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PAPEL DA ÓXIDO NÍTRICO SINTASE INDUZÍVEL NO CÓRTEX CEREBRAL DE MODELOS EXPERIMENTAIS DA ACIDEMIA METILMALÔNICA

TESE DE DOUTORADO

Leandro Rodrigo Ribeiro

Santa Maria, RS, Brasil
2012

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Leandro Rodrigo Ribeiro

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Luiz Fernando Freire Royes
Co-Orientadora: Prof[a]. Dr[a]. Michele Rechia Fighera

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Universidade Federal de Santa Maria
Centro De Ciências Naturais E Exatas
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elaborada por
Leandro Rodrigo Ribeiro

Como requisito parcial para a obtenção do grau de
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"O título nada vale,
a superioridade real é que tem valor."

(Allan Kardec)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
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PAPEL DA ÓXIDO NÍTRICO SINTASE INDUZÍVEL NO CÓRTEX CEREBRAL DE MODELOS EXPERIMENTAIS DA ACIDEMIA METILMALÔNICA

Autor: Leandro Rodrigo Ribeiro
Orientador: Luiz Fernando Freire Royes
Co-Orientadora: Michele Rechia Fighera
Local e data da defesa: Santa Maria, 15 de Dezembro de 2012.

A Acidemia Metilmalônica é um erro inato do metabolismo caracterizado bioquimicamente e clinicamente pelo acúmulo tecidual de ácido metilmalônico (MMA) e disfunção neurológica, incluindo convulsões e déficit cognitivo. Além disso, dados clínicos sugerem que quadros infecciosos podem precipitar crises metabólicas e causar as alterações neurológicas observadas nos pacientes com essa acidemia. Desde que o MMA causa complicações neurológicas, e que a inflamação pode contribuir para a ocorrência de convulsões e déficits cognitivos em vários modelos animais, é possível sugerir que mediadores inflamatórios, como a enzima Óxido Nítrico Sintase Induzível (iNOS), facilitem as convulsões induzidas por MMA. A iNOS é uma das três isoformas da enzima Óxido Nítrico Sintase (NOS), que gera o óxido nítrico (NO), uma molécula gasosa simples, sinalizadora e um radical livre. A iNOS é induzida em sítios de lesão/inflamação, mas também se expressa constitutivamente em algumas células, como nos neurônios. Estudos em modelos experimentais já demonstraram que o NO gerado no sistema nervos central (SNC), pelas isoformas endotelial e neuronal da NOS, tem envolvimento nas convulsões induzidas por MMA. Contudo, até o presente momento são escassos os dados na literatura avaliando a relação da iNOS em modelos experimentais da Acidemia Metilmalônica. Os resultados publicados no artigo mostraram que camundongos C57BL/6 nocaute para iNOS, ao serem injetados agudamente com MMA (2 µmols/2 µL, via intracerebroventricular), apresentam uma duração menor das convulsões, sem alteração significativa na amplitude média das ondas eletroencefalográficas (EEG); não aumentam os níveis de nitrito e nitrato (NOx) comparado aos animais injetados com solução salina, mas têm uma redução parcial nos níveis de 3-nitrotirosina (3-NT) comparado aos animais selvagens que também foram tratados com MMA; semelhantemente, mostram uma inibição parcialmente menor na atividade da enzima Na^+,K^+-ATPase; mas não exibem diferença na inibição da atividade da succinato desidrogenase (SDH) no córtex cerebral quando comparados aos camundongos selvagens que também receberam MMA. Os resultados apresentados no manuscrito submetido mostram que ratos Wistar, após serem injetados cronicamente com MMA (do 5º ao 28º dia de vida, duas vezes ao dia, com doses variando de 0,76 à 1,67 mmol/g em função da idade do animal, via subcutânea), apresentam um reduzido índice de reconhecimento em teste de memória/aprendizado espacial, mas não demonstram ansiedade no teste do labirinto em cruz elevado; têm uma redução no número de neutrófilos, mas um aumento no número de leucócitos mononucleares no sangue; e além disso mostram aumento nos níveis de interleucina-1beta (IL-1β), do fator de necrose tumoral-alfa (TNF-α), de iNOS e de 3-NT no córtex cerebral. Considerando os dados apresentados nos dois estudos, concluiu-se que o MMA pode causar convulsões, estresse nitrosativo e inibição da enzima Na^+,K^+-ATPase no córtex cerebral de camundongos por mecanismos relacionados à produção de NO via iNOS; e que o MMA também pode causar déficit neurocognitivo, alteração do sistema imunológico no sangue, e aumento de citocinas pró-inflamatórias, levando ao aumento na expressão da iNOS e estresse nitrosativo.

Palavras-chave: Óxido Nítrico Sintase Induzível. Acidemia metilmalônica. Metilmalonato. Córtex Cerebral. Convulsão. Neuroinflamação.

ABSTRACT

Doctoral Thesis
Postgraduate Program in Biological Science: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE IN CEREBRAL CORTEX OF EXPERIMENTAL MODELS FOR METHYLMALONIC ACIDEMIA

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Place and date of defense: Santa Maria, December 15th, 2012.

Methylmalonic acidemia is an inborn error of metabolism characterized clinically and biochemically by tissue accumulation of methylmalonic acid (MMA) and neurological dysfunction, including convulsion. Furthermore, clinical data suggest that infections conditions can precipitate metabolic crisis and cause neurological changes observed in patients of acidemia. Provided that the MMA cause neurological complications, and that the inflammation can contribute to the occurrence of convulsions and cognitive deficit in several animal models, it is possible to suggest that inflammatory mediators, such as inducible nitric oxide synthase (iNOS), facilitate MMA-induced convulsions. The iNOS is one of three isoforms of nitric oxide synthase (NOS), which generates nitric oxide (NO), a simple gaseous signaling molecule and free radical. The iNOS is induced at injury/inflammation sites, but is also constitutively expressed on some cells, such as in neurons. Studies in experimental models have already demonstrated that NO generated in the central nervous system (CNS), by endothelial and neuronal isoforms of NOS, is involved in MMA-induced convulsions. However, until the present moment are scarce the data in the literature evaluating the relationship of iNOS in experimental models of Methylmalonic Acidemia. The results published in the article has shown that iNOS knock-out C57BL/6 mice, when injected acutely with MMA (2 μmols/2 μl, intracerebroventricularly), have a shorter duration of seizures, no significant change in the mean amplitude of electroencephalographic waves (EEG); not increase the levels of nitrite and nitrate (NOx) compared to animals injected with saline, but have a partial reduction in the levels of 3-nitrotyrosine (3-NT) compared to wild animals that were also treated with MMA; similarly, show a partially lower inhibition of Na^+,K^+-ATPase, but exhibit no difference in succinate dehydrogenase (SDH) inhibition on cerebral cortex compared to wild mice which also received MMA. The results submitted in the manuscript has shown that Wistar rats, after being injected chronically with MMA (from 5th to 28th day of life, twice daily, with doses ranging from 0.76 to 1.67 mmol/g depending on the age of the animal, via subcutaneous) showed a reduced index of recognition in spatial learning/memory test, but show no anxiety at elevated plus maze test; they have a reduction in neutrophils, but an increase in the number of mononuclear leukocytes in the blood; and in addition show increased levels of interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), iNOS and 3-NT in the cerebral cortex. Considering the data presented in both studies, it was concluded that the MMA can cause seizures, nitrosative stress and inhibition of Na^+,K^+-ATPase activity in cerebral cortex of mice by mechanisms related to NO production via iNOS; and that the MMA can also cause neurocognitive deficits, altered immune system in blood and increase of pro-inflammatory cytokines, leading to increased expression of iNOS and nitrosative stress.

Keywords: Inducible Nitric Oxide Synthase. Methylmalonic acidemia. Methylmalonate. Cerebral Cortex. Convulsion. Neuroinflammation.

LISTA DE FIGURAS E TABELAS

LISTA DE ABREVIATURAS E SIGLAS

3-NT	3-nitrotirosina
7-NI	7-nitroindazol
AdoCbl	5'-desoxiadenosilcobalamina
ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato, Trifosfato de adenosina
ATPase	Família de enzimas que catalisam a hidrólise da adenosina trifosfato para originar adenosina difosfato (ADP)
Ca^{14}	Carbono-14
Ca^{2+}	Cálcio
CaM	Calmodulina
cbl	Vitamina $B_{12,}$ cobalamina
cblA	Variante da Acidemia Metilmalônica devido à mutação no gene 607481
cblB	Variante da Acidemia Metilmalônica devido à mutação no gene 607568
cblC	Variante da Acidemia Metilmalônica devido à mutação no gene 609831
cblD	Variante da Acidemia Metilmalônica devido à mutação no gene 611935
cblF	Variante da Acidemia Metilmalônica devido à mutação no gene 612625
EC	*Enzyme Commission Numbers*, esquema de classificação numérica para as enzimas
EEG	Eletroencefalográfico, eletroencefalográfica(s), eletroencefalograma, eletroencefalografia
EIMs	Erros Inatos do Metabolismo
eNOS, NOS-3	Óxido Nítrico Sintase Endotelial
ERN	Espécie reativa de nitrogênio
ex.	Exemplo
FAD	Dinucleótideo de flavina e adenina
FMN	Mononucleotídeo de flavina
GC	Guanilato ciclase
GC-MS	Cromatografia gasosa e espectrometria de massa
i.c.v.	Intracerebroventricular

IL-1β	Interleucina-1beta
iNOS, NOS-2	Óxido Nítrico Sintase Induzível
K^+	Potássio
L-NAME	N_ω-nitro-L-arginina metil éster
LPS	Lipopolissacarídeos
MCM	Metilmalonil-CoA mutase
MeCbl	Metilcobalamina
MIM	*Mendelian Inheritance in Man*, McKusick, base de dados que cataloga todas as doenças humanas que tenham uma componente genética
MMA	Metilmalonato, ácido metilmalônico
MS-MS	Espectrometria de massa em *tandem*
MTR	Metionina sintase, 5-metiltetrahidrofolato-homocisteína S-metiltransferase
MUT	Gene 609058 que codifica a enzima metilmalonil-CoA mutase
MUT^-	Perda parcial na atividade da metilmalonil-CoA mutase
MUT°	Perda total na atividade da metilmalonil-CoA mutase
Na^+	Sódio
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido
nNOS, NOS-1	Óxido Nítrico Sintase Neuronal
NO	Óxido nítrico
NOS	Óxido Nítrico Sintase
NOx	Nitrito e nitrato
OH-Cbl	Hidroxicobalamina
$ONOO^-$	Peroxinitrito
QI	Coeficiente de Inteligência
RNA	Ácido ribonucleico
s.c.	Subcutânea
SDH	Succinato desidrogenase
SNC	Sistema nervoso central
TNF-α	Fator de necrose tumoral-alfa

SUMÁRIO

1 INTRODUÇÃO

1.1 Metabolismo

Os organismos vivos são sistemas altamente ordenados, que apresentam uma multiplicidade de componentes biomoleculares interagindo entre si, de maneira integrada. Essa maquinaria complexa, composta por milhares de reações bioquímicas e processos de transporte, é responsável pelo funcionamento, sobrevivência e reprodução das células. A integração entre as cadeias de reações e suas ramificações pode levar à síntese ou degradação de compostos orgânicos e, ao mesmo tempo, maximizar o retorno do fluxo de energia através da matéria viva. Em um sentido amplo, essas redes bioquímicas são responsáveis pelas funções metabólicas básicas que alimentam a maquinaria molecular e os mecanismos internos da vida. Da mesma forma, vias complexas de sinalização resultam na transferência de informações para maximizar a eficiência e resposta celular (CAETANO-ANOLLES et al., 2009). Tanto as vias metabólicas quanto as de sinalização são sistemas altamente adaptados, desenvolvidos durante bilhões de anos de evolução, mas algumas vezes podem haver falhas em determinados genes, causando alterações na expressão ou atividade de enzimas ou transportadores e, consequentemente, na etapa metabólica da qual fazem parte.

1.2 Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIMs) são baseados ou em deficiências enzimáticas, que levam ao acúmulo de substratos e carência de produtos da reação enzimática afetada, ou na deficiência de transportadores, resultando no acúmulo de substratos em certos compartimentos celulares (ILLSINGER; DAS, 2010). Este bloqueio nas rotas metabólicas, além de induzir acúmulo de substâncias tóxicas e/ou a falta de substâncias essenciais, pode gerar distúrbios no desenvolvimento físico e mental (OBERHOLZER et al., 1967).

O termo EIMs foi empregado pela primeira vez no início do século XX (1908), pelo médico britânico Archibald Edward Garrod (1857-1936), um pioneiro nos estudos sobre

alcaptonúria e outros EIMs. Hoje, os EIMs também são chamados de doenças metabólicas congênitas ou doenças metabólicas hereditárias. Devido aos avanços nos estudos genéticos e técnicas de diagnóstico, já foram catalogados mais de 700 EIMs (ILLSINGER; DAS, 2010). Analisados individualmente são considerados raros, mas coletivamente são responsáveis por significantes níveis de morbidade e mortalidade, apresentando uma incidência de aproximadamente 1:800 nascidos vivos (PAMPOLS, 2010).

A maioria dos EIMs são doenças associadas a um único gene, e eles podem ser classificados em três grupos principais: (i) doenças que dão origem à intoxicação, através do acúmulo de compostos intracelulares ao longo do tempo; (ii) doenças envolvendo o metabolismo energético; e (iii) doenças envolvendo o metabolismo de moléculas complexas (SAUDUBRAY; CHARPENTIER, 1995; SAHOO et al., 2012).

As doenças que causam intoxicação aguda ou crônica englobam as acidemias orgânicas (ex.: metilmalônica, propiônica, isovalérica), aminoacidopatias (ex.: fenilcetonúria, homocistinúria, tirosinemia), doenças do ciclo da ureia, intolerância ao açúcar (ex.: galactosemia, intolerância hereditária a frutose), intoxicação por metais (ex.: doença de Wilson, doença de Menkes, hemocromatose), e porfirias. Todas estas doenças compartilham similaridades clínicas: não interferem no desenvolvimento embrionário e apresentam um período livre de sintomas e sinais clínicos da intoxicação, que podem ser agudos (vômito, coma, falha renal, complicações tromboembólicas) ou crônicos (falha no crescimento, atraso no desenvolvimento, cardiomiopatia, *ectopia lentis*) (SAUDUBRAY; SEDEL; WALTER, 2006).

1.3 Acidemias Orgânicas

O termo “acidúria orgânica” ou “acidemia orgânica” é utilizado para designar EIMs que ocorrem devido à deficiência, total ou parcial, na atividade de alguma enzima envolvida no metabolismo de aminoácidos, lipídeos ou carboidratos. Isto leva ao acúmulo de um ou mais ácidos orgânicos nos líquidos biológicos e tecidos dos pacientes afetados (CHALMERS; LAWSON, 1983).

O recém-nascido afetado com uma acidemia orgânica geralmente parece bem logo após o nascimento e nos primeiros dias de vida. Os sintomas das acidemias orgânicas são frequentemente manifestados durante crises agudas precipitadas por estresse catabólico.

Durante esses episódios ocorre um déficit energético, com a consequente mobilização dos estoques de carboidratos, ácidos graxos e proteínas. Estes sofrem catabolismo, mas devido ao bloqueio na rota metabólica, ácidos orgânicos e outros compostos se acumulam nos órgãos, tecidos e fluídos corporais. Neste contexto, tem sido sugerido que alguns desses metabólitos podem agir como toxinas endógenas e serem neurotóxicos (MCLAUGHLIN et al., 1998).

As apresentações clínicas e laboratoriais que denunciam uma possível acidemia orgânica são: encefalopatia tóxica, pobre alimentação, acidose metabólica, cetose, acetonúria, vômito, inapetência, suor com odor característico, hipo/hiperglicemia, hiperamonemia, neutropenia, baixos níveis de bicarbonato, dano aos gânglios da base com distúrbios do movimento, distonia-parkinsonismo, polineuropatia, paraplegia espástica, ataxia, e outros sintomas neurológicos como regressão neurológica, tremores, movimentos coreatetóticos, atraso no desenvolvimento psicomotor, retardo mental, convulsões, tônus anormal, letargia e progressão para o coma (SEASHORE, 1993; SCRIVER et al., 2000; SAUDUBRAY; SEDEL; WALTER, 2006).

Na Holanda, as acidemias orgânicas tem prevalência de 1 para cada 2.200 habitantes, enquanto na Arábia Saudita, onde a taxa de consanguinidade é elevada, é de pelo menos 1 para cada 740 nascimentos (HOFFMANN, 1994; RASHED, M. et al., 1994). Dentre as acidemias, encontram-se a Acidemia Isovalérica, Acidemias Láticas, Deficiência da 3-metilcrotonil CoA Carboxilase, Deficiência de Biotinidase, Acidemia 3-metilglutacônica, Acidemia 3-hidroxi 3-metilglutárica, Acidemia Glutárica Tipo I, Acidemia Propiônica, e Acidemia Metilmalônica (SAUDUBRAY; CHARPENTIER, 1995; WAJNER et al., 2001).

1.4 Acidemia Metilmalônica

1.4.1 Definição

O erro inato do metabolismo conhecido como Acidemia Metilmalônica (MIM 251000, sinônimo a Acidúria Metilmalônica) foi descrito pela primeira vez por Oberholzer e colaboradores em 1967, quando observaram crianças em estado crítico, com profunda cetoacidose metabólica e retardo no desenvolvimento, as quais apresentavam grandes quantidades de metilmalonato (MMA) no sangue e na urina (OBERHOLZER et al., 1967).

Esta acidemia compreende um grupo de doenças genéticas autossômicas recessivas, caracterizadas pela deficiência na atividade da metilmalonil-CoA mutase (MCM, (R)-2-metil-3-oxo propanoil-CoA CoA-carbonil mutase, EC 5.4.99.2), ou defeitos na captação, transporte ou síntese de seu cofator, a 5'-desoxiadenosilcobalamina (AdoCbl), formada a partir da vitamina B_{12} (cobalamina, cbl) (OBERHOLZER et al., 1967; CHANDLER et al., 2009). Essa deficiência enzimática geralmente é causada pela mutação no gene 609058 (MUT), que codifica a MCM, o que pode levar a uma perda parcial (MUT^{-}) ou total (MUT°) na atividade da mesma (MANOLI; VENDITTI, 1993; CHANDLER; VENDITTI, 2005; TANPAIBOON, 2005).

A MCM é uma enzima presente na matriz mitocondrial e é responsável pela isomerização de metilmalonil-CoA, gerado na rota de metabolismo dos aminoácidos isoleucina, metionina, treonina e valina, e ácidos graxos de cadeia ímpar e colesterol, em succinil-CoA, um intermediário do Ciclo de Krebs (Figura 1) (ACQUAVIVA et al., 2005; LEE et al., 2008). Quando sua atividade está diminuída, há o acúmulo primário de MMA (entre 1 e 2,5 mM no sangue), e o acúmulo secundário de outros metabólitos formados por tiólise espontânea, como propionato, 3-hidroxipropionato, 2-metilcitrato e cetonas de cadeia longa, nos órgãos, tecidos e fluídos corporais (FENTON; ROSENBERG, 1995; OKUN et al., 2002). Neste caso, por não haver a combinação com homocistinúria, o termo utilizado é Acidemia Metilmalônica isolada (MORATH; HORSTER; SAUER, 2012).

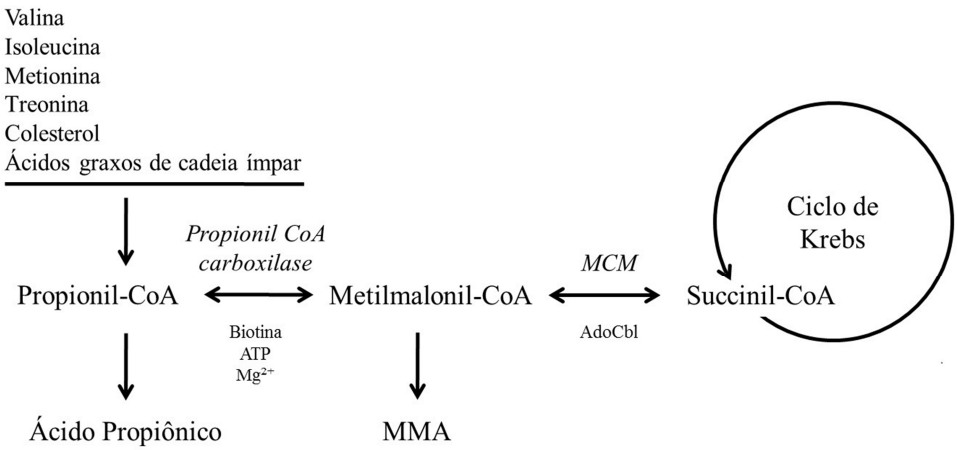

Figura 1 - Rota metabólica da qual faz parte a enzima MCM (FENTON; ROSENBERG, 1995).

Há outras variações desta acidemia, como as relacionadas à síntese da AdoCbl, o cofator para MCM. Conhecidas como cblA (MIM 251100) e cblB (MIM 251110), essas variantes são caracterizadas, respectivamente, pela mutação no gene 607481, que codifica a enzima envolvida na translocação da cbl para dentro da mitocôndria, onde ocorrem os passos finais da síntese da AdoCbl; ou pela mutação no gene 607568, que codifica a cob(I)alamina adenosiltransferase (EC 2.5.1.17), a qual catalisa a etapa final da síntese de AdoCbl (DOBSON et al., 2002a; DOBSON et al., 2002b).

Já outro grupo da Acidemia Metilmalônica é combinado à homocistinúria. Quando há interferência na ingestão, captação, absorção, transporte intestinal, fornecimento, ou metabolismo precoce da cbl, existe uma perturbação na síntese de AdoCbl e/ou de metilcobalamina (MeCbl), o cofator da metionina sintase (5-metiltetrahidrofolato-homocisteína S-metiltransferase, MTR, EC 2.1.1.13), ocorrendo então o acúmulo de homocisteína. A este grupo pertencem a cblC (MIM 277400), cblD (MIM 277410) e cblF (MIM 277380), causadas pela mutação no gene 609831, 611935 e 612625, respectivamente (COELHO et al., 2008; RUTSCH et al., 2009; LIU et al., 2010).

1.4.2 Incidência

Muitos estudos têm verificado a incidência desta acidemia em diversos países. Estima-se que 1:6.900 nascidos vivos é afetado pela Acidemia Metilmalônica na Arábia Saudita, 1:50.000 no Japão, e varia de 1:115.000 na Itália até 1:169.000 na Alemanha (COULOMBE; SHIH; LEVY, 1981; RASHED, M. S.; RAHBEENI; OZAND, 1999; SHIGEMATSU et al., 2002; DEODATO et al., 2006).

1.4.3 Apresentação Clínica e Laboratorial

Quando o quadro laboratorial se instala precocemente, até os 21 dias pós-natal, o paciente geralmente vai a óbito ou apresenta maior morbidade (OGIER; CHARPENTER; SAUDUBRAY, 1990), mas quando o distúrbio ocorre de 2 a 24 meses de vida, os pacientes apresentam um prognóstico melhor, com alterações metabólicas menos intensas, desde a

ausência de comprometimento neurológico até cetoacidose leve e ataxia (VAN DER MEER et al., 1994).

Os sinais clínicos geralmente começam nos bebês após uma gestação e parto normais. Passado um intervalo inicial livre de sintomas, que varia de horas até as primeiras semanas depois do nascimento, estes sinais surgem e progridem de inespecíficos sintomas gastrintestinais, tais como pobre sucção, recusa alimentar, vômitos, perda excessiva de peso, distensão abdominal, para manifestações neurológicas progressivas. Estas incluem postura e movimentos anormais, hipotonia generalizada, letargia e convulsões, muitas vezes com um padrão eletroencefalográfico (EEG) de surto-supressão. Se não for prontamente e adequadamente tratada, leva ao coma, edema cerebral, insuficiência respiratória, hipotermia, e os pacientes morrem em poucos dias ou desenvolvem lesão cerebral permanente (DEODATO et al., 2006).

Os pacientes também podem apresentar sinais e sintomas como falha no desenvolvimento, discinesia, acidose lática, desidratação, hepatomegalia, trombocitopenia, anemia, pancitopenia, hiper/hipoglicemia, degeneração seletiva dos núcleos da base e do córtex cerebral, hipomielinização, e encefalopatia, culminando em falha múltipla de órgãos e déficit neurológico permanente. A acidose metabólica (cetoacidose severa, aumento do ânion gap), neutropenia, hipo/normo/hiperglicinemia, e hiperamonemia são as principais características laboratoriais, e o MMA é excretado em grandes quantidades na urina (CHALMERS; LAWSON, 1983; MANOLI; VENDITTI, 1993; KOLKER et al., 2003; DEODATO et al., 2006; KANAUMI et al., 2006; LEE et al., 2008).

Em longo prazo, a principal complicação é o comprometimento neurológico. Isto ocorre devido a: lesões simétricas dos gânglios da base, principalmente localizadas bilateralmente no globo pálido; atrofia cerebral e cerebelar; gliose reativa; hipomielinização ou atraso na mielinização; hemorragia cerebelar multifocal; anormalidades na substância branca; pequenos focos hemorrágicos e necróticos no núcleo caudato, cerebelo e tronco encefálico; alterações esponjosas espalhadas pelo córtex cerebral, substância branca, núcleos do tronco encefálico e córtex cerebelar; dilatação dos ventrículos; alargamento dos sulcos; mudanças na capsula interna; calcificação, atrofia e necrose dos gânglios da base; afinamento do corpo caloso (HEIDENREICH et al., 1988; BRISMAR; OZAND, 1994; ENNS et al., 1999; BIANCHERI et al., 2001; KANAUMI et al., 2006; RADMANESH et al., 2008).

Doenças febris, alta ingestão proteica e pequenas infecções são condições conhecidas por induzir um estado catabólico e precipitar os sintomas listados anteriormente, característicos de crises metabólicas agudas nos pacientes com a Acidemia Metilmalônica. A

sobrevivência às crises reincidentes tem aumentado nas últimas décadas, contudo as complicações que se sucedem são bastante relevantes. Logo, a terapia apropriada deve ser empregada o mais cedo possível, evitando que esses episódios ocorram repetidamente e levem ao retardo psicomotor e atraso no desenvolvimento (HORSTER; HOFFMANN, 2004; HORSTER et al., 2007).

Como complicações secundárias pode-se citar a deficiência intelectual, que nem sempre está presente, mas tem relação com o estado de descompensação, e pode depender da magnitude e duração da hiperamonemia (MANOLI; VENDITTI, 1993). A hiperamonemia ocorre devido ao aumento no *pool* de ésteres de acil-CoA mitocondrial, que apresentam um efeito inibitório sobre a N-acetilglutamato sintetase, reduzindo a conversão de amônia para ureia em 70% dos pacientes com esta acidemia (FENTON; ROSENBERG, 1995; MORATH et al., 2008). Num estudo recente, observou-se que apenas 50% dos indivíduos com MUT°, 85% com MUT^{-}, 48% com cblA, e 70% com cblB apresentavam um QI acima de 90 (HORSTER et al., 2007).

Outras complicações secundárias são o risco para desenvolvimento de nefrite túbulo-intersticial com a progressiva insuficiência renal; pancreatite aguda ou crônica; falha no crescimento tanto pela má nutrição quanto pela doença crônica; aumento da susceptibilidade a infecções severas pela queda na função imune; e atrofia do nervo óptico (WONG et al., 1992; MANOLI; VENDITTI, 1993; KAHLER et al., 1994; WILLIAMS et al., 2009).

1.4.4 Diagnóstico

O diagnóstico definitivo da Acidemia Metilmalônica começa pela análise de metabólitos excretados na urina ou acumulados no plasma. Isto pode ser mensurado pelo método da cromatografia líquida ou gasosa, associada à espectrometria de massa. Pacientes com a acidemia apresentam grandes quantidades de MMA, e também, de metilcitrato, 3-hidroxipropionato, lactato e outros derivados do propionil-CoA, como a propionilglicina (DEODATO et al., 2006; FOWLER; LEONARD; BAUMGARTNER, 2008).

Bebês normais apresentam concentrações de MMA na urina que variam de acordo com a idade, com valores médios de 1,1, 5,2 e 0,8 mmol/mol de creatinina, até os 30 dias, 1-6 meses, e de 6-12 meses de idade, respectivamente (BOULAT et al., 2003). Já os bebês que apresentam Acidemia Metilmalônica leve têm concentrações de MMA que variam entre 10 e

20 mmol/mol de creatinina, e os que apresentam um distúrbio severo podem ter concentrações de até 20.000 mmol/mol de creatinina (FOWLER; LEONARD; BAUMGARTNER, 2008).

A mensuração da homocisteína no plasma sanguíneo é essencial para o diagnóstico diferencial da Acidemia Metilmalônica. A partir disto, verifica-se a possibilidade da acidemia estar combinada a um defeito no metabolismo ou transporte da cobalamina, ou a causas nutricionais (FOWLER; JAKOBS, 1998; FOWLER; LEONARD; BAUMGARTNER, 2008).

Existe também um perfil anormal de acilcarnitina no plasma, com a propionilcarnitina como o éster de carnitina predominante, formada pela transferência do grupamento acil do propionil-CoA. Assim, a carnitina livre é reduzida, e a relação de propionilcarnitina para carnitina livre é aumentada (DEODATO et al., 2006). Para mensurar os níveis destas, utiliza-se o método da espectrometria de massa em *tandem* (MS-MS) (MORATH; HORSTER; SAUER, 2012).

São recomendadas, além das análises dos níveis de ácidos orgânicos na urina e no sangue com o uso de cromatografia gasosa e espectrometria de massa (GC-MS), outras avaliações, tais como: verificar na química sérica os níveis de Na^+, K^+, Cl^-, glicose, ureia, aspartato aminotransferase (AST), alanina aminotransferase (ALT), fosfatase alcalina, bilirrubina, triglicerídeos, e colesterol; a contagem das células sanguíneas com diferencial; os gases no sangue venoso ou arterial; a concentração de amônia plasmática; a urinálise formal; e a quantificação de aminoácidos plasmáticos (MANOLI; VENDITTI, 1993). A partir daí são realizados os testes para se estabelecer qual o subtipo de Acidemia Metilmalônica afeta o paciente. Primeiramente, verifica-se a resposta dos indivíduos afetados quando administrados com vitamina B_{12}, preferencialmente hidroxicobalamina (OH-Cbl), 1 mg/dia/5 dias, pela via intramuscular ou intravenosa. Após estas administrações, novamente são verificados os níveis de metabólitos relacionados à acidemia, tanto no plasma quanto na urina. Quedas nos níveis destes metabólitos, superiores a 50%, são indicadores de uma resposta benéfica à OH-Cbl (FOWLER; LEONARD; BAUMGARTNER, 2008).

Um ensaio padronizado por Morrow e colaboradores (1975) também é utilizado para, indiretamente, mensurar a atividade da MCM. A partir de fibroblastos (células constituintes do tecido conjuntivo) do indivíduo com sinais da acidemia, são geradas culturas de células. Nestas, é adicionado o propionato radioativo (ácido propiônico marcado com carbono-14, C^{14}) e, após sua ativação intramitocondrial à propionil-CoA e metabolismo, observa-se a incorporação do C^{14} em macromoléculas subsequentes desta rota metabólica. Caso o C^{14} não seja encontrado nestas, significa que há um bloqueio metabólico, mas não define que este ocorra na MCM (MORROW et al., 1975). Para verificar se o bloqueio é no passo catalisado

pela MCM, o mesmo ensaio bioquímico pode ser realizado na presença/ausência do cofator para esta enzima, a AdoCbl (BAUMGARTNER, 1983).

Após exames complementares, é realizado o teste de análise da mutação. Atualmente, o diagnóstico molecular deste EIM verifica mais de 200 mutações somente para o gene MUT, que codifica a MCM (WORGAN et al., 2006; LEMPP et al., 2007).

1.4.5 Tratamento

O tratamento emergencial do recém-nascido com esta acidemia é composto principalmente de reidratação e promoção de anabolismo. A acidose é controlada pela reidratação, sendo que a terapia com bicarbonato só deve ser utilizada no caso do pH não normalizar. Simultaneamente, a maioria destes casos neonatais é beneficiado por uma rápida remoção das toxinas, como uma transfusão de sangue, a qual é bem sucedida para assegurar a retirada parcial de MMA acumulado (OGIER DE BAULNY; SAUDUBRAY, 2002; DEODATO et al., 2006).

Uma dieta com alto teor calórico, mas baixa ingestão de proteína deve ser instituída. As proteínas a serem ingeridas devem ser essenciais para o crescimento, e a restrição dietética de aminoácidos propiogênicos é um importante tratamento para reduzir os metabolitos circulantes (MANOLI; VENDITTI, 1993; CHANDLER; VENDITTI, 2010). A energia é suprida pela administração parenteral de glicose (80-120 kcal/kg/dia) e pela infusão nasogástrica dos alimentos altamente calóricos. Além disto, a insulina também pode ser utilizada para indução do anabolismo (DEODATO et al., 2006).

Geralmente aqueles pacientes que possuem o fenótipo MUT^{-}, ou aqueles com defeitos na síntese da AdoCbl, apresentam boas respostas clínicas quando suplementados com vitamina B_{12}, enquanto os pacientes com o fenótipo MUT° não respondem ao tratamento (TANPAIBOON, 2005; DEODATO et al., 2006). Logo, diariamente são realizadas injeções intramusculares de OH-Cbl, em doses ajustadas individualmente, de acordo com a idade e peso do paciente (~1 mg/dia) (MANOLI; VENDITTI, 1993).

A administração intravenosa de L-carnitina (50-500 mg/kg/dia) é utilizada para tamponar o acúmulo intramitocondrial de propionil-CoA, e aumentar a excreção de propionilcarnitina. Logo, a menor quantidade intracelular de CoA pode ser o mecanismo pelo

qual a suplementação de carnitina beneficia alguns dos pacientes (MANOLI; VENDITTI, 1993; DEODATO et al., 2006).

Alguns antibióticos, como Neomicina e Metronidazol, são utilizados para reduzir a produção de propionato pela flora intestinal. Entretanto, esta terapia crônica pode desencadear um agravante: o indivíduo pode ser "repovoado" com uma flora resistente. Isso pode representar uma séria ameaça infecciosa, especialmente perigosa para indivíduos com a Acidemia Metilmalônica isolada, uma vez que a maioria das mortes está relacionada à descompensação metabólica, muitas vezes precipitada por agentes infecciosos (HORSTER; HOFFMANN, 2004; DEODATO et al., 2006).

Alguns estudos têm mostrado que a acidemia leva ao estresse oxidativo, depleção de glutationa e inibição específica de complexos da cadeia respiratória. Isto sugere um potencial benéfico do tratamento com antioxidantes, como vitamina C, vitamina E, e coenzima Q_{10}; e outras terapias que atuem a nível mitocondrial (ATKURI et al., 2009; CHANDLER et al., 2009; DE KEYZER et al., 2009).

Além disso, o transplante hepático, ou hepático e renal, tem sido realizado de forma limitada, numa tentativa de melhorar a estabilidade metabólica através da provisão de uma atividade enzimática específica de um órgão (NAGARAJAN et al., 2005; KASAHARA et al., 2006). Entretanto, esta intervenção traz riscos significativos, pré- e pós-operatórios (LEONARD; WALTER; MCKIERNAN, 2001).

Métodos alternativos têm sido desenvolvidos recentemente para o tratamento de modelos experimentais da acidemia. Por exemplo, no trabalho desenvolvido por Hu e colaboradores (2009), a utilização de determinados fármacos, como a cisplatina, zidovudina ou adefovir, causou aumento na indução do RNA mensageiro da enzima MCM (HU et al., 2009). Já no trabalho experimental desenvolvido por Buck e colaboradores (2012), o transplante de células progenitoras fetais foi eficaz em reduzir metabólitos acumulados na acidemia (BUCK et al., 2012). Enquanto isso, o grupo dos pesquisadores Chandler e Venditti desenvolveu tratamentos genéticos para modelos da acidemia, baseados em adenovírus ou vírus adeno-associados, que também induzem a expressão da enzima MCM, dessa forma, reduzindo os níveis dos metabólitos circulantes e aumentando a expectativa de vida (CHANDLER et al., 2007; CHANDLER; VENDITTI, 2008; CARRILLO-CARRASCO et al., 2010; CHANDLER; VENDITTI, 2010; 2012).

1.4.6 Modelos Experimentais

Os modelos experimentais são essenciais para o entendimento dos processos fisiopatológicos envolvidos na Acidemia Metilmalônica, uma vez que conseguem reproduzir, pelo menos em parte, as manifestações encontradas nos seres humanos. Tanto modelos experimentais *in vivo* quanto *in vitro* têm sido desenvolvidos para o estudo desta acidemia (Tabela 1).

Tabela 1 - Alguns modelos experimentais para o estudo da Acidemia Metilmalônica.

in vivo	**Agudo**	Cerebral (DE MELLO et al., 1996), Sistêmico (STEWART; WALSER, 1980; NAKAI et al., 1991)
	Crônico	Sistêmico (WAJNER et al., 1988; MELLO et al., 1994), Farmacológico (KRAHENBUHL et al., 1990)
	Outros	Genético (PETERS et al., 2003)
in vitro	**Cultura de células**	Neurônios (MCLAUGHLIN et al., 1998)
	Órgãos isolados	Cérebro (WAJNER et al., 1992), Fígado (ARINZE; WATERS; DONALDSON, 1979)
	Outros	Sinaptossomas (LOPEZ-LAHOYA et al., 1981), Mitocôndrias (MIRANDOLA et al., 2008)

Alguns destes estudos mostraram que o MMA agindo sobre os hepatócitos pode inibir a gliconeogênese (ARINZE; WATERS; DONALDSON, 1979); aumentar os níveis de citrulina e aspartato, reduzir a atividade da carbamoil-fosfato sintetase I, e os níveis de acetil CoA e CoA livre (STEWART; WALSER, 1980); assim como diminuir os níveis de glutationa e a atividade do citocromo c oxidase, e causar um aumento do tamanho das mitocôndrias (CHANDLER et al., 2009).

Já no sistema nervoso central (SNC), o MMA foi capaz de inibir a captação de glicina nas sinapses inibitórias glicinérgicas da medula espinhal (LOPEZ-LAHOYA et al., 1981);

aumentar a produção de lactato e a captação de glicose, e diminuir a formação de CO_2 a partir de glicose e acetato (WAJNER et al., 1992); reduzir a incorporação de acetato nos lipídios cerebrais (DE MELLO et al., 1997); diminuir a relação ATP/ADP, o potencial de membrana e a concentração interna de K^+, e aumentar a concentração interna de Na^+ e Ca^{2+} em culturas de neurônios cerebrais (MCLAUGHLIN et al., 1998); e diminuir a atividade da Na^+,K^+-ATPase (WYSE et al., 2000).

Ainda no SNC, os modelos animais apresentaram inibição dos complexos I, II, I-III, e II-III da cadeia respiratória (BRUSQUE et al., 2002; PETTENUZZO et al., 2006); aumento nos níveis de AMPc, possivelmente pela ativação de receptores β-adrenérgicos (LOUREIRO et al., 2005); aumento na produção de óxido nítrico, carbonilação proteica e peroxidação lipídica (RIBEIRO et al., 2005; ROYES et al., 2005); redução no consumo de oxigênio pelas mitocôndrias cerebrais quando se utiliza o glutamato ou glutamato/aspartato como substrato, e inibição da α-cetoglutarato desidrogenase (MELO et al., 2012).

Além disto, em outros estudos o MMA conseguiu inibir: a atividade da succinato desidrogenase (SDH) e da β-hidroxidobutirato desidrogenase no fígado e cérebro (DUTRA et al., 1993); o complexo II-III no estriado, hipocampo, coração, fígado e rim, e o complexo I-III no fígado e rim (PETTENUZZO et al., 2006); a lactato desidrogenase no fígado e cérebro (SAAD et al., 2006); e o transporte de succinato pelo carreador de dicarboxilato mitocondrial no músculo e no cérebro (MIRANDOLA et al., 2008).

Estes resultados sugerem possíveis mecanismos pelos quais o MMA leva as alterações comportamentais evidenciadas em outros experimentos. Por exemplo, quando injetado cronicamente e de forma sistêmica, o MMA pode alterar o comportamento de ratos em testes de habituação, memória e aprendizado (DUTRA et al., 1991; PETTENUZZO et al., 2003a); e quando injetado de forma aguda, diretamente em uma estrutura cerebral, pode causar alterações EEG e convulsões (DE MELLO et al., 1996; MALFATTI et al., 2003).

1.5 Convulsão, Crise Epiléptica e Epilepsia

O termo convulsão refere-se a contrações musculares anormais e excessivas, geralmente bilaterais, que podem ser sustentadas ou descontinuadas. Já uma crise epiléptica pode ser definida como uma ocorrência de sinais e/ou sintomas desencadeados quando neurônios cerebrais disparam excessivamente e/ou sincronicamente, sendo identificado pela

análise de EEG. Por fim, a epilepsia é basicamente caracterizada por ser uma doença cerebral com pré-disposição para estas crises epilépticas, o que desencadeia consequências neurobiológicas, cognitivas, psicológicas e sociais (BLUME et al., 2001; FISHER et al., 2005).

Embora os EIMs sejam raros causadores de epilepsia, as convulsões são sintomas frequentes em doenças metabólicas, e geralmente são desencadeadas durante os episódios de descompensação aguda. Assim, se a Acidemia Metilmalônica for tratada apropriadamente, as convulsões tornam-se raras, refletindo dano cerebral permanente (WOLF; BAST; SURTEES, 2005). Um estudo realizado na China recentemente analisou pacientes que, além da acidemia, apresentavam epilepsia. Nestes pacientes foram observadas convulsões do tipo parcial, tônico-clônica generalizada, tônica, mioclônica, e espasmos epilépticos; além de alterações no EEG interictal, como lentificação da atividade das ondas, descargas epileptiformes focais ou multifocais, descargas epileptiformes generalizadas, hipsarritmia, e um padrão atípico de surto-supressão (MA et al., 2011).

Em modelos experimentais, o primeiro trabalho a demonstrar que o MMA causa crises convulsivas foi publicado em 1996. Neste estudo, de Mello e colaboradores apresentaram evidências de que a injeção intraestriatal de MMA causa rotações contralaterais e convulsões clônicas de um modo dose-dependente, com envolvimento do sistema glutamatérgico (DE MELLO et al., 1996).

1.6 Memória

A memória é a capacidade de armazenar informações que possam ser recuperadas e utilizadas posteriormente (LENT, 2004). Elas podem ser classificadas de acordo com o tempo que duram (de trabalho, de curta ou de longa duração), e quanto ao conteúdo (declarativa/explicita, não-declarativa/implícita ou operacional) (GOLDMAN-RAKIC, 1996; SQUIRE; ZOLA, 1996; ALBRIGHT; KANDEL; POSNER, 2000; LEES; JONES; KANDEL, 2000; MCGAUGH; IZQUIERDO, 2000; CURTIS; D'ESPOSITO, 2003; SQUIRE; KANDEL, 2003; RANGANATH; BLUMENFELD, 2005).

As pessoas com deficiência de vitamina B_{12} apresentam aumento nos níveis de MMA e homocisteína no soro sanguíneo. Estudos comprovaram que existe uma associação entre o aumento desses marcadores séricos e déficits cognitivos, incluindo a memória (LEWIS et al.,

2005; MCCRACKEN et al., 2006; TANGNEY et al., 2011). O déficit cognitivo e neurológico do paciente é mais grave quando a Acidemia Metilmalônica se instala precocemente, há presença de hiperamonemia no momento do diagnóstico, e/ou ela está associada a um histórico de convulsões (O'SHEA et al., 2012).

Os modelos experimentais da Acidemia Metilmalônica apresentaram déficits em testes de aprendizado/memória (DUTRA et al., 1991). Além disso, em testes de memória/aprendizado espacial (labirinto aquático de Morris), ratos injetados cronicamente com MMA, durante um período de grande proliferação celular e sinaptogênese em várias estruturas envolvidas no aprendizado/memória, têm deficiência na aquisição de um novo paradigma de localização espacial, indicando um comportamento perseverativo (PETTENUZZO et al., 2003a; PETTENUZZO et al., 2003b).

1.7 Neuroinflamação

Os estudos mostram que tanto convulsões quanto algumas alterações cognitivas podem apresentar envolvimento com a neuroinflamação (CHOI; KOH, 2008; RAVIZZA; BALOSSO; VEZZANI, 2011; THEOHARIDES; ZHANG, 2011; HEIN; O'BANION, 2012; SALIM; CHUGH; ASGHAR, 2012).

O termo ‘inflamação’ se refere a processos celulares caracterizados por mudanças na vasculatura local, ativação de células do sistema imune residente, infiltração de células polimorfonucleares (neutrófilos no caso de inflamação aguda) ou mononucleares (macrófagos, linfócitos e células plasmáticas no caso de inflamação crônica), e produção de citocinas (STREIT; MRAK; GRIFFIN, 2004; GRAEBER; LI; RODRIGUEZ, 2011). Isto ocorre como uma resposta do sistema imunológico a danos celulares e teciduais causados por infecções ou estímulos nocivos de origem química ou física (HAANEN; VERMES, 1995).

A ‘neuroinflamação’ é a inflamação observada em diversas doenças do SNC, o que pode contribuir para o dano tecidual, perda de neurônios e disfunção, ou para regeneração neuronal e reparo tecidual (RIVEST, 2009). Este processo é uma combinação complexa de respostas agudas e crônicas das células do SNC, incluindo neurônios, células da glia (micróglia e astrócitos) e leucócitos infiltrantes. Isto desencadeia um aumento de proteínas moduladoras do sistema imunológico presentes na superfície das células, e também o aumento

na síntese e liberação de mediadores pró-inflamatórios, incluindo citocinas, quimiocinas, óxido nítrico e prostanóides (HEIN; O'BANION, 2009).

Os pacientes da Acidemia Metilmalônica, principalmente da variante cblB, podem apresentar neutropenia. Com esta redução no número de neutrófilos, que são a maior parte das células brancas sanguíneas (defesa primária do organismo), estes indivíduos tornam-se mais susceptíveis a infecções e desenvolvimento de sepse neonatal, o que facilita o aparecimento da neuroinflamação (CHURCH et al., 1984; MANOLI; VENDITTI, 1993; GUERRA-MORENO; BARRIOS; SANTIAGO-BORRERO, 2003; SEMMLER et al., 2008).

1.8 Óxido Nítrico Sintase Induzível

Durante o processo inflamatório, agentes do sistema imunológico, como as citocinas pró-inflamatórias (interleucina-1 beta e fator de necrose tumoral-alfa, por exemplo), induzem a transcrição e expressão da enzima Óxido Nítrico Sintase Induzível (iNOS ou NOS-2) (CATTELL; JANSEN, 1995; AKTAN, 2004; PACHER; BECKMAN; LIAUDET, 2007). Mas além da iNOS ter sua expressão induzida quando há um estímulo pró-inflamatório (ZHENG et al., 1993; CAMPBELL; SAMIMI; CHIANG, 1994; IADECOLA et al., 1995), outras pesquisas sugerem que ela também é expressa constitutivamente em diversos tecidos de mamíferos, inclusive no SNC (PARK; PARK; KRISHNA, 1996; STARKEY; GRANT; HAGAN, 2001; BUSKILA et al., 2005). A iNOS é uma isoforma da família de enzimas da Óxido Nítrico Sintase (NOS, EC 1.14.13.39). Além dela, outras duas isoformas estão presentes nos mamíferos: a Óxido Nítrico Sintase Neuronal (nNOS ou NOS-1) e a Endotelial (eNOS ou NOS-3) (KNOWLES; MONCADA, 1994).

As isoformas da NOS apresentam algumas distinções. A localização do gene que codifica a nNOS é no cromossomo 12; da iNOS no 17; e da eNOS no 7 (KISHIMOTO et al., 1992; XU et al., 1993; XU et al., 1994). A isoforma neuronal é expressa em neurônios do SNC e periférico, e alguns outros tipos celulares, com as funções de modular a plasticidade sináptica, regulação central da pressão sanguínea, relaxamento do músculo esquelético, e vasodilatação por nervos nitrérgicos periféricos (SCHUMAN; MADISON, 1991; TOGASHI et al., 1992; FORSTERMANN et al., 1994). A isoforma induzível da NOS pode ser expressa em diversos tipos de células, como resposta a lipopolissacarídeos (LPS, um dos componentes principais da membrana exterior de bactérias gram-negativas), citocinas e outros agentes,

tendo um papel citostático sobre células-alvo, o que pode contribuir para a patofisiologia de doenças inflamatórias (GREEN et al., 1990; LI et al., 1991; NATHAN, C. F.; HIBBS, 1991). E a isoforma endotelial é expressa, como o próprio nome indica, principalmente em células endoteliais, objetivando manter os vasos sanguíneos dilatados, controlar a pressão sanguínea, e ter outros efeitos vasoprotetores e anti-ateroscleróticos (Tabela2) (KUBES; SUZUKI; GRANGER, 1991; DIMMELER; ZEIHER, 1999; AICHER et al., 2003).

Tabela 2 - Distribuição celular das isoformas da NOS (VILLANUEVA; GIULIVI, 2010).

Isoforma	Tipo de expressão	Tipos de célula
nNOS	**Constitutiva**	Neurônios, cardiomiócitos, músculo liso gastrointestinal, queratinócitos, mácula densa, neutrófilos, músculo esquelético, epitélio tubular, células de músculos lisos vasculares, hepatócitos
iNOS	**Induzível**	Macrófagos e células do músculo liso da via respiratória, macrófagos alveolares, condrócitos, células endoteliais, células de Kupffer, fibroblastos de pulmão, mastócitos, neutrófilos, músculo esquelético, células epiteliais tipo II, células de músculos lisos vasculares
	Constitutiva	Epitélio respiratório, mucosa do cólon, túbulos corticais, neurônios, hepatócitos, queratinócitos
eNOS	**Constitutiva**	Células do endotélio, células epiteliais dos brônquicas, eosinófilos, células epiteliais da mucosa nasal humana, fibroblastos, mucosa gastrointestinal, hepatócitos, linfócitos, neutrófilos, músculo esquelético, sincitiotrofoblastos da placenta humana, células alveolares tipo II

As isoformas da NOS também apresentam características em comum (Figura 2). Todas elas utilizam a L-arginina como substrato, e oxigênio molecular e fosfato de nicotinamida adenina dinucleotídeo reduzido (NADPH) como co-substratos. Os cofatores das isoformas são dinucleótideo de flavina e adenina (FAD), mononucleotídeo de flavina (FMN) e (6R-)5,6,7,8-tetrahidro-L-biopeterina (BH_4). As enzimas são homodímeros e transferem os elétrons do NADPH via FAD e FMN no domínio carboxi-terminal da redutase para o domínio amino-terminal da oxigenase (que também liga o BH_4, oxigênio e a L-arginina). No sítio

heme, os elétrons são utilizados para reduzir e ativar o O_2 e para oxidar a L-arginina para L-citrulina e óxido nítrico (NO) (ALDERTON; COOPER; KNOWLES, 2001; STUEHR; POU; ROSEN, 2001).

Além disso, apesar de todas as isoformas se ligarem a calmodulina (CaM), na nNOS e eNOS, esta ligação se dá por um aumento do cálcio intracelular (entre 200 e 400 nM), enquanto que para a iNOS, concentrações muito inferiores de cálcio (menos de 40 nM) levam à esta ligação com a CaM e, consequentemente, ativam a enzima (FORSTERMANN; SESSA, 2012).

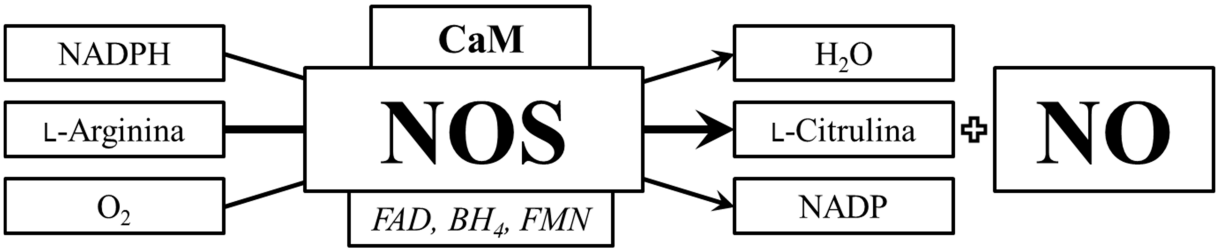

Figura 2 - Esquema representativo da formação de NO pelas isoformas da NOS.

1.8.1 Óxido Nítrico

As isoformas da NOS sintetizam o NO em dois passos: primeiro a NOS hidroxila a L-arginina para N^{ω}-hidroxi-L-arginina (a qual permanece ligada a enzima), e depois ela oxida a N^{ω}-hidroxi-L-arginina para L-citrulina e NO (NOBLE et al., 1999; STUEHR; POU; ROSEN, 2001). O NO é uma molécula gasosa simples, um mensageiro intercelular onipresente em todos os vertebrados, que modula o fluxo sanguíneo, formação de trombos e atividade neuronal. Além disto, a produção biológica do NO é um importante mecanismo de defesa (PACHER; BECKMAN; LIAUDET, 2007).

Por ter somente um elétron desemparelhado, o NO apresenta forte ligação ao ferro de grupamentos heme, sendo isto crucial para sua atividade biológica de ativar a guanilato ciclase (GC), ou retardar a respiração mitocondrial ao ligar-se à citocromo-c oxidase, por exemplo. Entretanto, quando o NO reage com o radical superóxido ($O_2^{\bullet -}$), provindo de enzimas como a NADPH oxidase e xantina oxidase, é gerado peroxinitrito ($ONOO^-$), uma espécie reativa de nitrogênio (ERN) altamente oxidante. Logo, passam a ocorrer processos

patológicos que resultam em substancial oxidação (como a nitração de proteínas) e potencial destruição de constituintes celulares, conduzindo à disfunção de processos celulares essenciais, rompimento da sinalização celular, e a indução de morte celular através de necrose ou apoptose (VIRAG et al., 2003; PACHER; BECKMAN; LIAUDET, 2007).

Durante o processo inflamatório, a iNOS não apresenta um aumento substancial na sua atividade e produção de NO se comparada as outras isoformas da NOS. Mas, por apresentar atividade mesmo com concentrações normais de cálcio, e pela indução de sua expressão, a iNOS passa a ser uma grande fonte de NO (NATHAN, C.; XIE, 1994b; a).

Já o papel do NO durante as convulsões ainda não é claro: Enquanto alguns autores acreditam que ele é um anticonvulsivante endógeno (MAGGIO et al., 1995; MOAZZAMI; EMAMZADEH-FARD; SHABANI, 2012; SHAFAROODI et al., 2012), outros sugerem que esta molécula apresenta um papel pró-convulsivo (VAN LEEUWEN; DE VRIES; DZOLJIC, 1995; REHNI et al., 2009; BEAMER et al., 2012).

Há estudos que mostram o envolvimento do NO gerado pelas isoformas da NOS no sistema nervoso central de modelos agudos da Acidemia Metilmalônica. As convulsões e a carbonilação proteica induzidas pela injeção intraestriatal de MMA são moduladas de maneira bifásica pela administração prévia de um inibidor não-específico da NOS, o N_{ω}-nitro-L-arginina metil éster (L-NAME): Em baixas doses, o L-NAME se mostrou protetor; mas em uma dose elevada, ele potencializou o efeito do MMA (ROYES et al., 2005). Já o inibidor específico da nNOS, 7-Nitroindazol (7-NI), potencializou as convulsões e a carbonilação proteica induzidas pelo MMA; enquanto o substrato para NOS, L-arginina, atenuou os efeitos do mesmo (ROYES et al., 2007).

1.9 Justificativa

Sabendo que a isoforma induzível da óxido nítrico sintase (iNOS) exacerba o processo inflamatório e condições neurodegenerativas, seria interessante especificar sua influência no sistema nervoso central de modelos experimentais da Acidemia Metilmalônica.

1.10 Problemática

A iNOS possui relação com sintomas neurológicos estudados em modelos experimentais da Acidemia Metilmalônica?

1.11 Objetivos

1.11.1 Objetivo Geral

Investigar o envolvimento da iNOS nas convulsões e na atividade de enzimas do córtex cerebral de camundongos injetados com MMA via intracerebroventricular (i.c.v.); e investigar se a injeção subcutânea (s.c.) e crônica de MMA em ratos jovens altera parâmetros comportamentais e/ou inflamatórios no córtex cerebral.

1.11.2 Objetivos do Artigo

Avaliar o efeito da injeção i.c.v. de MMA em camundongos selvagens e nocaute para iNOS sobre:

- As convulsões comportamentais e EEG;
- A produção de óxido nítrico;
- A nitração de resíduos de tirosina;
- A atividade da Na^+,K^+-ATPase;
- A atividade da SDH.

1.11.3 Objetivos do Manuscrito

Avaliar o efeito da injeção s.c. crônica de MMA em ratos jovens sobre:

- A memória e a ansiedade;

- A contagem de células brancas sanguíneas;
- A concentração de citocinas pró-inflamatórias;
- A expressão da iNOS;
- A nitração de resíduos de tirosina.

2 CONVULSÕES INDUZIDAS POR METILMALONATO SÃO ATENUADAS EM CAMUNDONGOS NOCAUTE PARA ÓXIDO NÍTRICO SINTASE INDUZÍVEL - ARTIGO

2.1 Título Original

Methylmalonate-induced seizures are attenuated in inducible nitric oxide synthase knockout mice.

2.2 Autores

Leandro Rodrigo Ribeiro, Michele Rechia Fighera, Mauro Schneider Oliveira, Ana Flávia Furian, Leonardo Magno Rambo, Ana Paula de Oliveira Ferreira, André Luiz Lopes Saraiva, Mauren Assis Souza, Frederico Diniz Lima, Danieli Valnes Magni, Renata Dezengrini, Eduardo Furtado Flores, D. Allan Butterfield, Juliano Ferreira, Adair Roberto Soares dos Santos, Carlos Fernando Mello, Luiz Fernando Freire Royes.

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Methylmalonate-induced seizures are attenuated in inducible nitric oxide synthase knockout mice

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ABSTRACT

Methylmalonic acidemias consist of a group of inherited neurometabolic disorders caused by deficiency of methylmalonyl-CoA mutase activity clinically and biochemically characterized by neurological dysfunction, methylmalonic acid (MMA) accumulation, mitochondrial failure and increased reactive species production. Although previous studies have suggested that nitric oxide (NO) plays a role in the neurotoxicity of MMA, the involvement of NO-induced nitrosative damage from inducible nitric oxide synthase (iNOS) in MMA-induced seizures are poorly understood. In the present study, we showed a decrease of time spent convulsing induced by intracerebroventricular administration of MMA (2 μmol/2 μL; i.c.v.) in iNOS knockout ($iNOS^{-/-}$) mice when compared with wild-type ($iNOS^{+/+}$) littermates. Visual analysis of electroencephalographic recordings (EEG) showed that MMA injection induced the appearance of high-voltage synchronic spike activity in the ipsilateral cortex which spreads to the contralateral cortex while quantitative electroencephalographic analysis showed larger wave amplitude during MMA-induced seizures in wild-type mice when compared with iNOS knockout mice. We also report that administration of MMA increases NOx (NO_2 plus NO_3 content) and 3-nitrotyrosine (3-NT) levels in a greater extend in $iNOS^{+/+}$ mice than in $iNOS^{-/-}$ mice, indicating that NO overproduction and NO-mediated damage to proteins are attenuated in iNOS knockout mice. In addition, the MMA-induced decrease in Na^+, K^+-ATPase activity, but not in succinate dehydrogenase (SDH) activity, was less pronounced in $iNOS^{-/-}$ when compared with $iNOS^{+/+}$ mice. These results reinforce the assumption that metabolic collapse contributes for the secondary toxicity elicited by MMA and suggest that oxidative attack by NO derived from iNOS on selected target such as Na^+, K^+-ATPase enzyme might represent an important role in this excitotoxicity induced by MMA. Therefore, these results may be of value in understating the pathophysiology of the neurological features observed in patients with methylmalonic acidemia and in the development of new strategies for treatment of these patients.

1. Introduction

Methylmalonic acidemia comprise a group of inherited metabolic disorders caused by either a deficiency of the mitochondrial enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2), or defects

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in the synthesis of 5′-deoxyadenosylcobalamin, the cofactor of MCM. Deficient MCM activity, which physiologically catalyses the reaction of methylmalonyl CoA to succinyl CoA, leads to the primary accumulation of methylmalonyl CoA, and a secondary accumulation of other metabolites, such as succinate, propionate, 3-hydroxypropionate, and 2-methylcitrate (Fenton and Rosenberg, 1995; Okun et al., 2002; Kolker et al., 2003). The major long-term complications are chronic renal failure, cardiomyopathy and neurological deficits, including lethargy, hypotonia/hypertonia, myoclonus, psychomotor delay/mental retardation (Fenton and Rosenberg, 1995; Touati et al., 2006; Morath et al., 2007). Furthermore, infants with this inborn error of metabolism may become debilitated and septic rather quickly, however, the presence of sepsis not exclude consideration of other possibilities such development of convulsion (Burton, 1998).

In this context, it has been shown that intrastriatal administration of MMA besides causing convulsive behavior, increases protein carbonylation and thiobarbituric acid reacting substances (TBARS) (Malfatti et al., 2003; Royes et al., 2006) indicating the involvement of reactive species in the genesis and/or propagation of convulsions elicited by this organic acid. Accordingly, MMA-induced convulsions are exacerbated by ammonia (Marisco et al., 2003), which also increases tissue lipoperoxidation. These data are corroborated by findings that MMA induces dose-dependent lipoperoxidation in vitro (Fontella et al., 2000) and ex vivo (Fontella et al., 2000; Malfatti et al., 2003; Marisco et al., 2003; Fighera et al., 2003) following by impairment of Na^+, K^+-ATPase activity (Wyse et al., 2000; Royes et al., 2006), a key enzyme activity in the maintenance of ionic gradients.

Furthermore, recent findings from our group have suggested a differential involvement of nitric oxide synthase (NOS) on convulsive behavior and oxidative damage to proteins elicited by MMA. While the intrastriatal injection of NG-Nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, exerts a biphasic modulation of MMA-induced convulsive activity and protein carbonylation (Royes et al., 2005), a striatal NO depletion elicited by 7-nitroindazol (7-NI) exacerbates seizures, protein carbonylation and Na^+, K^+-ATPase activity inhibition induced by MMA (Royes et al., 2007).

Inducible nitric oxide synthase (iNOS, EC 1.14.13.39) is one of three NOS isoforms generating NO by conversion of L-arginine to L-citruline (Pacher et al., 2007). iNOS is the isoform which contributes to exacerbation of inflammatory and degenerative conditions thought the excessive NO production and consequent reactive nitrogen species generation (RNS) (Madrigal et al., 2006; Pacher et al., 2007). In line of this view, genetic animal models have contributed significantly to understand aetiopathologies of epilepsies (Buchhalter, 1993; Burgess and Noebels, 1999). Recently, it has been demonstrated that iNOS knockout mice reach the kindled status induced by PTZ more slowly when compared to wild-type mice and it is also different from other mice strains (De Sarro et al., 1996; De Luca et al., 2005, 2006).

Since pharmacological and neurochemical evidence support that inflammation may be a common factor contributing or predisposing, to occurrence of seizures in various forms of epilepsy of different etiologies (Vezzani and Granata, 2005) it is rather possible that iNOS knockout mice present decreased MMA-induced seizure and oxidative stress when compared with wild-type littermates. Therefore, the current study was designed to investigate the possible mechanisms involved in the toxicity induced by MMA in $iNOS^{-/-}$ versus $iNOS^{+/+}$mice.

2. Experimental procedures

2.1. Animal and reagents

Experiments were conducted using $iNOS^{+/+}$ and ($iNOS^{-/-}$) mice, kept in a controlled room temperature (22 ± 2 °C) and humidity (60–80%) under a 12 h light/dark cycle (lights on 6:00 A.M.). iNOS knock-out mice were on the C57BL/6 background, constructed as described previously (MacMicking et al., 1995). The mice used at the beginning of this study were male and female from 63 to 80 days old and weighted 24–30 g. Animal utilization reported in this study have been conducted in accordance with the policies of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996. All efforts were made to reduce the number of animals used, as well as minimize their suffering. All reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Placement of cannula and behavioral evaluation

Wild-type and iNOS knockout mice (n = 8–10 in each group) were anesthetized with ketamine (100 mg/kg, i.p.) and xilazine (30 mg/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted into the right lateral ventricle (coordinates relative to bregma: AP 0 mm, ML 0.9 mm, V 1.8 mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure.

After a recovery period of three days, animals received intracerebroventricular injection of NaCl (2 μmol/2 μL) or MMA (2 μmol/2 μL). All intracerebroventricular injections were performed by using a needle (30 gauge) protruding 1 mm below a guide cannula. All drugs were injected over 1-min period by using a Hamilton syringe, and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula. The dose of MMA used in the present study was selected based on pilot dose–response experiments.

Immediately after the NaCl or MMA injections the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 10 equal areas. The open field session lasted 20 min and during this time the mice were observed for the appearance of convulsive behavior, defined by the occurrence of myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected site. In addition the animals were observed for appearance of generalized tonic–clonic convulsive episodes characterized by whole-body clonus involving all four limbs and tail followed by sudden loss of upright posture and autonomic signs, such hyper- salivation and defection respectively. The onset time for the first convulsive episode (characterized by appearance of myoclonic jerks and clonic movements) and the sum of the duration of all convulsions presented by mice during the behavioral evaluation period (total time spent convulsing) was recorded using a stopwatch according de Mello et al. (1996).

2.3. Placement of cannula and electrodes and EEG recordings

A subset of animals (n = 6 in each group) were anesthetized with ketamine (100 mg/kg, i.p.) and xilazine (30 mg/kg, i.p.) and surgically implanted with a cannula and electrodes under stereotaxic guidance for the purpose of EEG recording. The guide cannula was glued to a multipin socket and inserted into the right ventricle through a previously opened skull orifice. Two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP −4.5 and L 2.5), along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket and fixed to the skull with dental acrylic cement. The EEG recordings were performed 7 days after surgery.

The procedures for EEG recording were carried out as previously described by Cavalheiro et al. (1992). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 cm × 25 cm × 60 cm) for at least 30 min before the EEG recordings. Animals were then connected to the lead socket in a swivel inside a Faraday's cage. EEG was recorded using a digital encephalographer (Neuromap EQSA260, Neurotec LTDA, Itajubá, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After baseline recording, NaCl or MMA were administered and mice were observed for 30 min for the appearing of behavioral convulsions, as described above. EEG recordings were visually analyzed for seizure activity, which were defined by isolated sharp waves (≥1.5 X baseline); multiple sharp waves (≥2 X baseline) in brief spindle episodes (≥1 s, ≥5 s); multiple sharp waves (≥2 X baseline) in long spindle episodes (≥5 s); spikes (≥2 X baseline) plus slow waves; multispikes (≥2X baseline, ≥3 spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s). EEG spikes amplitude was calculated as variations of values (μV) before and after drug administration. Rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded.

2.4. Tissue processing for neurochemical analyses

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. Cerebral cortex was dissected on an inverted ice-cold Petri dish and homogeneized in cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM EDTA and 320 mM sucrose. The homogeneized was then divided in aliquots for subsequent neurochemical analyses, as described below.

2.5. Assay of NOx (NO_2 plus NO_3) as a marker of NO synthesis

For NOx determination, an aliquot (200 μL) was homogenized in 200 mM Zn_2SO_4 and acetonitrile (96%, HPLC grade). After, the homogenate was centrifuged at 16,000 × *g* for 20 min at 4 °C and supernatant was separated for analysis of the NOx content as described by Miranda et al. (2001). The resulting pellet was suspended in NaOH (6 M) for protein determination.

2.6. Slot blot assay for 3-nitrotyrosine

3-Nitrotyrosine immunoreactivity is a marker of oxidative nitric oxide damage and was determined as previously described by Joshi et al. (2006). Briefly, sample (5 μL) (normalized to 4 μg/mL), 5 μL of 12% SDS and 5 μL of modified Laemmli buffer containing 0.125 M Tris base pH 6.8%, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature, and the membranes were developed as described above except a 1:2000 dilution of anti-3-NT polyclonal antibody was used. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).The 3-NT blot had a faint background that was corrected in image analysis.

2.7. Na^+, K^+-ATPase activity measurements

Assay of Na^+, K^+-ATPase activity was performed according Wyse et al. (2000). Briefly, the reaction medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 50 μg of protein in the presence or absence of the Na^+, K^+-ATPase inhibitor ouabain (1 mM), in a final volume of 350 μL. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μL of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+, K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

2.8. Succinate dehydrogenase (SDH) activity measurements

For SDH activity assay, a sample (500 μL) was centrifuged at 1000 × *g* for 10 min and the resulting supernatant was centrifuged at 12,000 × *g* for 20 min. All procedures were performed at 4 °C. The pellet was suspended in 270 mM potassium PO_4 buffer, pH 7.2, containing 250 mM sucrose, 5 mM $MgCl_2$, 20 mM glucose, and 0.85% NaCl (buffer B) and frozen for 24 h. The protein content was adjusted to 1 mg/mL with buffer B. Succinate dehydrogenase activity was assayed as previously described by Dutra et al. (1993) using 2,6-dichorophenolindophenol (DCIP) as the electron acceptor in the presence of phenazine methosulfate. The reaction mixture (1500 μL) contained 50 mM potassium PO_4 buffer, pH 7.5, 1.5 mM KCN, 30 μM DCIP, 3 μg rotenone, 5 mM sodium succinate, 0.5 mM phenazine methosulfate. The mixture was preincubated for 10 min at 37 °C and the reaction started by the addition of the mitochondrial fraction (50 μg of protein). The reduction of DCIP was measured spectrophotometrically by monitoring the fall of absorbance at 600 nm for 30 s.

2.9. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976), using bovine serum albumin (1 mg/mL) as standard.

2.10. Statistical analysis

Statistical analysis was carried out by one- or two-way analysis of variance (ANOVA) when appropriated. Post hoc analysis was carried out, when appropriate, by the Student–Newman-test. *P* and *F* values are presented only if $P < 0.05$.

3. Results

The effect of intracerebroventricular administration of MMA on behavioral convulsions in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice is shown in Fig. 1. Behavioral and statistical analysis revealed that iNOS knockout ($iNOS^{-/-}$) had not effect on latency for the first convulsion induced by MMA when compared with wild-type littermates (Fig. 1A). However, the time spent in convulsive episodes in $iNOS^{-/-}$ mice was significant lower when compared with wild-type littermates [$F(1,37) = 6.29$; $P < 0.05$, Fig. 1B]. Furthermore, behavioral and EEG recordings revealed a similar convulsive behavior after administration MMA between male and female group of animals (*data not shown*), suggesting that possible changes in hormonal secretion at all levels of the reproductive neuroendrocrine axis in both groups of animals had not effect on convulsive episodes induced by this organic acid. EEG recordings also confirmed behavioral seizures elicited by MMA in $iNOS^{-/-}$ and $iNOS^{+/+}$ mice.

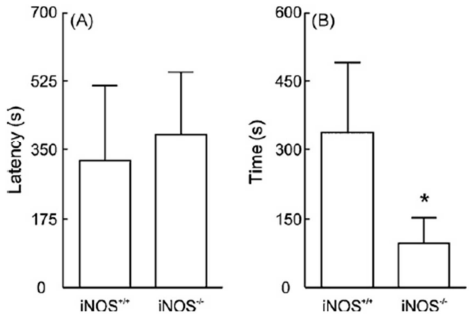

Fig. 1. (A) Latency for onset and (B) total time spent in seizures induced by MMA administration (2 μmol/2 μl; i.c.v.) in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. $^*P < 0.05$ compared with $iNOS^{+/+}$ mice. Data are mean + S.E.M. for $n = 8$–10 in each group.

The EEG recordings before and after MMA injection (2 μmol/2 μL; i.c.v) in $NOS^{+/+}$ mice is shown in Fig. 2A and B. The following

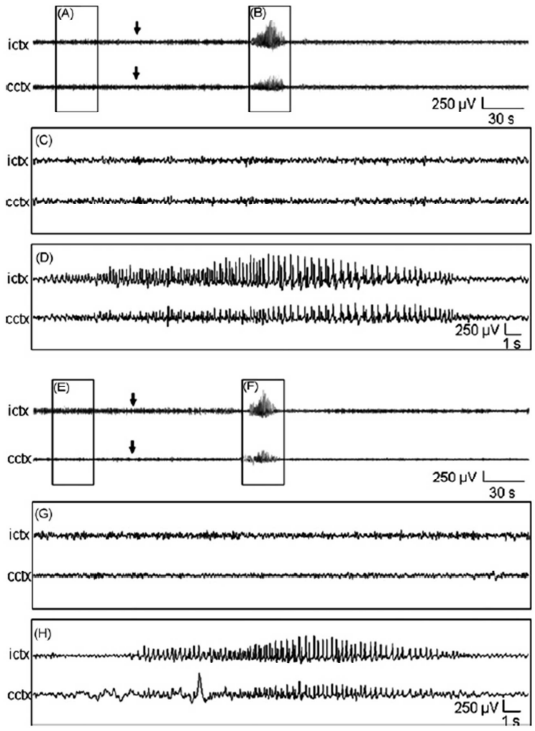

Fig. 2. Representative electroencephalographic recordings obtained in ipsi (ictx) and contralateral cortex (cctx) before (A) and after intracerebroventricular of MMA (2 μmol/2 μl) in $iNOS^{+/+}$ mice (B). The arrow indicates MMA administration and the expanded waveforms from the EEG recording outline by boxes (A and B) are shown in (C and D) respectively. Representative electroencephalographic recordings obtained before (E) and after (F) the intracerebroventricular of MMA (2 μmol/2 μl) in $iNOS^{-/-}$ mice. The typical seizure sequences observed after MMA injection were accompanied by the behavioral alterations described in the Results section. The arrow indicates MMA administration and the expanded waveforms from the EEG recording outline by boxes (E) and (F) are shown in G and H respectively.

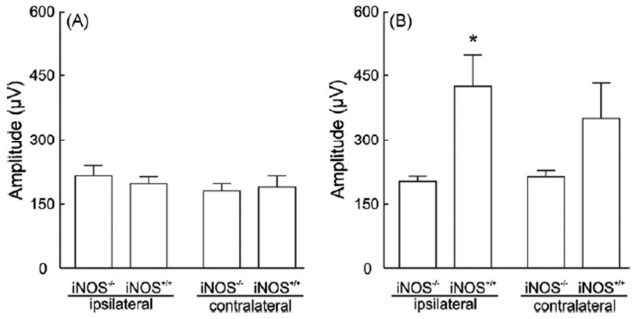

Fig. 3. Quantitative analysis of EEG recordings for wave amplitude in ispilateral and contralateral cortex of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (A) before and (B) after MMA injection (2 μmol/i.c.v.). $^{*}P < 0.05$ compared with wild-type (iNOS$^{-/-}$) mice. Data mean + S.E.M. for $n = 6$ in each group.

behavioral repertoire observed in iNOS$^{+/+}$ mice occurred concomitantly with electrographic recorded seizures: generalized seizures were characterized by the appearance of 2–3 Hz high-amplitude activity. These epileptic discharges (interictal spikes) were defined as abnormal paroxystic in the cerebral cortex and consisted of high-amplitude biphasic sharp transients. Furthermore, EEG recordings revealed that MMA induced the appearance of high-voltage synchronic spike clusters in the ipsilateral cortex which spread to the contralateral cortex in wild-type mice (Fig. 2D).

The Fig. 2E and F showed the representative EEGs before and after MMA injection in iNOS$^{-/-}$ mice, respectively. EEG recordings confirmed previous behavioral analysis since showed a similar latency for the first convulsion between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (Fig. 1A). On the other hand, EEG recordings revealed a decrease of ictal activity in iNOS$^{-/-}$ mice (Fig. 2H) when compared with wild-type littermates (Fig. 2D). This wave pattern alteration observed in iNOS knockout mice corroborated with quantitative analyses of EEG recordings that showed a significant decrease in the amplitude of seizure spikes in ipsilateral cortex of iNOS$^{-/-}$ mice (202 μV) when compared with ipsilateral cortex of iNOS$^{+/+}$ mice (424 μV) after period of observation (20 min) [$F(1,11) = 9.14$; $P < 0.05$, Fig. 3B].

Fig. 4 shows the effect of intracerebroventricular injection of MMA on cerebral NOx production in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Statistical analysis revealed that MMA increased NOx levels in cerebral cortex of wild-type iNOS$^{+/+}$, but not in iNOS$^{-/-}$ mice [$F(1,37) = 19.24$; $P < 0.05$; Fig. 4A]. In addition, quantitative image analysis of NO-mediated nitrative damage to proteins (3-NT) revealed a significant increase of 3-NT immunoreactivity in iNOS$^{+/+}$ when compared with iNOS$^{-/-}$ mice [$F(1,37) = 8.69$ $P < 0.05$, Fig. 4B] after MMA injection .

Considering that Na^+, K^+-ATPase activity correlates with time spent in MMA-induced convulsions (Royes et al., 2006) and this enzyme is also sensitive to NO overproduction (Moro et al., 2005), we also investigated whether there are differences in MMA-induced Na^+, K^+-ATPase activity inhibition in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Statistical analysis showed that intracerebroventricular injection of MMA decreased Na^+, K^+-ATPase activity in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice [$F(1,37) = 22.42$; $P < 0.05$, Fig. 5A]. In addition, statistical comparison between groups showed a higher inhibition of Na^+, K^+-ATPase activity in iNOS$^{+/+}$ mice when compared with iNOS$^{-/-}$ mice, reinforcing the idea that oxidative attack of select target such Na^+, K^+-ATPase represents a important role in the propagation of MMA-induced convulsive behavior (Royes et al., 2006).

Since it has been suggested that MMA induces convulsions through impairment of mitochondrial function (de Mello et al., 1996; Royes et al., 2003) and considering that SDH activity is especially sensitive to NO overproduction (Giulivi, 2003; Guix et al., 2005) we investigate whether iNOS knockout mice present altered sensitivity to SDH inhibition by MMA. Statistical analysis revealed that MMA injection induced a significant decrease in SDH activity of similar magnitude in both groups of mice [$F(1,37) = 5.96$; $P < 0.05$, Fig. 5B].

4. Discussion

In the present study we show that iNOS knockout mice present decreased MMA-induced seizure susceptibility compared with

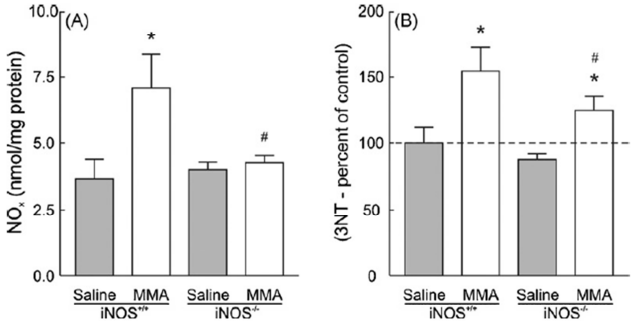

Fig. 4. The effect of MMA injection (2 μmol/2 μl; i.c.v.) on NOx content (NO_2 plus NO_3 levels; A) and 3-Nitrotyrosine immunoreactivity (B) from cerebral cortex of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. $^{*}P < 0.05$ compared with wild-type mice treated with saline; $^{\#}P < 0.05$ compared with wild-type mice treated with MMA. Data are mean + S.E.M. for $n = 8$–10 in each group.

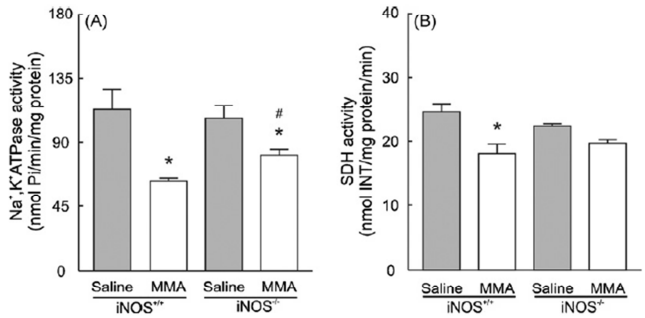

Fig. 5. The effect of MMA injection (2 μmol/2 μl; i.c.v.) on Na^+, K^+-ATPase activity (A) and SDH activity (B) from cerebral cortex of $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. $^*P < 0.05$ compared with wild-type mice treated with saline; $^\#P < 0.05$ compared with wild-type mice treated with MMA. Data are mean + S.E.M. for $n = 8–10$ in each group.

wild-type littermates, suggesting the participation of iNOS in the convulsive behavior elicited by this organic acid. We also report that intracerebroventricular administration of MMA increases NOx and 3-NT levels in a greater extend in $iNOS^{+/+}$ mice than in $iNOS^{-/-}$ mice, indicating that NO overproduction and NO-mediated damage to proteins are attenuated in iNOS knockout mice. In addition, we show that MMA-induced decrease in Na^+, K^+-ATPase activity, but not in SDH activity, is less pronounced in $iNOS^{-/-}$ mice when compared with wild-type littermates.

Recently, experimental findings from our group have evidenced the participation of NO in MMA-induced seizures and oxidative damage to proteins (Royes et al., 2005, 2007). In this context, the administration of low doses of the non-selective NOS inhibitor NG-Nitro-L-arginine methyl ester attenuates MMA-induced convulsions and protein carbonylation in rat striatum, while high doses of L-NAME have no effect on these parameters (Royes et al., 2005). Moreover, the administration of 7-nitroindazol, a preferential neuronal NOS inhibitor, increased seizures and protein carbonylation induced by MMA (Royes et al., 2007), suggesting a differential contribution of NOS isoforms to MMA-induced seizures and protein carbonylation.

Although it is believed that NO exerts a import role on neuronal hyperexcitability evidenced in several seizure disorders, (De Sarro et al., 1996; Paoletti et al., 1998; Borowicz et al., 2000; Itoh et al., 2004; de Vasconcelos et al., 2004; Kato et al., 2005), it is difficult to make a clear conclusion on the involvement of this free radical in epilepiform activity. The determining factor for such a discrepancy is not known, but one might argue that methodological differences may account for it. Another interesting possibility is that the effect of NO on convulsions may vary with the model of seizure employed and/or particular brain structures studied (Libri et al., 1997). Thus, since the role of NO in the pathophysiology of convulsions induced by MMA is not completely defined, the genetic animals models as $iNOS^{-/-}$ mice may be may be considered a valid genetic animal model to investigate the role of iNOS in the convulsive behavior elicited by this organic acid. In this context, experimental findings described by De Luca et al. (2006) demonstrated that $iNOS^{-/-}$ mice reach the kindled status induced by pentylenetetrazole (PTZ) more slowly and presented lower levels of glutamate and higher levels of GABA when compared than $iNOS^{+/+}$ after PTZ-induced kindling.

In the present study, we show novel data indicating a role for iNOS-derived NO in the convulsive episodes induced by MMA, since the total time spent in MMA-induced seizures was significantly shorter in $iNOS^{-/-}$ when compared with $iNOS^{+/+}$ mice. These results indicate that there are clear differences in the relative contribution of NOS isoforms to MMA-induced seizures, and, in light of these results, one may suggest that nNOS-derived NO may be protective, while iNOS-derived NO may be pro-convulsant. Although a number of studies have shown that there is no constitutive expression of iNOS in brain (Zheng et al., 1993; Campbell et al., 1994; Iadecola et al., 1995), other studies have suggested that iNOS is not only inducible, but also expressed constitutively on several cell types and tissues, including the brain (Park et al., 1996; Starkey et al., 2001; Buskila et al., 2005). In fact, in the cerebral cortex, a brief application of glutamate triggered a rapid (1–2 min) and massive iNOS-dependent NO production, which may suggest that constitutively expressed iNOS in the brain may contribute to physiological and pathological processes in this tissue (Buskila et al., 2005).

On the other hand, although genetic animals have contributed significantly to our understanding of the aetiopathologies of epilepsy (Buchhalter, 1993; Burgess and Noebels, 1999), the exact underlying mechanism involving iNOS-dependent NO production in this model of neurological disease are poorly know. Recently, de Luca and colleagues (2006), have suggested that the inability of $iNOS^{-/-}$ mice to increase the NO levels following PTZ administration indicate that this free radical plays a pro-epileptogenic role of some types of epilepsy.

Therefore, since the neurons are capable of rapid release of small amounts of NO serving as neurotransmitter and astrocytic NO production has been demonstrated mainly as slow reaction to various stress stimuli (Mander et al., 2005), it is reasonable to propose that initial NO production might to be a counteracting response to convulsive episodes, while a massive iNOS-dependent NO production by astrocytes may bear important implications for maintenance of convulsive behavior evidenced in this model of organic aciduria. In agreement of this view, recent experimental findings from our group have demonstrated that while striatal NO depletion exacerbates seizures, protein carbonylation and Na^+, K^+-ATPase activity inhibition, the increase of NO production induced by L-arginine injection attenuates MMA-induced behavioral, electroencephalographic and neurochemical deleterious effects (Royes et al., 2007).

The present study also revealed a role for NO derived from iNOS in MMA-induced decrease in Na^+, K^+-ATPase activity. This enzyme has been considered a target especially sensitive to free radical damage (Jamme et al., 1995; Morel et al., 1998), including NO-mediated damage (Moro et al., 2005), and a decrease in its activity has been associated with the appearance and/or propagation of seizures induced by MMA (Malfatti et al., 2003; Royes et al., 2007). In fact, recent studies from our group have demonstrated that duration of convulsive episodes induced by injection intrastriatal of MMA and glutaric acid (GA) correlates with Na^+, K^+-ATPase

activity inhibition (Royes et al., 2006; Fighera et al., 2006). Therefore, since the MMA-induced increase in NOx and 3-NT levels and the decrease in Na^+, K^+-ATPase activity was larger in $iNOS^{+/+}$ mice than in $iNOS^{-/-}$ mice, we suggest that nitrosative attack by iNOS-derived NO play a role, at least in part, in MMA-induced decrease in Na^+, K^+-ATPase activity. Moreover, it is plausible to propose that iNOS knockout mice presented less severe seizures than wild-type mice because MMA-induced decrease in Na^+, K^+-ATPase activity was smaller in this mice cohort.

A significant body of evidence has demonstrated that MMA compromises mitochondrial functions (Dutra et al., 1993; Fleck et al., 2004; Maciel et al., 2004), leading to decreased CO_2 production (Wajner et al., 1992) and O_2 consumption (Toyoshima et al., 1995), decreased ATP/ADP ratio (McLaughlin et al., 1998), phosphocreatine content (Royes et al., 2003) and succinate-supported O_2 consumption (Maciel et al., 2004; Kowaltowski et al., 2006). Furthermore, brain mitochondrial swelling experiments demonstrate that MMA is an important inhibitor of succinate transport by dicarboxylate carries (Mirandola et al., in press), suggesting that mitochondrial dicarboxylate carrier inhibition by MMA has important physiopathological implications, such impairment of neuronal energy metabolism and mitochondria-derived reactive species. In the line of this view, the results presented in this report revealed that extend of MMA-induced SDH activity inhibition was similar in both wild-type and iNOS knockout mice. In addition, these results suggest that the differences found in MMA-induced seizures in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice are not due differences in SDH inhibition and reinforce the assumption that MMA-induced convulsive behavior is mediated by generation of reactive species (Fighera et al., 1999, 2003; Marisco et al., 2003; Malfatti et al., 2003) and secondary excitotoxicity (de Mello et al., 1996; Royes et al., 2003). In line of this view, considering that hyperactivation of glutamate receptors, especially the NMDA subtype, are involved in the convulsive behavior elicited by MMA (de Mello et al., 1996; Royes et al., 2003) and stimulates iNOS enzyme (Iravani et al., 2004; Mander et al., 2005), it might be possible that the activation of iNOS regulates somehow synaptic activity facilitating the propagation of convulsive episodes induced by MMA. On the other hand, it is important to point out that the presently observed MMA-induced convulsive state might also be interpreted as a consequence of oxidative-stress-induced by iNOS pathways after MMA injection, since the induction of enzymes such iNOS and cyclooxygenase-2 (COX-2) are responsible for a great portion of the neurological damage produced in several models of stress and epilepsy (Vezzani and Granata, 2005; Madrigal et al., 2006; Kamida et al., 2007). However, further studies are needed to clarify this point.

In summary, the present study reinforces the significant participation of NO in the excitotoxicity induced by MMA and reports novel data not only about the role of iNOS-derived NO in MMA-induced seizures and concomitant nitrosative damage elicited by this organic acid, but also show that there is a role for iNOS in acute exposure to excitotoxic agents. We think that these results may be of value in understating the pathophysiology of the neurological features observed in patients with methylmalonic acidemia and in the development of new strategies for treatment of these patients.

Acknowledgements

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3 ADMINISTRAÇÃO CRÔNICA DE METILMALONATO EM RATOS JOVENS ALTERA MARCADORES NEUROINFLAMATÓRIOS E MEMÓRIA ESPACIAL - MANUSCRITO

3.1 Título Original

Chronic administration of methylmalonate on young rats alters neuroinflammatory markers and spatial memory.

3.2 Autores

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Abstract: The methylmalonic acidemia is an inborn error of metabolism (IEM) characterized by methylmalonic acid (MMA) accumulation in body fluids and tissues, causing neurological dysfunction, mitochondrial failure and oxidative stress. Although neurological evidence demonstrate that infection and/or inflammation mediators facilitate metabolic crises in patients, the involvement of neuroinflammatory processes in the neuropathology of this organic acidemia is not yet established.
In this experimental study, we used newborn Wistar rats to induce a model of chronic acidemia via subcutaneous injections of methylmalonate (MMA, from 5th to 28th day of life, twice a day, ranged from 0.72 to 1.67 µmol/g as a function of animal age). In the following days (29th - 31st) animal behavior was assessed in the object exploration test and elevated plus maze. It was performed differential cell and the number of neutrophils counting in the blood; and the cerebral cortex levels of interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT) were measured.
Behavioral tests showed that animals injected chronically with MMA have a reduction in the recognition index (R.I.) when the objects were arranged in a new configuration space, but do not exhibit anxiety-like behaviors. The blood of MMA-treated animals showed a decrease in the number of polymorphonuclear and neutrophils, and an increase in mononuclear and other cell types. Concomitantly, MMA increased levels of IL-1β, TNF-α, and expression of iNOS and 3-NT in the cerebral cortex of rats.
The overall results indicate that chronic administration of MMA increased pro-inflammatory markers in the cerebral cortex, reduced immune system defenses in blood, and coincide with the behavioral changes found in

young rats. This leads to speculate that, through mechanisms not yet elucidated, the neuroinflammatory processes during critical periods of development may contribute to the progression of cognitive impairment in patients with methylmalonic acidemia.

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Cover Letter

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Prof. W.J. Schwaeble

November 30th, 2012

Dear Professor

It is a pleasure to send you our manuscript entitled *"***Chronic administration of methylmalonate on young rats alters neuroinflammatory markers and behavioral parameters***"*, which we would like to submit for publication in Immunobiology. I would like to declare that the work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. Beside, all authors participated in the research and/or article preparation. I also like to state that I have read and have abided by the statement of ethical standards for manuscripts submitted to Immunobiology. Accordingly, I would like to declare that all animal experimentation reported in this study have been conducted in accordance with the policies of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

I look forward to hearing from you in a next future.

Sincerely Yours,

Dr. Michele Rechia Fighera

Manuscript

Chronic administration of methylmalonate on young rats alters neuroinflammatory markers and spatial memory

Methylmalonate induces neuroinflammation in rats

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Keywords

Cerebral cortex, memory, methylmalonate, neuroinflammation, rat.

List of abbreviations

3-NT: 3-nitrotyrosine; BBB: blood-brain barrier; BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium; BSA: bovine serum albumin; CNS: central nervous system; COX-2: cyclooxygenase-2; ELISA: enzyme-linked immunosorbent assay; EPM: elevated plus maze; HPLC-UV: high performance liquid chromatography/ultraviolet; IEM: inborn error of metabolism; IL-1β: interleukin-1 beta; iNOS: inducible nitric oxide synthase; MCM: Methylmalonyl-CoA mutase; MMA: methylmalonic acid, methylmalonate; NMDA: *N*-methyl-D-aspartate; NFκB: nuclear factor kappa B; OET: objects exploration test; PBS: phosphate buffered saline; R.I.: recognition index; s.c.: subcutaneously; SDS: sodium dodecyl sulfate-polyacrylamide gels; SEM: standard error of the mean; TBS: Tris–borate saline; TBS-T: Tween 20 in Tris–borate saline; TNF-α: tumor necrosis factor-alpha.

Abstract

The methylmalonic acidemia is an inborn error of metabolism (IEM) characterized by methylmalonic acid (MMA) accumulation in body fluids and tissues, causing neurological dysfunction, mitochondrial failure and oxidative stress. Although neurological evidence demonstrate that infection and/or inflammation mediators facilitate metabolic crises in patients, the involvement of neuroinflammatory processes in the neuropathology of this organic acidemia is not yet established.

In this experimental study, we used newborn Wistar rats to induce a model of chronic acidemia via subcutaneous injections of methylmalonate (MMA, from 5th to 28th day of life, twice a day, ranged from 0.72 to 1.67 μmol/g as a function of animal age). In the following days (29th – 31st) animal behavior was assessed in the object exploration test and elevated plus maze. It was performed differential cell and the

number of neutrophils counting in the blood; and the cerebral cortex levels of interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT) were measured.

Behavioral tests showed that animals injected chronically with MMA have a reduction in the recognition index (R.I.) when the objects were arranged in a new configuration space, but do not exhibit anxiety-like behaviors. The blood of MMA-treated animals showed a decrease in the number of polymorphonuclear and neutrophils, and an increase in mononuclear and other cell types. Concomitantly, MMA increased levels of IL-1β, TNF-α, and expression of iNOS and 3-NT in the cerebral cortex of rats.

The overall results indicate that chronic administration of MMA increased pro-inflammatory markers in the cerebral cortex, reduced immune system defenses in blood, and coincide with the behavioral changes found in young rats. This leads to speculate that, through mechanisms not yet elucidated, the neuroinflammatory processes during critical periods of development may contribute to the progression of cognitive impairment in patients with methylmalonic acidemia.

Introduction

Methylmalonyl-CoA mutase (MCM) deficiency or its cofactor, 5-deoxyadenosylcobalamin, leads to accumulation of methylmalonic acid (MMA) (Chandler, et al., 2009, Oberholzer, et al., 1967). This condition is known as methylmalonic acidemia, an inborn error of metabolism (IEM) with incidence of approximately 1:50,000 births (Shigematsu, et al., 2002). Extracellular accumulation of MMA acts as a potent neurotoxic metabolite which causes excitotoxicity (Kolker, et al., 2000), breakdown of mitochondrial energetic metabolism (Chandler, et al., 2009) and oxidative stress in the central nervous system (CNS) (Fernandes, et al., 2011). In line of

this view, it has been demonstrated that MMA accumulation plays a role in neurological alterations including failure to thrive and psychomotor delay in patients with this organic acidemia (Manoli and Venditti, 1993). Furthermore, the neurological crises evidenced in patients with methylmalonic acidemia are typically precipitated by a high intake of protein or minor infections inducing a catabolic state during critical periods of development (Horster and Hoffmann, 2004).

Neuroinflammation is closely related to pathologies of the CNS, which can lead to neuronal death as well as to learning and memory deficits. Neuroinflammatory response is characterized by a breakdown of the blood-brain barrier (BBB), activation of the microglia, infiltration of peripheral immune cells and increases in cytokine release (Lucas, et al., 2006), which can lead to neuronal death (Glass, et al., 2010) and learning or memory deficit (Hein and O'Banion, 2012). For example, Chen and coworkers showed that convulsions induced by kainate raise the expression of cyclooxygenase-2 (COX-2) in the brain of rats; this enzyme is expressed in most of tissues during inflammatory response, which can lead to chronic lesions development (Chen, et al., 1995). Furthermore, systemic or central bacterial lipopolysaccharide (LPS) injections activate microglia, potently block neuronal differentiation and disrupt the integration of neurons into existing hippocampal circuitry (Ekdahl, et al., 2003, Monje, et al., 2003, Belarbi, et al., 2012).

Although the studies have shown that neuroinflammation has a main role in human neurological diseases, such as epilepsy (Aronica and Crino, 2011), autism (Vargas, et al., 2005), multiple sclerosis (Lu, et al., 2010), Alzheimer's (Venneti, et al., 2009), Huntington's (Moller, 2010) and Parkinson's disease (Chung, et al., 2010), there are no studies in clinic and experimental literature about the relation between methylmalonic acidemia and the above-mentioned condition. Therefore, the objective of

the present work was to verify if the experimental model of MMA chronic injection in young rats alters the inflammation markers in cerebral cortex and/or in behavioral parameters.

Materials and Methods

Animals and reagents

The present study utilized Wistar rats with 5 days of life. Pregnant rats and/or the babies were kept in laboratory-controlled conditions (12:12h light-dark cycle, lights on at 07am.; 24 ± 1ºC; 55% relative humidity) with free access to water and food (Supra; Santa Maria, RS, Brazil). The utilization of animals reported in this study was conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) policies, revised in 1996. All possible efforts were made in order to reduce the number of animals utilized, as well as to minimize the animal suffering. All reagents were purchased from Sigma (St. Louis, MO, USA), except the antibodies, which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Experimental design

The pregnant rats were placed in individual cages during the final days of gestation. Forty eight hours after labor, only a number of eight babies (especially male) were selected to continue in cages. On the fifth day, the babies received the treatment (saline solution or MMA). On the twenty-first day, the rats were weaned. The treatment finished on the twenty-eighth day. The behavioral tests occurred at days 29, 30 and 31; after this procedure, the rats were euthanized to biochemistry analysis (Figure 1).

Drug treatment

Methylmalonic acid was diluted in saline and buffered to pH 7.4 with NaOH 6M. MMA was subcutaneously (s.c.) applied twice a day, from the fifth to the twenty-eighth day of life, in order to reproduce a methylmalonic acidemia. MMA doses were based on previous studies by Dutra and coworkers (1991) (Dutra, et al., 1991). Therefore, from the fifth to the twelfth day of life the animals received 0.72 µmol of MMA by gram of body weight, from the thirteenth to the nineteenth day the rats received 0,89 µmol/g, and from the twentieth to the twenty-eighth day 1,67 µmol/g (Figure 1). According to these protocols, MMA concentration reaches similar levels to the ones found in the plasma (2.0 a 2.5 mM) and in the brain (1 a 2 µmol/g cerebral) of patients with acidemia (Dutra, et al., 1991). The control group animals were s.c. injected with saline (0.9 %). All solutions were prepared so that the animals received 10 µl of solution by gram of body weight in each injection.

Objects exploration test (OET)

OET was adapted from Cippitelli and coworkers (Cippitelli, et al., 2010). Approximately 12 hours after the last drug injection, the animals were acclimatized (section 1) during ten minutes to the open-field. The field consisted of a square box without ceiling, with 40 centimeters of width and 20 cm of height, painted in white, divided in 12 areas and visually uniform.

One day after the acclimatization, the animals were once again put in the field (section 2), which contained two identical objects (A = A') placed in a linear configuration for training. The objects were clean and counterbalanced between each animal.

Section three, named of recognition test with spatial alteration, took place 4 hours after the second section, to test spatial learning and memory. On this section, one of the objects (A') was moved to a diagonal configuration. After the test, once again the objects were clean and counterbalanced between the animals.

According to the protocol, ten minutes after section three, section four was initiated, named of object recognition test. On this section, one of the objects was replaced by a new object (A ≠ B) still on a diagonal configuration on the field space.

In order to avoid that the animals were guided by smell, the field and the objects were clean with ethanol solution 30% and dried after each section. Each section lasted 10 minutes and was recorded to later behavioral analysis. The criteria analyzed in section one was the number of crossing and rearing; on the other sections, it was analyzed the percentage of time each object was explored, which is also measured by the recognition index (R.I.) of memory on the moved (A') or new (B) object. The mentioned procedure is calculated by the formula: (time of investigation of the moved or new object x 100) / (time of investigation of both objects) (Bevins and Besheer, 2006).

Elevated plus maze (EPM)

On the following day of OET, in the morning, the animals were submitted to the elevated plus maze test. The maze consists of a wood structure 50 cm from the floor, with two opposite open arms (50 cm of length x 10cm of width each), which cross with two opposite enclosed arms (with the same dimensions of the open arms, but containing walls with 40cm of height).

Initially, the animals were put on the crossroad of the maze in a similar way (File and Gonzalez, 1996). The animals could explore the maze during 5 minutes.

During this period of time, the animals were recorded and, subsequently, it was analyzed the following behaviors: percentage of the total number of entrances in the enclosed and open arms and time (seconds) of exploration of each arm.

The apparatus was cleaned with ethanol solution 30% and dried with clean cloths after each animal made the test.

Right after this test, the animals were euthanized. The blood was collected and the cerebral cortex was removed and immediately frozen in liquid nitrogen for posterior biochemical analysis.

Differential blood cell count

The total number of neutrophils in blood was determined through the differential count by the method of May–Grünwald–Giemsa (Bins, et al., 1989). The total number of leukocytes was determined in Neubauer chamber after the dilution (1:20; v/v) in Turk solution (Gencian Violet 0,01%; glacial acetic acid 1% in distilled water).

Determination of cytokines

The content of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) was determined in cerebral cortex, which were homogenized in phosphate buffered saline (PBS, pH 7.4) containing bovine serum albumin (BSA, 10 mg/mL), EGTA 2 mM, EDTA 2 mM and PMSF 0.2 mM. The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) kit, commercially provided by R&D Systems (Minneapolis, MN, USA), in accordance with the manufacturer protocols, and the results are expressed in pg/mg of protein.

Western blot

Western blot technique was conducted with small modifications on the method described by Casu and coworkers (Casu, et al., 2007). The cerebral cortex was homogenized in rate 1:5 (w/v) with ice-cold A buffer (10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM NaF, 10 µg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 g for 45 minutes at 4 ºC.

The supernatant (S1), denominated cytosolic fraction, was reserved for posterior processing. The pellet (P1) was resuspended in the same used above volume of ice-cold buffer B (10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM NaF, 10 µg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, and 1 % Triton-X in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 g for 45 minutes at 4 ºC. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 100 µL of ice-cold buffer C (50 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM NaF, 10 µg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, 420 mM NaCl, and 25 % glycerol in 20 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 g for 45 minutes at 4 ºC. The supernatant (S3) was considered the nuclear fraction (Medeiros, et al., 2007).

The protein concentration in the cytosolic and nuclear fractions was determined using the Bradford method (1976) (Bradford, 1976). Equivalent amounts of protein (80 or 20 µg for cytosolic or nuclear fractions, respectively) were added to 0.2 volumes of concentrated loading buffer (200 mM Tris, 10 % glycerol, 2 % SDS, 2.75 mM β-mercaptoethanol, and 0.04 % bromophenol blue) and boiled for 10 min. Proteins were

separated in 12 % sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE) and transferred to polyvinilidene difluoride membranes.

Ponceau staining (data not shown) served as a loading control (Romero-Calvo, et al., 2010). Western blot analysis of inducible nitric oxide synthase (iNOS) was carried out in cytosolic fractions. Membranes were processed using a SNAP i.d. system (Millipore, Billerica, MA, USA). First, the membrane was blocked with 1 % BSA in Tris–borate saline (TBS), then incubated for 10 min with specific primary antibody diluted 1:300 in TBS. iNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA catalog numbers sc-372 and sc-8310). Blots were washed three times, with 0.05 % Tween 20 in Tris–borate saline (TBS-T) followed by incubation with adjusted alkaline phosphatase-coupled secondary antibody (1:3.000, anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium (BCIP/NBT; Millipore). Membranes were dried, scanned, and quantified with the ImageJ program.

High performance liquid chromatography/ultraviolet (HPLC-UV) detection

The determination of 3-nitrotyrosine (3-NT) and tyrosine levels were performed by HPLC-UV, based on the method of Erdal and coworkers (2008) (Erdal, et al., 2008). Basically, the cerebral cortex was homogenized in five volumes of Tris-HCl 30 mM (pH 7.4), and a part was hydrolyzed in HCl 12N (1:1) at 100 ºC during 12 hours. The samples were then filtered through a membrane (0.45 µm pore size, Millipore®) before the injection in the HPLC-UV equipment (Shimadzu®).

The analytical column was a 5 µm particle and 100 Å pore size Phenomenex® ODS-2 C18 reverse-phase column (4.6 × 250 mm, Allcrom, BR). The mobile phase was

50 mM sodium acetate, 50 mM sodium citrate and 8% (v/v) methanol, pH 3.1 (corrected with 12 N HCl). The HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/min and UV detector set at 274 nm. 3-NT levels were expressed as 3-NT (μM)/total tyrosine (μM).

Protein quantification

The protein content was colorimetrically determined by the method of Bradford (1976) (Bradford, 1976), or by the method of Lowry and coworkers (1951) (Lowry, et al., 1951). BSA (1 mg/mL) was the pattern used.

Statistical Analysis

Data from behavioral and neurochemistry experiments were analyzed by unpaired *t* test or Two-way ANOVA test when appropriated, and were expressed as means and standards error of the mean (S.E.M.). Statistical analyses were performed using the SPSS (statistical Package for the Social Sciences) software in a PC-compatible computer. The value of *t* or F are presented only if $P < 0.05$.

Results

Chronic MMA induces spatial memory deficits

During all the period of the chronic injection of drugs, the animals were weighted and, similar to the original protocol (Wajner, et al., 1988), there was no weight differences between the groups (Figure 2), indicating no malnutrition or failure to thrive.

On the following morning after the last drug injection, the animals of both groups were acclimatized to the open field (section 1, Figure 1) in order to initiate the

OET protocol. It was verified no difference between groups in the number of crossing (t = 0.2775, Figure 3A) or rearing (t = 0.9153, Figure 3B).

In the morning of the following day (section 2, Figure 1) the animals were put in open field with two identical objects disposed in a linear configuration. On this evaluation it was verified that the animals did not show preference by any of the objects (Figure 3C).

Section 3 was made four hours after section 2. Section 3 aimed at verifying if there was any type of spatial recognition impairment. When the objects were positioned in a diagonal configuration, the animals treated with MMA present a smaller R.I. of the dislocated objected if compared to the control group (t = 2.929, $P < 0.01$; Figure 3D).

Following the protocol, section 4 was made 10 minutes after the end of section 3. On section 4, the objected which was moved before was substituted by a new one. However, there was no difference between the groups in this object recognition test (t = 0.3670, Figure 3E).

On the following day, the animals were tested in the morning in the EPM (Figure 1).

The EPM test has been used to verify the occurrence of anxiogenic or anxiolytic effects in various experimental models (File, et al., 1998, Pinton, et al., 2011), which could influence in others cognitive tests, such as OET. Table 1 presents the results on the analyzed criteria in the EPM test. There was no difference between groups regarding the time (in seconds) at which the animals were in the open or closed arms (t = 0.9272), or the percentage of entries into the open or closed arms (t = 0.6234). It proves that there were no alterations in anxiety-like behavior.

Chronic MMA alters the number of leukocytes

After the EPM, the animals were euthanized and were collecting the samples (Figure 1).

In the blood there was a count of the total leukocytes, in which was possible to perceive a reduction in the number of polymorphonuclear cells and an increase in the mononuclear cells in the group treated with MMA ($t = 5.505$, $P < 0.0001$; Figure 4A and B, respectively).

When the differential count was made in blood, it was possible to perceive a reduction in the neutrophils number and an increase in the number of other types of blood leukocytes in MMA-treated group ($t = 2.612$, $P < 0.05$; Figure 4C and D, respectively).

Chronic MMA induces an increase on inflammatory markers in the cerebral cortex

The cerebral cortex was frozen in liquid nitrogen and after homogenized in the ideal buffer for each biochemical analysis.

The measurement of the two pro-inflammatory cytokines IL-1β e TNF-α was made by the ELISA technique. As a result, it was observed an increase both in the IL-1β ($t = 3.199$, $P < 0.01$; Figure 5A) levels as in the TNF-α in cerebral cortex of the animals treated with MMA ($t = 5.357$, $P < 0.0001$; Figure 5B).

Knowing that the inflammatory cascade also leads to the induction of iNOS (Aktan, 2004), it was utilized the western blot method to quantify the immunoreactivity of this enzyme. Once again, the group treated with MMA presented an increase in the iNOS ($t = 2.402$, $P < 0.05$; Figure 6A) levels in the cerebral cortex.

Besides that, the iNOS causes a quick and excessive production of nitric oxide, which can result in nitrosative stress, another marker associated to inflammatory

processes (Schopfer, et al., 2003). Therefore, making use of the detection method by HPLC-UV, it was possible to measure the 3-NT levels, in which was verified an increase in the levels of this marker ($t = 2.718$, $P < 0.05$; Figure 6B) in the cerebral cortex of the group treated with MMA.

Discussion

Patients with methylmalonic acidemia usually present acute clinical features early in life resulting from metabolic decompensation, with recurrent vomiting, dehydration, respiratory distress and neurological symptoms, including psychomotor delay, irritability, lethargy, hypotonia, convulsions and coma. Most children survive to the first acute metabolic crisis, but develop long-term complications including neurological deficits (Baumgarter and Viardot, 1995, Leonard, 1995, Horster, et al., 2007). Although it is believed that these abnormalities occur as result of the primary metabolic impairment, the underlying mechanism of brain damage and neurological deficits in methylmalonic acidemia is poorly understood.

It is known that patients and experimental models of this IEM exhibit neuronal damage and changes in several areas of the central nervous system, as well as in several other neurological diseases (Melo, et al., 2011, Radmanesh, et al., 2008). Often this is related to oxidative stress and neuronal death, causing cognitive impairment and neuroinflammatory processes (Lee, et al., 2009, Cameron and Landreth, 2010, Hein and O'Banion, 2009). Recent articles in experimental models have elucidated the role of inflammatory mediators in models of acute seizures by MMA (Salvadori, et al., 2012, Ribeiro, et al., 2009). However, it is not known if MMA itself might increase these mediators.

We conducted this study with rats until the 31st day of life, a period of development with proven synaptogenesis and cell proliferation in several brain structures involved in learning and memory. We found that the chronic MMA administration (5 º -28 º day of life) at doses that raise its concentration in the blood and in the brain (Dutra, et al., 1991) causes memory deficits in spatial recognition, decreased number of polymorphonuclear cells (neutrophils) while increasing blood mononuclear leukocytes, and induces increased levels of pro-inflammatory cytokines (IL-1β and TNF-α), iNOS immunoreactivity and consequent nitrosative stress in the cerebral cortex.

This is the first study to investigate the effect of this experimental protocol on learning/memory through the OET in the days succeeding injections of MMA, and showed that the accumulation of the same may cause a decrease in R.I. when the objects are arranged in a spatial configuration different of the original. In previous studies by Pettenuzzo and collaborators (Pettenuzzo, et al., 2003, Pettenuzzo, et al., 2003), there was a deficiency in the purchase of a new paradigm of spatial localization in Morris water maze test of learning/memory, performed approximately two weeks after the probe trial, indicating a perseverative behavior. It also demonstrated that the damage MMA-caused on the CNS can be observed even in a long period of time after its last administration (more than one month and half). Thus, despite differences (temporal and techniques) between protocols performed in this and the aforementioned studies, it was found a similarity in the case of the inflexibility behavioral presented by the animals of MMA-injected group, which can result from damage caused by even at the CNS (Pettenuzzo, et al., 2003, Royes, et al., 2006). It should be remembered that several subcortical structures are involved in the acquisition, consolidation and evocation of memory (Izquierdo and Medina, 1997, Izquierdo, et al., 2006, McGaugh, 2000), and

these could also suffer consequences arising from the accumulation of MMA, as has been observed in experimental models of this acidemia (Wajner, et al., 1988, Pettenuzzo, et al., 2003, Vasques, et al., 2006, Malfatti, et al., 2003). However, this is only a supposition and would require more experiments to verify the possibility of neuroinflammatory or neurodegenerative processes in other brain structures.

Out of CNS, the blood presents as first line of defense of the organism the innate immune system. The neutrophils are polymorphonuclear leukocytes of immune system and have the function of phagocyte microorganisms or particles (Kobayashi and DeLeo, 2009). Patients with variants of methylmalonic acidemia show reduction in the number of these cells, a condition called neutropenia (Watkins and Rosenblatt, 2011, Guerra-Moreno, et al., 2003). In addition, Hutchinson and colleagues elegantly demonstrated that MMA causes a suppression of granulopoietic progenitor cell proliferation in marrow culture, suggesting that it could be one cause of neutropenia in patients (Hutchinson, et al., 1985). On the other hand, treatment with MMA also induced an increase in other types of leukocytes, peripheral blood mononuclear cells, such as lymphocytes and monocytes. It should be pointed out that on the total white cell count did not differ between groups (data not shown), and this large number of mononuclear cells may be only a "relative leukocytosis" due to the lower number of neutrophils.

In the CNS, neutrophils are usually not present because they do not cross the blood brain barrier. At this place, the first and foremost immune defense is the microglia cells (Ransohoff and Brown, 2012). They are one of the responsible for the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, which are accepted as modulators of neurotransmission within the brain (Merrill, 1992). In the present study we showed that MMA administration induced the increase of pro-inflammatory cytokines (IL-1β and TNF-α). Current evidence indicates that cytokines,

particularly IL-1β, increase neuronal excitability by activating IL-1 receptors (Vezzani, et al., 1999, Bernardino, et al., 2005). The neuronal IL-1R1 stimulation induces Src kinase-mediated tyrosine phosphorylation of the NR2B subunit in *N*-methyl-D-aspartate (NMDA) receptor. As a consequence, IL-1β facilitates NMDA receptor-mediated Ca^{2+} influx into neurons, promoting excitotoxicity (Viviani, et al., 2003). Considering that IL-1β can also inhibit glutamate uptake in astrocytes (Hu, et al., 2000) and increase its glial release possibly via TNF-α production (Bezzi, et al., 2001) it is plausible to propose that increase of pro-inflammatory cytokines result in elevated extracellular glutamate levels and toxicity in this model of organic acidemia. In agreement of this view, a considerable body of evidence has demonstrated that excessive glutamate receptor stimulation, in particular the NMDA receptor, has been implicated as a major pathway that leads to MMA-induced toxicity (de Mello, et al., 1996, de Mattos-Dutra, et al., 2000).

Furthermore, TNF-α and IL-1β acts in their respective receptors and cause activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a transcription factor that migrates to the cell nucleus and promotes the expression of iNOS (Aktan, 2004). The iNOS is one of three isoforms of nitric oxide synthase (EC 1.14.13.39) which, even though it does not present a greater specific activity than the other isoforms, can be highly expressed during the inflammatory process and, for presenting activity even at normal concentrations of intracellular calcium, can cause an increased production of NO (Hemmens and Mayer, 1998, Nathan and Xie, 1994). NO is a free radical that when reacting with superoxide anion ($O_2^{\bullet-}$, also formed during the inflammatory process by enzymes such as NADPH oxidase) generates the highly reactive peroxynitrite ($ONOO^-$) (Chung, 2006). This free radical then can nitrate tyrosine residues to 3-NT in proteins, a condition known as nitrosative stress, and can

also induce lipid peroxidation, and DNA damage (Chung, 2006, Ischiropoulos and Beckman, 2003). In the present study we revealed that chronic MMA administration induced increase in iNOS expression and consequent nitrosative stress in the cerebral cortex of rats.

In the literature, until the present moment, only the study of Goyenechea and colleagues (2012) (Goyenechea, et al., 2012) investigated the effect of chronic injections of MMA on pro-inflammatory markers but in renal cortex, one of the organs affected by acidemia. Among the results obtained in the study it was found, for example, increased levels of TNF-α and a positive correlation between the levels of mRNA of TGF-β, as well as urinary excretion of MMA, which can be important in inflammation and renal damage. Other studies using the same model of this acidemia observed small amounts of ganglioside related to synaptogenesis in the cerebellum and cerebrum (Wajner, et al., 1988, Brusque, et al., 2001), and reduced activity of the enzyme Na^+,K^+-ATPase activity in cerebral cortex (Wyse, et al., 2000). These effects may be due to inhibitory effects on several metabolic pathways and/or by oxidative stress caused by MMA (Wyse, et al., 2000, Mirandola, et al., 2008, Royes, et al., 2005, Wajner and Coelho, 1997). In another model of this acidemia, [U-14C]acetate incorporation into the lipids of cerebral cortex was reduced by MMA, which may explain the hypomyelinization and/or demyelinization characteristic of patients and, together with the findings of this paper, we can hypothesize that is mediated by immune system (de Mello, et al., 1997, Mayo, et al., 2012). Moreover, experimentally or clinically, cytokines interfere directly or indirectly in the process of memory consolidation, synaptic plasticity and/or neurogenesis, and chronic expression of neuroinflammatory mediators potentially implies in neuronal damage leading to cognitive impairment (Bossu, et al., 2012).

Although the results of the present study may suggest an association between memory deficits and increased pro-inflammatory markers in the cerebral cortex of animals treated with MMA, we are only aware that these data do not point out if/how a direct association actually exists. Changes in these pro-inflammatory markers may influence behavior by affecting neurotransmission, endocrine system, neuronal plasticity and brain circuitry (Bossu, et al., 2012), mechanisms and functions which evaluation is beyond the scope of this study. Therefore, it is interesting that more experiments are performed to question the assumption that the neuroinflammatory process is linked to pathophysiology of methylmalonic acidemia.

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Figure legends

Figure 1 Experimental design

Female Wistar rats which gave birth (day 0) were separated into individual cages some days before. On day 2 rats were selected in each litter 8 (preferably male) to remain in the cages. On day 5 the animals began receiving twice a day s.c. injections of MMA (n = 4/cage, 0.72 mmol/g) or saline solution (n = 4/cage). On day 13 and 20 the dose of MMA was increased (0.89 mmol/g and 1.67 mmol/g, respectively). On day 21 weaning of animals occurred with the removal from their mothers. On day 28 the animals received the last two injections and on days 29 to 31 were subjected to behavioral tests (OET and EPM). After testing the EPM, in the morning of day 31, the animals were euthanized, blood was collected and the cerebral cortex was removed and frozen for posterior biochemical analysis.

Figure 2 Treatment effects on the body weight of the animals

From 5th to 28th day, the animals were weighed (g) to daily adjust of doses of saline or MMA to be injected. There was no difference in weight gain between the two groups. Data represents mean ± standard error of the mean (SEM).

Figure 3 Treatment effects on OET

Was observed the number of crossing (A) and rearing (B) during the acclimatization of the animals (section 1, 29th day) in the open field. The next day (30th day), the animals returned to the open field and the preference for each one of the similar objects was checked (section 2) by the exploration time (in seconds) (C). Four hours later, one of the objects in the open field was placed in a diagonal position (section 3) and the R.I. by the percentage of time exploring the objects was assessed (D). Ten minutes later, the animals returned to the open field containing two different objects (section 4) and the R.I. was accessed again (E). Data represents mean ± SEM, except for the graph of time for exploration of similar objects (C), which is represented by the median ± interquartile range. According to unpaired t test, *$P < 0.05$ was considered significant.

Figure 4 Treatment effects on differential blood cell count

The levels of polymorphonuclear (A) and mononuclear cells (B) were estimated in a Neubauer chamber, and the result is expressed as a percentage of total leukocytes. The cellular differentiation estimated the number of neutrophil granulocytes (C) and other leukocytes types (D), and is displayed as percentage of total cells. Data represents mean ± SEM. According to unpaired t test, *$P< 0.05$ was considered significant.

Figure 5 Treatment effects on the levels of pro-inflammatory cytokines

Levels of IL-1β (A) and TNF-α (B) in the cerebral cortex of rats were estimated by ELISA and are expressed in pg/mg of protein. Data represents mean ± SEM. According to unpaired t test, $*P < 0.05$ was considered significant.

Figure 6 Treatment effects on induction of iNOS and nitrosative stress

The levels of iNOS (A) in the cerebral cortex of rats were estimated by Western blot (representative bands of groups in the box) and are expressed as percentage of control. The levels of 3-NT (B) in the cerebral cortex of rats were determined by HPLC-UV and are expressed in µmols by the total of tyrosine residues. Data represents mean ± SEM. According to unpaired t test, $*P < 0.05$ was considered significant.

Figure 1
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Figure 2
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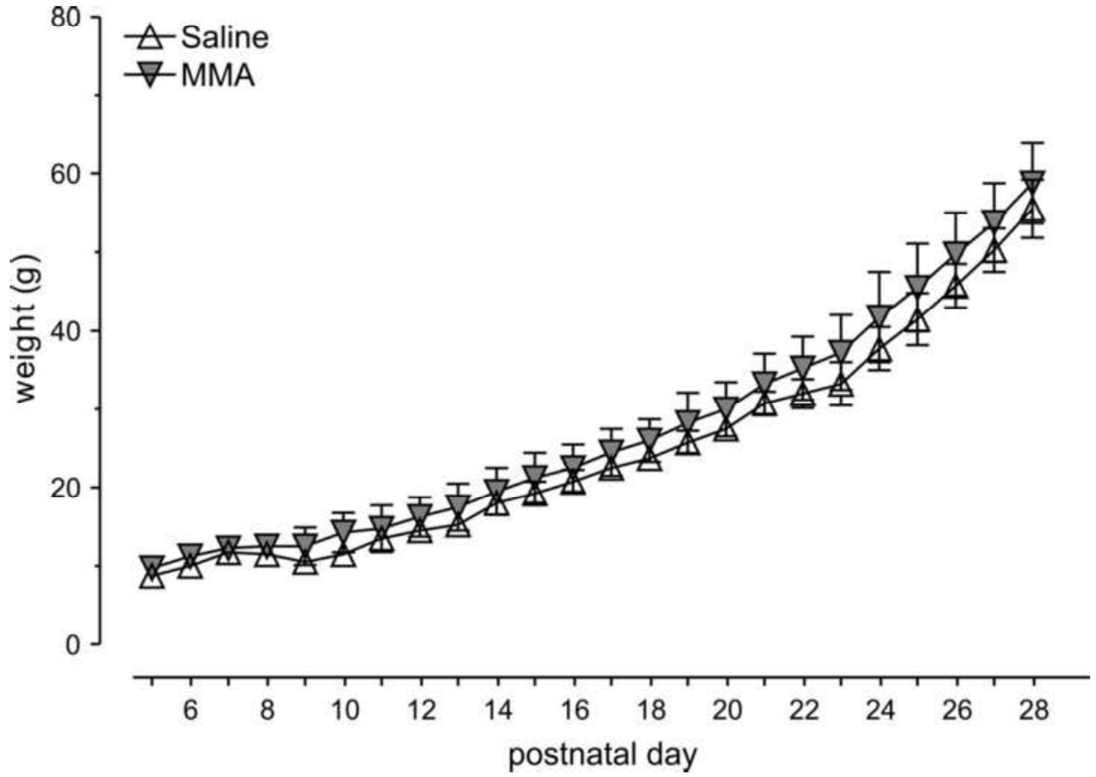

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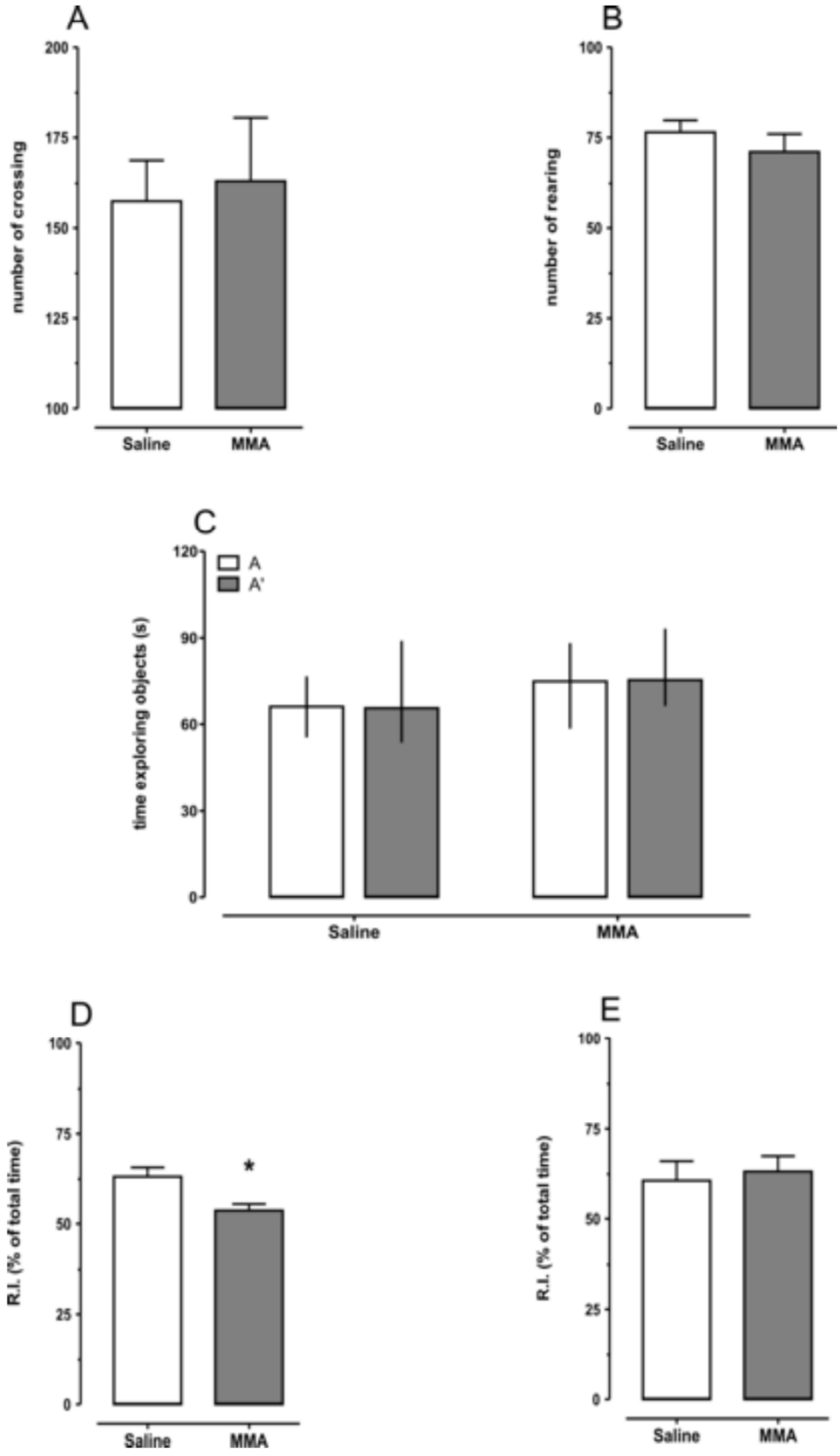

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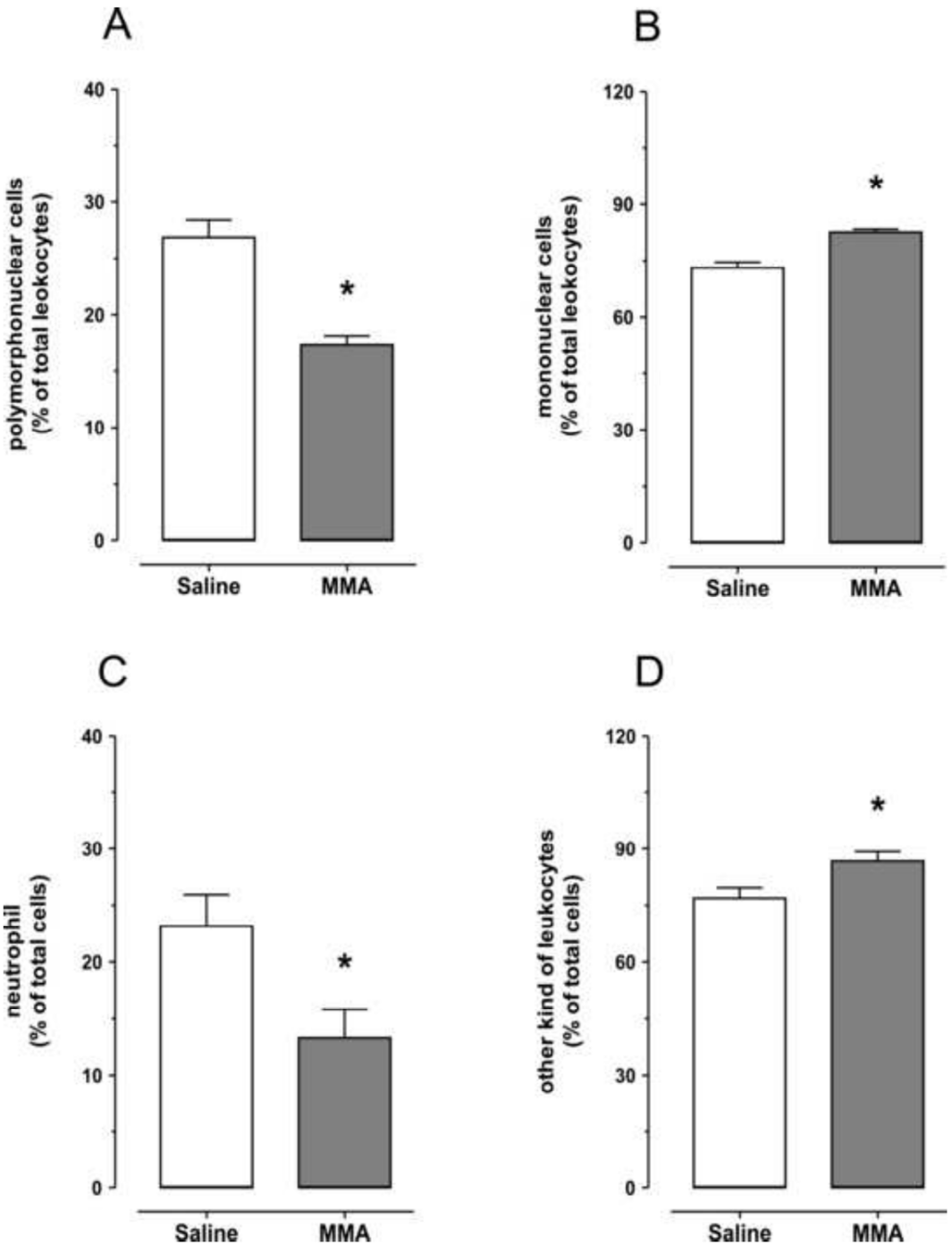

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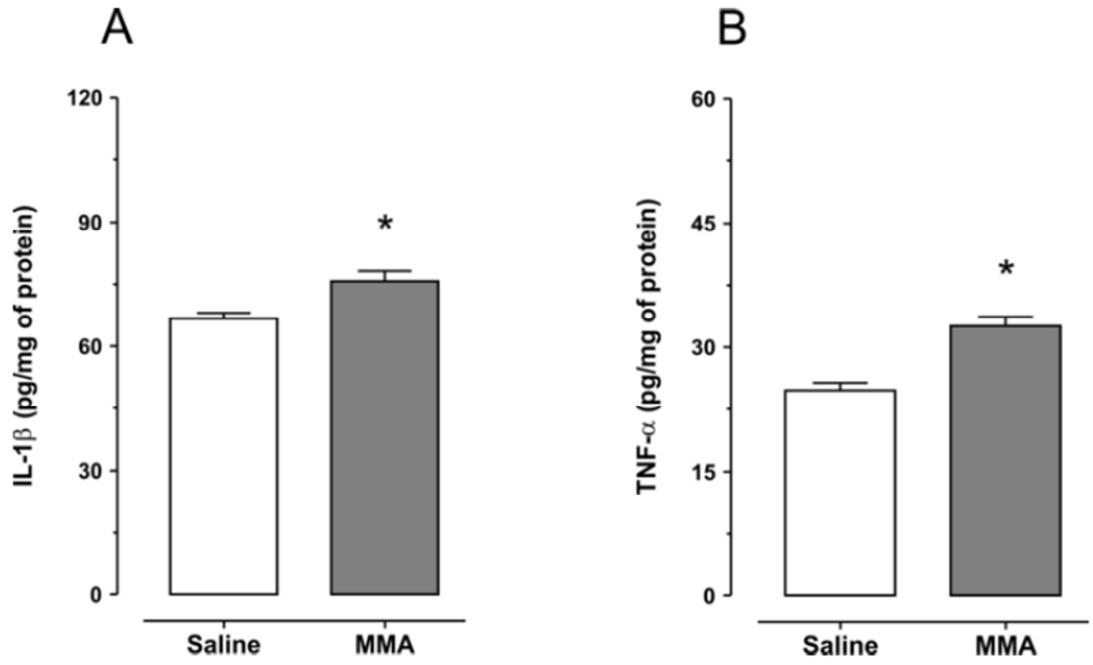

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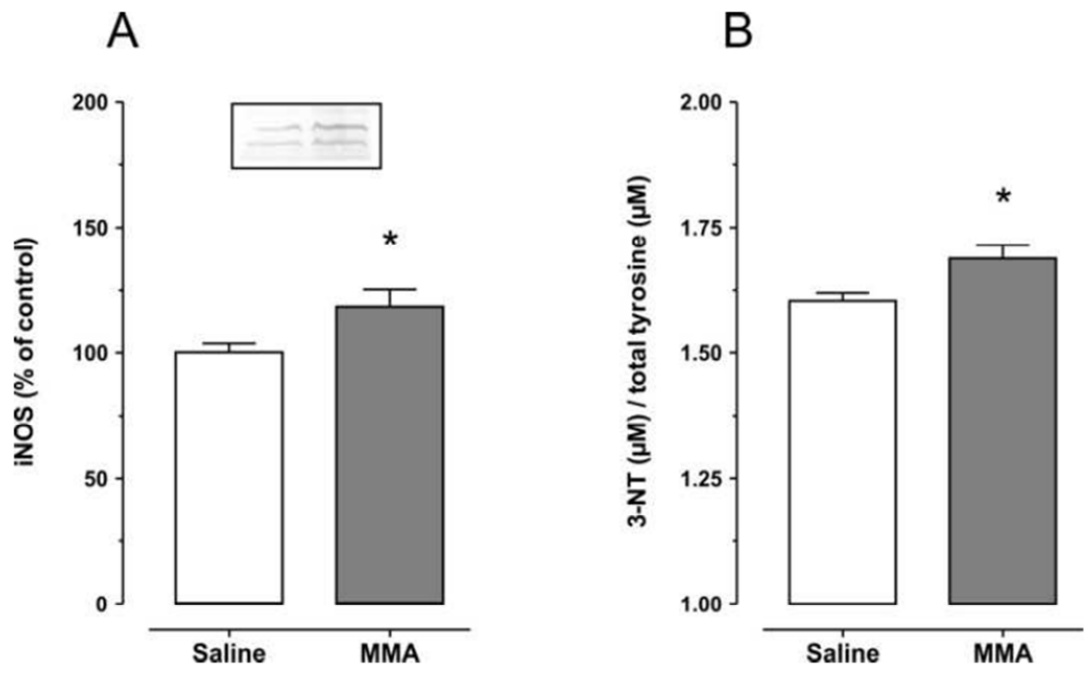

Table 1

Table 1 Assessment of the behavior in the EPM test

Parameter evaluated in EPM	Saline	MMA
Entries into open arms (%)	23,1±3,340	20,1±3,455
Entries into closed arms (%)	76,9±3,340	79,9±3,455
Time spent into open arms (s)	23,8±4,352	18,3±4,030
Time spent into closed arms (s)	241,5±6,979	247,2±5,427

EPM test was conducted on the 31st day of the experimental protocol. Data represent mean ± SEM. According to unpaired t test, there were no differences between the groups.

4 DISCUSSÃO

A Acidemia Metilmalônica é um EIM, uma acidemia orgânica que causa intoxicação aguda ou crônica devido à deficiência, parcial ou total, na atividade da MCM, ou defeitos na captação, transporte ou síntese da AdoCbl, o cofator da enzima, levando ao acúmulo primário de MMA e outros metabólitos nos órgãos, tecidos e fluídos corporais (MANOLI; VENDITTI, 1993; FENTON; ROSENBERG, 1995; SAUDUBRAY; SEDEL; WALTER, 2006). Os sinais clínicos da acidemia podem demorar a aparecer, variando de horas até semanas depois do nascimento, e progridem de sintomas gastrintestinais inespecíficos para manifestações neurológicas como as convulsões, podendo culminar em coma, morte ou lesão cerebral permanente, com degeneração seletiva dos núcleos da base e do córtex cerebral, e o consequente déficit neurológico (MANOLI; VENDITTI, 1993; DEODATO et al., 2006).

Atualmente, o tratamento dos pacientes com Acidemia Metilmalônica inclui medidas terapêuticas como remoção das toxinas por transfusão de sangue, restrição dietética para aminoácidos propiogênicos, alta ingestão calórica, administração de L-carnitina para tamponamento do propionil-CoA, utilização de antibióticos para reduzir a produção de propionato pela flora intestinal, utilização de antioxidantes, transplante hepático para prover atividade enzimática da MCM, e suplementação com vitamina B_{12} para algumas variantes da acidemia (MANOLI; VENDITTI, 1993; OGIER DE BAULNY; SAUDUBRAY, 2002; DEODATO et al., 2006). Considerando que estes tratamentos não são completamente eficazes na prevenção das manifestações clínicas, se faz necessária a busca por outras "estratégias" que possam auxiliar no tratamento desta acidemia.

No artigo intitulado "Convulsões Induzidas por Metilmalonato são Atenuadas em Camundongos Nocaute para Óxido Nítrico Sintase Induzível", publicado em 2009 no periódico *International Journal of Developmental Neuroscience*, utilizamos animais com genes "nocauteados" para a iNOS. Esta técnica foi desenvolvida pelos geneticistas Martin J. Evans, Mario Capecchi e Oliver Smithies, laureados em 2007 com o Prêmio Nobel de Medicina ou Fisiologia, e baseia-se em inativar um determinado gene substituindo-o ou interrompendo-o com um pedaço artificial de DNA, o que causa alterações no fenótipo do animal, como em características bioquímicas, e representa uma importante ferramenta de pesquisa (MANIS, 2007). Dessa forma, os camundongos nocaute para iNOS não expressam esta enzima e, portanto, produzem NO somente pelas outras isoformas da NOS. Este foi o

meio para se estudar o papel da iNOS nas convulsões induzidas pela injeção i.c.v. de MMA em camundongos, um modelo experimental agudo da Acidemia Metilmalônica.

Este estudo mostrou que camundongos nocaute para iNOS permaneceram menos tempo em convulsão, quando injetados i.c.v. com MMA, evitando um aumento na amplitude média EEG durante o período de avaliação. Os animais nocaute não apresentaram aumento nos níveis de nitrito e nitrato (NOx), metabólitos estáveis do NO (MIRANDA; ESPEY; WINK, 2001); mas tiveram uma redução parcial no dano nitrosativo, mensurado pela 3-nitrotirosina (3-NT). Além disto, os camundongos nocaute tiveram menor inibição na atividade da Na^+,K^+-ATPase, uma enzima que contribui para manutenção do gradiente eletroquímico responsável pelos potencias de ação e repouso, e captação e liberação de neurotransmissores (STAHL; HARRIS, 1986); mas não houve diferença na inibição da SDH, uma enzima presente na membrana mitocondrial interna que faz parte do Ciclo de Krebs e da cadeia transportadora de elétrons (YANKOVSKAYA et al., 2003).

Já no manuscrito intitulado "Administração Crônica de Metilmalonato em Ratos Jovens Altera Marcadores Neuroinflamatórios e Memória Espacial", submetido em 2012 para o periódico *Journal of Neuroinflammation*, recriamos o modelo experimental da Acidemia Metilmalônica desenvolvido pelo professor Moacir Wajner (WAJNER et al., 1988), com a injeção subcutânea e crônica de MMA em ratos durante um período de desenvolvimento, para causar o acúmulo do mesmo, em concentrações semelhantes à encontrada em pacientes recém-nascidos. O objetivo deste estudo era verificar se este modelo da acidemia poderia desencadear processos neuroinflamatórios, como induzir a expressão da iNOS.

Observou-se através deste estudo que o tratamento crônico com MMA desencadeou um déficit de memória/aprendizado espacial dos ratos. O tratamento com MMA também causou uma redução no número de neutrófilos, as primeiras células sanguíneas leucocitárias que deveriam chegar nas áreas de inflamação para defender o organismo (WITKO-SARSAT et al., 2000); mas levou à um aumento de leucócitos mononucleares, que também fazem parte do sistema imunológico, como os linfócitos e monócitos. Ademais, o MMA induziu um aumento nos níveis da interleucina-1beta (IL-1β) e do fator de necrose tumoral-alfa (TNF-α), que são citocinas pró-inflamatórias; assim como um aumento na expressão da iNOS e no dano nitrosativo, verificado pelos níveis elevados de 3-NT.

Como dito anteriormente, uma das características da injeção i.c.v. de MMA é o aparecimento de convulsões (DE MELLO et al., 1996). Logo, através dos achados do primeiro estudo foi comprovado que existe uma relação entre a produção de NO pela isoforma induzível da NOS e a duração das convulsões, estresse nitrosativo e redução na

atividade da Na^+,K^+-ATPase. Inclusive, um prejuízo ao funcionamento desta enzima pode ser consequência do estresse nitrosativo e também pode ser a causa do desenvolvimento e da propagação de episódios convulsivos (MORO et al., 2005; FURIAN et al., 2007).

Apesar dos animais injetados com MMA de modo crônico (via s.c.) não apresentarem esse tipo de manifestação comportamental, um conjunto de evidências farmacológicas e neuroquímicas suporta a ideia de que a inflamação do SNC pode contribuir para a ocorrência de convulsões em várias formas de epilepsia e modelos animais de crises convulsivas (VEZZANI; GRANATA, 2005). Além disso, a inflamação está relacionada à diversas doenças do SNC, incluindo o traumatismo crânioencefálico, acidente vascular cerebral, epilepsia, esclerose múltipla, doença do neurônio motor, doenças do movimento, doença de Alzheimer, e doenças psiquiátricas, como a depressão, ansiedade e esquizofrenia. A maioria dos mediadores inflamatórios são muito pouco expressos no SNC saudável, mas eles são rapidamente induzidos por neurônios, astrócitos, micróglia e oligodendrócitos, em resposta ao dano tecidual ou infecções (LUCAS; ROTHWELL; GIBSON, 2006).

Assim sendo, o segundo estudo evidenciou que o MMA pode induzir um aumento de marcadores neuroinflamatórios, incluindo citocinas que desencadeiam a expressão da iNOS; o consequente dano nitrosativo; e outras alterações no sistema imunológico e no SNC. A relevância disto se dá pela ligação com o primeiro estudo: o aumento nos níveis da iNOS, desencadeado pelo acúmulo de MMA, pode facilitar o processo fisiopatológico e as manifestações neurológicas/comportamentais encontradas tanto na clínica quanto nos modelos animais da Acidemia Metilmalônica.

Embora os pacientes com Acidemia Metilmalônica apresentem alterações comportamentais e bioquímicas, tais como convulsões e alterações no sistema imunológico (CHURCH et al., 1984; FENTON; ROSENBERG, 1995), compatíveis com os modelos experimentais estudados, é complicado extrapolar os resultados apresentados com animais para o ser humano. Contudo, apesar das evidências apresentadas serem de grande valia para o entendimento da fisiopatologia desta doença, bem como no estabelecimento de novas condutas para tratamento dos pacientes, outros estudos em animais e seres humanos são necessários para avaliar as implicações clínicas da utilização de inibidores específicos da iNOS ou de anti-inflamatórios.

5 CONCLUSÃO

De acordo com os resultados obtidos, pode-se concluir que:

A iNOS está envolvida nas convulsões induzidas por MMA em camundongos selvagens e nocaute, pois aumenta os níveis de óxido nítrico, que podem causar parte do dano nitrosativo e da inibição da Na^+,K^+-ATPase.

O tratamento crônico com MMA em ratos jovens pode causar um déficit de memória/aprendizado espacial devido ao processo neuroinflamatório e dano nitrosativo encontrado no córtex cerebral.

Portanto, se os resultados apresentados neste estudo ocorrerem em humanos, a iNOS pode ser um possível alvo terapêutico para o tratamento/prevenção da neuropatologia dos pacientes com Acidemia Metilmalônica.

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