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VANESSA KAPPEL DA SILVA

EFEITOS DO CANABIDIOL SOBRE PARÂMETROS MITOCONDRIAIS E APOPTÓTICOS EM HIPOCAMPO DE RATOS TRATADOS COM FERRO NO PERÍODO NEONATAL

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Orientadora: Profª. Drª. Nadja Schröder

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RESUMO

O acúmulo de ferro no cérebro tem sido observado tanto no envelhecimento normal quanto em muitas doenças neurodegenerativas. Nossos estudos anteriores mostraram que a sobrecarga de ferro cerebral resulta em déficits de memória persistentes, acompanhados por estresse oxidativo. A elevada taxa metabólica do sistema nervoso torna as mitocôndrias essenciais para células nervosas. Essas organelas têm como função o controle da homeostasia do ferro em seu interior e o gerenciamento das espécies reativas de oxigênio. Uma vez que ocorre desregulação nessas atividades, o funcionamento das mitocôndrias fica comprometido, resultando em falhas no aporte energético principalmente para as sinapses. O funcionamento inadequado de circuitos neurais pode culminar na ativação de vias de morte celular, uma característica bastante associada às doenças neurodegenerativas. No presente trabalho analisamos os efeitos da sobrecarga de ferro neonatal sobre as deleções no complexo I do DNA mitocondrial; sobre os mecanismos de metilação e hidroximetilação do DNA mitocondrial; sobre proteínas envolvidas no metabolismo de ferro mitocondrial (Ferritina mitocondrial e Mitoferrina 2), sobre a atividade enzimática da Succinato desidrogenase e Creatina quinase, envolvidas no aporte energético para as células; e sobre proteínas envolvidas nas vias apoptóticas, como a Caspase 8, Caspase 9, Caspase 3, Citocromo c, APAF1 e PARP em hipocampos de ratos adultos. Além disso, investigamos os efeitos do canabidiol (CBD), principal componente não psicotrópico da *Cannabis sativa*, na reversão dos efeitos induzidos pelo ferro sobre todos os parâmetros analisados. Ratos machos receberam veículo ou ferro carbonila (30 mg/kg) do 12º ao 14º dia pós-natal e na idade adulta foram tratados com veículo ou CBD (10 mg/kg) durante 14 dias. O tratamento com ferro induziu o aumento das deleções do DNA mitocondrial e das proteínas envolvidas na via intrínseca da apoptose, enquanto induziu a redução de metilação e hidroximetilação no DNA mitocondrial, bem como da atividade enzimática e da ferritina mitocondrial, proteína de armazenamento de ferro. O CBD reverteu os efeitos induzidos pelo ferro, recuperando os níveis de hidroximetilação, de ferritina mitocondrial, da atividade da Succinato dehidrogenase, e das proteínas apoptóticas Caspase 3, Caspase 9, PARP e APAF1 a níveis comparáveis com o controle. Os resultados sugerem que o ferro pode afetar mecanismos de funcionamento mitocondrial e desencadear vias de morte celular por apoptose. A reversão de alguns desses efeitos pelo CBD indica o seu potencial neuroprotetor.

Palavras-chave: Ferro, mitocôndria, apoptose, doenças neurodegenerativas, canabidiol.

ABSTRACT

Brain iron accumulation has been observed both in normal aging and in many neurodegenerative diseases. In previous studies, we have described that brain iron overload results in persistent memory deficits, accompanied by oxidative stress. The high metabolic rate of the nervous system makes mitochondria essential for nerve cells. These organelles control iron homeostasis in its interior and the management of reactive oxygen species. When deregulation in these activities occurs, mitochondrial functioning is compromised, resulting in failures in the energy supply mainly for the synapses. Inadequate functioning of neural circuits may culminate in the activation of cell death pathways, a feature strongly associated with neurodegenerative diseases. In the present study we analyzed the effects of neonatal iron overload on complex I deletions in the mitochondrial DNA ; on methylation and hydroxymethylation of mitochondrial DNA; on mitochondrial proteins involved on iron homeostasis, on the enzymatic activity of Succinate Dehydrogenase and Creatine Kinase, enzymes involved in the cells energy supply; and on proteins involved in apoptotic pathways, such as Caspase 8, Caspase 9, Caspase 3, Cytochrome c, APAF1, and PARP in the hippocampus of adult rats. In addition, we investigated the effects of cannabidiol (CBD), the main non-psychotropic component of *Cannabis sativa*, in reversing iron-induced effects on all parameters analyzed. Male rats received vehicle or iron carbonyl (30 mg / kg) from the 12^{th} to the 14^{th} postnatal day and were treated with vehicle or CBD (10mg / kg) for 14 days in adulthood. Iron treatment induced increased deletions of mitochondrial DNA and expression of proteins involved in apoptosis, while induced reductions of methylation and hydroxymethylation, enzymatic activity and mitochondrial ferritin, an iron storage protein. CBD reversed iron-induced effects, recovering hydroxymethylation levels, mitochondrial ferritin, Succinate dehydrogenase activity, apoptotic proteins Caspase 3, Caspase 9, PARP and APAF1 at levels comparable to controls. These results suggest that iron can affect mechanisms of mitochondrial functioning and trigger cell death pathways by apoptosis. The reversal of some of these effects by CBD indicates its neuroprotective potential.

Keywords: Iron, mitochondria, apoptosis, neurodegenerative disorders, cannabidiol.

LISTA DE ABREVIATURAS E SIGLAS

5hmC: 5- hidroximetilcitosina

5mC: 5- metilcitosina

Acetil CoA: Acetilcoenzima A

ADP: Adenosina difosfato (sigla do inglês *Adenosine diphosphate*)

APAF1: Fator 1 de ativação da protease apoptótica (sigla do inglês *Apoptotic protease activating factor-1*)

Aß: Beta amilóide (sigla do inglês *Amyloid beta*)

ATP: Trifosfato de adenosina (sigla do inglês *Adenosine triphosphate*)

BAK: Sigla do inglês *Bcl-2 homologous antagonist/killer*

BAX: Proteína X associada a Bcl-2 (sigla do inglês *Bcl-2-associated X protein*)

Bcl-2: Linfoma de células B- 2 (sigla do inglês *B cell lymphoma- 2*)

Bcl-XL: Linfoma de células B extra grande (sigla do inglês *B-cell lymphoma-extra large*)

BH3-only: Sigla do inglês *Bcl-2 homology domain 3-only*

BID: Agonista de domínio de morte interagindo com BH3 (sigla do inglês *BH3 interacting domain death agonist*)

BIM: Sigla do inglês *Bcl-2 interacting mediator of cell death*

CBD: Canabidiol

CpG: Nucleotídeo citosina e guanina conectados por ligação fosfodiéster

DA: Doença de Alzheimer

DH: Doença de Huntington

DIABLO: Proteína de ligação direta ao IAP com baixo pI (sigla do inglês *Direct IAP-binding protein with low pI*), também conhecida como SMAC

DISC: Complexo de sinalização indutor de morte (sigla do inglês *Death-inducing signaling complex*)

DNA: Ácido desoxirribonucleico (sigla do inglês *Deoxyribonucleic acid*)

DNM1L: Proteína semelhante à dinamina-1 (sigla do inglês *Dynamin 1-like*)

DNMT1: DNA metiltransferase 1 (sigla do inglês *DNA methyltransferase 1*)

DNMT3a: DNA metiltransferase 3 alfa (sigla do inglês *DNA methyltransferase 3 alpha*)

DP: Doença de Parkinson

FADD: Proteína com domínio de morte associado à Fas (sigla do inglês *Fas-associated death domain protein*)

FasL: Ligante Fas (sigla do inglês *Fas ligand*)

FasR: Receptor Fas (sigla do inglês *Fas receptor*)

GFAP: Proteína glial fibrilar ácida (sigla do inglês *Glial fibrillary acidic protein*)

H_2O_2: Peróxido de hidrogênio

HtrA2: Proteína A2 dependente de alta temperatura (sigla do inglês *High temperature requirement protein A2*), também conhecida como Omi

MCI: Comprometimento cognitivo leve (sigla do inglês *Mild Cognitive Impairment*)

Mcl1: Proteína de diferenciação celular da leucemia mielóide induzida (sigla do inglês *Induced myeloid leukemia cell differentiation protein*)

MOMP: Permeabilização da membrana externa mitocondrial (sigla do inglês *Mitochondrial outer membrane permiabilization*)

MPTP: 1-metil-4-fenil-1,2,3,6-tetraidropiridina (sigla do inglês *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*)

NADH: Nicotinamida adenina dinucleotídeo (sigla do inglês *Nicotinamide adenine dinucleotide*)

ND1: NADH desidrogenase subunidade 1 (sigla do inglês *NADH dehydrogenase subunit 1*)

ND2: NADH desidrogenase subunidade 2 (sigla do inglês *NADH dehydrogenase subunit 2*)

ND3: NADH desidrogenase subunidade 3 (sigla do inglês *NADH dehydrogenase subunit 3*)

ND4: NADH desidrogenase subunidade 4 (sigla do inglês *NADH dehydrogenase subunit 4*)

ND4L: NADH desidrogenase subunidade 4L (sigla do inglês *NADH dehydrogenase subunit 4L*)

ND5: NADH desidrogenase subunidade 5 (sigla do inglês *NADH dehydrogenase subunit 5*)

ND6: NADH desidrogenase subunidade 6 (sigla do inglês *NADH dehydrogenase subunit 6*)

NOXA: Gene que codifica a proteína *homology 3 (BH3)-only member*, membro da família Bcl-2

Omi: Endoprotease regulada por estresse (sigla do inglês *Stress-regulated endoprotease*), também conhecida como HtrA2

OPA1: Proteína atrofia óptica 1 (sigla do inglês *Optic atrophy 1*)

PAR4: Proteína de resposta apoptótica prostática 4 (sigla do inglês *Prostate apoptosis response 4*)

PARP: Enzima poli (ADP-ribose) polimerase (sigla do inglês *Poly (ADP-ribose) polymerase*)

PUMA: Modulador de apoptose regulado pela P53 (sigla do inglês *P53 upregulated modulator of apoptosis*)

RNA: Ácido ribunucleico (sigla do inglês *Ribonucleic acid*)

SH SY5Y: Linhagem celular de neuroblastoma humano

SMAC: Segundo ativador mitocondrial de caspases (sigla do inglês *Second mithocondria-derived activator of caspases*), também conhecido como DIABLO

TET: Translocação dez-onze (sigla do inglês *Ten-eleven translocation*)

THC: Tetra-hidrocanabinol (sigla do inglês *Tetrahydrocanabinol*)

TNF: Fator de necrose tumoral (sigla do inglês *Tumor necrosis factor*)

TNFR1: Receptor 1 do fator de necrose tumoral (sigla do inglês *Tumor necrosis factor receptor 1*)

TNFα: Fator de necrose tumoral alfa (sigla do inglês *Tumor necrosis factor α*), também conhecido como TNF

TRADD: Domínio de morte associado ao Receptor tipo 1 do fator de necrose tumoral (sigla do inglês *Tumor necrosis factor receptor type 1 associated DEATH domain*)

UPS: Sistema ubiquitina-proteassoma (sigla do inglês *Ubiquitin Proteasome System*)

XIAP: Proteína inibidora de apoptose ligada ao X (sigla do inglês *X-linked inhibitor of apoptosis protein*)

SUMÁRIO

CAPÍTULO 1

1.1 REFERENCIAL TEÓRICO

1.1.1 Acúmulo de ferro cerebral associado à neurodegeneração

O envelhecimento é um processo fisiológico caracterizado por um gradual e duradouro declínio funcional que afeta vários sistemas, como, por exemplo, o circulatório, digestivo, respiratório e o nervoso (Dziechciaz e Filip, 2014). Uma vez que afeta múltiplos órgãos, o envelhecimento torna-se fator de risco para uma série de doenças (McHugh e Gil, 2018). O aumento da expectativa de vida da população amplia a necessidade de pesquisas avaliando os mecanismos envolvidos na biologia do envelhecimento, visando o retardo e/ou a prevenção das patologias relacionadas à idade. A saúde mental é uma preocupação importante atualmente, tendo em vista a alta prevalência de declínio cognitivo observada no envelhecimento (Freitas *et al*., 2017). Embora declínio cognitivo seja comum em idades mais avançadas, a relação entre envelhecimento e doenças neurodegenerativas (como o caso da doença de Alzheimer - DA, doença de Parkinson – DP, comprometimento cognitivo leve - MCI, entre outras doenças) ainda permanece obscura (Lin *et al*., 2017). A etiologia dessas doenças ainda não está completamente estabelecida, mas danos oxidativos mediados por metais têm sido propostos como contribuintes significativos para os processos de neurodegeneração (Salvador *et al*., 2010).

Muitos metais, tais como ferro, cobre, magnésio e zinco, são essenciais em diversos processos fisiológicos do organismo (Chen *et al*., 2016). Uma vez que acúmulo ou deficiência de metais está relacionado a muitas patologias (Fraga, 2005), torna-se necessário amplo controle na homeostasia desses metais por meio da regulação de mecanismos de captação, armazenamento e secreção (Salvador *et al*., 2010). Nos mamíferos, o ferro é um metal essencial, participando de funções biológicas vitais, tais como transporte de oxigênio, respiração mitocondrial, síntese e reparo do DNA (sigla do inglês *Deoxyribonucleic acid*), proliferação e diferenciação celular e metabolismo dos fosfolipídios, entre inúmeras outras (Duzek *et al*., 2016).

Em homeostasia, o ferro no cérebro é crucial para manutenção do aporte energético dos tecidos neurais, além de estar envolvido na síntese e metabolismo da mielina e neurotransmissores (Belaidi e Bush, 2016). A perda dessa regulação leva ao acúmulo de ferro, que tem sido observado tanto no processo de envelhecimento cerebral normal quanto em muitas patologias que acometem o sistema nervoso (Ward *et al*., 2014). No envelhecimento

saudável, é visto acúmulo desse metal em diversas regiões cerebrais e tipos celulares, enquanto que, em muitas doenças neurodegenerativas, o acúmulo de ferro é muito maior e em regiões cerebrais específicas (Ward *et al*., 2014). Alguns estudos já relataram que neurônios, astrócitos e microglia apresentam acúmulo de ferro em regiões específicas como nos gânglios da base, assim como no córtex e hipocampo, alvos preferenciais nos processos neurodegenerativos como DA, DP e DH (Doença de Huntington) (revisado por Zecca *et al*., 2004; revisado por Li e Reichmann, 2016). Esse acúmulo de ferro observado no envelhecimento tem efeito tóxico para as células cerebrais devido à formação de radicais livres via Reação de Fenton, podendo ocasionar peroxidação lipídica dos ácidos graxos que compõem as membranas celulares dos neurônios, danos oxidativos a proteínas constitutivas e ao DNA, além de poder levar à morte celular (Mills *et al*., 2010; Wessling-Resnick, 2017).

Apesar do excesso de ferro e sua relação com as doenças neurodegenerativas ser amplamente reconhecido, os seus mecanismos não estão completamente estabelecidos (Dusek *et al*., 2016). A principal estratégia de proteção para evitar sobrecarga de ferro no cérebro é a barreira hemato-encefálica, que limita a passagem de ferro do sangue para o cérebro por meio de sistemas de transporte seletivos altamente regulados (Mills *et al*., 2010). Essa barreira passa por processo de amadurecimento até os seis meses de vida do ser humano, período no qual ainda não possui completa capacidade de regular a transferência de moléculas entre o sangue e o cérebro (Collard, 2009). Além disso, estudos anteriores já observaram que nessa fase ocorre aumento nos receptores de transferrina na barreira, o que ocasionaria uma maior captação de ferro (Taylor e Morgan, 1990). O processo de maturação da barreira e a capacidade de regulação do ferro ocorrem em um período em que o cérebro passa por etapas de desenvolvimento fundamentais dependentes de ferro (Collard, 2009). Foi demonstrado que a etapa neonatal é crítica para o estabelecimento do teor de ferro normal no cérebro adulto e, uma vez absorvido, o ferro não retorna mais para o plasma, sendo redistribuído dentro do encéfalo (Dwork *et al*., 1990).

Fredriksson e colaboradores (1999) demonstraram pela primeira vez que o tratamento sistêmico com ferro durante o período de rápido desenvolvimento cerebral ocasiona acúmulo de ferro nos gânglios da base, além de causar disfunções neurocomportamentais em camundongos. Também foi observado que ratos tratados com ferro entre o 10º e o 12º dia de vida pós-natal apresentam hipoatividade motora, bem como déficits no aprendizado e memória nas tarefas comportamentais de labirinto radial de oito braços e esquiva inibitória (Schröder *et al.,* 2001). Estudos realizados por nosso grupo de pesquisa verificaram que a memória de reconhecimento e a memória aversiva também são prejudicadas em ratos tratados

com ferro no período neonatal (Schröder *et al*., 2001; de Lima *et al*., 2005; 2007; Perez *et al*., 2010; Rech *et al*., 2010; Fagherazzi *et al*., 2012; Garcia *et al*., 2013). De Lima e colaboradores (2005) verificaram que o ferro induz aumento significativo na peroxidação lipídica na substância negra, no córtex e no hipocampo, bem como aumento de danos oxidativos às proteínas nestas mesmas regiões cerebrais em ratos adultos. Constataram ainda uma diminuição da atividade da superóxido dismutase (enzima antioxidante) na substância negra, no córtex e no hipocampo (de Lima *et al.*, 2005). Esses resultados sugerem que o ferro pode estar exercendo seus efeitos deletérios sobre a cognição através do aumento dos níveis de estresse oxidativo cerebral (de Lima *et al.*, 2005). Além disso, foi verificado que tratamento com quelantes de ferro previnem déficits de memória e estresse oxidativo em ratos idosos (de Lima *et al*., 2008).

Análises imunohistoquímicas demonstraram que o tratamento de ratos com ferro no período neonatal induz o aumento de proteínas envolvidas na cascata de apoptose, Caspase 3 e Par4 (sigla do inglês *Prostate apoptosis response 4*), em hipocampo e córtex de ratos adultos (Miwa *et al*., 2011). Provavelmente, isso se justifica pelo fato do ferro possuir um grande potencial de causar danos oxidativos nas células, verificando-se então um possível aumento na suscetibilidade para a neurodegeneração (Miwa *et al*., 2011). Também foi verificado em ratos adultos e velhos tratados com ferro no período neonatal aumento da proteína GFAP (sigla do inglês *Glial fibrillary acidic protein*), indicando gliose reativa, sugerindo que o tratamento com ferro induz à perda de células nervosas (Fernandez *et al*., 2011). Nosso estudo anterior verificou o papel deletério do ferro durante o período neonatal de ratos (da Silva *et al*., 2014). Nesse trabalho, pudemos observar que o ferro causou modificações nas proteínas envolvidas na dinâmica mitocondrial, reduzindo os níveis da proteína de fissão mitocondrial, DNM1L (sigla do inglês *Dynamin 1-like*), e de fusão mitocondrial, OPA1 (sigla do inglês *Optic atrophy 1*), no hipocampo e córtex, respectivamente (da Silva *et al*., 2014). Além disso, constatamos uma elevação nos níveis da proteína apoptótica Caspase 3 no hipocampo e córtex, seguido de uma redução nos níveis da proteína marcadora de viabilidade sináptica, sinaptofisina, no hipocampo (da Silva *et al*., 2014). Analisando o sistema UPS (sigla do inglês *Ubiquitin Proteasome System*) em hipocampos de ratos tratados com ferro neonatal, observamos aumento de proteínas poliubiquitinadas e esse mau funcionamento do sistema UPS pode estar relacionado ao comprometimento cognitivo induzido pelo ferro (Figueiredo *et al*., 2016). Foi observado também que o ferro causa decréscimo na atividade da acetilcolinesterase (Perez *et al*., 2010) e afeta regulação de proteínas responsáveis pela sua homeostasia (Dornelles *et al*., 2010). Esses

dados nos levam a sugerir que o potencial oxidativo do ferro altera o funcionamento mitocondrial, o que pode contribuir para déficits em produção de energia, declínio de viabilidade sináptica e consequentemente, morte celular.

Tendo em vista todos os aspectos abordados a respeito do uso ferro no período neonatal em ratos, esse se torna um adequado modelo animal de neurodegeneração. Isso permite estudos mais amplos visando um maior e melhor entendimento de todos os processos biológicos que contribuem nessas patologias que acometem o cérebro. Um importante foco de estudo são as mitocôndrias, uma vez que essas organelas são essenciais para o sistema nervoso.

1.1.2 Funcionamento mitocondrial

As mitocôndrias são organelas multifuncionais que desempenham papéis importantes na vida e morte das células, fornecendo ATP (sigla do inglês *Adenosine triphosphate*) para processos metabólicos por meio da fosforilação oxidativa (Oettinghaus *et al*., 2012). Elas também participam em uma série de outros processos, como por exemplo, controle de radicais livres, biossíntese de aminoácidos, lipídios e nucleotídeos, homeostase de cálcio e ferro, sinalização celular e apoptose (Herst *et al*., 2017). Essas organelas são altamente dinâmicas, sendo ativamente transportadas nas células e podendo mudar rapidamente a estrutura interna e a distribuição de acordo com as condições fisiológicas (Wang *et al*., 2009).

O encéfalo é um órgão composto por células altamente diferenciadas que povoam diferentes regiões anatômicas, possuindo alta demanda energética para manter potenciais de membrana e propagar sinais elétricos, liberar e recaptar neurotransmissores na fenda sináptica e realizar plasticidade sináptica (Grimm e Eckert, 2017). Apesar de representar apenas 2% da massa corporal do ser humano, consome 20% do total de oxigênio utilizado pelo corpo, sendo os neurônios as células que mais requerem energia (Schönfeld e Reiser, 2013). A fosforilação oxidativa é responsável por mais de 90% da energia gerada no cérebro, tornando as mitocôndrias essenciais para as células nervosas (Schönfeld e Reiser, 2013). Consistente com isso, as mitocôndrias estão presentes em grande número nas células nervosas, preferencialmente nos sítios pré e pós-sinápticos, uma vez que a maior parte do ATP cerebral é consumida para promover a transmissão sináptica (Harris *et al*., 2012).

Se por um lado as mitocôndrias produzem energia necessária para a sobrevivência celular, por outro lado, induzem a formação de espécies reativas de oxigênio, que podem ser prejudiciais quando produzidas em excesso (Golpich *et al*., 2017). O fato de o tecido cerebral

requerer grande aporte energético contribui para a formação de espécies reativas de oxigênio, favorecendo o estresse oxidativo (Grimm e Eckert, 2017). No cérebro em envelhecimento é observada uma redução da função mitocondrial, associada a um acúmulo de dano nessas organelas e de espécies reativas de oxigênio (Chakrabarti *et al*., 2011). Devido ao importante papel exercido pelas mitocôndrias no cérebro, é de se esperar que quaisquer disfunções no metabolismo dessas organelas possam causar alterações no suporte energético celular, ocasionando sérias consequências para a função e sobrevivência neuronal (Yin *et al*., 2016). Estudos recentes têm relevado que a função mitocondrial prejudicada desempenha um papel fundamental no envelhecimento e na patogênese de doenças neurodegenerativas (revisado por Golpich *et al*., 2017).

Muitos processos neurodegenerativos são marcados por deficiência mitocondrial, acúmulo de ferro cerebral e estresse oxidativo, situações que estão inter-relacionadas (Horowitz e Greenamyre, 2010). Logo, torna-se essencial a compreensão do papel fisiológico que as mitocôndrias desempenham na homeostasia do ferro celular para um melhor entendimento dessa relação em condições de envelhecimento e doença (Horowitz e Greenamyre, 2010).

1.1.2.1 Metabolismo do ferro mitocondrial

Uma vez que o ferro é essencial para a sobrevivência celular, esse metal precisa ser altamente regulado para manter as condições fisiológicas no organismo. Devido a sua propriedade oxidativa, o ferro livre pode reagir com oxigênio e catalizar a formação de espécies reativas de oxigênio, que são amplamente tóxicas para o organismo (Richardson *et al*., 2010). Logo, uma vez na circulação, o ferro é transportado no sangue e fluidos associado à proteina transferrina e, quando requerido pelas células, é internalizado para uso, enquanto o ferro em excesso é armazenado associado à proteína ferritina (Singh *et al*., 2014).

As mitocôndrias são os principais locais de utilização de ferro nas células, empregando esse metal na síntese do grupamento heme e na síntese de *clusters ferro-sulfur* (responsáveis por funções como transferência de elétrons, catálise enzimática e regulação da expressão gênica, entre outras) (Stehling *et al*., 2014). Logo, essas organelas desempenham um papel central na sobrevivência celular, não somente fornecendo energia como também participando da homeostasia do ferro celular. Com a liberação de elétrons que ocorre devido à fosforilação oxidativa e a necessidade de influxo de ferro para suprir suas demandas metabólicas, as mitocôndrias acabam tornando-se um local de grande produção de espécies

reativas de oxigênio, necessitando mecanismos rígidos de controle da homeostasia e toxicidade desse metal (Horowitz e Greenamyre, 2010).

Embora o excesso de ferro seja estocado primariamente no citoplasma da célula, a maior parte do ferro metabolicamente ativo das células é processada nas mitocôndrias (Yang *et al*., 2013). No entanto, ainda não está completamente elucidado como as mitocôndrias regulam a homeostasia do ferro em seu interior. A absorção do ferro para a matriz mitocondrial ocorre com o auxílio de proteínas localizadas na membrana interna, as mitoferrinas, pertencentes à família transportadora mitocondrial, também conhecida como família de proteínas transportadoras de soluto (Rouault, 2016). Existem dois tipos de mitoferrinas, a mitoferrina 1, que é altamente expressa em tecido hematopoiéticos (baço, medula óssea e fígado), sendo essencial para a assimilação do ferro eritróide, e a mitoferrina 2, que é expressa na maioria dos tecidos, possivelmente com função de manter os níveis adequados de ferro em células não eritróides (Ren *et al*., 2012).

O controle da expressão da proteína mitoferrina 1 está relacionado à necessidade de síntese de hemoglobina nas células eritróides (Chen *et al*., 2009). Quando os níveis de grupamento heme estão altos, ocorre desestabilização da mitoferrina 1, reduzindo a captação de ferro pela mitocôndria (Chen *et al*., 2009). No entanto, ainda permanece obscuro como é feita a regulação da expressão da mitoferrina 2 na maioria dos tecidos (Horowitz e Greenamyre, 2010). Apesar das mitoferrinas estarem envolvidas no transporte de ferro para as mitocôndrias, existem poucos estudos analisando o papel dessas proteínas em doenças neurodegenerativas. Estudos observaram um aumento da expressão de mitoferrinas na ataxia de Friedreich, DP e esclerose lateral amiotrófica, o que pode contribuir para o acúmulo de ferro mitocondrial nessas doenças (Huang *et al*., 2009; Carroll *et al*., 2011; Hadzhieva *et al*., 2013). Esses relatos sugerem um envolvimento das mitoferrinas na patogênese de doenças com acúmulo de ferro mitocondrial, podendo se tornar foco de abordagens futuras, visando uma expressão reduzida das mesmas para prevenção de acúmulo de ferro e consequentemente redução de sintomas nos pacientes (Ren *et al*., 2012).

Para evitar toxicidade, o ferro que entra na mitocôndria tem dois caminhos: ou é imediatamente utilizado ou é armazenado associado a uma proteína com função e estrutura muito similar à ferritina citosólica, chamada de ferritina mitocondrial (Horowitz e Greenamyre, 2010). Diferentemente da ferritina citosólica, que é amplamente expressa nos tecidos, a expressão da ferritina mitocondrial é tecido-específica, apresentando altos níveis em tecidos com alto consumo de energia, como o caso de testículos, cérebro, coração e pâncreas, e baixíssimos níveis em órgãos de armazenamento de ferro, como fígado e baço (Gao e

Chang, 2014). Essa distribuição tecidual indica que a ferritina mitocondrial pode exercer um efeito protetivo na mitocôndria contra o estresse oxidativo dependente de ferro em células caracterizadas por alta atividade metabólica e consumo de oxigênio (Santambrogio *et al*., 2007).

Apesar de a função e a regulação da ferritina mitocondrial ainda não estarem completamente elucidadas (Arosio e Levi, 2010), é de se presumir que uma proteína envolvida na homeostasia do ferro esteja relacionada à patogênese de doenças neurodegenerativas. No entanto, só recentemente estudos envolvendo a ferritina mitocondrial nas doenças neurodegenerativas começaram a surgir. Wang e colaboradores (2011) demonstraram aumento na expressão da ferritina mitocondrial no córtex de pacientes com DA e papel neuroprotetor contra estresse oxidativo induzido por peróxido de hidrogênio (H_2O_2). Um estudo mostrou que bloqueio na expressão da ferritina mitocondrial aumentou a neurotoxicidade induzida por Aβ (sigla do inglês *Amyloid beta*), o estresse oxidativo e a apoptose, apresentando resultados opostos quando da superexpressão dessa proteína (Wu *et al*., 2013). Outro estudo indicou que a deleção da ferritina mitocondrial exacerbou a apoptose e os prejuízos neurológicos ocasionados pela infusão de Aβ em camundongos (Wang *et al*., 2017). Em um modelo de DP, foi visto que a ferritina mitocondrial preveniu o dano neuronal induzido pela neurotoxina MPTP (sigla do inglês *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*) (You *et al*., 2016), enquanto que a superexpressão de ferritina mitocondrial protegeu as células de neuroblastoma humano SH-SY5Y do estresse oxidativo e preveniu contra o aumento da α-sinucleína induzido por H_2O_2 (Guan *et al*., 2017). Em geral, esses estudos sugerem que a ferritina mitocondrial pode atuar como um neuroprotetor contra a toxicidade ocasionada por ferro em certas doenças neurodegenerativas.

Uma vez que as mitocôndrias são a principal fonte de espécies reativas de oxigênio, elas tornam-se os principais alvos do dano oxidativo. Dentre as diversas alterações funcionais que essas organelas podem sofrer, um mecanismo central que medeia essas disfunções é o dano ao DNA mitocondrial, tornando-se um foco de estudo nas pesquisas relacionadas ao envelhecimento normal e às doenças relacionadas à idade (Keogh e Chinnery, 2015).

1.1.2.2 DNA Mitocondrial

As mitocôndrias são as únicas organelas do ser humano, além do núcleo, a possuírem material genético próprio e independente, sendo uma herança materna devido à eliminação de mitocôndrias masculinas durante a embriogênese precoce (Phillips *et al*., 2014). O DNA mitocondrial consiste em uma pequena molécula circular dupla-fita com aproximadamente 16.600 pares de bases, sendo encontradas de 2 a 10 cópias em cada organela, associadas a proteínas, formando agregados chamados nucleóides (Keogh e Chinnery, 2015). As mitocôndrias contêm uma variedade relativamente abundante de proteínas, no entanto, possuem apenas 37 genes que codificam 13 proteínas da cadeia respiratória, 2 RNAs (sigla do inglês *Ribonucleic acid)* ribossômicos e 22 RNAs transportadores (Lee e Han, 2017). Isso se deve ao fato de a maior parte das proteínas mitocondriais serem codificadas pelo DNA nuclear e só depois serem importadas para os locais corretos na mitocôndria (Lee e Han, 2017).

Já foi observado que ocorre um acúmulo de mutações nas moléculas de DNA mitocondrial com o envelhecimento e que várias dessas mutações e deleções têm sido associadas com fenótipos clínicos (Lagouge e Larsson, 2013). Uma série de fatores ocasiona essa vulnerabilidade do DNA mitocondrial. Primeiramente, o genoma mitocondrial não está compactado na forma de nucleossomo como o genoma nuclear, deixando o material genético mais exposto; apresenta proximidade com a cadeia transportadora de elétrons, que é a maior fonte de espécies reativas de oxigênio; oferece menor eficiência no sistema de reparo para alguns tipos de danos, além de erros nos mecanismos de replicação; possui poucas (ou até mesmo nenhuma) regiões não-codificantes, o que aumenta as chances de mutações em regiões que codificam elementos essenciais (Gao *et al*., 2009; Santos *et al*., 2013). O aumento das mutações no DNA mitocondrial é uma das características observadas nas doenças neurodegenerativas, uma vez que o genoma mitocondrial codifica elementos importantes da cadeia de transporte de elétrons, que é a responsável pelo suprimento energético através da síntese de ATP, bastante comprometido no envelhecimento (Wallace, 2005; Picard e Turnbull, 2013).

Associação entre DA, DP, DH e esclerose múltipla com modificações no DNA mitocondrial já foram observadas. Nesses casos, foi verificado que estresse oxidativo elevado está associado a maiores danos ao genoma mitocondrial e diminuição da capacidade respiratória e foi observado que essas mutações e/ou deleções no DNA mitocondrial ocorrem em regiões cerebrais características dessas doenças neurodegenerativas (Hirai *et al*., 2001;

revisão em Yang *et al*., 2008; Siddiqui *et al*., 2012; Müller *et al*., 2013). O DNA mitocondrial afetado pelo estresse oxidativo vai contribuir para uma disfunção ainda maior nessa organela, que pode resultar em morte celular (Gao *et al*., 2009). No entanto, mais investigações são importantes para determinar o papel dos danos ao material genético mitocondrial e maneiras de reparo em doenças neurodegenerativas, visando melhores estratégias preventivas e terapêuticas a essas patologias.

As espécies reativas de oxigênio são geradas em diversos locais e situações nas células, sendo principalmente produzidas na cadeia transportadora de elétrons mitocondrial, especialmente nos complexos I e III (Sanz *et al*., 2006). O complexo I tem sido relatado como o mais afetado ao longo do envelhecimento, principalmente pelo fato de ser o complexo mitocondrial com mais subunidades (são sete NADH desidrogenases: ND1, ND2, ND3, ND4, ND4L, ND5 e ND6) codificadas pelo DNA mitocondrial (Greenamyre *et al*., 2001; Pollard *et al*., 2016). Estudos têm sido realizados analisando duas regiões específicas do genoma mitocondrial, que codificam para proteínas do complexo I. Uma dessas regiões está dentro do gene *ND1* e é raramente afetada por deleções ao longo do envelhecimento, enquanto a outra região está dentro do gene *ND4*, sendo afetada em larga escala (Van Tuyle *et al*., 1996). A figura 1 mostra esquematicamente o genoma mitocondrial, incluindo as regiões que sofrem maiores deleções.

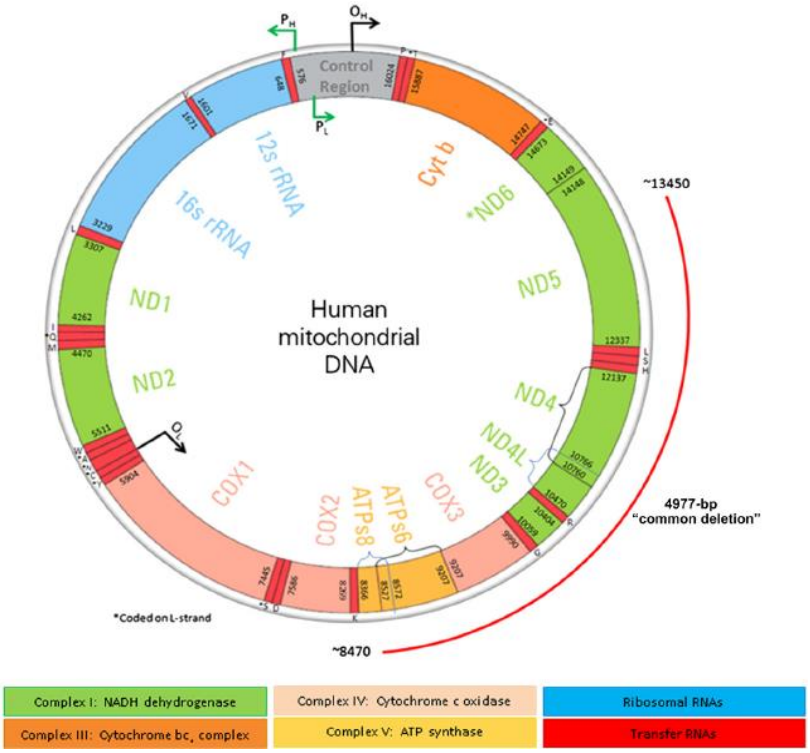

Figura 1: Esquema do genoma mitocondrial e localização das regiões "comuns" de deleção. Em verde estão representados os genes do complexo I; em laranja, os genes do

complexo III; em rosa, os genes do complexo IV; em amarelo, os genes do complexo V; em azul, os genes dos RNA ribossômicos; em vermelho, os genes dos RNA de transferência. (Adaptado de Philips *et al*., 2014).

Ochoa e colaboradores, em 2011, verificaram que o estresse oxidativo associado à idade ocasiona um aumento de deleções no DNA mitocondrial em cérebro de ratos velhos. Em seres humanos, verificou-se que na substância negra de controles idosos e pacientes com DP foi encontrado um alto nível de deleção no DNA mitocondrial, que foi associado à deficiência na cadeia respiratória (Bender *et al*., 2006). Aumento de deleção no DNA mitocondrial também foi observado na substância negra de pacientes com DP e hipocampo de pacientes com DA (Muller *et al*., 2013). Logo, nesses estudos foi possível verificar associação entre deleção no DNA na região codificadora do complexo I mitocondrial e envelhecimento e patologias associadas à idade.

Até pouco tempo atrás, mutações e deleções eram os únicos mecanismos analisados na maquinaria genética mitocondrial (Pirola *et al*., 2013). Embora haja um melhor entendimento das modificações epigenéticas ocorridas no genoma nuclear e de suas consequências, os conhecimentos sobre a epigenética mitocondrial ainda são primitivos (Ghosh *et al*., 2017). Modificações no genoma mitocondrial foram propostas por Vanyushin e colaboradores, em 1971, mas apenas recentemente estudos abordando essas questões começaram a ser realizados extensivamente.

A epigenética refere-se ao estudo de modificações herdáveis ao longo da etapa de divisão celular que regulam fortemente a expressão de informações genéticas específicas, sem afetar a sequência de DNA (Waddington, 1942, reimpresso em 2012). No núcleo, os mecanismos epigenéticos regulam a função e estrutura de um complexo de DNA e histonas chamado cromatina, sendo a metilação do DNA e a acetilação de histonas as principais alterações epigenéticas (Chen *et al*., 2012). Uma vez que o DNA mitocondrial não possui histonas, a metilação acaba tornando-se um importante foco nos estudos de mecanismos epigenéticos mitocondriais.

Durante muito tempo, a metilação do DNA mitocondrial foi questão de debate principalmente pela crença de que não poderia haver metilação uma vez que metiltransferases não poderiam acessar as mitocôndrias em vertebrados (Iacobazzi *et al*., 2013). Só recentemente, com avanços tecnológicos, é que se identificou a metilação como parte fisiológica das mitocôndrias, com estudos demonstrando a presença de DNA metiltransferases DNMT1 e DNMT3a (siglas do inglês *DNA methyltransferase 1 / 3 alpha*) dentro das mitocôndrias (Chestnut *et al*., 2011; Shock *et al*., 2011). Essas metiltransferases são similares

às enzimas presentes no núcleo e são responsáveis por adicionar um grupo metil à posição 5' da base citosina (5-metilcitosina, 5mC) (Gosh *et al*., 2015). Também foi reportada a presença de uma quantidade significativa de 5-hidroximetilcitosina (5hmC) no DNA mitocondrial, no entanto, o exato mecanismo dessa síntese ainda não está esclarecido (Shock *et al*., 2011). Não se sabe se a família das enzimas TET (sigla do inglês *Ten-eleven translocation*), que catalisam a hidroxilação de 5mC para 5hmC no núcleo, seriam funcionais nas mitocôndrias (Dzitoyeva *et al.*, 2012).

Estudos têm identificado padrões de metilação e hidroximetilação no DNA nuclear no envelhecimento e em diferentes doenças neurodegenerativas. Em amostras *post-mortem* de cérebro humano, a metilação do DNA nuclear aparece correlacionada com o envelhecimento, sendo a 5hmC abundante no córtex e cerebelo e altamente suscetível às modificações associadas ao envelhecimento (revisado por Dzitoyeva *et al*., 2012). Em hipocampos de pacientes com DA, foi observada diminuição da metilação e hidroximetilação do DNA global, enquanto foi observada hipometilação do íntron 1 do gene da α-sinucleína na substância negra, putamen e córtex de pacientes com DP (Jowaed *et al*., 2010). Apesar dos padrões de epigenética do DNA nuclear já estarem sendo estudados em doenças neurodegenerativas, os padrões de epigenética mitocondrial ainda não tem sido muito analisados nessas patologias (Iacobazzi *et al*., 2013). Em 2012, Dzitoyeva e colaboradores investigaram a epigenética mitocondrial no sistema nervoso central de mamíferos usando modelo animal de camundongos envelhecidos e neurônios primários de camundongos em cultura e demonstraram que o envelhecimento diminui o conteúdo de 5hmC no DNA mitocondrial no córtex frontal.

Evidências indicam que o perfil de metilação do DNA não é permanente, mudando ao longo do envelhecimento, observando-se uma perda gradual do total de metilcitosina na maioria dos tecidos vertebrados no envelhecimento (Richardson, 2003). Uma vez que em dinucleótidos CpG (nucleotídeo citosina e guanina ligados por ligação fosfodiéster) a citosina é a base preferida para a metilação do DNA, enquanto a guanina é a base atingida pela oxidação, percebe-se que o dano oxidativo do DNA pode modular os perfis de hipometilação e hipermetilação e causar impacto na expressão gênica (Zawia *et al*., 2009).

O estresse oxidativo é capaz de afetar o indivíduo de diferentes formas ao longo do envelhecimento. As mitocôndrias possuem papel de destaque nessas cirscunstâncias, pois são as principais fontes e alvos das espécies reativas de oxigênio. O aumento do estresse oxidativo cerebral pode levar à oxidação de proteínas, lipídeos e DNA, alterando o funcionamento mitocondrial, afetando diretamente as reservas energéticas celulares. Quando o acúmulo de

danos chega a um determinado limite, o funcionamento celular está tão comprometido que começam a ser desencadeados mecanismos de morte celular (Grimm e Eckert, 2017). A morte celular é uma das principais características observadas no envelhecimento e nas patologias associadas à idade (Okouchi *et al*., 2007).

1.1.3 Declínio energético e Apoptose

As mitocôndrias preservam as reservas energéticas celulares a partir de um rigoroso controle sobre dois processos metabólicos intimamente ligados: o ciclo de Krebs e a cadeia transportadora de elétrons (Basha e Poojary, 2014). No ciclo de Krebs, também chamado de ciclo do ácido tricarboxílico ou ciclo do ácido cítrico, a acetilcoenzima A (acetil-CoA) formada durante o catabolismo de carboidratos, aminoácidos e ácidos graxos é oxidada em dióxido de carbono (Quijano *et al*., 2016). Já a cadeia transportadora de elétrons é composta por quatro complexos enzimáticos (complexos I, II, III e IV) localizados na membrana interna, que culminam na formação de ATP a partir de ADP (sigla do inglês *Adenosine diphosphate*) (Gille e Reichmann, 2011). Além do ciclo de Krebs e da cadeia transportadora de elétrons, a enzima creatina quinase também é essencial para a homeostase energética, uma vez que essa enzima catalisa a transferência reversível de grupo fosforil entre ATP e creatina, principalmente em tecidos de elevado consumo energético (Pilla *et al*., 2003).

Mehan e colaboradores (2017) demonstraram uma redução de Succinato desidrogenase e ATP em estriado, córtex e hipocampo de modelo animal de DH. Um estudo demonstrou que o estresse oxidativo ocasionado por administração de L-tirosina compromete o metabolismo energético, ocasionando redução de Creatina quinase, Succinato desidrogenase, Citrato sintase e inibindo atividade dos complexos da cadeia transportadora de elétrons no córtex, hipocampo e estriado de ratos (Teodorak *et al*., 2017). Outro estudo observou redução de enzimas relacionadas ao ciclo de Krebs e de enzimas dos complexos da cadeia transportadora de elétrons em cérebros de ratos expostos ao pesticida *chlorpyrifos* e ao estresse induzido pelo frio para indução de estresse oxidativo (Basha e Poojary, 2014). Zhang e colaboradores, em 2011, observaram redução de Creatina quinase em modelo animal e em cérebros de pacientes com DH.

Uma vez que ocorre produção excessiva de espécies reativas de oxigênio, a função mitocondrial sofre prejuízo, levando a um declínio energético, fator contribuinte para o processo de envelhecimento (Mammucari e Rizzuto, 2010). Esse aumento de dano oxidativo e redução energética durante o envelhecimento leva a progressiva perda de função em células e

tecidos, representando uma ameaça para as células, que reagem a esse insulto ativando mecanismos de defesa como a morte celular (Paradies *et al*., 2010). As mitocôndrias contribuem na eliminação de células que não conseguem continuar suas funções vitais através da mediação de estímulos apoptóticos (Mammucari e Rizzuto, 2010).

A apoptose, um tipo de morte celular programada, tem importância fundamental devido seu papel crítico no desenvolvimento e na homeostase de organismos multicelulares (Pérez-Garijo, 2017). Esse tipo de morte ocorre fisiologicamente durante as etapas de desenvolvimento e envelhecimento, sendo delicadamente balanceado e regulado (Jin e El-Deiry, 2005). O número de células no organismo é mantido relativamente constante devido ao balanço entre a apoptose e a divisão celular, permitindo que células defeituosas ou doentes sejam removidas e substituídas (Shahin, 2017).

Esse processo é caracterizado pelo encolhimento celular, condensação da cromatina, fragmentação do DNA, formação de bolhas na membrana e formação de corpos apoptóticos que serão rapidamente fagocitados por macrófagos ou por células vizinhas, evitando assim uma resposta inflamatória nos tecidos da região (Kaczanowski, 2016). A apoptose é dependente de energia oriunda do ATP para a síntese protéica e ativação de sinais, como a formação do apoptossomo e reações de fosforilação mediadas por proteínas quinase (Okouchi *et al*., 2007). Como é necessário um limite mínimo de ATP para que uma célula sofra apoptose, em casos de depleção grave de energia, a morte celular apoptótica pode ser substituída por necrose (Okouchi *et al*., 2007). No entanto, o tipo de morte celular (apoptose ou necrose) vai depender da natureza do sinal de morte celular, do tipo de tecido, do estágio de desenvolvimento e do meio fisiológico (Elmore, 2007). A desregulação nos níveis de apoptose resulta na patogênese de uma série de doenças, como câncer, infecções virais, isquemia, doenças autoimunes e doenças neurodegenerativas, sendo que algumas dessas situações apresentam apoptose insuficiente, enquanto outras apresentam apoptose excessiva (Shahin, 2017).

As doenças neurodegenerativas são caracterizadas pela perda progressiva de células em populações específicas de neurônios (Jellinger, 2001). Na DA, é observada morte de neurônios hipocampais e corticais; na DP ocorre a morte de neurônios da substância negra; a DH envolve a morte de neurônios do estriado (Mattson, 2000). O papel da morte celular por apoptose nas doenças neurodegenerativas não se encontra totalmente elucidado e é possível que tanto morte celular apoptótica quanto não apoptótica coexistam em cérebros de pacientes afetados (Jin e El-Deiry, 2005). Estudos têm apresentado aumento de marcadores apoptóticos em doenças neurodegenerativas, mostrando que esse tipo de morte celular pode contribuir

para as condições neuropatológicas dessas doenças (Alberghina e Colangelo, 2006; Ghavami *et al*., 2014).

1.1.3.1 Vias de ativação da apoptose

Existem duas principais vias de ativação apoptótica: a via extrínseca ou do receptor de morte e a via intrínseca ou mitocondrial (Alberghina e Colangelo, 2006). No entanto, já foi evidenciado que essas duas vias estão ligadas e que as moléculas de uma via podem influenciar na outra (Elmore, 2007). Nessas duas vias, proteases cisteína-aspartil específicas (caspases) clivam substratos celulares e isso resulta na fragmentação de proteínas intracelulares, levando ao processo de morte celular (Green e Llambi, 2015).

A via apoptótica extrínseca envolve sinalização através de receptores transmembranas que pertencem a família de receptores TNF (sigla do inglês *Tumor necrosis factor*) (Tower, 2015). Esses receptores compartilham um domínio protéico intracelular envolvido na sinalização, chamado de "domínio de morte", responsável por transmitir o sinal de morte da superfície celular para vias de sinalização intracelular (Elmore, 2007). Já os ligantes que se associam nos receptores de morte celular pertencem a superfamília de citocinas TNF (Okouchi *et al*., 2007). A sequência de eventos que define a fase extrínseca da apoptose é melhor caracterizada para os pares de ligantes e receptores de morte FasL / FasR e TNF-α / TNFR1 (Elmore, 2007). Nesses casos, após a interação do ligante ao seu receptor, proteínas adaptadoras citoplasmáticas como as FADD (sigla do inglês *Fas-associated death domain protein*) são recrutadas para o domínio de morte e ligam ainda pró-Caspase 8 ou pró-Caspase 10, formando um complexo chamado DISC (sigla do inglês *Death-inducing signaling complex*), resultando na clivagem autocatalítica das caspases iniciadoras (Flusberg e Sorger, 2015). Algumas vezes, a formação do complexo DISC é fraca e insuficiente para induzir apoptose diretamente via Caspase 8, logo, ocorre a ativação de uma proteína pró-apoptótica chamada Bid (sigla do inglês *BH3 interacting domain death agonist*) que envolve sinalização apoptótica via mitocôndria (Okouchi *et al*., 2007).

A via apoptótica intrínseca é o mecanismo mais comum de apoptose nos vertebrados, sendo ativada em resposta a diferentes estímulos celulares, incluindo danos ao DNA, privação de fatores de crescimento, hipóxia, estresse oxidativo, entre outros (Green e Llambi, 2015). Esses estímulos não mediados por receptores produzem sinais intracelulares que alteram a expressão e estabilidade de proteínas citoplasmáticas da família Bcl-2 (sigla do inglês *B cell lymphoma- 2*), responsáveis pela regulação da integridade da mitocôndria (Mukhopadhyay *et*

al., 2014). Como a família Bcl-2 possui membros pró-apoptóticos e anti-apoptóticos, uma mudança no equilíbrio dessas proteínas leva ao acúmulo de proteínas pró-apoptóticas na membrana externa das mitocôndrias (Ghavami *et al*., 2014). Isso resulta na formação de poros de permeabilidade na membrana e na perda do potencial de membrana mitocondrial, resultando na liberação das moléculas Citocromo c, Smac/DIABLO (siglas do inglês *Second mithocondria-derived activator of caspases* / *Direct IAP-binding protein with low pI*) e HtrA2/Omi (siglas do inglês *High temperature requirement protein* A2 / *Stress-regulated endoprotease*) do espaço intermembrana para o citosol, desencadeando a via apoptótica intrínseca (Suhaili *et al*., 2017). O Citocromo c interage com a APAF1 (sigla do inglês *Apoptotic protease activating factor-1*) e pró-Caspase 9, formando o apoptossomo, essencial para a ativação da Caspase 9, enquanto que Smac/DIABLO e HtrA2/Omi promovem a apoptose por inibição de proteínas inibidoras de apoptose (Mukhopadhyay *et al*., 2014).

As caspases iniciadoras tanto da via intrínseca quanto da via intrínseca convergem para uma etapa comum de execução de apoptose, que ocorre pela ativação de Caspase 3, 6 e 7 (Okouchi *et al*., 2007). Uma vez ativadas, essas caspases executoras são responsáveis pela clivagem de vários substratos vitais para as células, ativando proteases e nucleases, causando as modificações bioquímicas e morfológicas observadas nas células apoptóticas (Elmore, 2007). Finalizando o processo de apoptose, são formados os corpos apoptóticos, que, devido a modificação da fosfatidilserina do citoplasma para a superfície externa da membrana celular, são reconhecidos pelos macrófagos e fagocitados (Jin e El-Deiry, 2005). A Figura 2 mostra esquematicamente as vias extrínseca e intrínseca de apoptose, desde a fase de iniciação até a fase de ativação de caspases efetoras de apoptose.

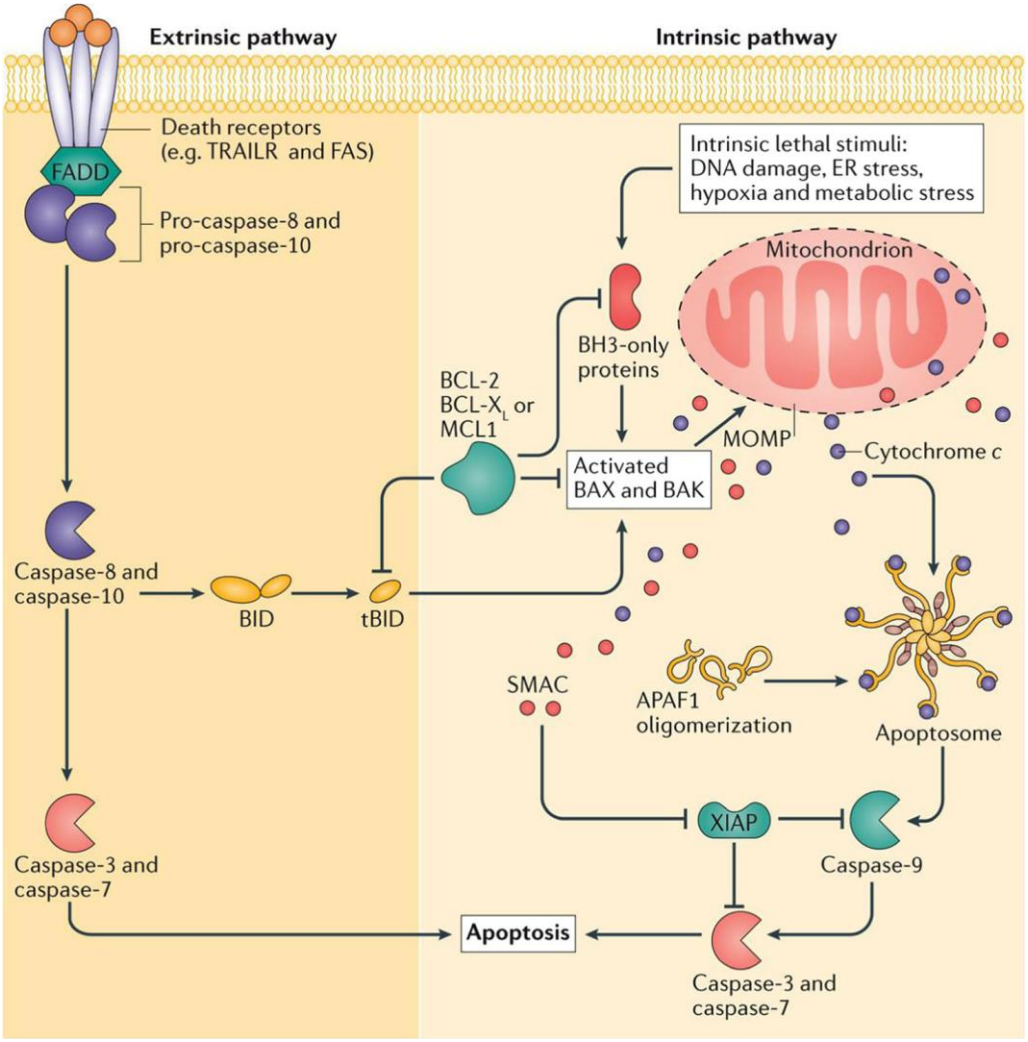

Figura 2: Visão geral das vias intrínseca e extrínseca da apoptose. Na via de apoptose extrínseca, após a ligação de ligantes específicos aos receptores de morte, pertencentes à superfamília dos receptores de necrose tumoral (TNFr), ocorre o recrutamento de proteínas adaptadoras aos domínios de morte dos receptores (FADD) e a ligação de pró-Caspases 8 e/ou 10. Esse complexo formado (DISC) cliva e ativa essas caspases, que irão clivar e ativar as caspases efetoras 3 e/ou 7, levando à apoptose. Já na via intrínseca, estresse celular desencadeia a ativação de proteínas *BH3-only*, que irão ativar as proteínas pró-apoptóticas Bax e Bak, resultando na permeabilização da membrana externa mitocondrial (MOMP). As proteínas anti-apoptóticas da família Bcl-2 neutralizam isso. A permeabilização da membrana permite a liberação de proteínas intermembranas SMAC e Citocromo c para o citosol. O Citocromo c interage com a APAF1, formando o apoptossoma, que ativa a Caspase 9. A ativação da Caspase 9 irá ativar as Caspase efetoras 3 e/ou 7, levando à apoptose. A SMAC facilita a apoptose devido ao bloqueio da proteína inibidora de apoptose (XIAP). A interligação entre as vias extrínseca e intrínseca ocorre uma vez que a Caspase 8 ativada promove a ativação da proteína pró-apoptótica Bid, que desenvolve a sinalização apoptótica via mitocôndria (Adaptado de Suhaili *et al*., 2017).

1.1.3.2 Moléculas envolvidas na apoptose

Os mecanismos de apoptose são altamente complexos e sofisticados, envolvendo uma cascata de eventos moleculares que contam com a participação de diferentes tipos de proteínas. Sendo essas proteínas essenciais nesse processo de morte celular, estudos são realizados visando um melhor entendimento de toda maquinaria apoptótica desenvolvida por essas proteínas, contribuindo para a compreensão do envolvimento da apoptose ao longo do desenvolvimento e em situações patológicas como as doenças neurodegenerativas (Calissano *et al*., 2009; Venderova e Park, 2012; Obulesu e Lakshmi, 2014). Os grupos de moléculas que abrangem a cascata de apoptose incluem os membros da família de receptores TNF, moléculas da família Bcl-2, caspases, proteínas adaptadoras que controlam a ativação ou inativação de caspases, bem como fatores de transcrição e proteínas envolvidas na regulação do ciclo celular, entre outras (Kajta, 2004).

A família Bcl-2 é composta por mais de 30 proteínas, sendo dividida em três grupos de acordo com a atividade e estrutura: proteínas pró-apoptóticas, proteínas anti-apoptóticas e proteínas BH3-*only* (sigla do inglês *Bcl-2 homology domain 3-only*) (Okouchi *et al*., 2007). A classe pró-apoptótica inclui as proteínas Bax (sigla do inglês *Bcl-2-associated X protein*) e a Bak (sigla do inglês *Bcl-2 homologous antagonist/killer*); a classe anti-apoptótica inclui as proteínas Bcl2, Bcl-XL (sigla do inglês *B-cell lymphoma-extra large*) e Mcl1 (sigla do inglês *Induced myeloid leukemia cell differentiation protein*); a classe BH3-*only* inclui as proteínas Bid, Bim (sigla do inglês *Bcl-2 interacting mediator of cell death*), Puma (sigla do inglês *P53 upregulated modulator of apoptosis*) e Noxa (Green e Lllambi, 2015). A família Bcl-2 é responsável pelo controle da permeabilidade da membrana mitocondrial, sendo a suscetibilidade à apoptose determinada pela relação entre proteínas pró e anti-apoptóticas (Elmore, 2007). Ao receber estímulos nocivos, as proteínas BH3-*only* ativam as proteínas pró-apoptóticas Bax e Bak, levando à formação de poros na membrana mitocondrial, que irá desencadear o processo de apoptose (Okouchi *et al*., 2007). As proteínas anti-apoptóticas Bcl-2 são as responsáveis pela manutenção da integridade da membrana mitocondrial por meio da interação e inibição das proteínas Bax e Bak e das proteínas BH3-*only*, apesar desses mecanismos de proteção não serem bem compreendidos (Suhaili *et al*., 2017). Interação entre as vias extrínseca e intrínseca é observada através da Caspase 8 (Elmore, 2007). Nesses casos, o complexo DISC é fracamente formado e a Caspase 8 ativada por ele não é capaz de acionar as caspases efetoras; logo, a Caspase 8 cliva a proteína Bid, que age sobre proteínas pró-apoptóticas e induz apoptose por via intrínseca (Suhaili *et al*., 2017).

Caspases são enzimas proteolíticas com resíduo cisteína no seu sítio catalítico que clivam proteínas em resíduos aspartato e são consideradas indispensáveis para promover a cascata que leva à apoptose (Troy e Salvesen, 2002). Quatorze tipos de caspases já foram identificadas nos mamíferos, sendo que, de acordo com suas funções, elas podem ser classificadas em três diferentes grupos: as caspases inflamatórias, as caspases iniciadoras de apoptose e as caspases efetoras de apoptose (Elmore, 2007). As caspases inflamatórias incluem as Caspases 1, 4, 5, 11, 12, 13 e 14 e são responsáveis pela iniciação dos processos inflamatórios, não apresentando papel na apoptose (Jin e El-Deiry, 2005). Caspases iniciadoras abrangem as Caspases 2, 8, 9 e 10 e são responsáveis por mediar os sinais apoptóticos oriundos dos receptores de morte (Caspases 8 e 10) ou das mitocôndrias (Caspases 2 e 9) (Ghavami *et al*., 2014). Compõem o terceiro grupo as caspases efetoras 3, 6 e 7, envolvidas em processos posteriores da cascata de apoptose, que levam aos processos finais de morte celular e são comuns às vias extrínseca e intrínseca (Jin e El-Deiry, 2005).

Essas proteases são sintetizadas na forma inativa, como zimogênios, sendo ativadas em decorrência de estímulos apoptóticos (Okouchi *et al*., 2007). As caspases iniciadoras são cataliticamente ativadas pela interação com proteínas adaptadoras, enquanto as caspases efetoras são ativadas pelas caspases iniciadoras e induzem à morte celular a partir de clivagem de diversos substratos (Alberghina e Colangelo, 2006). Essa cascata proteolítica, na qual uma caspase pode ativar outras caspases, amplifica a via de sinalização apoptótica e leva a uma rápida morte celular (Elmore, 2007).

PARPs (sigla do inglês *Poly (ADP-ribose) polymerase*) constituem uma ampla família de 18 proteínas nucleares que compartilham a capacidade de catalizar a transferência de ADP-ribose para proteínas alvo (Amé *et al*., 2004). Essas proteínas estão envolvidas em uma variedade de funções celulares essenciais, tais como reparo de DNA, estrutura da cromatina, ciclo celular, transcrição, morte celular, entre outras, sendo as proteínas responsáveis por esses processos os principais substratos para a PARP (Barkauskaite *et al*., 2013). A PARP1 é o membro mais estudado dessa família, tendo sido observado três domínios principais: domínio de ligação ao DNA N-terminal, domínio de automodificação central e domínio catalítico C-terminal (Virág *et al*., 2013). Na ausência de danos ao DNA, a PARP1 possui atividade insignificante, sendo seletivamente ativada por quebras ao DNA (Ha e Snyder, 2000). A indução de morte celular por apoptose e, consequentemente, ativação de caspases, leva à clivagem da PARP, o que ocasiona a separação do domínio de ligação ao DNA do domínio catalítico (Gerö e Szabó, 2008). Em consequência da clivagem da PARP1 por caspases ativas, ocorre a perda da capacidade enzimática dessa proteína, prevenindo

super-ativação dessa enzima para reparos desnecessários e preservando a demanda de ATP necessário à apoptose (Luo e Krauss, 2012). Devido a esse processo de clivagem da PARP1 pelas caspases, essa proteína é utilizada como um marcador de apoptose (Ha e Snyder, 2000).

A APAF1 é uma proteína monomérica citosólica que, em situações normais, encontra-se inativa (Harlan *et al*., 2006). Quando a célula sofre estímulos nocivos, ocorre a permeabilização da membrana mitocondrial, ocorrendo a liberação de moléculas importantes, como o Citocromo c (Elmore, 2007). Uma vez presente no citosol, o Citocromo c irá se ligar à APAF1, ocasionando uma modificação conformacional nessa proteína e levando à formação do complexo molecular apoptossoma (Harlan *et al*., 2006). Esse apoptossoma recruta a pró-Caspase 9, promove a sua ativação, formando a Caspase 9, que irá desencadear a cascata de proteólise e culminará na morte celular (Ashraf *et al*., 2007). A análise da atividade da APAF1 mostra que essa proteína constitui um elemento central na maquinaria de morte mitocondrial, uma vez que várias proteínas interagem direta ou indiretamente com ela e por ser o núcleo físico da maquinaria apoptótica, o apoptossoma (Campioni *et al*., 2005). Pode-se dizer, então, que a APAF1 é a responsável por interligar os sinais apoptóticos oriundos da mitocôndria com a via das caspases, promovendo assim a morte celular (Ashraf *et al*., 2007). Enquanto o papel da APAF1 na apoptose está bem estabelecido, outras funções dessa proteína não estão claramente identificadas (Harlan *et al*., 2006).

As moléculas envolvidas nos mecanismos de morte celular são de amplo interesse nas doenças neurodegenerativas, visando melhor entendimento da maquinaria que leva à perda de células nervosas. Kudo e colaboradores (2012) mostraram que tratamento com Aβ aumenta os níveis de Bim e diminui os níveis de Bcl-2, levando à ativação de Bax e morte neuronal em cultura de células hipocampais e *in vivo.* Além disso, inibição de Bax ocasiona significante prevenção de morte neuronal induzida por Aβ, mostrando que Bax pode ser um alvo terapêutico na DA. Em um estudo de 2017, foi observado que ratos expostos a sevoflurano para indução de déficits cognitivos apresentaram super-expressão do gene da Caspase 3, seguido de aumento de níveis de PARP clivada no hipocampo (Ling *et al*., 2017). Foi verificado que células expressando huntingtina mutante e *knock-out* para APAF1 apresentaram menos agregados proteicos que as células que expressam níveis normais de APAF1, mostrando evidências do envolvimento da APAF1 na agregação proteica da DH (Sancho *et al*., 2011). Shang e colaboradores (2009) demonstraram elevados níveis de caspases ativadas 3, 8 e 9 em um modelo animal de estresse oxidativo, sendo observada redução da atividade dessas caspases com a eliminação do indutor de estresse oxidativo.

Em cérebros de pacientes com MCI e DA foram encontrados elevados níveis de Caspase 3, o que poderia estar contribuindo para a perda neuronal e sináptica desses pacientes e elevados níveis de Bcl-2, que poderia estar agindo como mecanismo compensatório para proteger da morte celular (Bader Lange *et al*., 2008; Sultana *et al*., 2010). Analisando cérebros de pacientes com PD, foram encontrados níveis elevados de receptor TNF subtipo I e proteína adaptadora TRADD (sigla do inglês *Tumor necrosis factor receptor type 1 associated death domain*), proteínas Bid amplamente ativadas, Citocromo c liberado da mitocôndria e atividade da Caspase 3 aumentada, resultados que podem explicar em parte o declínio cognitivo associado à PD (Jiang *et al*., 2012). Todos esses achados demonstram que as vias apoptóticas tem um importante papel no desenvolvimento das doenças neurodegenerativas e tornam-se informações importantes para o desenvolvimento de estratégias de retardo e prevenção dessas patologias.

Os estudos atualmente têm visado analisar o completo funcionamento de toda a maquinaria envolvida nos processos neurodegenerativos, uma vez que a partir de uma melhor compreensão de todo o processo é possível a busca por estratégias mais efetivas de prevenção e tratamento para essas patologias. Atualmente, muitas pesquisas estão sendo realizadas com a *Cannabis sativa*, uma planta utilizada tanto para fins medicinais quanto como droga de abuso. Uma substância em crescente estudo é o canabinóide natural Canabidiol, o qual algumas pesquisas já têm sugerido apresentar um potencial terapêutico em várias patologias, entre elas as relacionadas ao sistema nervoso.

1.1.4 Canabidiol

A *Cannabis sativa* é uma planta que tem sido cultivada pelo homem há milhares de anos, sendo a primeira evidência do seu uso encontrada na China, onde era utilizada como fibra, comida e medicamento (Zuardi, 2006). Houve um declínio da utilização dessa droga para fins médicos até que os principais princípios ativos fossem identificados, isolados e elucidados, o que ocorreu apenas na década de 1960 (Crippa *et al*., 2010). A *C. sativa* possui diversos compostos, incluindo mais de 60 canabinóides diferentes que interagem com o sistema endocanabinóide no cérebro, tornando a separação de componentes um trabalho difícil e demorado (Schoeler e Bhattacharyya, 2013). Os dois principais canabinóides encontrados nessa planta são o Δ9-Tetra-hidrocanabinol (THC) e o Canabidiol (CBD) (Vukadinovic *et al*., 2013). Enquanto o THC é o principal canabinóide psicoativo dessa planta, combinando propriedades terapêuticas com importantes efeitos adversos, o CBD é não

psicoativo, bem tolerado e apresenta um espectro de propriedades terapêuticas (Fernández-Ruiz *et al*., 2013).

O CBD, que pode representar até 40% dos extratos da *C. sativa*, foi isolado pela primeira vez no final dos anos 1930, mas sua estrutura só foi elucidada na década de 1960 (Scuderi, *et al*., 2009; Fernández-Ruiz *et al*., 2013). Desde o início dos estudos, realizados na década de 1970, um grande número de efeitos farmacológicos do CBD tem sido descrito (Scuderi, *et al*., 2009). Uma série de ações tem sido demonstrada para o CBD em estudos clínicos e pré-clínicos, incluindo anticonvulsivantes, sedativas, hipnóticas, ansiolíticas, antipsicóticas, anti-inflamatórias e neuroprotetoras (Zuardi, 2008). No entanto, os mecanismos responsáveis por esses efeitos continuam controversos e obscuros, sendo propostas numerosas vias de sinalização (Campos *et al*., 2017).

A maioria dos efeitos exercidos pelos fitocanabinóides é mediada através de ação antagonista ou agonista em receptores específicos (Zuardi, 2008). Os tecidos de mamíferos expressam pelo menos dois tipos de receptores para canabinóides: CB_1 e CB_2 (Scuderi *et al*. 2009). Diferentemente do THC, tem sido demonstrado que o CBD se liga com baixa afinidade aos receptores CB1 e CB2 (Scuderi *et al*., 2009), portanto, modos de ação do CBD independentes de CB1 e CB2 têm sido investigados. Evidências indicam que o CBD se liga em receptores vanilóides tipo 1 (Bisogno *et al.*, 2001) e receptores serotoninérgicos 5-HT1A (Campos e Guimarães, 2008), o que poderia explicar alguns dos seus efeitos. Porém, o CBD pode exercer alguns efeitos que não são intercedidos por receptores, tais como: efeitos no sistema imunológico, no sistema circulatório e efeitos neuroprotetores (Zuardi, 2008).

Nos últimos anos, nosso grupo de pesquisa tem estudado o CBD, observando a capacidade desse composto em reverter o prejuízo de memória ocasionado pela sobrecarga de ferro (Fagherazzi *et al*., 2012) bem como alterações moleculares induzidas pelo tratamento neonatal com ferro (da Silva *et al*., 2014). Esses efeitos tornam o CBD uma droga com possível ação terapêutica em muitas doenças, entre elas as patologias que acometem o sistema nervoso. Nosso trabalho com o CBD resultou, ao longo do doutorado, na publicação de um capítulo entitulado "*Cannabidiol and Neuroprotection: Evidence from Preclinical Studies*" no livro "*Handbook of Cannabis and Related Pathologies*", abordando os efeitos neuroprotetores do CBD. A seguir, será apresentado esse trabalho realizado, visando melhor embasamento teórico sobre o envolvimento desse composto em patologias neurodegenerativas.

1.1.4.1 “Canabidiol e Neuroproteção: Evidências de Estudos Pré-Clínicos”

CHAPTER

83

Cannabidiol and Neuroprotection: Evidence from Preclinical Studies

N. Schröder, V.K. da Silva*, J.E.C. Hallak**, A.W. Zuardi**, J.A. de Souza Crippa***

*Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University, Porto Alegre, Rio Grande do Sul, Brazil

**Department of Neuroscience and Behavior, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Sao Paulo, Brazil

SUMMARY POINTS

- This chapter focuses on the potential use of cannabidiol (CBD), one of the chemical compounds found in *Cannabis sativa*, as a neuroprotective agent, for the treatment of neurodegenerative disorders.
- Evidence from preclinical studies indicates that CBD displays antioxidant, antiinflammatory, and antiapoptotic properties.
- CBD was also shown to hinder excitotoxicity, and protect mitochondria against mitochondrial toxins.
- Evidence supports the view that CBD may increase progenitor cell proliferation and neurogenesis.
- CBD was shown to improve and ameliorate damage observed in animal models of neurodegeneration associated to hypoxic-ischemic brain injury, multiple sclerosis, brain iron overload, Alzheimer's, Parkinson's, and Huntington's disease.
- Since neurodegeneration is considered a multifactorial process, and CBD acts on multiple pharmacological targets implicated on neurodegeneration, CBD may constitute a promising therapeutic agent for the prevention/treatment of neurodegenerative disorders.

KEY FACTS OF HYPOXIA-ISCHEMIA

- Hypoxia-ischemia (HI) encephalopathy results from stroke, which is a focal disruption of blood supply to a part of the brain, or from global ischemia, which can affect the entire brain.
- HI encephalopathy may also result from perinatal asphyxia in neonates, and may cause long-term neurologic sequelae or death.
- Physiopathology of HI includes energy failure due to reductions in cerebral blood flow and oxygen.
- Energy failure may lead to excitotoxic damage.
- Oxidative stress, mitochondrial failure, and apoptotic cell death are also associated to HI.

KEY FACTS OF ALZHEIMER'S DISEASE

- Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder, affecting 7–10% of individuals over 65 years of age, and 50–60% of people over 85 years of age.
- Features include memory loss, followed by a progressive deterioration of other domains of cognitive functioning.
- Degeneration affects primarily the hippocampus and the neocortex.
- Neuropathological hallmarks of AD are neuritic plaques and neurofibrilary tangles.

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- Neurofibrillary tangles are filamentous inclusions, formed of hyperphosphorylated tau.
- Neuritic plaques are extracellular deposits of aggregated amyloid-β peptide, which results from aberrant cleavage of amyloid precursor protein.

KEY FACTS OF IRON INVOLVEMENT IN NEURODEGENERATIVE DISORDERS

- Iron is the most abundant metal in the human brain.
- It progressively accumulates in selective brain regions affected by neurodegenerative disorders (NDD) such as PD, AD, among others.
- Due to its redox states, iron can catalyze free radical formation, leading to oxidative stress.
- Iron accumulation in the hippocampus and the basal ganglia has been related to impairments in spatial, aversive, and recognition memory in rodents.
- In humans, performance in cognitive tests negatively correlates with iron deposition, assessed by resonance magnetic imaging, in brain regions such as the hippocampus, cortical areas, and basal ganglia.

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid precursor protein
BV2	Immortalized murine microglial cell line
CA1	Cornu ammonis area 1
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
COX2	Cyclooxygenase-2
Cu,Zn SOD	Copper and zinc superoxide dismutase
CX3CR1	Chemokine (C-X3-C motif) receptor 1
DNA	Deoxyribonucleic acid
DNM1L	Dynamin-1-like protein
FCCP	Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone
GABA	γ-Aminobutyric acid
GSK3β	Glycogen synthase kinase 3 beta
HD	Huntington's disease
HI	Hypoxic-ischemic
5HT1A	5-Hydroxytryptamine (serotonin) receptor 1A
iNOS	Inducible oxide nitric synthase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NDD	Neurodegenerative disorders
NMDA	*N*-Methyl-D-aspartate
3NP	3-Nitropropionic acid
NO	Nitric oxide
6-OHDA	6-Hydroxydopamine
PC12	Pheocromocytoma cells
PD	Parkinson's disease
PPARγ	Peroxisome proliferator-activated receptor-γ
PS1	Presenilin-1
ROS	Reactive oxygen species
S100B	S100 calcium binding protein B
SHSY5Y	Human neuroblastoma cells type
SOD	Superoxide dismutase
THC	Δ^9-Tetrahydrocannabinol
TNFα	Tumor necrosis factor alpha

INTRODUCTION

There are two main neuroactive components in *Cannabis sativa*: the psychoactive Δ^9-Tetrahydrocannabinol (THC), and the nonpsychoactive Cannabidiol (CBD). CBD was firstly isolated during late 1930s, but its structure was only elucidated by Mechoulam and Shvo (1963). Nowadays, many researchers are studying this compound, and some of its pharmacological effects are already reported, including antiinflammatory, antioxidative, anxiolytic, antipsychotic, and neuroprotective effects, although its mechanisms of action are not completely elucidated (Zuardi, 2008). In view of these effects, CBD has been considered as an interesting compound with potential therapeutic application in a number of neurological and neuropsychiatric disorders. Studies have been conducted to verify the effects of CBD in nervous system pathologies. In recent years, preclinical in vitro and in vivo studies have investigated the potential of CBD in experimental models, focusing mainly on neurodegeneration associated to hypoxic-ischemic (HI) injury and Alzheimer's disease (AD). The purpose of this chapter is to provide a comprehensive overview on the main studies reporting the effects of CBD in the context of HI injury and AD, as well as other relevant experimental models of neurodegeneration, and to discuss its putative mechanisms. Studies in which CBD was used in combination with other cannabinoid compounds were not included in this chapter.

EXCITOTOXICITY AND HYPOXIC-ISCHEMIC BRAIN INJURY

Hampson, Grimaldi, Axelrod, and Wink (1998) analyzed the effects of CBD in rat cortical neuron cultures exposed to glutamatergic excitotoxicity. CBD was able to block *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor-mediated neurotoxicity, and to protect neurons against reactive oxygen species (ROS)-

induced cell death. The results also indicated that the effects of CBD were independent of cannabinoid receptors. From these results, the authors proposed that CBD could be a useful therapeutic agent for the treatment of neurological disorders associated to excitotoxicity and oxidative stress, such as cerebral ischemia. Therapeutic options to treating HI brain injury are very limited, and in vitro and in vivo preclinical studies indicate that CBD would be a candidate to be tested to treat this condition. Accordingly, Castillo, Tolón, Fernández-Ruiz, Romero, and Martinez-Orgado (2010) investigated the effects of CBD in HI immature brain of newborn mice, as a model of neonatal HI. Forebrain slices from newborn mice underwent glucose and oxygen deprivation, and the effects of CBD on acute and apoptotic cell death, excitotoxicity, inflammatory markers, and inducible oxide nitric synthase (iNOS) expression were evaluated. By using selective antagonists, they also analyzed cannabinoid receptors CB1 and CB2, and adenosine A_{1A} and A_{2A} receptors in mediating these effects. They observed that CBD was able to reduce acute and apoptotic damage in immature brain slices by reducing glutamate and interleukin-6 concentration, and tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and iNOS expression. These effects were reversed by CB2 and A_{2A} antagonists, suggesting that CBD-induced neuroprotection in HI cell death was mediated by CB2 and adenosine receptors.

Hayakawa et al. (2007) examined the neuroprotective effects of CBD, using a 4 h middle cerebral artery occlusion model of ischemia in mice. Results indicated that both pre- and postischemic treatment with CBD decreased infarct volume, reversed the reduction in cerebral blood flow, and increases in myeloperoxidase activity and number of myeloperoxidase positive cells, which are markers of neutrophil response, induced by ischemia. Thus, this study provided evidence that CBD conferred long-lasting neuroprotection, through an antiinflammatory mechanism against ischemic brain damage. The effect of CBD on behavioral end-point of neuroprotection was assessed in the rota-rod test for motor coordination, showing that CBD was able to significantly improve motor coordination in mice submitted to ischemia.

The effects of CBD were also verified in newborn piglets submitted to acute HI (Lafuente et al., 2011). In this study, the authors found that CBD recovered brain activity, assessed by amplitude-integrated electroencephalography, reduced neuronal damage and astrocytosis in the cortex, decreased the percentage of TNF-α positive cells, and normalized general neurobehavioral changes induced by ischemia. CBD administration displayed a neuroprotective effect, which included both neurons and astrocytes, possibly mediated by an antiinflammatory effect in the newborn brain. Pazos et al. (2013) have also analyzed the effects of CBD on HI brain injury using a HI model in newborn pigs. CBD was able to prevent all the alterations induced by HI, increasing the number of viable neurons, recovering brain activity, reducing excitotoxicity assessed by glutamate/*N*-acetylaspartate ratio, oxidative stress assessed by glutathione/creatine ratio and protein carbonylation, and inflammation assessed by brain levels of interleukin-10. The effects of CBD were reversed by CB2 and $5HT_{1A}$ antagonists, suggesting that these receptors are implicated in CBD's neuroprotective profile.

The same group has also analyzed the effects of CBD on neurobehavioral parameters in rats, 30 days after HI injury. Once again, neuroprotective effects of CBD were associated to reductions in excitotoxicity, oxidative stress, and inflammation. Neuroprotection was further confirmed by magnetic resonance imaging and histological evaluation to measure the extension of brain damage. At the functional level, results showed that CBD improved motor and coordination deficits tested in the rota-rod, and short-term object recognition memory (Pazos et al., 2012).

Neuroprotection against excitotoxic insult was also investigated using a diverse model of glutamatergic excitotoxicity in retinal neurons (El-Remessy et al., 2003). Intravitreal injection of NMDA in rats induced a dose- and time-dependent accumulation of nitrite/nitrate, lipid peroxidation, and nitrotyrosine (mark of peroxynitrite), and a dose-dependent apoptosis and loss of inner retinal neurons. CBD injected immediately before the intravitreal injection of NMDA significantly attenuated NMDA-induced tyrosine nitration and apoptosis. Given the involvement of excitotoxicity in retinal cell death observed in glaucoma, CBD has been proposed as a candidate therapy for its treatment.

NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders (NDD) are characterized by progressive loss of neurons in selected areas of the nervous system, which determine their clinical presentation. Although the etiology of NDD is not completely elucidated, there is a general consensus that they are multifactorial diseases that involve oxidative stress, iron accumulation, mitochondrial dysfunction leading to energetic and subsequent synaptic failure, protein misfolding, and formation of protein aggregates, neuroinflammation, excitotoxicity, and apoptosis (Duncan, 2011; Li, O, Li, Jiang, & Ghanbari, 2013).

In spite of tremendous effort put into the investigation of therapeutic agents to treat NDD, no effective disease-modifying drugs that can interrupt the progressive course of the disease are currently available. Thus, due to the already characterized pharmacological properties of CBD, this compound may be promising for the treatment of NDD.

Alzheimer's Disease

In order to investigate cellular and molecular alterations related to AD, many in vitro studies, in which cells are exposed to amyloid-β peptide, have been performed. Iuvone et al. (2004) investigated the effects of CBD in reversing amyloid-β peptide-induced cell death in cultured rat pheocromocytoma PC12 cells. Pretreatment with CBD significantly increased cell survival, and decreased ROS production, lipid peroxidation, as well as the active form of caspase 3 (a key enzyme in the effector phase of apoptosis) appearance, DNA fragmentation, and reversed increases in intracellular calcium induced by amyloid-β exposure. Those results suggested that antioxidant and antiapoptotic properties may underlie CBD-mediated neuroprotection against amyloid-β toxicity. Another study using PC12 neuronal cells reported that CBD inhibits hyperphosphorylation of tau in amyloid-β-stimulated cells. CBD dose-dependently reversed amyloid-β-induced glicogen synthase kinase 3β (GSK-3β) phosphorylation, and reduction in the expression of β-catenin, suggesting that the effects of CBD in rescuing amyloid-β-challenged PC12 cells are mediated through the Wnt/β-catenin pathway (Esposito, De Filippis, Carnuccio, Izzo, & Iuvone, 2006). In the same line, recently, Janefjord, Mååg, Harvey, and Smid (2014) have shown that CBD was able to protect SH-SY5Y cells against amyloid-$\beta_{1\text{-}42}$ neurotoxicity.

Esposito et al. (2011) investigated the effects of CBD on the release of inflammatory mediators induced by amyloid-β challenge in rat primary astroglial cultures. CBD antagonized the enhanced release of the proinflammatory molecules, nitric oxide (NO), interleukin-1β, TNFα, and S100B (S100 calcium binding protein B), and the blockade of peroxisome proliferator-activated receptor-γ (PPARγ) reversed this effect. In the same study, the authors stereotaxically inoculated human amyloid-β into the hippocampus of adult rats that were subsequently treated with chronic CBD. CBD rescued CA1 pyramidal neurons integrity, and downregulated reactive gliosis, as shown by a significant decrease of glial fibrillary acidic protein immunostaining. PPARγ antagonism completely abolished CBD neuroprotective effects. Remarkably, CBD restored neurogenesis in dentate gyrus of amyloid-β injected rat hippocampi through PPARγ activation. These findings provide evidence that the neuroprotective effects of CBD against AD neuroinflammation may be related to PPARγ activation. Along this line, the effects of CBD in a cellular model of AD were investigated using SHSY5Y^{APP+} cells (Scuderi, Steardo, & Esposito, 2013). CBD induced the ubiquitination of amyloid precursor protein (APP), with subsequent reduction of amyloid-β production. In addition, CBD was able to reduce apoptosis, increasing survival of SHSY5Y^{APP+} neurons. Results also indicated that these effects were related to selective activation of PPARγ. PPARs have been recently related to AD (Kitamura et al., 1999), and studies have proposed that CBD translocates into the nucleus, thereby interacting with PPARs (O'Sullivan, Sun, Bennett, Randall, & Kendall, 2009).

Martín-Moreno et al. (2011) investigated the effects of CBD in primary rat microglial cultures. CBD promoted microglial cell migration, and decreased NO generation in the culture media of lipopolysaccharides-stimulated microglia. Migration of microglial cells that may subserve a beneficial function as a requisite for phagocytosing aggregated amyloid-β. They also demonstrated that CBD could rescue spatial memory deficits, tested in the Morris water maze, in amyloid-β-injected mice, and to significantly reduce interleukin-6 mRNA expression, which was markedly increased by amyloid-β injection.

Cheng, Low, Logge, Garner, and Karl (2014) analyzed the effects of chronic CBD in a transgenic mouse model of AD. They used APP/PS1 mutant mice and these animals underwent a behavioral test battery. CBD reversed deficits in social recognition and object recognition displayed by APP/PS1 mice, without affecting fear-associated memory and anxiety-related behavior.

While in vitro and ex vivo studies are useful in the search of cellular and molecular mechanisms associated to CBD's neuroprotective actions, in vivo behavioral studies are essential to identify the potential of this drug in reversing neurofunctional deficits associated with the disease. Table 83.1 summarizes findings from the main behavioral studies investigating the effects of CBD in the context of preclinical models of NDD.

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by a selective degeneration of dopaminergic neurons of the substantia nigra pars compacta, that leads to motor deficits, including tremor, rigidity, bradykinesia, and postural instability (Kim, Ko, Dawson, & Dawson, 2005). The effects of CBD were investigated in the 6-hydroxy-dopamine (6-OHDA) rat model of PD (García-Arencibia et al., 2007). Results showed that CBD administration immediately after the lesion, but not 1 week later, was able to recover 6-OHDA-induced dopamine depletion, and upregulate mRNA levels for Cu,Zn-superoxide dismutase (SOD), a key enzyme in endogenous defenses against oxidative stress, and a representative parameter of the degree of neuronal injury caused by oxidative stress in PD.

Although only a few studies investigated the effects of CBD in preclinical models, CBD was already tested in patients suffering from PD. Zuardi et al. (2009) investigated safety, tolerability, and efficacy of CBD on PD patients. In this study they observed that CBD was able to decrease psychotic symptoms scores evaluated

TABLE 83.1 Summary of Studies Testing the Effects of Cannabidiol (CBD) on Behavioral Parameters

Experimental Model	Regimen of Treatment	Effects	Receptor(s) Involved	References
Ischemic mouse	Pre/postischemia, 1 or 3 mg/kg, intraperitoneally	Improved motor coordination	Not investigated	Hayakawa et al. (2007)
Neonatal hypoxic-ischemic rat	Acute (10 min after ischemia), 1 mg/kg, subcutaneous	Improved motor coordination deficits and short-term recognition memory	Not investigated	Pazos et al. (2012)
Mouse model of Alzheimer's disease (hippocampal amyloid-β injection)	Chronic, 24 h after procedure (first week: daily injection, second and third weeks: 3 days/week), 20 mg/kg, intraperitoneally	Rescued spatial memory deficits	Not investigated	Martín-Moreno et al. (2011)
Mouse model of Alzheimer's disease (APP/PS1 mutant mice)	Chronic (daily/3 weeks prior behavioral tests and 8 weeks in total duration), 20 mg/kg, intraperitoneally	Reversed social recognition and object recognition deficits	Not investigated	Cheng et al. (2014)
Mouse model of multiple sclerosis	Chronic, after infection (daily/10 days), 5 mg/kg, intraperitoneally	Improved motor deficits	Not investigated	Mecha et al. (2013)
Rat model of cognitive impairment induced by iron overload	Acute and chronic, in adult age (daily/2 weeks), 5 or 10 mg/kg, intraperitoneally	Rescued recognition memory deficits	Not investigated	Fagherazzi et al. (2012)

CBD improved motor or cognitive parameters when administered acute or chronically to rodent models of neurodegenerative disorders or hypoxic-ischemia.
APP/PS1, amyloid precursor protein/presenilin-1.

by questionnaires in PD patients, but this compound did not affect any cognitive or motor function. Given these positive results, further studies investigating the neuroprotective effects of CBD in PD are warranted.

Huntington's Disease

Huntington's disease (HD) is an inherited NDD characterized by loss of striatal projection neurons, characterized by chorea, cognitive, and psychiatric disturbances involving the basal ganglia and cerebral cortex. HD has been modeled in rodents by lesioning the striatum with the inhibitor of the mitochondrial complex II, 3-nitropropionic acid (3NP) (Kumar, Kalonia, & Kumar, 2010). Administration of CBD completely reversed 3NP-induced reductions in γ-aminobutyric acid (GABA) contents and mRNA levels for substance P, markers of striatal GABAergic projection neurons, neuronal-specific enolase, and SOD-2. This study demonstrated that CBD provided neuroprotection, mediated by cannabinoid receptor-independent antioxidant properties, against 3NP-induced striatal damage, which may be relevant for HD (Sagredo, Ramos, Decio, Mechoulam, & Fernández-Ruiz, 2007).

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory and degenerative disease affecting the central nervous system, characterized by demyelination and axonal degeneration (Messina & Patti, 2014). Mecha et al. (2013) investigated the effects of CBD using a viral model of MS. It was observed that CBD reduced the expression of inflammatory molecules, such as vascular cell adhesion molecule-1, chemokines, and the proinflammatory cytokine interleukin-1β, and attenuated microglia activation. CBD has also improved motor deficits. Some of the anti-inflammatory effects were blocked by an A_{2A} adenosine receptor antagonist. This study highlights the potential of CBD for the treatment of neuropathologies with an inflammatory component, such as MS.

OTHER MODELS OF NEURODEGENERATION

Cognitive Impairment Associated to Brain Iron Overload

Rats and mice treated with iron show severe memory impairment, and our research group has been using this animal model to investigate the involvement of iron in NDD-related cognitive impairment, as well as a tool for the search of compounds with therapeutic potential (for a review, see Schröder, Figueiredo, & de Lima, 2013). We have tested the effects of CBD in reversing cognitive dysfunction associated with brain iron overload (Fagherazzi et al., 2012). Male adult rats, treated with iron, received acute or chronic injections of CBD in adulthood, and were tested in the object recognition task. We showed

that CBD rescued recognition memory deficits in iron-overloaded animals (Fig. 83.1).

Subsequently, we decided to investigate the cellular and molecular mechanisms associated with CBD's ability in restoring the damage caused by iron loading in rats, using the same dose previously shown to ameliorate memory deficits associated with iron loading (da Silva et al., 2014). We analyzed proteins involved in mitochondrial fusion and fission mechanisms, which are crucial processes for mitochondrial recycling and survival of healthy mitochondria, synaptophysin, a transmembrane protein of synaptic vesicles, often regarded as a synaptic marker, and caspase 3, an apoptosis-related protein. Iron overload resulted in mitochondrial dynamics imbalance,

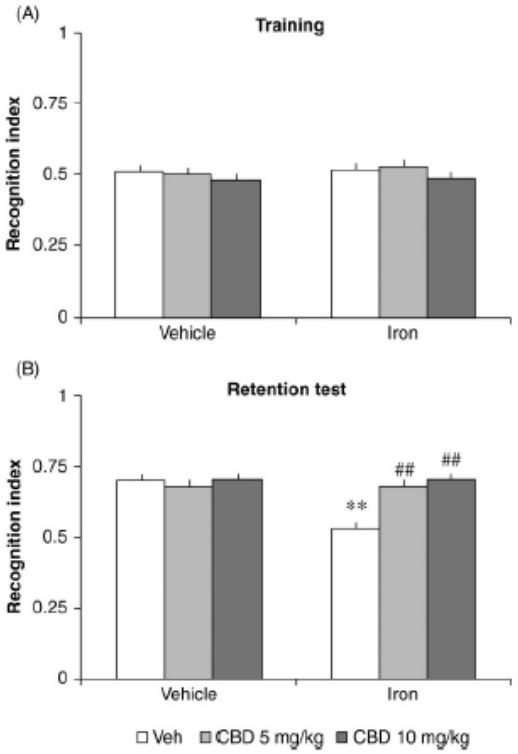

FIGURE 83.1 Effects of chronic CBD on iron-induced recognition memory deficits. Effects of chronic cannabidiol *(CBD)* on iron-induced recognition memory deficits. Recognition indexes in (A) training and (B) retention test session are expressed as mean ± SEM. A daily single injection of vehicle or CBD (5.0 or 10.0 mg/kg) was administered for 14 days. Animals were trained in the object recognition task 24 h after the last injection. $N = 12–15$ per group. Differences between vehicle–vehicle versus other groups are indicated as $^{**}p < 0.001$; difference between iron–vehicle versus iron–CBD is indicated as $\#\#p < 0.001$. *Adapted from* Springer and Psychopharmacology *(Berlin)*, 219*(4)*, *2012, 1133–1140, "Memory-rescuing effects of cannabidiol in an animal model of cognitive impairment relevant to neurodegenerative disorders." (Fagherazzi et al., 2012, Fig. 3). With kind permission from Springer Science and Business Media.*

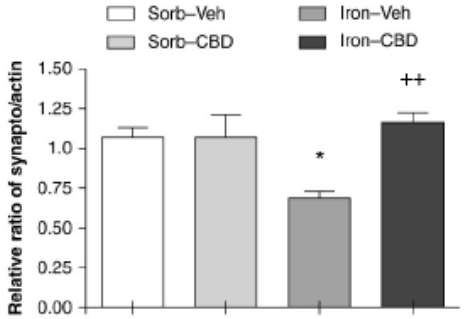

FIGURE 83.2 Effects of chronic CBD on Synaptophysin expression in hippocampus of iron-treated rats. Western blot of synaptophysin in the hippocampus of 3-month-old rats treated with sorbitol *(Sorb)* or iron neonatally, and treated with vehicle *(Veh)* or cannabidiol *(CBD)* chronically; 25 µg of protein, normalized to β-actin, were separated on SDS–PAGE and probed with antisynaptophysin. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD posthoc. Data expressed as mean ± SEM. $N = 4–6$/group. $^{*}p < 0.05$, differences between Sorb–Veh versus iron–Veh; $^{++}p < 0.01$, difference between iron–Veh versus iron–CBD. *Adapted from* Springer and Molecular Neurobiology, 49*(1)*, *2014, 222–233, Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: implications for neuroprotection (da Silva et al., 2014, Fig. 5a). With kind permission from Springer Science and Business Media.*

possibly triggering synaptic loss, expressed as a reduction of synaptophysin levels, and apoptotic cell death, related to increased caspase 3 levels. We found that CBD rescued iron-induced effects, bringing hippocampal synaptophysin levels (Fig. 83.2) and caspase 3 (Fig. 83.3) back to values comparable to the control group. We also showed that CBD was able to restore hippocampal levels of mitochondrial fusion protein, dynamin 1-like (DNM1L) in iron-treated rats. Altogether, our results suggest that CBD should be considered as a potential molecule with memory-rescuing and neuroprotective properties to be used in the treatment of cognitive deficits observed in NDD.

The neuroprotective potential of CBD was evaluated in an animal model of sciatic nerve transection in neonatal rats (Perez et al., 2013). In neonatal rats, transection of a peripheral nerve leads to an intense retrograde degeneration of both motor and sensory neurons, which is prominently related to neuronal loss resulting of apoptotic processes. Neuronal counting revealed both motor and sensory neuron rescue following treatment with CBD. Administration of CBD decreased the astroglial and microglial reaction, and number of apoptotic cells, mostly located in the spinal cord intermediate zone. CBD also induced synaptic preservation within the spinal cord, revealed by synaptophysin staining.

Recently, the effects of transdermal delivery CBD against alcohol-induced neurodegeneration were studied in a rodent model of alcohol use disorder

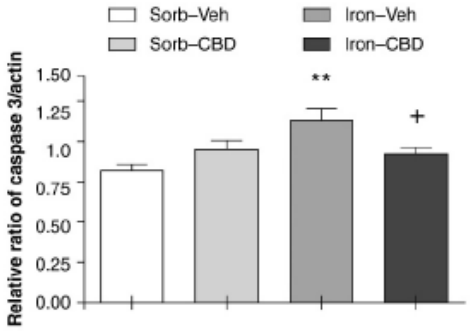

FIGURE 83.3 Effects of chronic CBD on caspase 3 expression in hippocampus of iron-treated rats. Western blot of caspase 3 in the hippocampus of 3-month-old rats treated with sorbitol *(Sorb)* or iron neonatally, and treated with vehicle *(Veh)* or cannabidiol *(CBD)* chronically; 25 µg of protein, normalized to β-actin, were separated on SDS–PAGE and probed with anticaspase 3. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD posthoc. Data expressed as mean ± SEM. $N = 4–6$/group. $^{**}p < 0.01$, differences between Sorb–Veh versus other groups; $+p < 0.05$, difference between Iron–Veh versus Iron–CBD. *Adapted from* Springer and Molecular Neurobiology, *49(1), 2014, 222–233, Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: implications for neuroprotection (da Silva et al., 2014; Fig. 7a). With kind permission from Springer Science and Business Media.*

(Liput, Hammell, Stinchcomb, & Nixon, 2013). Ethanol exposure resulted in neurodegeneration as indicated by the presence of Fluoro-Jade B positive cells along the entorhinal cortex. Treatment with CBD by either intraperitoneal injection or a second generation transdermal CBD gel resulted in a reduction in Fluoro-Jade B positive cells in the entorhinal cortex following binge ethanol treatment. Although the mechanisms involved in protection against alcohol-induced neurodegeneration are not clear, previous studies had indicated that impairment in mitochondrial function, and subsequent oxidative stress, are likely causal factors contributing to alcohol-induced neurodegeneration. It is possible that CBD attenuates oxidative stress caused by impairments in the mitochondrial electron transport chain.

SUMMARY OF THE MECHANISMS OF ACTION OF CBD

Using in vitro and ex vivo models, it is possible to investigate the molecular and cellular pathways targeted by CBD that mediate its neuroprotective effects. Some studies aimed to elucidate whether CBD's effects were related to its interaction with membrane receptors. Thus, the possibility of blocking CBD's effects by using cannabinoid receptor, adenosine receptor, and serotonin receptor antagonists was investigated. Remarkably, many studies have reported effects of CBD in specific intracellular pathways, which are probably receptor-independent effects. Table 83.2 presents a summary of the in vivo studies investigating the neuroprotective effects of CBD, indicating doses and regimen of treatment, and dependence on membrane receptors, when applicable.

Many studies report the antioxidant properties of CBD in brain tissue or neural cell cultures, using different molecular markers of oxidative stress, such as glutathione/creatine ratio, reduction of oxidative damage to proteins and lipids, assessed by levels of protein carbonylation and lipid peroxidation, respectively, ROS production, and upregulation of enzymatic antioxidant defense, such as SOD. The chemical structure of CBD, including a resorcinol, confers potent antioxidant effects (Mechoulan, Peters, Murillo-Rodriguez, & Hanus, 2007). CBD is also well known for its antiinflammatory actions. In vitro and in vivo studies have shown that CBD reduces numerous inflammatory markers, including cytokines, such as interleukin-6, TNF-α, and interleukin-1β, chemokines, and vascular cell adhesion molecule-1. Reductions in enzymes related to the inflammatory pathway, such as COX-2, and NOS, as well as attenuation of microglia activation were also demonstrated. Excitotoxicity and apoptotic cell death were also demonstrated to be hindered by CBD.

To characterize the transcriptional effects of CBD, Juknat et al. (2012) treated BV-2 microglial cells with CBD, and performed microarray analysis. They identified 1204 differentially expressed genes in response to CBD treatment, which were associated with many different functions, including stress, inflammatory response, membrane transport, adhesion and migration, cell cycle, and proliferation. CBD repressed the expression of an important subset of proinflammatory genes, especially the chemokine ligands, and the chemokine receptor CX-3CR1.

Intracellularly CBD was shown to interact with the GSK-3β pathway, thereby inhibiting hyperphosphorylation of tau, and also to activate nuclear receptors of the PPAR family. Increasing evidence also supports the view that CBD may have protective effects against mitochondrial damage. CBD restored hippocampal levels of a mitochondrial fusion protein, DNM1L, in iron-loaded rats, and increased the activity of mitochondrial complexes (I, II, II–III, and IV) in the rat brain, raising the possibility that CBD modulates brain energy production (Valvassori et al., 2013). CBD was shown to protect SH-SY5Y or cultured hippocampal neurons against the mitochondrial toxin FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) (Ryan, Drysdale, Lafourcade, Pertwee, & Platt, 2009).

Interestingly, Campos et al. (2013) have shown that CBD was able to significantly increase hippocampal progenitor proliferation and neurogenesis, in mice submitted to chronic unpredictable stress. Experiments

TABLE 83.2 Summary of In Vivo/Ex Vivo Studies Testing Cellular and Molecular Effects of CBD.

Experimental Model	Regimen of Treatment (Effective Dose)	Effects	Receptor(s) Involved	References
Ischemic mouse	Doses injection pre/postischemia, 1 or 3 mg/kg, intraperitoneally	⇩ Infarct volume, ⇧ myeloperoxidase activity	Cannabinoid receptor type 1 and 2-independent	Hayakawa et al. (2007)
Neonatal hypoxic-ischemic pig	Two doses (15/240 min after ischemia), 0.1 mg/kg, intravenous	⇧ Brain activity, ⇩ neuronal damage/astrocytosis in the cortex, ⇩ tumor necrosis factor α positive cells	Not investigated	Lafuente et al. (2011)
Neonatal hypoxic-ischemic pig	Acute (30 min after ischemia), 1 mg/kg, intravenous	⇧ Number of viable neurons, ⇧ brain activity, ⇩ excitotoxicity, ⇩ Oxidative stress, ⇩ Inflammation	Effects dependent on cannabinoid receptor type 2 and serotonin receptor 1A	Pazos et al. (2013)
Neonatal hypoxic-ischemic rat	Acute (10 min after ischemia), 1 mg/kg, subcutaneous	⇩ Excitotoxicity, ⇩ oxidative stress, ⇩ inflammation, ⇩ brain damage	Not investigated	Pazos et al. (2012)
Retinal neurotoxicity in rat	Acute before induced excitotoxicity, 2 mg/kg, intravenous	⇩ Nitrotyrosine formation, ⇩ apoptosis	Not investigated	El-Remessy et al. (2003)
Rat model of Alzheimer's disease (hippocampal amyloid-β injection)	Chronic, after surgery (daily/15 days), 10 mg/kg, intraperitoneally	⇧ Neurons integrity, ⇩ reactive gliosis, ⇧ neurogenesis	Not investigated	Esposito et al. (2011)
Mouse model of Alzheimer's disease (hippocampal amyloid-β injection)	Chronic, 24 h after procedure (first week: daily injection, second and third weeks: 3 days/week), 20 mg/kg, intraperitoneally	⇩ Interleukin 6 expression	Not investigated	Martín-Moreno et al. (2011)
Rat model of Parkinson's disease (6-OHDA)	Chronic, 16 h after procedure (daily/2 weeks) 3 mg/kg, intraperitoneally	⇧ Dopamine, ⇧ levels of Cu,Zn-superoxide dismutase	Cannabinoid receptor-independent	García-Arencibia et al. (2007)
Rat model of Huntington's disease	Chronic, 4 h after first procedure (daily/5 days), 5 mg/kg, intraperitoneally	⇩ Striatal degeneration	Effects dependent on cannabinoid receptor type 1/transient receptor potential vanilloid 1/adenosine A_{2A}-receptor-independent	Sagredo et al. (2007)
Mouse model of multiple sclerosis	Chronic, after infection (daily/10 days), 5 mg/kg, intraperitonealy	⇩ Expression of inflammatory molecules, ⇩ microglia activation	Effects dependent on A_{2A} adenosine receptor	Mecha et al. (2013)
Rat model of cognitive impairment induced by iron overload	Chronic in adult age (daily/2 weeks), 10 mg/kg, intraperitoneally	⇩ Hippocampal caspase 3, ⇧ hippocampal synaptophysin, ⇧ hippocampal dynamin-1-like protein	Not investigated	da Silva et al. (2014)
Rat model of sciatic nerve transection	Chronic, after transection (daily/5 days), 15 or 30 mg/kg, intraperitoneally	⇧ Motor and sensory neuron intregality, ⇩ astrogliosis and microgliois, ⇩ apoptotic cells, ⇧ synaptic preservation	Not investigated	Perez et al. (2013)
Rat model of alcoholism	Chronic, after 3rd ethanol dose (transdermal: daily/3 days; intraperitoneally: twice a day/3 days), 2.5 or 5%, transdermal gel; 20 mg/kg, intraperitoneally	⇩ Fluoro-Jade B positive cells in the entorhinal cortex	Not investigated	Liput et al. (2013)
Energetic metabolism in rat	Acute and chronic (daily/2 weeks), 15, 30, or 60 mg/kg, intraperitoneally	⇧ Activity of mitochondrial complexes (I, II, II–III and IV), ⇧ creatine kinase	Not investigated	Valvassori et al. (2013)
Mouse model of chronic unpredictable stress	Chronic, 2 h after daily stress (daily/2 weeks), 30 mg/kg, intraperitoneally	⇧ Neurogenesis	Not investigated	Campos et al. (2013)

Summary of studies designed to investigate cellular and molecular aspects of neuroprotective effects of CBD. CBD displays antiapoptotic, antiinflammatory, and antioxidant properties. CBD also reduces excitotoxicity, reactive astrocytosis, and improves brain activity and neuronal survival, synaptic and mitochondrial parameters, and neurogenesis. (6-OHDA: 6-hydroxydopamine; CBD, cannabidiol.)

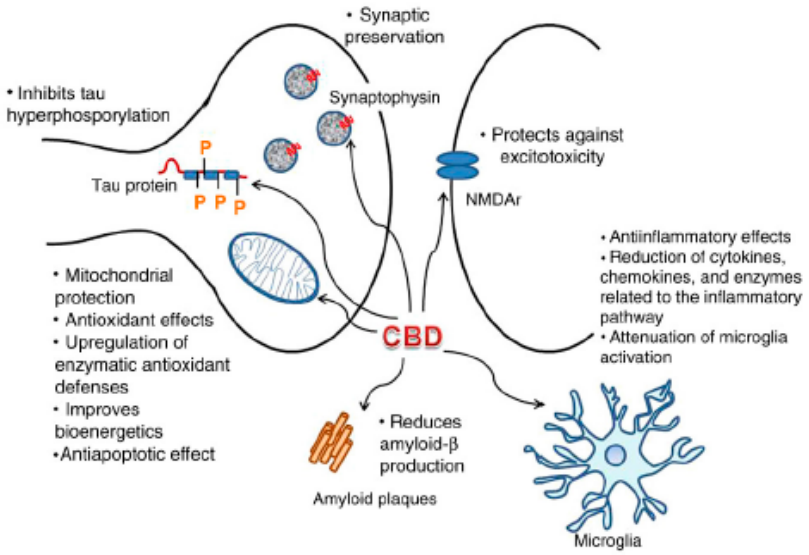

FIGURE 83.4 Summary of the main mechanisms of action of CBD. Using in vitro and ex vivo approaches, studies have shown that cannabidiol *(CBD)* exhibits antioxidant, antiinflammatory, and antiapoptotic effects. CBD was also demonstrated to exert a protective action on mitochondria, thus preserving energy production. CBD prevents synaptic loss, demonstrated by the maintenance of the synaptic marker, synaptophysin. CBD also prevents hyperphosphorylation of tau and amyloid-β production, and protects against excitotoxic cell death. *NMDAr*, NMDA receptor.

conducted with hippocampal progenitor cells in culture showed that CBD promoted progenitor proliferation and cell cycle progression, and mimicked the proliferative effect of CB1 and CB2 cannabinoid receptor activation, supporting the view that CBD may promote neurogenesis in the hippocampus.

Fig. 83.4 summarizes the putative mechanisms of action of CBD.

CONCLUSIONS

Despite the fact that the mechanisms of action of CBD are not fully understood, a great deal of evidence suggests that, by protecting against oxidative damage and neuroinflammation, and possibly by protecting the mitochondria, thereby maintaining energy supply, CBD may provide resistance against synaptic loss, and subsequent cell death. Neurodegeneration is considered a multifactorial process, involving oxidative stress, energetic failure, and synaptic dysfunction that may ultimately culminate in cell death. CBD combines multitarget pharmacological properties that might make it a promising candidate compound for the prevention/treatment of NDD.

Since CBD has been already tested in humans (Consroe et al., 1991; Zuardi et al., 2009) showing safety, with no toxic side effects, and while preclinical tests continue, in order to provide a better understanding of mechanisms, clinical tests with CBD should proceed, perhaps as a multitarget drug for the treatment of NDD.

MINI-DICTIONARY

Excitotoxic damage Damage resulting from excessive glutamate release and membrane depolarization associated to stimulation of NMDA receptors, which mediate an increase in intracellular calcium levels. Calcium activates enzymes involved in the catabolism of proteins, phospholipids, and nucleic acids.
Oxidative stress Imbalance between ROS production and a biological system's ability to remove or repair damage caused by ROS.
Apoptotic cell death A type of programmed cell death, characterized by ordered cellular destruction without inflammatory response, having an important role in development and tissue homeostasis; however, deregulation in this process results in pathological conditions.
Rota-rod Equipment used to evaluate motor behavior, including balance, coordination, and motor learning in rodents, particularly to test experimental drugs' effects, analyze brain damage, and diseases. In this performance test, rodents are placed on a rotating rod with a controlled speed.
Astrocytosis An abnormal increase in the number of astrocytes, in response to all forms of central nervous system injury and disease.
Object recognition Behavioral test used to evaluate the recognition memory in rodents, based on the proportion of time that the animal explores a novel object, when presented together with a familiar object.
PPARs Ligand-activated transcription factors belonging to the nuclear receptor superfamily. They play an essential role in energy homeostasis and other important biological processes, such as inflammation, cell proliferation, and differentiation.

APP/PS1 mutant mice Mice carrying mutations in amyloid precursor protein (APP) and presenilin-1 (PS1) genes, known as a double transgenic AD model.

6-OHDA model of PD 6-hydroxydopamine is a neurotoxic synthetic compound involved in massive destruction of nigrostriatal dopaminergic neurons, widely used to induce parkinsonism in laboratory animals.

Fluoro-Jade B positive cells Fluoro-Jade staining, an anion derived from fluorescein and highly acidic, is a specific marker for neurons that are in the process of degeneration.

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1.2 OBJETIVOS

1.2.1 Objetivo Geral

Este trabalho teve como objetivo investigar os efeitos do tratamento neonatal com ferro sobre o metabolismo do ferro mitocondrial, bem como sobre o DNA mitocondrial e seus mecanismos epigenéticos. Também foram avaliadas enzimas relacionadas ao metabolismo energético e marcadores moleculares de apoptose em cérebros de ratos tratados com ferro. Também nos propusemos a caracterizar os mecanismos relacionados aos efeitos neuroprotetores do CBD sobre os parâmetros moleculares analisados neste modelo animal.

1.2.2 Objetivos Específicos

- Avaliar o efeito do tratamento com ferro no período neonatal sobre a expressão gênica e proteica das proteínas envolvidas no metabolismo de ferro mitocondrial (Ferritina mitocondrial e Mitoferrina 2) no hipocampo de ratos.

- Avaliar o efeito do tratamento com ferro no período neonatal sobre as deleções no DNA Mitocondrial do Complexo I no hipocampo de ratos.

- Avaliar o efeito do tratamento com ferro no período neonatal sobre a metilação e hidroximetilação do DNA mitocondrial no hipocampo de ratos.

- Avaliar o efeito do tratamento com ferro no período neonatal sobre a atividade enzimática das enzimas Succinato desidrogenase e Creatina quinase (envolvidas no metabolismo energético) no hipocampo de ratos.

- Avaliar o efeito do tratamento com ferro no período neonatal sobre a expressão proteica das proteínas da cascata apoptótica (Citocromo c, APAF1, Caspase 3, Caspase 8, Caspase 9 e PARP) no hipocampo de ratos.

- Avaliar o efeito do tratamento crônico com CBD na idade adulta sobre a expressão gênica e proteica das proteínas envolvidas no metabolismo de ferro mitocondrial

(Ferritina mitocondrial e Mitoferrina 2) no hipocampo de ratos tratados com ferro no período neonatal.

- Avaliar o efeito do tratamento crônico com CBD na idade adulta sobre as deleções no DNA Mitocondrial do Complexo I no hipocampo de ratos tratados com ferro no período neonatal.

- Avaliar o efeito do tratamento crônico com CBD na idade adulta sobre a metilação e hidroximetilação do DNA mitocondrial no hipocampo de ratos tratados com ferro no período neonatal.

- Avaliar o efeito do tratamento crônico com CBD na idade adulta sobre a expressão proteica das proteínas da cascata apoptótica (Citocromo c, APAF1, Caspase 3, Caspase 8, Caspase 9 e PARP) no hipocampo de ratos tratados com ferro no período neonatal.

- Avaliar o efeito do tratamento crônico com CBD na idade adulta sobre a atividade enzimática das enzimas Succinato desidrogenase e Creatina quinase (envolvidas no metabolismo energético) no hipocampo de ratos.

CAPÍTULO 2

2.1 ARTIGO CIENTÍFICO 1

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Research report

Novel insights into mitochondrial molecular targets of iron-induced neurodegeneration: Reversal by cannabidiol

Vanessa Kappel da Silva[a,b], Betânia Souza de Freitas[a], Victória Campos Dornelles[a], Luiza Wilges Kist[c], Maurício Reis Bogo[c], Milena Carvalho Silva[d], Emílio Luiz Streck[d], Jaime Eduardo Hallak[b,e], Antônio Waldo Zuardi[b,e], José Alexandre S. Crippa[b,e], Nadja Schröder[a,b,*]

[a] Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul, 90619-900 Porto Alegre, RS, Brazil
[b] National Institute of Science and Technology for Translational Medicine (INCT-TM), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasília, Brazil
[c] Laboratory of Genomics and Molecular Biology, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul, 90619-900 Porto Alegre, RS, Brazil
[d] Laboratory of Bioenergetics, Graduate Program in Health Sciences, Health Sciences Unit, University of Southern Santa Catarina (UNESC), 88806-000 Criciúma, SC, Brazil
[e] Department of Neuroscience and Behavioral Sciences, Ribeirão Preto Medical School, University of São Paulo, 14048-900 São Paulo, SP, Brazil

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ABSTRACT

Evidence has demonstrated iron accumulation in specific brain regions of patients suffering from neurodegenerative disorders, and this metal has been recognized as a contributing factor for neurodegeneration. Using an experimental model of brain iron accumulation, we have shown that iron induces severe memory deficits that are accompanied by oxidative stress, increased apoptotic markers, and decreased synaptophysin in the hippocampus of rats. The present study aims to characterize iron loading effects as well as to determine the molecular targets of cannabidiol (CBD), the main non-psychomimetic compound of *Cannabis sativa*, on mitochondria. Rats received iron in the neonatal period and CBD for 14 days in adulthood. Iron induced mitochondrial DNA (mtDNA) deletions, decreased epigenetic modulation of mtDNA, mitochondrial ferritin levels, and succinate dehydrogenase activity. CBD rescued mitochondrial ferritin and epigenetic modulation of mtDNA, and restored succinate dehydrogenase activity in iron-treated rats. These findings provide new insights into molecular targets of iron neurotoxicity and give support for the use of CBD as a disease modifying agent in the treatment of neurodegenerative diseases.

1. Introduction

The etiology of neurodegenerative diseases has not been completely elucidated, but it is widely accepted that oxidative damage, linked to accumulation of transition metals, can contribute to neurodegeneration (Salvador et al., 2010; Kim et al., 2015). Progressive iron accumulation in the brain has been described during the normal aging process. Remarkably, in neurological diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases, iron accumulates in brain areas relevant to disease-associated neurodegenerative processes (Stankiewicz and Brass, 2009; Mills et al., 2010). For instance, it has been demonstrated that iron selectively accumulates in the *substantia nigra pars compacta* in PD patients (Dexter et al., 1991; Sofic et al., 1991), while it builds up around and within amyloid plaques and neurofibrillary tangles in brains from AD patients (Connor et al., 1992; Lovell et al., 1998).

In previous studies, aiming to examine the mechanisms of iron neurotoxicity in neurodegenerative diseases, we have established an animal model of brain iron loading, with oral administration of iron during the neonatal period, which is the period of maximal iron uptake by the brain (Taylor and Morgan, 1990). Iron neonatal treatment induces emotional memory deficits, tested in the inhibitory avoidance task (Schröder et al., 2001; Fagherazzi et al., 2012; Figueiredo et al., 2016) as well as recognition memory impairments (de Lima et al., 2005; Fagherazzi et al., 2012; Figueiredo et al., 2016). These memory deficits are accompanied by increased thiobarbituric acid reactive species (TBARS), protein carbonylation and superoxide production (Dal-Pizzol et al., 2001; de Lima et al., 2005), increased levels of apoptotic markers,

* Corresponding author at: Department of Physiological Sciences, Faculty of Biosciences, Pontifical Catholic University, Av. Ipiranga, 6681 Prédio 12D, Sala 301, 90619-900 Porto Alegre, RS, Brazil.
E-mail address: nadja.schroder@pucrs.br (N. Schröder).

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Par4 and caspase 3 (Miwa et al., 2011, da Silva et al., 2014), and reactive astrogliosis (Fernandez et al., 2011). In addition, recent studies have shown that iron overload leads to accumulation of ubiquitinated proteins (Figueiredo et al., 2016), decreased levels of synaptophysin, as well as alterations in the levels of DNM1L, a protein critically involved in mitochondrial fission (da Silva et al., 2014), resembling common alterations observed in neurodegenerative disorders. Kaur et al. (2007) reported that mice, treated with iron for eight days during the neonatal period with a dose four times higher than the one used in our model, developed histological and neurochemical alterations relevant to PD pathology, i.e., increased oxidative stress, decreased striatal dopamine content and nigral tyrosine hydroxylase (TH) positive cells.

Neurons are highly differentiated cells with high energy requirements, which mainly come from mitochondria, warranting neuronal survival and many essential functions, including axonal growth and branching, generation of action potentials, and synaptic transmission and plasticity (Lin and Sheng, 2015). Therefore, it has been suggested that dysfunctions in these organelles play a role in the pathogenesis of neurological disorders (Mattson et al., 2008). Mitochondrial DNA damage has already been related to aging and neurodegenerative diseases and contribute to mitochondrial disruption, which in turn may lead to cell injury, particularly in the central nervous system (CNS) (Siddiqui et al., 2012; Mao et al., 2012; Grünewald et al., 2016). Recently, studies have shown that mtDNA is also subject of epigenetic regulation, including methylation (5mC) and hydroxymethylation (5hmC) (Manev and Dzitoyeva, 2013). Increases and decreases of DNA methylation have been observed during aging, but most of the studies are limited to nuclear DNA (Richardson, 2003). Mitochondrial epigenetics are in nascent form and should be better and extensively studied (Manev et al., 2012).

Mitochondria maintain cellular energy reserves, which are extremely important to the CNS, by keepping respiratory chain and Krebs cycle under strict control (Basha and Poojary, 2014). Creatine kinases catalyze reversible transfer of phosphoryl groups between ATP and creatine, mainly in high energetic consumption tissues, being essential to energy homeostasis (Pilla et al., 2003). Mitochondrial dysfunction compromises energetic metabolism, resulting in overproduction of reactive oxygen species (ROS) and bioenergetic failure to the cells, contributing to many neurodegenerative diseases (Arun et al., 2016).

Since mitochondria are the major sources of cellular iron utilization, these organelles play a key role in maintaining iron homeostasis (Napier et al., 2005). Iron entry across the mitochondrial inner membrane requires one of two homologous proteins of the mitochondrial solute carrier family, called mitoferrin 1 and mitoferrin 2 (Paradkar et al., 2009). Mitoferrin 1 is the main mitochondrial iron importer in haematopoietic tissues, while mitoferrin 2 contributes to iron acquisition in non-erythroid tissues (Shaw et al., 2006; Paradkar et al., 2009). In 2001, Levi and coworkers (Levi et al., 2001) identified an iron storage protein inside mitochondria, mitochondrial ferritin, with a similar structure to the cytosolic ferritin. It has been described that mitochondrial ferritin is expressed preferentially in tissues with high oxygen consumption and has a role in protecting mitochondria from oxidative damage induced by free iron rather than storing iron (Levi and Arosio, 2004; Santambrogio et al., 2007). Although it has been proposed that both mitochondrial disruption and iron accumulation are involved in the pathophysiology of neurodegenerative disorders, there is restricted information about the regulation of mitochondrial mechanisms of iron transport and storage in these diseases.

Cannabidiol (CBD) is the main non-psychotropic constituent of *Cannabis sativa*, corresponding to approximately 40% of plant extract (Campos et al., 2012; Zuardi, 2008). Evidence indicates that CBD possesses antioxidant, antiapoptotic, and neuroprotective properties (Hampson et al., 1998; Iuvone et al., 2004; García-Arencibia et al., 2007; Castillo et al., 2010, Pazos et al., 2012). We have previously shown that CBD completely reverses iron-induced memory deficits (Fagherazzi et al., 2012) and normalizes hippocampal levels of caspase 3, synaptophysin, and mitochondrial fission protein DNM1L in rats with brain iron overload (da Silva et al., 2014).

The aim of the present study was to characterize the effects of iron loading on mitochondrial physiology by measuring mtDNA deletions and mtDNA epigenetic modifications in the hippocampal formation, a brain region critically involved in learning and memory, known to be primarily affected in AD. We also wanted to determine if neonatal iron loading would hinder mitochondrial iron handling, by altering the expression of mitoferrin 2 and mitochondrial ferritin later in life. Relevant functional parameters of energy metabolism, succinate dehydrogenase and creatine kinase activities, were also analyzed in the hippocampus of iron-loaded rats. Considering that CBD proved to ameliorate iron-induced memory deficits, which are relevant in the context of aging and neurodegenerative disorders, and the demand for neuroprotective treatments, we further investigated possible targets of CBD action on iron-induced mitochondrial alterations.

2. Material and methods

2.1. Animals

Pregnant Wistar rats (CrlCembe:WI) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE), Pontifical Catholic University, Porto Alegre, RS, Brazil. After birth each litter was adjusted within 48 h to eight rat pups, and to contain offspring of both genders in about equal proportions, and kept at standard laboratory conditions. At the age of 3 weeks, pups were weaned and the males were selected and raised 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 experimental procedures were performed in accordance to the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil) and approved by the Institutional Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA 14/00409). All efforts were made to minimize the number of animals.

2.2. Treatments

2.2.1. Neonatal iron treatment

The neonatal iron treatment has been described in detail elsewhere (da Silva et al., 2014; Fagherazzi et al., 2012). Briefly, 12-day-old rat pups received orally a single daily dose of vehicle (5% sorbitol in water) (control group) or 30 mg/kg of body weight of Fe^{2+} (iron carbonyl, Sigma-Aldrich, São Paulo, Brazil) via a metallic gastric tube, over 3 days (postnatal days 12–14).

2.2.2. Cannabidiol

Adult (3 month-old) rats, treated neonatally with vehicle or iron, as described above, received a daily intraperitoneal injection of vehicle (Tween 80–saline solution 1:16 v/v) or CBD (10 mg/kg, approximately 99.9% pure; kindly supplied by BSPG-Pharm, Sandwich, UK) for 14 consecutive days. Drug solutions were freshly prepared immediately prior to administration (Fagherazzi et al., 2012; da Silva et al., 2014).

Rats were euthanized by decapitation at 24 h after the last injection of CBD treatment. Brains were quickly dissected and hippocampi were isolated and stored at −80 °C for subsequent RT-qPCR, Western Blotting, and enzymatic activity assays. For analyses of mtDNA methylation and hydroxymethylation, hippocampal mitochondria were freshly extracted according to a commercial protocol (XIT™ Mitochondrial DNA, G-Biosciences, St. Louis, USA) and mtDNA was isolated.

2.3. Molecular analyses

2.3.1. Real-time PCR analysis of mitochondrial Complex I deletion

The assays were performed as previously described by Ochoa et al. (2011) with adaptations. Two different regions of the mitochondrial genome were studied, one that is rarely affected by deletions (*nd1*) and another that is frequently deleted (*nd4*) both in humans and in rats (Van Tuyle et al., 1996; He et al., 2002). Total DNA was isolated from hippocampus using the Qiagen® DNeasy tissue kit (Hilden, GER) in accordance with the manufacturer's instructions. DNA purity (Abs 260/280 nm ~1.8) and concentration were determined by Nanodrop® (Thermo Fisher Scientific Inc, Waltham, USA). Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, USA), 0.25 ng/μL DNA, and mitochondrial DNA primers for *nd1* (forward primer CGCC CCAACCCTCTCC, reverse primer GTATGCCTAGGTTGAGGTTGATAAGG) and *nd4* (forward primer CATTTTCCTGATCGAACCCCTCTAT, reverse primer AGTTTTCCTCGTTGGGTTGTGATAA) on the 7500 Real-time PCR System (Applied Biosystems, Foster City, USA). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and showed in all cases one single peak. All real time assays were carried out in sextuplicate and, in all cases, a reverse transcriptase negative control was included. Both reactions (that of *nd1* and that of *nd4*) propagated with high efficiency (close to 1). The relative deletion was calculated using XCq *nd4*/XCq *nd1*. Similar efficiency values between the two reactions mean that Ct values can be used as a measure of input DNA and to quantify the relative amount of *nd1* to *nd4* because Ct does not depend on the dilution series.

2.3.2. mtDNA 5hmC and 5mC content

The 5mC content of mitochondrial DNA was measured using a methylated DNA quantification kit (MethylFlash™ Methylated DNA Quantification Kit, Epigentek, Farmingdale, USA). 100 ng of DNA from each sample were used in the 96-well plate and quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer. The 5hmC content was measured using a hydroxymethylated DNA quantification kit (MethylFlash™ Hydroxymethylated DNA Quantification Kit, Epigentek, Farmingdale, USA). 200 ng of DNA from each sample were used in the 96-well plate and quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer (adapted from Dzitoyeva et al., 2012).

2.3.3. Western Blot analyses

Proteins were extracted as previously described by da Silva et al. (2014). The supernatant was collected and the protein content was determined using Bradford assay (Bradford, 1976). Aliquots were stored at −20 °C.

Fifty μg of protein was separated on a 10% SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% albumin in TBS containing 0.05% Tween 20 and were incubated overnight with one of the following antibodies: anti-Mitocondrial ferritin (Abcam, Cambridge, UK) at 1:700; anti-Mitoferrin 2 (Abcam, Cambridge, UK) at 1:500, and anti-Tubulin (Abcam, Cambridge, UK) at 1:20000. Goat polyclonal anti-rabbit IgG H&L (HPR) (Abcam, Cambridge, UK) secondary antibody was used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected bands molecular weight and confirm antibodies target specificity. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200). Total blotting protein levels of samples were normalized according to each sample's Tubulin levels (adapted from da Silva et al., 2014).

2.3.4. RT-qPCR analysis

The gene expression of *Ftmt* (mitochondrial ferritin) and *Mitoferrin*-2 were determined by RT-qPCR. The total RNA was isolated from hippocampus with TRIzol® Reagent (Life Technologies, Carlsbad, USA) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280 nm ~2.0) and concentration were determined by Nanodrop® and after treated with Deoxyribonuclease I (Sigma-Aldrich, São Paulo, Brazil) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, USA) from 1 μg of the total RNA, following the manufacturer's instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, USA) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, Foster City, USA). Primers were designed by authors (*Ftmt:* forward primer TCCTGGACTTGCATA CTCTGGCCTCAG, reverse primer GCTTGTCGAAAAGATACTCCGCTA GG; *Mitoferrin 2*: forward primer AACACCCAGGAGTCCCTGGCCTTG, reverse primer CATGCGATGGCTGTGGAGGGGATC). The PCR cycling conditions were the same as described above in *Real-time PCR analysis of mitochondrial Complex I deletion* section. All real time assays were carried out in quadruplicate and, in all cases, a reverse transcriptase negative control was included. *Hprt1* was used as reference gene for normalization. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (adapted from da Silva et al., 2014).

2.3.5. Sample preparation for enzymatic activity determination

For the determination of succinate dehydrogenase and creatine kinase enzyme activities, hippocampi were homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C and the supernatants were used for enzyme activity determination. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard (adapted from Schuck et al., 2013).

2.3.6. Succinate dehydrogenase activity

Succinate dehydrogenase activity was measured according to the method of Fischer et al. (1985) by following the decrease in absorbance due to the reduction of 2,6-di-chloroindophenol (2,6-DCIP) at 600 nm with 700 nm as reference wavelength (ε = 19.1 mM − 1 cm − 1) in the presence of phenazine methasulphate (PMS). The reaction mixture, consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM 2,6-DCIP, was preincubated with 40–80 μg homogenate protein at 30 °C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone and 40 μM 2,6-DCIP were added and the reaction was initiated by adding 1 mM PMS. The reaction was monitored for 5 min. The activity of succinate dehydrogenase is expressed as nmol.Min − 1. mg protein − 1 (adapted from Teodorak et al., 2017).

2.3.7. Activity of creatine kinase

Total creatine kinase activity in brain homogenates, pretreated with 0.625 mM lauryl maltoside, was measured. The reaction mixture consisted of 60 mM Tris–HCl, 7 mM phosphocreatine, 9 mM MgSO4, pH 7.5, and approximately 0.4–1.2 μg protein in a final volume of 100 μL. After 15 min of pre-incubation at 37 °C, the reaction was started by adding ADP 3.2 mmol. The reaction was stopped after 10 min by adding 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by adding 100 μL 2% α-naphtol and 100 μL 0.05% diacetyl in a final volume of 1 mL and finally read spectrophotometrically after 20 min at 540 nm (adapted from Teodorak et al., 2017).

2.4. Statistical analysis

The results were expressed as mean ± S.E.M and were analyzed using SPSS 20.0 software. Levene's Test of Equality of Variances was used in order to test the assumption of homogeneity of variance. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, when necessary. In all comparisons, *p* values less than 0.05 were considered to indicate statistical significance.

3. Results

We first decided to investigate the impact of excessive iron on the integrity of mitochondrial DNA, by quantifying the frequency of *nd1* and *nd4* mitochondrial genes, which code Complex I proteins. Statistical comparisons of relative mtDNA deletion levels demonstrated a statistically significant difference among the groups ($F_{(3,20)} = 165.59$, $p < 0.0001$, Fig. 1). When groups were compared using Tukey's *post hoc* test, results revealed that neonatal iron treatment induced a significant increase in mtDNA deletion levels, when compared to the control group (sorb-veh, $p < 0.0001$). The iron-treated group that received CBD in the adulthood had also significantly higher deletion levels when compared to the control group ($p < 0.0001$).

We next decided to further investigate the effects of iron loading and CBD treatment on epigenetic modifications of mtDNA. Interestingly, statistical comparisons have indicated that both methylation and hydroxymethylation are diminished by iron treatment. Accordingly, ANOVA revealed a significant difference among the groups regarding mtDNA methylation ($F_{(3,12)} = 39.72$, $p < 0.0001$, Fig. 2A) and hydroxymethylation ($F_{(3,16)} = 11.31$, $p < 0.0001$, Fig. 2B). Tukey's *post hoc* comparison indicated that the group that received iron in the neonatal period and vehicle in the adulthood presented a significantly reduced mtDNA methylation in comparison to the control group ($p < 0.0001$). The group that received iron in the neonatal period and CBD (iron-CBD) in the adulthood has also presented significantly reduced methylation in relation to the control group ($p < 0.0001$). Additionally, the iron-CBD group was not statistically different from the iron-veh group ($p = 0.142$). Statistical analyses of mtDNA hydroxymethylation also revealed that rats treated with iron in the neonatal period presented significantly reduced hydroxymethylation in comparison to the control group (Sorb-veh; $p = 0.001$). Interestingly, CBD in adulthood reversed this effect, since iron-treated rats that received CBD presented higher hydroxymethylation levels than the iron-veh group ($p = 0.001$) and similar levels in comparison to the control group ($p = 0.98$).

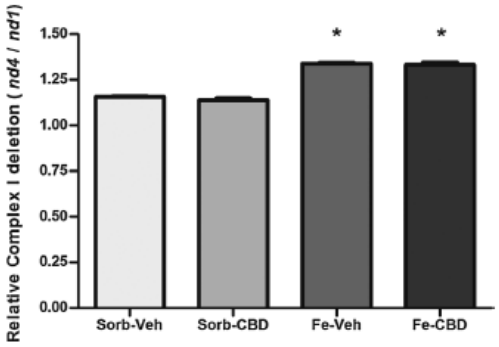

Fig. 1. Iron significantly increased relative mtDNA deletions in the hippocampus. Relative deletion in complex I deleted mtDNA in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in adulthood (at 3 months of age). Samples were obtained from 6 animals in each group and analyses were carried out in sextuplicate. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. * $p < 0.0001$ indicates significantly higher mtDNA deletion levels in iron-vehicle (Fe-Veh) and iron-CBD (Fe-CBD) groups in comparison to the sorbitol-vehicle (Sorb-Veh) group.

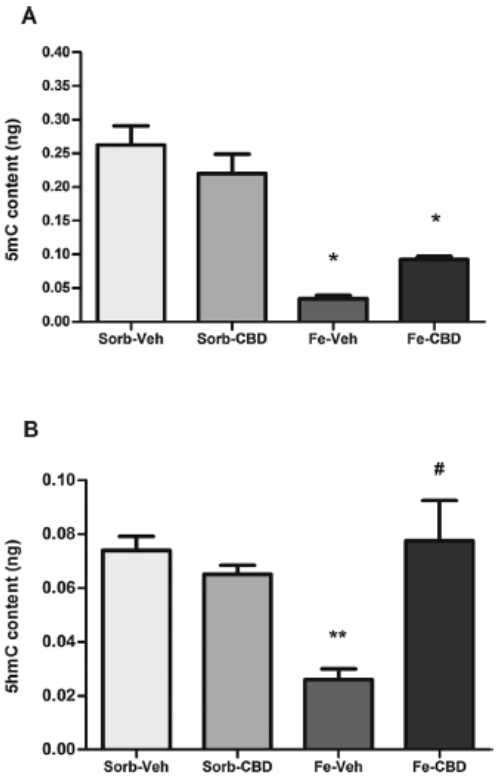

Fig. 2. Iron significantly decreased mtDNA methylation and hydroxymethylation. 5-methylcytosine (5mC) (A) 5-hydroxymethylcytosine (5hmC) (B) content in mtDNA from hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in adulthood (at 3 months of age). DNA was extracted and equal amounts of mtDNA were analyzed with 5mC and 5hmC enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. $N = 6$ per group. * $p < 0.0001$ indicates significantly lower levels of mtDNA methylation in iron-vehicle (Fe-Veh) and iron-CBD (Fe-CBD) groups in comparison to controls; ** $p < 0.001$ indicates significantly lower levels of mtDNA hydroxymethylation in iron-vehicle (Fe-Veh) group in comparison to controls; # $p < 0.001$ indicates significantly higher levels of mtDNA hydroxymethylation in the iron-CBD (Fe-CBD) in comparison to the iron-vehicle (Fe-Veh) group.

We also aimed to investigate the long-term consequences of neonatal iron loading and adult treatment with CBD on mitochondrial iron handling, by measuring protein levels and gene expression of mitochondrial proteins involved in iron metabolism, i.e. the mitochondrial iron transporter, mitoferrin 2, and mitochondrial ferritin. Statistical comparison using one-way ANOVA showed no significant differences among the groups when protein levels of mitoferrin 2, measured by western blot ($F_{(3,19)} = 1.55$, $p = 0.233$; Fig. 3A), or its gene expression ($F_{(3,16)} = 2.70$, $p = 0.080$; Fig. 3B) were compared. In contrast, when mitochondrial ferritin levels where compared, statistically significant differences among the groups were revealed, both in protein levels ($F_{(3,15)} = 20.49$, $p < 0.0001$) and gene expression ($F_{(3,13)} = 9.32$, $p = 0.001$). *Post hoc* comparisons of protein levels between groups indicated that neonatal iron treatment induced a significant reduction in ferritin levels in comparison to the control group (sorb–veh) ($p < 0.0001$; Fig. 4A), and in comparison to the group that received sorbitol in the neonatal period and CBD when adult ($p = 0.001$; Fig. 4A). Moreover, the iron-treated group that received vehicle in the

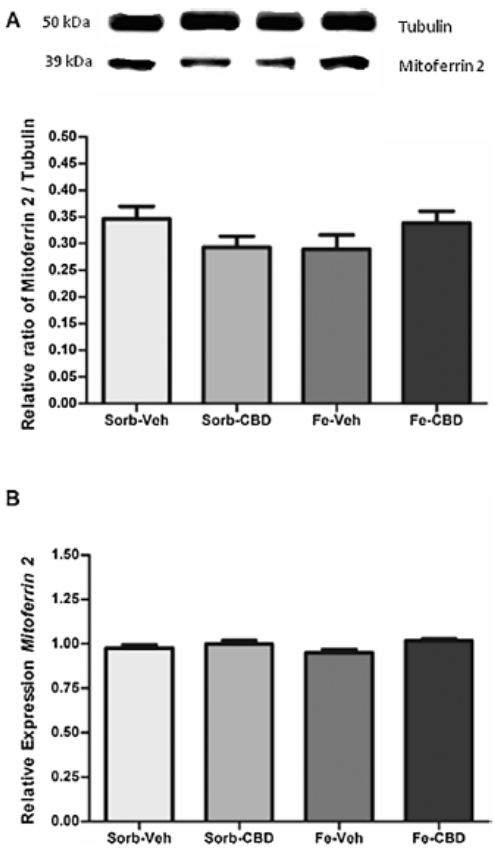

Fig. 3. Iron or CBD treatments did not alter Mitoferrin 2 expression.
(A) Western Blotting of Mitoferrin 2 and (B) relative *Mitoferrin 2* gene expression in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Western blotting samples were performed with 50 µg of protein levels, normalized to Tubulin, separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for Mitoferrin 2 and Tubulin are shown. RT-qPCR samples were carried out in quadruplicate and *hprt1* was used as reference gene for normalization. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. $N = 5–6$ per group. No significant differences were found among groups.

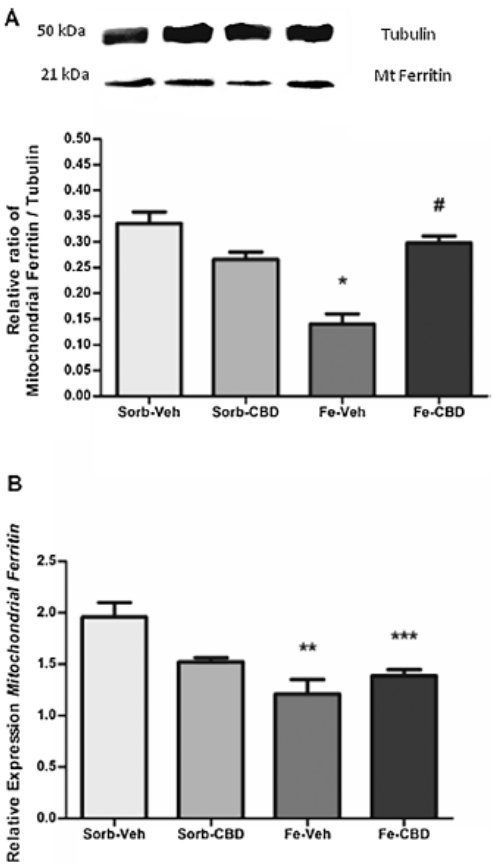

Fig. 4. Iron significantly decreased Mitochondrial ferritin protein levels and mRNA expression.
(A) Western Blotting of Mitochondrial ferritin and (B) relative *Mitochondrial ferritin* gene expression in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Western blotting samples were performed with 50 µg of protein levels, normalized to Tubulin, separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for Mitochondrial ferritin and Tubulin are shown. RT-qPCR samples were carried out in quadruplicate and *hprt1* was used as reference gene for normalization. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc test. Data expressed as mean ± S.E.M. $N = 4–5$ per group. * $p < 0.0001$ indicates a significant reduction in Mitochondrial ferritin protein levels in the iron-vehicle (Fe-Veh) group in comparison to controls; # $p < 0.0001$ indicates that the iron-CBD (Fe-CBD) group shows significantly higher levels of Mitochondrial ferritin in comparison to the iron-vehicle group (Fe-Veh); ** $p < 0.001$ indicates a significant reduction in *Mitochondrial ferritin* mRNA expression in the iron-vehicle (Fe-Veh) group compared to controls; *** $p < 0.01$ indicates a significant reduction in *Mitochondrial ferritin* mRNA expression in the iron-CBD (Fe-CBD) group compared to controls.

adult age also presented significantly lower ferritin levels when compared to the iron-treated group that received CBD in the adulthood ($p < 0.0001$; Fig. 4A). In addition, the iron-CBD group presented no significant differences when compared to the control group. At the gene expression level, results were comparable. *Post hoc* analyses revealed that ferritin gene expression was decreased in iron-treated rats that received vehicle in the adulthood in comparison to the control group ($p = 0.001$; Fig. 4B). However, the group that received iron in the neonatal period and CBD in the adulthood (iron-CBD) also presented significantly reduced ferritin gene expression in comparison to the control group ($p = 0.007$) and was not statistically different from the group that received iron in the neonatal period and vehicle in the adult age ($p = 0.609$).

We also analyzed the effects of neonatal iron loading and adult treatment with CBD on important parameters of energy metabolism in hippocampus of rats, namely succinate dehydrogenase and creatine kinase activities. Statistical comparison using one-way ANOVA has revealed significant differences in succinate dehydrogenase activity among the groups ($F_{(3,17)} = 4.518$, $p < 0.017$). *Post hoc* comparisons of enzymatic activities between groups indicated that neonatal iron treatment induced a significant decrease in succinate dehydrogenase activity in comparison to the control group, that received sorbitol in the neonatal period and vehicle in adulthood ($p = 0.022$; Fig. 5A), and in comparison to the group that received sorbitol in the neonatal period and CBD when adult ($p = 0.046$; Fig. 5A). There were no statistically significant differences in succinate dehydrogenase activity between the group that received iron in the neonatal period and CBD in the adulthood (iron-CBD) and the control group ($p = 0.742$). Although creatine kinase activity was also reduced in the iron-vehicle group, this effect did not reach statistical significance ($F_{(3,14)} = 2.815$, $p = 0.078$; Fig. 5B).

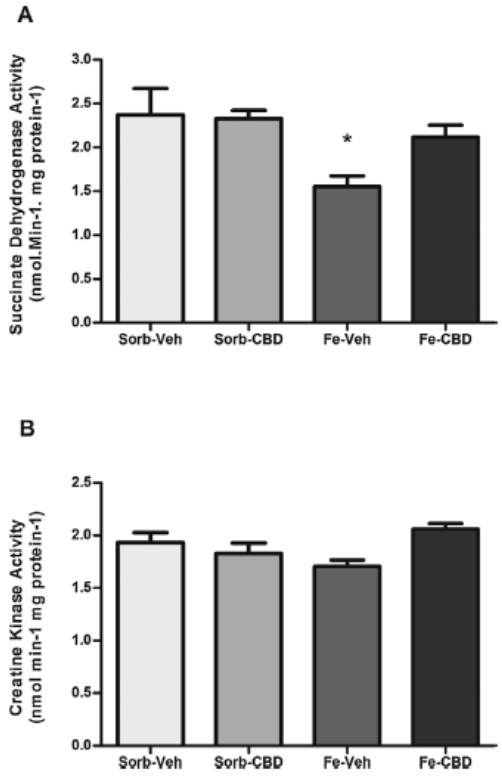

Fig. 5. Iron significantly decreased Succinate dehydrogenase activity without affecting Creatine kinase activity.

(A) Succinate dehydrogenase and (B) Creatine kinase activities in the hippocampus of rats treated with sorbitol or iron neonatally and treated with vehicle or CBD chronically in the adulthood (3 months of age). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M, $N = 4$–6 per group, for independent experiments performed in duplicate. * $p < 0.05$ indicates a significantly reduced succinate dehydrogenase activity in the iron-vehicle (Fe-Veh) in comparison to sorbitol-vehicle (Sorb-Veh) and sorbitol-CBD (Sorb-CBD) groups.

4. Discussion

Compelling evidence suggest that iron accumulation is involved in the pathogenesis of neurodegenerative diseases, mainly due to its ability to generate highly toxic free radicals, inducing oxidative damage (Li and Reichmann, 2016). Mitochondrial metabolism represents a main source of free radical production and this organelle has been recently linked to cellular iron homeostasis. Here we showed for the first time that iron overload in the neonatal period induced higher relative mtDNA deletions in the hippocampus of adult rats in comparison to controls, implicating iron excess in mitochondrial dysfunctions.

Previous studies have shown that mtDNA deletions accumulate with aging in rats (Parkinson et al., 2014; Cahif et al., 2013; Loshchenova et al., 2015) and humans (Kazachkova et al., 2013). In addition, increased mtDNA deletions in *substantia nigra* have been found in PD patients (Bender et al., 2006) and in hippocampal neurons from AD patients (Müller et al., 2013). MtDNA damage, including deletions most likely occur during repair of oxidatively damaged molecules (Krishnan et al., 2008). We have previously characterized that iron overload induces oxidative stress (de Lima et al., 2005, Dal-Pizzol et al., 2001), including increased superoxide production in mitochondrial fraction (de Lima et al., 2005) in cortex, *substantia nigra*, and hippocampus. High levels of ROS may lead to double strand breaks which, if repaired incorrectly, result in mtDNA deletions (Greaves et al., 2012). Although several evidences suggest that CBD may possess antioxidant properties, in the present conditions, CBD treatment later in life was not able to reverse iron-induced mtDNA deletions.

Interestingly, iron administered in the neonatal period produced dramatic decreases in mtDNA methylation and hydroxymethylation in the hippocampus of adult rats. Despite the increasing concentration of studies on epigenetic mechanisms in CNS physiology and pathology, the great majority has focused on nuclear DNA epigenetics. Dzitoyeva et al. (2012) investigated mitochondrial epigenetics in the mammalian CNS using a mouse model of aging and mouse primary neurons in culture, and demonstrated that aging decreases mtDNA 5hmC in the frontal cortex. Although mitochondrial epigenetic modulation is obscure and more studies are required to understand mechanisms of regulation and functioning, we can speculate on a role played by iron excess, since iron accumulates in the CNS with aging and in neurodegenerative diseases. Devall et al. (2014) suggested that mitochondrial dysfunctions associated to AD might be related to an altered pattern of epigenetic modulations. CBD was able to modify mitochondrial hydroxymethylation profile of rats treated neonatally with iron, bringing 5hmC content to levels comparable to controls. One study (Pucci et al., 2013) investigated the possible epigenetic regulation of skin differentiation genes by phytocannabinoids, including CBD. They showed that CBD reduced the expression of all the genes tested by increasing DNA methylation, supporting the view that CBD's mechanism of action may include epigenetic modulation.

Taking into account the present findings showing that iron overload leads to mtDNA injury, we sought to further investigate on how this organelle handled excessive iron. We found that mitochondria failed to increase ferritin expression in response to iron overload, while no alterations were observed in the iron transporter protein, mitoferrin 2. Mitochondrial ferritin has been recently described as a specific mitochondrial iron storage protein that contributes to cellular iron homeostasis. Despite its large similarity to cytosolic ferritin, little is known about physiological functions and regulation of this protein (Arosio and Levi, 2010; Wang et al., 2011). It has been suggested that mitochondrial ferritin expression is not primarily related to iron storage, but to protection of mitochondria against oxidative damage (Levi and Arosio, 2004; Santambrogio et al., 2007; Arosio and Levi, 2010). Due to the fact that we have not used a mitochondrial protein to normalize protein content, we cannot completely rule out the possibility that reductions in mitochondrial ferritin levels observed might be related to iron-induced changes in cellular mitochondrial content rather than specific changes in this protein. Although abnormalities of mitochondrial function and iron metabolism have been reported in neurodegenerative diseases, only recently, investigations relating mitochondrial ferritin and neurodegenerative disorders started to appear. Wang et al. (2011) reported that mitochondrial ferritin expression was increased in the cerebral cortex of DA patients, and that H_2O_2 alone or in combination with β-amyloid increased mitochondrial ferritin in human neuroblastoma cell line. Wu et al. (2013) showed that knocking mitochondrial ferritin expression down enhanced Aβ-induced neurotoxicity, oxidative stress, and cell apoptosis and opposite results were obtained with overexpression of this protein. Recently, it was demonstrated that mitochondrial ferritin deletion in mice exacerbated the effects of Aβ infusion on learning and memory and increased apoptosis (Wang et al., 2017). Interestingly, in the present study, we found reduced expression of mitochondrial ferritin in rats that received iron in the neonatal period. The precise mechanisms that regulate mitochondrial ferritin expression are poorly understood. We, hypothesize that the insult of iron overload in the neonatal period may have induced early alterations, possibly increasing mitochondrial ferritin expression early in life, leading to a decreased expression when iron intake was normalized throughout life. It is possible that the inability to keep increased mitochondrial ferritin levels may contribute to iron-induced mitochondrial damages, which subsidize mtDNA deletions observed in the present study, as well as increased levels of apoptotic markers

induced by iron previously described (da Silva et al., 2014). Studies investigating the ontogenetic pattern of mitochondrial ferritin expression under iron loading conditions would contribute to better characterize the role of this protein in iron homeostasis.

Our results showed that chronic CBD in adulthood was able to rescue mitochondrial ferritin protein levels in iron-treated rats. The mechanisms by which CBD was able to modulate mitochondrial ferritin levels remain unknown. We propose that the ability of CBD in recovering ferritin levels in iron-treated rats may account, at least in part, to its anti-oxidant and neuroprotective effects.

As expected, iron-induced mitochondrial alterations resulted in functional bioenergetic failures as well. Succinate dehydrogenase, an enzyme that links the tricarboxylic cycle with the electron transport chain, showed reduced activity in hippocampus of iron-treated rats, whereas, a tendency of reduction in creatine kinase, an enzyme that plays a key role in energy transfer in cells with high energy flux requirements, was also observed. Studies have shown that oxidative damage changes bioenergetic metabolism, decreasing Krebs cycle enzymes and electron transport chain complexes activities in some regions of CNS in rats (Basha and Poojary, 2014; Mehan et al., 2017; Teodorak et al., 2017). CBD was able to partially reverse iron-induced succinate dehydrogenase's activity reduction. Although this is the first evidence that CBD improves the activity of succinate dehydrogenase, a recent study has shown that CBD enhances mitochondrial bioenergetics, and modulates glucose metabolism via the pentose-phosphate pathway, preserving both energy and the redox balance in an *in vitro* model of oxygen-glucose-deprivation/reperfusion (OGD/R) in mouse hippocampal neuronal cell line (Sun et al., 2017). Furthermore, *in vivo* acute and chronic CBD increased the activity of the mitochondrial complexes (I, II, II–III, and IV) and creatine kinase in the rat brain (Valvassori et al., 2013). The dose of CBD used in the present study was lower than those utilized by Valvassori and coworkers, possibly explaining why in the present study CBD did not alter creatine kinase activity.

In conclusion, here we observed that iron overload in the neonatal period changes mitochondrial function, increasing mtDNA deletions, reducing epigenetic modulation of mtDNA, decreasing mitochondrial ferritin levels, and decreasing succinate dehydrogenase activity, which may altogether compromise proper cellular functioning, and contribute to neurodegeneration. Considering that the neonatal period is critical for iron uptake by the CNS and iron-fortified infant formulas contain much higher iron content than the breast milk, the long-term effects of iron supplementation still need to be determined. It has been proposed that elevated iron intake during the neonatal period in humans may represent a risk factor to the development of neurodegenerative diseases later in life (Hare et al., 2015).

We showed for the first time that CBD was able to restore hippocampal epigenetic modulation of mtDNA and to increase mitochondrial ferritin levels, which may be directly related to its neuroprotective properties. Also, CBD rescued succinate dehydrogenase activity in iron-treated rats, contributing for bioenergetic recovery, thereby promoting neural cell survival. These findings provide new insights in the molecular targets of CBD, and give support for its use as disease modifying agent in the treatment of neurodegenerative diseases.

Disclosures

AWZ, JECH and JAC are co-inventors (Mechoulam R, JC, Guimarães FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders". JAC and JECH received travel support from and are medical advisors of BSPG-Pharm.

All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

All authors have read and approved the final version of the manuscript.

Role of the funding source

The funding sources had no role in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

Conflict of interest

The authors declare that there are no conflict of interest.

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2.2 ARTIGO CIENTÍFICO 2

Article

Antiapoptotic effects of cannabidiol in an experimental model of cognitive decline induced by brain iron overload

Running title: Effects of cannabidiol in a model of iron overload

Vanessa Kappel da Silva[a,b], Betânia Souza de Freitas[a], Rebeca Carvalho Lacerda Garcia[a], Ricardo Tavares Monteiro[a], Jaime Eduardo Hallak[b,c], Antônio Waldo Zuardi[b,c], José Alexandre S. Crippa[b,c], Nadja Schröder[a,b,*]

a Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul, 90619-900 Porto Alegre, RS, Brazil.

b National Institute of Science and Technology for Translational Medicine (INCT-TM), Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Brasília, Brazil.

c Department of Neuroscience and Behavior, Ribeirão Preto Medical School, University of São Paulo, 14048-900 São Paulo, SP, Brazil.

*** Corresponding author (current address)**

Nadja Schröder Email: nadja_s@terra.com.br
Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde
Universidade Federal do Rio Grande do Sul
Rua Sarmento Leite, 500 – 90050-170 Porto Alegre, RS, Brazil.
Telephone number: +55 51 99139-4308

Abstract

Iron accumulation in the brain has been recognized as a common feature of both normal aging and neurodegenerative diseases. Cognitive dysfunction has been associated to iron excess in brain regions in humans. We have previously described that iron overload leads to severe memory deficits, including spatial, recognition, and emotional memory impairments in adult rats. In the present study we investigated the effects of iron overload on proteins involved in apoptotic pathways, such as Caspase 8, Caspase 9, Caspase 3, Cytochrome c, APAF1, and PARP in the hippocampus of adult rats, in an attempt to establish a causative role of iron excess on cell death in the nervous system, leading to memory dysfunction. Cannabidiol (CBD), the main non-psychotropic component of *Cannabis sativa*, was examined as a potential drug to reverse iron-induced effects on the parameters analyzed. Male rats received vehicle or iron carbonyl (30 mg/kg) from the 12^{th} to the 14^{th} postnatal days and were treated with vehicle or CBD (10 mg/kg) for 14 days in adulthood. Iron increased Caspase 9, Cytochrome c, APAF1, Caspase 3 and cleaved PARP, without affecting cleaved Caspase 8 levels. CBD reversed iron-induced effects, recovering apoptotic proteins Caspase 9, APAF1, Caspase 3 and cleaved PARP to the levels found in controls. These results suggest that iron can trigger cell death pathways by inducing intrinsic apoptotic proteins. The reversal of iron effects by CBD indicates that it has neuroprotective potential through its anti-apoptotic action.

Keywords: Cannabidiol; neuroprotection; intrinsic pathway; apoptosis; iron.

Introduction

Iron accumulation has been described in normal ageing in several brain regions and cell types. However, in neurological disorders such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases, iron accumulates in selective brain areas such as the hippocampus, substantia nigra, cortex, and basal ganglia, regions relevant to disease-associated neurodegenerative processes[1,2,3].

The exact mechanisms that underlie neurotoxicity induced by iron and other metals are not completely understood. In previous studies, we have established an animal model of brain iron loading, with oral administration of iron during the neonatal phase, period of maximal iron uptake by the brain[4] to better characterize the effects of iron excess on brain function. We have previously described that iron overload, induces severe and persistent long-term impairments in spatial, recognition, and emotional memories[5, 6, 7, 8, 9, 10,11]. In molecular analyses, we found lipid peroxidation and oxidative damage associated with iron excess[6], increased apoptotic markers, Par4 and Caspase 3[12,13], accumulation of ubiquitinated proteins[14], and reactive gliosis[15]. Moreover, iron treatment in the neonatal period decreased acetylcholinesterase activity in the striatum[8] and affected the regulation of iron homeostasis proteins in the hippocampus, cortex, and striatum of aged rats[16]. In addition, there was a decrease in synaptophysin levels, a marker of synaptic viability, and changes in DNM1L levels, a protein critically involved in mitochondrial dynamics in the hippocampus of iron-treated rats[13]. We have also demonstrated that iron chelation prevented memory impairments and oxidative stress in aged rats, supporting the concept that cognitive deficits associated with aging might be related to iron accumulation in the brain[17].

Apoptosis is a major form of programmed cell death that has been implicated in neurodegenerative disorders[18]. Studies have consistently reported deregulations in the expression of apoptotic proteins in the brains of both PD and AD patients and in experimental

models of neurodegenerative disorders (for a review, see [19, 20]). Among the stimuli known to trigger apoptosis are alterations of the redox balance and oxidative damage[21, 22].

Cannabidiol (CBD) is a compound currently being investigated as a potential therapeutic option for neurodegenerative disorders. CBD is the main non-psychotropic constituent of *Cannabis sativa*, corresponding to about 40% of the plant extract[23]. Evidence indicates that CBD has antioxidant, antiapoptotic, and neuroprotective properties[24, 25, 26, 27, 28, 29]. Our previous studies showed that CBD is able to improve iron-induced memory deficits[10] and regulate markers of synaptic viability and mitochondrial dynamics in the hippocampus of iron-overloaded rats[13].

The aim of the present study was to characterize the effects of iron loading on proteins critically involved with apoptotic processes in the hippocampal formation. Considering that CBD was able to restore memory in iron-treated rats, we examined possible neuroprotective effects of CBD against iron-induced deregulation of apoptotic players.

Material and methods

Animals

Pregnant Wistar rats (CrlCembe:WI) were obtained from the *Centro de Modelos Biológicos Experimentais* (CeMBE) of the Pontifical Catholic University in Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48h to eight rat pups including offspring of both genders in about equal proportions and kept at standard laboratory conditions. At the age of three weeks, pups were weaned and the males were selected and raised 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 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) and approved by the Institutional Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA 14/00409). All efforts were made to minimize the number of animals and their suffering.

Treatments

Neonatal iron treatment

The neonatal iron treatment has been described in detail elsewhere[10, 13]. Briefly, 12-day-old rat pups received a single oral daily dose of vehicle (5% sorbitol in water, control group) or 30 mg/kg of body weight of Fe^{2+} (iron carbonyl, Sigma-Aldrich, São Paulo, Brazil) via a metallic gastric tube, over 3 days (postnatal days 12-14).

Cannabidiol

Adult (three-month-old) rats, treated neonatally with either vehicle or iron as described above, received a daily intraperitoneal injection of vehicle (Tween 80 – saline solution 1:16 v/v) or CBD (10 mg/kg, approximately 99.9% pure; kindly supplied by BSPG-Pharm, Sandwich, UK) for 14 consecutive days. Drug solutions were freshly prepared immediately prior to administration[10, 13].

Rats were euthanized by decapitation at 24h after the last injection of CBD. Brains were quickly dissected and hippocampi were isolated and stored at -80ºC for subsequent Western Blotting or enzyme-linked immunosorbent (ELISA) assays.

Molecular Analyses

Western Blotting Analysis

Proteins were extracted as previously described by da Silva and coworkers[13]. The supernatant was collected and the protein content was determined using Bradford assay[30]. Aliquots were stored at - 20 °C.

Fifty µg of protein were separated on a 10% SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% albumin in TBS containing 0.05% Tween 20 and incubated overnight with one of the following antibodies: anti-Caspase 3 (Abcam, Cambridge, UK) at 1:500; anti-Caspase 9 (Abcam, Cambridge, UK) at 1:500; anti-PARP (Abcam, Cambridge, UK) at 1:200; anti-Cleaved-Caspase 8 (Cell Signaling, Danvers, USA) at 1:600 and anti-Tubulin (Abcam, Cambridge, UK) at 1:20000. Goat polyclonal anti-rabbit IgG H&L (HPR) (Abcam, Cambridge, UK) secondary antibody was used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected bands molecular weight and confirm antibodies target specificity. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200). Total blotting protein levels of samples were normalized according to each sample's Tubulin protein levels (adapted from [13]).

Enzyme Linked Immunosorbent Assay (ELISA)

We used sandwich-ELISA commercial kits to measure hippocampal APAF1 and Cytochrome c proteins (LSBio, Seattle, USA). Briefly, hippocampi were finely minced and homogenized in 750 µL of PBS with a glass homogenizer on ice. Cells were lysed by 3 cycles of freeze (-20°C) / thaw (room temperature). Homogenates were centrifuged at 5000×g for 5 minutes and the supernatant was collected for assaying. One hundred µL of standard, blank and samples were tested in duplicate and the optical density was determined using a

microplate reader set to 450 nm. The standard curve demonstrated a direct relationship between optical density and APAF1 or Cytochrome c concentrations. Results were expressed as nanograms of APAF1 or Cytochrome c per μg of protein obtained from tissue homogenates. Total protein was measured by Bradford's method using bovine serum albumin as protein standard[30].

Statistical analysis

The results were analyzed using SPSS 20.0 and expressed as means ± S.E.M. Levene's test of equality of variances was used in order to test the assumption of homogeneity of variance. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, when necessary. In all comparisons, *p* values below 0.05 were considered as indicative of statistical significance.

Results

Aiming to evaluate the effects of iron loading in the neonatal period on apoptosis, we first examined the effects of iron treatment on Caspase 3, which is a caspase of the final common pathway of apoptosis, activated both by extrinsic and intrinsic apoptotic pathways, and cleaved PARP, one of several known cellular substrates cleaved by Caspase 3.

Statistical comparisons using ANOVA revealed significant differences among the groups when protein levels of Caspase 3 were measured by western blot ($F_{(3, 16)} = 7.74$, $p < 0.01$, Fig. 1A). *Post hoc* comparisons between groups indicated that neonatal iron treatment significantly increased Caspase 3 in comparison to the control group ($p < 0.01$). CBD was able to completely reverse iron-induced effects on Caspase 3, since measures from the iron-CBD group were statistically different from those of the iron-vehicle group ($p < 0.01$), but not from the control group ($p= 0.995$). In order to confirm the activation of Caspase 3 we

quantified the ratio of cleaved PARP to total PARP. One way ANOVA revealed a significant difference among the groups ($F_{(3, 15)} = 22.96$, $p < 0.0001$, Fig. 1B). Neonatal iron treatment significantly increased cleaved PARP in comparison to the control group ($p < 0.0001$), while CBD treatment in adulthood reversed this effect. The iron-CBD group presented significantly lower cleaved PARP in relation to total PARP than the iron-vehicle group ($p < 0.0001$) and was not significantly different from the control group ($p = 0.949$).

We also analyzed the effects of neonatal iron loading and adult treatment with CBD on Caspases 8 and 9, initiator caspases to extrinsic and intrinsic apoptotic pathways, respectively. The ANOVA revealed no significant differences among the groups when protein levels of cleaved-Caspase 8 were measured by western blot ($F_{(3, 14)} = 0.44$, $p = 0.726$, Fig. 2A). However, statistical comparisons using one-way ANOVA revealed significant differences in Caspase 9 protein levels among the groups ($F_{(3, 12)} = 19.05$, $p < 0.0001$, Fig. 2B). *Post hoc* comparisons between groups indicated that neonatal iron treatment induced a significant increase in Caspase 9 protein levels in comparison to the control group, which received sorbitol in the neonatal period and vehicle in adulthood ($p < 0.0001$). The reversion effects of CBD were also observed, considering that the group that received iron in the neonatal period and CBD in adulthood (iron-CBD) showed statistically significant differences in Caspase 9 levels in comparison to the group that received iron in the neonatal period and vehicle in adult age ($p = 0.001$), and this group was not significantly different from the control group ($p = 0.281$).

In order to confirm the involvement of the intrinsic apoptotic pathway, we then decided to investigate the effects of excessive iron on Cytochrome c levels, a protein released when mitochondrial membrane permeability is altered, involved in apoptosis initiation. Statistical comparisons of Cytochrome c levels demonstrated a significant difference among the groups ($F_{(3, 12)} = 13.80$, $p < 0.0001$, Fig. 3). When groups were compared using Tukey's

post hoc test, results revealed that neonatal iron treatment induced a significant increase in Cytochrome c levels when compared to the control group (Sorb-Veh, $p < 0.05$). The iron-treated group that received CBD in adulthood had also significantly higher Cytochrome c levels when compared to the control group ($p = 0.001$), suggesting that CBD was not able to reverse the effects of iron loading on Cytochrome c protein levels.

We also aimed to investigate the long-term consequences of neonatal iron loading and adult treatment with CBD on APAF1 protein levels, an adapter protein responsible for the formation of apoptosome and activation of pro-caspases in the intrinsic pathway. A statistical comparison of APAF levels using one-way ANOVA showed significant differences among the groups ($F_{(3, 18)} = 39.9$, $p < 0.0001$, Fig. 4). *Post hoc* comparisons between groups indicated that neonatal iron treatment significantly increased APAF1 levels in comparison to the control group, which received sorbitol in the neonatal period and vehicle in adulthood ($p < 0.0001$). The iron-treated group that received CBD in adult age showed statistically significant differences in APAF1 when compared to the group that received vehicle in adult age ($p < 0.0001$) and no significant differences were observed in comparison to the control group ($p = 0.829$), indicating that CBD was able to completely reverse iron-induced effects on APAF1.

Discussion

The present results showed that neonatal iron-treatment led to significant changes in the concentration of apoptotic proteins, increasing all intrinsic apoptotic pathway proteins analyzed. Iron has the ability to exchange single electrons with many different substrates and, as a result of the participation of iron in Fenton chemistry, this metal can lead to the generation of reactive oxygen species (ROS)[31]. ROS triggers oxidative stress, inducing lipid peroxidation and DNA damage that can lead to impaired cell viability and initiation of signaling pathways crucial for cell survival and cell death[32].

Aiming to gain a better understanding of the mechanisms involved and trying to establish a possible causative role of iron overload in apoptosis, we have investigated key players in the apoptotic pathway in the hippocampus of adult rats submitted to iron overload in the neonatal period. Previous studies performed by our research group have shown that neonatal iron treatment induces lipid peroxidation and increases mitochondrial superoxide generation in the hippocampus, cortex, and substantia nigra[6, 33] and protein carbonylation in the substantia nigra[34]. In line with the present results, in previous studies we have also observed an increase in apoptotic markers, Caspase 3 and Par4 in the brains of iron-treated rats[12, 13]. Corroborating the present findings, You and colleagues[35] found that excess of iron in the substantia nigra increased oxidative stress levels, promoting apoptosis through the Bcl-2 / Bax pathway and the activated Caspase 3 pathway in an animal modelo f PD. In cultures of hippocampal slices exposed to iron, ROS formation and lipid peroxidation were increased, in association with Cytochrome c and Caspase 3-dependent apoptotic pathways[36]. Iron overload in the neonatal period induces severe hippocampus-dependent memory deficits, indicating hippocampal dysfunction[5, 6, 7, 8, 9, 11, 14], while studies performed in humans have correlated iron accumulation in selective brain regions with poor performance in cognitive tests (for a review, see [37]). Based on these findings, we suggest that iron-induced increased apoptosis might lead to functional deficits observed in our animal model and in patients, implicating iron in the pathogenesis of memory dysfunction associated to aging and neurodegenerative disorders.

The present findings show that iron-overload led to increases in APAF1, Caspase 3, Caspase 9, Cytochrome c, and cleaved PARP levels. Although we have not performed a direct measurement of apoptosis, increased cleaved PARP levels have been considered a marker of apoptosis because this protein is the substrate of activated caspases[38]. In agreement, upregulation of Caspase 3 gene expression in a model of cognitive impairment induced by

sevoflurane was associated with increased cleaved PARP levels[39]. While Caspase 3 is an effector caspase, being part of the final common pathway of apoptosis, Cytochrome c, APAF 1, and Caspase 9 integrate the intrinsic apoptotic pathway. Interestingly, no alterations in cleaved-Caspase 8 levels were found, confirming that there was no activation of extrinsic apoptosis pathways. On the other hand, we found increases in Caspase 9, Cytochrome c and APAF1 levels, suggesting that the intrinsic pathway is most significantly affected by iron overload. Since mitochondria are the main source of ROS, they are expected to become an important target of oxidative damage, which could explain functional alterations in these organelles in pathological conditions[40]. Moreover, mitochondria play a key role in regulating the intrinsic apoptotic pathway, and there is evidence indicating that iron affects mitochondrial homeostasis[13, 41], thus supporting the concept that iron effects are most pronounced in the intrinsic pathway. Nonetheless, more studies on the effects of iron on the extrinsic pathway are warranted.

Nowadays, many studies are being performed with CBD aiming to analyze its therapeutic properties and mechanisms of action. In this study, we showed the neuroprotective effects of the adult treatment with CBD on apoptotic markers in rats treated neonatally with iron. We observed that CBD was able to rescue APAF1, Caspase 9, Caspase 3, and cleaved PARP levels. Only Cytochrome c levels were not rescued to control levels. Notwithstanding, taken together the present findings suggest that CBD was able to protect from apoptosis by reducing Caspase 3 and cleaved PARP levels, proteins that participate in the effector phase of apoptosis, which culminates in cell death.

Although the mechanisms of action of CBD have not been completely elucidated, among the actions proposed for CBD is its antioxidant capacity[42]. In 2016, Chen and colleagues[43] found that CBD treatment was able to protect cells in cultures exposed to H_2O_2 to generate oxidative stress against apoptotic, inflammatory, and oxidative activities, suggesting

that CBD acts by modulating these pathways. Using a mouse model of ischemia, investigators found that CBD attenuated oxidative damage, increased antioxidant defenses, improved mitochondrial function and energetic metabolism, and regulated apoptotic markers in hippocampal neurons[44]. Previously, we studied the effects of CBD in rats submitted to iron overload and observed that CBD recovered mitochondrial dynamic and synaptic viability, besides reducing Caspase 3 in the hippocampus of adult rats[13]. Since we could observe the anti-oxidant, anti-apoptotic, and mitochondrial preservation properties related to neuroprotection, it is clear that no single mechanism will explain the remarkable pharmacological profile of CBD[45]. Therefore, the mechanism of action of CBD must include the modulation of several pathways that, together, improve cellular metabolism and confer neuroprotection, which may account for rescuing the functional deficits observed in this model[10].

In summary, we have shown that iron treatment in the neonatal period disrupts the apoptotic intrinsic pathway. This finding may place iron excess as a central component in neurodegenerative processes since many neurodegenerative disorders are accompanied by iron accumulation in brain regions. Moreover, indiscriminate iron supplementation to toddlers and infants, modeled here by iron overload in the neonatal period, has been considered a potential environmental risk factor for the development of neurodegenerative disorders later in life[46]. Our findings also strongly suggest that CBD has neuroprotective effects, at least in part by blocking iron-induced apoptosis even at later stages, following iron overload, which puts CBD as a potential therapeutic agent in the treatment of neurodegenerative diseases.

Disclosures

AWZ, JECH and JAC are co-inventors (Mechoulam R, JC, Guimarães FS, AZ, JH, Breuer A) of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.:

WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders". JAC and JECH received travel support from and are medical advisors of BSPG-Pharm.

All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

All authors have read and approved the final version of the manuscript.

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The funding sources had no role in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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Figure captions

Figure 1 – Iron treatment increases and CBD restores Caspase 3 and cleaved-PARP levels

(A) Western Blotting of Caspase 3 and (B) ratio of cleaved PARP to total PARP in the hippocampus of rats treated neonatally with sorbitol or iron and treated with vehicle or CBD chronically in the adulthood (3 months of age). Fifty µg of protein were separated on SDS-PAGE and probed with specific antibodies, normalized to Tubulin. Representative Western Blots for Caspase 3, cleaved PARP, total PARP and Tubulin are shown in the upper panel. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. N = 4 - 6 per group. * $p < 0.01$ differences between sorbitol-vehicle (Sorb-Veh) vs iron-vehicle (Fe-Veh); ** $p < 0.0001$ differences between Sorb-Veh vs Fe-Veh . # $p < 0.01$ difference between Fe-Veh vs iron-CBD (Fe-CBD); ## $p < 0.0001$ difference between iron-vehicle vs iron-CBD.

Figure 2 – Iron treatment increases and CBD restores Caspase 9 levels, without affecting cleaved-Caspase 8

(A) Western Blotting of cleaved-Caspase 8 and (B) Caspase 9 in the hippocampus of rats treated neonatally with sorbitol or iron and treated with vehicle or CBD chronically in the adulthood (3 months of age). Fifty µg of protein were separated on SDS-PAGE and probed with specific antibodies and normalized to Tubulin. Representative Western Blots for cleaved-Caspase 8, Caspase 9 and Tubulin are shown in the upper panel. Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. N = 4-5 per group. * $p < 0.0001$ indicates a significant increase in Caspase 9 protein expression in the iron-vehicle (Fe-Veh) group compared to controls. # $p \leq 0.001$

indicates a significant decrease in Caspase 9 protein expression in the iron-CBD (Fe-CBD) group compared to Fe-Veh.

Figure 3 – Iron treatment increases Cytochrome c levels

Cytochrome c protein expression by ELISA assay in the hippocampus of rats treated neonatally with sorbitol or iron and treated with vehicle or CBD chronically in the adulthood (3 months of age). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc. Data expressed as mean ± S.E.M. N = 4 per group. * $p < 0.05$ indicates a significant increase in Cytochrome c protein expression in the iron-vehicle (Fe-Veh) group compared to controls. ** $p \leq 0.001$ indicates a significant increase in Cytochrome c protein expression in the iron-CBD (Fe-CBD) group compared to controls.

Figure 4 – Iron treatment increases and CBD restores APAF1 levels

APAF1 protein expression by ELISA assay in the hippocampus of rats treated neonatally with sorbitol or iron and treated with vehicle or CBD chronically in the adulthood (3 months of age). Statistical analysis was performed using one-way ANOVA followed by Tukey HSD post-hoc test. Data expressed as mean ± S.E.M. N = 5-6 per group. * $p < 0.0001$ indicates a significant increase in APAF1 protein expression in the iron-vehicle (Fe-Veh) group compared to controls. # $p < 0.0001$ indicates a significant decrease in APAF1 protein expression in the iron-CBD (Fe-CBD) group compared to Fe-Veh.

Figure 1

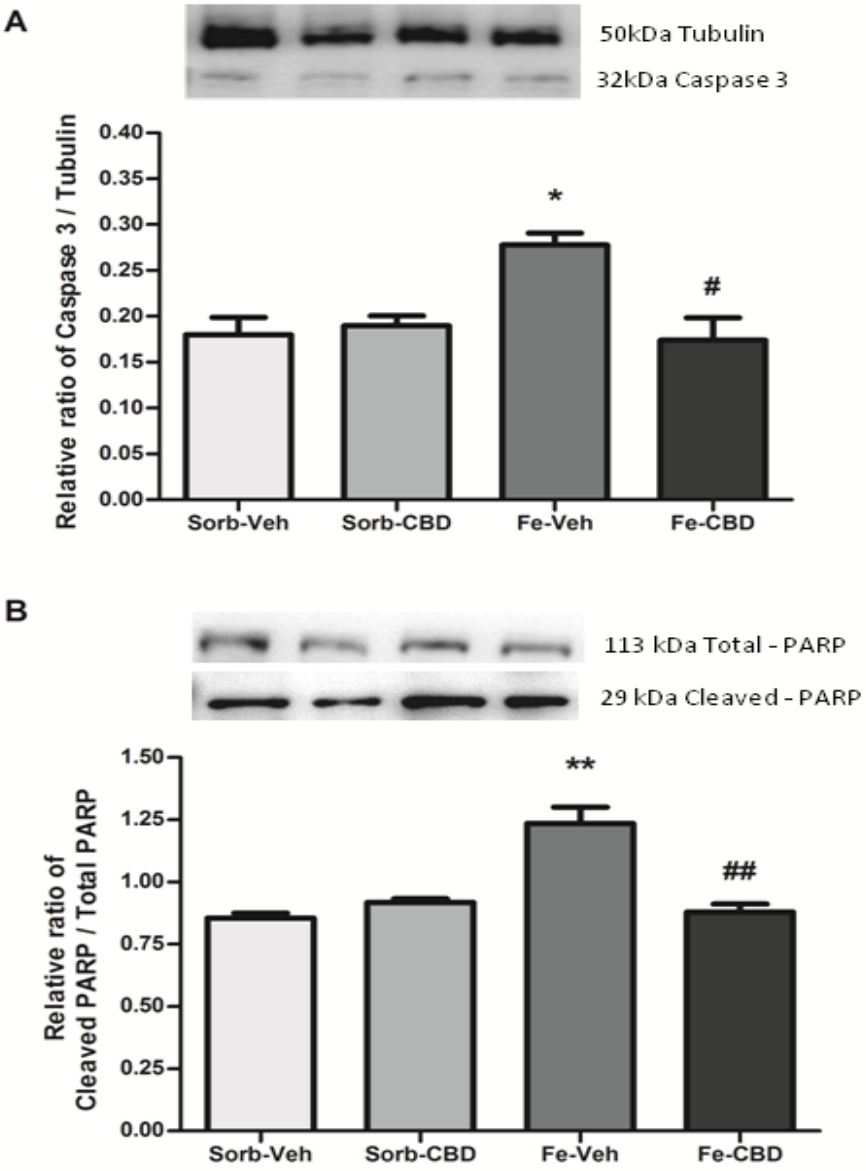

A
50kDa Tubulin
32kDa Caspase 3
Relative ratio of Caspase 3 / Tubulin
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
#
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD
B
113 kDa Total - PARP
29 kDa Cleaved - PARP
Relative ratio of Cleaved PARP / Total PARP
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
##
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD

Figure 2

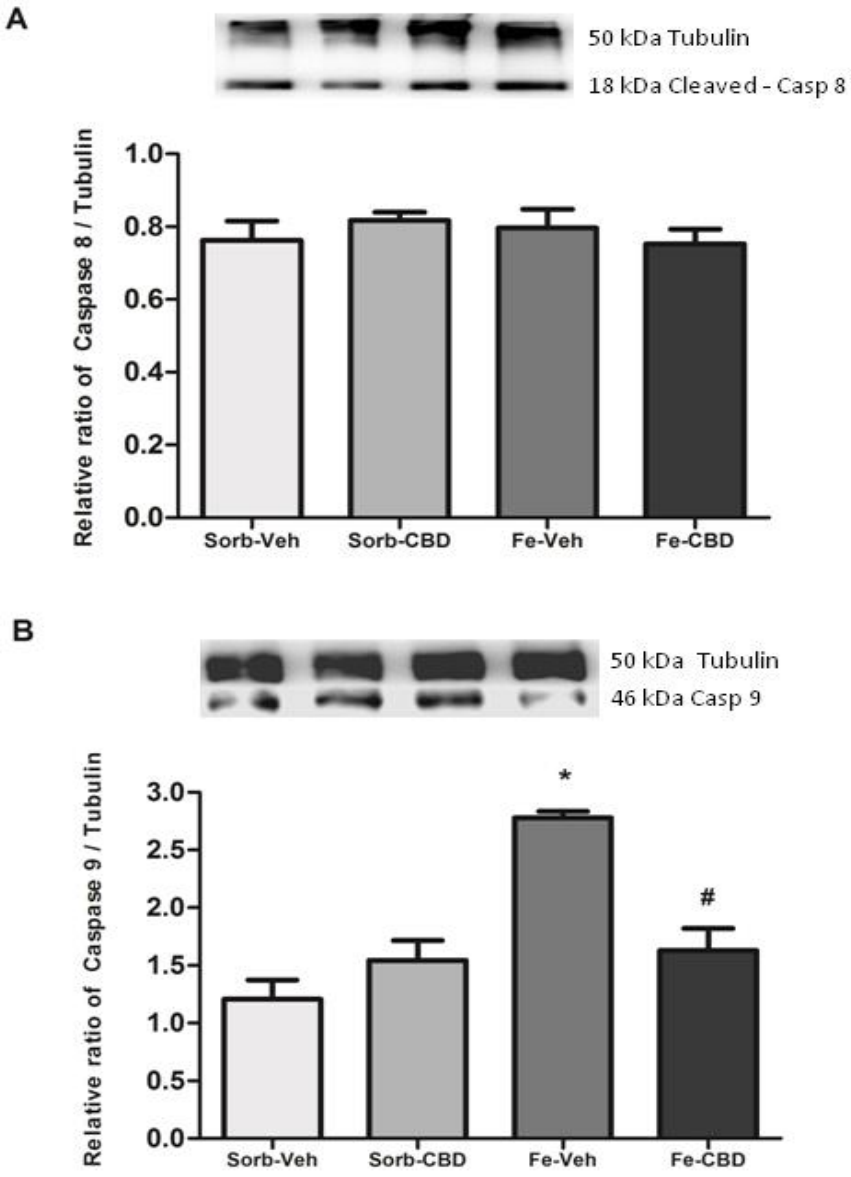

A
50 kDa Tubulin
18 kDa Cleaved - Casp 8
Relative ratio of Caspase 8 / Tubulin
1.0
0.8
0.6
0.4
0.2
0.0
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD
B
50 kDa Tubulin
46 kDa Casp 9
Relative ratio of Caspase 9 / Tubulin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD

Figure 3

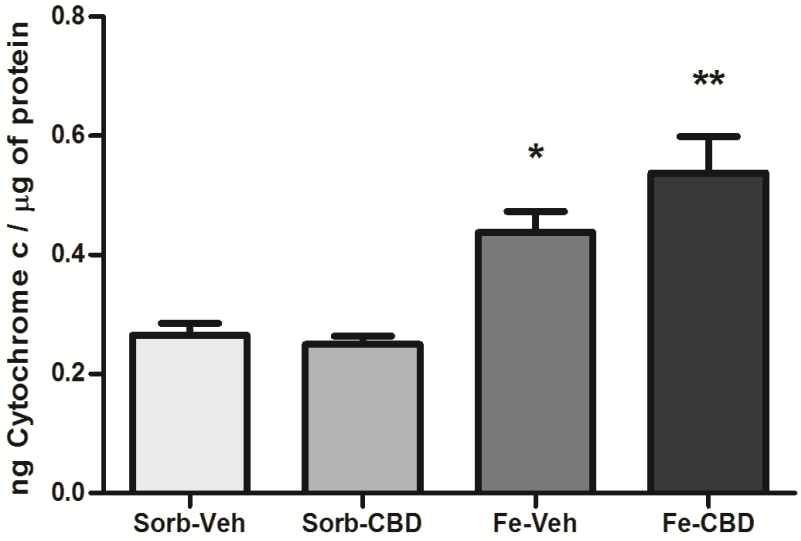
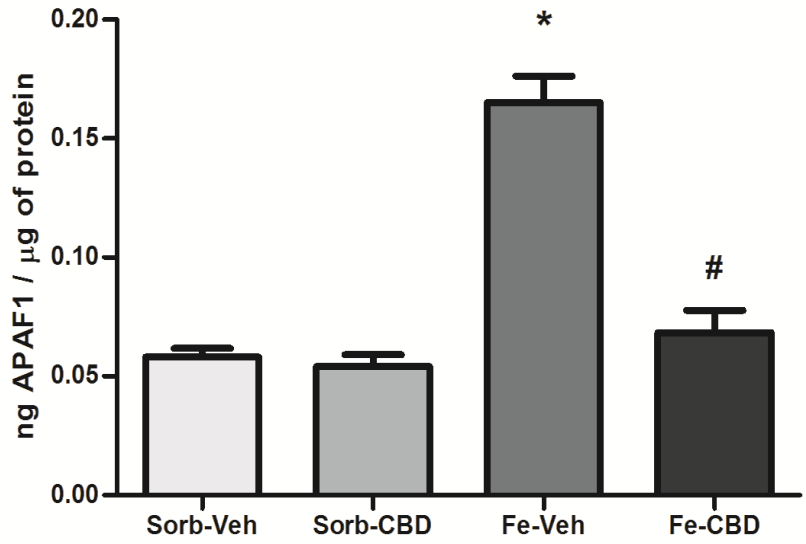

ng Cytochrome c / µg of protein
0.8
0.6
0.4
0.2
0.0
*
**
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD

Figure 4


0.20
0.15
0.10
0.05
0.00
ng APAF1 / µg of protein
*
#
Sorb-Veh
Sorb-CBD
Fe-Veh
Fe-CBD

CAPÍTULO 3

3.1 CONSIDERAÇÕES FINAIS

O ferro é um metal essencial para muitos processos biológicos como, por exemplo, para o transporte de oxigênio, síntese de DNA e geração de ATP (Bogdan *et al*., 2016). A homeostasia desse metal deve ser altamente regulada, pois, tanto a deficiência quanto o excesso de ferro podem ser nocivos ao organismo e estar associados a doenças neurológicas (Biasiotto *et al*., 2016). O excesso de ferro tem sido observado durante o envelhecimento cerebral normal e em doenças neurodegenerativas e, embora a etiologia dessas doenças não esteja completamente elucidada, acredita-se que o potencial oxidativo do ferro via reação de Fenton esteja relacionado à neurodegeneração (Ward *et al*., 2014). Apesar de ser aceita a relação do excesso de ferro nas doenças neurodegenerativas, os mecanismos de ação pelos quais esse metal causa seus efeitos deletérios ainda não estão completamente elucidados.

Nosso grupo de pesquisa tem analisado os efeitos da sobrecarga de ferro em ratos durante o período neonatal, tendo encontrado prejuízos na memória de reconhecimento e na consolidação de memória aversiva (Schröder *et al*., 2001; de Lima *et al*., 2005; 2007; Perez *et al*., 2010; Rech *et al*., 2010; Fagherazzi *et al*., 2012; Garcia *et al*., 2013). Em análises moleculares, já observamos que a sobrecarga de ferro induz aumento de proteínas apoptóticas (Miwa *et al*., 2011; da Silva *et al*., 2014), gliose reativa (Fernandez *et al*., 2011), ubiquitinação de proteínas (Figueiredo *et al*., 2016), alterações em marcadores de viabilidade sináptica e dinâmica mitocondrial (da Silva *et al*., 2014), que podem estar relacionados com o aumento de danos oxidativos ocasionados pelo ferro (de Lima *et al*., 2005).

Visando ampliar o conhecimento a respeito dos mecanismos de ação relacionados ao excesso de ferro cerebral e assim contribuir para um melhor entendimento do estresse oxidativo nas doenças neurodegenerativas, esse trabalho teve como objetivo analisar o acúmulo de ferro sobre mecanismos mitocondriais. Nosso olhar voltado às mitocôndrias deve-se ao fato dessas organelas serem de suma importância para o aporte energético do sistema nervoso, além do envolvimento dessa organela em manter a homeostasia do ferro celular e o equilíbrio de espécies reativas de oxigênio. Uma vez que o acometimento dessas patologias neurológicas vem aumentando progressivamente com o envelhecimento da população, analisamos o potencial terapêutico do CBD nesses ratos com sobrecarga de ferro, na expectativa de contribuir na busca de estratégias de prevenção e tratamento para essas doenças.

Inicialmente, analisamos as deleções do DNA mitocondrial do complexo I nos hipocampos de ratos tratados com ferro no período neonatal. Pudemos observar que o ferro provocou um aumento significativo nas deleções do DNA mitocondrial, sendo que o tratamento com CBD na idade adulta não foi capaz de recuperar esses danos causados. Também analisamos os mecanismos epigenéticos do DNA mitocondrial desse nosso modelo animal. Verificamos que o ferro ocasionou uma redução nos padrões de metilação e hidroximetilação e o CBD, apesar de não apresentar eficácia para reverter o conteúdo de 5mC para níveis similares ao grupo controle, foi capaz de reverter o conteúdo de 5hmC do DNA mitocondrial após tratamento com ferro. Embora estudos analisando o perfil do DNA mitocondrial em doenças neurodegenerativas sejam realizados, a sua contribuição no processo ainda não está muito bem compreendida (Pinto e Moraes, 2014). Quanto à epigenética mitocondrial, estudos ainda são primitivos tanto em sua fisiologia quanto em patologias (Manev *et al*., 2012). Porém, em um estudo pioneiro, Dzitoyeva e colaboradores (2012) demonstraram que o próprio envelhecimento diminui a hidroximetilação do DNA mitocondrial no córtex frontal de camundongos e neurônios primários de camundongos em cultura. Em nosso modelo de sobrecarga de ferro, pudemos observar alterações no genoma, que possivelmente estão relacionadas ao estresse oxidativo induzido por esse metal. Essas modificações no DNA mitocondrial podem afetar diretamente a cadeia respiratória, comprometendo o metabolismo dessas organelas e desencadeando funcionamento celular inadequado. A reversão do padrão de 5hmC após o tratamento com CBD nos leva a propor a epigenética como mecanismo de ação para esse composto.

A investigação do perfil gênico e proteico das proteínas Mitoferrina 2 e Ferritina mitocondrial foi realizada na tentativa de entender como a mitocôndria faz a manipulação do ferro no seu interior, uma vez que existe restrita informação a esse respeito em situações de envelhecimento e doença. Hipocampos de ratos com sobrecarga de ferro apresentaram redução nos níveis gênicos e proteicos da Ferritina mitocondrial, enquanto que o tratamento na idade adulta com CBD restaurou os níveis proteicos da Ferritina mitocondrial, apesar de em níveis gênicos não ter apresentado alterações. Já em relação à Mitoferrina 2, o tratamento com ferro no período neonatal, bem como o tratamento com CBD na idade adulta, não alterou a expressão dessa proteína tanto em nível gênico quanto em nível proteico. Observa-se que a Ferritina mitocondrial é expressa preferencialmente em tecidos com alta atividade metabólica e apresentaria papel na proteção das mitocôndrias contra danos oxidativos ocasionados por ferro livre ao invés de armazenamento de ferro (Levi e Arosio, 2004). É possível que a sobrecarga de ferro no período neonatal tenha induzido alterações precoces nos níveis dessa

proteína e a incapacidade de manter o aumento desses níveis ao longo da vida pode contribuir para danos mitocondriais induzidos pelo ferro. Os mecanismos de modulação da Ferritina mitocondrial permanecem desconhecidos, mas propomos que esteja relacionado a suas propriedades antioxidantes e neuroprotetoras. Existem mecanismos finamente regulados pela disponibilidade de ferro que controlam a expressão de proteínas de transporte de ferro celular, como o receptor de transferrina, por exemplo. Entretanto, os mecanismos que regulam a expressão de proteínas relacionadas ao transporte do ferro na mitocôndria não estão esclarecidos. Nossos resultados indicaram que a sobrecarga com ferro no período neonatal não alterou a expressão da Mitoferrina 2 no hipocampo dos ratos na idade adulta. Outros estudos são necessários para que os mecanismos que regulam a expressão desta proteína sejam melhor compreendidos.

Nossos estudos prévios observaram que o ferro ocasiona alterações em proteínas envolvidas na dinâmica mitocondrial em cérebros de ratos (da Silva *et al*., 2014). Somando-se a isso, nesse trabalho pudemos verificar as modificações promovidas pelo ferro no genoma mitocondrial. Logo, investigamos a atividade enzimática de enzimas envolvidas na produção energética celular para analisar se essas transformações mitocondriais culminariam em um prejuízo energético para a maquinaria celular. Não foram encontradas diferenças estatísticas na atividade da enzima Creatina quinase; no entanto, a atividade da Succinato desidrogenase mostrou-se reduzida após tratamento neonatal com ferro. Apesar de não apresentar diferenças estatísticas com o grupo ferro, o tratamento com CBD também não apresentou diferenças com o grupo controle, sugerindo uma reversão parcial na atividade da enzima após administração desse composto. O efeito negativo do ferro na Succinato desidrogenase possivelmente se dá pela sua capacidade oxidativa, podendo ser comprovado, pelo menos em parte, o mecanismo antioxidante do CBD. O comprometimento de enzimas responsáveis pela bioenergética celular implica em mais disfunções celulares, que, em último caso, irão desencadear mecanismos de morte celular (Grimm e Eckert, 2017).

Marcadores de morte celular foram analisados para verificarmos se as alterações mitocondriais induzidas pelo ferro poderiam culminar em morte celular no sistema nervoso, característica clássica das patologias neurodegenerativas. Visando constatar que mecanismos de morte celular via apoptose haviam sido ativados, verificamos a expressão das proteínas Caspase 3 e PARP. Encontramos níveis aumentados de Caspase 3 em ratos tratados com ferro no período neonatal. Como analisamos a Caspase 3 total, e não somente a clivada, buscamos observar a razão entre PARP clivada e PARP total, uma vez que esta proteína é um dos principais substratos da Caspase 3, verificando que o tratamento com ferro também aumenta

os níveis dessa proteína clivada, sendo indicativo de ativação da via apoptótica. Análises seguintes objetivaram verificar qual via apoptótica estaria sendo ativada: a extrínseca, pela ativação da Caspase 8, ou a intrínseca, pela ativação da Caspase 9. Não foram observados resultados significativos nos níveis da Caspase 8 clivada, enquanto que a Caspase 9 apresentou aumento em sua expressão em ratos tratados com ferro. Duas outras proteínas da via intrínseca, Citocromo c e APAF1, também foram investigadas. Verificou-se que o tratamento com ferro induziu a elevação das expressões de ambas as proteínas. Nossos estudos prévios já nos mostravam indicativo de ativação de vias apoptóticas induzida pelo ferro, ao verificarmos expressão de Caspase 3 elevada (da Silva *et al*., 2014). Apesar de o tratamento com CBD na idade adulta não ter revertido os níveis elevados do Citocromo c, todos os outros parâmetros apresentaram reversão da expressão para níveis similares ao controle, estabelecendo um possível mecanismo anti-apoptótico para o CBD.

Em conclusão, esse trabalho permitiu maiores esclarecimentos acerca dos mecanismos utilizados pelo ferro para a indução de uma gama de efeitos deletérios, como por exemplo, déficits de memória e morte celular. Uma sobrecarga de ferro no período neonatal, momento em que o cérebro está mais suscetível e passa por etapas fundamentais de desenvolvimento, permite uma série de modificações celulares que, em estágios posteriores, como no caso do envelhecimento, poderão desencadear situações patológicas. As mitocôndrias são organelas extremamente dependentes desse metal para o desenvolvimento de suas funções e esse trabalho nos permitiu observar que elas se tornam um dos principais alvos de ataque quando ocorre perda da homeostasia do ferro celular. A compreensão do funcionamento de todos os mecanismos envolvidos com essas organelas, tanto em níveis fisiológicos quanto em níveis patológicos, é essencial para o entendimento de condições relacionadas ao envelhecimento e doenças, além de permitir estratégias mais efetivas de prevenção e tratamento. O CBD, atualmente, é um foco de estudo para as doenças relacionadas ao sistema nervoso. Apesar de vários potenciais terapêuticos propostos para esse composto, ainda não se tem a compreensão completa dos mecanismos de ação pelos quais ele age no organismo. Temos estudado o CBD há alguns anos na expectativa de um melhor entendimento dessa droga. Nossos resultados nesse trabalho sugerem que o CBD pode exercer seu papel neuroprotetor a partir de mecanismos antioxidantes, anti-apoptóticos e talvez até epigenéticos. Como muitos tópicos abordados nesse trabalho ainda possuem estudo primitivo, necessitamos que mais abordagens sejam realizadas para possuirmos maior entendimento de toda a mecânica que envolve a sobrecarga de ferro e o uso terapêutico do CBD.

3.2 CONCLUSÕES

- Hipocampos de ratos tratados com ferro no período neonatal apresentaram redução nas expressões gênica e proteica da Ferritina mitocondrial, proteína responsável pelo armazenamento de ferro nas mitocôndrias. Já as expressões gênica e proteica da Mitoferrina 2, proteína responsável pelo transporte de ferro para dentro das mitocôndrias, não sofreram modificações. Logo, observou-se que uma sobrecarga de ferro ocasiona alterações no metabolismo do ferro mitocondrial.

- O tratamento crônico com CBD na idade adulta foi capaz de reverter a expressão proteica de Ferritina mitocondrial em hipocampos de ratos tratados com ferro neonatal, embora não tenha apresentado mesma capacidade de reversão a nível gênico. A Mitoferrina 2 não apresentou modificações nos hipocampos após tratamento crônico com CBD. Verificou-se, então, que o CBD tem ação sobre o metabolismo do ferro mitocondrial.

- As deleções no DNA mitocondrial do Complexo I sofreram aumento em hipocampos de ratos tratados com ferro no período neonatal, mostrando a capacidade desse metal em ocasionar danos mitocondriais a nível gênico.

- A administração crônica de CBD na idade adulta não foi capaz de reverter o aumento das deleções no DNA mitocondrial do Complexo I em hipocampo de ratos tratados com ferro no período neonatal.

- Hipocampos de ratos tratados com ferro neonatal apresentaram redução nos padrões de metilação e hidroximetilação do DNA mitocondrial, sugerindo possível efeito do ferro na epigenética mitocondrial.

- CBD crônico na idade adulta mostrou capacidade de reversão dos efeitos causados pelo ferro sobre a hidroximetilação mitocondrial em hipocampos de ratos, embora não tenha sido eficaz para reverter os padrões de metilação. Mostrou-se aqui uma potencial ação do CBD sobre mecanismos epigenéticos mitocondriais.

- Administração neonatal de ferro acarretou redução da atividade enzimática da Succinato desidrogenase em hipocampos de ratos, apesar de nenhum efeito ter sido observado na atividade enzimática da Creatina quinase. Logo, o ferro mostrou ter impacto sobre a bioenergética celular.

- Tratamento crônico com CBD na idade adulta apresentou capacidade de reversão parcial da atividade enzimática da Succinato desidrogenase em hipocampos de ratos após o tratamento neonatal com ferro, enquanto nenhum efeito foi verificado na atividade enzimática da Creatina quinase. Aqui, o CBD revelou-se apto em recuperar a bioenergética celular, mesmo que de forma parcial.

- Ferro administrado neonatalmente ocasionou aumento da expressão proteica das proteínas apoptóticas Citocromo c, APAF1, Caspase 3, Caspase 9 e PARP clivada nos hipocampos de ratos, enquanto a expressão proteica da Caspase 8 clivada não foi alterada. Percebeu- se aqui a ação do ferro sobre proteínas apoptóticas envolvidas na via intrínseca e na via efetora.

- CBD administrado cronicamente na idade adulta reverteu a expressão proteica das proteínas apoptóticas Caspase 3, Caspase 9, APAF1 e PARP em hipocampos de ratos neonatalmente tratados com ferro.Verificou-se o efeito anti-apoptótico do CBD.

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ANEXO A – Comprovante de aprovação do projeto pela Comissão de Ética para o Uso de Animais (CEUA)

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 64/2014 - CEUA

Porto Alegre, 01 de setembro de 2014.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 14/00409, intitulado **"Efeitos do Δ9-Tetra-Hidrocanabinol e do Canabidiol sobre parâmetros mitocondriais e sinápticos em ratos tratados com ferro no período neonatal".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
90	Rattus norvegicus	11/2014 – 07/2015

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profa. Dra. Nadja Schröder
FABIO
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6681 – P. 99 – Portal Tecnopuc – sala 1512
CEP: 90619-900 – Porto Alegre/RS
Fone: (51) 3353-6365
E-mail: ceua@pucrs.br