights into the functional significance and therapeutic potential of this dysregulation. For this purpose, Wistar rats received, via the intracerebroventricular (ICV) route, the miR-196b-5p inhibitor (0.4 nmol/2µL) 24 hours before being submitted to Status Epilepticus induced by Lithium-Pilocarpine. We observed a significant depletion of miR-196-5p hippocampal levels. The effect of this depletion was evaluated in terms of molecular (expression of epileptogenesis markers and the SLC9A6 target), histochemical (marking of Fluoro-Jade positive neurons) and behavioral parameters (assessment of self-sustained seizures through latency, severity, and frequency). As a control group, wistar rats were used that received, via ICV, 2µL of phosphate saline buffer (PBS). The animals that received the miR-196b-5p inhibitor (0.4 nmol/2µL) did not show a significant change in the latency time for the onset of SE, as well as in the severity and frequency of seizures during 90 minutes of SE. Regarding the analysis of neurodegeneration, we did not observe a significant difference in Fluoro-Jade positive cells between the groups. Regarding epileptogenesis, we did not observe a significant difference in the expression of GFAP (astrogliosis), BDNF (neuroplasticity) and TNF-a (neuroinflammation) markers. The joint analysis of these data indicates that miR-196 depletion does not have anticonvulsant and antiepileptogenic potential. On the other hand, we observed that animals administered the miR-196b-5p inhibitor (0.4 nmol/2µL) showed an even greater increase in the levels of neuropeptide Y (NPY) transcripts, indicating a possible regulatory role of miR- 196b about NPY. A decrease in the levels of miR 146a-5p transcripts was also observed in animals administered the miR-196b-5p inhibitor (0.4 nmol/2µL), indicating a possible decrease in neuroinflammation. In conclusion, our data suggest that the LNA-type inhibitor is a useful tool for functional assays of miRs inhibition, however, the inhibition of miR-196b-5p, at the doses and times used here, did not alter the epileptogenic processes investigated.

Keywords: Epilesy; MicroRNAs; Functional assay;

Introduction

Temporal lobe epilepsy (TLE), a neurological disorder that affects about 50 million people worldwide, is characterized by recurrent spontaneous limbic seizures (SRSs) because of hypersynchronous neuronal activity. The epileptic condition arises because of a progressive process of structural and physiological changes that begins when susceptible people are exposed to specific environmental insults, such as Status Epilepticus (SE). This process – Epileptogenesis – drives, therefore, the conversion of a normal brain into an epileptic one, capable of initiating an epileptic seizure (1). Some structural changes have already been elucidated, such as axonal rearrangements, aberrant neurogenesis, astrogliosis, neuroinflammation, and a reorganization in gene expression. Later, some genes undergo a consistent overexpression in response to the epileptogenic insult, which has been used as biomarkers of the different biological processes that occur during epileptogenesis. For example, the Glial Acid Fibrillary Protein (GFAP) for astrogliosis, the Neuropeptide Y (NYP) for neurogenesis, the Brain-Derived Neurotrophic Factor (BDNF) for neuroplasticity, and Tumor Necrosis a-Factor (TNF-a) for neuroinflammation. The clinical treatment of epilepsy is a significant challenge. Currently, the first line of choice to abort seizures is the administration of antiepileptic drugs (AEDs), such as phenytoin, phenobarbital, carbamazepine, benzodiazepines, and valproic acid, also known as primary AEDs (2). However, one-third of the patients undergoing this pharmacological treatment do not achieve complete remission of seizures due to a drug-resistant mechanism to be elucidated (3). Furthermore, pharmacoresistant patients have a two to ten times greater risk of sudden Unexpected Death (SUDEP) (4). However, a therapeutic alternative for drug-resistant patients is the surgical resection of the epileptogenic structures (5). Regardless, the individual after surgery has several adverse effects, such as memory loss and cognitive deficit, and in most cases, several neuropsychiatric diseases appear (6, 7). Thus, there is an emerging need for a therapeutic approach capable of reducing the frequency and severity of seizures with fewer side effects. Efforts have been directed towards approaches to manipulate the expression of regulatory genes, such as microRNAs (miRNAs).

MicroRNAs (miRs) are small non-coding RNAs of 17-22 nucleotides that post-transcriptionally regulate gene expression. miRs function by base complementarity with their target genes. The mRNA can be degraded when the miR has perfect complementarity to the 3' untranslated region (3’ UTR) region of the target gene, or the translation process of this mRNA can be interrupted when there is partial complementarity in this binding.

Several reports have revealed the involvement of miRs in epileptogenic processes (8). Most of them are based in functional studies of loss of function by Knockdown assays. An oligonucleotide antisense molecule (ASO) inhibits the miR and raises the target genes’ levels (9). There are currently several types of ASO with modifications to improve transfection, internalization efficiency, improve resistance against endonucleases (10). A newer generation (third generation) ASO known as LNA (locked nucleic acid) is known to have a chemical modification in its ribose structure, where there is a bond between 2'O and 4'C, which confers stability in complementarity and better base pairing speed and better resistance against endonucleases (11) thus proving to be an excellent tool for miR inhibition.

In a previous study using Microarray coupled to the RT-qPCR approach, we identified several miRs dysregulated in the acute phase of epileptogenesis, such as miR 196b-5p, which was overexpressed in 24-hour after the PILO-induced SE in rats (12). Here, we depleted the miR-196-5b using an LNA-based inhibitor, administered one hour before the SE induction. We assessed behavioral, histological, and molecular parameters to characterize the effect of miR-196-5p depletion in the epileptogenic process.

Methods

Bioinformatics

For selection of the miR-196b-5p target gene, bioinformatics tools were used to choose the predicted target. We used TargetScan Human 8.0, miRDB, DIANA-microT-CDS v5.0, platforms (table 1). Among the predicted target genes found, SLC9a6 was chosen because it is involved in events that are important because it is a gene involved in solute transport and participates in glutamate-induced

excitability (13, 14), a valuable neurotransmitter in the pathophysiology of epilepsy.

Animals

Adult male Wistar rats (230-320 g) were obtained from the central bioterium of the Federal University of Alagoas. All animals were housed in 50-60% humidity and maintained on a 12h light/dark cycle with free access to food and water. Before the experimentation, the animals were acclimated for at least 1 week. the Ethics Committee of the Federal University of Alagoas approved all experimental procedures (protocol nº 84/2016).

Intracerebroventricular injections

miR-196b-5p LNA inhibitor (Exiqon miRCURY LNA) was used to specifically inhibit miR-196b-5p. For this, the molecule was injected into the right lateral ventricle of the rats using the following bregma coordinates: AP = -0.8 mm, L = -1.5 mm, DV = -3.5 mm. Each mouse was injected with 2μL of inhibitor, 0.4 nmol per microsyringe, dissolved in PBS at a rate of 0.5 μL/min; the needle remained in the brain for an additional 2 min. After 24 h, the rats underwent SE induced by lithium-pilocarpine. As a control, 2μL of PBS were used, following the same parameters used for the animals that received the inhibitor.

Status Epilepticus Induction

A total of 24 rats were randomly divided into four groups: *Naiive* group (n= 6), Sham group (n= 6) PBS-SE group (n= 6), and INHIBITOR-SE group targeting LNA-inhibitor of miR-196b-5p (n = 6). Briefly, rats in the PBS-SE and INHIBITOR-SE miR-196b groups were injected intraperitoneally first with 127 mg/kg lithium chloride (LiCl) and 16h later with 30 mg/kg pilocarpine. In addition, 30 min before pilocarpine treatment, rats were administered intraperitoneally with 1 mg/kg scopolamine methyl bromide to reduce the peripheral cholinergic effects of pilocarpine. The SE was defined as self-sustained seizure behavior or intermittent seizures of less than 5 minutes. SE rats have screened following Racine's (1972) classification: type 0 immobility, type 1 facial automatisms, type 2 head, and neck myoclonias, type 3 anterior paw clonies, type 4 elevation on hind paws, and class 5 elevation and falling. To stop the seizures, we used intraperitoneal administration of 5 mg/kg diazepam in the rats 90 min after the

onset of SE.

Tissue preparation and Quantitative Real-Time PCR Total RNA

The rats were sacrificed by rapid decapitation. For real-time polymerase chain reaction (RT-qPCR), the hippocampus was microdissected on the ice-chilled plate, stored in liquid nitrogen, and then on ice at -80 °C. RNA was isolated from samples of the left hippocampus using Trizol (Invitrogen, CA, USA), following the manufacturer's protocol. For SLC9A6 expression analysis, the total RNA was treated with DNase I (Ambion, TX, USA) for 30 minutes to avoid amplifying genomic DNA. Total RNA (1 µg) was reverse transcribed to single-stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RT-qPCR was carried out on a StepOnePlus PCR System (Applied Biosystems). Reactions were performed using 2µL of cDNA, specific forward (F) and reverse (R) primers, and GoTaq qPCR Master Mix (Promega - USA). The target gene expression was normalized to the Actina/Rplp1, as previously determined in our previous study as the best combination of reference genes for expression analysis in the hippocampus. For miR-196b-5p expression analysis, RT-qPCR was performed by using the TaqMan MicroRNA Assay (Life Tech) to assess the expression of miR-196b-5p. In the reverse transcription (RT) step, cDNA was generated from 1 µg of total RNA using Taqman MicroRNA reverse transcription kit (Life Tech) according to the manufacturer's instructions. Real-time PCR was carried out on a StepOnePlus PCR system (Applied Biosystems) using TaqMan Universal PCR Master Mix (Life Tech) according to the supplier's instructions. The miR expression was normalized by the U6snRNA as described previously. Relative fold change was determined by the 2-ΔΔCt method. The absence of contamination was confirmed by PCR amplification in the absence of cDNA. Each assay was performed in triplicate, and the mean values were used for further analysis.

Histopathology

The rats were anesthetized with intraperitoneal sodium thiopental and fixed with 150mL of 4% paraformaldehyde. Then, the rat brain was removed and fixed in 4% paraformaldehyde for 24h. After, the tissues were embedded in tissue-tek O.C.T (Sakura) and sliced into 30µm tissue sections. The neurodegeneration was

detected by Fluoro-Jade B staining. After staining the slides, the sections were captured for analysis through the EVOS M5000 microscope (Thermofisher). The analysis was performed in three regions of the hippocampus: CA1, CA3 and hilus of the dentate gyrus.

Statistical analysis

First, data were analyzed by the normality (Kolmogorov). Statistical analysis was performed using ANOVA and unpaired t-test. Bonferroni/Dunn's Post-hoc comparisons. Mean differences were statistically significant when $p<0,05$.

Results

Knockdown of miR-196b-5p

Anti miR196b injection decreased the levels of miR-196b-5p after 24h of SE-induced animals. (Figure 2A). miR-196b in the INHIBITOR-SE group was significantly lower compared to the PBS-SE group (P=0,0317). To confirm the performance of miR-196b inhibition, we also verified the relative expression of SLC9A6, selected due to its association with epilepsy, higher predictions among prediction algorithms, and lack of validation data on microRNA-gene interactions. Through miR-196b-5p inhibition, the relative expression of SLC9A6 demonstrated a significant increase in the INHIBITOR-SE group compared to the PBS-SE group ($p = 0.0337$), and thus confirming the first-time validation performed, through RT-qPCR, that the administration of 0.4nmol/2mL of the miR-196b-5p inhibitor upregulates SLC9A6 gene (Fig. 2b).

Inhibition of miR-196b-5p has no effect on seizures.

To verify the convulsive effects of miR 196b-5p inhibition, we evaluated total seizures, frequency, latency. during SE. As seen in figure 3A, there was not statistically significant difference between the groups. The total of each type of seizures presented during the SE was also analyzed. As a reference to the Racine scale, no statistically significant difference was observed in the number of scores 2, 3, 4 and 5 seizures in the Inhibitor group when compared to the PBS group (Figure 3B-E). Type 1 seizures were not analyzed due to the difficulty of viewing the video recordings.

Gene expression of epileptogenic targets

To investigate the effect of miR 196b-5p inhibition on epileptogenesis, we performed the relative expression of hallmarks of epileptogenic processes (miR 146a-5p, GFAP, BDNF, NPY and TNFA) 24h after SE induction. In figure 4A we can see the relative expression of BDNF gene, we observed overexpression in the following comparisons: inhibitor and SHAM ($p \leq 0.0008$), inhibitor and NAIVE ($p \leq 0.0008$), PBS and SHAM ($p \leq 0.0159$), PBS and NAIVE ($p \leq 0\ .0196$), there was no statistic difference found between the inhibitor group and PBS (Fig. 4A). In Figure 4B, we observed an overexpression of NPY in rat hippocampi 24h after induced SE in the inhibitor group when compared with the PBS ($p \leq 0.0002$), SHAM ($p \leq 0.0001$) and NAIVE ($p \leq 0.0001$) groups. The relative expression of TNFa (Fig. 4C) reveals a decrease in its transcripts in the NAIVE group when compared to the inhibitor group ($p \leq 0.0302$) and PBS group ($p \leq 0.0190$). For GFAP gene (fig. 4D), which revealed an increased expression in the inhibitor group when compared to the SHAM and NAIVE groups ($p \leq 0.0062$ and $p \leq 0.0001$, respectively), there was also an overexpression in the PBS group when compared to the NAIVE ($p \leq 0.0073$) there was no statistically significant difference found between the inhibitor group and PBS. In figure 4E we can see the relative expression of miR-146a-5p, we observed the decrease in the levels of transcripts in the group that received the miR-196b-5p inhibitor when compared to the animals that received only PBS ($p \leq 0.0190$) and the naive group also showed lower expression when compared to the SE+PBS group ($p \leq 0.0037$).

Effect of inhibition on neurodegeneration

Figure 5 shows the effects of administration of 196b-5p inhibitor on neurodegeneration in the hippocampus of the animals The presence of neurons in neurodegeneration (FJ+) was observed in all hippocampal regions analyzed (CA1, CA3 and hilus of the dentate gyrus) 24 after SE induction in the PBS and inhibitor groups (5A1-5B3). There was not statistically difference in neuronal death between the two groups for any hippocampal subregion (5C-E), The SHAM group did not present, statistically, neurons marked by FJ, in fact, demonstrating that stereotactic surgery did not promote neurodegeneration.

Discussion

Approximately 35% of patients suffer from AED refractoriness (15, 16). Surgical resection of the epileptogenic structure is an alternative for these cases; however, there are serious adverse effects, including impairment of cognitive functions to the onset of neuropsychiatric diseases (17, 18). In this sense, therapy based on the manipulation of genes, such as miRs, is currently on the rise as it is a useful tool to overcome the difficulties of traditional pharmacology (8, 19) with several clinical trials based on gene manipulation for numerous pathologies (20) as it is an approach that has shown positive effects without leaving sequelae or adverse effects on patients. Some miRs have already been considered markers of various pathophysiological processes of epilepsy, such as neuroinflammation, astrogliosis and neuronal plasticity (21). Several studies have identified dysregulated miRs in patients and experimental models of epilepsy (22-24). A recent work carried out by our research group showed several miRs differentially expressed in epilepsy through microarray hybridization coupled with individual validation by RT-qPCR (12). Loss-of-function studies of MiRs are crucial to understand involvement in pathophysiology, and the use of ASO is essential for miRs depletion. Among the types of ASO used for miR depletion, LNA has been one of the most used due to its chemical modification qualities that confer greater stability. Thus, this work aimed to carry out the functional modulation of miR 196b-5p, since this miR showed overexpression in the acute phase of epileptogenesis and there are no reports about its role in the pathophysiology of epilepsy, therefore, the effect of its modulation is of great importance for understanding its functional role in the ELT. We investigated the effect of miR 196b-5p inhibition using an LNA-type inhibitor 24h before SE induction in animals using Lithium-Pilocarpine. We were able to obtain a partial depletion of miR 196b-5p levels and an increase in the expression of its predicted target gene, SLC9A6, thus confirming that inhibition had occurred. Regarding the behavioral analysis of SE, the injection of 0.4nmol/2mL of the miR 196b-5p inhibitor did not affect self-sustained seizures, nor did we observe effects on the neurodegeneration of neurons with Fluorojade. The analysis of the expression of markers involved in epileptogenesis (GFAP, BDNF, and TNF-a) showed no significant difference. However, NPY expression showed a significant increase in the inhibitor group,

and miR 146a-5p, a neuroinflammation marker, showed a decreased expression in the inhibitor group.

Validation of miR-196b-5p inhibition

LNA-type miR inhibitors consist of essential modifications that improve the affinity to the mature miR, resulting in less inhibitor degradation by improving stability against endonucleases. (11). We then performed the intracerebroventricular injection of 0.4nmol/2uL of the miR-196b-5p LNA inhibitor to decrease the high levels of this miR found in an animal model in the acute phase of epileptogenesis in a lithium-pilocarpine model (8). We verified the partial depletion of miR 196b-5p, analyzed through RT-qPCR. There are no studies on the role of miR 196b in epileptogenesis, this being the first work concerning to the functional role in epilepsy.

miR 196b-5p is well described in the literature for its involvement in cell proliferation processes and tumors (25, 26). In fact, miR 196b-5p has shown high potential to be a biomarker for patients with seizures diagnosed with low-grade glioma (27-29). However, there are no reports of functional assays linking miR-196b-5p with epilepsy, and therefore, its function in pathophysiological mechanisms is still unclear.

Through bioinformatics programs, we used SLC9A6 as the predicted target gene of miR-196b-5p. To confirm the tools' prediction, we analyzed of their transcript levels through RT-qPCR and observed the significantly higher expression of SLC9A6. There is a need for additional tests such as analysis of protein levels through Western Blotting and validation of the 3'UTR region by luciferase assay.

SLC9A6, also known as NHE6, is essential for the transport of solutes between the blood-brain barrier and several cells of the central nervous system (30). The expression of SLC9A6 had not yet been studied in animals submitted to SE, however, it is known through studies with patients that a mutation in the SLC9A6 gene is involved in neurological diseases, including epilepsy (13, 31-33). In fact, it is seen that this gene plays a crucial role in neural arborization and synapse generation (34). Because dendritic abnormalities are one among many structural changes that occur in epilepsy (35) it may be that SLC9A6 really can

be involved with epileptogenic processes and its modulation can be a key point to modify alterations that occur in epileptogenesis, therefore, additional studies should be performed to evaluate this possible interaction using specific markers.

Behavioral analysis of self-sustaining crises

The relationship between miR 196b-5p and seizures in patients is reported in the study by You et al., (2012), who analyzed the association of miR expression with seizure rate, demonstrating a 67-fold increase in miR- 196b in preoperative patients. Here we observed that the inhibition of miR 196b-5p 24 hours before SE induction by Lithium-Pilocarpine showed no effect on the latency, frequency, or severity of the animals' seizures during the 90 minutes of self-sustained seizures. There are studies of functional modulation, with antagomir, which show behavioral changes when analyzing the SE, either in the aspect of the peak observed in the EEG or latency for the onset of the first seizure (36). Even so, other studies with functional modulation in epilepsy also failed to verify statistical difference in seizures during SE, however, in the chronic phase of epilepsy, it was seen that the depletion of miRs caused some effect during epileptogenesis as they were able to see changes such as a decrease in the severity of seizures and increased latency time for CREs (37-40), thus suggesting that the assessment in the acute phase may not reflect the general effect of inhibition on epileptic seizures.

Gene expression of transcripts involved in epileptogenic processes.

Processes such as neuroinflammation, astrogliosis, and aberrant neurogenesis are part of the progress of epileptogenesis. In this way, we evaluated the expression of genes associated with epileptogenic processes. We had an overexpression of NPY after miR-196b-5p inhibition in the SE group. There is still no prediction or validation of the involvement of miR 196b-5p with NPY; however, it is known that the two participate in critical physiological processes for epilepsy, such as cell proliferation (41), and the fact that is the only gene involved in epileptogenesis to have increased expression when miR 196b-5p was inhibited suggests an association between the two. The increase in NPY transcripts suggests a protective effect since this gene is associated with inhibitory processes in excitatory synaptic transmissions, including glutamatergic

transmissions (42). There was no significant change in transcript levels for the other analyzed genes involved in the epileptogenesis process, such as GFAP, BDNF, and TNF-a.

miR-146a-5p, well described as an important marker of neuroinflammatory processes (43) and reported as a possible serum marker for drug resistance in patients (44), had its transcripts reduced after miR-196b-5p inhibitor injection. Functional modulation studies confirm the participation of miR-146a-5p in neuroinflammatory processes (45) and as a possible treatment for pharmacoresistant (19). It is noteworthy that the LNA-type inhibitor used here was specific for miR-196b-5p. The inhibition of miR-196b-5p may have influenced molecular pathways adjacent to miR-146a-5p. This statement can be considered, since some genes are targets of the two miRs, as is the case of FAS, cell surface death receptor, an important marker of apoptosis (46, 47).

Hippocampal neurodegeneration

The classic drugs used to combat seizures, such as phenytoin, cannot demonstrate a neuroprotective effect, bringing risks to the endogenous protection of the patient's central nervous system (48). Thus, assessing neurodegeneration can be a valid point for new therapies. Functional tests of miR 196b-5p loss and gain of function showed that this miR is related to cell proliferation, causing risks of cell migration causing metastases (49, 50). However, using the Fluoro-Jade technique, we evaluated the hippocampal neurodegeneration of the CA1, CA3, and hilus of the dentate gyrus. We did not observe a statistically significant difference in any subarea of the hippocampus.

Conclusions and perspectives

In summary, this work was the first to perform miR inhibition using an LNA-type inhibitor in animals subjected to Status Epilepticus induced by lithium-pilocarpine. Inhibition with intracerebroventricular injection 24h before SE effectively decreased the levels of miR-196b-5 and consequently increased the levels of its target gene, SLC9A6, thus confirming the inhibition through RT-qPCR With the injection of 0.4nmol/2mL of inhibitor 24h before SE, we did not obtain any difference in latency, severity, or frequency of seizures during 90 minutes of

SE, nor did we verify any difference when observing neurons positively stained with Fluoro-Jade. Regarding the expression of epileptogenesis markers, we observed an overexpression of NPY, indicating a possible regulatory role of miR-196b on this gene. We also saw a difference in the levels of miR-146a-5p transcripts, an important marker of neuroinflammation, which decreased in the expression of the group that received 0.4nmol/2mL of inhibitor 24h before SE, possibly because it indirectly modulated some miR-146a-5p pathway. We did not obtain significant results for other markers such as GFAP, BDNF and TNF-a. Thus, the data indicate that miR-196 depletion does not have anticonvulsant and antiepileptogenic potential within the analyzes performed here. Therefore, additional studies using the miR-196b-5p inhibitor at other concentrations and therapeutic schemes may be of great importance to assess whether the concentration used in this study is the most effective.

Figures

Figure 1 – *Experimental design*

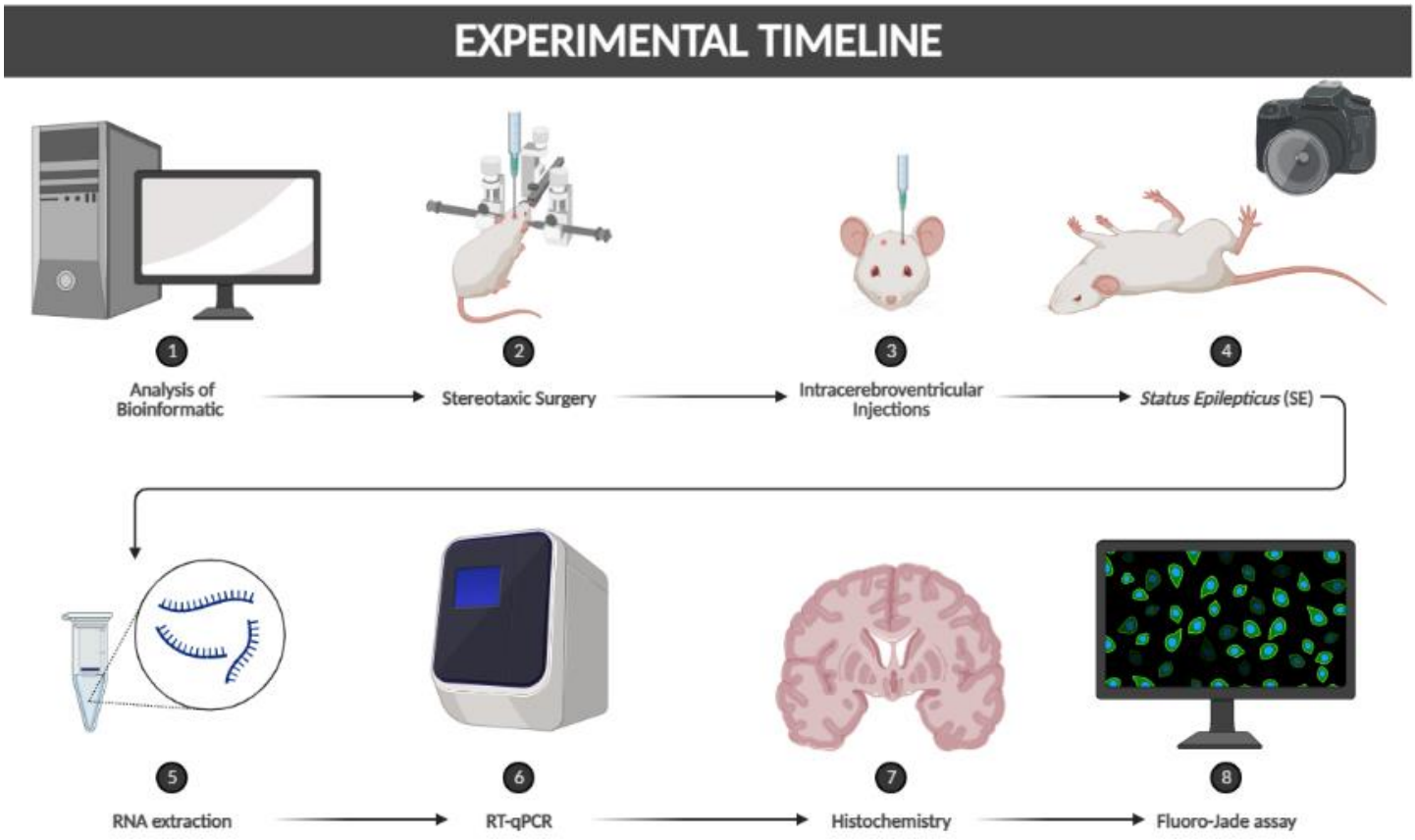
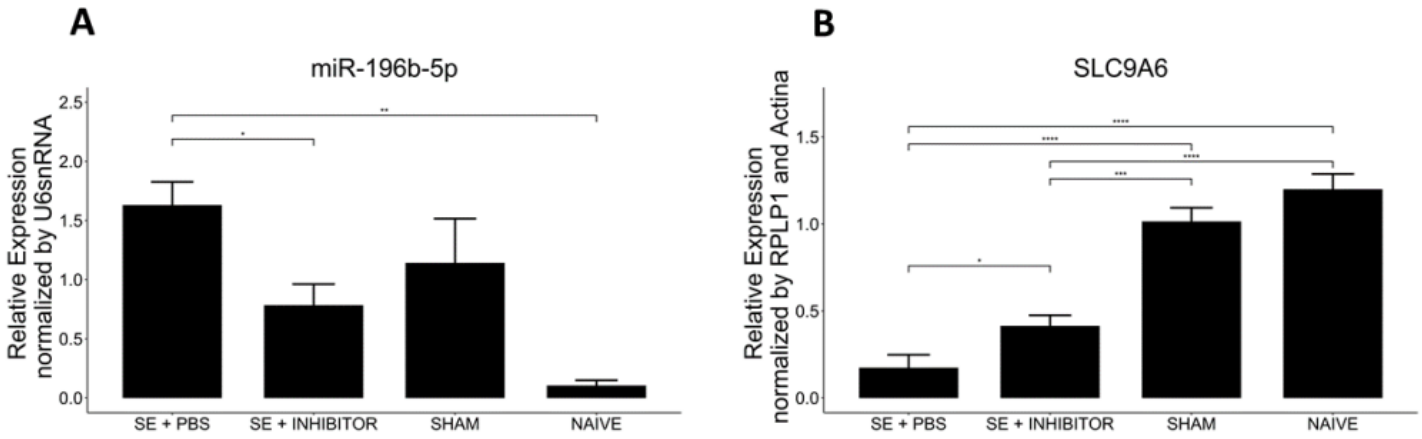
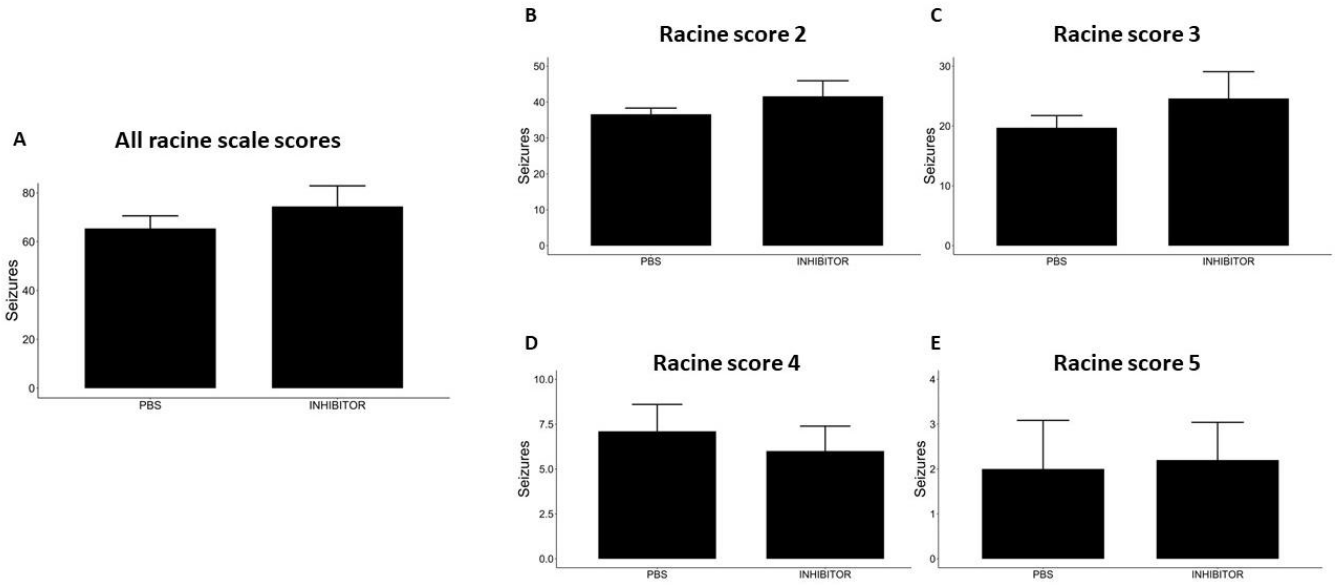


Figure 2 – ***Knockdown.***



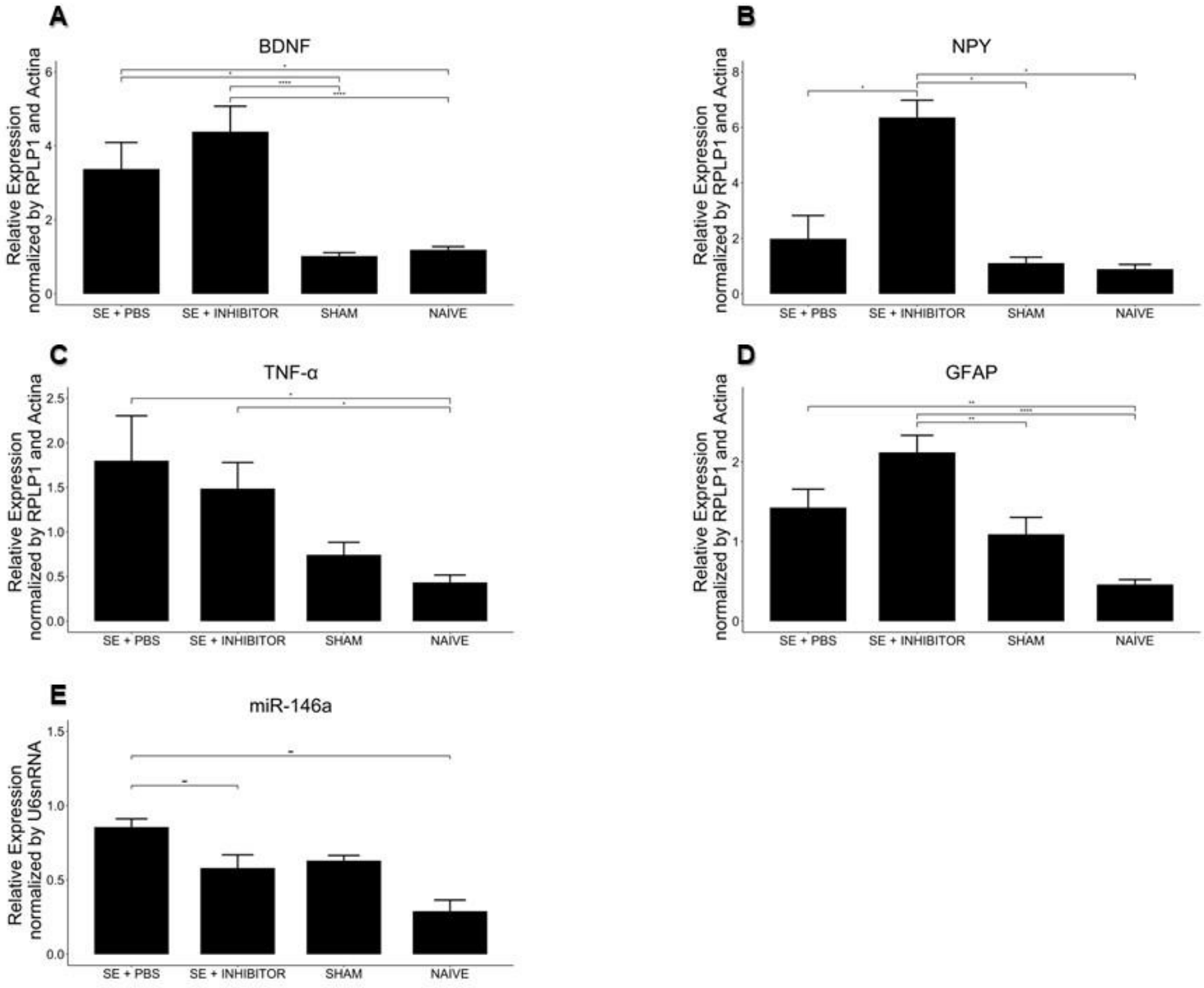
Legend: Figure 2 – Differential gene expression: 2A) Relative expression of microRNA 196b after 24 hours of the SE-induced. 2B) Relative expression of SLC9A6 after 24 hours of the SE-induced. Test t and Analysis of variance (ANOVA) was used for comparison between groups. Post-hoc comparisons were performed with Dunns' multiple comparisons test. **p≤0.05; ***p≤0.01; ***p≤0.001, ****p≤0.0001*

Figure 3 – ***Inhibition of miR-196b-5p has no effect on seizures.***



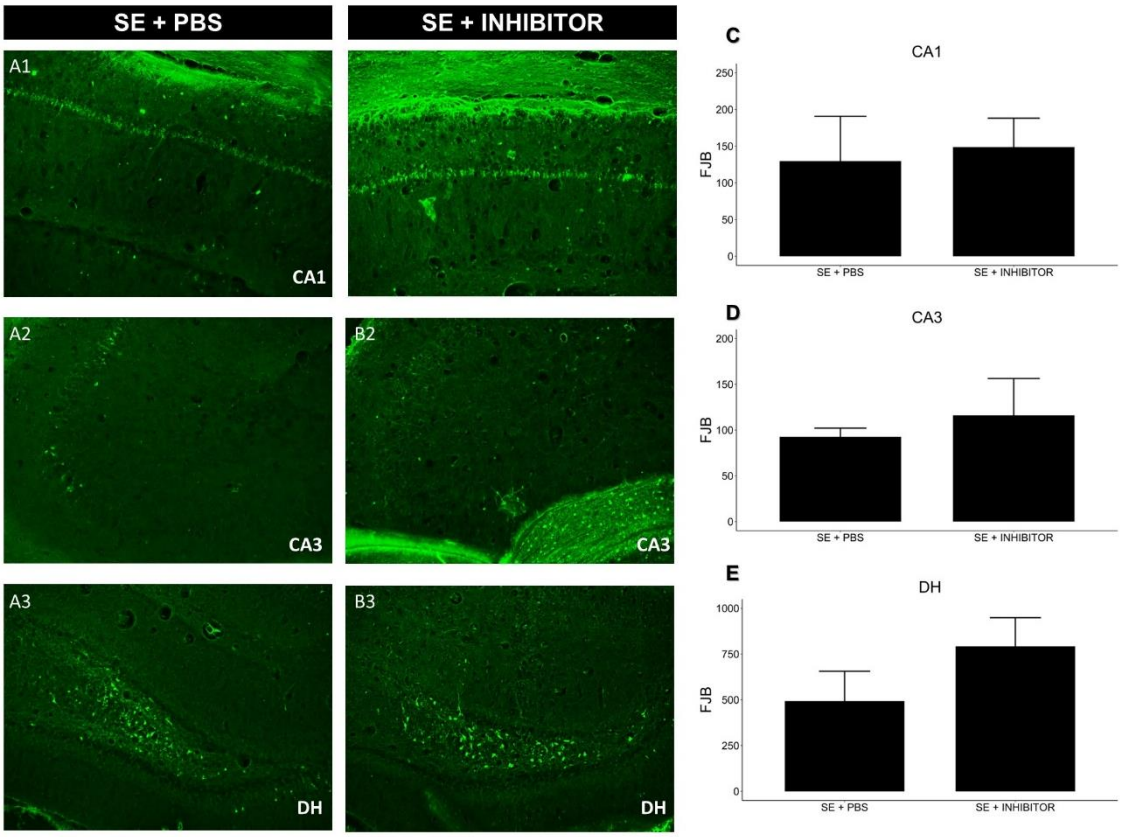
Legend*:* Figure 3 – Seizures analysis during SE-induction: 3A) Seizures parameters during SE when injected 0.4 nmol/2 µL of inhibitor or 2 µl of PBS. They did not differ. 3B-E) Racine scale during SE when injected 0.4 nmol/2 µL of inhibitor They did not differ. Test t and Analysis of variance (ANOVA) was used for comparison between groups. Post-hoc comparisons were performed with Dunns' multiple comparisons test. Inhibitor (n=10) and PBS (n=11).

Figure 4 – Gene expression of epileptogenic targets



Legend: Figure 4 – Differential gene expression: 4A) Relative expression of BDNF after 24 hours of the SE-induced. 4B) Relative expression of NPY after 24 hours of the SE-induced. 4C) Relative expression of TNF-α after 24 hours of the SE-induced. 4D) Relative expression of GFAP after 24 hours of the SE-induced4E) Relative expression of microRNA 146a after 24 hours of the SE-induced. Test t and Analysis of variance (ANOVA) was used for comparison between groups. Post-hoc comparisons were performed with Dunns' multiple comparisons test. **p≤0.05; ***p≤0.01; ***p≤0.001, ****p≤0.0001*

Figure 5 – ***Inhibition of miR-196b-5p has no effect on seizures.***



Legend: Figure 5 – Graphs and photomicrographs (hippocampus) 24 h after SE-induced in rat given PBS or inhibitor. 4A1-B3) Photomicrographs show the occurrence of neurodegeneration labeling in CA1, CA3 and Hilus of the dentate gyrus of the hippocampus of the PBS (A1-A3) and inhibitor (B1-B3) groups. 4C) Number of fluorojade-labeled neurons in the CA1 region of the hippocampus 4D) Number of fluorojade-labeled neurons in the CA3 region of the hippocampus 4E) Number of fluorojade-labeled neurons in the Hilus od the dentate gyrus region of the hippocampus. Scale of photomicrographs:

Table 1 – Targets gene predicted by microRNA target prediction algotithms

Ortholog of target gene	Gene description
HOXC8	homeobox C8
HOXB8	homeobox B8
HOXA7	homeobox A7
RP1-170O19.20	Uncharacterized protein
HAND1	heart and neural crest derivatives expressed 1
HOXA9	homeobox A9
NR6A1	nuclear receptor subfamily 6, group A, member 1
HMGA2	high mobility group AT-hook 2
HOXB7	homeobox B7
HOXA5	homeobox A5
EPC2	enhancer of polycomb homolog 2 (Drosophila)
FAM222B	family with sequence similarity 222, member B
VSNL1	visinin-like 1
HOXB6	homeobox B6
LCOR	ligand dependent nuclear receptor corepressor
DDX19A	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19A
BLOC1S6	biogenesis of lysosomal organelles complex-1, subunit 6, pallidin
SOCS2	suppressor of cytokine signaling 2
ZCCHC3	zinc finger, CCHC domain containing 3
GAN	gigaxonin
TSPAN12	tetraspanin 12
ING5	inhibitor of growth family, member 5
LIN28B	lin-28 homolog B (C. elegans)
CCNJ	cyclin J
PRDM5	PR domain containing 5
ZBTB26	zinc finger and BTB domain containing 26
NME4	NME/NM23 nucleoside diphosphate kinase 4
FOXM1	forkhead box M1
ZNF385D	zinc finger protein 385D
NCS1	neuronal calcium sensor 1
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
PSMD11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11
RXFP2	relaxin/insulin-like family peptide receptor 2
LETMD1	LETM1 domain containing 1
ZMYND11	zinc finger, MYND-type containing 11
CALM1	calmodulin 1 (phosphorylase kinase, delta)
TMX1	thioredoxin-related transmembrane protein 1
TOX3	TOX high mobility group box family member 3
RCC2	regulator of chromosome condensation 2
SLC9A6	solute carrier family 9, subfamily A (NHE6, cation proton antiporter 6), member 6
CADM2	cell adhesion molecule 2
SSR1	signal sequence receptor, alpha
PPP6R2	protein phosphatase 6, regulatory subunit 2
HMGA1	high mobility group AT-hook 1
ADRB3	adrenoceptor beta 3
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)
EPS15	epidermal growth factor receptor pathway substrate 15

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