UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

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PAPEL DA MITOCÔNDRIA E DAS INTERAÇÕES ENTRE MITOCÔNDRIA E RETICULO ENDOPLASMÁTICO NA DOENÇA DE HUNTINGTON: ESTUDO EM UM MODELO ANIMAL

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

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Porto Alegre, RS

2021

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AGRADECIMENTOS

À Dra Bianca, minha incrível orientadora, finalizo o mestrado agradecendo a grande sorte de ter uma orientadora tão atenciosa, que soube sempre respeitar o meu tempo, com a maior paciência sentou comigo na bancada e me explicou cada detalhe dos protocolos, esteve sempre presente e disposta a encarar todo esse trabalho em meio a uma pandemia. Muito obrigada!

SUMÁRIO

PARTE I

RESUMO

A doença de Huntington (DH) é uma doença neurodegenerativa herdada de forma dominante e causada por uma expansão de repetição de bases de nucleotídeos CAG no gene *huntingtin,* resultando na síntese de uma proteína huntingtina mutante (mHTT). A tríade sintomatológica da DH inclui disfunções motoras, cognitivas e psiquiátricas. A primeira região cerebral a apresentar anormalidades é o estriado, sendo relatada uma degeneração de neurônios GABAérgicos em estágio avançando da doença. Outros achados, como perda de função de células gliais, bem como uma ativação de astrócitos, contribuem para um fenótipo neurotóxico na DH. Além disso, vários eventos celulares e moleculares desempenham um papel importante na patologia da DH. Em destaque estão as possíveis alterações mitocondriais, uma vez que a mitocôndria é uma organela essencial para diversos processos celulares, como a produção de ATP, metabolismo do cálcio e homeostase redox. Neste contexto, já é bem estabelecido que doenças neurodegenerativas apresentam disfunção mitocondrial, podendo alterar os processos envolvidos na dinâmica mitocondrial e nas interações dessa organela com outros constituintes celulares, em particular o retículo endoplasmático (RE). Assim, no presente estudo um modelo experimental animal para a DH induzido pela administração intraperitoneal do ácido 3-nitropropiônico (3-NP, 20 mg/kg) durante 3 dias consecutivos, investigamos parâmetros chave relacionados à mitocôndria no período de 7, 14, 21 e 28 dias após indução. Nossos resultados mostraram alterações na homeostase redox em estriado, com aumento da peroxidação lipídica 28 dias após a administração de 3-NP, e diminuição de defesas antioxidantes enzimáticas e não-enzimáticas em diferentes tempos. Também observamos disfunção do metabolismo bioenergético, com redução do estado 3, estado 4 e não-acoplado em 7 dias após a administração de 3-NP, aumento dos estados 4 e não acoplado e capacidade de reserva em 28 dias, e diminuição da atividade dos complexos da cadeia respiratória em 7 e 28 dias. Observamos alterações na dinâmica mitocondrial através do aumento da expressão proteica da mitofusina 1 (MFN1) em 7, 14 e 28 dias, uma importante proteína para a fusão mitocondrial, sugerindo uma resposta compensatória ao insulto oxidativo. Além disso, nossos resultados sugerem que em 28 dias após a administração 3-NP ocorreram alterações na interação mitocôndria-RE, com o aumento do conteúdo proteico de canal aniônico voltagem dependente 1 (VDAC1) e da proteína reguladora de glicose 75 kDa (GRP75). Também houve um aumento inicial observado no conteúdo da proteína sinaptofisina, seguido de diminuição, sugerindo uma resposta transitória da atividade sináptica, buscando a manutenção da neurotransmissão normal. Nossos achados mostram que um prejuízo grave na função mitocondrial, com alteração na dinâmica mitocondrial, homeostase do cálcio e redox, bem como um dano na interação mitocôndria-RE os quais estão envolvidos na fisiopatologia do dano estriatal observado na DH.

Palavras-chave: Ácido 3-nitropropiônico, déficits metabólicos, disfunção mitocondrial, doença neurodegenerativa, estriado.

ABSTRACT

Huntington's disease (HD) is a dominantly inherited neurodegenerative condition caused by a CAG nucleotide base repeat expansion in the *huntingtin* gene, resulting in the production of a mutant huntingtin protein (mHTT). The symptomatologic triad of HD confers motor, cognitive and psychiatric dysfunctions. In the initial stage, patients present with abnormalities in the striatum caused by mHTT, whereas degeneration of GABAergic neurons is observed in an advanced stage of the disease. Other findings, such as glial cells dysfunctions, particularly reactive astrocytes, contribute to a neurotoxic phenotype in HD. On the other hand, several cellular and molecular events have been shown to play a role in HD pathology. In this scenario, mitochondrial dysfunction has a key role since mitochondria are essential organelles for various cellular processes, such as ATP production, calcium metabolism and redox homeostasis. Furthermore, it is suggested that disrupted mitochondrial dynamics and alterations in the interactions of mitochondria with other cellular constituents, in particular the endoplasmic reticulum (ER), are common findings in neurodegenerative diseases. Thus, in the present study an experimental animal model for HD induced by intraperitoneal administration of 3-nitropropionic acid (3-NP, 20 mg/kg) for 3 consecutive days, we investigated key parameters related to mitochondria in the period of 7, 14, 21 and 28 days after induction. Our results showed changes in redox homeostasis in striatum, with an increase in lipid peroxidation 28 days after 3-NP treatment, and a decrease in defenses enzymatic and non-enzymatic antioxidant at different periods after 3-NP exposure. We also observed bioenergetic dysfunction, with reduced state 3, state 4 and non-coupled at 7 days, augmented state 4 and non-coupled state and reserve capacity at 28 days, and decreased activity of the respiratory chain complexes at 7 and 28 days. We observed changes in mitochondrial dynamics through increased protein expression of mitofusin 1 (MFN1) at 7, 14, and 28 days, an important protein for mitochondrial fusion, suggesting a compensatory response to 3-NP induced oxidative insult. Furthermore, our results demonstrated that, at 28 days after 3-NP administration, alterations in mitochondria-ER interactions occurred, with increased protein content of voltage-dependent anion channel 1 (VDAC1) and 75 kDa glucose regulated protein (GRP75). Additionally, an initial increase of synaptophysin protein content followed by a reduction of this content was seen, indicating a transient response aiming the maintenance of a normal neurotransmission. Our findings show that impaired mitochondrial function, with changes in mitochondrial dynamics, calcuim and redox homeostasis, as well as disturbances in mitochondria-endoplasmic reticulum interactions are involved in the pathophysiology of striatal damage seen in DH.

Keywords: 3-Nitropropionic acid, metabolic deficits, neurodegenerative disease, striatum, mitochondrial dysfunction.

LISTA DE ABREVIATURAS

Ácido 3-nitropropiônico	3-NP
Ácido desoxirribonucleico	DNA
Ácido gama-aminobutírico	GABA
Canal aniônico voltagem dependente 1	VDAC1
Catalase	CAT
Citosina, adenina e guanina	CAG
Dinamina-2	DMN2
DNA Mitocondrial	mtDNA
Doença de Huntington	DH
Espécies reativas de nitrogênio	ERNs
Espécies reativas de oxigênio	EROs
Fator nuclear kappa B	NF-κB
Fosforilação oxidativa	OXPHOS
Glutationa peroxidase	GPx
Glutationa redutase	GR
Glutationa reduzida	GSH
Glutationa S-transferase	GST
Huntingtin	*HTT*
Membranas associadas às mitocôndrias	MAMs
Mitofusina 1	MFN1
Mitofusina 2	MFN2
Nicotinamida adenina dinucleotídeo	NADH
Óxido nítrico	NO
Peroxidação lipídica	LPO
Proteína atrofia óptica 1	OPA1
Proteína huntingtina mutante	mHTT
Proteína quinase ativada por mitogênio	MAPK
Proteína reguladora de glicose 75 kDa	GRP75
Proteína reguladora de glicose 78 kDa	GRP78

Proteína relacionada à dinamina 1	DRP1
Receptor de inositol 1,4,5-trifosfato	IP3R
Retículo endoplasmático	RE
Succinato desidrogenase	SDH
Superóxido dismutase	SOD
Trifosfato de adenosina	ATP

1. INTRODUÇÃO

1.1. Doença de Huntington

A doença de Huntington (DH) é uma doença genética de caráter autossômico dominante, causada por uma repetição dos três nucleotídeos citosina, adenina e guanina (CAG), no gene *huntingtin* (*HTT*) localizado no cromossomo 4, levando à produção de uma proteína huntingtina mutante (mHTT) (McColgan e Tabrizi, 2018). A DH tem uma prevalência que varia de 10,6 a 13,7 indivíduos por 100.000 nas populações ocidentais, ao passo que na população oriental tem uma incidência muito menor, de 1 a 7 por milhão. Essa discrepância entre prevalências está relacionada com as diferenças genéticas no gene HTT, onde as repetições de CAG mais longas estão presentes em populações com alta incidência (Bates *et al.*, 2015; Morrison, Harding-Lester e Bradley, 2011) .

A progressão da DH ocorre na maioria dos casos na faixa etária da meia idade, ou seja, cerca de 40 anos. Contudo, em aproximadamente 5% dos relatos de casos, o surgimento dos primeiros sinais e sintomas em pacientes ocorre na infância ou juventude, sendo caracterizada uma DH juvenil (Quarrell *et al.*, 2013). Relatos também mostram que pode haver início tardio da doença, por volta dos 70-80 anos de vida, cujo desenvolvimento é considerado insidioso (Snowden, 2017).

A DH é uma doença degenerativa cerebral, resultando no acúmulo da proteína mHTT, que é neurotóxica e leva à morte neuronal massiva (Snowden, 2017). A região cerebral mais vulnerável é o estriado, pois apresenta proeminente perda celular e atrofia nas regiões do caudado e putâmen. Ao longo do curso da doença a atrofia cerebral atinge outras regiões, resultando em uma perda de conectividade e função entre o estriado e as outras partes do cérebro (Snowden, 2017). A tríade de sintomas característicos da doença são disfunções motoras, cognitivas e psiquiátricas (Goh *et al.*, 2018).

A mHTT é encontrada em diversas células do sistema nervoso dos pacientes diagnosticados com DH, e a alteração neuronal já descrita com relação à toxicidade da mHTT é a degeneração de neurônios GABAérgicos no estriado em estágio avançado da doença (Vonsattel e DiFiglia, 1998). Além disso, exames neuropatológicos em tecidos de pacientes com DH demonstrou astrogliose, com um aumento significativo no número de astrócitos reativos (Gray, 2019; Vonsattel *et al.*, 1985).

Os pacientes com DH apresentam distúrbios motores, com movimentos involuntários, distonia, coreia, rigidez e distúrbios orais. Os transtornos cognitivos observados incluem

disfunção executiva e apatia, que podem ocasionar problemas comportamentais e psiquiátricos, tais como, depressão, ansiedade, psicose, irritabilidade e agitação. Outras complicações, mais relacionadas com a progressão tardia da DH, também são relatadas, como distúrbios do sono, perda de peso e problemas de deglutição, podendo resultar em engasgo (Loi *et al.*, 2018; Papoutsi *et al.*, 2014).

A diferença no fenótipo clínico da doença pode estar relacionada com a expansão da repetição de CAG, como foi observado em um estudo na DH juvenil, em que indivíduos com uma alta ou baixa expansão demonstraram diferenças na progressão da doença. Os pacientes com alta taxa de expansão apresentam sintomas mais críticos, como um comprometimento graveda marcha, atraso na fala e convulsões. Por outro lado, um aumento no comportamento obsessivo ocorreu de forma mais significativa em pacientes com baixa taxa de expansão (Fusilli *et al.*, 2018).

Achados de ressonância magnética demonstraram que a região do estriado é a primeira a apresentar perda de volume total, comparando pacientes com DH e indivíduos que não apresentavam a doença. Foi possível observar um desgaste extenso em toda a região cortical, com preservação relativa da parte frontal anterior e regiões temporais laterais (Tabrizi *et al.*, 2009). Quando correlacionadas as mudanças estruturais com as avaliações clínicas, o hipocampo não pareceu apresentar uma relação significativa com as manifestações clínicas ou com a carga genética (idade x [comprimento de repetição e CAG-35,5]) presente na DH (Coppen *et al.*, 2016).

A proteína huntingtina (HTT) está associada com diversas organelas, incluindo núcleo, retículo endoplasmático (RE), complexo de Golgi e mitocôndria (Zuccato, Valenza e Cattaneo, 2010). Além disso, a HTT apresenta interação com outras proteínas, como o canal aniônico voltagem dependente 1 (VDAC1), localizado na membrana externa da mitocôndria, podendo ser uma região física de contato entre mitocôndria e HTT. O receptor de inositol-1,4,5-trifosfato (IP3R), localizado na membrana do RE, também mostrou interagir com a HTT (Giacomello, Hudec e Lopreiato, 2011).

Apesar da descoberta da mutação genética da DH ter ocorrido há mais de 20 anos, o tratamento ainda é focado no manejo sintomático. Os sintomas motores, como a coreia, caracterizada por movimentos involuntários arrítmicos, rápidos e abruptos, responde a medicamentos que reduzem a neurotransmissão dopaminérgica (Wyant, Ridder e Dayalu, 2017). Para a sintomatologia psíquica, inibidores seletivos da recaptação da serotonina e inibidores da recaptação da serotonina-norepinefrina são considerados opções de tratamento de

primeira linha (Stahl e Feigin, 2020).

Observações em pacientes demonstraram que a disfunção neuronal ocorre antes do desenvolvimento de sinais motores, que são utilizados para o diagnóstico clínico na DH. Dessa forma, a sintomatologia clínica apresentada é secundária à disfunção ou perda neuronal progressiva (Tabrizi *et al.*, 2009). Tal observação pode colaborar para o direcionamento futuro de intervenções terapêuticas. Contudo, até o presente momento não há a elucidação do mecanismo exato subjacente à morte dos neurônios do estriado, bem como ainda não há uma terapia eficaz para prevenir a progressão da DH (Yang *et al.*, 2020).

1.2. Modelo animal induzido com ácido 3-nitropropiônico

O ácido 3-nitropropiônico (3-NP) é uma toxina produzida pelo metabolismo do 3-nitropropanol em várias espécies de fungos, como *Apergillus flavus*, Astragalus, Arthirinium e a partir de plantas indigofera. É importante ressaltar que o 3-NP possui a capacidade de permear a barreira hematoencefálica (Danduga *et al.*, 2018). Essa toxina tem sido amplamente estudada como modelo de indução experimental para a DH em animais de laboratório, pois leva ao aparecimento de sintomas semelhantes encontrados em pacientes diagnosticados com a doença. Isso se deve principalmente ao aparecimento de lesões bilaterais na região do estriado no cérebro induzidas pelo 3-NP (Chakraborty *et al.*, 2014), além da perda neuronal na região cortical, disfunção motora e cognitiva, dano oxidativo, liberação de citocinas pró-inflamatórias, alterações na neurotransmissão, depleção de ATP e apoptose neuronal (Liu *et al.*, 2018).

Uma hipótese para a seletividade do 3-NP em determinadas regiões cerebrais está relacionada com áreas mais sensíveis ou com elevada expressão de receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) (Borlongan, 1997), pois a administração de 3-NP resulta em uma despolarização da membrana com ativação desses receptores (Centonze *et al.*, 2006). Neste contexto, estudos *in vivo* e *in vitro* demonstraram que a administração de antagonistas de receptores NMDA atenuam os efeitos causados pela indução do 3-NP (Domenici *et al.*, 2018).

Já foi relatado que o 3-NP causa disfunção mitocondrial por diferentes vias moleculares, levando à geração de espécies reativas de oxigênio (EROs) e consequente dano oxidativo. Estudos demonstraram que a indução de DH por 3-NP leva ao desequilíbrio entre oxidantes e antioxidantes enzimáticos e não enzimáticos, com níveis elevados de peroxidação lipídica (LPO) e de óxido nítrico (NO) no estriado, acompanhado de diminuição no conteúdo de

glutationa reduzida (GSH) e inibição da atividade das enzimas catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx) e glutationa redutase (GR) (Abdelfattah *et al.*, 2020).

Déficits locomotores e comportamentais são características em modelos de DH, indicando degeneração estriatal. Já foi evidenciada em modelos da DH em roedores com indução por 3-NP uma supressão na atividade locomotora central e periférica (Sayed *et al.*, 2019). Através da inibição da atividade da enzima succinato desidrogenase (SDH) do complexo II, o 3-NP prejudica o metabolismo energético, que também pode causar degeneração dos neurônios estriatais (Jamwal e Kumar, 2016), Outros trabalhos ainda demonstraram que o 3-NP induz a ativação de vias de sinalização relacionadas a proteínas MAPK e ao fator nuclear de transcrição kappa B (NF-κB) na microglia (Yang *et al.*, 2020).

1.3. Função mitocondrial

Mitocôndrias são organelas responsáveis pela coordenação da homeostase bioenergética e celular, controlando a produção de energia na forma de ATP, principalmente através da fosforilação oxidativa (OXPHOS). As mitocôndrias também têm papel fundamental na homeostase intracelular do cálcio (Ca^{2+}), juntamente com outras organelas, como o RE, e participam de diferentes vias de sinalização de apoptose, bem como na β-oxidação de ácidos graxos, na esteroidogênese, no metabolismo de aminoácidos, na biossíntese do heme, na gliconeogênese e cetogênese (Formosa e Ryan, 2018; Tomar e Elrod, 2020).

Os neurônios apresentam uma alta demanda energética, consumindo a maior parte do ATP gerado para manter a atividade neural, como a neurotransmissão e a plasticidade sináptica. Desta forma, a disfunção mitocondrial é um processo comumente relacionado a vários distúrbios neurodegenerativos, como a DH a partir da mHTT e também através da indução experimental em modelo animal pelo 3-NP (Figura 1). Além disso, as mitocôndrias participam da defesa neuronal, sinalização *downstream* e morte neuronal, destacando a sua importância nos neurônios e tecidos cerebrais (Farshbaf; Ghaedi, 2017; Bravo- Sagua *et al*., 2017; Carmo *et al*., 2018).
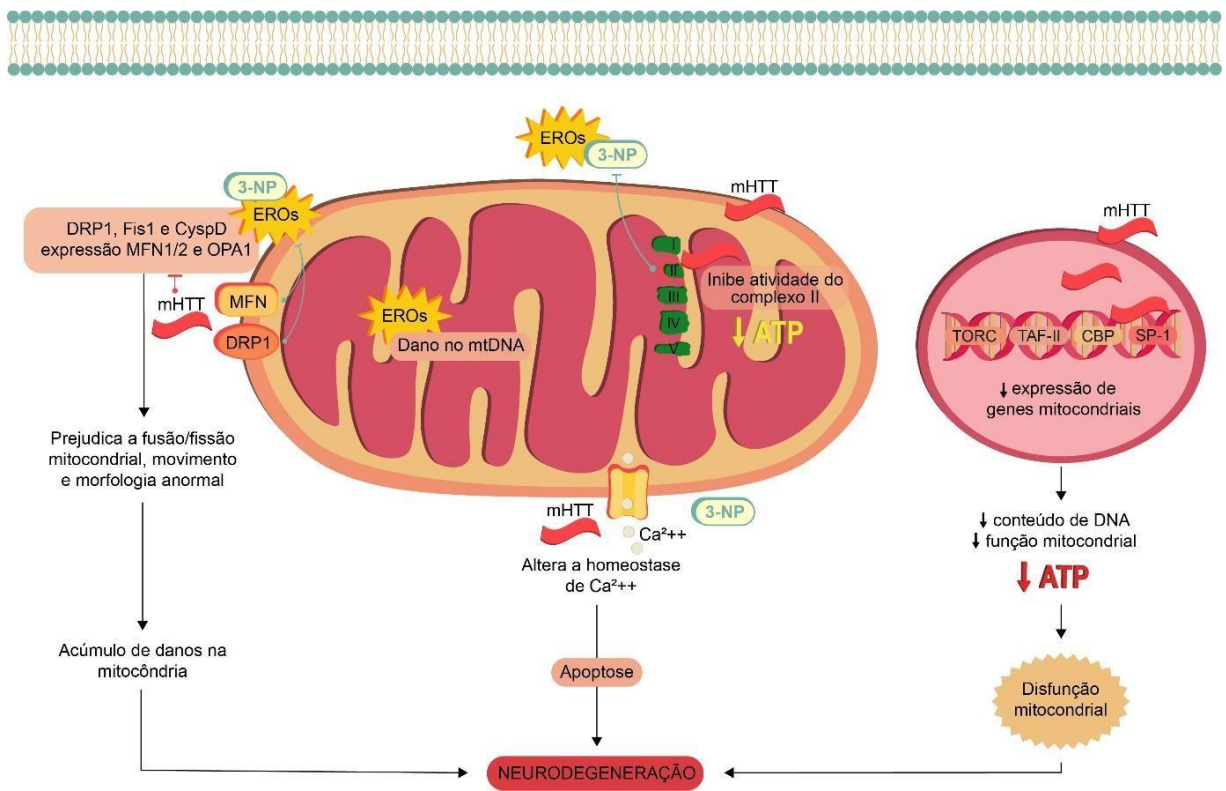
Figura 1- Disfunção mitocondrial causada pela proteína huntingtina mutante (mHTT) e pelo ácido 3- nitropropiônico (3-NP). Adaptado de Chaturvedi e Beal, 2013.

O crescente interesse na investigação sobre mitocôndrias como alvo terapêutico para um amplo espectro de doenças humanas apresenta um grande número de estudos pré-clínicos, demonstrando os benefícios de vários agentes farmacológicos com relação a diferentes aspectos funcionais da mitocôndria (Webb, Sideris e Biddle, 2019). No entanto, ainda não há aplicação clínica de um composto direcionado para as mitocôndrias aprovado pelos órgãos de regulamentação. Um dos motivos para essa dificuldade de aplicação clínica é resultante do escasso conhecimento sobre os mecanismos precisos de como esses compostos alteram as funções mitocondriais, sendo na regulação de funções fisiológicas básicas, bem como na fisiopatologia de doenças. A interação dessa organela com outros compartimentos celulares, como o RE, núcleo e o lisossomo também precisa ser melhor elucidada (Javadov; Kozlov; Camara, 2020).

1.3.1. Homeostase redox

Em condições fisiológicas, EROs e espécies reativas de nitrogênio (ERNs) são

produzidas como resultado do metabolismo, tendo papéis essenciais em diversos processos celulares, como na divisão celular, sinalização, homeostase e autofagia. A homeostase redox tem por função tanto a produção, quanto eliminação dessas espécies, coordenando assim o bom funcionamento celular (Fão e Rego, 2021).

As mitocôndrias são consideradas as principais fontes de produção de EROs, principalmente durante a produção de ATP, onde uma pequena parte dos elétrons que escapam da cadeia respiratória interagem com moléculas de oxigênio produzindo radicais livres. Uma dessas espécies reativas é o superóxido ($O_2^{\bullet-}$), que pode reagir com outras biomoléculas além de formar peróxido de hidrogênio, hidroperoxil, entre outros (Kumar e Ratan, 2016). Um desequilíbrio redox envolve a desregulação de várias defesas antioxidantes, como enzimas e proteínas, paralelamente ao aumento na produção de radicais livres (Sbodio, Snyder e Paul, 2019).

Marcadores de dano oxidativo, como oxidação de proteínas, peroxidação lipídica e danos ao DNA já foram relatados em células e tecidos de modelos de DH, bem como em amostras de pacientes (Paul e Snyder, 2019). A glutationa está envolvida na manutenção redox, atuando como um doador de elétrons em reações oxidorredutoras, mantendo o equilíbrio entre glutationa reduzida (GSH) e glutationa oxidada (GSSG) para constituir um ambiente controlado de homeostase redox (Ushioda e Nagata, 2019). A administração sistêmica de 3-NP em modelos animais já demonstrou alterações significativas em importantes marcadores de estresse oxidativo, como redução nos níveis de GSH e na atividade de enzimas antioxidantes, bem como aumento de LPO, contribuindo para o dano estriatal (Suganya e Sumathi, 2017).

Em condições estressantes, o catabolismo do heme celular para monóxido de carbono, ferro e biliverdina é mediado pela enzima heme oxigenase-1 (HO-1). Há um consenso sobre os efeitos benéficos da regulação positiva de HO-1 no cérebro. Ao converter o heme pró-oxidante em antioxidantes, biliverdina e bilirrubina, as enzimas HO-1 e biliverdina redutase podem ajudar a restaurar a homeostase redox tecidual (Shipper *et al*., 2018).

1.3.2. Metabolismo energético

Em células humanas grande parte das rotas metabólicas que produzem ATP e equivalentes reduzidos ocorre na mitocôndria, a única organela celular que possui genoma próprio, o DNA mitocondrial (mtDNA), o qual é responsável por codificar algumas proteínas essenciais para a formação dos complexos proteicos necessários para o fluxo de elétrons que,

em conjunto, constituem a cadeia respiratória (Wallace, 2015).

A OXPHOS é o processo de transferência de elétrons através da cadeia respiratória, mais especificamente pelos complexos proteicos que tem a capacidade de aceitar e doar elétrons, inseridos na membrana mitocondrial. Os complexos I e II transferem elétrons para a ubiquinona a partir de NADH e $FADH_2$, respectivamente, sendo reduzida a ubiquinol. O ubiquinol transfere os elétrons para o complexo III, que então os transfere para o citocromo *c*. Finalmente, o complexo IV transfere os elétrons do citocromo *c* para o oxigênio, reduzindo-o à H_2O (Nelson; Cox, 2018).

O fluxo de elétrons pelos complexos I, III e IV resulta em um bombeamento de prótons pela membrana interna da mitocôndria, gerando assim uma diferença na concentração de prótons entre a matriz mitocondrial, mais alcalina, com relação ao espaço intermembrana. Com isso, é formado um gradiente de prótons que fornece energia para a síntese de ATP pela ATP-sintase a partir de ADP e Pi (Bagkos, Koufopoulos e Piperi, 2014; Lippe *et al.*, 2019).

O excesso de mitocôndrias disfuncionais ocasiona um desequilíbrio celular, visto que diversas funções fisiológicas não ocorrem sem ATP, tais como reações enzimáticas, processos de transcrição, de tradução e de expressão de genes e proteínas, bem como biossíntese, liberação e captação de neurotransmissores (Picard *et al.*, 2018).

Déficits no metabolismo energético cerebral e na captação de glicose são relatados em doenças neurodegenerativas, demonstrando que há uma ligação entre a disfunção mitocondrial e o dano neurológico (Garabadu *et al.*, 2019). No caso da DH, estudos clínicos e em modelos animais examinaram a interação entre o metabolismo da glicose prejudicado com o desenvolvimento da doença, porém há a necessidade de uma melhor comprovação de como ocorre essa associação (Montojo, Aganzo e González, 2017).

Estratégias terapêuticas que viabilizam sobrevivência neuronal na DH podem promover modulação através do metabolismo energético. A morte neuronal na DH pode correr através do bloqueio irreversível da enzima succinato desidrogenase (complexo II) da cadeia respiratória, resultando em sobrecarga de radicais livres, com perda de potencial de membrana e consequente redução na produção de ATP, levando a uma falha na bioenergética e apoptose neuronal (Ahmed *et al.*, 2021).

1.3.3. Dinâmica mitocondrial

As mitocôndrias são organelas dinâmicas, cruciais em eventos de biogênese, remodelação e renovação. Os processos de fusão e fissão participam dessa atividade e em

conjunto ajudam a manter forma, tamanho e número de mitocôndrias, garantindo assim a qualidade dessas organelas celulares (Garza-Lombó *et al.*, 2020).

A fusão mitocondrial é mediada por proteínas GTPase relacionadas à dinamina, sendo elas a mitofusina 1 (MFN1) e 2 (MFN2), presentes na membrana externa da mitocôndria. A MFN2 possui uma função celular adicional, também sendo encontrada em locais de contato entre a mitocôndria e o RE. Com relação à membrana interna, a GTPase proteína da atrofia óptica 1 (OPA1) completa o sistema de fusão, e diversas isoformas dessa proteína são encontradas em diferentes tecidos. A proteína OPA1 também é fundamental para manter a arquitetura normal das cristas mitocondriais, garantindo o funcionamento adequado do transporte de elétrons na membrana interna da mitocôndria (Dorn, 2019; Whitley, Engelhart e Hoppins, 2019).

Diversos fatores coordenam a fissão, e dentre os mais estudados estão a proteína relacionada à dinamina 1 (DRP1), uma grande GTPase necessária para a constrição mitocondrial, estimulada por cardiolipina, e a dinamina-2 (DNM2), que executa a cisão final. Estudos demonstram que a DNM2 é dispensável para o processo de fissão mitocondrial quando esse evento ocorre em células nocautes para DNM2, em que a DRP1 possui capacidade de constrição e de corte da membrana (Kamerkar *et al.*, 2018).

Alterações na fissão mitocondrial já foram relatadas na DH, pois um aumento na fragmentação mitocondrial dependente de DRP1 foi observado em cultura de células estriatais de animais mutantes. Avaliações através de microscopia também demonstraram aumento na fissão mitocondrial em cultura estriatais de mutantes para DH em relação ao grupo controle. O aumento na atividade de DRP1 foi proposto como um potencial mecanismo de fissão mitocondrial subjacente na DH (Cherubini, Lopez-Molina e Gines, 2020).

1.3.4. Interação entre mitocôndria e retículo endoplasmático

O RE é uma importante organela envolvida na homeostase celular e no controle de qualidade de proteínas, mediando a síntese e exportação de proteínas e glicoproteínas. Alterações no RE podem ocasionar acúmulo de proteínas, resultando em um processo chamado de estresse de RE, sendo esse um contribuinte chave conhecido para a neurodegeneração (Jiang, Chadwick e Lajoie, 2016b; Jiang, Chadwick e Lajoie, 2016a). Também envolvido no metabolismo de lipídeos e carboidratos, assim como no armazenamento de Ca^{2+}, o RE é rico em proteínas responsáveis pela homeostase do Ca^{2+}, como calreticulina, calnexina e as

proteínas reguladoras de glicose 75 kDa (GRP75) e 78 kDa (GRP78). Desequilíbrios celulares, como alterações na homeostase do Ca^{2+} e no estado redox, também podem comprometer a capacidade do RE de manter o controle de qualidade do dobramento de proteínas (Stankov, Stanimirov e Mikov, 2013; Veeresh *et al.*, 2019).

Nos sítios de contato entre RE e mitocôndria ocorrem eventos redox, possuindo grande significado tanto para a fisiologia celular quanto para doenças, pois determinam o balanço metabólico de geração de energia. O mecanismo mais conhecido que influencia esse balanço é a transferência de Ca^{2+}. Neste particular, já foi descrito que a transferência de Ca^{2+} do RE para a mitocôndria ocorre em um contato formado pelas proteínas IP3R (proteína do RE) e VDAC (proteína mitocondrial). Neste contato, há ainda a participação da GRP75, que ancora a proteína IP3R à VDAC (Fan e Simmen, 2019). Por outro lado, a interação entre RE e mitocôndria, e até mesmo entre outras organelas, precisa ser melhor investigada, considerando o envolvimento de diversas proteínas nos sítios de contato.

1.3.5. Estresse de retículo endoplasmático

A ativação da resposta a proteínas mal dobradas (do inglês, *unfolded protein response*, UPR) é mediada por três vias de estresse de RE, a proteína-1 que requer inosol (IRE1), o fatorde transcrição de ativação 6 (ATF6), e a quinase do RE similar à proteína quinase R (PERK), PERK, ATF6 e IRE1 possuem dois domínios: um no lúmen do RE, que é ativado pelo acúmulo de proteínas mal dobradas, e outro no citosol, que ativa efetores de sinalização à jusante. Quando a capacidade da UPR é excedida, eventos pró-apoptóticos são desencadeados, como a regulação positiva de genes responsáveis pela morte celular. A proteína CHOP (proteína homóloga C/EBP, também chamada DDIT3), é estimulada pelos componentes da UPR, e a superexpressão desses genes induz apoptose por proteínas da família BCL-2 (Xiang *et al.*, 2017;Xu, Bailly-Maitre e Reed, 2005).

A CHOP tem função reguladora em diversos processos celulares, além dos eventos apoptóticos, tais como autofagia, inflamação, diferenciação e proliferação celular. Em condições basais, ou seja, não estressoras, apresenta como localização, principalmente, o citoplasma. Quando em condições de estresse, a CHOP é translocada para o núcleo, resultando em uma ligação ao DNA, permitindo assim a regulação da expressão de genes (Chiribau *et al.*, 2010; Yang *et al.*, 2017). Já a GRP78 é a proteína chaperona mais bem estudada, e quando ocorre um aumento de proteínas deformadas no RE, ela dissocia-se dos sensores transmembranares e inicia o processo de UPR (Muneer, Mozammil e Khan, 2019). Proteínas

mal dobradas e estresse de RE são características de diversas doenças neurológicas com alta incidência na população humana, como doença de Parkinson, Alzheimer e DH (Vidal *et al.*, 2011).

Estudos usando diferentes modelos para a DH apresentaram um desequilíbrio na homeostase do RE, com ativação de vários mecanismos como resposta ao estresse para manter a qualidade celular. O direcionamento de estudos para aprimorar o entendimento da UPR e de outras vias de estresse de proteínas mal dobradas em DH é uma abordagem promissora para o desenvolvimento de tratamentos mais eficazes (Jiang, Chadwick e Lajoie, 2016b).

1.4. Justificativa

Estudos em modelos animais e amostras de tecido de pacientes já demonstraram que disfunção bioenergética mitocondrial e produção aumentada de radicais livres estão envolvidas na patogênese da DH. No entanto, outras funções mitocondriais importantes para a homeostase celular, tais como a dinâmica e as interações dessa organela com outros constituintes celulares, em particular o RE, precisam ainda ser melhor investigadas nessa doença. Considerando essas observações, no presente estudo investigamos diferentes processos mitocondriais em um modelo animal para a DH induzido pela administração de 3-NP, a fim de contribuir para o entendimento da fisiopatologia dessa doença e auxiliar no estabelecimento de novas estratégias terapêuticas que poderão melhorar o prognóstico de vida dos indivíduos afetados.

2. OBJETIVOS

2.1. Objetivo geral

Investigar a respiração, a dinâmica (fusão e fissão) e a biogênese mitocondrial, além de estresse de RE, interações das membranas associadas às mitocôndrias (MAMs) e parâmetros de estresse oxidativo no estriado de ratos Wistar submetidos à injeção de 3-NP como um modelo da DH.

2.2. Objetivos específicos

i. Avaliação da respiração mitocondrial: parâmetros respiratórios estado 3, estado 4 e a razão de controle respiratório (RCR) medidos pelo consumo de oxigênio;

ii. Avaliação de atividades enzimáticas da cadeia respiratória mitocondrial: atividades dos complexos II-III, II+ SDH e IV;

iii. Avaliação de estresse oxidativo: medida de substâncias reativas ao ácido tiobarbitúrico (TBA-RS), das concentrações de glutationa reduzida (GSH), e da atividade das enzimas antioxidantes glutationa peroxidase (GPx), glutationa redutase (GR), glutationa S-transferase (GST), superóxido dismutase (SOD) e catalase (CAT);

iv. Avaliação da fusão e fissão mitocondrial: determinação do conteúdo proteico da mitofusina 1 e proteína 1 relacionada à dinamina (DRP1);

v. Avaliação da sinalização sob condições de estresse: Avaliação do conteúdo proteico de heme oxigenase- 1 (HO-1);

vi. Avaliação do estresse de RE: determinação do conteúdo da proteína regulada por glicose de 78 kDa (GRP78);

vii. Avaliação da integridade das membranas associadas à mitocôndria (MAMs): determinação do conteúdo proteico das proteínas marcadoras de MAMs: canal de ânion dependente de voltagem 1 (VDAC1; proteína mitocondrial) e proteína de 75 kDa regulada por glicose (GRP75; proteína citosólica de choque térmico que liga e regula tanto IPR3 quanto VDAC1);

PARTE II

Capítulo I

Bioenergetics and redox homeostasis disruption, mitochondrial-ER crosstalk and dynamics alterations in rat model of Huntington's disease induced by 3-nitropropionic acid

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Bioenergetics and redox homeostasis disruption, mitochondrial-ER crosstalk and dynamics alterations in rat model of Huntington's disease induced by 3-nitropropionic acid

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ABSTRACT

Huntington's Disease (HD) is a neurodegenerative disorder caused by a CAG nucleotide base repeat expansion in the *huntingtin* gene, leading to the accumulation of a mutant protein huntingtin (mHTT). mHTT has been shown to be neurotoxic, resulting in neuronal death principally in the striatum, but during the progression of the disease other brain regions are affected. Treatment for HD is limited and based on management of symptoms. Therefore, in the present study we used an HD animal model developed with 3-nitropropionic acid (3-NP) administration in Wistar rats to better elucidate mechanisms of neurodegeneration observed in HD. We evaluated parameters of mitochondrial function and interaction of mitochondria with endoplasmic reticulum (ER) in the striatum 7, 14, 21, and 28 days after administration of 3-NP. Our results showed that 3-NP induced disturbances in redox homeostasis with increased of malondialdehyde (MDA) levels in 28 days, and reduced of glutathione (GSH) concentration in 21 and 28 days, also decrease the activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST). Moreover, impairment of mitochondrial respiration, with reduction of respiratory chain complex activity in 7 and 28 days. In addition, we found a reduce of state 3, state 4, and non-coupled in 7 days, but in 28 days we observed an increase state 4, non-coupled, and reserve capacity. Furthermore, 3-NP impaired mitochondrial dynamics and interactions between ER and mitochondria by increasing protein expression of mitofusin-1 (MFN1) in all times tested, voltage-dependent anion-selective channel 1 (VDAC-1) in 28 days and chaperone glucose-regulated protein 75 (GRP75) in 28 days. Finally, 3-NP induced a loss of the synaptic protein synaptophysin in 28 days, indicating impairment of neuronal viability. In conclusion, it may be presumed that mitochondrial dysfunction plays a key role in the pathophysiology of striatum abnormalities observed in HD.

KEYWORDS: Huntington's disease; 3-Nitropropionic acid; Striatum; Redox homeostasis; Mitochondria; Bioenergetics; Mitochondria-endoplasmic reticulum interactions.

Availability of Data and Material Not applicable

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

Ethics Approval International, national, and institutional guidelines for the care and use of animals were followed. The experimental protocol was approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Universidade Federal do Rio Grande do Sul, (RS, Brazil), number 34695. This article does not contain studies with human participants.

Consent to Participate Not applicable

Consent for Publication Not applicable

Code Availability Not applicable

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansive CAG repeat in the *huntingtin* gene (*HTT*), leading to massive neuronal loss in striatum. HD is characterized by a combination of motor, cognitive, and psychiatric symptoms, as well as atrophy of the basal ganglia and cerebral cortex [1]. The *HTT* product, the huntingtin protein, has many functions in human neurons and is associated with various organelles, such as nucleus, endoplasmic reticulum (ER), Golgi complex and mitochondria [2]. In addition, the huntingtin protein interacts with other proteins, like the voltage-dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane, that can be a physical contact site between mitochondria and huntingtin protein [3].

Although the pathogenic mechanism of HD remains elusive, preventing the development of new effective therapeutics, mitochondrial dysfunction and oxidative stress has been demonstrated to contribute to HD pathology. Both processes have a critical role in the disease's neuropathogenesis and progression, and may modulate pathomechanisms that drive many features of the HD [4]. Alterations in bioenergetics were described in HD, by reduced complex II and III activities [5], and inhibition of the succinate dehydrogenase enzyme caused by 3-nitropropionic acid (3-NP) [6]. Furthermore, intraperitoneal administration of 3-NP in rats induced striatal lesions, resulting in neurochemical and behavioral features that mimic the alterations observed in HD patients [7]. Therefore, the 3-NP induced HD model has a great potential to cause similar symptomatology and molecular alterations seen in affected individuals [8].

Another potential mechanism evidenced to participate in HD pathophysiology is ER stress. Different cellular and animal models of HD suggest a correlation between HD and ER stress [9]. The origin of this alteration has similar features in various multifactorial neurodegenerative diseases, and recent data demonstrate the toxic consequences of protein misfolding and aggregation in HD on the ER stress induction [10]. In this regard, it should be considered that the interaction sites between mitochondria and ER have a great importance to normal cellular physiology, as well as for neurodegenerative diseases such as HD, since redox events may occur in their contact sites, which are fundamental to the metabolic balance of ATP generation [11].

Therefore, the present study investigated redox homeostasis and mitochondrial bioenergetics, as well as mitochondrial processes that have not been investigated in HD so far, as mitochondrial dynamics and communication between mitochondria and ER in striatum of rats receiving 3-NP administration, a well stablished HD animal model. We expect our results may contribute to the understanding of HD pathophysiology and contribute for the develop of new therapeutic strategies for this disorder.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. 3-NP solution was prepared on the same day of the experiments.

Animals and ethical approval

Wistar rats (120 - 150g) were obtained from the Center for Reproduction and Experimentation of

Laboratory Animals (CREAL) of the Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were maintained on a 12:12-h light/dark cycle (lights on 07:00 am–07:00 pm) in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and a 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil).

The experimental protocol was approved by the local Ethical Committee for the Care and Use of Laboratory Animals of the Universidade Federal do Rio Grande do Sul (# 34695). The experiments followed the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, publication no. 80–23, revised 2011), the Directive 2010/63/EU and the International Guiding Principles for Biomedical Research Involving Animals. All efforts were made to minimize the number of animals used and their suffering.

3-Nitropropionic acid (3-NP) administration

Thirty-five-day-old female Wistar rats were used because animals of this age correspond to the period of life in which symptomatology is clearly seen in the affected patients [12]. The animals received an intraperitoneal (i.p) administration of 3-NP (20 mg/kg body weight; 1 injection per day) or PBS (control) for a period of three consecutive days [13]. The animals were euthanized 7, 14, 21 or 28 days after the last 3-NP injection.

Tissue preparation

The brain was rapidly removed and placed on a Petri dish on ice, and the striatum was dissected. For the evaluation of redox homeostasis parameters, the striatum was homogenized in nine volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C [14]. The pellet was discarded and the supernatant containing mitochondria and other cell organelles was used to measure the biochemical parameters. Protein content was quantified for data normalization according to Lowry et al [15].

For the evaluation of respiratory chain complexes activities, the striatum was homogenized (1:20, w/v) in SET buffer (250 mM sucrose, 2.0 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Trizma base), pH 7.4, and centrifuged for 10 min at $800 \times g$. The supernatants were kept at -80 °C until the biochemical analysis. In addition, striatum crude homogenates were prepared for the determination of respiratory parameters at an approximate protein concentration of 3 mg.mL^{-1}, as described by Makrecka-Kuka et al [16].

For western blot analysis, striatum was homogenized in a RIPA buffer containing protease and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM aprotinin, and 1% protease inhibitor cocktail) and centrifuged at $10,000 \times g$ for 10 min at 4 °C. Supernatant protein concentrations were determined by the method of Lowry et al. [14], then the samples were denatured in 4× Laemmli buffer (250 mM Tris, 8 % SDS, 40 % glycerol, and 0.002 % bromophenol blue, pH 6.7) and 10 % 2-mercaptoethanol. These samples were then heated at 98 °C for 10 min and used for western blotting.

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels according to the method described by Yagi [17] with slight modifications. One hundred microliters of tissue supernatants were treated with

200 µL of 10% trichloroacetic acid and 300 µL of 0.67% thiobarbituric acid in 7.1% sodium sulfate and incubated for 2 h in a boiling water bath. After cooling, the mixture was extracted with 400 µL of butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. MDA levels were expressed as nmol MDA/mg protein.

Antioxidants defenses

Nonenzymatic antioxidant defenses were determined by measuring reduced glutathione (GSH) concentration. GSH concentration were measured according to Browne and Armstrong [18]. One hundred eighty-five microliters of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 µL of o-phthaldialdehyde (1 mg/mL) were added to 30 µL of sample (0.3–0.5 mg of protein) previously deproteinized with metaphosphoric acid. After incubating this mixture at 25 °C in a dark room for 15 min, the fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol GSH/mg protein.

The activities of the following antioxidant enzymes were also measured: glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST). The specific activity was calculated and expressed as U/mg protein.

GPx activity was measured according to Wendel [19] using tertbutyl hydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH, and tissue supernatants.

GR activity was measured according to Carlberg and Mannervik [20] using GSSG and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM EDTA, 1 mM GSSG, 0.1 mM NADPH, and tissue supernatants.

SOD activity was assayed according to Marklund [21] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on superoxide, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM EDTA, pH 8.2, 80 U/mL CAT, 0.38 mM pyrogallol, and tissue supernatants.

CAT activity was assayed according to Aebi [22] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and tissue supernatants.

GST activity was measured according to Guthenberg and Mannervik [23] with slight modifications. GST activity was determined by the rate of formation of dinitrophenyl-S- glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants.

Mitochondrial respiratory parameters

Oxygen consumption was measured in striatum crude homogenates (2 mg tissue·mL^{-1}) through substrate-uncoupler inhibitor titration (SUIT) protocol [16]. NADH-linked substrates (5 mM pyruvate, 0.5 mM malate and 10 mM glutamate) were first added to the chamber, followed by 500 μM ADP (state 3 respiration PMG), in addition, 10 mM of succinate (FADH$_2$-linked substrate) was added (state 3 respiration PMG + S). To evaluate state 4 respiration, 1 μg.mL^{-1} oligomycin was added to the chamber. Next, 1.5 μM CCCP (three pulses of 0.5 μM) was titrated to determine electron transfer system (ETS) capacity (non-coupled respiration PMG + S). One micromolar rotenone (complex I inhibitor) was used to obtain the non-coupled respiration stimulated by succinate (non-coupled respiration S). Finally, antimycin A was added to the chamber for the determination of residual oxygen consumption (ROX). The reserve capacity was obtained through the difference between non-coupled respiration PMG to State 3 PMG + S. All parameters were corrected by ROX. The real-time oxygen fluxes were calculated using DatLab7 (Oroboros Instruments) and expressed as pmol O$_2$ flux·s^{-1} ·mg protein^{-1}.

Respiratory chain complex activities

The activities of the various complexes of the respiratory chain were measured in the homogenates of the striatum in the presence of approximately 30 μg of protein. Succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate: cytochrome c oxidoreductase (complex II–III) activities were determined according to Fischer et al [24]. The cytochrome c oxidase (complex IV) was assayed according to Rustin et al [25]. The activities of the respiratory chain complexes were calculated as nmol.min^{-1}.mg protein^{-1}.

Western blot analysis

Equal amounts of protein (30 μg/well) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After verifying protein loading and electroblotting efficiency through Ponceau S staining, the membrane was blocked in Tween-Tris-buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, 0.9 % NaCl, and 0.1 % Tween-20) containing 5 % albumin. Membranes were then incubated overnight at 4 °C with the primary antibodies anti- heme oxygenase-1 (ab13248), anti-mitofusin-1 (MFN1) (ab57602), anti-dynamin-related protein 1 (DRP1) (ab56788), anti-GRP75 (ab2799), anti-VDAC1 (ab154856), anti-GRP78 polyclonal antibody (ab21685), and anti-synaptophysin (ab8049) from Abcam, Cambridge Science Park Cambridge, UK; separately in TTBS, at different working dilutions as suggested by the manufacturers, and finally washed with TTBS. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary anti-body (1:10,000; Santa Cruz®, sc-2030 and sc-2031, respectively) was then incubated with the membranes for an additional 2 h, following by washed again, and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis was performed with ImageJ software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a ratio relative to the β-actin (1:20,000, Protein tech Group, Inc., HRP-conjugated 60008) internal control.

Statical analysis

The data were analyzed using Student's *t* test for unpaired samples or one-way analysis of variance (ANOVA) followed by the post hoc Tukey multiple range test when the F value was significant, using the GraphPad 8.0.2 software. Only significant values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All data presented here are the result of three or more independent experiments, performed

in duplicate or triplicate, and the mean was used for statistical analysis. Data were expressed as mean ± SD forabsolute values.

RESULTS

3-NP disturbs redox homeostasis in rat striatum

Oxidative stress is defined as an unbalance between reactive oxygen species (ROS) production and the antioxidant enzymes [26]. The brain is vulnerable to oxidative stress, especially due to its high oxygen consumption that leads to ROS [27]. We initially evaluated the effect of 3-NP administration, which consists of an animal model of HD, on MDA levels in the striatum of rats euthanized 7, 21 and 28 days after the injection. We verified that 3-NP increased MDA levels only 28 days after 3-NP injection, as compared to control animals that received PBS [P < 0,01] (Fig. 1A).

GSH is an important non-enzymatic antioxidant defense, acting as a cofactor of essential antioxidant enzymes and scavenging free radicals [28]. GSH concentrations were then determined, and we observed decreased GSH levels 21 days [P < 0.05] and 28 days [P < 0.05] after 3-NP administration (Fig. 1B). Moreover, we investigated the effects of 3-NP on the activities of important antioxidant enzymes. As shown in Fig. 1, 3-NP administration decreased activities of GPx in 7 days [P < 0.001], 21 days [P < 0.001], and 28 days [P < 0.001] (Fig. 1C), SOD in 7 days [P < 0.05], 21 days [P < 0.05] and 28 days [P < 0.01] (Fig. 1E), as well as GST in 7 days [P < 0.05], 21 days [P < 0.05] and 28 days [P< 0.01] (Fig. 1G). We also observed a decrease of GR activity in striatum only at 21 days after 3-NP administration [P < 0.05] (Fig. 1D), whereas a reduction in CAT activity was observed at 21 days [P < 0.05], and 28 days [P < 0.05] (Fig. 1F).

3-NP alters mitochondrial respiration in rat striatum

In the HD mice model, the respiratory capacity changes when comparing different brain regions and age-related, using high resolution respirometry [29]. In the next set of experiments, we determined whether 3-NP could alter mitochondrial respiration 7 (Fig. 2) or 28 days (Fig. 3) after 3-NP treatment, using the SUIT protocol [16]. We evaluated mitochondrial respiration in striatum crude homogenates because this preparation presents full mitochondrial functionality in an integrated cellular system, which is similar to an *in vivo* situation [30]. We verified that 3-NP decreased state 3 in striatum 7 days after 3-NP administration when respiration was supported only by NADH-linked substrates (pyruvate, malate and glutamate) [P < 0.01] (Fig. 2A) as well as with succinate (FADH$_2$-linked substrate) [P < 0.001] (Fig. 2B). 3-NP also decreased state 4 [P < 0.05] (Fig. 2C), non-coupled respiration driven by PMG and succinate [P < 0.01] (Fig. 2D), whereas non-coupled respiration was also reduced only when supported by succinate [P < 0.001] (Fig. 2E). Reserve capacity was not significantly altered (Fig. 2F).

On the other hand, state 3 respiration was not significantly changed in striatum 28 days after treatment (Fig. 3). In contrast, non-coupled state [P< 0.05] (Fig. 3D) and state 4 [P< 0.01] (Fig. 3C) were augmented by 3-NP when respiration was supported by PMG and succinate. Moreover, an increase in reserve capacity was observed [P < 0.01] (Fig. 3F).

3-NP decreases respiratory chain complex activities in rat striatum

Mitochondrial respiratory chain complexes play crucial role in regulating mitochondrial function [31]. We then determined the activities of the respiratory chain complexes II, II–III and IV in striatum at 7 and 28 days after 3-NP administration. Figure 4 shows a decreased activity of complex II in 7 days [$P< 0.05$] (Fig 4A), and 28 days [$P< 0.01$] (Fig. 4B), of complex II-III in 7 days [$P< 0.01$] (Fig. 4E), and 28 days [$P< 001$] (Fig. 4F). SDH activity was significantly decreased by 3-NP at both periods, 7 days [$P< 0.001$] (Fig. 4C), and 28 days [$P< 0.05$] (Fig. 4D). Our results also demonstrated that complex IV activity was decreased only at 28 days [$P< 0.01$] (Fig 4H) after 3- NP last injection.

3-NP did not change heme oxygenase-1 levels in rat striatum

Considering the redox homeostasis alterations demonstrated above, we measured the protein levels of heme oxygenase-1 (HO-1), an important antioxidant enzyme whose expression is modulated during oxidative stress [32,33]. Figure 5 shows that 3-NP did not change the content of this enzyme.

3-NP disrupts mitochondrial dynamics and ER-mitochondria crosstalk in rat striatum

Disturbances in mitochondrial dynamics and ER-mitochondria crosstalk are often associated with bioenergetics impairment and oxidative stress [34,35]. Thus, we investigated the effects of 3-NP on MFN1 and DRP1 content, the main proteins implicated in mitochondrial fusion and fission, respectively, as well as VDAC1 and GRP75, components of ER-mitochondria contacts, 7, 14 and 28 days after treatment. We found that 3-NP increased MFN1 protein levels in 7 days [$P < 0.05$], 21 days [$P < 0.05$], and 28 days [$P < 0.05$] (Fig. 6C and D), but did not alter DRP1 levels (Fig. 6A and B), suggesting that mitochondrial fusion processes are increased. Regarding the ER-mitochondria communication, 3-NP increased VDAC1 [$P< 0.001$] (Fig. 7A and B) and GRP75 [$P< 0.05$] (Fig. 7C and D) only 28 days after 3-NP administration. GRP78, a chaperone involved in ER stress and unfolded protein response (UPR) [36], was not altered by 3-NP (Fig. 7E and F).

3- NP alters synaptophysin levels in rat striatum

A striatal synaptic loss in HD is related with loss of striatal medium-sized spinous neurons [37]. Synaptophysin is the major integral membrane protein of synaptic vesicles [38]. Figure 8 depicts that 3-NP caused variable effects on the of this protein depending on the period evaluated. At 7 days, we found that 3-NP increased synaptophysin protein levels [$P < 0.05$], while at 14 days no significant changes were detected. On the other hand, 3-NP significantly reduced synaptophysin levels 28 days after treatment [$P < 0.05$] (Fig. 8A and B).

DISCUSSION

Neuropathological findings of HD include degeneration of basal ganglia, mainly the striatum, and cortical atrophy. Although the mechanisms underlying the basal ganglia abnormalities are not totally established, mounting evidence has shown that 3-NP administration in rats replicates biochemical, behavioral and striatum degeneration similarly to HD patients [39]. Therefore, in the present study we used 3-NP treatment in Wistar rats as HD model to study the role of mitochondrial processes and functions in the neurodegeneration observed in this disorder.

We initially showed that 3-NP induced oxidative stress in striatum of rats at different ages, corroborating

previous data demonstrating that this mechanism is involved in HD pathophysiology [40,41]. Oxidative stress occurs when the endogenous system has a reduced capacity to fight against the oxidative attack of target biomolecules [42,43]. 3-NP inhibited the activity of the important antioxidant enzymes SOD, CAT, GPx, GST and GR. In addition, lipid peroxidation, a process where free radicals, especially the peroxyl radical, oxidize polyunsaturated fatty acid molecules [44], is also induced by 3-NP. Specifically, we found that 3-NP augmented MDA levels, a final product of lipid peroxidation. Furthermore, GSH concentrations, the most abundant non-enzymatic antioxidant defense in the brain, were decreased by 3-NP. The alterations on GSH levels seen in our model could be explained by iron accumulation in the brain that is commonly seen in neurodegenerative diseases, such as HD, which may lead to oxidative stress and degeneration [45]. In this regard, an important regulator of the cellular production of iron from heme is HO-1, a stress protein responsible for the degradation of heme to generate carbon monoxide, iron, and biliverdin-IXα, which is further converted to bilirubin-IXα [46]. Owing to this, we evaluated the effects of 3-NP on the content of this enzyme, but we did not find a significant alteration. In contrast, previous study showed reduced levels of HO-1 in another HD model based on the administration of quinolinic acid [47]. Besides the difference in the substance used for the induction of the HD model (quinolinic acid versus 3-NP), another factor that may explain these controversial results on HO-1 levels is that we injected 3-NP intraperitoneally whereas quinolinic acid was injected directly into the striatum of rats.

ATP production deficiency and ROS accumulation are mechanisms that often a result from disturbances in the expression or altered activity of mitochondrial respiratory chain complexes [48]. Thus, we determined whether 3-NP could change mitochondrial oxygen consumption, evaluated by high-resolution respirometry, and respiratory chain complex activities. We verified that 3-NP caused differential effects on mitochondrial respiration in striatum of rats at 7 and 28 days after 3-NP treatment. We found a reduction of state 3 (ADP-stimulated), state 4 (non-phosphorylating) and non-coupled (CCCP-stimulated) respiration supported by pyruvate, malate and glutamate or succinate 7 days after 3-NP administration. However, at 28 days, we found that 3-NP increased state 4 and non-coupled respiration, and reserve capacity. The increased state 4 respiration observed at 28 days could be explained by an uncoupling effect induced by 3-NP treatment. In this regard, it has been shown that 3-NP augments RNA transcript levels of the uncoupling protein-2 (UCP-2) in striatum adult rats [49]. Moreover, it should be considered that another study verified that the mitochondrial respiration was not altered in the *vastus lateralis* muscle from individuals affected by HD with expansion mutation carriers [50]. Our results also demonstrated alterations in the activities of respiratory chain complexes, with decreased activity of complexes II, II-III and IV, as well as of SDH activity in both periods evaluated (7 and 28 days), corroborating previous findings showing a reduction of complex IV activity in striatum and cerebral cortex of Huntington R6/2 transgenic mice [51]. Our findings are also in line with the classical inhibition of complex II caused by 3-NP administration, that mimics the HD pathogenesis and has been widely reported in human and animal models [52,53]. Although some of our findings are in line with studies in the literature, it is also possible to observe controversial results and further studies are necessary to elucidate the possible alterations on mitochondrial respiration in HD.

Liot and collaborators [54] evidenced that 3-NP has a dual mechanism that cause mitocondrial damage: the first is an effect on mitocondrial bioenergetics resulting in ATP production impairment and ROS increase, and the second event occurs through the activation of N-methyl-D-aspartate (NMDA) glutamatergic receptors, which further participate in ROS rise and may induce mitochondrial fragmentation with consequent neuronal cell death

[54]. Consistent with this study, we observed abnormal mitochondrial dynamics in our model with increased MFN1 content. However, no change was seen in DRP1. Many studies diverge from the significance of the fission/fusion ratio in the pathogenesis of HD. In this regard, previous data showed high mRNA levels of MFN1 without changes in protein content in striatum of end-stage Huntington R6/2 transgenic mice [55]. On the other hand, elevated fission has been suggested to be involved in HD since inhibition of DRP1 was shown to reverse the phenotype of mitochondrial fragmentation and improve survival in HD mice [56]. It is possible that these differential results occurred due to the sustained elevation of truncated mutant huntingtin levels found in the transgenic mice (permanent alteration), which does not occur in our chemical model based on 3-NP injection. Furthermore, it is known that oxidative stress may modulate mitochondrial dynamics, so it may be speculated that ROS induced by 3-NP may change expression levels and cause post translational modifications in MFN1 [57].

Mitochondria are dynamic organelles and interact with other organelles. These contact sites between organelles are called mitochondria-associated membranes (MAMs). The best communication characterized so far is that occurring between mitochondria and ER, responsible for a variety of processes, including Ca^{2+} flux [58]. Therefore, we evaluated the content of two proteins involved in this communication, namely VDAC1 and GRP75, as well as of GRP78, an ER stress marker. We found that 3-NP augmented the levels of VDAC1 and GRP75, which can act as a compensatory mechanism to increase calcium transfer from ER to mitochondria as a consequence of the reported activation of NMDA receptor provoked by 3-NP [59]. However, GRP78 content was not modified. These data indicate a disruption of mitochondria-ER crosstalk, but more studies are necessary to clarify whether Ca^{2+} transfer between mitochondria-ER is modified in this animal model of HD.

Finally, we verified variable effects of 3-NP on synaptophysin levels at different periods. We initially verified an increase in synaptophysin levels at 7 days followed by a reduction at 28 days after the administration of 3-NP. These findings imply that there is an initial adaptive response of neurons in order to cope with the toxic effects elicited by 3-NP followed by a significant reduction of synaptophysin levels, which could be related to a late stage of HD progression, with synapse degeneration and reduced neuronal viability. Thus, our results show that the periods evaluated provide an overview of the worsening trend of symptoms induced by 3-NP.

CONCLUSION

Several studies in the literature diverge on which period after 3-NP administration better mimics HD symptoms. In this context, studies evaluating behavioral and biochemical parameters at 14 days [60–62], 30 days [63], and 21 days after 3-NP injection [64] can be found. Despite of this, to our knowledge, this is the first study to show that the communication of mitochondria with ER is possibly disturbed in HD, as a consequence of bioenergetic dysfunction and oxidative stress. In conclusion, we evidence here a severe impairment of mitochondrial function that possibly underlies the striatum degeneration observed in HD. Our data further show that the communication of mitochondria with ER is an important intracellular function, contribuiting to the whole cellular homeostasis, and may lead to the development of pharmacological strategies to improve the prognosis of HD patients.

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FIGURES

Figure 1
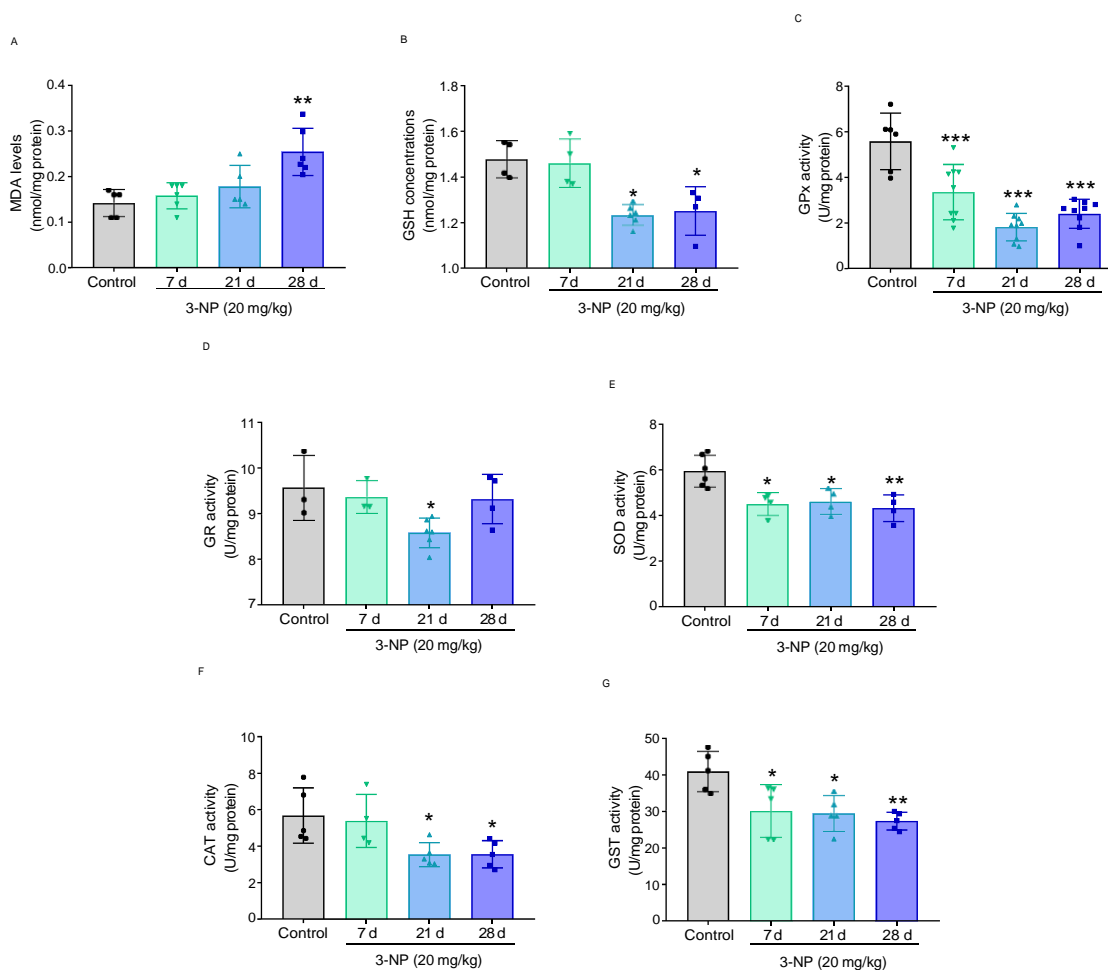
Figure 1. Effect of 3-nitropropionic acid (3-NP) on malondialdehyde (MDA) levels (A), reduced glutathione (GSH) concentrations (B), and glutathione peroxidase (GPx) (C), glutathione reductase (GR) (D), superoxide (SOD) (E), catalase (CAT) (F), and glutathione S-transferase (GST) (G) activities in rat striatum. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d), 21 (21 d), and 28 days (28 d). Values are mean ± standard deviation for three to six independent experiments (animals) expressed as nmol/mg protein or U/mg protein. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared to control (one-way ANOVA as described in the text, followed by Tukey's multiple comparions test).

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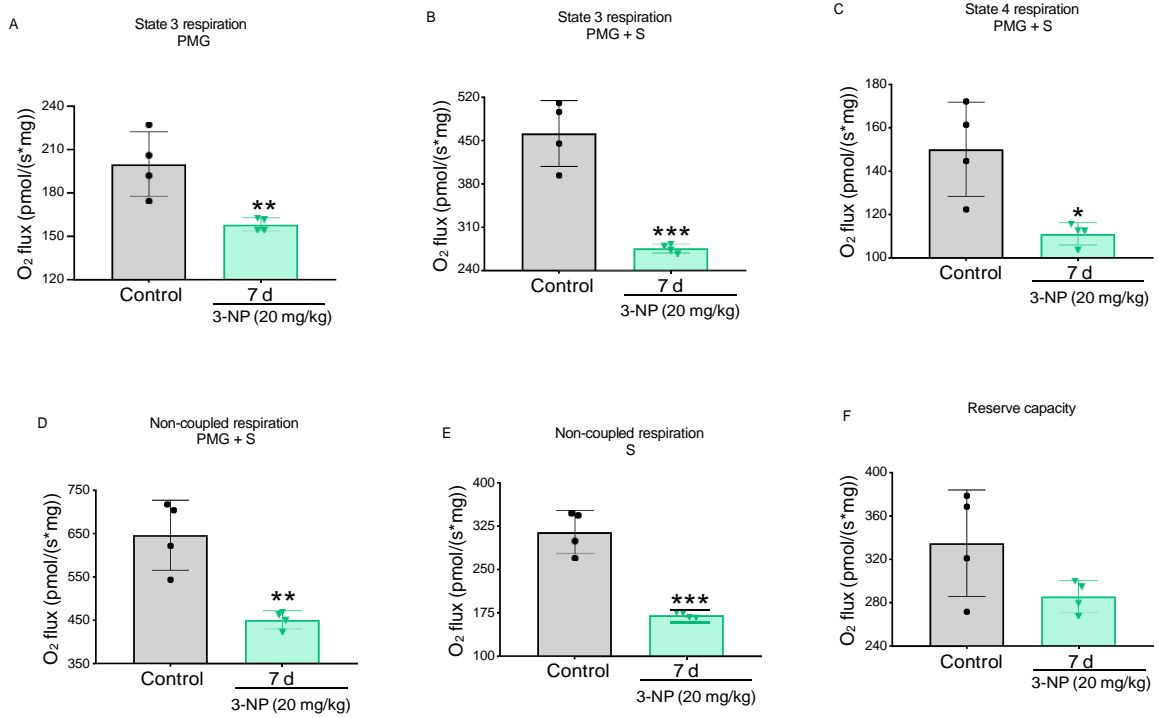
Figure 2

Figure 2. Effects of 3-nitropropionic acid (3-NP) on respiratory parameters in rat striatum using the substrate-uncoupler inhibitor titration (SUIT) protocol. State 3 (ADP-stimulated) (A and B) and Non-coupled (CCCP-stimulated) (C and D), State 4 (non-phosphorylating) (E), and reserve capacity (F). Pyruvate (5 mM), malate (0.5 mM) plus glutamate (10 mM) (A, B and C) and succinate (10 mM) (B, C and D) were used as substrates. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 days (7 d). Values are means ± standard deviation for four to five independent experiments (N) expressed as pmol O_2. s^{-1}. mg of protein^{-1}. One-way ANOVA is described in the text. *P< 0.05, **P< 0.01, ***P< 0.001 compared to control (Tukey's range test).

Figure 3
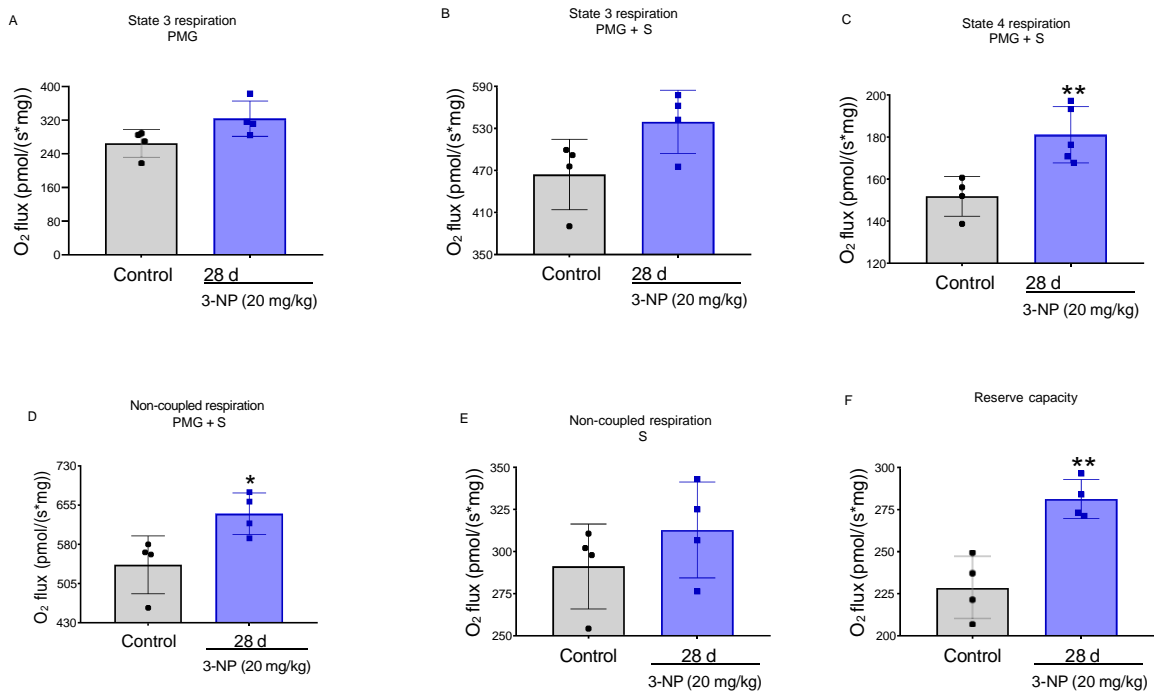
Figure 3. Effects of 3-nitropropionic acid (3-NP) on respiratory parameters in rat striatum using the substrate-uncoupler inhibitor titration (SUIT) protocol. State 3 (ADP-stimulated) (A and B) and Non-coupled (CCCP-stimulated) (C and D), State 4 (non-phosphorylating) (E), and reserve capacity (F). Pyruvate (5 mM), malate (0.5 mM) plus glutamate (10 mM) (A and B) and succinate (10 mM) (B) were used as substrates. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 28 days (28 d). Values are means ± standard deviation for four to five independent experiments (N) expressed as pmol O_2. s^{-1}. mg of protein^{-1}. One-way ANOVA is described in the text. *$P < 0.05$, **$P < 0.01$ compared to control (Tukey's range test).
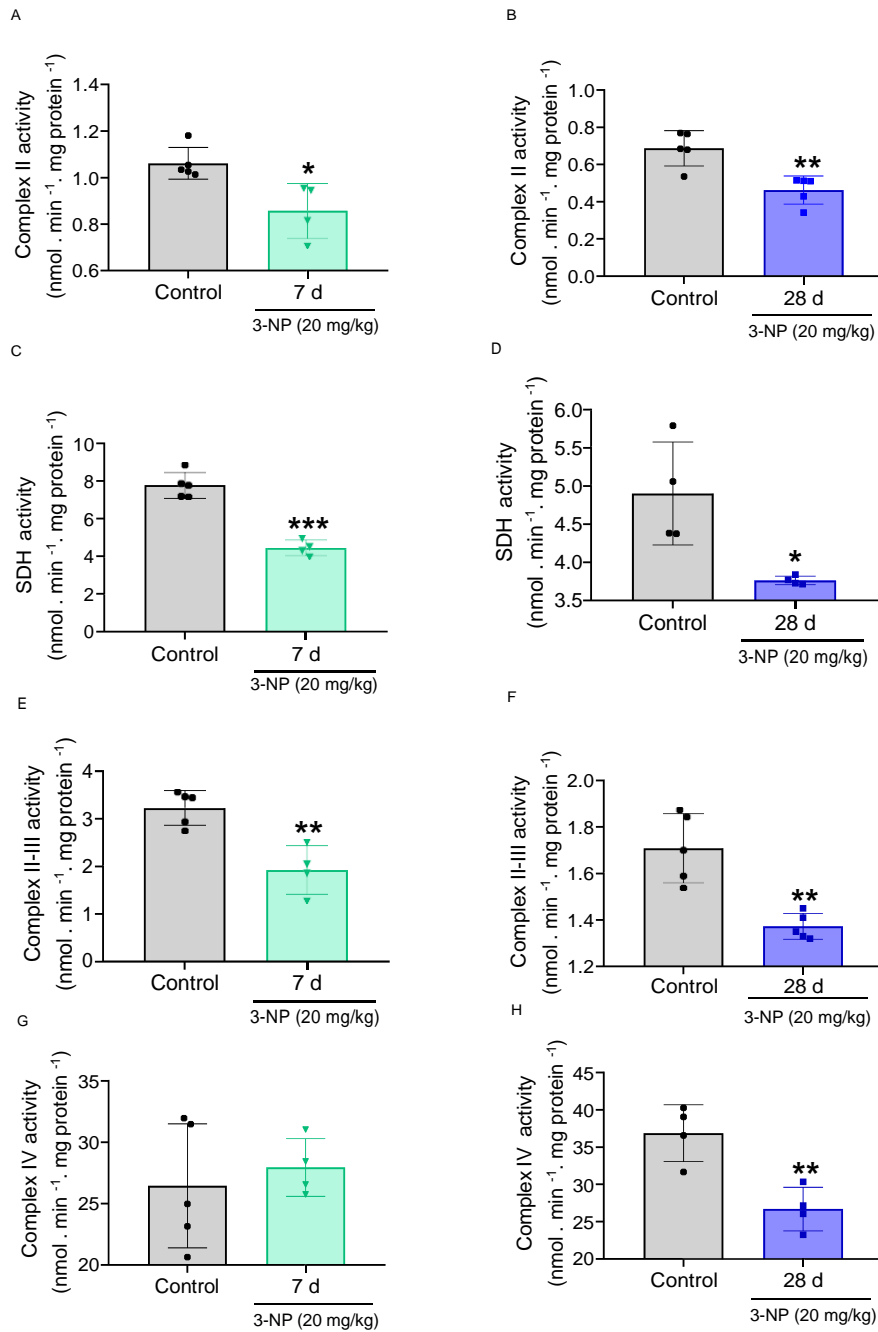
Figure 4. Effects of 3-nitropropionic acid (3-NP) on the activity of the respiratory chain complexes II (A and B), SDH (C and D), II- III ((E and F) and IV (G and H) in rat striatum. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d) and 28 days (28 d). Values are mean ± standard deviation of four to five independent experiments (animals) performed in triplicate and expressed as nmol.min^{-1}.mg protein^{-1}. Student's *t* test for unpaired samples is described in the text. *P< 0.05, **P< 0.01, ***P< 0.001 compared to control.

Figure 5
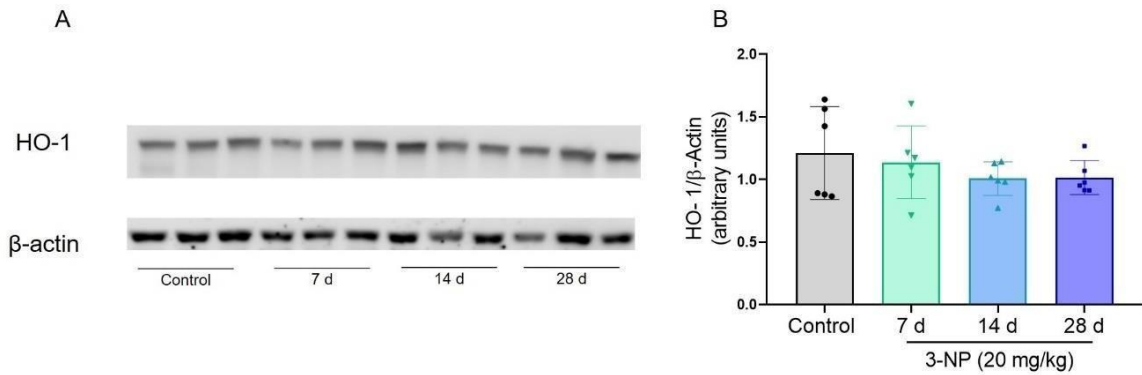
Figure 5. Effect of 3-nitropropionic acid (3-NP) on heme oxygenase-1(HO-1) protein levels in rat striatum (A and B). Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d), 14 (14 d), and 28 days (28 d). Values are mean ± standard deviation for six independent experiments (animals) expressed as arbitrary units, normalized by the content of the protein β-actin. *P < 0.05 compared to control (one-way ANOVA as described in the text, followed by Tukey's multiple comparisons test and Student's t test for unpaired samples).

Figure 6
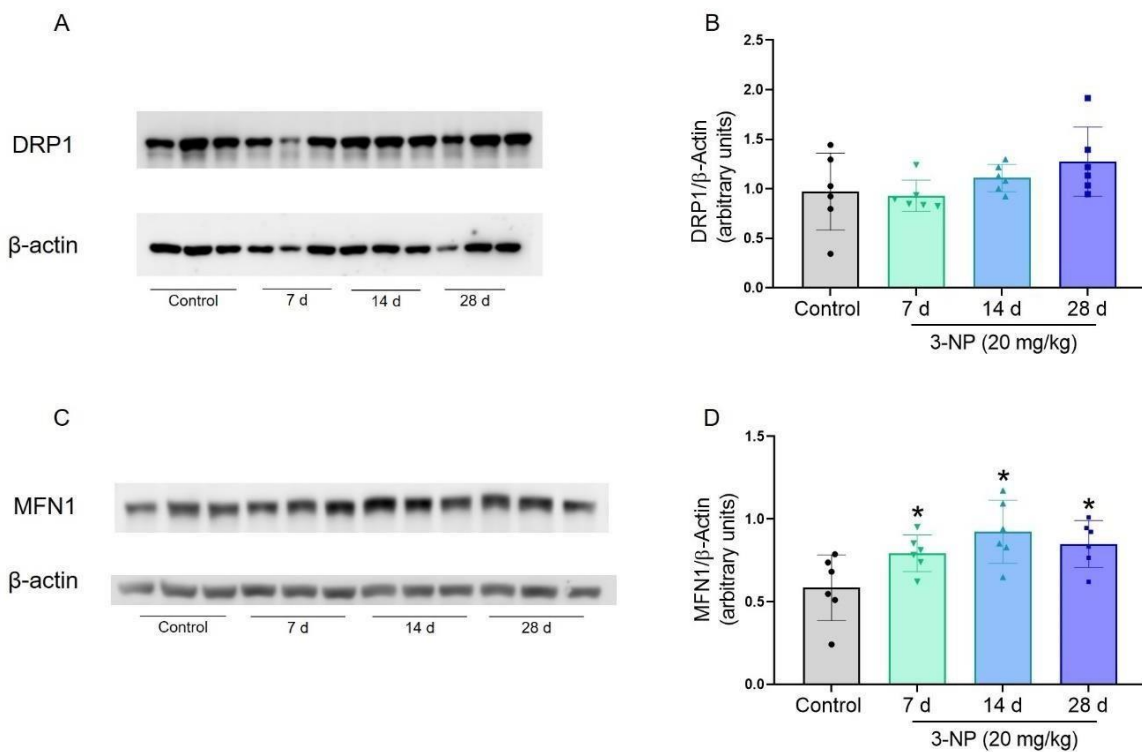
Figure 6. Effect of 3-nitropropionic acid (3-NP) on dynamin-related protein 1 (DRP1) (A and B) and mitofusin-1 (MFN1) (C and D) levels in rat striatum. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d), 14 (14 d), and 28 days (28 d). Values are mean ± standard deviation for six independent experiments (animals) expressed as arbitrary units, normalized by the content of the protein β-actin. *P < 0.05 compared to control (one-

way ANOVA as described in the text, followed by Tukey's multiple comparisons test and Student's t test for unpaired samples).

Figure 7
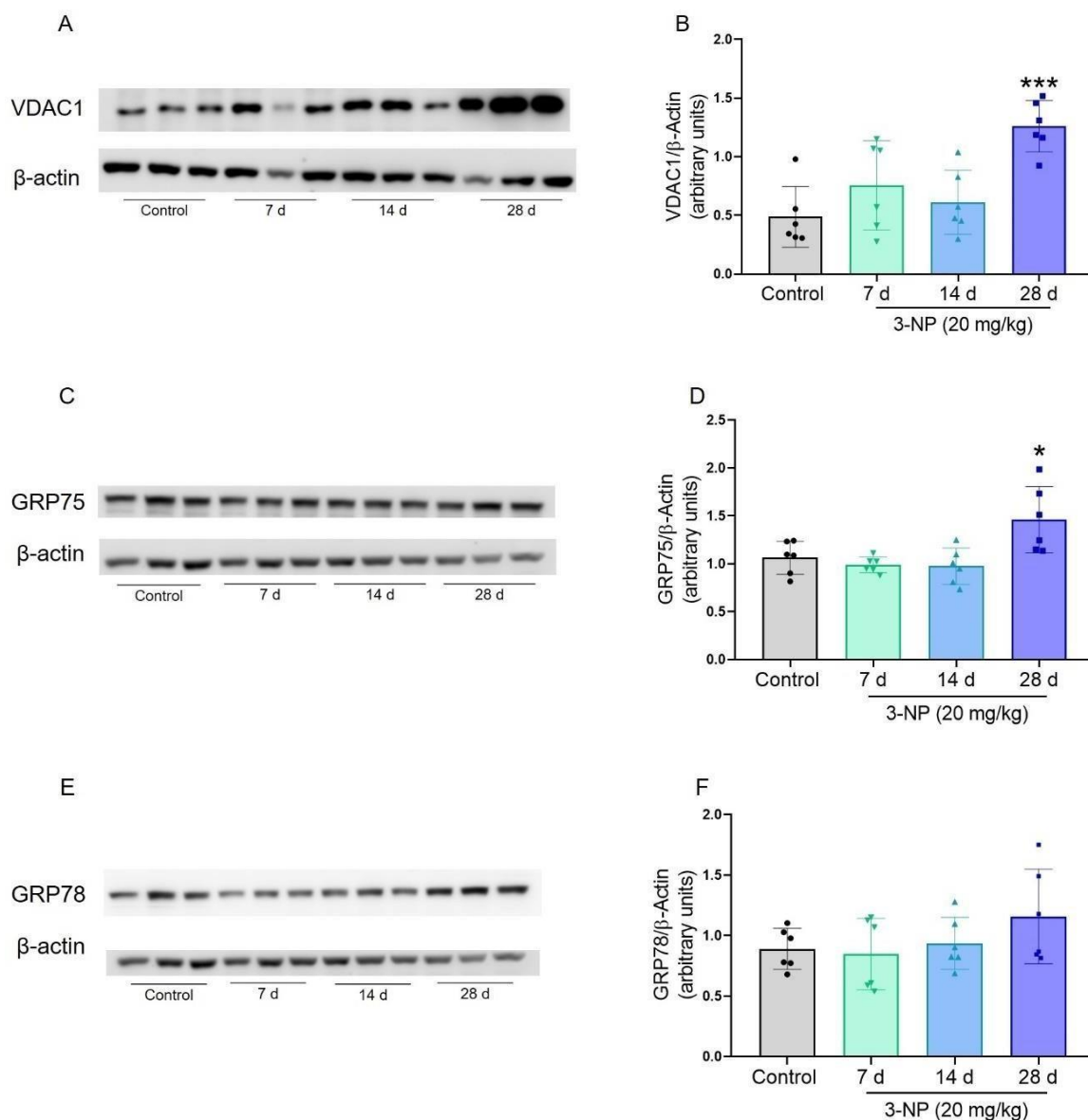
Figure 7. Effect of 3-nitropropionic acid (3-NP) on voltage-dependent anion-selective channel 1 (VDAC-1) (A and B), chaperone glucose-regulated protein 75 (GRP75) (C and D), and chaperone glucose-regulated protein 78 (GRP78) (E and F) levels in rat striatum. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d), 14 (14 d), and 28 days (28 d). Values are mean $\pm$ standard deviation for six independent experiments (animals) expressed as arbitrary units, normalized by the content of the protein β-actin. *$P < 0.05$, ***$P < 0.001$ compared to control (one-way ANOVA as described in the text, followed by Tukey's multiple comparisons test and Student's t test for unpaired samples).

Figure 8

Figure 8. Effect of 3-nitropropionic acid (3-NP) on synaptophysin (A and B) levels in rat striatum. Rats were injected with PBS or 3-NP (20 mg/kg) and euthanized at 7 (7 d), 14 (14 d), and 28 days (28 d). Values are mean ± standard deviation for six independent experiments (animals) expressed as arbitrary units, normalized by the content of the protein β-actin. *P < 0.05 compared to control (one-way ANOVA as described in the text, followed by Tukey's multiple comparisons test and Student's t test for unpaired samples).
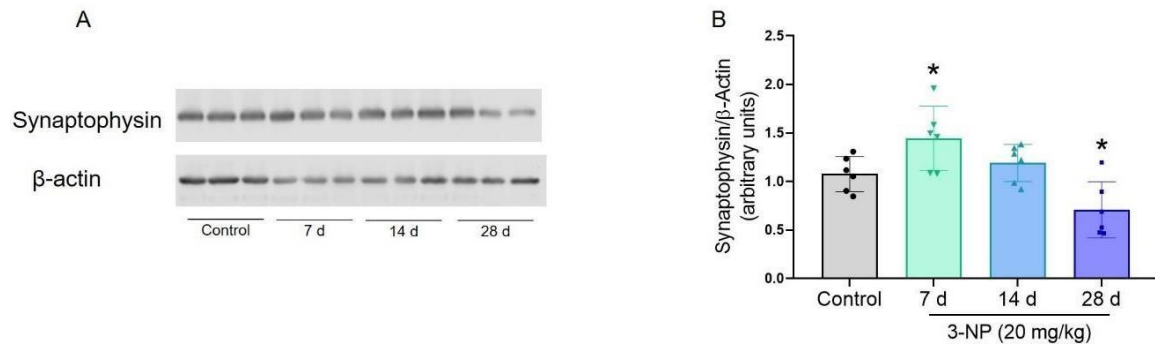
PARTE III

1. DISCUSSÃO

A DH, caracterizada pela primeira vez em 1872 por George Huntington como uma coreia hereditária, apresenta acúmulo de mHTT como o principal mecanismo responsável pelas alterações neuronais, muitas vezes levando à morte celular. Dentre os efeitos ocasionados por essa proteína mutada, podem ser citadas alterações na transcrição, na tradução e na transmissão sináptica (Bates *et al.*, 2015; McColgan e Tabrizi, 2018). Além disso, alguns mecanismos da patogênese da DH estão relacionados à função mitocondrial, como alteração na homeostase do Ca^{2+}, dinâmica mitocondrial, produção em excesso de EROs, alterações na captação de glutamato pelos astrócitos e plasticidade sináptica, com excitotoxicidade causada por ativação exacerbada de receptores NMDA pós-sinápticos (Jimenez-Sanchez *et al.*, 2017). Dessa forma, uma investigação mais ampla dos mecanismos neurotóxicos, em específico aqueles relacionados à mitocôndria e sua interação com outras estruturas celulares, em destaque o RE, pode contribuir para a elucidação de mecanismos patológicos ainda não detalhados e também para o desenvolvimento de alternativas terapêuticas mais eficazes para o tratamento da DH.

Em nosso estudo visamos inicialmente o desenvolvimento de um modelo animal da DH através da administração de 3-NP (20 mg/kg) durante três dias consecutivos em ratos Wistar, como descrito anteriormente por Sandhir et al. (2014). Os parâmetros foram então avaliados no estriado dos animais em quatro diferentes tempos (7, 14, 21 e 28 dias) após o término do tratamento com 3-NP, o que possibilitou observar diferentes estágios de indução da doença. Verificamos que o 3-NP alterou a homeostase redox através da indução de peroxidação lipídica, pois observamos um aumento nos níveis de MDA 28 dias após a administração de 3-NP, bem como uma diminuição substancial dos níveis de GSH, um antioxidante não enzimático, em 21 e 28 dias após o tratamento com 3-NP. Além disso, houve diminuição da atividade das enzimas antioxidantes GPx, GST e SOD nos três tempos avaliados (7, 21 e 28 dias), redução da atividade da CAT em 21 e 28 dias, e diminuição da atividade da GR somente 21 dias após administração do 3-NP. Estes resultados sugerem uma grave alteração no sistema antioxidante, de forma mais direcionada aos tempos mais prolongados de avaliação, ou seja, em 21 e 28 dias. Para avaliar de maneira mais ampla o dano oxidativo causado pelo 3-NP, medimos o conteúdo de HO-1, uma importante enzima com expressão modulada durante o estresse oxidativo (Waza *et al.*, 2018). No entanto, não foi encontrada diferença significativa no nível dessa enzima.

Outro mecanismo importante estudado é o dano à cadeia respiratória causado pelo 3-

NP, que ocorre através da inibição da atividade da SDH do complexo II (Jamwal e Kumar, 2016). Neste sentido, dados demonstram que a mitocôndria contribui significativamente com a produção de celular de EROs. Considerando isso, avaliamos a atividade dos complexos da cadeira respiratória, bem como da enzima SDH, e foi possível observar uma diminuição na atividade dos complexos II, II-III e IV, além da atividade da SDH, sugerindo um prejuízo no metabolismo energético no estriado de animais submetidos ao 3-NP. Apesar de os complexos I e III da cadeia respiratória serem descritos como as principais fontes mitocondriais de EROs (Larosa e Remacle, 2018), também já foi descrito que o complexo II quando inibido é capaz de gerar EROs (Damiano *et al.*, 2013). A produção de EROs pelas mitocôndrias defeituosas pode ocorrer de forma mais exacerbada quando estimuladas por inibidores do complexo II (Johri e Beal, 2012), como o 3-NP.

O mecanismo exato de geração de EROs pelo complexo II não foi totalmente elucidado (Hwang *et al.*, 2014). Algumas hipóteses demonstram que a produção de EROs é consequência de uma dissociação do complexo II, este sendo formado por quatro subunidades SDH (A, B, C e D). Já foi visto que uma queda de pH pode resultar na dissociação das subunidades SDHA e SDHB das subunidades SDHC e SDHD, mas a atividade da SDH é mantida. Essa dissociação do complexo II leva a um aumento na formação de EROs e indução de morte celular via estresseoxidativo (Grimm, 2013; Lagadic-Gossmann, Huc e Lecureur, 2004). Além disso, Wilhelm e colaboradores (2014) demonstraram através do coeficiente de correlação de Pearson que a atividade da SDH foi negativamente correlacionada aos níveis de EROs em estriado de ratos em um modelo animal para DH induzido pelo 3-NP, com uma inibição da atividade de SDH e aumento dos níveis de EROs. Dessa forma, é possível concluir que o estresse oxidativo causadopelos EROs gerados através de um dano à cadeia respiratória mitocondrial desempenha um papel fundamental no dano estriatal causado pelo 3-NP (Wilhelm *et al.*, 2014).

Com o intuito de avaliar melhor o processo oxidativo mitocondrial, utilizamos uma técnica de medida da respirometria de alta resolução feita no equipamento Oroboros. A aplicação do protocolo SUIT com diferentes substratos e inibidores para os complexos da cadeia respiratória permitiu registrar dados e traçar um quadro integrado da função mitocondrial (Makrecka-Kuka, Krumschnabel e Gnaiger, 2015). Observamos diminuição na taxa de consumo de O_2 nos estados 3 e 4, e na respiração desacoplada, usando diferentes substratos (piruvato, malato, glutamato e succinato) 7 dias após o tratamento com 3-NP, sem alterar a capacidade de reserva. Por outro lado, ao contrário do que foi detectado 7 dias após o tratamento com 3-NP, em 28 dias observamos um aumento no consumo de O_2 no estado 4 e na respiração

desacoplada, usando os mesmos substratos, além de um aumento na capacidade reserva. Esses resultados sugerem um desacoplamento mitocondrial causado pelo 3-NP.

Outro estudo avaliou a respiração mitocondrial em córtex cerebral, fígado, músculo sóleo e coração em modelo animal mutante para HD, também através de respirometria de alta resolução, e demonstrou diferenças significativas na atividade complexo II, capacidade máxima desacoplada após adição de FCCP e consumo de oxigênio ligado à produção de ATP (O_2ATP) em córtex cerebral e fígado dos animais mutantes. Ainda foi avaliada a respiração mitocondrialem músculo vasto lateral de pacientes com DH e não houve diferença estatística entre portadores da mutação de expansão da DH e os indivíduos controles (Buck *et al.*, 2017).

Dessa forma, nossos resultados mostram que alguns parâmetros da respiração mitocondrial estão alterados de forma distinta nos diferentes estágios de progressão do dano causado pelo 3-NP. Mais estudos devem ser realizados para o melhor entendimento da disfunção mitocondrial causada pelo 3-NP nos parâmetros respiratórios.

Para avaliarmos com maior propriedade uma importante característica das mitocôndrias, a dinâmica mitocondrial, quantificamos proteínas essenciais para esse processo, como a MFN1, responsável pela fusão, e DRP1, principal proteína relacionada à fissão mitocondrial. Os nossos resultados demonstraram que o 3-NP altera o processo de fusão, com um aumento significativo de MFN1, sem mudanças em DRP1. Alterações nesses processos já são relatados, mas com certa divergência, em diferentes amostras, como tecidos de estriado de modelo animal para DH e culturas celulares de linfoblastos de pacientes com DH (Hering *et al.*, 2017).

O 3-NP já foi descrito como um ativador do receptor NMDA (CENTONZE *et al.*, 2006), induzindo dois eventos como resposta ao dano causado por 3-NP em mitocôndrias de neurônios corticais de ratos por microscopia de fluorescência 3D. O primeiro evento caracteriza-se por um leve aumento de EROs, com uma queda dramática e rápida de ATP, mas sem alteração na morfologia mitocondrial. O segundo evento após horas de exposição resulta em uma ativação de receptores NMDA, com um dramático aumento de EROs associado à fragmentação mitocondrial e morte de células neuronais (Liot *et al.*, 2009). Nossos dados demonstram uma um aumento na fusão, podendo caracterizar uma resposta compensatória, pois as mitocôndrias alongadas apresentam maior eficiência na produção de ATP, o que pode auxiliar na recuperação ao dano causado pelo estresse oxidativo (Shutt e McBride, 2013). Especulamos que nossos achados não evidenciaram fragmentação mitocondrial, como no estudo supracitado,

porque o 3-NP em nosso modelo está sendo injetado intraperitonealmente nos animais, diferentemente da exposição direta de neurônios ao composto tóxico.

A comunicação mitocôndria-RE desempenha importantes funções, principalmente na coordenação da função dessas organelas em processos como a sinalização do Ca^{2+} (Marchi *et al.*, 2018). Considerando isso, que o 3-NP leva à ativação dos receptores tipo NMDA, também presentes em mitocôndria de neurônios (NMDAm) (Korde e Maragos, 2012), e que os níveis de Ca^{2+} tem importante influência na bioenergética mitocondrial (Korde e Maragos, 2021), avaliamos importantes proteínas da comunicação mitocôndria-RE, bem como de estresse de RE. Foi observado um aumento do conteúdo proteico de VDAC1 e GRP75 nos animais avaliados 28 dias após administração de 3-NP, porém não foi encontrada diferença significativa no conteúdo de GRP78. Esses achados sugerem uma alteração no metabolismo do Ca^{2+}. No entanto, investigações adicionais precisam ser realizadas para melhor entender a alteração na comunicação entre essas organelas, como a avaliação de IP3R, proteína do RE, e a transferência de Ca^{2+} propriamente dita.

Com o objetivo de melhor compreender o dano neuronal causado pelo 3-NP em diferentes estágios da maturação cerebral, avaliamos os níveis proteicos da sinaptofisina, uma importante proteína para a atividade sináptica. Observamos que em 7 dias após tratamento com 3-NP ocorreu um aumento nos níveis de sinaptofisina. Além disso, não detectamos diferenças no tempo de 21 dias após administração de 3-NP, enquanto em 28 dias foi vista uma diminuição do conteúdo dessa proteína no estriado. Assim, presumimos que no estágio inicial do dano causado pelo 3-NP ocorre um aumento de sinaptofisina como um mecanismo compensatório visando à manutenção dos processos de sinapse, seguido de uma estabilização da expressão e finalizando com uma resposta de declínio da densidade sináptica, podendo ser essa transição uma característica da neuropatologia da doença.

Uma resposta transitória de eventos sinápticos já foi relatada em neurônios corticais de um modelo animal de DH (R6/2), ocorrendo uma redução progressiva da atividade sináptica em um estágio mais avançado da DH. A observação demonstrou uma diminuição na frequência de corrente pós-sináptica excitatória acompanhada por diminuições nas proteínas chaves pré-sináptica e pós-sináptica, sinaptofisina e PSD95, em animais mais velhos (11-15 semanas), acompanhando o avanço do dano na DH. Quando avaliados animais mais jovens (3-4 semanas), foram vistos níveis similares entre os animais R6/2 e os animais do grupo controle (Cepeda *et al.*, 2003).

A HTT possui um papel importante na conectividade sináptica, estando associada a terminais pré- e pós-sinápticos (Jimenez-Sanchez *et al.*, 2017). Neste particular, alterações na função sináptica e na plasticidade já foram descritos na DH (Smith-Dijak, Sepers e Raymond, 2019). Contudo, ainda é necessário estabelecer se o acúmulo de mHTT o evento primário responsável pelas alterações sinápticas.

O 3-NP já é bem estabelecido como um indutor químico para modelos animais de HD por causar danos que mimetizam a sintomatologia da doença, porém os protocolos utilizados diferem bastante com relação a tempo de administração e avaliação dos parâmetros (Brouillet, 2014; Ramaswamy, McBride e Kordower, 2007). Em nosso estudo, foi observado que o tempo que melhor demonstra o estágio degenerativo da DH é o de 28 dias após a administração de 3-NP em uma concentração de 20 mg/kg, caracterizado por proeminente disfunção mitocondrial, com prejuízo na homeostase redox e do cálcio, dano na dinâmica mitocondrial, bem como uma degeneração sináptica. A utilização de compostos químicos para mimetizar o padrão de neurodegeneração encontrada em pacientes DH é amplamente realizada, sendo o ácido quinolínico (AQ) e o ácido caínico outros compostos administrados desde a década de 1970 com o intuito de descobrir os mecanismos patogênicos da HD (Kosior e Leavitt, 2018).

Estudos com o ácido caínico mostraram semelhança com as lesões encontradas na DH, porém é considerado um modelo imperfeito de DH por apresentar significativa diminuição dos níveis de somatostatina e perda de neurônios da somatostatina (Flint Beal *et al.*, 1985). Por outro lado, as lesões produzidas pelo ácido quinolínico, agonista do receptor NMDA, fornecem um modelo melhor da DH porque resultam em economia relativa de somatostatina e neuropeptídeo Y, apesar de depleções significativas de níveis de GABA e substância P. Já foi demonstrado, no entanto, que na DH há aumento nas concentrações de somatostatina e neuropeptídeo Y (Beal *et al.*, 1991). O modelo 3-NP parece ser confiável para estudar a DH, devido à capacidade deste composto em atravessar a barreira hematoencefálica, sendo possível a realização de uma administração sistêmica. Com isso, tal modelo com 3-NP simula um processo posterior de morte celular observado na DH, bem como o comprometimento mitocondrial (Ramaswamy, McBride e Kordower, 2007).

2. CONCLUSÃO

Nossos achados em conjunto evidenciam que a indução da DH em modelo animal utilizando o 3-NP ocasiona um extenso dano mitocondrial, com ação em diferentes processos,

como a homeostase redox e do Ca^{2+}, essa última, em especial, sendo causada por alterações na interação entre mitocôndria e RE. Além disso, nossos dados demonstram um estado de hiperfusão mitocondrial sugerindo uma tentativa de adaptação ao estresse e uma resposta sináptica transitória, buscando uma possível sobrevida neuronal em resposta ao dano induzido pelo 3-NP. Por fim, podemos sugerir também que a tentativa de reverter o dano induzido pelo 3-NP não ocorre, mas sim um agravamento da lesão com o decorrer do tempo.

3. PERSPECTIVAS

i. Melhor avaliar a interação mitocôndria-RE através da quantificação de importantes proteínas, como o IP3R, no modelo de indução de DH por 3-NP;

ii. Melhor estudar as modificações pós-traducionais das proteínas que atuam na dinâmica mitocondrial, como DRP1;

iii. Avaliar a homeostase do cálcio em mitocôndrias isoladas de estriado após a administração de 3-NP;

iv. Avaliar os efeitos de antagonistas glutamatérgicos (por exemplo, MK-801) em mitocôndrias isoladas e homogeneizado de estriado após a administração de 3-NP;

v. Avaliar o dano causado pelo 3-NP em córtex cerebral;

vi. Avaliar o efeito protetor da administração de bezafibrato, um agonista de receptores PPAR que induz biogênese mitocondrial, frente ao dano causado pelo 3-NP.

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ANEXO I

Molecular Neurobiology - Submission guidelines

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• Book chapter

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