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EFEITO DO USO DE PERAMPANEL NA CAPACIDADE COGNITIVA DE RATOS WISTAR SUBMETIDOS À SOBRECARGA DE FERRO NO PERÍODO NEONATAL

Porto Alegre
2024

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Tese apresentada ao Programa de Pós- Graduação em Ciências Biológicas: Fisiologia do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Fisiologia.

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"É muito melhor lançar-se em busca de conquistas grandiosas, mesmo expondo-se ao fracasso, do que alinhar-se com os pobres de espírito, que nem gozam muito nem sofrem muito, porque vivem numa penumbra cinzenta, onde não conhecem nem vitória, nem derrota."

Theodore Roosevelt

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RESUMO

DA SILVA, J. A. C. **Efeito do uso de Perampanel na capacidade cognitiva de ratos *Wistar* submetidos à sobrecarga de ferro no período neonatal.** 2023. 95p. Tese (Doutor em Fisiologia) – Programa de pós-graduação em Ciências Biológicas – Universidade Federal do Rio Grande do Sul, 2023.

No decorrer da vida, diversos insultos independentes interagem e resultam em lesões subsequentes de DNA e apoptose celular no Sistema Nervoso Central. Na composição de um evento de morte neuronal, alguns distúrbios pontuais já são bem esclarecidos, tais como as alterações na função mitocondrial, nos níveis de antioxidantes e radicais livres, na vulnerabilidade neuronal, na concentração de glutamato na fenda sináptica e de cálcio intracelular $[Ca^{2+}]^{i}$. Existem evidências que em momentos de estresse neuronal, o receptor ionotrópico de glutamato, AMPA, sofre a ação de diversos fatores intrínsecos que alteram sua permeabilidade aos íons Ca^{2+}, o que soma positivamente no processo neurodegenerativo. Nesse sentido, o antagonista alostérico de receptores AMPA como o fármaco Perampanel (PER), parece agir de maneira favorável contra essa permeabilidade ao cálcio do receptor, porém, o fármaco carece de ensaios moleculares que elucidem seu mecanismo de ação frente a diferentes agressões neuronais. Sendo assim a presente tese, teve como objetivo fazer uma revisão da literatura sobre o papel dos receptores AMPA permeáveis ao cálcio, nas doenças neurodegenerativas, neuroinflamação e neurotoxicidade. Também foi objetivo desta tese avaliar o efeito do uso subcrônico de PER sobre o déficit cognitivo e expressão de marcadores proteicos do receptor AMPA no hipocampo, principal estrutura responsável pela formação da memória, de ratos *Wistar* adultos tratados com sobrecarga de ferro no período neonatal, modelo de neurotoxicidade utilizado pelo nosso grupo de pesquisa. Referente aos ensaios experimentais, como principais resultados, foi verificado que sobrecarga de ferro gerou um déficit cognitivo identificado no teste de esquiva inibitória e reconhecimento de objeto novo, revertido parcialmente pela administração do PER. Além disso, através dos ensaios de RT-PCR, evidenciou-se que a sobrecarga de ferro aumentou a expressão relativa dos genes GRIA1 e DGL4, responsáveis pela transcrição das proteínas GluA1 e PSD-95, importantes para inserção de receptores AMPA permeáveis ao cálcio na membrana neuronal, e um componente dos processos neurodegenerativos. A administração do PER não interferiu na transcrição das proteinas, mas sim, na fosforilação da Serina 845 da subunidade GluA1, revertendo a indução da fosforilação mediada pela sobrecarga de ferro no período neonatal. Concluindo, a partir dos dados e da revisão elaborada, é possível, traçar um paralelo entre os efeitos neurotóxicos da sobrecarga de ferro e o aumento de receptores AMPA permeáveis ao cálcio, bem como, estabelecer um caminho pelo qual o fármaco PER apresenta efeito neuroprotetor no modelo estudado.

Palavras-chave: memória, neurodegeneração, perampanel, sobrecarga de ferro.

ABSTRACT

DA SILVA, J. A. C. **Effect of the use of Perampanel on the cognitive capacity of Wistar rats subjected to iron overload in the neonatal period.** 2023. XXp. Thesis (Doctor in Physiology) – Postgraduate program in Biological Sciences – Federal University of Rio Grande do Sul, 2023.

Over the life course , diverse independent insults begin to interact and result in subsequent DNA damage and cell apoptosis. In the composition of a neuronal death event, some specific disruptions are already well understood, such as changes in mitochondrial function, levels of antioxidants and free radicals, neuronal vulnerability, concentration of glutamate in the synaptic cleft, and intracellular calcium [Ca2+]I. There is evidence that, in moments of neuronal stress, the ionotropic glutamate receptor, AMPA, is influenced by several intrinsic factors that alter its function and permeability to Ca2+ ions, positively contributing to the neurodegenerative process. In this sense, the AMPA receptors allosteric antagonist , Perampanel (PER), appears to act favorably against this receptor's calcium permeability. However, the drug lacks molecular assays to elucidate the mechanism of action against different neuronal disturbances. Therefore, the present thesis aims to review the literature on the role of calcium-permeable AMPA receptors in neurodegenerative diseases, neuroinflammation, and neurotoxicity. Additionally, it aims to evaluate the effect of subchronic use of PER on cognitive deficit and expression of AMPA receptor protein markers in the hippocampus, the main structure responsible for memory formation, of adult Wistar rats treated with iron overload in the neonatal period, a model of neurotoxicity used by our research group. Regarding the experimental trials, the main results indicate that iron overload generated a cognitive deficit identified in inhibitory avoidance and object recognition tests, partially reversed by the administration of PER. Furthermore, through RT-PCR assays, it was evidenced that iron overload increased the relative expression of the GRIA1 and DGL4 genes, responsible for the transcription of the GluA1 and PSD-95 proteins, important for the insertion of calcium-permeable AMPA receptors in the neuronal membrane, and a component of neurodegenerative processes. PER administration did not interfere with protein transcription but rather with the phosphorylation of Serine 845 of the GluA1 subunit, reversing the induction of phosphorylation mediated by iron overload in the neonatal period. In conclusion, based on the data and the review prepared, it is possible to draw a parallel between the neurotoxic effects of iron overload and the increase in AMPA receptors permeable to calcium. Additionally, it is possible to establish a pathway through which the drug PER has a neuroprotective effect in the exprimental model.

Keywords: memory, neurodegeneration, perampanel, iron overload.

LISTA DE FIGURAS

LISTA DE SIGLAS

ABP - Proteína de ligação de receptores AMPA, sigla do inglês *AMPAR binding protein*

ACh – Acetilcolina

AKAP79 - Proteína de ancoragem a Kinase 79

AMPAR - Receptor ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionico

AMPc - Adenosina monofosfato cíclico

APP - Proteína precursora amiloide

ATD – Domínio amino terminal

ATP – Adenosina Trifosfato

AVC - Acidente Vascular Cerebral

Aβ - Beta-amiloide

CaMKII - Proteína-quinase dependente de cálcio-calmodulina

ChaT - Colina acetiltransferase

CNG - Canal dependente de nucleotídeos cíclicos

CP-AMPAR - Receptores AMPA permeáveis ao cálcio

DA – Doença de Alzheimer

DAE - Droga antiepilética

DAG – Diacilglicerol

DMT1 - Transportador de metal divalente 1

DNA - Ácido Desoxirribonucleico

DP – Doença de Parkinson

ECD – Domínio extracelular

ELA - Esclerose Lateral Amiotrófica

EMA - Agência Europeia de Medicamentos

EROS – Espécies reativas do oxigênio

FTH1 - Cadeia pesada de ferritina 1

FTL - Cadeia leve de ferritina

GluA1 – Subunidade A1 de receptor AMPA

GluA2 – Subunidade A2 de receptor AMPA

GluA3 – Subunidade A3 de receptor AMPA

GluA4 – Subunidade A4 de receptor AMPA

GluRs- Receptores de glutamato

GRIP -Proteína que interage com o receptor de glutamato

H_2O_2 – Peróxido de hidrogênio

iGluRs - Receptores glutamatérgicos ionotrópicos

IP3 – Inositol trifosfato

LBD - Ligand-binding domains

LTD - Depressão de longa duração

LTP - Potenciação de longa duração

MD - Tálamo mediodórtrico

mGluRs - Receptores glutamatérgicos metabotrópicos

MRI - Imagem quantitativa por ressonância magnética

NMDA - N-Metil-D-Aspartato

PDZ – Acrônimo de PSD95, DlgA e Zo-1

PER – Perampanel, 2-(2-oxo-1-fenil-5-piridin-2-il-1,2-di-hidro-piridin-3-il) benzonitrilo

PIP2 - Fosfatidilinositol-bifosfato

PKA - Proteína quinase A

PKC - Proteína quinase C

RE – Retículo endoplasmático

RL's – Radicais livres

RNA - Ácido Ribonucleico

RNAm - Ácido Ribonucleico mensageiro

RyRs – Receptores rianodínicos

S818 – Serina 818

S831 – Serina 831

S845 – Serina 845

S880 – Serina 880

SAP97 - Proteína 97 associada à sinapse

SNC – Sistema Nervoso Central

T840 – Treonina 840

TFR1 - Receptor de membrana transferrina 1

TM2 – Domínio transmembranar 2

TNF Fator de necrose tumoral-alfa

TARPs - Proteínas reguladoras transmembranares

VOC - Canais de Cálcio Voltagem Dependente

Y876 – Tirosina 876

LISTA DE SÍMBOLOS

α – Alfa

ß – Beta

γ - Gama

LISTA DE ANEXOS

SUMÁRIO

1. CAPÍTULO 1

1.1 REVISÃO DA LITERATURA

1.1.1 Envelhecimento e doenças neurodegenerativas

As alterações fisiológicas intrínsecas ao envelhecimento são sutis e inaptas a gerar qualquer incapacidade na fase inicial, embora, com o passar dos anos, venham a causar níveis crescentes de limitações funcionais e cognitivas (IRWIN et al., 2016; ROESLER e SCHRÖDER, 2011, SANCHETI et al., 2013). São um fenômeno natural, embora, progressivo, que aumentam o risco para desordens degenerativas, caracterizadas pela deposição de proteínas anormais, danos no DNA (AKASAKA e RUAN, 2016; NIEDZIELSKA et al., 2015;), declínio mitocondrial (FEDERICO et al., 2012), e/ou agregação de proteínas oxidadas e complexos oligoméricos independentes (HROUDOVA et al., 2014, IRWIN et al., 2016), culminando na deterioração neuronal (SOLOWAY et al., 2013). Esse ambiente de estresse oxidativo e mau funcionamento mitocondrial são dois mecanismos interdependentes, que desempenham um papel central no envelhecimento do cérebro (SANTOS et al., 2013).

Algumas hipóteses são levantadas referentes à origem de algumas doenças do Sistema Nervoso Central (SNC), as quais, têm o aparecimento insidioso durante o envelhecimento, tais como, a Doença de Alzheimer (DA) e a Doença de Parkinson (DP). A hipótese mais antiga sobre a DA surgiu no início da década de 80 e descreveu a importância da função colinérgica nos processos de aprendizagem e memória, assim como, a diminuição na concentração da colina acetiltransferase (ChAT), enzima responsável pela síntese da acetilcolina (ACh), no córtex e no hipocampo, assim como, uma redução variável dos neurônios colinérgicos localizados no núcleo basal de Meynert (DAVIES e MALONEY 1976; KÁSA et al., 1997).

Outra hipótese, que emergiu na década de 80, foi da excitotoxicidade do glutamato na DA. Essa, prevê que, em condições específicas, tais como, na alteração do metabolismo energético celular, ocorre uma excessiva ativação de receptores de N-Metil-D-Aspartato (NMDA), podendo alterar a homeostase de cálcio, levando a um aumento das concentrações intracelulares deste íon, capaz de iniciar o processo de apoptose neuronal (DANYSZ, 2000).

O trabalho que pela primeira vez propôs a sequência de eventos denominada “hipótese da cascata amiloide” foi publicado no início da década de 90 e postula que o peptídeo beta-amiloide (Aβ) e/ou os produtos de clivagem da sua proteína precursora, uma glicoproteína integral, denominada, proteína precursora amiloide (APP), são neurotóxicos e podem levar à formação das placas senis, resultando em morte neuronal. Esses, podem causar diversas lesões filamentosas intraneuronais e extracelulares no córtex límbico, assim como, no córtex cerebral

associadas aos agregados anormais de fibras citoplasmáticas, ocorrendo, em grande escala, na maioria dos pacientes com DA avançada (GIROTRA et al., 2021).

Nos últimos anos, com a ampla discussão a respeito da hipótese da cascata amiloide, um número crescente de evidências sugere que os íons metálicos endógenos, particularmente, os que possuem atividade redox, tais como, cobre (II) e ferro (III), além de certos íons não redox-ativos, como o zinco (II), podem contribuir na evolução de doenças neurodegenerativas, favorecendo a agregação de Aβ e aumentando a sua toxicidade. Por exemplo, a oxidação do ferro por meio da reação de Fenton gera anormalidades no RNA, que, na DA, causa grande redução na síntese protéica, enquanto, o radical hidroxila provoca diversos danos às biomoléculas atacando as bases nitrogenadas e a desoxirribose do DNA, reagindo com as cadeias laterais de aminoácidos e proteínas (ZHU et al., 2007). Tal processo, pode levar à formação de fragmentos proteicos não funcionais e também em derivados de lipídeos de membrana, convertendo sítios lipídicos específicos em novos centros de formação de radicais livres (RL's) (BARNHAN e BUSH, 2008).

Em que pesem todas as descobertas feitas nos últimos anos referentes à complexidade do processo neurodegenerativo, ainda, há muito a ser elucidado, como, por exemplo, a correlação entre o acúmulo de ferro, os receptores glutamatérgicos e o dano neuronal. A elucidação destes mecanismos pode auxiliar no desenvolvimento de novas terapias neuroprotetoras.

1.1.2 O papel do ferro nas doenças neurodegenerativas

No SNC, o ferro é o cofator de muitos processos metabólicos essenciais, desempenhando uma importante função na neurogênese, mielinização, desenvolvimento sináptico e metabolismo energético (AGRAWAL et al., 2017). Uma grande concentração de ferro é necessária para a execução desses processos, sendo o período neonatal a fase mais crítica de abastecimento do órgão, já que, é nesse momento que o transporte de ferro para o SNC atinge seus níveis mais altos (PRADO e DEWEY, 2014; RADLOWSKI e JOHNSON, 2013; RAMEL e GEORGIEFF, 2013).

Apesar do intenso tráfego de ferro na fase inicial da vida, a absorção de ferro pelo cérebro ainda ocorre, na idade adulta. Embora, em níveis, significativamente, inferiores, sendo alterado pelo ferro periférico, oriundo da alimentação. Todo esse processo da homeostase do metal é mantido através de um mecanismo bem regulado de absorção, armazenamento e secreção (VENKATARAMANI, 2021).

A deficiência de ferro é uma das mais comuns de nutrientes em todo o mundo (LOPES et al., 2021), afetando, aproximadamente, 1,3 bilhões de pessoas. Esse fato resultou no uso generalizado de alimentos enriquecidos com ferro, como bebidas lácteas, outros líquidos e alimentos sólidos, além da utilização de ferropolimaltose na suplementação infantil, a fim de, prevenir quadros anêmicos. À medida que, a prevalência da deficiência de ferro diminuiu em todas as idades, a dieta aumentada de ferro, acima dos níveis necessários, sugere ser a causa da toxicidade que o metal apresenta no SNC (AGRAWAL et al., 2017). As maiores quantidades de ferro são encontradas no fígado, considerado principal órgão de armazenamento de ferro (CHAI et al., 2015; SCHRÖDER et al., 2013).

O acúmulo de ferro no cérebro ocorre com o envelhecimento humano e correlaciona-se com alterações na função cognitiva (GHADERY et al., 2015). O déficit cognitivo parece estar relacionado a vários fatores, que incluem, o aumento da permeabilidade da barreira hematoencefálica, inflamação, redistribuição do metal no interior do cérebro e desregulação da homeostase do íon, que pode promover neurotoxicidade (DI DOMENICO et al., 2015; SCHRÖDER et al., 2013, WARD et al., 2014). O acúmulo de ferro (>10x da necessidade nutricional) pode induzir estresse oxidativo através da geração de espécies reativas de oxigênio (EROS) (OLIVEIRA et al., 2014; SIAN-HULSMANN et al., 2011) que, consequentemente, podem danificar o DNA e mtDNA (DNA mitocondrial), afetando a expressão do mesmo e, oxidando proteínas (KWOK, 2010; MELIS et al., 2013). Além disso, podendo interferir nas funções mitocondriais (JOMOVA et al., 2010, OLIVEIRA et al., 2014), além de catalisar a geração de estruturas oxidantes e peroxidação lipídica, causando morte celular (KRUSZEWSKI, 2003, WARD et al., 2014), pela apoptose (FARINA et al., 2013) e ferroptose (DIXON et al., 2012).

A ferroptose é uma forma recentemente reconhecida de morte celular regulada. Caracteriza-se morfologicamente pela presença de mitocôndrias menores que o normal com densidades de membrana mitocondrial condensada, redução ou desaparecimento de cristas mitocondriais e ruptura da membrana externa (XIE et al., 2016). Esse processo, é caracterizado pelo acúmulo de produtos de peroxidação lipídica e espécies reativas do oxigênio (EROS) derivadas do metabolismo do ferro, que podem ser inibidas farmacologicamente por quelantes de ferro (por exemplo, mesilato de deferoxamina e desferrioxamina) e inibidores de peroxidação lipídica (por exemplo, ferrostatina, liproxstatina e zileuton) (DIXON et al., 2014).

O ferro circulante existe na forma de ferro férrico [Fe^{3+}], ligado à transferrina. O [Fe^{3+}] é importado para células através do receptor de membrana transferrina 1 (TFR1) e depois se armazena no endossoma. No endosoma, [Fe^{3+}] é reduzido ao ferro ferroso [Fe^{2+}], pela atividade

da enzima ferro redutase. Finalmente, o transportador de metal divalente 1 (DMT1, também denominado SLC11A2) medeia a liberação de [Fe^{2+}] do endossoma para uma associação de ferro lábil no citoplasma (YANG e STOCKWELL, 2008; XIE et al., 2016).

O excesso de ferro é armazenado ligado à ferritina, um complexo protéico de armazenamento de ferro, incluindo a cadeia leve de ferritina (FTL) e a cadeia pesada de ferritina 1 (FTH1). A exportação de ferro é mediada pela proteína de membrana ferroportina (uma bomba de efluxo de ferro, também denominada SLC11A3), que pode oxidar [Fe2+] para [Fe3+]. Células mais sensíveis a ferroptose, convencionalmente expressam mais TRF1 e menos ferritina, aumentando a absorção e o armazenamento em ferro reduzido (YANG e STOCKWELL, 2008; XIE et al., 2016). Nesse sentido, o acúmulo de ferro relacionado com a idade pode ser um fator importante que contribui para processos neurodegenerativos (HARE et al., 2015).

Em uma abordagem mais ampla especula-se que a secreção e deposição da Aβ, assim como, a agregação das placas, ocorram como consequências do estresse oxidativo, contribuindo para a desregulação da homeostase metálica nas sinapses na DA (LEI et al., 2021). Da mesma forma, o desequilíbrio, principalmente, através do aumento da mobilização de Ferro intracelular, resulta em alterações do metabolismo da dopamina, desestabilizando protofibrilas proteicas como α-sinucleína contribuindo para a formação de corpos de Lewy na DP (RIEDERER et al., 2023).

A relação entre sobrecarga de ferro no período neonatal e a neurodegeneração também merece destaque (HARE et al., 2015). Estudos sugerem que a sobrecarga de ferro no período neonatal induz o estresse oxidativo no cérebro adulto (DAL-PIZZOL et al., 2001; DE LIMA et al., 2005, FERNANDEZ et al., 2011), além de, prejudicar a formação da memória (ARCHER et al., 2003, DE LIMA et al., 2005; FREDRIKSSON et al., 2003; ROGALSKA et al., 2009; SCHRÖDER et al., 2001). Investigações indicaram que, a absorção de ferro é máxima durante o período pós-natal, com um pico no dia 15 pós-parto (FREDRIKSSON et al., 2003).

Estudos realizados pelo nosso grupo de pesquisa comprovaram que a sobrecarga de ferro no período neonatal resulta em déficits de memória graves e persistentes em ratos adultos (SCHRÖDER et al., 2001; SILVA et al., 2012). Além disso, estudos clínicos com pacientes com comprometimento cognitivo leve verificaram um padrão para o acúmulo de ferro e sugerem que a deposição no cérebro tenha relevância clínica como biomarcador para cognição (SUN et al., 2017). A técnica de ressonância magnética quantitativa (MRI, do inglês Quantitative Magnetic Resonance Imaging), permitiu mapear o acúmulo de ferro nas regiões do tálamo, caudado e putâmen e determinou uma íntima relação com o déficit de memória visual,

acompanhando o avanço da idade (DARNAI et al., 2017). Os pesquisadores Agrawal et al., (2017), através da uma revisão sistemática de estudos com modelos animais e humanos, baseados na sobrecarga de ferro, fornecem evidências de que, a suplementação de ferro neonatal promove neurodegeneração na região da substância negra, relacionada a DP, no surgimento de proteínas malformadas na DA e de processos neuroinflamatórios.

Sendo assim, o acúmulo de ferro no SNC parece estar relacionado ao surgimento de estresse oxidativo e desbalanço energético neuronal, podendo ser, parte relevante do processo neurodegenerativo e influenciando nas concentrações e na toxicidade de outros íons e neurotransmissores.

1.1.3 Controle da homeostase do cálcio: transportadores de cálcio neuronal

A importância do cálcio para o nosso organismo advém de algumas de suas características fundamentais. O cálcio é capaz de ligar-se e desligar-se de proteínas muito rapidamente, mudando sua conformação e funcionamento. Além disso, os transportadores de cálcio apresentam alta eficiência, já que, sua quantidade fora das células é 10 bilhões de vezes maior que no interior, permitindo, um fluxo intenso, com a abertura de um número reduzido de canais (SUKUMARAN et al., 2021).

Apesar deste gradiente eletroquímico transmembranar favorável, o Ca^{2+} tem a sua entrada na célula restringida, pois é mediada por canais e transportadores específicos existentes na membrana plasmática. As oscilações na $[Ca^{2+}]^{i}$ devem-se não só à sua entrada ou saída da célula através da membrana celular, mas também à sua mobilização das reservas intracelulares (LA ROVERE et al., 2016).

Os íons Ca^{2+} são mensageiros universais, que regulam uma vasta gama de funções celulares importantes, como diferenciação, proliferação, crescimento, sobrevivência, apoptose, transcrição de genes e a excitabilidade da membrana (CAPIOD, 2016; LA ROVERE et al., 2016). A passagem do íon pelas membranas biológicas controla processos vitais para as células, como por exemplo, na plasticidade sináptica (BERRIDGE, 2011). Por sua vez, os neurônios exigem extrema precisão no controle da concentração de Ca^{2+} nos compartimentos para a função adequada.

A maquinaria de sinalização é complexa e inclui vários canais condutores de cálcio e um grande número de proteínas alvo dependentes de cálcio, incluindo cinases, fosfatases, fatores de transcrição e proteínas que induzem a fusão das vesículas sinápticas (BRINI et al.,

2014). Os canais de cálcio podem ser, principalmente, divididos em: Canais de Cálcio Voltagem Dependente (VOC) e Canais de cálcio ligante dependente.

As proteínas da família VOC pertencem a uma família maior de canais seletivos para íons dependentes da voltagem, de estrutura homo ou hetero-oligomérica, e constituídos por várias subunidades diferentes, estando o canal propriamente dito, normalmente, associado à subunidade α (ou α1) (CATTERALL et al., 2019). Nesta família, incluem-se os canais de K^+, Na^+ e Ca^{2+}. Enquanto, os canais de K^+ são estruturas homotetraméricas, os canais de Na^+ e de Ca^{2+} são heterotetraméricos.

O VOC contém um sensor de voltagem que consiste de quatro subunidades ou domínios internos repetidos (I-IV). Cada domínio contém seis regiões trans-membranares (S1-S6) em α–hélice. O domínio I é o responsável pela ativação do canal. A região S4, positivamente carregada, é parte do sensor de voltagem e os domínios situados entre as regiões S5 e S6 constituem o poro do canal (LA ROVERE et al., 2016). De acordo com as suas propriedades farmacológicas e eletrofisiológicas, podem distinguir-se seis tipos de canais de cálcio voltagem dependente: L, N, P, Q, R e T.

Além dos VOC, já mencionados, existem canais de cálcio dependentes de ligantes, como os canais dependentes de nucleotídeos cíclicos (CNGs), que transportam íons cálcio assim que são ativados por três moléculas de adenosina trifosfato (ATP), canais de Ca^{2+} dependentes de cálcio, como as ATPases transportadoras de Ca^{2+} e canais de Ca^{2+} dependentes de inositol trifosfato (IP3), localizados no retículo endoplasmático (RE) das células nervosas e ativados pelo aumento nos níveis intracelulares de IP3 (MALTSEV et al., 2017).

Por fim, os receptores rianodínicos (RyRs), que são uma família de canais de liberação do Ca^{2+} existente em organelas intracelulares, as quais possuem, reservas de cálcio (como o RE ou o retículo sarcoplasmático e as mitocôndrias). Tais moléculas, são estruturas complexas, com grandes domínios citoplasmáticos contendo numerosos sítios de ligação para agentes que controlam o estado de atividade do domínio que forma o canal. As interações existentes entre os domínios citoplasmático e intramembranar parecem controlar a função do canal (DULHUNTY et al., 2017).

O cálcio ainda, pode ser transportado, através de ATPases de Ca^{2+} da família P, que se localizam, quer na membrana plasmática, quer em membranas intracelulares (RE e mitocôndrias), e, catalisam o efluxo ou a captação de Ca^{2+}, respectivamente, usando a energia liberada da hidrólise do ATP. Por fim, o trocador Na^+-Ca^{2+}, que troca íons Na+ por íons Ca^{2+}, através da membrana plasmática. O referido transportador de cálcio é um componente essencial das vias de sinalização em vários tecidos (ROBERTSON et al., 2017).

Os diversos transportadores de cálcio fazem parte de um sistema complexo de sinalização em células neuronais, permitindo a ativação de diferentes processos dependentes de Ca^{2+} separados, espacialmente, porém, em diversos momentos, ao mesmo tempo. Os neurônios são extremamente sensíveis aos níveis de concentração de cálcio e até mesmo alterações sutis na homeostase de Ca^{2+} podem levar a consequências destrutivas alterando a atividade neuronal normal. Várias evidências sugerem que a desregulação de Ca^{2+} desempenha um papel importante no envelhecimento e na neurodegeneração (VERMA et al., 2022).

Desse modo, restaurar a homeostase da sinalização do cálcio apresenta um alvo atraente para a descoberta de fármacos para tratamento de doenças neurodegenerativas (VERMA et al., 2022). Tendo em vista que, apesar dos esforços de pesquisas ativas, esses distúrbios ainda são incuráveis, com a maioria dos medicamentos oferecendo apenas alívio sintomático (CACABELOS, 2017).

1.1.4 O glutamato e o controle da homeostase do cálcio

O glutamato é reconhecido como um dos mais importantes mediadores excitatórios, e, seus receptores, estão presentes na membrana plasmática de uma ampla variedade de neurônios e células (HERTZ e ROTHMAN, 2017). Além disso, esse neurotransmissor, sinaliza eventos em tecidos e órgãos periféricos, bem como, em células endócrinas (HENGJIA et al., 2016), destacando-se o seu papel fundamental na transdução de sinais celulares, formação da rede neuronal durante o desenvolvimento e em eventos como memória, aprendizado e plasticidade sináptica (HUANG et al., 2017).

Um dado interessante na literatura, indica uma longa história evolutiva dos receptores de glutamato. Pois, os mesmos vêm sendo observados em sítios de reconhecimento, de bactérias e plantas com a função de modulação fótica (EGBENYA, et al., 2021).

Por outro lado, o glutamato também está envolvido em processos patofisiológicos, incluindo, dor, trauma, epilepsia e esquizofrenia, dentre outros. De fato, tanto o glutamato, como outros aminoácidos excitatórios em altas concentrações são tóxicos para o sistema nervoso. Como exemplos da ativação excessiva dos receptores de glutamato (GluRs), podemos citar, os eventos observados em situações como na isquemia, trauma cerebral e epilepsia, que podem conduzir à morte de neurônios. Além disso, a exacerbação da estimulação de GluRs pode ocasionar também processos de morte celular em distúrbios neurodegenerativos crônicos, como esclerose amiotrófica lateral, doença de Huntington, DP e DA (ANDERSEN et al., 2021; JIANG et al., 2019).

Os GluRs podem ser divididos, de acordo com sua estrutura, em receptores glutamatérgicos ionotrópicos (iGluRs) e receptores glutamatérgicos metabotrópicos (mGluRs). Os iGluRs governam a transmissão sináptica excitatória rápida. Ativados pelo glutamato, eles promovem a transdução do sinal permitindo o influxo de cátions através de seus canais e, como resultado, rapidamente, despolarizam a membrana dos neurônios pós-sinápticos (WYLLIE e BOWIE, 2022).

A partir de técnicas de biologia molecular houve a clonagem das diversas subunidades dos iGluRs: 4 para AMPAR (receptor ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionico) (BOULTER et al., 1990; KEINANEN et al., 1990; SOMMER et al., 1990), 5 para cainato (BETTLER et al., 1990; HOLLMANN et al., 1989) e 5 para NMDA (IKEDA et al., 1992; MORIYOSHI et al., 1991). Todos os três tipos são permeáveis a cátions, entretanto, a permeabilidade ao Na^+ e ao Ca^{2+} varia de acordo com a família e composição da subunidade do receptor (MELDRUM, 2000).

O outro grupo dos receptores de glutamato, mas ionotrópicos, os receptores NMDA, participam da neurotransmissão excitatória no SNC, através de mecanismos distintos daqueles utilizados pelos GluRs do tipo AMPA. Os GluRs do tipo NMDA apresentam um bloqueio por Mg^{2+} voltagem-dependente e uma alta permeabilidade aos íons Ca^{2+} (SREBRO et al., 2016).

Já os mGluRs são ancorados na membrana celular por sete domínios transmembrânicos, são considerados proteínas grandes (854-1179 aminoácidos). Os 8 subtipos de mGluRs são classificados em 3 subgrupos com base na homologia da sequência de aminoácidos e critérios funcionais: grupo I (mGluR1 e mGluR5), grupo II (mGluR2 e mGluR3) e grupo III (mGluR4, mGluR6-8) (MAO e WANG, 2016; RITTER-MAKINSON et al., 2017).

Os receptores do grupo I, que inclui o mGluR1 e mGluR5 (variantes “splicing”: mGluR1a-e, mGluR5a,b), são ativados fortemente por quisqualato e são acoplados a proteína Gq. A sua ativação estimula a hidrólise do fosfatidilinositol-bifosfato (PIP2), gerando como segundos mensageiros o diacilglicerol (DAG) e IP3. A via do IP3, por sua vez, libera o Ca^{2+} de estoques intracelulares, enquanto o DAG facilita a ativação de proteína cinase C (PKC) (MAO and WANG, 2016). Os receptores do grupo II, mGluR2 e mGluR3, são acoplados à proteína Gi. A ativação desses receptores produz efeitos pleiotrópicos, incluindo a inibição da adenilato ciclase, inibição de canais de Ca^{2+} voltagem-dependentes e a ativação de canais de K^+, inibindo assim a produção de adenosina monofosfato cíclico (AMPc) (RITTER-MAKINSON et al., 2017). Com efeitos intracelulares similares ao grupo II está o grupo III, que inclui os receptores mGluR4, mGluR6, mGluR7 e mGluR8, são acoplados à proteína Gi/Go (VAN HOOK et al., 2017).

Evidências contundentes apoiam as contribuições da hiperativação de receptores glutamatérgicos ("excitotoxicidade") na neurodegeneração, tanto em eventos agudos, quando em processos crônicos de destruição neuronal (BERNARDO et al., 2017). Contudo, os ensaios terapêuticos neuroprotetores, geralmente, são orientados para canais de glutamato de tipo NMDA, altamente permeáveis ao Ca^{2+}. Porém, até o momento, não foi demonstrada grande eficácia terapêutica. Considerando que, a maioria dos canais de glutamato do tipo AMPA são impermeáveis ao Ca^{2+}, um conjunto de evidências confirmou que tipos, relativamente, incomuns do receptor, podem contribuir para lesões neuronais nessas condições (WERNER et al., 2017).

O receptor AMPAR funciona na escala de tempo do milissegundo e serve como um indicador de resistência sináptica. Evidências sugerem que aberrações na função AMPAR levam a uma ampla gama de distúrbios neurodegenerativos, como DA, DP (TRAYNELIS et al., 2010), esquizofrenia e epilepsia (ROGAWSKI, 2013).

Estruturalmente, AMPARs são estruturas tetraméricas de subunidades com uma arquitetura de domínio em camadas (SOBOLEVSKY, 2015). O domínio extracelular (ECD, extracellular domain), é composto de domínios amino-terminais (ATD, amino terminal domains), que desempenham um papel importante na montagem do receptor e os domínios de ligação ao ligando (LBD’s, ligand-binding domains), que ligam agonistas, antagonistas e moduladores alostéricos positivos, que se estende para fora da membrana pós-sináptica e fenda sináptica (figura 1, A). Embora, os detalhes estruturais do AMPAR são conhecidos, como as combinações possíveis entre as subunidades, GluA1, GluA2, GluA3 e GluA4, com cerca de 70% de homologia na sequência de peptídeos (COLLINGRIDGE et al., 2004), as descobertas sobre as estruturas das AMPARs ligadas às suas subunidades auxiliares estão apenas começando a emergir (TWOMEY et al., 2017).

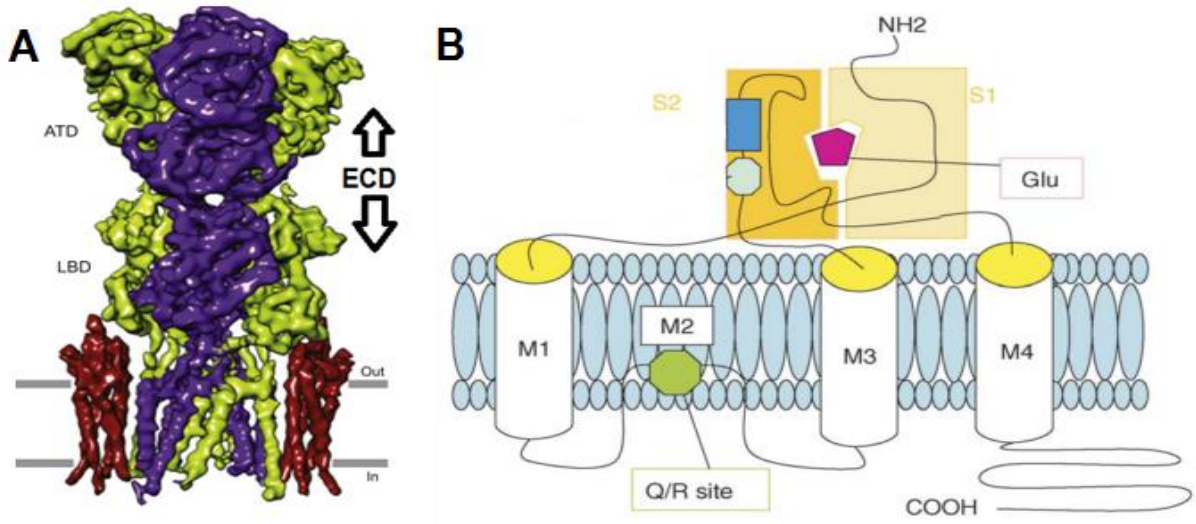

Figura 1: (A) Representação gráfica do domínio extracelular (ECD, extracellular domain), da estrutura amino-terminal (ATD, amino terminal domains) e dos domínios de ligação ao ligando (LBD's, ligand-binding domains) do receptor AMPA (Twomey et al., 2017); (B) Representação dos domínios membranares (M1-M4) e do sítio Q/R. Fonte: KWAK e WEISS (2006).

Enquanto as subunidades GluA1-GluA3 são, particularmente, expressas nas camadas externas do córtex cerebral, hipocampo e amígdala (VAN DER SPEK et al., 2022), a subunidade GluA4 está presente em quantidades mais baixas no SNC, exceto no cerebelo, onde esta subunidade também é abundante (ATANASOVA et al., 2019). Curiosamente, no hipocampo, a expressão das subunidades AMPAR's são diferentemente reguladas ao decorrer do desenvolvimento cerebral. No hipocampo maduro, a composição é, basicamente, de combinações entre GluA1-GluA2 ou GluA2-GluA3 (VAN DER SPEK et al., 2022), enquanto os AMPAR's contendo GluA4 são, principalmente, expressos no desenvolvimento pós-natal inicial (ATANASOVA et al., 2019).

Os receptores AMPA com maior permeabilidade aos íons Ca^{2+} são expressos em subpopulações neuronais discretas, esse número parece ser regulado positivamente em doenças como Esclerose Lateral Amiotrófica (ELA) (HOSAKA et al. 2021) e em eventos agudos como no Acidente Vascular Cerebral (AVC) (ACHZET et al., 2021). Além disso, ao contrário dos canais NMDA, os canais AMPA permeáveis ao Ca^{2+} não são bloqueados por Mg^{2+}, mas, são, altamente permeáveis, a outro cátion endógeno indesejável como o Zn^{2+} (CARRILLO et al., 2020).

A condutância de Ca^{2+} nos receptores AMPA difere marcadamente de acordo com a presença ou não da subunidade GluA2. Os receptores AMPA que contêm pelo menos uma subunidade GluA2 têm baixa condutância de Ca^{2+}, enquanto que aqueles que não possuem uma subunidade GluA2 são mais permeáveis ao Ca^{2+} (ISHIDA et al., 2017). As propriedades do

GluA2 são geradas pós-transcrição por edição do RNA no sítio Q/R no segundo domínio de membrana (M2), durante o qual um códon de glutamina (Q) é substituído por um códon de arginina (R) (OAKES et al., 2017). Essa discrepância ocorre em mais de 99% das transcrições de GluA2 e controla várias propriedades dos AMPAR's, incluindo sua permeabilidade a diferentes íons (OAKES et al., 2017).

A presença de um resíduo carregado positivamente, a arginina, no poro do canal, impede a permeabilidade ao Ca^{2+} (figura 1, B). As análises do RNA a partir de cérebros de ratos adultos demonstraram que quase todo o RNAm GluA2 nos neurônios é modificável, enquanto que nas subunidades GluA1, GluA3 e GluA4 a glutamina mantém a sua posição, demonstrando a permeabilidade aumentada ao Ca2+ nos receptores de AMPA sem a subunidade GluA2 (Oakes et al., 2017).

Em circunstâncias normais, a maioria dos neurônios possui poucos canais AMPA permeáveis ao Ca^{2+}, refletindo a presença de subunidades GluA2 editados com a presença da arginina. Além disso, os canais AMPA não são estáticos, mas passam por regulação dinâmica através de muitos mecanismos, indicando a importância da homeostase neuronal em tal regulação (KAMALOVA e NAKAGAWA, 2021).

Estudos baseados em eventos neuronais pró-inflamatórios, descobriram que, a presença do fator de necrose tumoral-alfa (TNF-alfa) (BEATTIE et al., 2002; OGOSHI et al., 2005) e/ou modificações na transcrição de RNAm do GluA2 (GORTER et al., 1997; PELLEGRINI-GIAMPIETRO et al., 1997) podem resultar na inserção de canais AMPA permeáveis ao Ca2+ na membrana de alguns neurônios.

Destarte, para que seja possível entender a correlação do surgimento dos receptores AMPA permeáveis ao cálcio na doença neurodegenerativa é necessário entender como é regulado o processamento dos receptores e, quais são os fatores e proteínas envolvidas na inserção do mesmo na membrana plasmática do neurônio.

1.1.5 Alvos moleculares no processamento do receptor AMPA

Em condições de atividade neuronal basal, os receptores AMPA medeiam a maioria das correntes excitatórias rápidas, influenciando diretamente na força da resposta sináptica (GIRALT et al., 2017). Por outro lado, os receptores NMDA, em potenciais de membrana próximos do repouso, permanecem inativos devido a um bloqueio de canal mediado pelo íon magnésio. Esse, dependente de uma despolarização de membrana inicial, desencadeado, parcialmente, pela ativação do receptor AMPA. A ativação do NMDAR e o influxo de cálcio

subsequente são críticos para a indução de diversas formas de plasticidade sináptica, como a potenciação de longa duração (LTP) e a depressão de longa duração (LTD) (GIRALT et al., 2017; YANG et al., 2017).

A interrelação entre os receptores ionotrópicos de glutamato, faz com que existam diversos alvos moleculares, promotores de distúrbios sinápticos, como por exemplo, possíveis modificações no tráfego e distribuição celular da AMPAR, pós-tradução e pós-transcrição celular, que, apesar de não estarem, diretamente, ligados aos efeitos excitatórios de longa duração, são fundamentais no processo de aprendizagem e formação de memória (OPAZO e CHOQUET, 2011).

1.1.5.1 Regulação pós-traducional do receptor AMPA

As propriedades e a função das AMPARs podem ser moduladas por modificações pós-traducionais, tais como glicosilação, palmitoilação e fosforilação.

A glicosilação é uma modificação protetora que pode ocorrer em 4 a 6 regiões diferentes localizadas nos domínios extracelulares de cada subunidade AMPAR. A N-glicosilação facilita a maturação do receptor e protege-os da degradação proteolítica (ZHENG et al., 2015).

A palmitoilação é uma acetilação gordurosa reversível que regula o tráfego de proteínas e a localização celular. Todas as subunidades AMPAR podem sofrer esse processo através da inserção de dois resíduos de cisteína em seu domínio transmembranar TM2 e em sua região C-terminal intracelular. A primeira palmitoilação no TM2 leva a um acúmulo de AMPAR no aparelho de Golgi, resultando em uma diminuição da expressão do receptor na superfície celular. Da mesma forma, palmitoilação no C-terminal contribui para a internalização dos receptores, reações conhecidas por estabilizar a expressão de AMPAR na superfície celular (HAN et al., 2015; HUGANIR e NICOLLI 2013; JEYIFOUS et al., 2016).

A fosforilação acontece, principalmente, nas subunidades GluA1 e GluA2. Até o momento, quatro locais de fosforilação foram relatados para a subunidade GluA1 (MAO et al., 2013; POUGNET et al., 2016), todos residentes no domínio C-terminal intracelular. O primeiro local identificado foi na serina 831 (S831) propenso a ser fosforilado pela proteína quinase C (PKC) (Jenkins et al., 2014) e pelo complexo proteína-cinase dependente de cálcio-calmodulina (CaMKII) (FRAIZE et al., 2017; WHITEHEAD et al, 2017). Além disso, na serina 845 (S845) foi identificada como um substrato da fosforilação pela proteína cinase A (PKA) (BABIEC et al., 2016). A serina 818 (S818) foi descoberta mais tarde como um substrato de PKC (JENKINS

et al., 2014), assim como a treonina 840 (T840) considerada o principal local de fosforilação regulatória dessa enzima em GluA1 (BABIEC et al., 2016).

A fosforilação destes resíduos é considerada importante para a regulação do tráfego e inserção sináptica das subunidades GluA1, e desempenha um papel importante em cadeias prototípicas de plasticidade sináptica no hipocampo, no surgimento da LTP e LTD, dependentes de NMDA. Esses eventos sinápticos de plasticidade estão associados à fosforilação e à desfosforilação, respectivamente, desses locais na GluA1 (PARK et al., 2016).

A subunidade GluA2 também é fosforilada em dois locais principais. Serina 880 (S880) é o local de fosforilação para PKC localizado no domínio C-terminal (SANTERRE et al., 2014), e outro no resíduo de tirosina (Y876), local que é um substrato para a família de proteínas Src-Tirosina cinases que produz um efeito semelhante à fosforilação no S880 (KOHDA et al., 2013). Assim, a fosforilação no GluA2 desempenha um papel na ligação diferencial do GluA2 às proteínas que contenham domínios que interagem com o receptor (KOHDA et al., 2013; SANTERRE et al., 2014), promotoras, por exemplo, da internalização dos receptores de superfície, que é um evento crítico para a indução de LTD (CORTESE et al., 2016; WERNER et al., 2017).

1.1.5.2 Interações específicas na subunidade do receptor AMPA

A inserção do receptor AMPA na membrana sináptica envolve uma regulação rigorosa, através de eventos que dependem da composição da subunidade do receptor e, de sinais específicos contidos no domínio c-terminal. As principais informações sobre mecanismos que regulam o tráfego dos AMPARs vieram da descoberta de que proteínas que contêm domínios PDZ (um acrónimo que combina as primeiras letras de três proteínas, PSD95, DlgA e Zo-1, descobertos, primeiramente, compartilhando esse domínio), que desempenham papéis gerais nas proteínas de membrana (SILVERMAN et al., 2007; WARD e KANNEDY, 2015).

Os domínios PDZ são sequências peptídicas modulares de interação proteína-proteína, que contêm três repetições de aproximadamente 90 aminoácidos (PONTING et al., 1997; SONGYANG et al., 1997). A maioria das interações mediadas pelos domínios PDZ ocorrem através do reconhecimento de uma sequência curta localizada no extremo c-terminal da proteína de ligação, promovendo o agrupamento de canais iônicos e receptores na membrana plasmática, bem como, o alinhamento de quinases e fosfatases para seus substratos (GARNER et al., 2000; SONGYANG et al., 1997).

Devido à localização do mRNA dos AMPAR (assim como de diversas proteinas de membrana) no corpo celular neuronal, é necessário um citoesqueleto microtubular para o transporte do mesmo até os longos processos dendríticos (HANLEY, 2014; ESTEVES DA SILVA et al., 2015). Embora, os dendritos apresentem microtúbulos ao longo de seu trajeto, as espinhas dendríticas são desprovidas de citoesqueleto microtubular, e são mais enriquecidos por filamentos de actina altamente móveis (FISCHER et al., 1998). Portanto, em algum momento, as organelas contendo os receptores AMPA, no tráfego ao longo das trilhas microtubulares, devem ser transferidas para o citoesqueleto baseado em actina para a entrega final nas sinapses (HANLEY, 2014). As miosinas são as principais proteínas motoras dependentes de actina que apresentam envolvimento no transporte de AMPAR, visto que, a diminuição da expressão da proteína Miosina VI em neurônios de ratos provocou um acúmulo de GluA1 no soma e reduziu a expressão superficial desta subunidade (ESTEVES DA SILVA et al., 2015).

A proteína 4.1N é uma proteína que interage com GluA1, pertencente a uma família de componentes de citoesqueleto multifuncionais e que é essencial para montagem e manutenção do citoesqueleto da actina, agindo como uma actina e permitindo a chegada da subunidade na superfície. A proteína 4.1N é altamente expressa em sinapses excitatórias, onde interage e se liga à região da membrana intracelular de GluA1. Assim, 4.1N serve como um adaptador para a ligação da subunidade ao citoesqueleto de actina, o que sugere que os AMPAR contendo GluA1 são entregues às membranas das sinapses através de filamentos de actina (COPITS e SWANSON, 2013; DOUYARD et al., 2007; LIN et al., 2009;).

As proteínas PDZ também interagem com a região c-terminal das subunidades GluA2, através da ligação de PDZ de tipo II, em oposição a GluA1 que está em conformidade com um ligante de PDZ de tipo I.

As proteínas GRIP e ABP, ambas contendo sete domínios PDZ consecutivos (BRAITHWAITE et al., 2002; LU e ZIFF, 2005; SRIVASTAVA e ZIFF, 1999) localizam-se em sinapses e em vesículas pós-Golgi, sugerindo um possível papel no tráfego dos AMPAR para os dendritos. Mutações do sítio de ligação GluA2/PDZ que bloqueiem seletivamente a sua ligação a ABP e a GRIP aceleram a endocitose do GluA2 em sinapses (OSTEN et al., 2000), identificando ABP e GRIP como âncoras que são cruciais para a acumulação sináptica do receptor (SANTOS et al., 2009).

1.1.5.3 Intermediários transmembranares do receptor AMPA

Os AMPARs contêm proteínas reguladoras transmembranares (TARPs), com suas subunidades auxiliares (SULLIVAN et al., 2017). Essas proteínas são subdivididas em γ-2 (ou stargazin), γ-3, γ-4, γ-8, γ-5, γ-7 (SUMIOKA et Al., 2010). A stargazin, foi a primeira subunidade auxiliar identificada do AMPARs e foi originalmente caracterizada como o gene mutante no mouse *stargazer*, que exibe um fenótipo atáxico e epiléptico, resultante da falta de funcionalidade dos canais AMPAR em células granulares cerebelares (SEMENOV et al., 2012; SHAIKH et al., 2016).

Estas proteínas contêm quatro domínios transmembranares e seu terminal carboxílico interage com os domínios PDZ do PSD-95 (CHEN et al., 2000). As TARPs parecem coexistir com os AMPARs no início da via sintética e controlam suas dobras, montagem e maturação, estabilizando e facilitando sua exportação a partir do retículo endoplasmático até sua inserção na membrana plasmática. Dessa forma, promovem a expressão na superfície dos AMPARs e são críticos para o agrupamento AMPARs em sinapses excitatórias através da sua interação com PSD-95 (CHEN et al., 2000; SANTOS et al., 2009; SUMIOKA et al., 2010).

Em um estudo, outra família de proteínas transmembranares, as cornichon, foram identificadas por interagir com as subunidades AMPARs (SCHWENK et al., 2009). De acordo com esse estudo, a maioria dos AMPARs no cérebro do rato co-existem com dois membros da família de proteínas cornichon transmembranares (homólogos de cornichon 2 e 3), no lugar das proteinas TARPs. Além disso, as proteinas Cornichon aparentemente aumentam a expressão superficial de AMPARs em células cultivadas (SCHWENK et al., 2009).

1.1.5.4 Inserção dos receptores AMPA

A localização na membrana dos AMPARs é um processo altamente dinâmico e seu controle é importante devido a sua influência na plasticidade sináptica e desenvolvimento. Um dos últimos passos na longa jornada de AMPARs para a sinapse é a sua entrega na membrana dendrítica especializada que constitui a função pós-sináptica terminal (GROC e CHOQUET, 2006). No entanto, a segmentação e a inserção precisa dos receptores são extremamente complicadas. Apesar do estudo intenso, ainda não está claro se os AMPARs são primeiramente inseridos na membrana plasmática extrasináptica ou diretamente nas sinapses. Algumas teorias sustentam a ideia de que os AMPARs são primeiro exocitados para a membrana plasmática em sítios extrasinápticos, seguido de sua difusão lateral na superfície neuronal até chegarem aos

dendritos para finalmente o aprisionamento nas sinapses através de ancoragem pelas PSDs (figura 2) (GROC e CHOQUET, 2006).

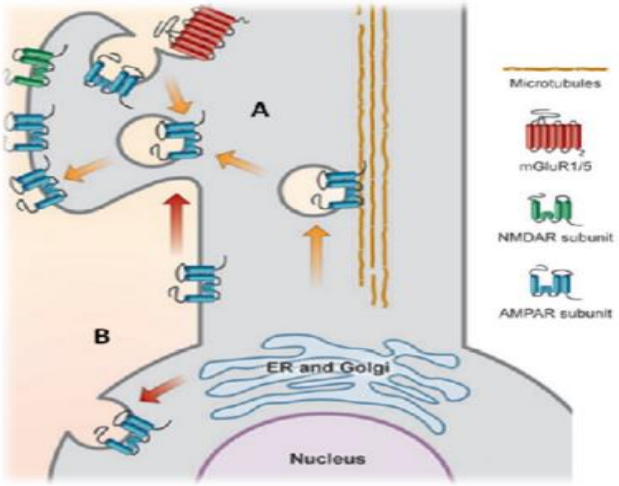

Figura 2: Modelo de ancoragem sináptica dos receptores AMPA. Fonte: GROC e CHOQUET (2006).

Além disso, muitos estudos mostraram que os mecanismos que regulam a inserção dos AMPARs são específicos para cada subunidade. Enquanto a inserção de GluA2 é rápida e ocorre constitutivamente em condições basais, sem a necessidade de atividade sináptica, a inserção de GluA1 é lenta, mas induzível, exigindo, por exemplo, a ativação de NMDARs (PASSAFARO et al., 2001). Embora, ambas as subunidades se concentrem em sinapses, o acúmulo das subunidades GluA2 nestas estruturas é mais rápida do que a subunidade GluA1. Assim, é dada a hipótese que essa diferença no processo de inserção, define que o GluA1 é inserido inicialmente em sítios extrasinápticos, enquanto que a subunidade GluA2 é inserida mais diretamente nas sinapses, ou que ambas as subunidades são inseridas em sítios extrasinápticos, mas o GluA2 difunde-se mais rapidamente na membrana e, assim, se acumulam mais rapidamente nas sinapses (GROC e CHOQUET, 2006).

Assim, dado que os AMPARs endógenos consistem, principalmente, em heterólogos GluA 1/2 ou GluA2/3 e que os sinais de tráfego GluA1 dominam GluA2 no controle da inserção (SHEPHERD e HUGANIR, 2007), um modelo simples foi proposto com base nessas regras de tráfico específicas da subunidade: Os receptores contendo as subunidades GluA2/3 estão continuamente fazendo um ciclo dentro e fora das sinapses, preservando o número total de AMPARs sinápticos (a via constitutiva), enquanto que, os receptores com subunidades GluA1/2 são adicionados em sinapses de uma forma dependente da atividade durante a plasticidade

sináptica (o caminho regulado) (MALINOW et al., 2002). Portanto, a via constitutiva pode manter resistência sináptica, apesar do turnover da proteína, e a via regulada pode atuar transitoriamente sobre a indução de plasticidade sináptica (SHEPHERD e HUGANIR, 2007).

Além do seu tráfico e inserção sináptica, a expressão de AMPARs na sinapse também depende da sua internalização regulada (STEINMETZ et al., 2016). Acredita-se que, esse processo ocorra através de endocitose mediada por clatrina, semelhante à endocitose estimulada dos receptores acoplados à proteína G.

1.1.6 Neuroproteção mediada pelo perampanel, antagonista não competitivo de AMPAR

O PER é um antagonista alostérico não competitivo do receptor AMPA, altamente seletivo, interferindo tanto nos receptores AMPA impermeáveis aos íons Ca^{2+}, quanto nos permeáveis (BARYGIN, 2016). Até à data, não há evidências de que o PER atue sobre outros canais de íons. Hanada et al. (2011), descreveram a seletividade do PER sobre AMPAR's através da diminuição do $[Ca^{2+}]^i$ em neurônios corticais de ratos, apresentando efeito sobre NMDAR's apenas nas doses acima de 30μM de PER, muito acima das concentrações necessárias para a inibição do receptor AMPA (0,23 μM) (CHEN, et al., 2014). Da mesma forma, a inibição das correntes $[Ca^{2+}]$ desenvolvidas por glutamato e kainato, na presença do PER, não diferem significativamente (BARYGIN, 2016).

O PER [2-(2-oxo-1-fenil-5-piridin-2-il-1,2-di-hidro-piridin-3-il) benzonitrilo] é uma nova estrutura química não quiral (figura 3). Sua fórmula molecular é C23H15N3O, com peso molecular de 349,4g/mol. Existe como um pó não higroscópico branco a ligeiramente colorido, é insolúvel em água e ligeiramente solúvel em soluções ácidas, com um pKa de 4.73. São conhecidas cinco formas polimórficas anidras e uma forma de hidrato.

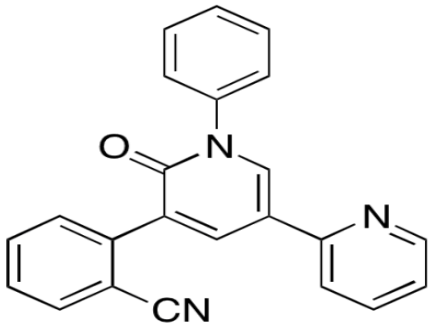

Figura 3: Estrutura química do Perampanel (PER). Fonte: Do autor.

O PER foi aprovado em 2012, pela Agência Europeia de Medicamentos (EMA), como um fármaco antiepilética (DAE), adicional para convulsões de início parcial em pacientes com idade igual ou superior a doze anos. A eficácia e a segurança do PER foram analisadas em três ensaios clínicos randomizados (FRENCH et al., 2012; FRENCH et al., 2013). As taxas de pacientes responsivos ao tratamento aumentou, paralelamente, à dose do fármaco, foram 28,5% (PER 4 mg), 35,3% (PER 8 mg) e 45,0% (PER 12 mg). Houve uma ótima tolerância nesses estudos, com um pequeno número de pacientes que sofreram de efeitos secundários, como sonolência, fadiga, tonturas, náuseas, quedas e efeitos adversos neuropsiquiátricos como agressão, depressão e irritabilidade (STEINHOFF et al., 2013), os mesmos sempre associados a pacientes com presença de polifarmácia.

Estudos de fase IV, dão suporte para a utilização de PER em pacientes com a Síndrome das Pernas Inquietas, associado ao uso de fármacos que agem na via dopaminérgica (GARCIA-BORREGUERO et al., 2017) e outras patologias baseadas na perda do controle do disparo neuronal como, no caso da doença de Lafora (LD), uma epilepsia mioclônica progressiva e fatal, de caráter autossômico recessivo, que atinge adolescentes previamente saudáveis. Nesse caso, o PER levou à remissão sustentada da mioclonia e das convulsões tônico-clônicas generalizadas (DIRANI et al., 2014; SCHORLEMMER et al., 2013). Em casos de mioclonia pós-hipóxia (sindrome de Lance-adams), o PER apresentou efeitos importantes e reprodutíveis (STEINHOFF et al., 2016), assim como, em casos de epilepsia mioclônica juvenil (FRENCH et al., 2013) e outros quadros epiléticos refratários (REDECKER et al., 2015).

Semelhante a outros antiepiléticos que são metabolizados predominantemente pelo fígado (AHMED e SIDDIQI, 2006), o PER é amplamente metabolizado através de enzimas CYP3A4 e CYP3A5, sendo a principal via a oxidação e subsequente a glucuronidação (REKTOR, 2013; ROGAWSKI e HANADA, 2013).

Como demonstrado por Wu et al. (2017), a neuroproteção proporcionada pelo PER apresenta íntima relação com os níveis elevados de glutamato e com áreas que apresentam maior aumento do influxo de Ca^{2+} para o citoplasma celular. No modelo de indução de estados epiléticos por lítio-pilocarpina, o PER apresentou neuroproteção parcial nas regiões da sub-região CA1 do hipocampo, o córtex piriforme, regiões que desempenham o papel de inicialização da crise, mediada por estímulos colinérgicos e neuroproteção total no tálamo mediodórtrico (MD), região que recebe os disparos hipotalâmicos e límbicos e propagam a manutenção do estado pela via glutamatérgica. Isso pode explicar por que PER confere maior controle de crises em pacientes com generalização secundária e processos tônico-clônicos (FRENCH et al., 2013; STEINHOFF et al., 2013).

O efeito do PER como neuroprotetor em modelos animais, foi descrito em diversos estudos. Como por exemplo na pesquisa de Kawakita et al. (2023), que avaliaram o efeito do PER em um modelo de lesão cerebral, no estudo o antagonista AMPAR foi capaz de diminuir citocinas pró-inflamatórias como as interleucinas-1β e -6 e suprimir as vias de apoptose neuronal (KAWAKITA et al., 2023). O efeito neuroproter do PER, diminuindo citocinas inflamatórias foi registrado em uma pesquisa pré-clínica de lesão cerebral, nela, houve uma inibição da necroptose e da neuroinflamação, diminuição da morte neuronal *in vitro* e *in vivo* (YANG et al., 2021), assim como, no estudo de Chen et al (2021), onde o PER, da mesma forma, inibiu a ativação da caspase3 e a expressão e a fosforilação dos fatores necroptóticos (CHEN et al., 2021).

Poucos estudos demonstram a eficácia do PER na neuroproteção de quadros demenciais, não permitindo suporte para seu emprego clínico nesse âmbito. Há apenas um relato de caso (DOLTON e CHOUDRY, 2014) e um estudo de Shah et al., (2016), que incluiu um subgrupo de pacientes com "dificuldades de aprendizagem", porém, o mecanismo de envolvimento da droga não foi especificado.

1.1.7 Justificativa

A utilização de quelantes de ferro demonstrou em diversos estudos, ser capaz de prevenir o aumento de RL e a morte celular, sugerindo uma estreita relação entre a peroxidação lipídica e o metabolismo do ferro (DIXON et al., 2013), em consonância a esse conhecimento Yang et al., (2008), elucidaram uma importante via de depleção da glutationa intracelular, pelo processo de ferroptose, mediado pelo sistema cistina/glutamato Xc^-, esgotando os níveis de glutationa nas células astrocitárias e gliomas, resultando na perda da proteção contra RL.

Em estudos prévios realizados por nosso grupo de pesquisa, foi demonstrado que o tratamento neonatal, em ratos com sobrecarga de ferro está associado a parâmetros aumentados de estresse oxidativo, como peroxidação lipídica e carbonilação de proteínas na idade adulta (DAL-PIZZOL et al., 2001). Mais recentemente, também foi comprovada a relação do ferro com a expressão de proteínas envolvidas na dinâmica de fissão e fusão mitocondrial, além de comprometer a função mitocondrial e induzir apoptose pela via intrínseca (mitocondrial) (DA SILVA et al., 2014; 2018a; 2018b).

A utilização de bloqueadores do influxo de Cálcio demonstrou diminuir a morte celular induzida por peróxido de hidrogênio (H_2O_2), mediador de processos de estresse oxidativo

(RAJASEKARAN e KALAIVANI, 2015), sugerindo que o acúmulo de cálcio intracelular auxilia no surgimento de vias apoptóticas pela sobrecarga de RL's. Um dos componentes relacionados à presença de distúrbios no influxo de cálcio é a inserção membranar de receptores AMPA permeáveis ao cálcio (CP-AMPAR), potencializando a toxicidade do íon. Fatores como a presença de citocinas como o TNF-alfa, na neuroinflamação e estresse oxidativo neuronal parecem mediar a diferenciação do receptor (KWAK e WEISS, 2006).

De fato, a contribuição das CP-AMPARs para a etiologia de doenças neurodegenarativas foi cuidadosamente revisada (LIU et al., 2017) e o potencial de utilizar AMPARs como estratégia terapêutica começa a ser explorado (HENLEY e WILKINSON, 2013; WAKAYAMA et al., 2017), dada a crescente evidência que implica a presença de CP-AMPARs no início de diversas patologias sinápticas (KWAK e WEISS, 2006; KOPPENSTEINER et al., 2017; RAJASEKARAN e KALAIVANI, 2015; SUYAMA et al., 2017). No entanto, para desenvolver efetivamente novas metodologias para modular a função CP-AMPAR, primeiro se faz necessária uma melhor compreensão dos mecanismos que levam à inserção de CP-AMPAR, bem como, os mecanismos pelos quais o CP-AMPAR funciona aberrantemente na presença de diferentes dirtúrbios neuronais. Ao fazê-lo, será possível sugerir abordagens através de inibidores dos receptores de AMPA.

Um estudo recente, demonstrou que a sobrecarga com ferro provocou um aumento nos níveis de AMPAR no córtex pré-frontal, bem como, um aumento nos níveis dos receptores NMDA no hipocampo e no córtex pré-frontal em ratos (HAN e KIM, 2015). Em outro estudo, sugeriu-se que, a ativação de receptores NMDA regulou o influxo de ferro intracelular por meio do transportador DMT1 (sigla dos termos em inglês, *Divalent Metal Transporter 1*) (WHITE et al., 2016). Assim, estudos abordando uma possível interação entre o sistema glutamatérgico e a sobrecarga com ferro contribuirão para o entendimento da participação desses fatores em doenças neurodegenerativas.

Portanto, o presente estudo se propõe a avaliar o efeito da sobrecarga de ferro neonatal sobre a expressão das subunidades do receptor AMPA em ratos e, verificar o efeito neuroprotetor do antagonista alostérico do receptor AMPA, PER, no déficit cognitivo e perda de memória, bem como, investigar seu possível mecanismo de ação.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Avaliar os efeitos da sobrecarga com ferro neonatal sobre a expressão das subunidades do receptor AMPA (AMPAR) e proteínas relacionadas à funcionalidade do AMPAR e investigar os efeitos do uso subcrônico de PER, um antagonista não competitivo de AMPAR sobre o déficit cognitivo induzido pela sobrecarga com ferro, em ratos Wistar Adultos.

1.2.2 Objetivos específicos

- Realizar uma revisão da literatura sobre o papel dos receptores AMPA permeáveis ao Ca2+ em doenças neurodegenerativas, tema relacionado ao referencial teórico do projeto.

- Avaliar o efeito da administração subcrônica de PER em ratos adultos que receberam ferro ou veículo no período neonatal, sobre o comportamento motor e exploratório em teste de campo aberto; sobre a memória através dos modelos de esquiva inibitória e teste de reconhecimento de objetos.

- Avaliar o efeito subcrônico com PER em ratos adultos sobre os níveis totais e as formas fosforiladas das subunidades GluA1 e GluA2 do receptor AMPA, após o tratamento com a sobrecarga de ferro no período neonatal.

- Avaliar o efeito subcrônico com PER em ratos adultos sobre a expressão gênica das subunidades GluA1 e GluA2 do receptor AMPA, após o tratamento com a sobrecarga de ferro no período neonatal.

- Avaliar o efeito uso subcrônico com PER em ratos na fase adulta sobre a expressão da proteína andaime PSD-95, e da proteína transmembranar Stargazin, após o tratamento com a sobrecarga de ferro no período neonatal.

2 CAPÍTULO 2

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MINI-REVIEW

The Role of Ca^{2+} Permeable AMPA Receptors in Neurodegeneration, Neurotoxicity, and Neuroinflammation

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Abstract: It is believed that degenerative conditions that give rise to neurological diseases may share an abnormal influx of Ca^{2+}, mainly through glutamate receptors. Current research on the glutamatergic system indicates that the *N*-methyl-D-aspartate receptor (NMDAR) is not the only receptor permeable to Ca^{2+}. Under certain conditions, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are able to rapidly and potently mediate a neurotoxic Ca^{2+} influx. AMPARs are encoded by four genes designated *GluR 1-4*. The presence of the edited GluA2 subunit makes the heteromeric AMPAR impermeable to Ca^{2+} (CI-AMPAR's). On the other hand, the lack of GluA2 or disruptions in its post-translational editing result in Ca^{2+}-permeable AMPA receptors (CP-AMPARs). In addition to triggering behavioral changes, the increase in CP-AMPARs is documented in several neurodegenerative, neuroinflammatory and neurotoxic conditions, demonstrating that AMPAR changes may play a role in the emergence and evolution of pathological conditions of the central nervous system (CNS). Seeking to better understand how CP-AMPARs influence CNS neuropathology, and how it may serve as a pharmacological target for future molecules, in this article, we summarize and discuss studies investigating changes in the composition of AMPARs and their cellular and molecular effects, to improve the understanding of the therapeutic potential of the CP-AMPAR in neurodegenerative, neurotoxic and neuroinflammatory diseases.

Keywords: AMPA receptors, calcium-permeable AMPA receptors (CP-AMPAR), neurodegenerative diseases, calcium, pharmacological target, neurotoxicity.

1. INTRODUCTION

Compelling evidence suggests that hyperactivation of glutamatergic receptors contributes to pathological processes in the Central Nervous System (CNS), in both acute events and chronic processes associated with neuronal damage. However, experimental anti-neurotoxic and neuroprotective therapeutic strategies, often focusing on highly Ca^{2+} permeable *N*-methyl-D-aspartate (NMDA) glutamate receptors, have not yet demonstrated significant therapeutic efficacy in the clinical setting. Although typically most α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (AMPARs) are impermeable to Ca^{2+}, a body of evidence suggests that unusual types of AMPARs may contribute to neuronal injury, which could justify the failure of therapies aimed at selectively blocking NMDA receptors [1].

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The structure of AMPAR has been well characterized and consists of combinations of GluA1, GluA2, GluA3, and GluA4 subunits, which show approximately 70% homology in the peptide sequence [2]. However, the understanding of the possible relationship between distinct receptor subunit assembly and neurodegenerative diseases is just beginning to emerge [3]. The Ca^{2+}-permeability of AMPAR (CP-AMPAR) varies, depending on whether the GluA2 subunit is present within the tetramer. The ability of GluA2 subunits to regulate AMPAR Ca^{2+}-permeability in turn depends on RNA editing. RNA editing is a post-transcriptional modification that alters a codon encoding glutamine (Gln; Q) to a codon encoding arginine (Arg; R) in the GluA2 sequence [4]. The positive charge under physiological pH and the bulkier side chain of arginine confers GluA2-containing AMPARs with their generic properties, such as impermeability to Ca^{2+}, linear current-voltage (I-V) relationship, and insensitivity to polyamines [5]. However, a subpopulation of AMPARs lacks the GluA2 subunit and displays Ca^{2+} permeability, sensitivity to polyamines, and inward rectification of current [6].

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An activity-induced switch in AMPAR phenotype from GluA2-lacking to GluA2-containing receptors was first observed at the parallel fiber to the stellate cell synapses in the cerebellum following high frequency presynaptic stimulation [7]. Evidence suggests that aberrations in AMPAR function might be associated with a wide range of neurological disorders, including Alzheimer's disease (AD) [8], schizophrenia, epilepsy [9], memory loss [10], movement disorders, Parkinson disease (PD) [11], Amyotrophic Lateral Sclerosis (ALS) [12] and neuroinflammatory processes [13].

Considering the variability in AMPAR structure, the diversity of pathways that can influence AMPAR assembly, and the emergence of forms permeable to calcium, we face a large gap in the knowledge of AMPAR participation in neurodegenerative disorders, making it important to better understand both the physiology of the receptor and its role in the pathogenesis of CNS diseases. Therefore, the present study aims to review critical aspects of AMPAR traffic control and synaptic insertion, and discuss the current evidence on the involvement of CP-AMPAR in ALS, PD, AD, toxicity, neuroinflammation, and cerebral ischemia.

2. CP-AMPAR AND NEURODEGENERATIVE DISORDERS

Structurally, AMPARs are tetrameric structures composed of GluA1-4 subunits with a layered domain architecture [14]. While the GluA1-GluA3 subunits are particularly expressed in the outer layers of the cerebral cortex, hippocampus, and amygdala [15], the GluA4 subunit is present in lower amounts throughout the CNS, but heavily expressed in the cerebellum [16]. Interestingly, in the hippocampus, the expression of AMPAR subunits is differentially regulated during brain development. While in the mature hippocampus, the composition is a combination between either GluA1-GluA2 or GluA2-GluA3 [17], AMPARs containing GluA4 are mainly expressed in the early postnatal period [18]. This information corroborates that AMPAR subunits with the highest prevalence in the regions that are the target of the main neurodegenerative and neuroinflammatory diseases, apparently, are the GluA1 and GluA2 subunits.

The GluA1 subunit undergoes two-step dependent synaptic trafficking, the first being the phosphorylation of Ser845 by cGMP-dependent protein kinase (PKG) and cGMP-dependent protein kinase II (cGKII), this process promotes the accumulation of receptors in the extra-synaptic sites [19]. Following the lateral diffusion to the synapse, the second step that begins with the phosphorylation of Ser818 by protein kinase C (PKC), which stabilizes AMPARs within the synapse [20], and both stages are stimulated by the inflammatory cascade. Other processes such as Ser567 and Ser831 phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) also contribute to synaptic uptake and extra-synaptic targeting, respectively [21].

The GluA2 subunit is phosphorylated primarily at two sites, Ser880, a phosphorylation site located at the C-terminal domain by PKC [22], and Tyr876, a site which is a substrate for the Src-Tyrosine kinase family of proteins that produces a similar effect to phosphorylation at Ser880 [23]. Thus, phosphorylation in GluA2 plays an important role in subunit binding to domain-containing proteins that have the function of internalizing surface receptors [1]. Ca^{2+} conductance through AMPAR differs markedly according to the presence or absence of the GluA2 subunit. AMPARs containing at least one GluA2 subunit have low Ca^{2+} conductance, whereas those that do not have a GluA2 subunit are permeable to Ca^{2+} [24]. The properties of GluA2 are introduced after transcription by RNA editing at the Q/R site in the second membrane domain (M2), during which a codon of glutamine (Q) is replaced by an arginine (R) codon [25]. This alteration occurs in over 99% of GluA2 transcripts and controls various properties of AMPAR including its permeability to different ions (Fig. **1**). The presence of the positively charged arginine residue in the canal pore avoids Ca^{2+} access. The analysis of mRNAs from adult mouse brains has demonstrated that almost all GluA2 mRNAs in neurons are

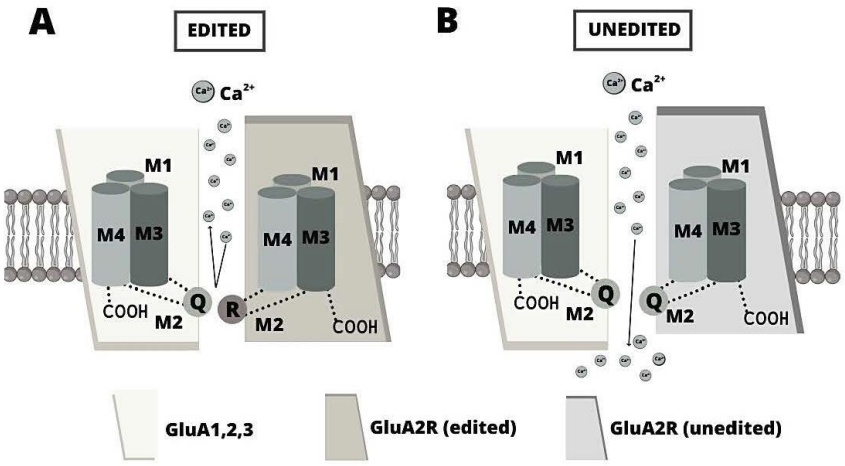

Fig. (1). The AMPA receptor with its four-membrane domain (M1-M4) and Ca^{2+} permeability. (**A**) An AMPA receptor with the GluA2 subunit edited at the Q/R site in M2, preventing calcium entry through the pore. (**B**) Ca^{2+} permeable AMPA receptors without the GluA2 subunit editing at the Q/R site, allowing calcium entry into the cell. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

modifiable, whereas in GluA1, GluA3, and GluA4 glutamine subunits maintain their position, which demonstrates an increase in Ca^{2+} permeability at AMPA receptors without the GluA2 subunit [25, 26].

A sustained increase in intracellular calcium, per se, can lead to neurodegeneration and cell death. However, calcium homeostasis deregulation can also affect the fate of proteins involved in the pathogenesis of the disease, as it is the case for tau and β-amyloid peptide (Aβ) in AD. In addition to the Aβ plaques, histopathological studies of the AD brain have revealed the presence of dramatic ultrastructural changes triggered by other lesions, the neurofibrillary tangles, which consist of aggregated hyperphosphorylated tau proteins [27]. The state of tau phosphorylation and proteolysis can be regulated by calcium-dependent mechanisms. CaMKII can phosphorylate tau [28], Cyclin-dependent kinase 5 (cdk5), another kinase involved in tau phosphorylation [29], is indirectly activated by the calcium-activated protease calpain (Fig. **2**).

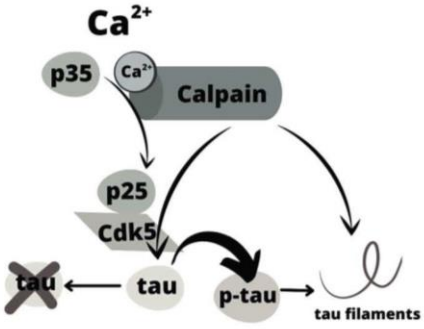

Fig. (2). Scheme of the tau phosphorylation pathway in Alzheimer's disease (AD) and the relationship to calcium-activated calpain. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

In addition to its impact on AD, Ca^{2+} accumulation appears to be involved in the dysregulation of dopamine levels in PD through increased production of reactive oxygen species (ROS) and cellular damage and in the neuroinflammation process in ALS, mainly through the induced loss of the blood-brain barrier (BBB) integrity, influencing autoimmune mechanisms and by stimulating neurodegenerative processes in central nerve cells [30].

One of the possible mechanisms for neurodegeneration caused by the insertion of CP-AMPARs into the neuronal membrane is Ca^{2+} accumulation. Therefore, several studies have shown that increased GluA1 subunit gene expression results in the synaptic insertion of a larger number of CP-AMPARs [31], demonstrating the unique relationship between receptor expression, traffic and insertion in a variety of animal models that investigate CNS disorders. Accordingly, the increased expression of GluA1 subunits, and the consequent increased CP-AMPAR, have been characterized in brain areas in studies using animal models of PD [32], AD [33], ALS [34] and neuroinflammation [35] (Fig. **3**), supporting the involvement of CP-AMPAR in neurodegeneration.

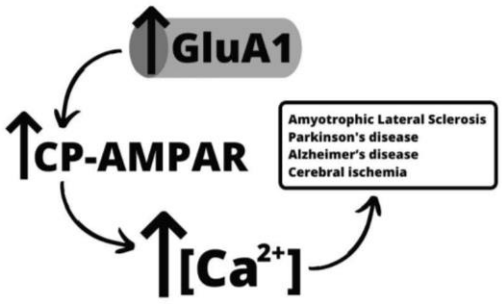

Fig. (3). Relationship between GluaA1, CP-AMPARs, calcium concentrations, and neurodegenerative diseases. The increased expression of GLUA1 increases the insertion of CP-AMPAR in the neuronal membrane, increasing intracellular calcium and establishing the set of components present in degenerative disorders. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

2.1. Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disease characterized by progressive muscle weakness due to the relatively selective degeneration of upper and lower motor neurons (MNs), usually leading to death within 2 to 5 years after diagnosis. While most cases are sporadic, approximately 10% are familial, and of these, a fraction is linked to dominant mutations in the superoxide dismutase 1 (SOD1) gene [36]. Neurodegeneration of MN subgroups in the brainstem, spinal cord, and motor cortex has characteristic marks of mitochondrial Ca^{2+} overload, free radical damage, excitotoxicity, and compromised axonal transport [37].

It has been suggested that the lesion-promoting event may be related to the rapid insertion of AMPA-like glutamate receptors into the plasma membrane, mainly stimulated by the inflammatory process mediated by TNF-α [38]. Interestingly, most of these AMPARs contain GluA1, characterizing increased Ca^{2+} permeability [39]. MNs show a substantial content of CP-AMPA channels [34]. In addition, MN populations that degenerate in ALS are characterized by low levels of Ca^{2+}-detecting proteins [40] and cytosolic Ca^{2+} buffering gates, such that the excitotoxically-induced Ca^{2+} loads are rapidly absorbed by mitochondria, with the consequent release of mitochondrial ROS [41]. Providing strong evidence that CP-AMPAR plays a neurotoxic role in ALS, Damme and coworkers [42] have demonstrated that the presence of SOD1 in astrocytes in contact with MNs results in an increased number of CP-AMPAR channels and accelerates the progression of neurodegeneration, given that positive regulation of the GluA2 expression by the astrocytes, when in the absence of SOD1, generates less vulnerability of the MNs. Changes in GluA2 glutamine/arginine (Q/R) by the negative regulation of adenosine deaminase (ADAR2), in MNs bearing the pathological marker TDP-43 (ALS) are associated with this failure. The process increases the number of CP-AMPARs [43], characterizing a predisposition to the disease [26] and the beginning of a neurotoxic vicious cycle, proving that lower GluA2 expression results in greater vulnerability of MN to neurotoxicity [42].

The chronic excitotoxicity mediated by CP-AMPARs and NMDA appears to initiate a vicious cycle of intracellular calcium deregulation leading to toxic Ca^{2+} overload and therefore to selective neurodegeneration [26]. CP-AMPARs are also involved in the increased vulnerability of MNs in patients with C9ORF72 mutations, the most common cause of the disease in family history cases [12]. Studies by Selvaraj and coworkers [37] have demonstrated, using the postmortem material of patients with C9ORF72 mutation, that the specific dysregulation of MNs is directly associated with an increase of GluA1 expression and the appearance of CP-AMPA. Other studies that sought to discover the origin of the change in receptor permeability have found, curiously, that mutations in the GluA2 subunit are not related to the onset of the disease. Furthermore, Gregory and coworkers [44] showed that C9orf72RE patient MNs displayed a vulnerability to AMPAR-mediated excitotoxicity due to a C9ORF72RE-dependent increase in CP-AMPAR expression through an abnormal increase in GluA1 subunit expression. However, altered AMPAR properties were not observed in human cortical neurons, suggesting that the C9ORF72RE mutation contributes to a selective AMPAR-associated mechanism of excitotoxicity in MNs. sALS and mutant C9ORF2 cases exhibited GluA1 upregulation, whereas mutant SOD1 cases displayed GluA2 down-regulation.

Considering the neurotoxic feature of the calcium-permeable receptors present in the ALS pathogeny, the effects of AMPAR antagonist drugs, such as Perampanel, a selective and noncompetitive AMPAR antagonist [45], 6-nitro-7-sulfamobenzo (f) quinoxaline-2,3-dione (NBQX) [46], and 1-naphthyl acetylspermine (NASPM) [47] have been investigated in animal models, showing potentially therapeutical results. In the motor cortex of patients with ALS, the pyramidal neurons of the corticospinal V layer (upper motor neurons) are progressively lost during the disease. The loss of layer II/III pyramidal neurons also occurs in the severe stages of the disease [48]. The loss of layer V pyramidal neurons was attenuated by AMPA/kainate or NBQX CP-AMPA receptor antagonists [49]. In an experiment using Talampanel, a noncompetitive CP-AMPA antagonist, Denes and coworkers [50] demonstrated a protection of MNs from Ca^{2+}-mediated degeneration by preventing Ca^{2+} influx through AMPAR in mSOD1 transgenic mice. The lack of treatment efficacy in early symptomatic mSOD1 mice could be attributed to the progressive nature of the degeneration, which might propagate from the upper to lower motor neurons, imposing an excitotoxic burden on ventrolateral spinal motor neurons ("dying forward"), or retrogradely from the neuromuscular junction ("dying back"), or may occur independently. Similar results have been reported by Patai and coworkers [51].

Candidate drugs that have been developed to treat ALS are largely based on studies in SOD1 transgenic mice [52], the most widely used ALS animal model, but virtually all these drugs have proven ineffective [53]. Therefore, new strategies that consider other types of treatment must be developed and the CP-AMPARS are showing promise. It is clear that MN survival depends critically on the interrelationships with neighboring cells and on the appearance of CP-AMPAR. In current studies, data provided evidence on the influence of AMPAR blockade on the evolution of ALS, demonstrating that decreased receptor expression or even the use of antagonists may decrease the resting phenotype of the disease.

2.2. Parkinson's Disease (PD)

PD is a chronic neurodegenerative disease characterized by tremor, rigidity, slowness of movement (bradykinesia), postural imbalance, and, finally, other nonmotor symptoms such as cognitive impairment and depression [54]. The age- and sex-adjusted incidence rate of PD is approximately 13.4 per 100,000, which increases rapidly at age 60. It is the second most common neurodegenerative disease after AD. With the aging of the population, the prevalence and incidence of PD is expected to increase by more than 30% by 2030 [55]. It is characterized by the loss of nigro-striatal dopaminergic neurons and aggregation of α-synuclein-rich inclusions, called Lewy bodies [56]. Treatment with the dopamine precursor L-DOPA improves the motor symptoms of PD and is the basis of antiparkinsonian therapy. However, motor complications of L-DOPA treatment, including L-DOPA-induced dyskinesia (LID), decrease adherence to this medication. One approach to controlling LID is to add specific agents that modulate the concentration of L-DOPA to reduce dyskinesias, such as metabotropic glutamate receptor (mGluR) drugs. Several mGlu5R negative allosteric modulators attenuate LID acutely in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA). Chronic administration of negative allosteric modulators of mGlu5R to MPTP-treated monkeys and 6-OHDA-lesioned mice also attenuates LID, maintaining the antiparkinsonian effect of L-DOPA [57]. Thus, the relationship between PD, the glutamatergic system and AMPARs is suggested, given that the activation or blockade of mGlu5R is already reported to likely induce new CP-AMPARs [58]. Mutation of mGlu5R, as performed in the research by Morin and coworkers [57], decreases the expression of the GluA1 subunit, and thus, the insertion of new CP-AMPAR receptors in the synapse [58].

A study by Kobylecki and coworkers [59], investigated the effects of the administration of the selective CP-AMPAR antagonist, IEM-1460, on abnormal involuntary movements in the 6-OHDA-lesioned rats and LID in the MPTP-lesioned nonhuman primates. Experiments have demonstrated the anti-dyskinetic effect of CP-AMPAR blockade, not only in 6-OHDA-injured mice, but also in the MPTP-lesioned non-human primates treated with L-DOPA, specifically in the initiation of LID due to the long-term use of L-DOPA. Subsequently, Kobylecki and coworkers [32], used animal models of PD and LID to elucidate the mechanism of action of CP-AMPAR blockade, both acute and chronically, evaluating the behavioral and molecular effects. In the group of nonhuman primates treated with MPTP, acute treatment with IEM-1460 dose-dependently reduced LID, without adverse effects on motor performance; associated with this result, the 21-day chronic treatment with IEM-1460 decreased the induction of involuntary movements by L-DOPA, without affecting motor performance. Chronic treatment with the CP-AMPAR antagonist reversed the L-DOPA-induced positive regulation of pre-pro-naphysine-A and normalized the pre-pro-enkephalin-B mRNA expression in the lateral striatum, indicating an inhibition of both

the behavioral and molecular basis of the disease, corroborating the interrelationship between the CP-AMPA receptors and PD neuronal control system.

Specific regions were also stimulated with AMPAR modulators to verify their relationship with PD. Lindenbach and coworkers [60] evaluating the primary motor cortex (M1) in 6-OHDA-lesioned rats, demonstrated that the onset of dyskinetic symptomatology can be attributed to this region, a state that was previously related only to the basal ganglia. Through microinfusions of D1, $GABA_A$, and AMPAR antagonists in the M1 region, the dyskinesia suppressor effect was verified, allowing the antagonist NBXQ blocking potential to be compared to other drugs. This was the first evidence of the role of AMPAR in the process of motor cortex-associated dyskinesia. Another process that relates the presence of CP-AMPAR to degeneration in PD is the Zn^{2+} influx in dopaminergic neurons [61]. In a study by Nakajima and coworkers, AMPA (1 mM) was injected in the SNpc of elderly rats and induced an increase in the levels of intracellular Zn^{2+}, followed by an increase in turning behavior in response to apomorphine and nigral dopaminergic degeneration. In the same study, a selective blocker of CP-AMPARs, 1-Naphthyl acetyl spermine (NASPM), blocked the increase in intracellular Zn^{2+} in the SNpc of rats, and resulted in rescue of nigral dopaminergic degeneration [61]. Further supporting the hypothesis that CP-AMPAR's are intimately related to Zn^{2+} influx, a study by Tamano and coworkers [11] showed results similar to those obtained by Nakajima and coworkers [61], in addition to describing the reversal of the turning behavior with the use of Zn^{2+} chelators. 6-OHDA-induced extracellular Zn^{2+} influx is completely blocked in the presence of CaEDTA, an extracellular Zn^{2+} chelator and CNQX, an AMPAR antagonist. Therefore, it is likely that AMPA-induced extracellular Zn^{2+} influx contributes to the pathogenesis of movement disorder *via* nigrostriatal dopaminergic neurodegeneration.

Protein misfolding and aggregation are key events in several neurodegenerative disorders such as PD and AD. Diogenes and coworkers [62] investigated the impact of alpha-synuclein aggregation on AMPA and NMDA receptor-mediated synaptic transmission in the rat hippocampus (CA3-CA1) and on long-term potentiation (LTP). They described that long-term treatment with alpha-synuclein oligomers, but not monomers or fibrils, caused a long-lasting increase in hippocampal basal synaptic transmission through NMDA receptor activation, triggering an enhanced contribution of CP-AMPAR to synaptic transmission and LTP impairment. Research to date on the influence of CP-AMPAR on PD symptoms indicates that they are involved in the LID process. Moreover, the lack of adverse motor effects makes them a potential target for antidyskinetic therapy.

2.3. Alzheimer's Disease (AD)

AD presents a preclinical course of many years, manifested mainly by the stochastic aggregation of Aβ in oligomers and successively in toxic plaques in the extracellular space of the brain [63]. The pathogenic activity of Aβ is strongly influenced by the concentration of the peptide. Thus, Bukke and coworkers [64], evaluated the influence of the glutamatergic system on the Aβ concentration, through AMPAR modulation. Findings indicated that the baseline levels of AMPAR activity and that AMPAR activity can decrease the concentration of Aβ, through peripheral and NMDAR-independent pathways, the only glutamatergic receptor associated with the disease so far.

Increased intracellular levels of oligomeric Aβ in the hippocampus induce a rapid increase in AMPAR-mediated synaptic transmission. GluA1, but not GluA2 knockdown, as well as the expression of S845-phosphomutant GluA1, blocked this effect. The administration of IEM-1460 also reversed the increase in the amplitude of excitatory postsynaptic currents. These findings suggest that the synaptic insertion of CP-AMPARs is a primary neuronal response to intracellular Aβ oligomers [31].

Cascelha and coworkers [65] demonstrated that both AMPAR and NMDAR are involved in the early disruption of Ca^{2+} homeostasis, induced by toxic oligomers in AD. Additionally, Megill and coworkers [66] demonstrated in a transgenic mouse model of AD that the increase of CP-AMPAR is directly related to the onset of the pathology. Sustained CP-AMPAR expression in the early stages of AD may accelerate excitotoxicity, thus spreading cognitive decline [67]. Gilbert and coworkers [68] investigated the relationship between Aβ, CP-AMPARs, and homeostatic synaptic plasticity (HSP). The results indicated that Aβ increases the expression of GluA2-lacking CP-AMPARs and induces an abnormal upregulation of AMPA receptor (AMPAR)-mediated synaptic currents and cell-surface AMPAR expression. This relationship may underlie the unbalanced neuronal network activity and seizures seen in the early stages of AD, as well as neuronal death and cognitive dysfunction in the later stages of AD.

Whitcomb and coworkers [31] studied the underlying toxic effects caused by increased intracellular levels of Aβ, an event that may be important during the early stages of AD. Their study showed that the administration of oligomerized Aβ induces a rapid increase in AMPAR-mediated synaptic transmission (EPSCA) and that this effect depends on postsynaptic calcium and PKA. An inhibitor of CP-AMPARs, IEM 1460, has been shown to reverse the increase in EPSCA amplitude, corroborating the relation between the rapid deposition of Aβ and the insertion of CP-AMPARs in the neuronal membrane.

In addition to being related to the neurodegenerative component, CP-AMPAR's apparently influenced social deficits, as demonstrated by Gascon and coworkers [69], who studied frontotemporal dementia (FTD), using a forebrain-specific FTD-associated mutant CHMP2B in mice. In the study, it was found that miR-124 downregulation causes dysregulation in AMPAR composition and a selective impairment in sociability. Furthermore, through an electrophysiological analysis it was suggested that FTD-related social behaviors are linked to an increase in Ca^{2+}-permeable AMPARs in excitatory synapses of pyramidal neurons.

As the main investigations involving AMPAR and AD are concentrated on the effect of pathological amyloid species on the receptor [70], the inverse relationship, the effect of AMPAR on Aβ, receives much less attention. In neurodegenerative diseases such as AD, the complexity of the

disease brings great difficulty in the application of therapy, with the aim of preventing or slowing the progression of the disease. Thus, new findings on the relationship between CP-AMPARs and AD open a new horizon of possibility, through prototypes of CP-AMPAR antagonists that can ameliorate cognitive deficits and prevent or slow the progression of neurodegeneration [65].

2.4. Metal-induced Neurotoxicity

One of the most used parameters to study the conformational differences of certain ionotropic receptors is the mean rectification index (RI), which is revealed by electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs). Several studies using intoxication models use this analysis to determine the influence of structural changes in receptors.

Ankolekar and Sikdar [35] studied the influence of chronic lithium (CLi) overload on AMPAR in cell cultures of hippocampal pyramidal cells obtained from rats in the early postnatal period and found that CLi decreased the mean amplitude and mean RI of AMPAR mEPSCs. Lowered mean RI indicates that the contribution of CP-AMPARs in synaptic events is higher in CLi-treated neurons. The authors proposed that the effect of lithium on the increase in CP-AMPARs is related to the decrease in PKA, GSK-3β, and glutamate, as the co-inhibition of PKA, GSK-3β, and glutamate reuptake is required to closely reproduce the effects of CLi on AMPAR mEPSCs. Lithium–induced alterations in AMPARs might have implications for CNS complications of lithium exposure in the perinatal period as well as in side effects associated with lithium therapy, including cognitive decline.

The decrease of GluA2 in AMPAR assembly, thus generating CP-AMPARs, is examined in several neurotoxicity studies, supporting the hypothesis that the increase in calcium permeable receptors is related to cell death. In previous studies, Ishida and coworkers [24], reported that long-term exposure of rat cortical neurons to lead acetate decreases GluA2 expression [71]. Subsequently, they demonstrated, again using cell cultures, the contribution of GluA2 noncontaining AMPARs to lead-induced neurotoxic events. The expression of four AMPAR subunits (GluA1, GluA2, GluA3, and GluA4) was reduced by lead exposure, but the decrease in GluR2 expression was more pronounced [24]. In addition, they found that cell death was prevented with the use of the glutamate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a nonselective AMPAR blocker) and, more significantly, by 1-naphthyl acetyl spermine (NAS, a specific CP-AMPAR blocker). Furthermore, a previous study has reported that lead enters adrenal medullary cells through the L-type voltage-dependent Ca^{2+} channel (VDCC) [72], Ishida and coworkers [24] demonstrated that similarly to VDCCs, CP-AMPARs, resulting from decreased GluR2 expression induced by lead exposure, would be permeable to lead. They also demonstrated the protective effects of CNQX against lead-induced activation of MAPKs, PKC, and neuronal cell death.

2.5. Neuroinflammation

One of the neurodegenerative pathways, mentioned in the previous section, generated by toxicants such as lead and lithium, is the neuroinflammation cascade and the release of proinflammatory molecules such as TNFα and NO, which apparently increase the traffic of CP-AMPAR, leading to calcium increase, which in turn contributes to the enhanced inflammatory environment [73]. TNFα is a pleiotropic cytokine with physiological and pathological functions that include regulation of inflammation, innate immunity, cancer, synaptic function, and neurogenesis [74]. TNFα exerts homeostatic control of synaptic strength by regulating AMPAR trafficking in the CNS [75].

Pribiag and Stellwagen cite the possible direct effects of pro-inflammatory cytokines on synapses: (1) increase excitatory synaptic strength through upregulation of cell surface AMPARs, (2) enhance calcium influx at the synapse, either through Ca^{2+}-permeable AMPARs or NMDARs, and (3) decrease inhibitory synaptic strength, through down-regulation of surface GABAARs/Glycine receptors or increased intracellular Cl^- concentration. The combined action of these effects is likely to alter network behavior by increasing overall neuron firing frequency [75]. Administration of exogenous TNFα to hippocampal neuron cultures induces cell surface expression of GluA2-lacking AMPARs, which are Ca^{2+}-permeable [36]. Moreover, TNFα-induced expression of CP-AMPAR's exacerbates neuronal death during acute ischemia and excitotoxicity [47].

Important work by Lewitus and coworkers [13], demonstrated that the acute treatment of striatal slices with TNFα reduced the ratio of AMPA to NMDA current amplitudes. In addition, they demonstrated a differential effect of TNFα in specific brain regions, with respect to the expression or internalization of the AMPA receptor. In GABAergic medium spiny neurons (MSNs), TNFα increase, through the regulation of DARPP-32 activity, leads to the internalization of AMPARs, in contrast to the synaptic insertion of AMPARs observed in pyramidal cells [13].

2.6. Cerebral Ischemia

It is not only neuroinflammatory processes that promote increased levels of CP-AMPAR; the acute injury associated with cerebral ischemia, possibly also leads to increased CP-AMPAR expression. Ischemia occurs when the blood supply to the brain is interrupted, for example, by occlusion following a stroke, or as a result of cardiac arrest [76]. The oxygen restriction that occurs during ischemia exposes neurons to metabolic stress, which causes widespread depolarization of the neuronal plasma membrane, massive release of the excitatory neurotransmitter glutamate and overexcitation of ionotropic glutamate receptors, resulting in a sustained elevation of intracellular Ca^{2+}, and consequently a delayed, selective cell death [77]. In hippocampal CA1 neurons, changes in synaptic AMPAR subunit composition, resulting in the expression of GluA2-lacking CP-AMPARs were observed. These lead to Ca^{2+} influx that contributes to delayed cell death hours to days later [5]. In an experimental model of cerebral ischemia, two distinct phases to this process have been described; an initial rapid trafficking phase, involving an NMDAR-dependent removal of GluA2 subunits from the plasma membrane [78] and a later phase in which GluA2 subunit mRNA expression and consequently protein levels are reduced [76].

Achzet and coworkers [79] investigated how oxidative stress underlies ischemia/reperfusion-induced internalization and demonstrated that GluA2 undergoes internalization and is subsequently degraded after ischemia/reperfusion. The subsequent increase in expression of AMPARs lacking GluA2, CP-AMPARs, results in excitotoxicity and, eventually, delayed neuronal death. They further demonstrated that ischemic/reperfusion-induced internalization and subsequent endocytic trafficking of GluA1 and GluA2 AMPAR subunits to late endosomes is mediated by an oxidative stress signaling pathway. The loss of GluA2-containing AMPARs in the plasma membrane following ischemia/reperfusion occurs in vulnerable areas of the brain, such as the hippocampus, leading to delayed neuronal death. The findings by Chen and coworkers [80] corroborate the study by Achzet and coworkers [79], indicating that the recruitment of Ca^{2+}-impermeable AMPAR to the synaptic surface, by PKA-dependent mechanism, is crucial for the neuroprotective effect of β -Caryophyllene (BCP) and protection against cognitive impairment after acute cerebral ischemia.

CONCLUSION

The subunit composition of synaptic AMPARs can undergo dynamic changes during physiological functioning as well as under pathological conditions. It involves changes in the levels of GluA2 subunits that are mediated *via* regulated AMPA receptor trafficking, modification of local protein synthesis, and altered gene transcription of GluA2 subunits. Changes in the configuration of AMPA receptor subunits have been associated with modifications in calcium permeability.

Through new studies involving the presence of CP-AMPARs as inducers of neuronal toxicity in neurodegenerative diseases or even as initiators of the degenerative process, new treatment possibilities are opened. Current evidence describes important behavioral and cellular results of some antagonists. For example, IEM-1460 attenuates epileptic seizures and may reduce visceral pain and CP-AMPAR activity related to schizophrenia; NASPM reduces oxidative stress and protects neurons following ischemia; AgTx-636 is an efficacious natural CP-AMPAR channel blocker with neuroprotective properties.

Studying CP-AMPARs as a central component of neurodegenerative and neurotoxic diseases will allow us to expand our knowledge about the appearance and the different ways in which such pathologies develop. Identifying the moment of emergence of aberrant forms of the AMPA receptors, how they are regulated, and mainly, how we can modify them, may be a crucial missing piece so that new advances in the treatment of neurodegenerative diseases can be obtained.

LIST OF ABBREVIATIONS

AD	=	Alzheimer's Disease
ALS	=	Amyotrophic Lateral Sclerosis
AMPA	=	α-Amino-3-hydroxy-5-Methyl-4-isoxazole-propionic Acid
AMPAR	=	α-Amino-3-hydroxy-5-Methyl-4-Isoxazo-lepropionic Acid Glutamate Receptor
Aβ	=	β-Amyloid Peptide
BBB	=	Blood-Brain Barrier
CaMKII	=	Ca^{2+}/Calmodulin-Dependent Protein Kinase II
cdk5	=	Cyclin-Dependent Kinase 5
cGKII	=	cGMP-Dependent Protein Kinase II
CNQX	=	6-Cyano-7-Nitroquinoxaline-2,3-Dione
CNS	=	Central Nervous System
CP-AMPAR	=	Ca^{2+}-Permeable AMPAR
D1	=	Dopamine Receptor 1
FTD	=	Frontotemporal Dementia
$GABA_A$	=	γ-Aminobutyric acid (GABA) Receptor A
GSK-3β	=	Glycogen Synthase Kinase-3 Beta
LID	=	L-DOPA-Induced dysKinesia
LTP	=	Long-Term Potentiation
M1	=	Primary Motor Cortex
MAPKs	=	Mitogen-Activated Protein Kinases
mEPSCs	=	Miniature Excitatory Postsynaptic Currents
mGluR	=	Metabotropic Glutamate Receptor
MNs	=	Motor Neurons
MPTP	=	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MSNs	=	Medium Spiny Neurons
NASPM	=	1-Naphthyl Acetylspermine
NBQX	=	6-Nitro-7-sulfamobenzo (f) quinoxaline-2,3-dione
NMDAR	=	*N*-methyl-D-Aspartate Receptor
NO	=	Nitric Oxide
6-OHDA	=	6-Hydroxydopamine
PD	=	Parkinson Disease
PKA	=	Protein Kinase A
PKC	=	Protein Kinase C
PKG	=	Protein Kinase G
RI	=	Rectification Index
ROS	=	Reactive Oxygen Species
SNpc	=	Substantia Nigra, Pars Compacta
SOD1	=	Superoxide Dismutase 1
TNFα	=	Tumor Necrosis Factor α
VDCC	=	L-type Voltage-Dependent Ca^{2+} Channel

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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3 CAPÍTULO 3

Artigo submetido para a publicação da revista **Molecular Neurobiology**: Neuroprotective Effect of Perampanel on Cognitive Function in Rats Exposed to Neonatal Iron Overload.

Original article

Neuroprotective Effect of Perampanel on Cognitive Function in Rats Exposed to Neonatal Iron Overload

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Abstract

Iron accumulation has been associated with the pathogenesis of neurodegenerative diseases and states of neuronal toxicity. As previously described by our research group, iron overload in the neonatal period induces persistent memory deficits, increases oxidative stress and apoptotic markers. The neuronal insult caused by iron excess generates an energetic imbalance that can alter glutamate concentrations and thus trigger a toxic state induced by this neurotransmitter, known as excitotoxicity. Several drugs block glutamatergic pathways and thus mitigate neurotoxicity, among them, Perampanel (PER), a reversible antagonist of glutamatergic AMPA receptors. In the present study, the neuroprotective effect of the subchronic use of PER in rats subjected to iron overload in the neonatal period was investigated. Recognition memory, locomotion, and aversive memory were evaluated, as well as the relative expression of genes such as DGL4, GRIA1, GRIA2, and CAC, which code important proteins for AMPA receptor metabolism. Male rats received vehicle or carbonyl iron (30 mg/kg) from the 12th to the 14th postnatal day and were treated with vehicle or PER (2 mg/kg) for 21 days in adulthood. The excess of iron in the neonatal period demonstrated its neurodegenerative potential, causing the recognition memory deficits in the new object recognition test and the impaired emotional memory, in the inhibitory avoidance test, where PER was able to significantly improve the rodents' memory. Furthermore, iron overload increased the expression of the GRIA1 gene and decreased the expression of the DGL4 gene, demonstrating the influence of metal accumulation on the metabolism of AMPA receptors. These results suggest that iron can trigger changes in the expression of genes important for the assembly and anchoring of AMPA receptors and that blocking AMPA receptors with PER is capable of partially reversing the cognitive deficit caused by iron overload.

Keywords: Memory, AMPA receptors, Neurotoxicity.

Introduction

Aging is a stochastic phenomenon that progressively and irreversibly affects all living organisms. It is characterized by a precise mosaic of cellular and tissue changes that gradually reduce the organism's adaptability to its environment [1]. This decline in the body's biochemical and physiological functions is the result of the interplay between genetic factors, the environment, lifestyle, and the extent to which these components can cause DNA damage and hinder its repair [2, 3].

Morphofunctional, histologic, and chemical disturbances also manifest within this system, primarily due to elevated levels of free radicals (FRs), resulting in oxidative stress [4, 5].

The progression of degenerative damage may be exacerbated by elevated levels of certain metals such as iron, that when present in excess, can increase the permeability of the blood-brain barrier, leading to central inflammation and neuronal toxicity [6]. This association is well established in neurodegenerative diseases, where high iron levels have been identified in key pathological features, including neurofibrillary tangles in Alzheimer's disease [6] and protein misfolding and aggregation in mitochondrial dysfunction [7].

Despite the large amount of evidence in the literature that correlates iron accumulation in the Central Nervous System (CNS) and neurodegenerative diseases [8] further investigations are still needed in order to elucidate the mechanisms underlying iron neurotoxicity. In previous studies, our research group established an animal model of iron administration in the neonatal period, when maximum iron absorption into the CNS occurs, and was able to describe that the insult caused in the process generated behavioral and cognitive impairments [9], damaging recognition and emotional memory [10]. Furthermore, it was found that iron accumulation in the neonatal period led to oxidative damage [11], increased expression of apoptosis markers [12,13], changes in mitochondrial function [13], changes in autophagy [14], and accumulation of ubiquitinated proteins in rodents [10].

In addition, several studies have reported profound changes in PD affected brains, including significant depletion of the antioxidant glutathione (GSH), reduced mitochondrial complex I activity, DNA oxidation, and elevated levels of free iron [15]. These findings are consistent with the work of Zhu et al. [5], who observed elevated iron levels in degenerating dopaminergic neurons, and the research of Castellani et al.

[16], who identified iron-reactive Lewy bodies along with aggregated proteins such as α-synuclein.

When generated in sufficient quantities, FRs can suppress defense mechanisms against oxidative stress, leading to metabolic and cellular perturbations, including DNA strand breaks and changes in intracellular Ca^{2+} levels. These changes result from increased permeability of calcium channels, including AMPA-type glutamatergic receptors (AMPARs). Importantly, there is growing evidence that Ca^{2+} permeability in AMPARs directly influences synaptic plasticity and contributes to the pathophysiology of various neuronal disorders [17].

The development of novel drugs targeting AMPA receptor antagonism is a growing field that aims to mitigate the harmful effects of calcium ion accumulation and provide neuroprotection [18]. Perampanel (PER) represents the first of a novel class of antiepileptic drugs that acts through non-competitive AMPAR antagonism. It has demonstrated efficacy and clinical tolerability in patients 12 years of age and older [18, 19]. Notably, this drug was recently approved in the U.S. for the treatment of epilepsy, including partial-onset seizures and primary tonic-clonic seizures, and as adjunctive therapy in the treatment of Lafora disease and Lance-Adams syndrome. It has also shown promise in reducing myoclonic seizures and providing neuroprotection [18, 20].

Given the clinical importance of understanding the mechanisms of action of new drugs in the field of neuroprotection, this study sought to investigate the neuroprotective capabilities of PER. Specifically, neonatal Wistar rats were exposed to elevated iron levels and the effect of iron accumulation on the expression of different subunits of the AMPA receptor was evaluated. It was also intended to evaluate whether PER would be able to attenuate memory impairments induced by iron in rats.

The study encompassed behavioral assessments of cognition in the animals, as well as an examination of the hippocampus to provide cellular insights into the mechanisms of neuroprotection and associated pathways. The study also evaluated the expression and phosphorylation levels of AMPA receptor subunits, GluA2 and GluA1, and assessed the mRNA expression of proteins such as PSD-95, a scaffolding protein for the AMPA receptor, and stargazer transmembrane interaction protein, a transmembrane protein responsible for anchoring and stabilizing AMPA receptors in the plasma membrane.

Materials and Methods

Animals

Twenty-three pregnant Wistar rats were obtained from the Centro de Reprodução e Experimentação em Animais de Laboratório (CREAL), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48h to contain eight rat pups including offspring of both genders. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 21 ± 1 °C and a 12/12 h light/dark cycle. At the age of 3 weeks, pups were weaned, and the males were selected and maintained in groups of three to five in individually ventilated cages with sawdust bedding. For postnatal treatments, animals were given standardized pellet food and tap water ad libitum. All behavioral experiments were performed at light phase between 09:00 a.m and 4:30 p.m. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Edition 2011) and the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted and approved by the Institutional Ethics Committee of the Federal University of Rio Grande do Sul (permit number: 35604). All efforts were made to minimize the number of animals and their suffering.

Experimental Design

With the aim of evaluating the effects of iron overload on the expression of AMPA receptor (AMPAR) subunits and proteins related to AMPAR functionality and investigating the effects of subchronic use of PER, a non-competitive AMPAR antagonist, on the cognitive deficit of animals, litters were randomly assigned (simple randomization) to receive either iron carbonyl or vehicle solution. Only male pups from each litter received the treatment from postnatal days 12th to 14th. All litters (those that received iron and those that received vehicle) were kept in the same room with their respective mothers under the same environmental conditions. Subsequently, at the age of 90 days, the two groups were further randomly divided into four groups, resulting in four experimental groups: Veh-Veh, Veh-Perampanel, Iron-Veh, and Iron-Perampanel. During treatments, all groups of rats were kept in the same room under the same environmental conditions. After 14 days of PER treatment, rats were tested in the

novel object recognition task and open field test. Seven days later, they were exposed to inhibitory avoidance memory task. Twenty-four hours after the completion of behavioral task, all rats were euthanized by decapitation, and their hippocampi were quickly removed and stored in − 80 °C freezer for posterior RT-PCR or western blot analysis. Experimental design is shown in **Fig. 1.**

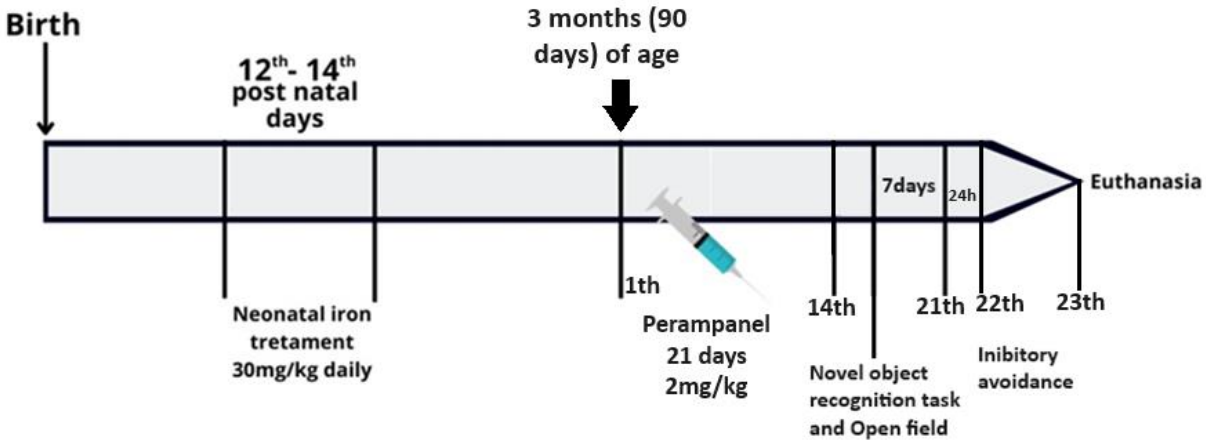

Fig 1. Experimental design. Groups of rats were treated with vehicle or iron (orally, 30 mg/kg) in the neonatal period at postnatal days 12th to 14th. In adulthood (3 months of age), they received gavage of perampanel (2.0 mg/kg) or vehicle for 21 days. After 14 days of Perampanel treatment, rats were tested in the novel object recognition task and open field. Seven days later, they were submitted to inhibitory avoidance memory task. Twenty-four hours after the completion of behavioral testing, animals were euthanized by decapitation, and their hippocampi were quickly isolated and stored in − 80 °C for RT-PCR and Western blot analysis.

Pharmacological Treatments

Neonatal Iron Treatment

The neonatal iron treatment was performed as previously described [13, 10]. Briefly, 12-day-old rat pups received a single oral daily dose (10 mL/kg solution volume) of vehicle (5% sorbitol in water, control group) or 30 mg/kg of body weight of Fe2+ (iron carbonyl, Sigma-Aldrich, São Paulo, Brazil) via a metallic gastric tube (gavage), over 3 days (postnatal days 12–14). Rats in each group (vehicle or iron) were derived from 5 to 6 different litters, to avoid a possible litter effect.

Perampanel

Adult (3-month-old) male rats, treated neonatally with vehicle or iron, as

described above, received a daily gavage of vehicle (Tween 80 – saline solution 1:16 v/v) or Perampanel (PER) (2 mg/kg, Fycompa®) for 21 consecutive days. Drug solutions were freshly prepared immediately prior to administration, with dissolving the tablet and calculating the dose. Rats were euthanized by decapitation at 24h after the last injection of PER treatment. Brains were quickly dissected and hippocampi were isolated and stored at -80ºC for subsequent RT-qPCR. The dose of PER was chosen based on previous animal studies [21, 22].

Open field

Open field exploratory activity was analyzed 24 h before object recognition training session. This behavioral session was also used as habituation to the field. An open field arena (40 cm × 45 cm × 60 cm), made of plywood with a front glass wall and floor divided into 12 equal squares by black lines, was used. The animals were placed in the lower left corner to then explore freely for 5 minutes. The experimental session was videotaped and subsequently analyzed by an experimenter blinded to the animals' experimental condition The latency to start locomotion, line crossings, number of rearings, and the number of fecal pellets were counted using a stopwatch and two manual counters during the experimental sessions. The evaluated parameters are indicative of the animals' locomotion and anxiety [23].

Novel Object Recognition Test

Animals were habituated to the environment, 24 h before the training session. In the training session, rats were placed in the same open rectangular field (45 × 40 × 60 cm), with sawdust covering its floor, in which they were exposed to two identical objects (A1 and A2), for 5 minutes. To evaluate the long-term memory retention (LTM), 24 h after the training session, the rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object (A) and a new object (B). These objects were placed in the same locations as in the training session. All objects used had similar textures, colors and sizes, but different shapes. The objects were positioned in two adjacent corners, 9 cm from the walls and between trials, the objects were washed with 10% ethanol [24].

All experimental sessions were videotaped and subsequently analyzed by an

experimenter blinded to the animals' experimental condition. Exploration of the objects was measured using two stopwatches to record the time spent exploring them during the experimental sessions. Exploration was defined as: smelling or touching the object with the nose or the forepaws. The recognition index was expressed by the equation TB/(TA+TB), where: TA = time spent exploring the familiar object (A) and TB = time spent exploring the new object [25, 26].

Inhibitory Avoidance Task

We used the single-trial, step-down inhibitory avoidance (IA) conditioning as an established model of fear-motivated memory. In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). The IA behavioral training and retention test procedures were described in previous reports [10]. The IA apparatus was a 50 × 25 × 25-cm3 acrylic box (Albarsh, Porto Alegre, Brazil) whose floor consisted of parallel stainless-steel bars (1-mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform, and their latency to step-down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a mild footshock (0.4 mA) and were removed from the apparatus immediately afterwards. A retention test trial was carried out 24 h after the training trial. The retention test trial was procedurally identical to training, except that no footshock was presented. Step-down latencies (in seconds) on the retention test trial (maximum 180 s) were used as a measure of IA retention. Behavioral procedures were performed by an experimenter blinded to animals' experimental condition.

Molecular Analysis

Twenty-four hours after the completion of inhibitory avoidance testing, rats were euthanized by decapitation and hippocampi were rapidly dissected. For organization purposes, and to ensure that the rapidly dissected hippocampal samples were immediately placed in the adequate solutions (RNA-later for RT-qPCR and protease inhibitor for WB) and snap-frozen. We used a procedure in which the left hemisphere was placed in a refrigerated RNA-later solution (Sigma-Aldrich, São Paulo, Brazil) for

RT-qPCR assays and the right hemisphere was placed in a protease inhibitor solution (Complete Mini, Roche Applied Science, Mannheim, Germany) also refrigerated for the Western blot tests. Samples were stored at -80 °C for subsequent analysis.

Western Blot Analysis

Proteins were extracted in 1X RIPA buffer (ThermoFisherScientific, Waltham, USA) containing protease inhibitor cocktail and sodium orthovanadate (1mM). After 10 min in ice, samples were centrifuged at 13,500 rpm for 10 min. The supernatant was collected and the protein content was determined using Bradford assay [27]. Aliquots were stored at −80 °C. Twenty-five micrograms of protein were separated on a 9% SDS polyacrylamide gel and transferred electrophoretically to a PVDF membrane (Immobilon-P, Millipore, Burlington, USA). Membranes were blocked with 5 % nonfat dry milk in TBS containing 0.1 % Triton X-100 and were incubated overnight with one of the following antibodies: anti-β-actin (cat. # ab6276, Abcam, Cambridge, UK) at 1:5,000, anti-phospho GluA1 (S845) (cat. # ab7632, Abcam, Cambridge, UK) at 1:1,000, anti-phospho GuA2 (S880) (cat. # ab52180) Abcam, Cambridge, UK) at 1:500, anti-GluA1 (cat. # ab31232, Abcam, Cambridge, UK) at 1:1,000, and anti-GluA2 (cat. # ab133477, Abcam, Cambridge, UK) at 1:1,000. Goat polyclonal anti-mouse IgG (cat. # A4416) at 1:10,000, and goat polyclonal anti-rabbit IgG (cat. # A0545) at 1:80,000 (both from Sigma-Aldrich, St. Louis, USA) secondary antibodies were used and detected using Millipore Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, USA). Prestained molecular weight protein markers (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards, Bio-Rad Laboratories, Hercules, USA) were used to determine the detected bands' molecular weight and confirm target specificity of antibodies. Images were obtained using an ImageQuant LAS 500 (GE Healthcare, Chicago, USA). The densitometry quantification was performed using ImageJ software (http://rsb.info.nih.gov/ij/). Phosphorylation levels were normalized by the respective total protein levels. Total blotting protein levels of samples were normalized according to each sample’s β-actin protein levels [13, 28].

RT-PCR

TRIZOL (Invitrogen) was used to extract and isolate total RNA according to the manufacturer's instructions. Total RNA quantity and quality were determined spectrophotometrically using a BioPhotometer Plus (Eppendorf, Germany). Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The cDNA from each sample was used as a template for gene expression analysis through real-time quantitative polymerase chain reaction (RT-qPCR) using GoTaq® Probe qPCR Master Mix (Promega) and specific primers for each gene: the transmembrane interacting proteins stargazer (CAC gene) and scaffold PSD-95 (DLG4 gene), GluA1 (GRIA1 gene) and GluA2 (GRIA2 gene) and the constitutive genes Gadp and Rpl13. Primers' sequences are listed in Table 1. RTqPCR conditions were optimized to yield an amplification efficiency of 95% -105%. Products were run on an agarose gel to verify the correct amplification length. The specificity of the amplified products was confirmed by analyzing the dissociation (melt) curves at the end of each reaction. The relative expression of he genes analyzed was calculated for each sample by the relative comparative (ΔΔCT) method. All samples were run in triplicates.

Table 1: List of PRIMERS used

Primers		Sequences
CACNG2	F	CTTCAAAGGTCTGTGCAAGC
	R	AAAGCAGGATCACACTCAGG
DLG4	F	GAGTGCTTCTCAGCCATCGT
	R	TAGGGGCCTGAGAGGTCTTC
GRIA1	F	CGAAGCGGATGAAGGGTTTCT
	R	TGGATTGCATGGACTTGGGG
GRIA2	F	GGGGAGGTGATTCCAAGGAAAA
	R	CACCAGCATTGCCAAACCAA
GAPDH	F	GTCTCCTCTGACTTCAACAGCG
	R	ACCACCCTGTTGCTGTAGCCAA
RPL13	F	CTCAAGGTGTTTGACGGCATCC
	R	TACTTCCAGCCAACCTCGTGAG

Statistical Analysis

Data were tabulated and analyzed using SPSS v software. 26.0 (IBM, Armonk, NY, USA). The assumptions of normality and homogeneity of variances were assessed with the Shapiro-Wilk test and Levene's test, respectively. Behavioral parameters evaluated in the open field, recognition indexes, latencies to step-down and data from Western blot and RT-PCR experiments are expressed as mean ± standard error (S.E.). Statistical comparisons were performed using two-way analysis of variance (2-way ANOVA), with neonatal treatment (vehicle or iron) and adult treatment (vehicle or perampanel) as independent factors, to identify possible interaction effects of iron treatment and PER. One-way ANOVA, followed by Tukey's post hoc test, was used to test differences between the experimental groups. In all comparisons, p values less than 0.05 were considered to indicate statistical significance.

Results

We aimed to investigating whether PER could recover memory deficits induced by iron overload. Firstly, animals were tested in the object recognition task. The comparison of recognition indexes using 2-way ANOVA indicated that the experimental groups did not show significant differences in the training session. No significant main effect of iron treatment ($F(1,38) = 0.03$, $p = 0.864$), PER ($F(1,38) = 0.00$, $p = 0.994$), nor interactions were observed ($F(1,38) = 0.177$, $p = 0.677$, Figure 2). However, when we compared the recognition indices in the long-term memory test session, we noticed a significant main effect of iron treatment in the neonatal period ($F(1,38) = 8.68$, $p = 0.005$), revealed by a significantly lower recognition index in these groups indicating that iron causes recognition memory impairments, according to previous results from our research group. No significant main effect of PER in the adulthood was found ($F(1,38) = 0.012$, $p = 0.912$). Although the recognition index of the iron-treated group that received PER (Fe-PER) in the adulthood was higher than the Fe-Veh group, interaction between iron and PER demonstrated a trend significance ($F(1,38) = 3.75$, $p = 0.060$, Fig 2). However, one-way ANOVA comparisons of retention test recognition indexes, indicated that the iron-treated group that received PER in adulthood (Fe-PER), showed no difference in comparison to the control group (Sorb-Veh; $p = 0.137$),

suggesting that treatment with PER in adulthood was capable of improving, increased the memory of animals treated with iron in the neonatal period (Fig.2).

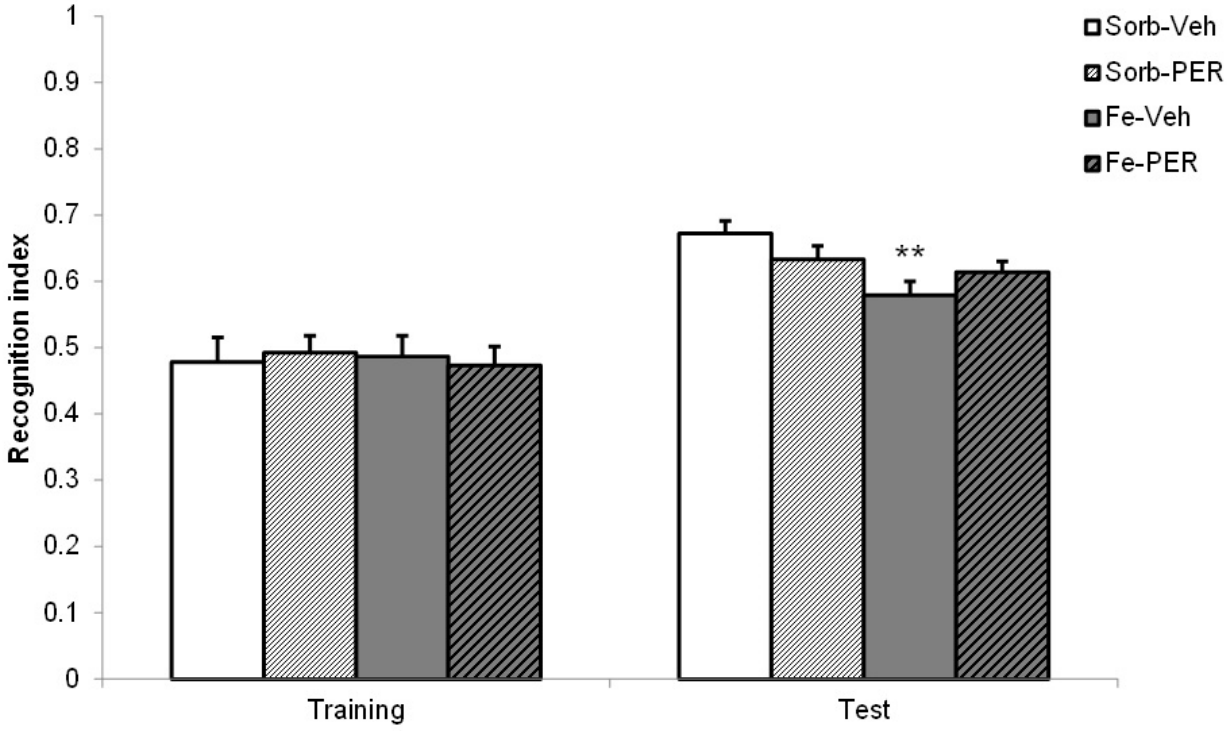

Fig.2 – Effect of PER on object recognition in rats treated neonatally with iron. Novel object recognition task was performed in rats treated neonatally with vehicle (Sorbitol, Sorb) or iron (30 mg/kg of Fe2+) and given vehicle (Veh) or PER (2.0 mg/kg) in adulthood (3 months of age) for 14 days. Sorb-Veh N = 12, Sorb-PER N = 9, Iron-Veh N = 11, Iron-PER N = 10. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or PER) as fixed factors. ** indicates a significant main effect of iron ($p = 0.005$). PER: Perampanel.

Next, we tested the animals in the inhibitory avoidance task, a type of emotionally regulated memory task. The comparison of latencies using 2-way ANOVA indicated that the experimental groups did not show significant differences in the training session. We observed no significant main effects of iron ($F(1,53) = 0.43$, $p = 0.515$), PER ($F(1,53) = 1.13$, $p = 0.293$), nor interaction ($F(1,53) = 0.59$, $p = 0.445$). However, when we compared the latencies in the long-term memory test session, we noticed a significant main effect of iron ($F(1,53) = 13.72$, $p = 0.001$), indicating that iron causes emotional memory impairment, confirming previous results from our research group. No significant main effect of PER in the adulthood ($F(1,53) = 0.79$, $p = 0.379$). However, 2-way ANOVA revealed a significant interaction between iron and PER

(F(1,53) = 5.70, p = 0.021), Moreover, comparing groups using one-way ANOVA indicated that the iron-treated group that received PER in adulthood (Fe-PER) showed no difference compared to the control group (Sorb-Veh; p = 0.200). These findings show that treatment with PER in the adulthood increased the emotional memory impairment induced by iron in the neonatal period (**Fig.3**).

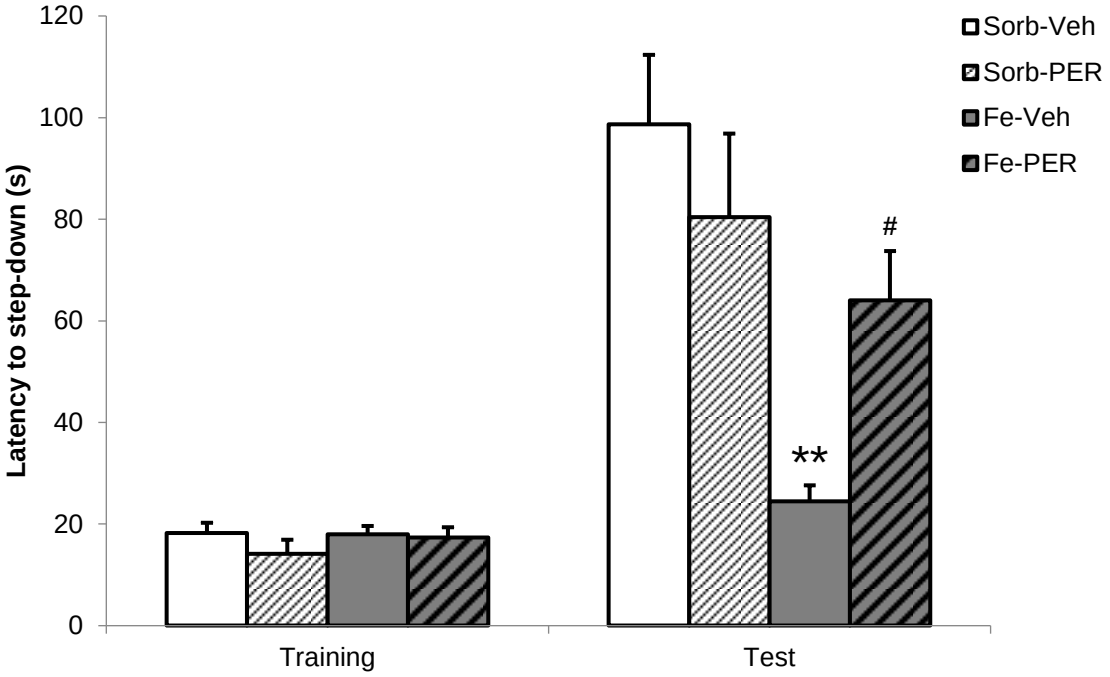

Fig.3 – **Effects of PER on inhibitory avoidance memory in rats treated neonatally with iron**. Inhibitory avoidance task was performed in rats treated neonatally with vehicle (Sorbitol, Sorb) or iron (30 mg/kg of Fe2+) and given vehicle (Veh) or PER (2.0 mg/kg) in adulthood (3 months of age) for 21 days. Sorb-Veh N = 16, Sorb-PER N = 14, Iron-Veh N = 14, Iron-PER N = 13. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA, with neonatal treatment (vehicle or iron) and adult treatment (vehicle or PER) as fixed factors. ** indicates a significant main effect of iron (p = 0.001); # indicates a significant interaction between iron x PER (p = 0.021). PER: Perampanel.

To control for possible motor, exploratory, or motivational alterations induced by iron treatment or PER we analyzed behavior in an open field. The comparisons of the parameters analyzed in the open field using ANOVA showed no statistically significant differences among the groups in the latency to start locomotion (F(3,38) = 2.25, p = 0.099), number of crossings (F(3,38) = 1.71, p = 0.182), number of rearings (F(3,38) =

2.68, p = 0.061), and number of fecal pellets produced during the session (F(3,38) = 2.13, p = 0.112) (**Table 2**).

Table 2 – Open field test.

Group	N	Latency to start locomotion (s)	Number of line crossings	Number of rearings	Number of fecal pellets
Sorb-Veh	12	9.72 ± 1.27	100.00 ± 2.94	31.75 ± 1.52	1.33 ± 0.43
Sorb-PER	9	7.31 ± 1.47	104.56 ± 6.82	31.56 ± 2.26	2.78 ± 0.68
Iron-Veh	11	12.12 ± 1.26.	89.73 ± 3.70	37.27 ± 0.98	1.54 ± 0.51
Iron-PER	10	8.58 ± 1.40	104.10 ± 7.51	31.40 ± 2.28	3.30 ± 0.97
p		0.099	0.182	0.061	0.112

Open-field behavior was analyzed during the habituation session for the object recognition task. Data are expressed as mean ± SEM. Data were analyzed by one-way ANOVA. No significant differences were observed when the latency to start locomotion, number of crossings, number of rearings, and fecal pellets produced during the session were compared.

To gain a better understanding on the mechanisms involved with the deleterious effects of iron excess on cognition and possible reversion effects of PER, we next decided to examine the levels of GLUA1 and GLUA2 AMPAR subunits and their phosphorylated forms. Two-way ANOVA showed no significant main effects of iron (F(1,16) = 0.40, p = 0.536) , PER (F(1,16) = 0.17, p = 0.683), nor interactions (F(1,16) = 0.032, p = 0.860) when analyzing total GLUA1 levels (**Fig 4B**). However, when comparing pGLUA1, a significant interaction (F(1,16) = 6.99, p = 0.0177) between iron and PER treatments was revealed (**Fig. 4A**). Although no main effect of iron (F(1,16) = 1.93, p = 0.184) or PER (F(1,16) = 1.12, p = 0.306) were observed, multiple comparisons of groups showed a significant difference between the group treated with iron in the neonatal period (Fe-Veh) and the control group (Sorb-Veh, p = 0.024), and a significant difference between the iron-treated group that received Vehicle (Fe-Veh) and the iron-treated group that received PER in the adulthood (Fe-PER, p = 0.041, **Fig 4A**). These findings suggest that iron increased pGLUA1 and PER was able to reverse this effect.

In relation to GLUA2, neither total GLUA2 levels nor p-GLUA2 were affected by iron or PER. Two way ANOVA analysis of total GLUA2 levels indicated no main effect of iron (F(1,13) = 0.18, p = 0.676), PER (F(1,13) = 0.48, p = 0.500), nor interaction (F(1,13) = 0.39, p = 0.544, **Fig 4D**). Likewise, no significant main effect of iron (F(1,15) = 1.03, p = 0.325), PER (F(1,15) = 0.34, p = 0.566), nor interaction (F(1,15) = 0.79, p = 0.388) were revealed when analyzing

p-GLUA2 (**Fig 4C**). Also, multiple comparison test revealed no significant differences among the groups.

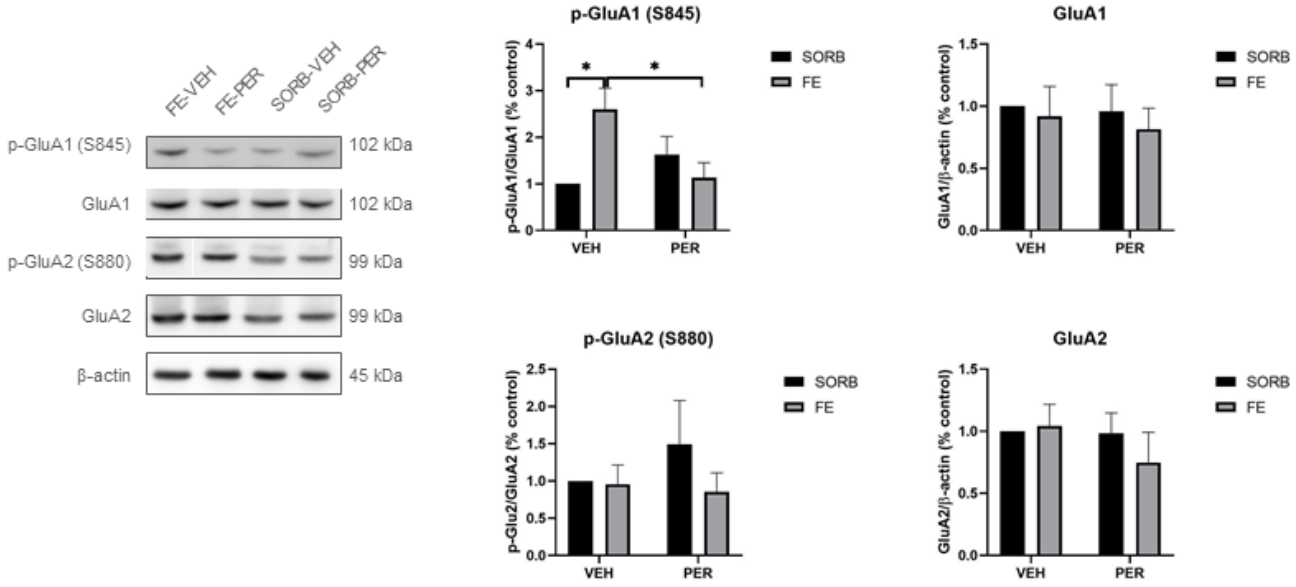

Fig 4 - Western blot of GLUA1 AMPA receptor subunit (A) and its phosphorylated form (S845) pGLUA1 in the hippocampus of rats treated with vehicle (Sorbitol, Sorb) or iron (Fe) neonatally and treated with vehicle (Veh) or Perampanel (PER) for 21 days in the adulthood (3 months of age); 25 µg of protein, normalized to β-actin, were separated on SDS-PAGE and probed with specific antibodies. Representative Western blots for GLUA1, pGLUA1, and β-actin are in the top panel. Statistical analysis was performed using two-way ANOVA, and Tukey multiple comparison test. Data expressed as mean ± SEM. pGLUA1 - Sorb-Veh N = 5, Sorb-PER N = 3, Fe-Veh N = 7, Fe-PER N = 5; GLUA1 – Sorb-Veh N = 4, Sorb-PER N = 3, Fe-Veh N = 5, Fe-PER N = 6; pGLUA2 - Sorb-Veh N = 4, Sorb-PER N = 3, Fe-Veh N = 7, Fe-PER N = 6; GLUA2 - Sorb-Veh N = 4, Veh-PER N = 3, Fe-Veh N = 5, Fe-PER N = 6. * Indicates significant differences between Sorb-Veh vs Fe-Veh vs Fe-PER ($p<0.05$)

.

We also sought to analyze mRNA expression of AMPAR subunits, GLUA1 and GLUA2, and scaffolding proteins related to AMPAR anchoring in the membrane, stargazin and PSD-95. As can be seen in **Fig.5**, the analysis of GRIA1 gene expression, indicated a significant main effect of iron treatment ($F(1,13) = 5.52$, $p=0.035$), which increased GRIA1 expression. No significant main effect of PER ($F(1,13) = 2.33$, $p=0.151$) nor interaction was observed

(F(1,13) = 1.48, p=0.245). On the other hand, GRIA2 mRNA expression was not affected by iron treatment (F(1,19) = 2.48, p=0.132). No significant main effect of PER (F(1,19) = 1.07, p=0.314) nor significant interaction was observed when analyzing GRIA2 mRNA expression (F(1,19) = 0.59, p=0.453).

Additionally, we found a significant main effect of iron on the mRNA expression of the DLG4 gene (F(1,14) = 13.44, p=0.03), in which iron treatment decreased the expression of this gene, that codes for the PSD-95 protein. No significant main effect of PER (F(1,14) = 3.61, p=0.078) nor interaction was found (F(1,14) = 0.74, p=0.403). Analysis of mRNA expression of CAC revealed no significant main effects of iron (F(1,14) = 0.64, p=0.437), PER (F(1,14) = 0.03, p=0.866), nor interaction (F(1,14) = 0.06, p=0.804).

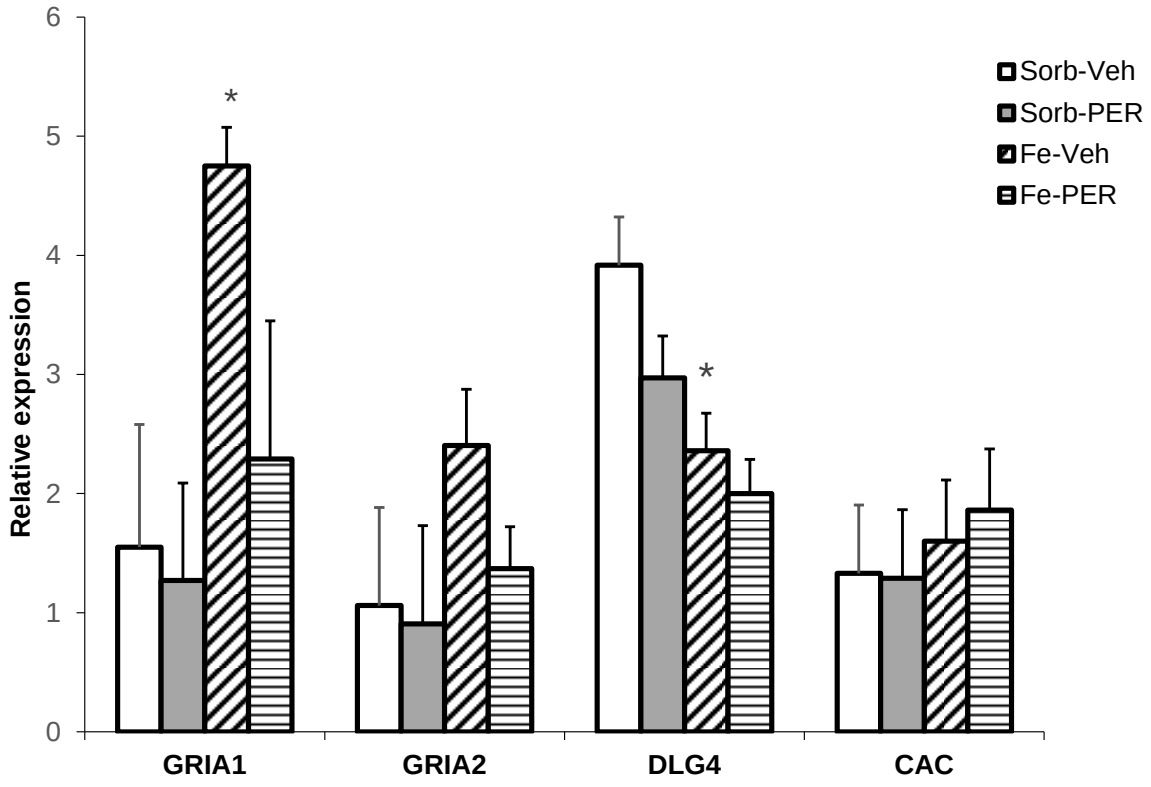

Fig.5 – mRNA expression of GRIA1, GRIA2, DLG4, and CAC in the hippocampus of adult rats treated neonatally with vehicle (Sorbitol, Sorb) or iron (30 mg/kg of Fe) and given vehicle (Veh) or PER (2.0 mg/kg) at old age only (19 months of age). **GRIA1** - Sorb-Veh N = 3, Sorb-PER N = 3, Fe-Veh N = 6, Fe-PER N = 5; **GRIA2** - Sorb-Veh N = 4, Sorb-PER N = 4, Fe-Veh N = 7, Fe-PER N = 8**; DLG4** - Sorb-Veh N = 3, Sorb-PER N = 4, Fe-Veh N = 5, Fe-PER N = 6; **CAC** - Sorb-Veh N = 4, Sorb-PER N = 4, Fe-Veh N = 5, Fe-PER N = 5. Data expressed as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA * Indicates significant main effects of neonatal iron treatment, GRIA1 ($p < 0.035$); DLG4 ($p = 0.030$). PER: Perampanel.

Discussion

The present findings show that iron overload in the neonatal period resulted in cognitive impairment in both behavioral tests, as previously demonstrated by our research group [9,24,14,29]. Our results also showed that subchronic treatment with Perampanel (PER) in the adulthood was able to reverse the memory impairments induced by iron overload. No motor or exploratory alterations that could interfere with memory acquisition, following iron or PER treatments were observed when open field results were analyzed.

The effect of PER on recognition memory corroborates the findings of Mohammad et al. [28], showing that PER improved object recognition ability in male rats in a pilocarpine model of status epilepticus. In addition, Alqahtani, et al [23], evaluating the anti-inflammatory effect of the co-administration of PER and Ketamine in a model of acute brain trauma, described an improvement in the percentage of spontaneous alternation and a higher percentage of discrimination index in the object recognition test with the association of PER. In the same study, it was found that the combination of the two substances reduced the central and peripheral expression of NF-κB and iNOS, substances whose concentrations are elevated in conditions of oxidative stress and mitochondrial damage, and an imbalance in CNS hyperexcitability. Mitochondrial failure, which can be caused by iron overload [29] has been linked to increased glutamate in the synaptic cleft, and consequent hyperexcitability.

A study by da Silva and coworkers [31], has revealed that iron accumulation during the neonatal period leads to mitochondrial damages. They also established a correlation between iron overload and the expression of proteins involved in mitochondrial fusion and fission mechanisms, such as DNM1L and OPA1. In addition, previous research reported increased expression of caspase-3, and decreased expression of the synaptic marker synaptophysin in the hippocampus of iron-treated rats [13].

CNS hyperexcitability is known to be a consequence of Aß oligomer accumulation, a key aspect of Alzheimer's disease pathophysiology. A study conducted by Bellingacci and coworkers [33] showed that administration of PER not only reversed neuronal Aß-induced hyperexcitability, but also improved cognitive deficits in Aβ oligomers-injected mice PER also decreased the expression of proinflammatory cytokines, particularly TNF-α, IL-1ß, and IL-6. In addition, it has been demonstrated that PER increases anti-inflammatory cytokines such as IL-10 and TGF-β1 [32]. It's worth noting that iron overload in the CNS has been associated with increased levels of TNF-α and IL-1ß [34], and the elevation of these proinflammatory cytokines is also observed in animal models with impaired memory tasks [35] and altered expression of AMPA receptors [36]. This correlation suggests a potential bidirectional influence between iron overload and AMPA receptor metabolism.

PER is a selective, non-competitive antagonist of the AMPA receptor, it has lower toxicity and greater reach, blocking receptors containing both GluA1 and GluA2 subunits, two subunits present in the different AMPA receptor assemblies [37]. This allows PER to interact in different conditions in which CNS injury alters the expression or transcription of any of the AMPAR subunits.

Several studies have consistently linked the expression of different AMPAR subunits and their associated proteins to neuronal and behavioral disorders [38,39]. The structure of AMPAR has been well characterized and consists of combinations of the GluA1, GluA2, GluA3 and GluA4 subunits, which have approximately 70% homology in the peptide sequence [40]. However, understanding of the possible relationship between the assembly of distinct receptor subunits and neurodegenerative diseases is only just beginning to emerge [41].

The Ca2+ permeability of AMPAR (CP-AMPAR) varies depending on whether the GluA2 subunit is present in the tetramer. The insertion of AMPARs, which are permeable to calcium ions and can thus promote neurotoxicity, results from a decreased expression of genes encoding the GluA2 subunit or increased expression of genes encoding the GluA1 subunit [42].

In our study, neonatal iron overload increase the relative expression of the GRIA1 gene, which is responsible for the expression of the GluA1 subunit of the AMPA receptor, and decrease the relative expression of the DLG4 gene, which is responsible for the synthesis of the PSD-95 protein, a scaffolding protein of the AMPA receptor. This effect of iron on the expression of GluA1 has already been documented in a study evaluating the effect of a diet rich in iron on young rats, in which it was found that at the end of one month of iron supplementation, there was a 70% increase in the expression of GluA1 in the prefrontal cortex. The authors suggested that both the glutamatergic and cholinergic pathways can be regulated by iron diet [43].

The correlation of the different AMPA subunits (GluA1 and GluA2) with the different scaffolding and cytoplasmic trafficking proteins has already been extensively studied, since a decrease in the expression of the AMPAR-GluA2 subunit in the hippocampus is accompanied by a decrease in postsynaptic density protein 95 (PSD-95), while the levels of the transmembrane regulatory protein stargazin increase [44]. As shown in the present study, iron overload significantly decreased the expression of the DLG4 gene, the gene encoding PSD-95, while it increased, although not significantly, the expression of GRIA2, the gene encoding the AMPAR-GluA2 subunit. Despite these findings, iron overload and the use of PER did not significantly affect the expression of the CAC gene, which encodes the stargazin protein.

In addition to changes in the synthesis of the various proteins and structures that regulate the insertion and function of AMPA receptors, during the process of neuronal toxicity, neuroinflammation and neurodegeneration, the expression of the receptor subunits may be

regulated by the administration of PER, as shown by Yang et al. [45], who demonstrated that the chronic use of PER can positively regulate the GluA2 subunit. However, in the present study, PER decreased, although not significantly, the expression of the gene encoding GluA2 and especially GluA1 in the groups exposed to iron overload.

Consistent with the above, several studies have shown that increased gene expression of the GluA1 subunit, as well as the relatively higher GluA1:GluA2 ratio [45] found in the present study, results in the synaptic insertion of a greater number of CP-AMPARs (Ca2+ ion-permeable AMPA receptors) [47], demonstrating a unique relationship between receptor expression, trafficking, and insertion in a variety of animal models of neurodegenerative disease [48].

The fact that iron overload, in addition to increasing the expression of the GluA1 subunit, increased its phosphorylation, reinforces the hypothesis that the injury caused by the metal not only altered the Glua1/Glua2 ratio, but also altered the functional characteristics of the subunit.

As demonstrated by western blot analyses, the injury caused by iron overload increased the phosphorylation of the GluA1 subunit, an effect reversed by the administration of PER. Despite the significant results, publications that studied the effect of PER on the phosphorylation of GluA1 receptors are scarce and inconclusive. A recent study by Zhai et al., (2023), evaluated the effect of the drug on the expression of PKC (protein that phosphorylates GluA1) and phosphorylation of GluA1, trying to explain the effect of PER on migraine, through the expression of PKC and other enzymes, without finding a clear correlation [49]. Kim et al., (2019), found that PER influences the phosphorylation of GluA1 depending on the exposure time, in the same way that it alters the expression of several enzymes that phosphorylate the GluA1 subunit, such as PKC and CAMKII [50].

Regarding the increase in GluA1 phosphorylation mediated by iron overload, we can suggest it causes an increase in the placement of the subunit in the plasma membrane through this process. Serine 845 phosphorylation decreases GluA1binding to the AP2 adapter protein, which internalizes the subunit and, with it, the AMPA receptor [51].

As is already known, the phosphorylation of the GluA1 and GluA2 subunits of AMPA receptors may be correlated with LTP, but probably not at serine 845 [52]. The phosphorylation of GluA1 serine 845 may be related to the expression of PKA [53], suggesting future studies that seek to correlate iron overload, the use of PER and the expression of enzymes that phosphorylate GluA1 subunits.

Through our mRNA expression results and behavioral testing, we established a clear correlation between neonatal iron overload, the relative expression of genes responsible for encoding AMPA receptor subunits and scaffold proteins, and cognitive impairment.

Evidently, exposure to iron overload during the neonatal period instigates a neurodegenerative condition, which is further exacerbated by the insertion of AMPA receptors permeable to calcium ions. This is facilitated by the increased relative expression of the GRIA1 gene, responsible for the GluA1 subunit, and its phosphorylation at serine 845.

CP-AMPARs are not blocked by extracellular cations and allow Ca2+ entry at all levels of receptor activation. In fact, CP-AMPARs are significantly increased in disease states that can trigger mitochondrial dysfunction and cell death [54] acting synergistically with the degenerative effects of iron overload. This may explain why PER, a non-selective AMPA receptor antagonist, was able to reverse the cognitive deficit produced by iron overload.

Conclusion

In conclusion, the present study shows that the administration of PER was able to reverse, at least in part, the cognitive deficit caused by iron overload in the rats neonatal period. Furthermore, it was observed for the first time that iron overload in the neonatal period increased the relative expression of the GRIA1 gene, that codes the GluA1 subunit of the AMPA receptor and increase the phosphorylation of serine 845. Since the increase in GRIA1 expression and consequent increased calcium permeability have been consistently reported in disease context, this may be one of the mechanisms underlying iron neurotoxicity later in life. Further molecular analyses attempting to elucidate the molecular mechanisms relying PER neuroprotective effects, especially analyzing the different proteins kinases capable of phosphorylating different sites on the AMPA receptor subunits.

Statements & Declarations

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Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

This study was approved by the Institutional Ethics Committee for the Use of Animals of the Federal University of Rio Grande do Sul (CEUA , #35604) and all experimental procedures were performed in accordance with the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil).

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

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4. CAPÍTULO 4

4.1 DISCUSSÃO

Em 2020, aproximadamente, 50 milhões de indivíduos em todo o mundo tinham uma doença neurodegenerativa, resultando, muitas vezes, em demência, um número que deverá aumentar para 152 milhões até 2060 (PAPADOPOULOS, 2020). A prevalência global de doenças neurodegenerativas que levam a esse estado, calculada por uma meta-análise que avaliou os principais estudos europeus de relevância (EURDEM), é de 1,6% e 1% para homens e mulheres, respectivamente, na faixa etária de 65 a 69 anos, aumentando para 11% e 12,6% na faixa etária de 85-89 anos. Dos diferentes tipos de demência, 62% dos casos são atribuíveis à doença de Alzheimer (DA) (LOBO et al., 2000; ZHANG et al, 2021).

Devido ao aumento exponencial da população na faixa etária avançada, as doenças neurodegenerativas passaram a ocupar, gradativamente, um lugar de maior destaque nas preocupações da saúde pública e nos programas de assistência especializada (GRABHER et al., 2018). Em especial, podemos citar o DA e a DP (HOU et al., 2019). Porém, apesar da notável importância das doenças neurodegenerativas no cuidado da saúde populacional, podemos citar dois grandes problemas da atualidade.

Primeiro, a desigualdade no acesso aos médicos, o que significa, que nem todos os pacientes com demência e outras doenças neurodegenerativas recebem acompanhamento médico sistemático e especializado e, portanto, as condições tratáveis podem ser negligenciadas ou mal geridas, levando ao declínio funcional acelerado, hospitalizações e aumento da mortalidade, acarretando, um aumento dos custos de saúde (FREDERIKSEN et al., 2020).

Segundo, a polifarmácia, presente em pacientes idosos, que gera interações e reações adversas e, uma relação risco-benefício desfavorável (FREDERIKSEN et al., 2020). Além disso, os tratamentos disponíveis no momento, para as diferentes doenças neurodegenerativas apresentam um impacto de modesto a nulo na qualidade de vida, visível, principalmente, na DP (ZHAO et al., 2021) e DA (BIRKS e HARVEY, 2018), visto que, a própria condição clínica dos pacientes diminui, vertiginosamente, a qualidade de vida (FREDERIKSEN et al., 2020).

O panorama citado acima, foi o plano de fundo para o desenvolvimento da ideia de pesquisa do presente trabalho, principalmente, pela necessidade de avançar no entendimento das vias subjacentes à patogênese das doenças neurodegenerativas, com foco em diferentes alvos, como os receptores AMPA, conforme descrito no referencial teórico e em nosso artigo de revisão.

Em um artigo de revisão, Richard Armstrong, pesquisador de Birmingham, Reino Unido, levantou-se algumas hipóteses para o surgimento de doenças neurodegenerativas e, além de descrever os fatores de risco, como alterações genéticas, ambientais e de hábitos, citou

algumas condições fisiopatológicas do SNC, ressaltando o impacto do estresse oxidativo e a correlação dele com as proteínas malformadas, vetores conhecidos da neurodegeneração (ARMSTRONG, 2020).

Pois bem, como descrito em nosso referencial teórico e reforçado nos presentes artigos e em publicações do nosso grupo de pesquisa, uma das formas de aumentar o estresse oxidativo e, assim, gerar uma cascata de neurodegeneração, é através do acúmulo de metais redox, como por exemplo, com a sobrecarga de ferro.

Através de nossos estudos, foi possível perceber o impacto do acúmulo de ferro, não apenas nos déficits cognitivos, como já era esperado, mas também, na expressão de diferentes subunidades do receptor AMPA. A influência mais notável foi no aumento da expressão relativa da subunidade GluA1, que, de acordo com a literatura, quando aumentado gera uma tendência em sobrepor receptores com características de maior permeabilidade aos íons cálcio. Ou seja, a sobrecarga de ferro, provavelmente, através do estresse oxidativo, foi capaz de modificar o perfil de receptores AMPA para um receptor que passa a auxiliar no processo neurodegenerativo.

Um dos principais achados em estudos experimentais da sobrecarga de ferro no período neonatal é o déficit de memória, causado nas tarefas comportamentais, como por exemplo, nos testes de esquiva inibitória e de reconhecimento de objeto novo. Ambos, são modelos que permitem a avaliação da memória na fase adulta, o que sugere que apesar do insulto causado pela sobrecarga do ferro ser em um evento distante do teste, no período neonatal, o estímulo neurotóxico desencadeia um processo neurodegenerativo que perdura por todo o desenvolvimento neuronal dos animais. Através da presente pesquisa, podemos incluir também nesse processo, o surgimento de receptores AMPA permeáveis ao cálcio, somando no dano neuronal causado pela sobrecarga de ferro.

A partir do referencial bibliográfico utilizado para dar embasamento teórico à pesquisa pôde-se documentar diversas proteínas, que de alguma maneira, interagem com a funcionalidade do receptor AMPA e suas subunidades. Dentre elas, a proteína de densidade pós-sináptica (PSD-95), essencial para a colocação da subunidade GluA2 na membrana plasmática (REDULESCU et al., 2021). A partir dos resultados experimentais encontrados em nossa pesquisa, notou-se que, a sobrecarga de ferro foi capaz de diminui a expressão relativa dessa proteína, o que pode ter influenciado na razão GluA1:GluA2, ou até resultar na ineficiência da colocação de GluA2 e estabilização do mesmo no local de montagem do AMPAR (SUMIOKA et al., 2010). Esse resultado corrobora com o achado anterior sobre a expressão relativa de GluA1, já que, a presença de receptores AMPA não permeáveis ao cálcio precisam,

necessariamente de, pelo menos, uma subunidade GluA2 na montagem, demonstrando que, a sobrecarga de ferro pode ter influenciado em ambas as subunidades do receptor.

O artigo de revisão publicado pelo nosso grupo de pesquisa detalha o papel dos receptores AMPA permeáveis ao cálcio nas doenças neurodegenerativas (DA SILVA e SCHRÖDER, 2023), demonstrando que o aumento do $[Ca^{2+}]^i$ mediado pelos receptores AMPA é um componente presente em desordens como na Esclerose Amiotrófica Lateral (YIN et al., 2002), na Doença de Parkinson (KOBYLECKI et al., 2010) e Doença de Alzheimer (WHITEHEAD et al., 2017), corroborando com os resultados nos testes de déficits cognitivos comportamentais.

Devido à característica de alta permeabilidade aos íons cálcio encontrada no receptor glutamatérgico NMDA, a maioria das pesquisas que estudam opções terapêuticas para doenças neurodegenerativas estudam as vias glutamatérgicas que utilizam esse receptor. Um plano diferente para buscar entender os processos degenerativos do SNC é através do receptor AMPA, que, como descrito em nosso artigo de revisão, passa a ser permeável ao cálcio em doenças neurodegenerativas (DA SILVA e SCHRÖDER, 2023).

Sendo assim, ao utilizar o fármaco Perampanel (PER) é possível avaliar a via glutamatérgica sobre a via dos receptores AMPA e, dessa forma, verificar o quanto os receptores influenciam no processo de neuroproteção.

Em nossas pesquisas foi possível verificar que o uso subcrônico de PER, apesar de, não afetar a expressão relativa de proteínas relacionadas e subunidades do receptor AMPA foi capaz de reverter o déficit cognitivo no modelo de esquiva inibitória, promovido pela sobrecarga de ferro. Bem como, foi capaz de reverter o aumento da fosforilação na Serina 845 da subunidade GluA1 induzida pela sobrecarga de ferro, sugerindo que o efeito neuroprotetor do PER pode estar relacionado com essa via. É possível que esse efeito esteja relacionado com o efeito do PER na diminuição de citocinas pró-inflamatórias (ALQAHTANI, et al., 2020), como demonstrado no estudo de Chen et al. (2017), em que o PER foi capaz de suprimir a expressão das citocinas pró-inflamatórias TNF-α e IL-1β, e aumentar os níveis das citocinas anti-inflamatórias IL-10 e TGF-β1, efeito que pode estar relacionado com a diminuição na atividade da PKA, enzima que fosforila a serina 845 da subunidade GluA1 (WIGERBLAND et al., 2017).

Futuros trabalhos podem ser desenvolvidos, a fim de avaliar o efeito da sobrecarga de ferro na expressão das enzimas como PKA e PKC que fosforilam em diferentes sítios a subunidade GluA1, bem como avaliar o efeito do PER, não apenas na fosforilação, mas sim nos mecanismos que a sustentam.

4.2 CONCLUSÃO

Com base em nossos resultados, pode-se verificar que o PER, um antagonista não competitivo do AMPAR, apresenta um efeito neuroprotetor parcial sobre o déficit cognitivo induzido pela sobrecarga com ferro no período neonatal, em ratos Wistar Adultos. Ainda, foi definido, pelos resultados, que a sobrecarga neonatal de ferro, altera os níveis de RNAm da subunidade GluA1 e da proteína PSD95, evidenciando uma possível via de indução para o surgimento de AMPAR permeáveis ao cálcio. Por fim, verificou-se que a administração subcrônica de PER diminui a fosforilação da subunidade GluA1 na serina 845, induzida pela sobrecarga de ferro, sugerindo a possível via de neuroproteção do fármaco.

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ANEXOS

ANEXO A – Carta de aprovação da comissão de ética no uso de animais UFRGS.

U F R G S
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 35604
Título: EFEITO DO USO DE PERAMPANEL NA CAPACIDADE COGNITIVA DE RATOS WISTAR SUBMETIDOS A SOBRECARGA DE FERRO NO PERIODO NEONATAL

Vigência: 09/07/2018 à 01/06/2022

Pesquisadores:

Equipe UFRGS:

NADJA SCHRODER - coordenador desde 09/07/2018
José Afonso Corrêa da Silva - Aluno de Doutorado desde 09/07/2018

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 17/09/2018 - Sala 330 do Anexo I do Prédio da Reitoria - Campus Centro - Av. Paulo Gama,100/ Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 30 ratas Wistar prenhes (para uso efetivo de 76 filhotes machos), ambos obtidos do Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL); de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 28 de Setembro de 2018

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Vice Coordenador da comissão de ética

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