UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

Neurotoxidade Neonatal do Metilmalonato é suficiente para iniciar déficit de memória em camundongos: envolvimento de marcadores inflamatórios e apoptóticos.

Dissertação

Patrícia Gabbi

Santa Maria, RS, Brasil
2014

Neurotoxidade Neonatal do Metilmalonato é suficiente para iniciar déficit de memória em camundongos: envolvimento de marcadores inflamatórios e apoptóticos.

Por

Patricia Gabbi

Dissertação apresentada ao curso de mestrado do
Programa de Pós - Graduação em Farmacologia, do centro de ciências da Saúde, da Universidade Federal de Santa Maria (UFSM,RS) com requisito parcial para obtenção do grau de

Mestre em Farmacologia

Orientador: Profª. Dr. Michele Rechia Fighera

Santa Maria, RS, Brasil
2014

Universidade Federal de Santa Maria

Centro de Ciências da Saúde

Programa de Pós Graduação em Farmacologia

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A comissão examinadora, abaixo assinada.

Aprova a Dissertação de Mestrado

Elaborada por

Patricia Gabbi

Como requisito parcial para obtenção do grau de

Mestre em Farmacologia

COMISSÃO EXAMINADORA

Prof.ª Dra. Michele Rechia Fighera (UFSM)

(Presidente/ Orientadora)

Prof.ª Dra. Roselei Fachinetto (UFSM)

Profª. Dra. Maria Rosa Chitolina Schetinger (UFSM)

Santa Maria , 28 de junho de 2014.

AGRADECIMENTOS

Primeiramente, gostaria de agradecer, a Deus e a minha família que sempre me apoiou e acreditou em mim mais do que eu mesma, e nunca mediu forças para que hoje eu pudesse chegar até aqui. Pai, mãe vocês são minha força e minha inspiração, sem vocês não teria chego onde estou.

Aos meus orientadores Micheli e Nando, pela oportunidade que me deram, me recebendo no laboratório sem ter experiência nesta área. Agradeço pela amizade, pela paciência, pela compreensão dos erros e pelos conhecimentos a mim passados. Minha eterna admiração e reconhecimento, muito obrigada! Espero um dia conseguir retribuir todo esse apoio a vocês!

Falando em laboratório, essa grande família que é o BioEx, vocês não são apenas colegas de trabalho, mas sim grandes amigos, Iuri, Busanello, Fred, Mandolate, Rogério, Maurão, Fê tiopental, Ale, Marla, Ana Paula, admiro todos, vocês são pessoas incríveis! Obrigada pela ajuda no laboratório, pela parceria, amizade. Agradecimento especial a Fernanda por ter me ajudado sempre que precisei! Iuri em especial por ter me ajudado no layout das figuras o meu muito obrigado! Josi uma amiga, irmã de todas as horas que sempre me apoiou e confiou em mim o meu muito obrigado! Ângela minha amiga de conversas de parceria que me apoio tanto nos momentos bons como ruim e me aguentou nessa etapa final o meu muito obrigado! Simoni amiga de todas as horas, sempre me apoiou e me deu força para continuar nessa etapa, muito obrigado! Em especial as colegas de trabalho por realizar trocas de plantão, sempre que precisei muito obrigado!

À Universidade Federal de Santa Maria por tornar possível o sonho da pós-graduação em uma universidade pública e de qualidade.

Muito Obrigada!!!

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Farmacologia: Departamento de Fisiologia e Farmacologia

Universidade Federal de Santa Maria, RS, Brasil.

Neurotoxidade Neonatal do Metilmalonato é suficiente para iniciar déficit de memória em camundongos: envolvimento de marcadores inflamatórios e apoptóticos

Autor: Patricia Gabbi

Orientador: Michele Rechia Fighera

Co-orientador: Luiz Fernando Freire Royes

Local e data de defesa: Santa Maria, 28 de junho de 2014

A acidemia metilmalônica (AM) é um erro inato do metabolismo (IEM) caracterizado pelo acúmulo do ácido metilmalônico (MMA) nos fluidos corporais e tecidos, causando disfunção mitocondrial, estresse oxidativo e neuroinflamação, resultando principalmente em disfunções neurológicas. Embora, estudos sugerem que a infecção ou mediadores da inflamação facilitem as crises metabólicas nos pacientes afetados, o envolvimento de processos neuroinflamatórios na patologia dessas acidemia orgânica ainda não foi estabelecido. Neste estudo, uma dose única intracerebroventricular de MMA (MMA 2.5μmol/g; dose encontrada no cérebro e fluidos corporais de pacientes afetados) foi administrada a filhotes de camundongos logo após o nascimento (P0). Além disso, o peso dos animais foi aferido até o dia dos testes comportamentais. A partir do 21° até 33° ou 40º até 52° dias de vida, os animais foram avaliados em tarefas comportamentais como o teste de labirinto radial e em cruz elevado. Os níveis de fator de necrose tumoral-alfa (TNF-α), DCFH, atividade da acetilcolinesterase (AchE) e os níveis de caspases foram determinados no córtex cerebral, estriado e hipocampo de camundongos com 21 e 40 dias de vida. Os testes do labirinto radial mostrou que os animais injetados com o MMA apresentaram um pior desempenho no teste de memória de trabalho, mas não no teste de memória de referência com 21 e 40 dias de vida. Os animais não apresentaram comportamento de ansiedade. Além disso, o MMA aumentou os níveis de TNF- α, a atividade AchE e a ativação das caspases 1, 3 e 8 no córtex cerebral, hipocampo e estriado dos camundongos com 21 e 40 dias de vida. Entretanto, a administração de MMA não causou alterações histológicas nas estruturas analisadas. Dessa forma, os resultados sugerem que uma administração única de MMA aumentou os níveis de mediadores pró-inflamatórios e a expressão de marcadores apoptóticos. Esses eventos podem estar associados com as mudanças comportamentais encontradas nos camundongos jovens. Assim, pode-se sugerir que, devido a mecanismos ainda não totalmente esclarecidos, o acúmulo de MMA durante períodos críticos de desenvolvimento pode causar processos neuroinflamatórios, que contribuem para a progressão da piora de memória nos pacientes com acidemia metilmalônica.

PALAVRAS CHAVES: Córtex cerebral, estriado, hipocampo, memória, metilmalonato, camundongos, inflamação e marcadores apoptóticos.

ABSTRACT

Master Dissertation

Graduating Program in Pharmacology Sciences: Department of Physiology and Pharmacology

Federal University of Santa Maria, RS, Brazil

Neonatal Nerurotoxicity of Methylmalonate is Sufficient to Trigger Memory Deficit in mice: Involvement of Inflammatory and Apoptotic Markers

Author: Patricia Gabbi

Advisor: Michele Rechia Fighera

Co-adivisor: Luiz Fernando Freire Royes

Date and place of defense: Santa Maria, 28th June, 2014

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, oxidative stress and neuroinflammation. 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, a single intracerebroventricular dose of MMA (MMA 2.5µmol /g, 12 hs after birth; at dose that raise its concentration in blood and in the brain from affected) was administered to mice pups at postnatal day 0 (P0) to induce an acute, transient rise of MMA levels in the central nervous system (CNS). In the following days (21^{st} – 33^{th} or 40^{th} – 52^{th}) animal behavior was assessed in the radial maze test and elevated plus maze. It was measured tumor necrosis factor-alpha (TNF-α), DCFH, Ache activity and caspase levels in the cerebral cortex, striatum and hipoccampus from mice with 21 e 40 days of life. Behavioral tests showed that animals injected with MMA have a reduction in the working memory test, but no in the reference test. The animals did not exhibit anxiety-like behaviors. Furthermore, MMA increased levels of TNF-α, AchE activity and activation of caspases 1, 3 and 8 in the cerebral cortex, hippocampus and striatum of mice with 21 and 40 days of life. The overall results indicate that a simple administration of MMA increased pro-inflammatory markers in the structure studied, increased apoptotic markers, and coincide with the behavioral changes found in young mice. This leads to speculate that, through mechanisms not yet elucidated, the transient metabolic insult with MMA may cause a neuroinflammatory processes during critical periods of development, contributing to the progression of cognitive impairment in patients with methylmalonic acidemia.

Keywords

Cerebral cortex, striatum, hippocampus, memory, methylmalonate, mice, inflammatory and apoptotic markers.

LISTA DE FIGURAS E TABELAS

Tabela 1 - Principais manifestações clínicas e laboratoriais dos erros inatos do metabolismo no período neonatal. 13

Figura 1 – Rota metabólica da enzima Metilmalonil-CoA mutase (MCM)..... 15

Figura 2 – Figura representativa do mecanismo de ação relacionado ao prejuízo cognitivo induzido pelo MMA 76

LISTA DE ABREVIATURAS

AdoCbl	5'-desoxiadenosilcobalamina
ATP	Adenosina trifosfato, Trifosfato de adenosina
ATPase	Família de enzimas que catalisam a hidrólise da adenosina trifosfato para originar adenosina difosfato (ADP)
AP	Antero posterior
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
cblE	Variante da Acidemia Metilmalônica devido à mutação no gene 612625
CO_2	Dióxido de carbono
DCFH	Diacetato de diclorofluoresceína
ERN	Espécie reativa de nitrogênio
ex.	Exemplo
EIM	Erro inato do metabolismo
H_2O_2	Peróxido de hidrogênio
IL-1β	Interleucina-1beta
IRC	Insuficiência renal crônica
K^+	Potássio
L	Lateral
MAP 2	Microtúbulo do tipo 2
MCM	Metilmalonil-CoA mutase

MMA	Metilmalonato, ácido metilmalônico
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
NO	Óxido nítrico
SNC	Sistema Nervoso Central
TNF-α	Fator de necrose tumoral-alfa
V	Vertical

SUMÁRIO

LISTA DE FIGURAS E TABELAS 7

LISTA DE ABREVIATURAS 8

APRESENTAÇÃO 11

INTRODUÇÃO 12

1.1 Erros Inatos do Metabolismo 12

1.2 Acidemias Orgânicas 13

1.3 Acidemia Metilmalônica 14

1.3.1 Definição 14

1.3.2 Incidência 16

1.3.3 Manifestações clínicas 16

1.3.4 Fisiopatologia 17

1.3.5 Diagnóstico 18

1.3.6 Tratamento 20

1.4 Biomarcadores na Acidemia Metilmalônica 21

1.4.1 Biomarcadores do estresse oxidativo, inflamatório e marcadores apoptóticos na acidemia metilmalônica 21

1.4.2 Marcadores apoptóticos e inflamação na acidemia metilmalônica 23

1.5 Memória 24

1.6 Objetivo 26

1.6.1 Objetivo Geral 26

1.6.2 Objetivos Específicos 26

2. ARTIGO CIENTIFICO 30

3. DISCUSSÃO 68

4. CONSIDERAÇÕES PARCIAIS 75

5. CONSIDERAÇÕES FINAIS 76

6. REFERÊNCIAS BIBLIOGRÁFICAS 78

APRESENTAÇÃO

No item **INTRODUÇÃO** está descrita uma revisão de literatura sobre os temas abordados nesta dissertação.

Os resultados que fazem parte desta dissertação estão sob a forma de artigo, o qual se encontra no item **ARTIGO CIENTÍFICO**. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo e representam a íntegra deste trabalho.

Os itens **DISCUSSÃO** e **CONCLUSÕES** da dissertação apresentam interpretações e comentários gerais sobre o artigo científico contidos no final neste trabalho.

O item **REFERÊNCIAS BIBLIOGRÁFICAS** refere-se somente as citações que aparecem nos itens **INTRODUÇÃO E DISCUSSÃO** desta dissertação.

INTRODUÇÃO

1.1 Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM), foram descritos pela primeira vez por Archibald Edward Garrod em 1908, quando foram realizados trabalhos em pacientes com diagnósticos de Alcaptonúria, albinismo, cistinúria e pentosúria. Estas alterações ocorriam principalmente nos filhos de pais consanguíneos que apresentavam sinais de artrite aguda e excretavam grande quantidade de ácido homogentísico na urina (Scriver 2008). Baseado na observação da maior prevalência consanguínea entre os pais dos pacientes, foi possível observar uma relação nas leis de Mendel (Oberholzer, Levin et al. 1967; Giugliani 1988).

Atualmente, a condição EIM é chamada de distúrbio hereditário autossômico. Tal distúrbio resultará em deficiência na atividade de determinadas enzimas, que leva ao bloqueio de uma ou mais rotas metabólicas específicas. Situação esta que, além de induzir acúmulo de substâncias tóxicas e/ou a falta de substâncias essenciais, pode gerar distúrbios no desenvolvimento físico e mental (Fenton 1995; Scriver 2001; Illsinger and Das 2010).

Com o passar dos anos os avanços médicos relacionados à genética e aos erros inatos vão aumentando, atualmente, mais de 700 EIM já foram descritos (Illsinger and Das 2010). Embora essas doenças sejam raras, são responsáveis por altos índices de mortalidade e morbidade com uma prevalência aproximadamente entre 1:800 recém nascidos (Pampols 2010; Sahoo, Franzson et al. 2012).

Essa doença apresenta-se clinicamente de maneira variável sendo geralmente de sintomatologia grave ou muitas vezes fatal. A tabela 1 apresenta os sintomas clínicos e laboratoriais ocorridos mais frequentemente nos EIM.

Tabela 1. Principais manifestações clínicas e laboratoriais dos erros inatos do metabolismo no período neonatal.

Manifestações clínicas	Manifestações laboratoriais
• Diarréia • Coma • Vômitos • Letargia • Ataxia • Convulsão de causa desconhecida • Mioclonias • Hipotonia muscular • Deficiência do crescimento • Hepatomegalia • Dificuldade alimentar • Dificuldade respiratória e apneia • Anormalidades oculares • Dismorfias • Taquipnéia/apnéia • Odor peculiar na urina ou no paciente	• Acidose metabólica • Hipoglicemia/hiperglicemia • Neutropenia • Cetonúria • Anemia • Trombocitopenia • Presença de substâncias redutoras na urina • Elevação transaminase • Hiperamonemia

(Scriver 2001)

1.2 Acidemias Orgânicas

As Acidúrias ou acidemias orgânicas são erros inatos do metabolismo causados, muitas vezes, pela deficiência de uma atividade enzimática, relacionada ao metabolismo dos aminoácidos, glicídios ou lipídios. Esta redução e/ou perda da atividade enzimática causa o acúmulo tecidual de um ou mais ácidos carboxílicos e/ou derivados nos tecidos e fluidos corporais nos indivíduos afetados. Entretanto, alguns desses metabólitos podem agir como toxinas endógenas e serem neurotóxicos (Chalmers RA 1982; Scriver 2001; Sahoo, Franzson et al. 2012; Schuck, Busanello et al. 2013).

A incidência das acidúrias orgânicas na população em geral ainda é pouco conhecida. No entanto, na Holanda, um país modelo para o estudo dos erros inatos do metabolismo (EIM), há a ocorrência de 1 para cada 2.200 habitantes afetado por alguma dessas doenças, enquanto na Arábia Saudita, onde a taxa de consanguinidade é elevada, observa-se a incidência de pelo

menos 1 para cada 740 nascimentos (Hoffmann 1994; Rahbeeni, Ozand et al. 1994). Por mais que tenha ocorrido um progresso muito grande em diagnosticar recém-nascidos com EIM, como as acidemias orgânicas, evidenciou–se que elas são ainda as doenças metabólicas mais frequentes na população (Scriver 1995; Wajner, Barschak et al. 2001). Dentre essas acidemias, são as mais comuns 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 and Charpentier 1995; Wajner, Barschak et al. 2001).

1.3 Acidemia Metilmalônica

1.3.1 Definição

Acidemia metilmalônica foi descrita pela primeira vez por Oberholzer et al. (1967), o qual caracterizou quantidades elevadas de ácido metilmalônico (MMA) no sangue e na urina de crianças que desenvolviam cetoacidose (Fenton 1995). A acidemia metilmalônica é um erro inato do metabolismo autossômico recessivo, caracterizado pelo acúmulo tecidual de ácido metilmalônico e de seus metabólitos como propionato, metilcitrato, β-OH propionato e cetonas da cadeia longas, devido á deficiência da atividade da enzima L-metilmalonil-CoA mutase (MCM) (EC 5.5.99.2) e defeitos na síntese 5´ deoxyadenosylcobalamin (AdoCbl), formada a partir da vitamina B_{12} (cobalamina, cbl) (Fenton 1995; Royes, Fighera et al. 2007; Chandler, Zerfas et al. 2009). A deficiência enzimática ocorre na maior parte pela mutação no gene 609058 (MUT), que codifica a MCM, o que pode levar a uma perda parcial (MUT^-) ou total (MUT^0) na atividade da mesma (Manoli and Venditti 1993; Chandler and Venditti 2005; Tanpaiboon 2005). Essa enzima MCM faz parte da rota de degradação do propionato e converte o metilmalonil-CoA a succinil-CoA (Figura 1); (Fenton 1995; Lee, Chien et al. 2008). A acidemia orgânica, apresenta uma incidência aproximada de 1:50.000 – 1:80.000 nascidos vivos (Fenton 1995; Scriver 2001; Deodato, Boenzi et al. 2006).

A acidemia metilmalônica é caracterizada bioquimicamente pelo acúmulo de MMA no plasma e líquido cefalorraquidiano (aproximadamente 3 mM), assim como na urina (1.000 – 10.000 mmol/mol de creatinina) dos pacientes (CP 1993–2005 (updated 2010 Sep 28).; Fenton 1995; Morath, Okun et al. 2008). Os níveis sanguíneos de MMA em pacientes normais são quase indetectáveis, já os afetados encontram-se próximo de 2.9 mM (34mg/dl). A excreção desses metabolitos geralmente não passa de 5mg (0,04 mmol) em 24horas, mas esses valores podem ser modificados durante as crises metabólicas (Fenton 1995; Wright and Jalan 2007; Cichoz-Lach and Michalak 2013).

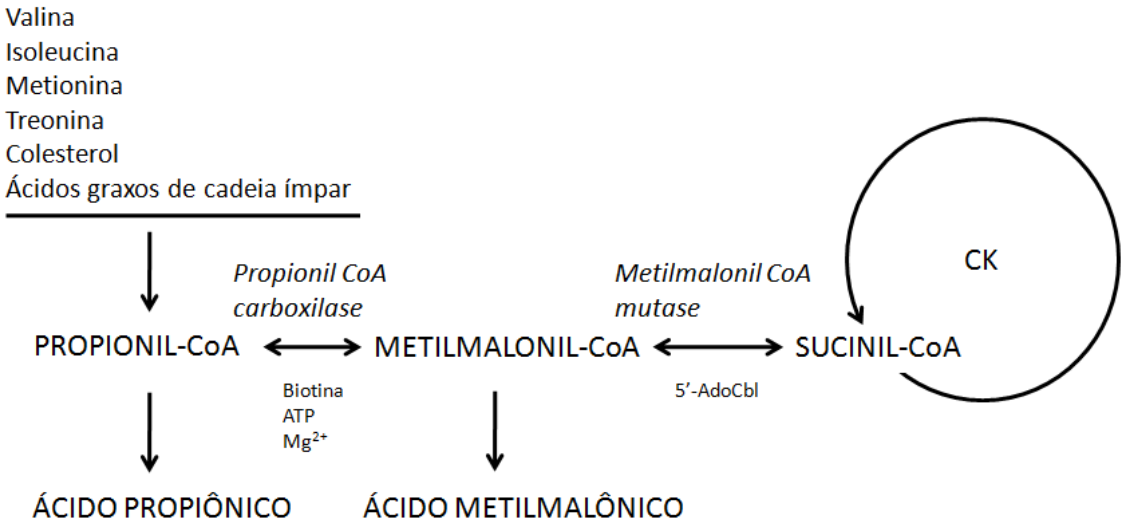

Figura 1: Metabolismo do propionil-CoA: metabólitos, cofator e enzima. A enzima metilmalonil-CoA mutase é responsável pela conversão do L-metilmalonil-CoA a succinil-CoA na rota de degradação de aminoácidos de cadeia ramificada, ácidos graxos de cadeia ímpar e do propionato (Scriver 2001).

A acidemia metilmalônica está relacionada a uma deficiência na enzima, podendo apresentar-se de duas formas: *mut(o)*, quando a enzima não tem qualquer atividade e que não responde a altas doses de cobalamina, e *mut(-)*, a enzima tem uma pequena reação quando o paciente recebe uma suplementação de cobalamina. Nos demais casos, a acidemia se deve a defeitos na síntese, ativação (cblA, cblB, cblC, cblD e cblE) ou transporte da cobalamina. A deficiência desta atividade enzimática leva ao acúmulo de ácido

metilmalônico (MMA) e de metabólitos secundários, tais como os ácidos propiônico, tíglico, 3-hidroxipropiônico, 2-metilcítrico, bem como propionil-CoA e propionilcarnitina (DS 2001; Cornejo, Manriquez et al. 2003; Sauer, Okun et al. 2008).

1.3.2 Incidência

Atualmente 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, variando de 1:115.000 na Itália até 1:169.000 na Alemanha (Coulombe, Shih et al. 1981; Rashed, Rahbeeni et al. 1999; Shigematsu, Hirano et al. 2002; Deodato, Boenzi et al. 2006), no Brasil este valor é 1:48.000 (Fenton.W.A 2001).

1.3.3 Manifestações clínicas

Os sinais clínicos podem apresentar-se nas primeiras horas de vida ou nas primeiras semanas até 21 dias de vida, estes sinais surgem e progridem inespecíficamente, dentre eles podemos citar: diminuição sucção recusa alimentar, vômitos, perda excessiva de peso, distensão abdominal e manifestações neurológicas progressivas (Lehnert, Sperl et al. 1994; van der Meer, Poggi et al. 1994). Estas manifestações neurológicas incluem: postura e movimentos anormais, hipotonia generalizada, letargia e convulsões, muitas vezes com um padrão eletroencefalográfico (EEG) de surto-supressão. Caso esses sintomas não sejam prontamente e adequadamente tratados, podem levar ao coma, edema cerebral, insuficiência respiratória, hipotermia, déficit cognitivo e os pacientes morrem em poucos dias, ou desenvolvem lesão cerebral permanente (Deodato, Boenzi et al. 2006).

Ao longo do tempo, se os pacientes não forem adequadamente tratados, começam a desenvolver problemas neuropatológicos, incluindo atrofia cerebral, desmielinizacão, anormalidades nos gânglios da base e na substância branca (Brismar and Ozand 1994; DS 2001; Deodato, Boenzi et al. 2006; Harting, Seitz et al. 2008). Os gânglios da base apresentam alterações no globo pálido e durante as crises de descompensacão metabólica ou por infecção, ocorre um aumento nos níveis de metabólitos tóxicos (Deodato, Boenzi et al. 2006;

Harting, Seitz et al. 2008). Além dos problemas neurológicos, os pacientes apresentam encefalopatia hepática e insuficiência renal crônica (IRC), os quais podem levar a complicações graves.

Quando esses problemas ocorrem mais tardiamente (de 2 a 4 anos de vida), os pacientes apresentam um prognóstico melhor, tanto do ponto de vista da morbidade quanto da mortalidade. As alterações metabólicas tardias são menos intensas do que as que acometem os pacientes logo após o nascimento (J; Kamoun P; Saudubray JM. 1994).

1.3.4 Fisiopatologia

A deficiência da atividade da enzima L-metilmalonil-CoA mutase resulta em um acúmulo de MMA e de outros metabólitos secundários (Fenton, 2001). A inibição do complexo piruvato carboxilase (Gregersen 1981) é impedida pela metilmalonil – CoA, assim como o N- acetilglutamato sintase é um ativador alostérico da carbonil fosfato sintase (Lehnert, Sperl et al. 1994; Filipowicz, Ernst et al. 2006; Morath, Okun et al. 2008), enzima responsável pelo primeiro passo do ciclo da uréia. Um bloqueio nessa síntese leva ao acúmulo de amônia provocando a ocorrência da hiperamonemia nesses pacientes (Stewart and Walser 1980; Morath, Okun et al. 2008). A inibição da enzima, β hidroxibutirato desidrogenase (Dutra, Dutra-Filho et al. 1993) e lactato desidrogenase (Saad, Mirandola et al. 2006) pelo metilmalonil CoA, podem contribuir para o aparecimento de alguns sintomas como hipoglicemia, acidemia láctica, cetoacidose (Ogier de Baulny and Saudubray 2002).

O mecanismo fisiopatológico do dano cerebral característico dos pacientes afetados pela acidemia metilmalônica ainda é pouco conhecido. Entretanto, observa-se que o acúmulo de ácidos orgânicos intracerebral está relacionado à neurodegeneração em pacientes acometidos por esta acidemia. (Melo, Kowaltowski et al. 2011). Quantidades de acido lático aumentadas no globo pálido são observadas nas crises agudas de pacientes com acidemia metilmalônica, podendo assim inferir uma possível inibição *in vivo* da ação mitocondrial do piruvato (Trinh, Melhem et al. 2001), bem como uma liberação aumentada de lactato e redução de CO_2 em cérebro de ratos jovens (Wajner, Dutra et al. 1992) este lactato pode contribuir para o dano cerebral, este acumulo do ácido láctico e o aumento da pCO_2 tecidual podem provocar

acidose grave. Sendo assim alterações no PH intracelular podem inibir a receptação do glutamato pelas células da glias, mesmo ocorrendo níveis normais de ATP levando ao dano neurotóxico (Swanson, Farrell et al. 1995).

Estudos experimentais da doença evidenciaram efeitos tóxicos do MMA, sobre metabolismo mitocondrial e na produção de ATP. Tais efeitos são atribuídos á inibição de complexos da cadeia respiratória, enzimas do Ciclo de Krebs e do complexo Na^+, K^+-ATPase (Wyse, Streck et al. 2000; Brusque, Borba Rosa et al. 2002; Pettenuzzo, Ferreira Gda et al. 2006; Mirandola, Melo et al. 2008). Schuck e colaboradores (2004) demostraram que creatina quinase mitocondrial é inibida pelo MMA em córtex cerebral de ratos. Este mesmo estudo mostrou ainda presença de danos cognitivos em ratos tratados cronicamente com este ácido, efeito este revertido pela administração concomitantemente de ácido ascórbico (McLaughlin, Nelson et al. 1998; Pettenuzzo, Schuck et al. 2003; Schuck, Rosa et al. 2004). Estudos utilizando culturas de células de córtex cerebral e estriado de cérebro de embriões de ratos mostraram que a exposição destas células ao MMA por 6, 12 e 24 horas causaram morte neuronal, com concentrações de 1 mM e 10 mM, sendo que as células corticais são as principais atingidas (McLaughlin, Nelson et al. 1998; Jafari, Braissant et al. 2013).

Outros estudos têm mostrado que alteração na fosforilação de proteínas do citoesqueleto diminuiu o conteúdo de gangliosídeos cerebrais, bem como, o estresse oxidativo *in vivo* e *in vitro.* Esses resultados sugerem que estes eventos podem estar envolvidos na patogênese de diferentes doenças neurodegenerativas, bem como, nos danos cerebrais que são observados m alguns erros inatos do metabolismo, incluindo a acidemia metilmalônica. (Fighera, Queiroz et al. 1999; Okun, Horster et al. 2002; Wajner, Latini et al. 2004; Fernandes, Borges et al. 2011).

Apesar da investigação intensa e da gravidade dos sintomas apresentados pelos pacientes com acidemia metilmalônica, ainda não há na literatura trabalhos que relacionem as alterações fisiopatológicas com o déficit de aprendizado apresentado pelos pacientes, bem como ainda não está bem delimitado o mecanismo pelo qual o acúmulo dos ácidos orgânicos leva a neurotoxicidade.

1.3.5 Diagnóstico

O diagnóstico precoce continua sendo crucial para um melhor prognóstico para os pacientes afetados com acidemia metilmalônica. Sendo assim, um diagnóstico precoce em crianças assintomáticas, poderia prevenir o desenvolvimento de crises encefalopáticas ou reduzir as alterações neurológicas, em indivíduos adultos.

Atualmente o diagnóstico começa a partir da análise de metabólitos excretados na urina ou acumulados no plasma. Esse material será analisado por 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, Boenzi et al. 2006; Fowler, Leonard et al. 2008).

O quadro laboratorial é caracterizado principalmente por acidose metabólica, hipercetonemia, hiperamonemia, hipoglicemia, anemia, leucopenia, acidose lática, hiperglicinemia, neutropenia e trombocitopenia (Imen, Hanene et al. 2012; Zwickler, Haege et al. 2012). Podemos observar que as características da maioria dos sinais clínicos e bioquímicos nas acidúrias orgânicas de cadeia ramificada e na descompensacão do ciclo da uréia, é a perda de peso. Este é um sinal muito frequente que facilita um diagnóstico precoce e um tratamento imediato (Deodato F 2003). O índice de pacientes que sobrevivem varia entorno de 15 %, os quais apresentam convulsões, retardo mental e psicomotor, alterações comportamentais e neuropsiquiátricas, tais como déficit de atenção, quadros de agressividade e comportamento autista, (Hoffmann, Meier-Augenstein et al. 1993). O tônus muscular apresenta-se geralmente comprometido, e a hipotonia se acentua após crises de descompensação metabólicas (Fernandes 1990).

De acordo com a idade dos recém nascidos, podemos observar as concentrações de MMA no fluido cérebro espinhal entre 2.5 a 5 mM (Hoffmann, Meier-Augenstein et al. 1993) e na urina entre 1,1; 5,2 e 0,8 mmol/mol de creatinina, após 1-6 meses, e de 6-12 meses (Boulat, Gradwohl et al. 2003). No entanto os recém-nascidos que apresentam acidemia metilmalônica leve apresentam uma concentração variante entre 10 e 20 mmol/mol de creatinina e

em estagio mais grave podem atingir até 20.000 mmol/mol (Fowler, Leonard et al. 2008).

Além disso, estudos mostram que em caso de gravidez o diagnóstico pode ser rápido e confiável realizado através do líquido amniótico, ou pode ser analisado através da atividade enzimática em cultura de células amnióticas, ou por testes genéticos DNA a partir das células fetais (Saudubray 2002; Nyhan 2005; Venditti, De Rosa et al. 2005).

1.3.6 Tratamento

O tratamento da acidemia metilmalônica em neonatos é realizado através da utilização de um leite especial (Propinex ®) que possuem restrição de aminoácidos precursores do MMA (valina, isoleucina, leucina, metionina e treonina), bem como através de dieta com restrição proteica (0,5 a 1,5 g/kg/dia) em infantes e restrição dietética de aminoácidos propiogênicos se faz importante para reduzir os metabolitos circulantes (Manoli and Venditti 1993; Thomas 1994; Chandler and Venditti 2010).

Os recém-nascidos com cetoacidose são tratados através da retirada total de proteínas na dieta e uma terapia com bicabornato para auxiliar na retirada de toxinas do organismo. Pode-se ainda realizar uma transfusão sanguínea para a retirada parcial de MMA acumulado e também administrar soro glicosado para evitar o catabolismo de proteínas (Ogier de Baulny and Saudubray 2002; Deodato, Boenzi et al. 2006). Também há a possibilidade do tratamento com antibioticoterapia (metronidazol) para eliminar da flora intestinal concentrações plasmática de propionato que podem apresentar-se com níveis elevados (Thomas 1994; D'Angio, Ambati et al. 2001; Ogier de Baulny and Saudubray 2002; Cornejo, Manriquez et al. 2003; de Baulny, Benoist et al. 2005).

Outro método terapêutico utilizado consiste na administração parenteral de altas doses de vitamina B12, nas acidemias metilmalônicas por deficiência do cofator da enzima L-metilmalonil-CoA mutase (Ogier de Baulny and Saudubray 2002), além da administração de L-carnitina, propiciando a excreção urinária de proprionil-carnitina e redução da toxidade do proprionato (Burns 1996).

Atualmente, estudos vêm utilizando culturas de células neuronais para demonstrar que o uso de substâncias como o diazóxido, é capaz de proteger tecidos isquêmicos, prevenir lesões celulares e teciduais provocadas por MMA (Kowaltowski, Maciel et al. 2006). Outro método é os transplantes de células progenitoras fetais, o qual é eficaz em reduzir metabólitos acumulados na acidemia (Buck, Pennell et al. 2012). Além disso, tratamentos genéticos vêm sendo desenvolvidos para modelos com acidemia, baseado em adenovírus ou vírus adeno-associados (Chandler, Tsai et al. 2007; Chandler and Venditti 2008; Carrillo-Carrasco, Chandler et al. 2010; Chandler and Venditti 2010; Chandler and Venditti 2012).

Assim, novas estratégias terapêuticas estão sendo pesquisadas a fim de prevenir as crises encefalopáticas e os danos causados pelo acúmulo dos ácidos orgânicos característicos. Sendo assim, o conhecimento das bases fisiopatológicas e os biomarcadores envolvidos da acidemia metilmalônica auxiliará na elaboração de futuros tratamentos mais efetivos para esta doença.

1.4. Biomarcadores na Acidemia Metilmalônica

1.41. Biomarcadores do Estresse Oxidativo

O radical livre (RL) é definido como um átomo ou molécula que contém um ou mais elétrons desemparelhados (Southorn and Powis 1988; Halliwell 1989). Entretanto, existem compostos igualmente reativos aos RL que não têm necessariamente um desemparelhamento na camada de valência, classificados desta maneira como espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (Droge 2002). As ERO e ERN são por sua vez compostos altamente reativos que buscam estabilidade durante sua breve existência, reagindo com a matéria circundante, desta maneira causando danos às membranas celulares, proteínas e DNA, (Halliwell 2012). Durante o metabolismo celular são produzidas ERO, como por exemplo, o radical superóxido ($O_2^{\bullet-}$), peroxido de hidrogênio (H_2O_2) e radical hidroxil ($^{\bullet}OH$). Os níveis fisiológicos de ERO podem ser eliminados por um sistema antioxidante enzimático e não enzimático (Navarro and Boveris 2008). Entretanto um aumento na produção de ERO, uma diminuição na eficiência dos sistemas

antioxidantes ou ambos podem levar ao estresse oxidativo, que é caracterizado por uma oxidação de biomoléculas com consequente perda de suas funções biológicas (Halliwell and Whiteman 2004).

As EROS têm uma vida curta e sua recombinação química é quase imediata (Rice-Evans and Burdon 1993). Visto isso, torna-se muito difícil a medição imediata dos mesmos, o que torna bem aceito a medição de suas consequências. A determinação da formação de grupos carbonil (>C – O) é um método bastante utilizado para avaliar o dano das ERO as proteínas. Por sua vez, a peroxidação lipídica (LPO) é um processo fisiológico e continuo que ocorre nas membranas celulares. No entanto, este processo pode se tornar tóxico quando as defesas anti-oxidantes são insuficientes ou quando há uma produção intensa de EROs (Halliwell and Gutteridge 1995). A LPO produz aldeídos, gases hidrocarbonados e vários resíduos químicos, como o malondialdeído (MDA), dienos conjugados e 4-hidroxinonenal (4-HNE) (Hotz, Hoet et al. 1987). Desta forma, esta reação pode ser estimada pela medida de seus produtos, e é utilizada para medir indiretamente a produção de RL (Hotz, Hoet et al. 1987). Outro marcador de dano oxidativo não enzimático é a 2,7-dichlorodihydrofluorescein diacetate (DCFH) (LeBel, Ischiropoulos et al. 1992).

O estresse oxidativo pode desenvolver por parte da célula uma adaptação, afim de detoxificar as espécies reativas, bem como pode causar danos a alvos moleculares ao DNA, proteínas, carboidratos e lipídios; e até mesmo morte celular causada por necrose ou apoptose (Halliwell and Gutteridge 1995). Desta forma, o estresse oxidativo pode representar um mecanismo fundamental envolvido em doenças humanas, como as doenças neurodegenerativas (Halliwell 2007) e os EIM, como a acidemia metilmalônica.

Nesse contexto, foi mostrado que o MMA aumenta a produção de espécies reativas, e reduz as defesas antioxidantes em cérebro de ratos (Fontella, Pulrolnik et al. 2000; Brusque, Rotta et al. 2001; Indo, Davidson et al. 2007; M. 2011). Além disso, estudos mostraram um aumento na peroxidação lipídica (TBARS) e produção de radicais livres (oxidação de DCFH-DA) no cérebro dos animais após a administração do MMA.

De fato, outros estudos sugerem o envolvimento das espécies reativas nas convulsões induzidas pelo MMA, na medida em que as convulsões induzidas pela administração intra-estriatal deste ácido orgânico são atenuadas

pela administração de antioxidantes, como o gangliosídeo GM1 e o α-tocoferol (Fighera, Queiroz et al. 1999; Fighera, Bonini et al. 2003). Da mesma forma, a administração de amônia (um agente pró-oxidante) potencializa as convulsões induzidas pelo MMA (Marisco Pda, Ribeiro et al. 2003). Além disto, estudos *in vitro* e *in vivo* mostraram que o MMA inibe a atividade da enzima Na+,K+-ATPase (Wyse, Streck et al. 2000; Malfatti, Royes et al. 2003), responsável pela manutenção do gradiente de Na+ e K+ através das membranas celulares, o que é essencial para a manutenção do potencial de membrana (Jorgensen 1986; Stryer 1996). Também tem sido mostrado que, além do dano oxidativo, citocinas pró-inflamatórias estão envolvidas na inibição da atividade da enzima Na^+,K^+-ATPase e no dano celular (Wang, Xiao et al. 2003; Kreydiyyeh, Abou-Chahine et al. 2004; Kreydiyyeh and Al-Sadi 2004; Lu, Oveson et al. 2009)

1.4.2. Biomarcadores Inflamatórios e Apoptóticos

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 neurorregeneração e reparo tecidual (Rosales-Corral, Reiter et al. 2010). 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 and O'Banion 2009). Muitas das ferramentas de resposta inflamatória que visam patógenos, como proteases e espécies reativas de oxigênio, também danificam as células saudáveis (Kono, Orlowski et al. 2012; Galea and Brough 2013).

Sendo assim, a inflamação e as caspases sinalizam a apoptose e clivam esses substratos levando à condensação e fragmentação nuclear, sinalizando para estas células serem fagocitadas por macrófagos (Nicholson and Thornberry 1997; Boatright and Salvesen 2003).

As caspases constituem um novo alvo terapêutico para doenças do SNC. As principais representantes são as caspases 2, 8, 9 e 10, que são chamadas de iniciadoras e as caspases 3, 6 e 7, que são chamadas de executoras. As caspases 1, 4, 5, 11 e 12 estão envolvidas na ativação das

citocinas, sendo marcadores encontrados também nos processos neuroinflamatórios (Troy and Salvesen 2002; Friedlander 2003). De fato, a ativação da caspase 1 está relacionada com a ativação de TNF-α e interleucina 1β refletindo assim um aumento da resposta inflamatória (Skeldon, Faraj et al. 2014).

Nesse contexto, pacientes com 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 são mais susceptíveis a infecções e desenvolvimento de sepse neonatal, o que facilita o aparecimento da neuroinflamação (Church, Koch et al. 1984; Manoli and Venditti 1993; Guerra-Moreno, Barrios et al. 2003; Semmler, Hermann et al. 2008). Além disso, foi observado que o MMA induz morte celular e aumento de caspases em culturas de células cerebrais em embriões de ratos (Jafari, Braissant et al. 2013).

1.5 Memórias e Acidemia Metilmalônica

Memória é a capacidade que temos de armazenar informações que possam ser recuperadas e utilizadas posteriormente (Lent 2004). A memória pode ser classificada de acordo com o tempo de duração do trabalho, incluindo também memória de curta ou de longa duração, e quanto ao conteúdo pode ser declarativa/explicita, não declarativo/implícita ou operacional (Goldman-Rakic 1996; Squire and Zola 1996; Albright, Kandel et al. 2000; Lees, Jones et al. 2000; McGaugh and Izquierdo 2000; Curtis and D'Esposito 2003; Squire and Kandel 2003; Ranganath and Blumenfeld 2005).

A memória de curta duração serve para manter uma conversa ou uma leitura, por diversas vezes a memória de trabalho também é classificada dentro da memória de curta duração, porém sua função é específica. Ambas são processadas pela (ativação sináptica) dos neurônios do córtex pré-frontal anterolateral e orbito- frontal (Izquierdo, Barros et al. 2002) e do hipocampo córtex entorrinal (Izquierdo, Barros et al. 1998) respectivamente. Não requerem modificação na expressão genica ou síntese proteica, e por isso não causam mudanças permanentes. As memórias de longa duração podem ser de horas, dias e anos essas sim requerem mudanças na expressão genica e síntese

proteica para conservar a informação referente em diversas regiões do cérebro (Izquierdo, Barros et al. 1998; Izquierdo, Barros et al. 2002; Izquierdo 2004).

A memoria explicita ou declarativa esta relacionada a eventos de nossas vidas, fatos históricos marcantes, números de telefones, nomes de pessoas, entre outros, e esta armazenada no lobo temporal e diencéfalo. A memoria implícita ou procedimental se refere á lembrança de procedimentos que está relacionada ás habilidades, como dirigir, jogar bola, amarrar o cadarço, encontrada no cerebelo e gânglios da base (Izquierdo, Barros et al. 2002; Ranganath and Blumenfeld 2005; Squire, Wixted et al. 2007).

A formação do processo da memória no SNC ocorre em lugares e estruturas diferentes, envolvendo alguns eventos moleculares (Izquierdo, Barros et al. 2002). 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, Miller et al. 2005; McCracken, Hudson et al. 2006; Tangney, Aggarwal et al. 2011; O'Shea, Sloan et al. 2012). Estudos comportamentais observaram um déficit permanente de aprendizagem e desenvolvimento neuromotor causado pela MMA (Dutra, Wajner et al. 1991; Mello, Somer et al. 1994).

Além disso, um aumento na atividade da enzima acetilcolinesterase (AChE), também está relacionada a algumas doenças tais como, manifestação neurológicas referente a alteração da atividade motora, aprendizagem, comportamento e memória (Anglade and Larabi-Godinot 2010). Embora o papel do sistema colinérgico na acidemia metilmalônica ainda não está claro, a perda de neurônios colinérgicos está associada á ocorrência de convulsões e retardo mental em pacientes com erros inatos do metabolismo (Ratnakumari, Qureshi et al. 1994).

A formação da memória também é dependente de mudanças na eficiência sináptica que permite o fortalecimento das associações entre os neurônios. Esse fortalecimento das conexões sinápticas é alcançado através de um mecanismo chamado potencialização de longa duração (LTP) (Lynch 2004). Sabe-se, no entanto, que citocinas pro-inflamatórias e espécies reativas podem inibir a LTP em região de CA1 *in vitro* e no giro denteado *in vivo*

(Auerbach and Segal 1997). De fato, Ribeiro e colaboradores mostraram que a administração crônica de MMA induziu déficit de memória e aumento de citocinas inflamatórias no córtex de camundongos (Ribeiro, Della-Pace et al. 2013).

Nesse contexto, os processos neuroinflamatórios durante períodos críticos do desenvolvimento podem contribuir para a progressão da disfunção neuronal e, consequentemente com o déficit cognitivo nos pacientes com acidemia metilmalônica (Pettenuzzo, Wyse et al. 2003; Jafari, Braissant et al. 2013; Ribeiro, Della-Pace et al. 2013).

Considerando que o acúmulo de MMA leva a um aumento de dano oxidativo e marcadores inflamatórios, e segundo LYNCH (Lynch 2004) esses danos podem estar relacionados ao déficit cognitivo observado em diferentes patologias, a compreensão dos processos fisiopatológicos na acidemia metilmalônica pode contribuir para reduzir os danos neuroquímicos, e talvez minimizar o déficit de memória nos pacientes afetados.

1.6 Objetivo

1.6.1 Objetivo Geral

Investigar o efeito da administração, no período neonatal, de MMA sobre as alterações comportamentais, bem como, nos marcadores de estresse oxidativo, inflamatórios e de morte celular em camundongos.

1.6.2 Objetivo Específico

1- Analisar o efeito da administração única de MMA no período neonatal (P1) em camundongos com 21 dias e 40 dias de vida em relação:
2- Ao peso corporal dos filhotes ao longo do tempo
3- A atividade locomotora e exploratória dos animais
4- A memória de trabalho e avaliar o comportamento dos animais no teste de ansiedade (teste de cruz elevado).

5- Aos níveis de citocinas (TNF-α) e atividade acetilcolinesterase no hipocampo, córtex e estriado dos animais.
6- Aos marcadores de dano oxidativo 2,7 dihidro diclorofluoresceína diacetate (DCFH) no hipocampo, córtex e estriado dos animais.
7- Marcadores apoptóticos das caspase1, 3, e 8 e no hipocampo, estriado e córtex cerebral.
8- Morte neuronal através da analise histológica

Manuscrito

Neonatal Nerurotoxicity of Methylmalonate is Sufficient to Trigger Memory Deficit in mice: Involvement of Inflammatory and Apoptotic Markers

Patricia Gabbi[1,2†], Leandro Rodrigo Ribeiro[1,2†], Alexandra Seide Cardoso[1,2,6], Fernanda Haupental[1,2,6], Fernanda Silva Rodrigues[2,3,6], Alencar Kolinski Machado[1], Juliana Sperotto Brum[7,8], Marta Maria Medeiros Frescura Duarte[5], Ivana Beatrice Mânica da Cruz[1], Ana Flávia Furian[1], Mauro Schneider Oliveira[1], Adair Roberto Soares dos Santos[3,4], Luiz Fernando Freire Royes[1,2,3], Michele Rechia Fighera[1,2,3,6*]

Manuscrito Submetido para: Orphanet Journal of Rare Diseases

Submissão:

Article title: Neonatal Nerurotoxicity of Methylmalonate is Sufficient to Trigger Memory Deficit in mice: Involvement of Inflammatory and Apoptotic Markers
MS ID : 1647223462129974
Authors : Patricia Gabbi, Leandro Rodrigo Ribeiro, Alexandra Seide Cardoso, Fernanda Haupental, Fernanda Silva Rodrigues, Alencar Kolinski Machado, Juliana Sperotto Brum, Marta Maria Medeiros F. Duarte, Ivana Beatrice M. da Cruz, Ana Flávia Fúrian, Mauro Schneider Oliveira, Adair Roberto S. dos Santos, Luiz Fernando Freire Royes and Michele Rechia Fighera
Journal : Orphanet Journal of Rare Diseases

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e-mail: ojrd@biomedcentral.com
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2. ARTIGO CIENTIFICO

2.1 Neonatal Nerurotoxicity of Methylmalonate is Sufficient to Trigger Memory Deficit in mice: Involvement of Inflammatory and Apoptotic Markers

2.1.2 Título em português

Neurotoxidade Neonatal do Metilmalonato são suficientes para desencadear déficit de memória em camundongos: envolvimento inflamatório e marcadores apoptóticos**.**

2.2.2 Autores:

Patricia Gabbi[1,2†], Leandro Rodrigo Ribeiro[1,2†], Alexandra Seide Cardoso[1,2,6], Fernanda Haupental[1,2,6], Fernanda Silva Rodrigues[2,3,6], Alencar Kolinski Machado[1], Juliana Sperotto Brum[7,8], Marta Maria Medeiros Frescura Duarte[5], Ivana Beatrice Mânica da Cruz[1], Ana Flávia Furian[1], Mauro Schneider Oliveira[1], Adair Roberto Soares dos Santos[3,4], Luiz Fernando Freire Royes[1,2,3], Michele Rechia Fighera[1,2,3,6*]

[1]Programa de Pós-Graduação em Farmacologia, Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde; [2]Laboratório de Bioquímica do Exercício, Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, UFSM; [3]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica; [4]Programa de Pós-graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina; [5]Universidade Luterana do Brasil, Campus Santa Maria; [6]Departamento de Neuropsiquiatria; Centro de Ciências da Saúde, UFSM, [7]Programa de pós graduação em Medicina Veterinária UFSM, [8]Universidade Federal do Paraná UFPR.

[†]These authors contributed equally to this work.

*Corresponding author: Dra. Michele Rechia Fighera

Centro de Ciências da Saúde, Departamento de Neuropsiquiatria, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brasil. e-mail: mrfighera@yahoo.com.br

Abstract

Background: 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, oxidative stress and neuroinflammation. 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. **Methods:** In this experimental study, a single intracerebroventricular dose of MMA (MMA 2.5 µmol /g, 12 hs after birth; at dose that raise its concentration in blood and in the brain from affected) was administered to mice pups at postnatal day 0 (P0) to induce an acute, transient rise of MMA levels in the central nervous system (CNS). In the following days (21^{st} – 33^{th} or 40^{th} – 52^{th}) animal behavior was assessed in the radial arm maze test (RAM) and elevated plus maze. It was measured tumor necrosis factor-alpha (TNF-α), 2',7'- Dichlorofluorescein diacetate (DCFH), acetylcholinesterase (Ache) activity and caspase levels in the cerebral cortex, striatum and hipoccampus from mice with 21 e 40 days of life. **Results:** Behavioral tests showed that animals injected with MMA have a reduction in the working memory test, but no in the reference test. The animals did not exhibit anxiety-like behaviors. Furthermore, MMA increased levels of TNF-α, AchE activity and activation of caspases 1, 3 and 8 in the cerebral cortex, hippocampus and striatum of mice with 21 and 40 days of life. The

overall results indicate that a simple administration of MMA increased pro-inflammatory markers in the structures studied, increased apoptotic markers, and coincide with the behavioral changes found in young mice. **Conclusions:** This leads to speculate that, through mechanisms not yet elucidated, the transient metabolic insult with MMA may cause a neuroinflammatory processes during critical periods of development, contributing to the progression of cognitive impairment in patients with methylmalonic acidemia.

Keywords
Cerebral cortex, striatum, hippocampus, memory, methylmalonate, mice, inflammatory and apoptotic markers.

Introduction

Methylmalonic acidemia is an autosomal recessive inborn error of metabolism, characterized by tissue accumulation of methylmalonate (MMA) and its metabolites as propionate, metilcitrato and, β-OH propionate, due to deficiency of the enzyme activity of L-methylmalonyl-CoA mutase (MCM; EC 5.5.99.2) (Fenton 1995; Royes, Fighera et al. 2007; Chandler, Zerfas et al. 2009). The symptoms and signs of this acidemia are manly neurologic such as mental retardation, cerebral atrophy, convulsions and cognitive disability that usually occur in the first week of life after encephalopathic crises (Deodato, Boenzi et al. 2006; O'Shea, Sloan et al. 2012). In addition to the neurological manifestations, patients suffering from this disorder have vomiting, metabolic acidosis, dehydration, respiratory distress, muscular hypotonia, lethargy and, ketoacidosis (Lehnert, Sperl et al. 1994; Kolker, Schwab et al. 2003; Deodato, Boenzi et al. 2006; Morath, Okun et al. 2008). It is feasible that during these episodes, which usually follow infections or other metabolic stress conditions occurs an increase of MMA levels in tissue and body fluids (Richard, Alvarez-Barrientos et al. 2007). In fact, previous studies show that MMA has been considered as the major neurotoxin in methylmalonic aciduria. Although the

mechanisms underlying this acidemia are not well established, increasing evidence suggests that excitotoxicity (Kolker, Ahlemeyer et al. 2000; Brusque, Rotta et al. 2001; Okun, Horster et al. 2002; Malfatti, Perry et al. 2007), free radical generation (Fighera, Queiroz et al. 1999; Fighera, Bonini et al. 2003) and, inflammation (Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013) play a central role in the neuropathogenesis this acidemia.

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 and increase in cytokine release (Lucas, Rothwell et al. 2006), which can lead to neuronal death (Glass, Saijo et al. 2010) and learning or memory deficit (Hein and O'Banion 2012). For example, systemic or central bacterial lipopolysaccharide (LPS) injections activate microglia, potently block neuronal differentiation and disrupt the integration of neurons into existing hippocampal circuitry (Ekdahl, Claasen et al. 2003; Monje, Toda et al. 2003; Belarbi, Arellano et al. 2012). Furthermore (Rodrigues, Souza et al. 2013) showed that LPS- induced cytokines levels increase leads to cognitive deficit in experimental EIM model.

Although the studies have shown that neuroinflammation has a main role in human neurological diseases, such as epilepsy (Aronica and Crino 2011), autism (Vargas, Nascimbene et al. 2005), multiple sclerosis (Lu, Joseph et al. 2010), Alzheimer's (Venneti, Wiley et al. 2009), Huntington's (Moller 2010) and Parkinson's disease (Chung, Ko et al. 2010), there are little 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 acute injection in neonatal period alters the inflammation and cellular death markers in cerebral cortex, hippocampus and striatum of mice, as well as the behavioral parameters.

2.Experimental procedures

Ethics Statement

Laboratory experiments were performed in accordance with national and international legislations (National Council for the Control of Animal Experimentation [CONCEA] and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals-PHS Policy) and approved by the Ethics Committee for Animal Research of Universidad Federal de Santa Maria (UFSM; Permit Number: 112/2010). Indeed, animal handling and laboratory assays were carried out in such a way that all efforts were made to minimize suffering

Animals and reagents

The present study utilized pup male Swiss mice newborn. Pregnant Swiss mice were housed in individual cages and left undisturbed during gestation. Twenty-four hours after delivery, litters were culled to six male pups. Pups were fed by the mother since birth until 21 days of life when they were weaned. Animals were divided in order to have the same number of rats for each treatment in each cage. Animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (24 ± 1◦C, 55% relative humidity) colony room.

MMA administration and drug treatment

The MMA administration directly into the intraventricularlly cisterna magna between 12 hs after birth (P0) with MMA (2.5 μmol/g; pH 7.4) or vehicle sodium chloride (NaCl 0.9%) (Hoffmann, Meier-Augenstein et al. 1993; Brusque, Rotta et al. 2001). The external reference point used to locate cisterna magna was the intersection between bones, i.e the meeting point of bone sutures bregma, lambda and the interaural line. The animals were anesthetized and injected directly in the cisterna magna with a 30-gauge needle, the parameters used to inject were: anterior posterior (AP) = -2.7 mm (later the interaural line), vertical (V) = -1 mm (below dura mater), lateral (L) = 0, angle (Θ = 90º) (Consiglio and Lucion 2000). All drugs are injected within a period of 2

minutes using a hamilton syringe. The experimental protocol is described in the figure 1.

Physical development

All mice used in the experiments had assessed their behavioral development. For this, the weight of animals was weekly determined at the appropriate ages by one experimenter that was not aware of the subject condition.

Test behavioral

Open-field task

The locomotor activity was measured for 5 min in the open field. The apparatus consisted of a wooden box measuring 60 cm x 40 cm x 50 cm with a glass front wall. Its floor was divided by black lines into 12 equal squares. Animals were gently placed facing the rear left corner of the arena and the number of squares crossed with the four paws by two observer one of observer count exploratory activity and other locomotor activity of animals per for 5 min to evaluate motor activity and exploratory (Walsh and Cummins 1976). The testing room was dimly illuminated with indirect white lighting.

Radial arm maze test

The maze consists of a wooden eight RAM that was secured to a wooden base and elevated 100 cm from the floor. The radial arms maze were 35 cm in length, with outer arm walls 2.6 cm high, inner arm walls 15 cm high and 5.8 cm wide. The center well of the maze was 16.7 cm in diameter, the maze was situated in the middle with moderate luminosity (Olton 1972; Ros-Simo, Moscoso-Castro et al. 2013), the food wells at the end of each arm. Cornflakes chips were used as rein forcers that were placed in circular plastic Petri dishes, these dishes were attached to the ends of the maze arms, four

open Petri dishes, without lids, were used to house the obtainable cornflakes chips in the reinforced arms. In the non-reinforced arms four other Petri dishes with lids, that had several small holes drilled, had cornflakes chipped inside of them. This meant that the rats could not obtain them but allowed the odor of the cornflakes chips to permeate from the dishes preventing the rats from solving the task using the smell of the cornflakes, in a separate group of mice, RAM was assessed.

The 1st week of the procedure test training sessions were carried out for 12 days (D1 to D12, being D9 starting implementing the memory test). During this acquisition period, animals were trained to find the cornflakes chips in four randomly selected arms. Animals were subjected to three trials of 5 min/day or until the mouse collected all the packages (Jarrard 1983; Jarrard 1993). Three types of errors were scored: reference memory errors (RME), defined as the number of first entries into an unabated arm; “correct” working memory errors (CWME), defined as the number of reentries into a baited arm; and “incorrect” working memory errors (ICWME), defined as the number of reentries into an unabated arm (Schmitt, Deacon et al. 2003; Kosaraju, Gali et al. 2013).

Elevated plus maze task

Based on the design of File and Gonzalez (File and Gonzalez 1996; Haller and Alicki 2012) the maze consisted of two opposite closed arms (30 cm x 5 cm) enclosed with walls (15 cm in height) and two opposite open arms (also 30 cm x 5 cm, without edges) forming a plus shape. The whole apparatus had a central arena (5 cm x 5 cm) and was elevated to 80 cm above the floor by a tripod. Each rat was placed in the arena of the maze facing an open arm and observed for 5 min. The behaviors recorded were: total number of entries, the percentage of time spent on either arm, and percentage of time spent in the middle. The apparatus was cleaned thoroughly between the 5 min observation sessions with a 30% ethanol solution.

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, hippocampus in striatum was dissected on an inverted ice-cold Petri dish and homogeneized in cold 10 mM Tris–HCl buffer (pH 7.4). The homogeneized was then divided in aliquots for subsequent neurochemical analyses, as described below.

TNF-α immunoassay

The cerebral cortex, hippocampus and striatum was weighted and homogenized in a solution containing bovine serum albumin (BSA 10 mg/ml), EGTA (2 mM), EDTA (2 mM) and PMSF (0.2 mM) in phosphate-buffered saline (PBS, pH 7.4) using a Potter homogenizer. The homogenate was centrifuged (3000 g for 10 min) and cytokines were determined in supernatant. Cytokine levels were measured using a commercially available ELISA Kit from Rand Systems (Minneapolis, MN) using an antibody selective against mice TNF-α, according to the manufacturer's protocol. The results are expressed in pg/mg of protein for hippocampus homogenate assays. Absorbance was read at 405 nm. The detection limit was 4 mg/ml.

Measure of oxidation of DCFH

The production of reactive oxygen species and nitrogen was measured using 2 ', 7'- dichlorofluorescein diacetate (DCF-DA). The oxidant H_2O_2 derived from Fe_2 is the responsible main for the not enzyme oxidation of DCFH. The DCFH was prepared in 20 mmol / L sodium phosphate buffer, pH 7.4, containing 140 mmol / L KCI solution and incubated with 100 μ L of supernatant for 30 min at 37 ° C. The DCFDA is enzymatically hydrolyzed by intracellular esterases to form DCFH not fluorescent which is rapidly oxidized and form 2 ', 7'-dichlorofluorescein (DCF), highly fluorescent in the presence of reactive species. The fluorescence intensity of DCF is proportional to the amount of reactive species formed. The fluorescence was measured using excitation wavelengths and emission at 480 and 535 nm respectively. The calibration curve was constructed using standard DCF (0,25-10 mmol) and the levels of

reactive oxygen species were calculated as pmol DCF formed by mg protein (LeBel, Ischiropoulos et al. 1992) .

Acetylcholinesterase Activity

The activity of this enzyme was performed according to the method described by Ellman et al. (Ellman, Courtney et al. 1961). The hydrolysis of acetylcholine was evaluated at a concentration of 0.8 mM in 1 mL of solution containing 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of sample were added to the solution and pre-incubated for 3 min. The hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 3.2 min at intervals of 30 s to a temperature of 25 °C. the samples were analyzed in duplicate.

Histologic Protocol

After the RAM, the mice were killed for histologic analysis. Under deep anesthesia (ketamine hydrochloride, 200 mg/kg, i.p.) they were transcardially perfused with 100 mL of heparinzed saline (1000 UI/ml) followed by 100 mL of formaldehyde (4%) in PBS (0, 1 M), then the brains were carefully removed from the skull. Briefly, serial sections from bregma levels +2 a -4 (Paxinos 2001); (Figure 14), spaced 500 µm apart, were stained with hematoxylin and eosin, and digitally photographed using a stereomicroscope (Olympus BX51) with a digital camera (Olympus DP25). The images of striatum body and hippocampus were traced on each image by a pathologist, using a microscope at ×4 magnification.

Isolation and cell culture

For the isolation and cell culture, cerebral cortex, hippocampus and striatum of 12 mice pups were treated previously with saline or methylmalonate. The animals were euthanized and structures were removed using sterile

materials. After removal, the specimens were placed in Petri dishes containing 2 ml of Hanks solution glycosylated, with sectioned and slightly macerated.

Shortly thereafter, the materials were filtered and the content was prepared in 15 mL falcon tubes. In order, it was added 4 mL of cell culture medium Dulbecco's modified Eagle's medium (DMEM), with 10% Fetal Bovine Serum (FCS) and supplemented with 1% penicillin/streptomycin and 1% antifungal amphotericin B. The content was homogenized and centrifuged for 10 minutes at 2000 rpm, then the supernatant was removed and the cell pellet was suspended in complete culture medium.

Cells isolated from each frame of interest were prepared on sterile 96–well ELISA plates, with 200 µL per well, and kept at optimal cell culture conditions in CO_2 incubator at 37 °C and saturation of 5 % CO_2 for 24 hours to stabilize the cells. After the incubation period, the cultures were tested for apoptosis-related proteins, caspases 1,3 and 8, including starting caspases as well as effectors, using the Quantikine Immunoassay Kit Caspase Human® (*Quantikine Human Caspase Immunoassay*®) (PERES 2005).

Protein determination

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

Statistical analysis

Statistical analysis was carried out by two-way analysis of variance (ANOVA) or t- test when appropriated. Post-hoc was carried out by Student-Newman-Keuls (SNK) if necessary. Parametric tests are expressed as mean + S.E.M. and non-parametric tests are expressed as mean ± interquartile range. A probability of $p<0.05$ was considered significant.

Results

Physical development of animals

The physical development of animals determined by their weights during the treatment is presented in Figure 2A e 2B. Statistical analysis did not shown a significant difference between groups on weight of animals of 21 [$F(1,40)= 0.23$; $p>0.05$; Figure 2A] and 40 [$F(1,60)=1.77$; $p>0.05$; Figure 2B] days of life.

In the present study we showed that the administration of MMA did not decrease the number of rearing in the animals with 21 ($t = 1.325$; $p>0.05$) and crossing ($t = 1.420$; $p>0.05$; Figure 3A e 3B) and 40 days of life ($t = 0.963$; $p>0.05$) and ($t = 1.387$; $p>0.05$; Figure 3C and 3D) in the open field test, as compared with your respective control group.

Effect of MMA on anxiety

Statistical analysis of the percent of time and entries in open arm in the elevated plus maze did not show a significant drug (Saline or MMA) interaction in the animals with 21 ($t = 1.73$ and $t = 0.016$; respectively) and 40 days of life ($t = 0.43$ and $t = 1.59$; respectively). In addition, statistical analysis did not show a significant drug (Saline or MMA) interaction for the percent of time and entries in enclosed arm in the animals with 21 ($t =0.60$ and $t =1.36$; $p>0.05$; respectively) and 40 days of life ($t = 0.15$ and $t = 1.17$; $p>0.05$; respectively), indicating that the treatment had no effect on anxiety-like behavior (Table 1).

Effect of MMA on Radial Arm-Maze Task

To investigate whether MMA treatment affects working and reference memory formation, the pup mice were evaluated in the RAM task (RAM) whit 21 days and 40 days of life.

Statistical analysis revealed that MMA administration did not change the RME in animals with 21 [$F(1,32)= 0.30$; $p>0.05$; Figure 4A) and 40 days of life [$F(1,32)= 1.12$; $p>0.05$; Figure 5A) as compared to respective control group. However, statistical analysis showed that MMA injection increased CWME in group of animals with 21 [$F(1,32)= 42.00$, $p<0.05$; Figure 4B) and 40 days of life [$F(1,32)= 46.08$, $p<0.05$; Figure 5B). Furthermore, MMA injection increased ICWME in the animals with 21 [$F(1,32)= 33.68$, $p<0.05$; Figure 6C) and 40 days of life [$F(1,32)= 50.81$, $p<0.05$; Figure 4C and 5C, respectively).

Effect of MMA on the Oxidative Stress

The as same studies suggest that oxidative stress may play the important role in brain damage induced by MMA (de Mello, Freitas et al. 1996; Myhre, Andersen et al. 2003; Royes, Fighera et al. 2003), we decided to measure DCFH levels in the cerebral cortex, hippocampus and striatum of mice with 21 and 40 days of life.

Statistical analysis revealed that MMA injection induced an increase on DCFH levels in the cerebral cortex ($t = 4.3$, $p<0.01$; Figure 6A; $t= 4.41$, $p<0.01$, Figure 7A) and striatum ($t = 3.03$, $p<0.01$, Figure 6C; $t = 3.85$, $p<0.01$, Figure 7C) in animals with 21 and 40 days of life, respectively. However, MMA injection increased DCFH levels only in the hippocampus ($t=3.0$, $p<0.01$, Figure 7B) from mice with 40 days of life, but not in the hippocampus ($t = 0.09$, $p>0.05$, Figure 6B) from animals with 21 days of life.

Effect of MMA on the Inflammatory Biomarkers

Besides the involvement of oxidative stress (de Mello, Freitas et al. 1996; Myhre, Andersen et al. 2003; Royes, Fighera et al. 2003), same studies also suggest that inflammatory process may play an important role in the brain damage induced by MMA (Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013), suggesting a close link between these events. In this line of view, we decided to measure the levels of TNF-α in the cerebral cortex, hippocampus, and striatum of mice with 21 and 40 days of life (figure 8 and 9, respectively).

The statistical analysis revealed that MMA injection increased TNF- α levels from the animals with 21 and 40 days of life in the cerebral cortex ($t = 13.71$, $p<0.001$, Figure 8A; $t = 8.376$, $p<0.05$, Figure 9A), hippocampus ($t = 20.79$, $p<0.001$, Figure 8B; $t = 7.405$, $p<0.05$, Figure 9B) and striatum ($t = 9.754$, $p<0.001$, Figure 8C; $t = 5.096$, $p<0.05$; Figure 9C), respectively.

Since current studies suggest that the involvement of the cholinergic pathways in brain immune responses (Gnatek, Zimmerman et al. 2012; Affonso, Machado et al. 2013), we decide to determine the AChE activity in the cerebral

cortex, striatum and hippocampus of mice pup. The statistical analysis showed that MMA injection increased AChE activity of the animals with 21 and 40 days of life in the cerebral cortex [t = 5.9, p<0.0001, Figure 10A; t=5.7; p<0.0001; Figure 11A], striatum [t = 39.7, p< 0.0001; Figure 10C; t=7.5, p<0.0001, Figure 11C] and hippocampus [t = 32.73, p<0.0001, Figure 10B; t= 14.3, p<0.0001, Figure 11B], respectively.

Effect of MMA on the Apoptotic Markers

The figure 12 shows increase on number of apoptotic cells in the brain of mice pups after MMA injection (Jafari, Braissant et al. 2013). The statistical analysis revealed an increase on the activation of caspases-1, 3 and 8 in the cerebral cortex (t = 25.54; t=38.6; t=57.9; p<0.0001; Figure12A; respectively), striatum (t = 43.03; t=53.3; t=176.4; p<0.0001; Figure 12C; respectively), and hippocampus (t = 85.2; t=106.3; t=78.6; p<0.0001; Figure12B; respectively) in the animal with 21 days of life. Furthermore, the statistical analysis revealed an increase on the activation of caspases-1, 3 and 8 in the cerebral cortex (t = 5.1; t=4.3; t=4.2; p<0.0005; Figure 13 A; respectively), striatum (t = 5.6; t=8.1; t=9.3; p<0.0001; Figure 13C; respectively), and hippocampus (t = 39.9; t=42.2; t=57.4; p<0.0001; Figure 13B; respectively) in the animal with 40 days of life.

Effect of MMA on the Histologic Analysis

Figure 14 shows digitized images of the cerebral cortex, hippocampus and striatum. It was not observed significant differences of those regions between the experimental groups in the animals with 21 (A) and 40 (B) days of life.

Discussion

Patients with methylmalonic acidemia usually present acute clinical features early in life resulting from metabolic decompensation, including lethargy, coma, vomiting, muscular hypotonia, convulsions and psychomotor developmental delay. Most children survive to the first acute metabolic crisis,

but develop long-term complications including neurological symptoms (Leonard 1995; Horster, Baumgartner et al. 2007; Zwickler, Haege et al. 2012). Although, it is believed that these abnormalities occur as a 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 with methylmalonic acidemia and experimental models of this IEM exhibit neuronal damage and changes in several areas of the central nervous system (Hein, Stutzman et al. 2007; Magni, Oliveira et al. 2007; Morath, Okun et al. 2008; Pinar-Sueiro, Martinez-Fernandez et al. 2010; Melo, Kowaltowski et al. 2011). Often this is related to oxidative stress, neuronal death, and neuroinflammatory processes, causing neurological disabilities (Cameron and Landreth 2010; Olivera-Bravo, Fernandez et al. 2011; Jafari, Braissant et al. 2013). Recent studies have elucidated the role of inflammatory mediators in model of acute seizures and brain damage by apoptosis in the methylmalonic acidemia (Salvadori, Bandero et al. 2012; Jafari, Braissant et al. 2013; Li, Peng et al. 2014).

This current study was carried out with mice newborn pup with proven synaptogenesis and cell proliferation in several brain structures involved in learning and memory. We found that the acute MMA administration (1^{th} day of life) at dose that raise its concentration in blood and in the brain from affected patients (Hoffmann, Meier-Augenstein et al. 1993; Brusque, Rotta et al. 2001), causes memory deficit and increases levels of TNF-α and AChE activity in the cerebral cortex, hippocampus and striatum from mice pups with 21 and 40 days of age. In addition, an increasing DCFH and caspase levels, without histological changes, were observed.

It is important to observe that acute MMA administration had no effect on body weight, implying that acute injection did not cause malnutrition in animals with 21 days and 40 days of age (Figure 1 and 2). Similarly, the same treatment did not change mice pups performance in the open field task, as observed by a number of rearing and crossing at the testing session (Figure 3 and 4). In addition, MMA did not change the behavior of mice pups in the elevated plus maze task, indicating that this organic acid was not anxiogenic in animals with 21 days and 40 days of life (table 1).

As regards to the behavior of mice pups, this is the first study to investigate the effect of this experimental protocol on learning/memory through the radial test with a simple injection of MMA, showing that the administration of this acid in the neonatal period is sufficient to trigger working memory deficit, but not in the reference memory in this acidemia model. In fact, we observed that MMA potentiated the deficit of learning and memory compared to control group in animals with 21 days and 40 days of age in the working memory test. Furthermore, at the test of ICWME in the RAM, which was defined as the number of reentries into an unbaited arm (Schmitt, Deacon et al. 2003), the animals with 21 and 40 days of life showed an increase number of reentries in all days of the test, suggesting that cognitive deficit presented in methylmalonic acidemia patients can be related with accumulate MMA cerebral, mainly in the basal ganglia.

Working memory generally expresses a disturbance of executive function (Kirkby 1969; Devan, Goad et al. 1996) and this alteration is often related with lesions in the striatum and is interpreted as an inability to inhibit ongoing action or as a failure to initiate a next response (Devan, Goad et al. 1996). Interestingly, the difficulty in the working memory is one of the symptoms observed cognitive deficit in various inherited metabolic diseases involving the basal ganglia, including methylmalonic acidemia (Fenton.W.A 2001) as well as in other situations associated with basal ganglia lesions, as Huntington's disease (El Massioui, Ouary et al. 2001). Interestingly, we observed here that the acute administration of methylmalonic acid to mice during a period of rapid brain development resulted in a normal performance in the reference memory test (i.e., learning and spatial memory), suggesting that hippocampus is not directly related with cognitive deficit observed in this study. These results agree with studies that show the methylmalonic acidemic patients develop mainly bilateral striatal degeneration, but not in hippocampus, after catabolic or infectious events observed in neuroradiological imaging (Ibanez-Mico, Izquierdo-Fos et al. 2008; Bindu, Kovoor et al. 2010).

In recent study by Ribeiro and collaborators (Ribeiro, Della-Pace et al. 2013) there was a deficiency in the purchase of a new paradigm of spatial localization in the object recognition test (ROT) of learning/memory, performed approximately 4 days after the last MMA chronic administration (from 5^{th} to 28^{th}

day of life), indicating a worse in spatial memory. However, it demonstrated that MMA-caused spatial memory deficit could be due to long period of administration this acid (more than twenty days). Thus, despite differences (temporal and techniques) between protocols performed in this and the aforementioned study, 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, Schuck et al. 2003; Royes, Fighera et al. 2006). Furthermore, it should be remembered that several subcortical structures are involved in the acquisition, consolidation and evocation of memory (Izquierdo and Medina 1997; McGaugh 2000; Izquierdo, Bevilaqua et al. 2006), and these also suffer different consequences arising from the accumulation of MMA, as has been observed in others experimental models of this acidemia (Wajner, Brites et al. 1988; Malfatti, Royes et al. 2003; Pettenuzzo, Wyse et al. 2003; Vasques, Brinco et al. 2006).

In this line of view, inflammation and oxidative damage are also featured in the methylmalonic acidemia patients brain (Fighera, Queiroz et al. 1999; Fighera, Bonini et al. 2003; Harting, Seitz et al. 2008; Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013). TNF-α and IL-1b, key cytokines produced by activated microglia, play a role in the pathogenesis of methylmalonic acidemia (Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013). In the CNS, neutrophils are usually not present because they do not cross the blood brain barrier (BBB). 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 IL-1β and TNF-α, which are accepted as modulators of neurotransmission within the brain (Merrill 1992). However, in the present study we showed that MMA administration induced the increase of TNF-α in the cerebral cortex, hippocampus and striatum. Therefore, since the BBB is still in process of maturation during the neonatal period (Song, Son et al. 2002), which was administrated MMA, the observed increase of TNF-α in the CNS may be a consequence of the inflammatory response induced by acid, and this can act as an insult to the developing brain triggering behavioral alterations (Stolp, Johansson et al. 2011). Current evidence indicates that cytokines, particularly TNF-α, increase neuronal excitability by activating TNF receptors

(TNFR)(Tansey MG 2008). The cerebral TNFR stimulation induces excitotoxicity directly through activation of *N*-methyl-D-aspartate (NMDA) receptor (Zou and Crews 2005) and indirectly by inhibiting glial glutamate transporters on astrocytes (Choi 1988). As consequence, TNF-α facilitates NMDA receptor-mediated Ca^{2+} influx into neurons, promoting excitotoxicity (Viviani, Bartesaghi et al. 2003). Considering that TNF-α can inhibit glutamate uptake in astrocytes (Hu, Sheng et al. 2000) and increase its glial release (Bezzi, Domercq et al. 2001), it is plausible to propose that increase of this pro-inflammatory cytokine 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, Begnini et al. 1996; de Mattos-Dutra, Meirelles et al. 2000).

Furthermore, TNF-α 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 can promote elevated concentrations of intracellular calcium as well as a production of NO and H_2O_2 increase (Hemmens and Mayer 1998). These free radicals induce a nitrosative/oxidative stress that may result in increase of lipid peroxidation and DNA damage (Puttfarcken, Getz et al. 1993; Frantseva, Velazquez et al. 2000). In the present study we revealed that simple MMA administration induced increase in DCFH, consequently oxidative stress and neuroinflammation in the cerebral cortex, hippocampus and striatum of mice pups.

It has also been demonstrated that AChE has an important role in immune responses by rapidly hydrolyzing ACh (Kawashima and Fujii 2000), which is known to have anti-inflammatory actions and suppress the production of pro-inflammatory cytokines (Kawashima and Fujii 2003). Studies have demonstrated that the activation of the nicotinic receptors in macrophages reduces significantly the liberation of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-1ra, whereas the production of anti-inflammatory cytokines, such as IL-10, is not affected (Borovikova, Ivanova et al. 2000). In this way, inhibitors of AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory

cytokines and may attenuate inflammation by increasing the ACh concentration in the extracellular space (Nizri, Hamra-Amitay et al. 2006). Here, we observed an increase in the AChE activity in the striatum, hippocampus and cerebral cortex from mice pups. It is possible that this alteration leads to a decrease of ACh level contributing to the pro-inflammatory status. Although the role of cholinergic system in methylmalonic acidemia is still unclear, alterations in the AChE activity support the view that this system may contribute to the regulation of the immune responses in this acidemia and consequently with the neurological symptoms. In fact, the cholinergic neurons loss is associated the occurrence of seizures and mental retardation in patients with metabolism inherited diseases (Ratnakumari, Qureshi et al. 1994). These findings open the doors to the discovery of more specific mechanism, as for example the use of AChE inhibitors for the treatment of cognitive deficit in this acidemia.

Besides MMA injection be able to increase an inflammatory biomarkers levels, it induced caspase-1, -3 and -8 activation in the striatum, hippocampus and cerebral cortex from mice pups with 21 and 40 days. In fact, activation of caspase 1 may occur by its substrates, as TNF-α and IL-1β, and reflect an increase of cellular inflammatory response (Skeldon, Faraj et al. 2014). After TNF binding, its receptors can be internalized and this leads to activation of the executioner caspases, as caspase-8 through the extrinsic apoptosis pathway (Micheau and Tschopp 2003; Schneider-Brachert, Tchikov et al. 2004). The caspase-8, combined with its ability to induce apoptosis through the extrinsic pathway, also triggers the intrinsic apoptosis pathway by cleaving the pro-apoptotic Bcl-2 family members to initiate mitochondria-induced apoptosis (Gross, Yin et al. 1999; Zhao, Li et al. 2001). These events induce a subsequent reactive species increase and release of mitochondrial apoptogenic factors (such as cytochrome *c)* to the cytosol, consequently to an activation of caspase 3 and cellular dysfunction (Budihardjo, Oliver et al. 1999). Since oxidative stress and pro-inflammatory cytokines lead to activation of caspases by extrinsic and intrinsic apoptotic pathway (Hu, Sheng et al. 2000), it is plausible to propose that increased MMA-induced TNF-α and DCFH levels result in apoptotic pathway activation and consequently the cognitive deficit observed in this study. In agreement of the view, there is also evidence of the

activation of caspases 1, 3 and 8 in the brain of patients and in experimental model of Huntington's Disease induced by MMA (Ona, Li et al. 1999; Sanchez, Xu et al. 1999; Andreassen, Ferrante et al. 2000).

In another model of methylmalonic acidemia, also was observed that [U-14C]acetate incorporation into the lipids of cerebral cortex was reduced by MMA, which may explain the hypomyelinization and/or demyelization characteristic of patients and, together with the findings of this paper, we can hypothesize that is mediated by immune system (de Mello, Rubin et al. 1997; Mayo, Quintana et al. 2012). Moreover, experimentally or clinically, cytokines interfere directly or indirectly in the process of memory consolidation, synaptic plasticity and/or neurogenesis, and expression of neuroinflammatory and apoptotic mediators potentially implies in neuronal damage leading to cognitive impairment (Bossu, Cutuli et al. 2012).

On the other hand, the results presented in this report showed that MMA administration did not induce morphological changes in the analyzed cerebral structures. In fact, McLaughlin et al. (McLaughlin, Nelson et al. 1998) did not observe neuronal death after MMA exposure (5 or 50 µM), only with higher concentrations (500 µM, 1 mM and 10mM) in striatal and cortical cultures from embryonic rat brain for 24h, suggesting that MMA leads to dose-dependent cell death.

Furthermore, it is feasible that during encephalopathic episodes, which usually follow infections or other metabolic stress conditions, occurs an increase of MMA levels in the body fluids and potentiation of neurological manifestations (Richard, Alvarez-Barrientos et al. 2007; Salvadori, Bandero et al. 2012). Considering that methylmalonic acidemia patients develop cerebral degeneration and neurological symptoms manly after a metabolic decompensation, we may propose that maybe it is necessary a catabolic or infectious event to lead to morphological changes in this acidemia model. In agreement of this view, Salvadori and collaborators (Salvadori, Bandero et al. 2012) showed that prostaglandin E2 increased MMA-induced seizures; supporting a view that infections may precipitate and exacerbate neurologic dysfunction in patients with methylmalonic acidemia. However, it would require future experiments to verify the possibility whether infectious events could

potentiate a neurodegenerative processes in brain structures analyzed in this study. In this way, due methodologic differences that may account for the discrepant results in this and the aforementioned study, such as the dose or histologic technics, further in-depth studies are necessary to clarify the relation between MMA exposure and neuronal death.

Although the results of the present study may suggest an association between memory deficits and increased pro-inflammatory and apoptotic markers in the brain 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 and apoptotic markers may influence behavior by affecting neurotransmission, endocrine system, neuronal plasticity and brain circuitry (Bossu, Cutuli 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 and apoptotic process are linked to pathophysiology of methylmalonic acidemia.

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Tabela 1

	21 days of life		40 days of life	
Group	Saline	MMA	Saline	MMA
% NO.E	35.63 ± 8.464	35.81 ± 7.674	17.23 ± 4.186	26.77 ± 4.315
% NO.E.R	64.64 ± 8.488	57.46 ± 8.329	55.45 ± 10.66	53.52 ± 7.499
% T.O	18.84 ± 8.584	39.94 ± 8.661	4.981 ± 2.548	6.514 ± 2.540
% T.E	62.03 ± 14.81	37.75 ± 9.835	83.50 ± 4.001	77.48 ± 3.243

Tabela 1. Data are presented as means ± S.E.M. for n = 10-15 in each group. No significant differences between groups were detected. %T.O percent of time spent on open arms; %No.E.o percent of number of entries on open arms; %T.E percent of time spent on closed arms; % No.E.e percent of number of entries on encloses arms; effect of early postnatal acute MMA administration on anxiolytic-like behavior of mice pups with 21 and 40 days of life.

FIGURE LEGENDS

Figure 1. Experimental design

Representation of experimental design of MMA administration (2.5 µmol/g) intra cisterna magna in the birth day (PO). The behavioral and biochemical analysis was analyzed in the mice pups with 21 and 40 day of life. The histologic analysis was determined after RAM task (33 and 52 days of life).

Figure 2. Treatment effects on the body weight of the animals.

Effect of postnatal acute MMA administration on physical development of animals in 21 (A) and 40 (B) days of life. Data are presented as means ± S.E.M. for n =15 in each group. No significant differences between groups were detected.

Figure 3. Effect of postnatal acute MMA administration on the number of crossing (A) and rearing (B) of the animals with 21 days of life and on the number of crossing (C) and rearing (D) of the animals with 40 days of life. Data are presented as means ± S.E.M. for n = 10-15 in each group. No significant differences between groups were detected**.**

*Figure 4**.** Treatment effects on the RAM test of mice with 21 days of life.*

Acute treatment with MMA induced working memory deficit, but not on the reference memory, in mice pups with 21 days of life. Data expressed as means ± S.E.M. for n = 10-15 in each group. Significance was determined *P < 0.05 when compared to control group. (A) Reference memory errors (RME), (B) correct working memory errors (CWME) and (C) incorrect working memory errors (ICWME).

*Figure 5**.** Treatment effects on the RAM test of mice with 40 days of life.*

Acute treatment with MMA induced working memory deficit, but not on the reference memory, in mice pups with 40 days of life. Data expressed as means ± S.E.M. for n = 10-15 in each group. Significance was determined *P < 0.05 when compared to control group. (A) Reference memory errors (RME), (B)

correct working memory errors (CWME) and (C) incorrect working memory errors(ICWME).

Figure 6. MMA increases DCFH levels in mice with 21 days of life.
MMA increased the mitochondrial DCFH levels from cerebral cortex (A) and striatum(C), but not in the hippocampus (B). Significance was determined *P< 0.05 as compared to control. Data mean + S.E.M. for n = 10-15 in each group.

Figure 7. MMA increases DCFH levels in mice with 40 days of life.
MMA increased the mitochondrial DCFH levels from cerebral cortex (A), hippocampus (B) and striatum (C) in the mice pups with 40 days of life. Significance was determined *P< 0.05 as compared to control. Data mean + S.E.M. for n = 10-15 in each group.

Figure 8. MMA increases cytokine levels in mice with 21 days of life.
MMA increased the levels of cytokines TNF-α from cerebral cortex (A), hippocampus (B) and striatum (C) of mice. Significance was determined *P< 0.05 as compared to control. Data mean + S.E.M. for n = 10-15 in each group.

Figure 9. MMA increases cytokine levels in mice with 40 days of life.
MMA increased the levels of cytokines TNF-α from cerebral cortex (A), hippocampus (B) and striatum (C) of mice. Significance was determined *P< 0.05 as compared to control. Data mean + S.E.M. for n = 10-15 in each group.

Figure 10. Treatment effects on the AchE activity in mice with 21 days of life.
MMA increased the AchE activity from cerebral cortex (A), hippocampus (B) and striatum (C) of mice pups. Significance was determined *P <0.0001 as compared to control group. Data mean + S.E.M. for n = 10-15 in each group.

Figure 11. Treatment effects on the AchE activity in mice with 40 days of life.
MMA increased the AchE activity from cerebral cortex (A), hippocampus (B)

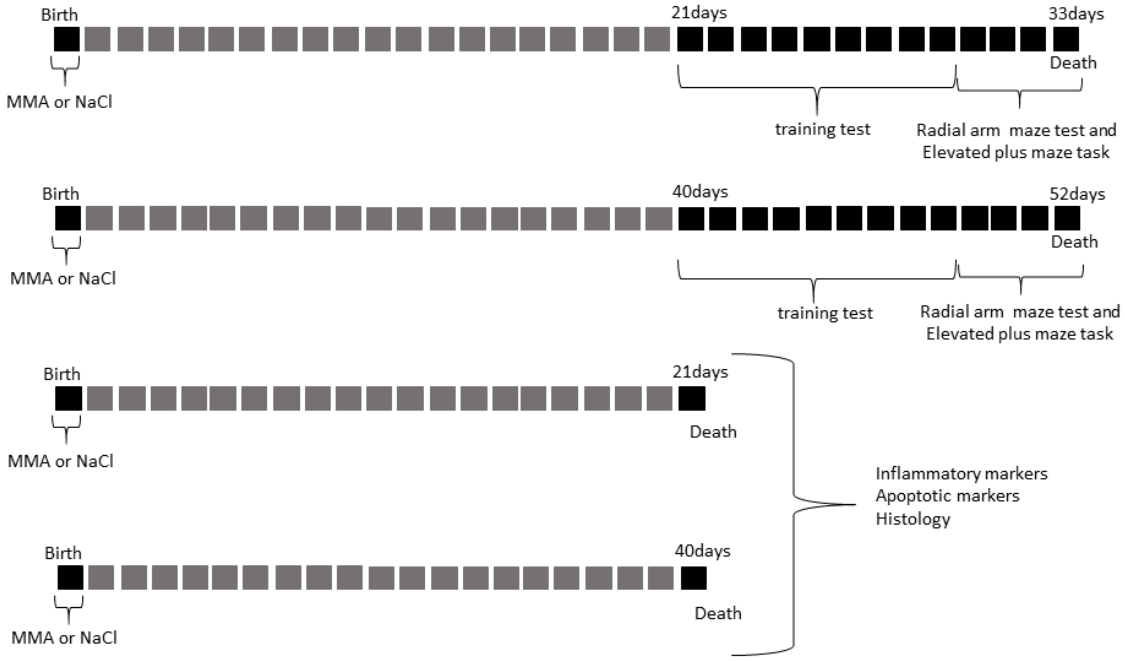
and striatum (C) of mice pups. Significance was determined *P <0.0001 as compared to control group. Data mean + S.E.M. for n = 10-15 in each group.

Figure 12. Evaluation of apoptotic markers in the mice with 21 days of life. Immunohistochemical staining for caspase-1, caspase-3 and caspase-8 after exposure MMA or NaCl in cerebral cortex (1), hippocampus (2) and striatum (3) in the mice pups. Significance was determined *P <0.0001 as compared to control group. Data mean + S.E.M. for n = 10-15 in each group.

Figure 13. Evaluation of apoptotic markers in the mice with 40 days of life. Immunohistochemical staining for caspase-1, caspase-3 and caspase-8 after exposure MMA or NaCl in cerebral cortex (1), hippocampus (2) and striatum (3) in the mice pups. Significance was determined *P <0.0005 as compared to control group. Data mean + S.E.M. for n = 10-15 in each group.

Figure 14. *Digitized images of the cerebral cortex, hippocampus and striatum.* Effect of postnatal acute MMA administration on the histological analysis mice pups in 21 (A) and 40 (B) days of life. No significant differences between groups were detected (n =15 in each group).

Figure 1



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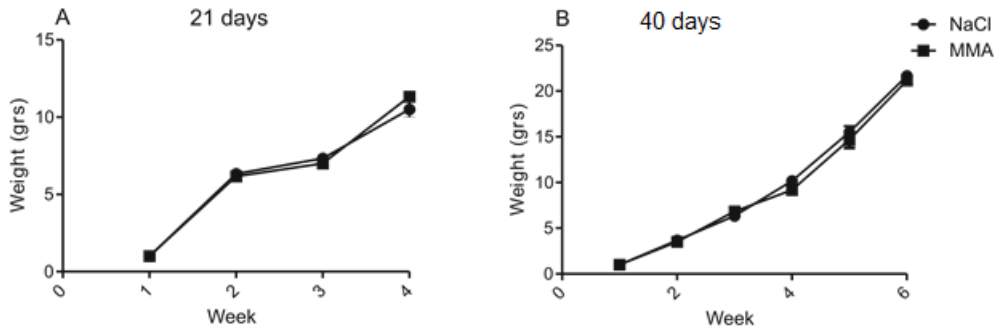

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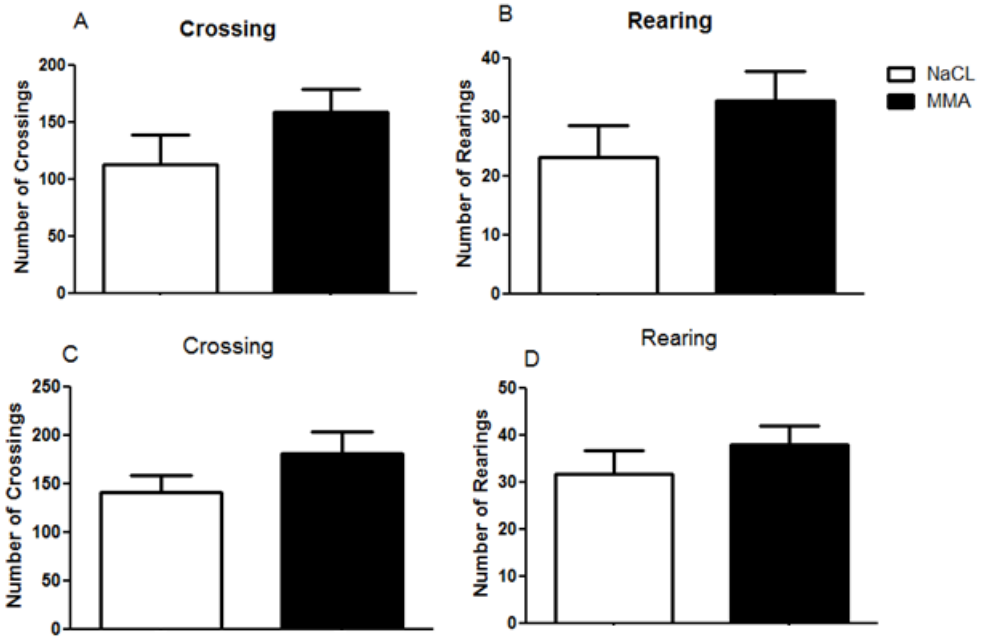

A
Crossing
Number of Crossings
200
150
100
50
0
B
Rearing
Number of Rearings
40
30
20
10
0
NaCL
MMA
C
Crossing
Number of Crossings
250
200
150
100
50
0
D
Rearing
Number of Rearings
50
40
30
20
10
0

Figure 4

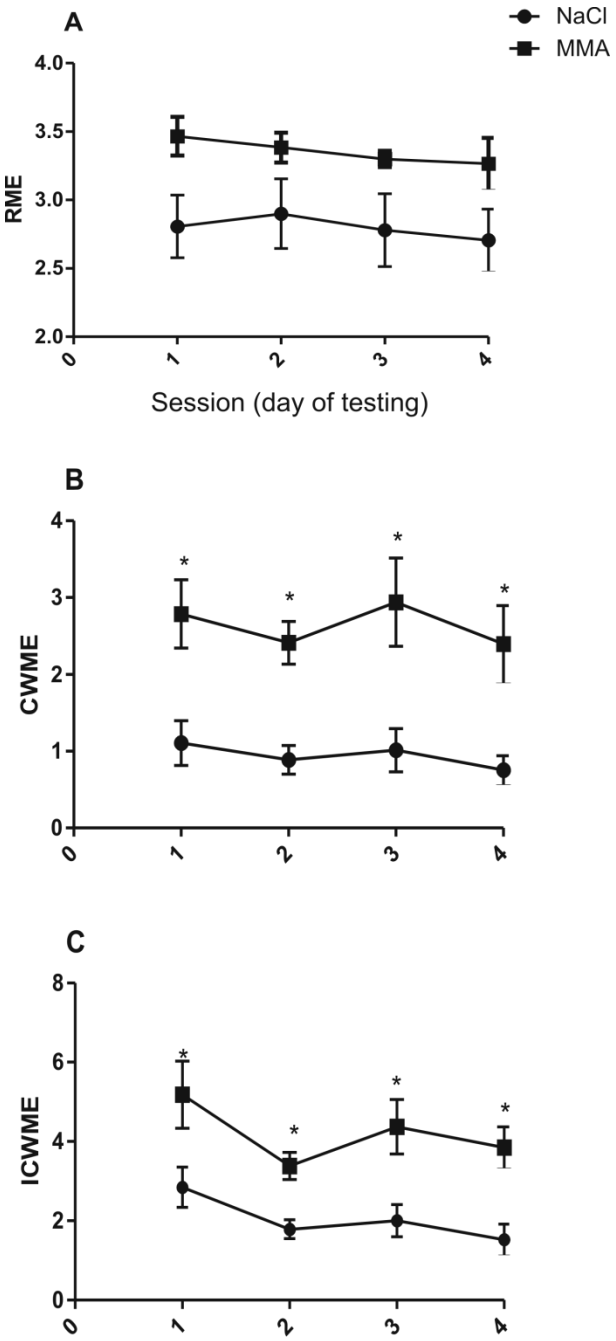

A
NaCl
MMA
RME
4.0
3.5
3.0
2.5
2.0
0
1
2
3
4
Session (day of testing)
B
CWME
4
3
2
1
0
C
ICWME
8
6
4
2
0

Figure 5

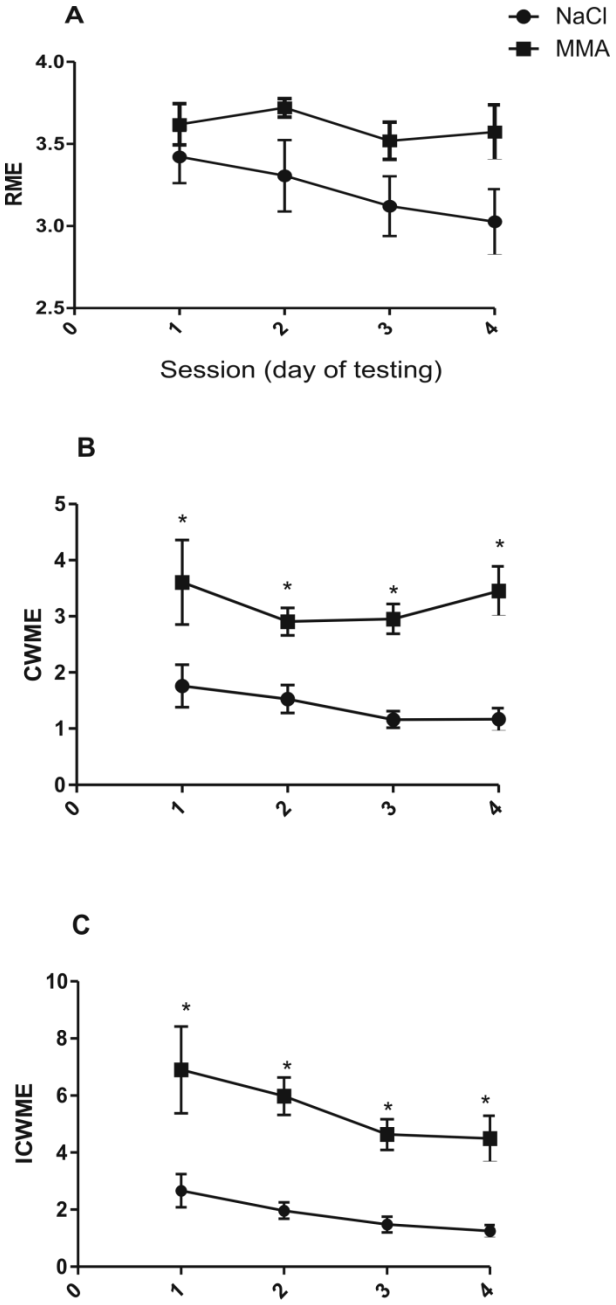

A
NaCl
MMA
RME
4.0
3.5
3.0
2.5
0
1
2
3
4
Session (day of testing)
B
CWME
5
4
3
2
1
0
*
C
ICWME
10
8
6
4
2
0

Figure 6

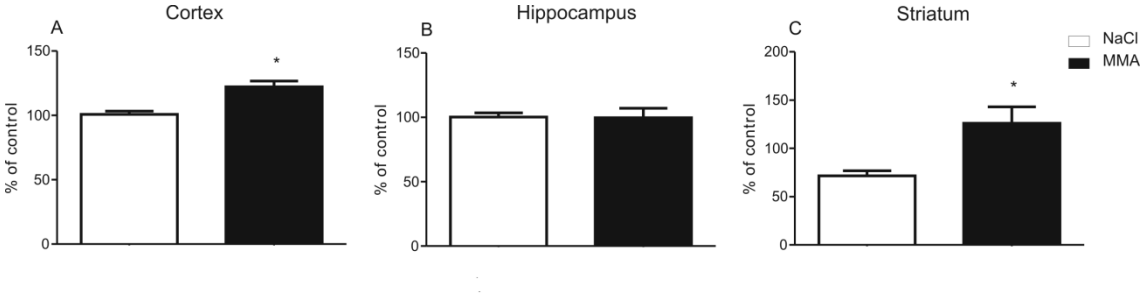


Figure 7

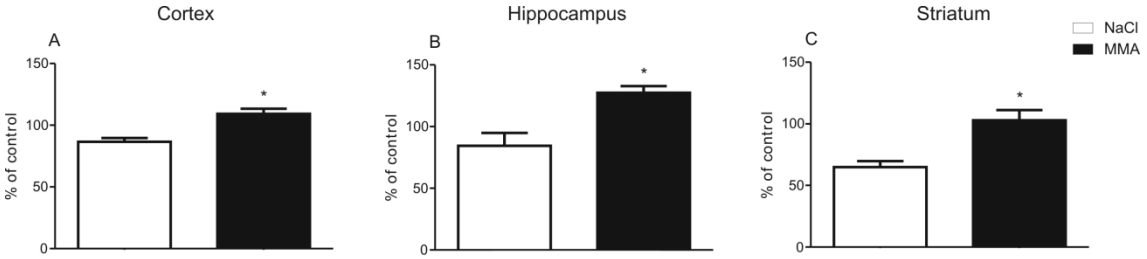
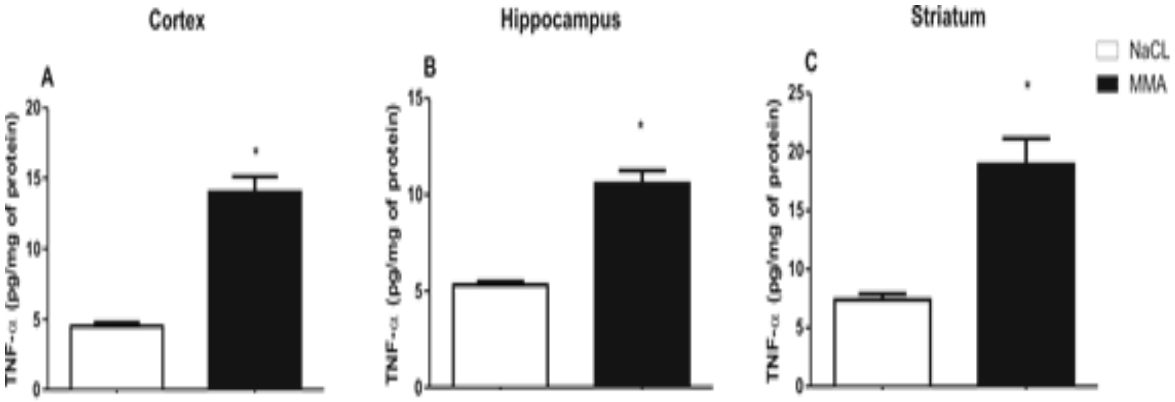


Figure 8



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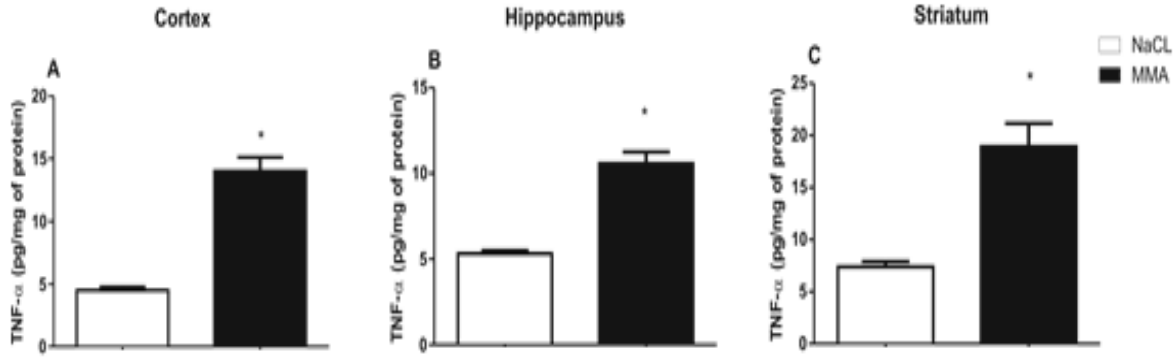

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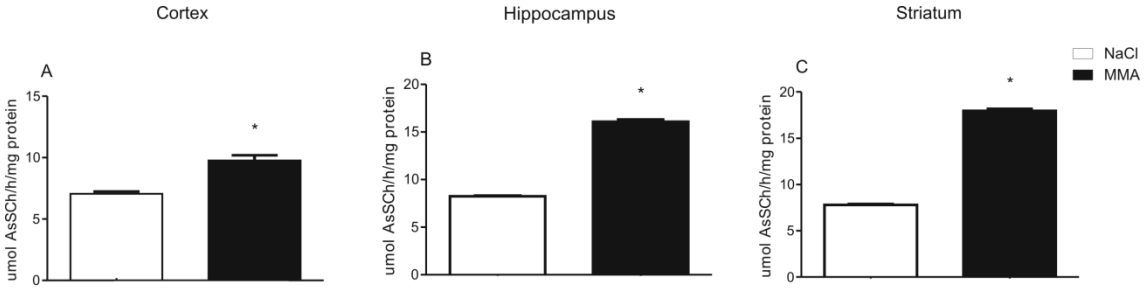
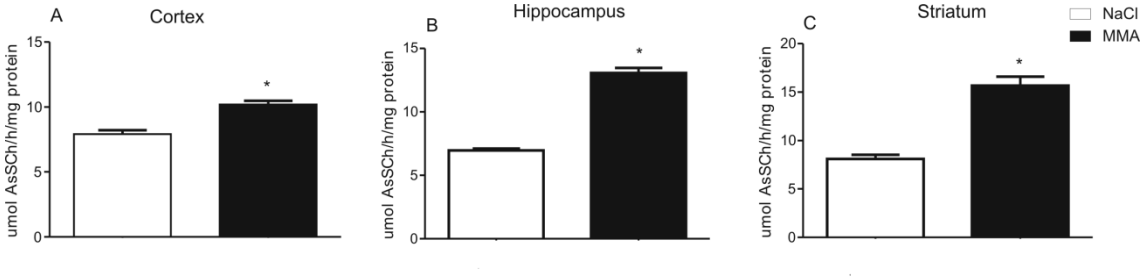


Figure 11



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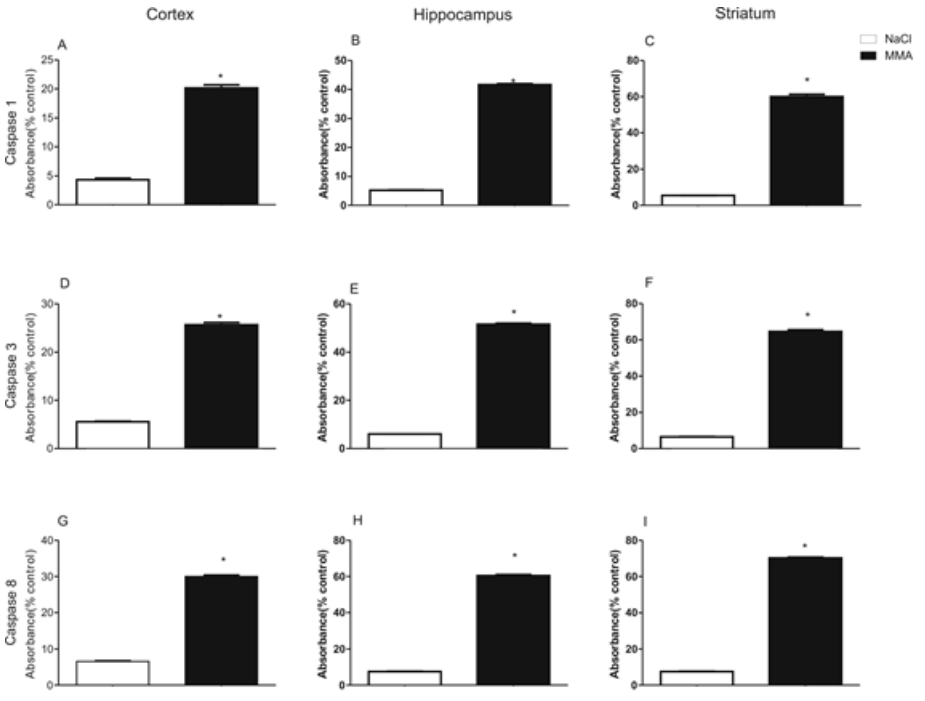


Figure 13

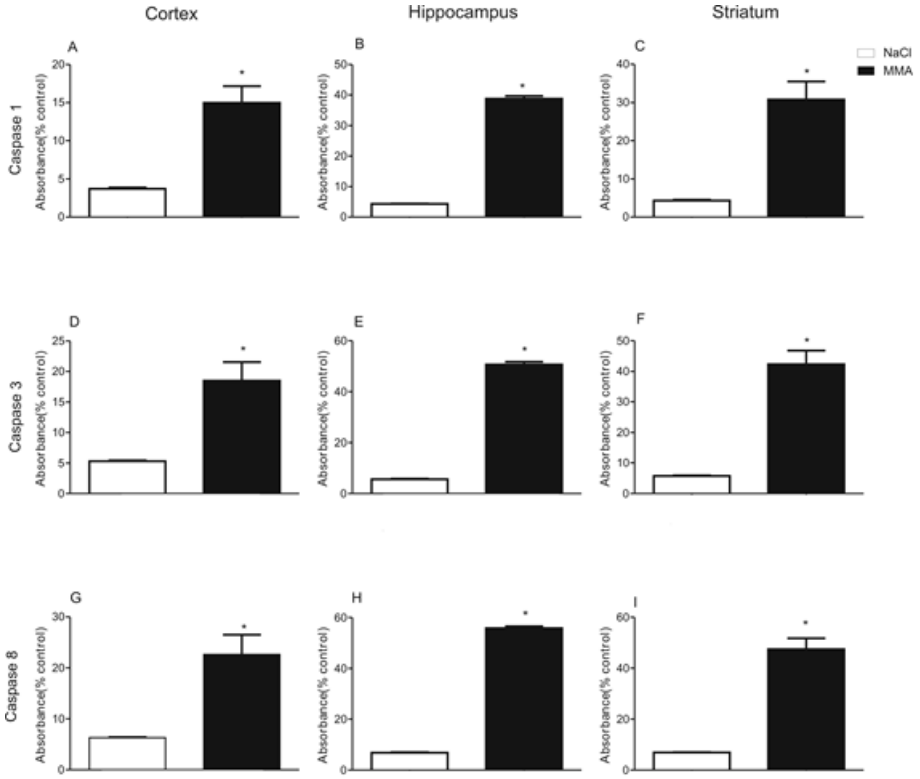
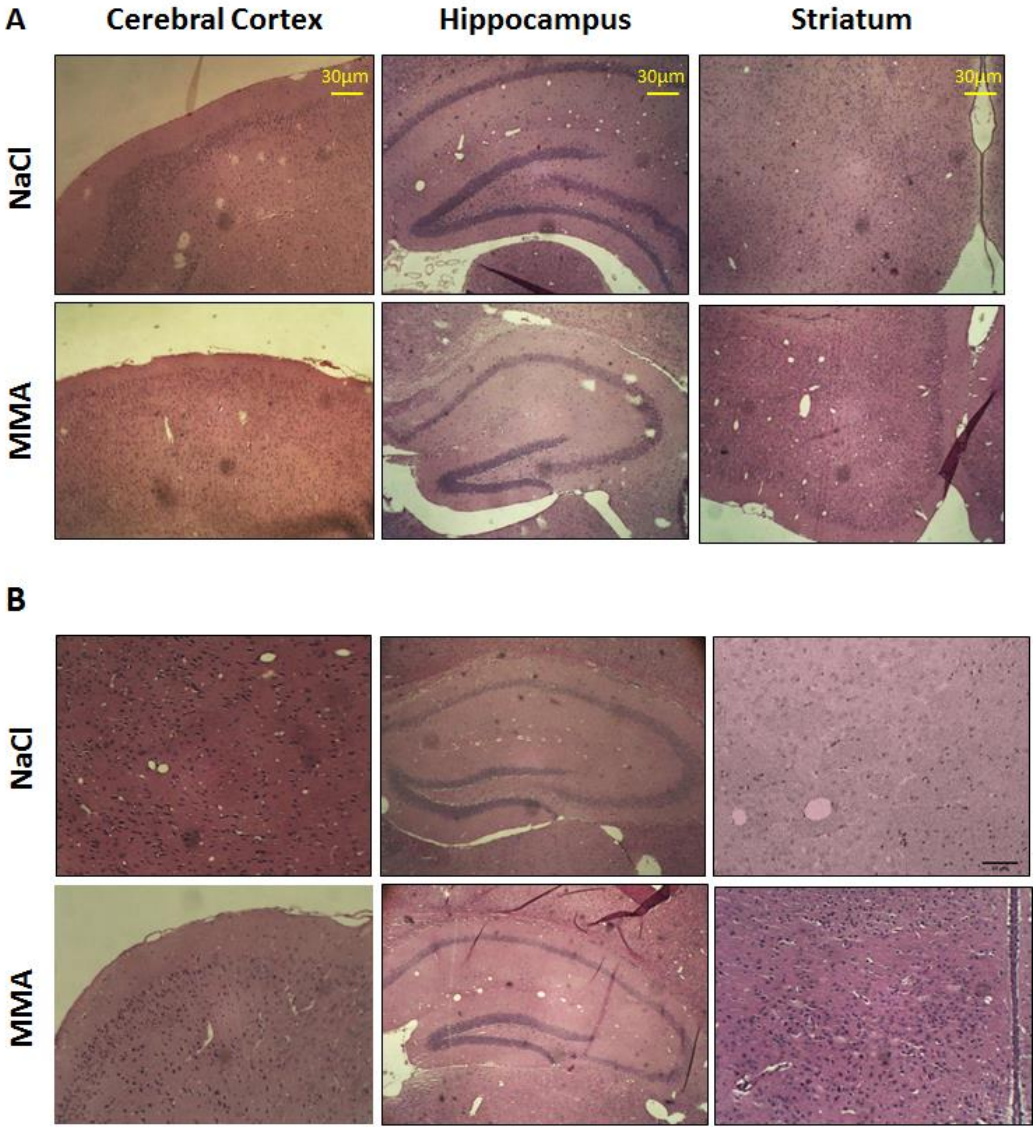

Cortex
Hippocampus
Striatum
NaCl
MMA
A
B
C
D
E
F
G
H
I
Caspase 1
Caspase 3
Caspase 8
Absorbance(% control)
*

Figure 14


A
Cerebral Cortex
Hippocampus
Striatum
NaCl
MMA
30µm
30µm
30µm
B
NaCl
MMA

3. DISCUSSÃO

A acidemia metilmalônica é um erro inato do metabolismo caracterizado bioquimicamente pelo acúmulo de MMA e de seus metabólitos como propionato e metilcitrato nos líquidos biológicos e tecidos corporais dos pacientes afetados (Fenton 1995; Royes, Fighera et al. 2007; Chandler, Zerfas et al. 2009). O acúmulo desse ácido tem sido considerado como um dos principais responsáveis pelas alterações neurológicas descritas na doença, as quais aparecem nos períodos iniciais do desenvolvimento cerebral, e geralmente após crises de descompensação metabólica, vacinações rotineiras ou quadros infecciosos (GOODMAN 2001). Aproximadamente 90% das crianças afetadas desenvolvem graves sintomas clínicos antes dos 36 meses de idade, e a ocorrência de crises encefalopáticas, as quais levam ao início dos sintomas, não tem sido reportadas após os 5 anos de vida (Fenton 1995; Wright and Jalan 2007; Cichoz-Lach and Michalak 2013). Dentre as alterações neurológicas apresentadas pelos pacientes com acidemia metilmalônica, pode-se citar o déficit de cognitivo (Lehnert, Sperl et al. 1994; van der Meer, Poggi et al. 1994). Embora os distúrbios neurológicos sejam prevalentes nesta acidemia, pouco é conhecido sobre o mecanismo pelo qual o acúmulo dos ácidos orgânicos causam essas alterações e a sua associação com processos inflamatórios. Nesse contexto, recentes estudos mostraram o papel dos mediadores inflamatórios em modelos de crise convulsiva e morte cerebral por apoptose na acidemia metilmalônica (Salvadori, Bandero et al. 2012; Jafari, Braissant et al. 2013; Li, Peng et al. 2014).

Interessantemente, os resultados do presente estudo mostraram, pela primeira vez, que a administração única de MMA (concentração encontrada no sangue e no cérebro de pacientes afetados; (Hoffmann, Meier-Augenstein et al. 1993; Brusque, Rotta et al. 2001), no período neonatal, causou um déficit de memória de trabalho e um aumento nos níveis de TNF-α e atividade da AchE, assim como, aumentou os níveis de DCFH e a ativação das caspases nos animais com 21 e 40 dias de vida no estriado, córtex e hipocampo. Estes dados sugerem um possível envolvimento do processo oxidativo e inflamatório na disfunção neurológica observada nos pacientes com acidemia metilmalônica .

Entretanto, o tratamento com MMA não causou alteração histológica significativa no hipocampo, córtex e estriado dos camundongos.

Dados adicionais mostraram que nenhum dos tratamentos apresentou efeito no peso corporal, ou seja, não causaram desnutrição dos animais (Figura 1 e 2). Isto é importante, uma vez que animais desnutridos podem apresentar um comportamento diferente em testes neurocomportamentais (Davis and Squire 1984; Seminotti, da Rosa et al. 2012). Logo, as alterações no teste do labirinto radial, observadas nos animais tratados com MMA, não foram devido a este efeito nutricional. Da mesma forma, o desempenho dos animais no teste de ansiedade (Tabela 1) e no teste de locomoção e exploração (Figura 3 e 4) não foi alterado por nenhum dos tratamentos.

Em relação ao comportamento dos filhotes de camundongos, este é o primeiro estudo que investiga o efeito de uma única injeção de MMA avaliando o aprendizado e a memória através do teste do radial, mostrando que a administração deste ácido, no período neonatal, foi suficiente para induzir um déficit na memória de trabalho, mas não na memória espacial.

A memória de trabalho geralmente é expressa por uma alteração na função executiva (Kirkby 1969; Devan, Goad et al. 1996) e essa alteração é muitas vezes relacionada com lesões no corpo do estriado e interpretada como a incapacidade de inibir a ação em curso ou como um fracasso para iniciar a próxima resposta, (Devan, Goad et al. 1996). Curiosamente, a dificuldade na memória de trabalho é um dos sintomas observados no déficit cognitivo em várias doenças hereditárias do metabolismo e naquelas que envolvem os glânglios da base, incluindo a acidemia metilmalônica (Fenton.W.A 2001) e a doença de Huntington (El Massioui, Ouary et al. 2001). Nesse contexto, o fato do MMA ter potencializado o déficit de memória de trabalho nos animais com 21 e 40 dias de vida, sugere que um acúmulo deste ácido orgânico, principalmente nos gânglios da base, pode estar associado ao déficit cognitivo nos pacientes com acidemia metilmalônica.

Interessantemente, nós observamos que a administração aguda do MMA em filhotes de camundongos, em um período de desenvolvimento cerebral, não alterou a desempenho dos animais no teste de memória de referência (aprendizado e memória espacial), sugerindo que o hipocampo não está relacionado diretamente com o déficit cognitivo observado neste estudo.

Estes resultados concordam com estudos que mostram que pacientes com acidemia metilmalônica apresentam degenerações estriatais, mas não no hipocampo, nos exames de imagem neuroradiológicos (Ibanez-Mico, Izquierdo-Fos et al. 2008; Bindu, Kovoor et al. 2010).

Um estudo recente de Ribeiro e colaboradores (Ribeiro, Della-Pace et al. 2013), demonstrou que existe uma deficiência de aprendizagem e memória em decorrência de um novo paradigma de localização espacial no teste de reconhecimento de objeto, por aproximadamente quatro dias após a administração crônica de MMA (5º a 28º dias de vida), indicando uma piora da memória espacial. No entanto, foi observado que este déficit de memória espacial causado pelo MMA está relacionado com o longo período de administração do ácido (por mais de 20 dias). Assim, apesar das diferenças (temporal e técnica) entre o protocolo realizado e o referido estudo, verificou-se uma similaridade em relação à alteração no comportamento dos animais (ou por piora na memória espacial ou de trabalho), sugerindo que essas alterações estão relacionadas aos danos causados no SNC pela administração de MMA (Pettenuzzo, Schuck et al. 2003; Royes, Fighera et al. 2006). Dessa forma, deve-se lembrar de que várias estruturas subcorticais estão envolvidas na aquisição, consolidação e evocação da memória (Izquierdo and Medina 1997; McGaugh 2000; Izquierdo, Bevilaqua et al. 2006) e também sofrem diferentes consequências decorrentes do acúmulo de MMA, como foi observado em outros modelos experimentais desta acidemia (Wajner, Brites et al. 1988; Malfatti, Royes et al. 2003; Pettenuzzo, Wyse et al. 2003; Vasques, Brinco et al. 2006).

Além disso, a inflamação e o dano oxidativo podem ser mecanismos comuns envolvidos nas alterações cerebrais dos pacientes com acidemia metilmalônica (Fighera, Queiroz et al. 1999; Fighera, Bonini et al. 2003; Harting, Seitz et al. 2008; Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013). Nesse contexto, TNF-α e IL-1β, citocinas produzidas pela micróglia ativada, desempenham um papel importante na patogênese da acidemia metilmalônica (Salvadori, Bandero et al. 2012; Ribeiro, Della-Pace et al. 2013). No SNC, a primeira e principal defesa imune são as células da glia (Ransohoff and Brown 2012). Essas, por sua vez, são responsáveis pela produção das citocinas pró-inflamatórias, incluindo a IL-1β e TNF-α, que são

considerados moduladores da neurotransmissão cerebral (Merrill 1992). Sendo assim, as citocinas inflamatórias podem influenciar vários componentes celulares no cérebro e ter um papel importante nos efeitos prejudiciais sobre o funcionamento da memória e plasticidade sináptica (Nguyen, Deak et al. 1998; Lynch 2004).

Nesse contexto, foi observado que a administração do MMA induziu um aumento do TNF-α no córtex cerebral, hipocampo e estriado nos animais com 21 e 40 dias de vida. De fato, desde que a barreira-hematoencefálica (Song, Son et al. 2002), ainda está em processo de maturação no período neonatal, quando foi administrado o MMA, pode-se sugerir que o aumento do TNF-α cerebral seja devido à resposta inflamatória induzida pelo ácido orgânico e o consequente desenvolvimento das alterações comportamentais, (Stolp, Johansson et al. 2011).

Nesta linha de visão, estudos mostraram que as citocinas pró-inflamatórias, especialmente o TNF-α, aumentam a excitabilidade neuronal pela ativação dos receptores para TNF (Tansey MG 2008). De fato, a estimulação do receptor do TNF cerebral causa excitotoxidade diretamente por ativação do receptor N-metil-D-aspartate (NMDA) (Zou and Crews 2005) e indiretamente por inibir os transportadores gliais de glutamato nos astrócitos (Choi 1988). Como consequência, o TNF-α facilita o influxo de cálcio mediado pelo receptor NMDA para dentro dos neurônios, promovendo assim um aumento da excitotoxidade neuronal (Viviani, Bartesaghi et al. 2003). Considerando-se que o TNF-α pode inibir a captação de glutamato em atrócitos (Hu, Sheng et al. 2000) e aumentar sua liberação glial (Bezzi, Domercq et al. 2001), é plausível propor que o aumento desta citocina pró-inflamatória resulta em níveis elevados de glutamato extracelular e toxicidade neste modelo de acidemia. Em concordância com estes dados, um considerável corpo de evidências tem mostrado que o excesso de estimulação do receptor glutamatérgico, em particular o receptor NMDA, é considerada a principal via relacionada a toxidade induzida pelo MMA (de Mello, Begnini et al. 1996; de Mattos-Dutra, Meirelles et al. 2000).

Além disso, a ação do TNF-α nos seus respectivos receptores causa ativação do fator nuclear kappa de potencializador de cadeia-leve de células β

ativadas (NFKβ), um fator de transcrição que migra para o núcleo das células e pode promover elevadas concentrações de cálcio intra-celular, assim como, a produção de óxido nítrico e aumento do peróxido de hidrogênio (Hemmens and Mayer 1998). Estes radicais livres induzem o estresse nitrosativo/oxidativo, podendo resultar em um aumento da peroxidação lipídica e dano ao DNA (Puttfarcken, Getz et al. 1993; Frantseva, Velazquez et al. 2000). Nesse contexto, foi observado que a administração de MMA induziu a um aumento do DCFH, e consequentemente, o estresse oxidativo e neuroinflamação observados no córtex cerebral, hipocampo e estriado dos filhotes de camundongos (Figura 7 e 8).

Além disso, também foi mostrado que a AchE possui uma importante função nas respostas imunitárias devido a hidrólise rápida da Ach (Kawashima and Fujii 2000), que é conhecida por possuir ação anti-inflamatória e suprimir a produção de citocinas pró-inflamatórias (Kawashima and Fujii 2003). Estudos têm mostrado que as ativações dos receptores nicotínicos em macrófagos reduziram significativamente a liberação de citocinas pró-inflamatórias, tais como o TNF-α, IL-1β e IL-1ra, ao passo que a produção de citocinas anti-inflamatórias, tais como a IL10 não é afetada (Borovikova, Ivanova et al. 2000). Deste modo, os inibidores da AchE reduzem a proliferação dos linfócitos e a secreção de citocinas pró-inflamatórias e podem atenuar a inflamação, aumentando a concentração de Ach no espaço extracelular (Nizri, Hamra-Amitay et al. 2006). Neste estudo, foi observado um aumento na atividade da AchE no corpo estriado, hipocampo e córtex cerebral de filhotes de camundongo. É possível que esta alteração conduza a uma redução do nível de Ach, e contribua para o estado pró-inflamatório. Embora o papel do sistema colinérgico na acidemia metilmalônica ainda não esteja bem claro, alterações na atividade da AchE apoiam o fato de que este sistema pode contribuir para a regulação das respostas imunes nesta acidemia e, consequentemente, nos sintomas neurológicos.

Cabe salientar que a perda de neurônios colinérgicos está associada à ocorrência de convulsões e retardo mental em pacientes com doenças inatas do metabolismo (Ratnakumari, Qureshi et al. 1994). Estes achados despertam para novas pesquisas que esclareçam um mecanismo mais específico para o

sistema colinérgico como, por exemplo, a utilização de inibidores da AchE para o tratamento do déficit cognitivo na acidemia metilmalônica.

Assim, além da injeção de MMA ser capaz de aumentar os níveis de biomarcadores inflamatórios desta doença, ela também induziu a ativação das caspases 1, 3 e 8 no corpo estriado, hipocampo e no córtex cerebral de camundongo com 21 e 40 dias. De fato, a ativação da caspase 1 pode ocorrer por seus substratos, como IL-1β e TNF-α, e refletem um aumento da resposta inflamatória celular (Skeldon, Faraj et al. 2014). Após a ligação do TNF, seus receptores podem ser interligados levando a ativação das caspases executoras, como a caspase 8, por meio da via apoptótica extrínseca (Micheau and Tschopp 2003; Schneider-Brachert, Tchikov et al. 2004). A caspase 8 combinada com sua capacidade de induzir a apoptose por meio da via extrínseca, também desencadeia a via da apoptótica intrínseca, por meio da clivagem dos membros da família dos pró-apoptóticos Bcl-2 para iniciar o processo apoptótico induzido pela mitocôndria (Gross, Yin et al. 1999; Zhao, Li et al. 2001). Estes eventos induzem a um aumento subsequente de EROS e liberação de fatores apoptogênicos mitocondriais (tais como citocromo c) para o citosol, consequentemente, levando uma ativação da caspase 3 e disfunção celular (Budihardjo, Oliver et al. 1999). Como o estresse oxidativo e citocinas pró-inflamatórias ativam as caspases por via apoptótica extrinseca e intrínseca (Hu, Sheng et al. 2000) é plausível propor que o aumento induzido pelo MMA nos níveis de TNF-α e DCFH resulta na ativação da via apoptótica e consequentemente, o déficit cognitivo observado neste estudo. Corroborando com estes dados, há também evidências da ativação das caspases 1, 3 e 8 no cérebro de pacientes e em modelo experimental da doença de Huntington após a administração de malonato (Ona, Li et al. 1999; Sanchez, Xu et al. 1999; Andreassen, Ferrante et al. 2000).

Em outro modelo de acidemia metilmalônica, também foi observado que a incorporação de acetato de lipídeos (U-14C) no córtex cerebral foi reduzida pelo MMA, o que pode explicar a hipomielinização e/ou desmielinização característica nos pacientes, juntamente com os resultados observados neste trabalho, podendo supor que são mediados pelo sistema imunológico (de Mello, Rubin et al. 1997; Mayo, Quintana et al. 2012). Além disso, experimentalmente ou clinicamente, as citocinas interferem de forma direta ou

indireta no processo de consolidação da memória, plasticidade ou neurogênese sináptica e expressão de mediadores neuroinflamatórios e apoptóticos, implicando um potencial dano neuronal e comprometimento cognitivo (Bossu, Cutuli et al. 2012).

Por outro lado, os resultados apresentados neste trabalho mostraram que a administração de MMA não induziu alterações histológicas nas estruturas cerebrais estudadas. De fato, McLaughlin et al. (McLaughlin, Nelson et al. 1998) também não observaram morte neuronal após a exposição do MMA nas concentrações de 5 ou 50 mM, mas apenas com concentrações mais elevadas (500 mM, 1mM e 10mM) no estriado e culturas corticais do cérebro de ratos embrionários após 24 horas de incubação, sugerindo que o MMA possui efeito dose-dependente para induzir a morte celular.

Além disso, é possível que durante os episódios de encefalopatia, que geralmente se seguem após as infecções ou outras condições de estresse metabólico, ocorra um aumento dos níveis de MMA nos fluídos corporais e potencialização das manifestações neurológicas (Richard, Alvarez-Barrientos et al. 2007; Salvadori, Bandero et al. 2012). Considerando que os pacientes com acidemia metilmalônica desenvolvem degeneração cerebral e sintomas neurológicos após uma descompensação metabólica, podemos também propor que talvez seja necessário um evento catabólico ou infeccioso para levar a alterações morfológicas neste modelo de acidemia. De acordo, Salvadori e colaboradores (Salvadori, Bandero et al. 2012) mostraram que a prostaglandina E2 aumentou as convulsões induzidas pelo MMA; reforçando a possibilidade de que as infecções podem piorar e agravar as disfunções neurológicas nos pacientes com acidemia metilmalônica. No entanto, futuros estudos serão necessários para verificar se eventos infecciosos poderão causar alterações nas estruturas cerebrais, principalmente, nas analisadas neste estudo. Sendo assim, devido ás diferenças metodológicas que podem ser responsáveis pelos resultados discrepantes no trabalho em questão, como a dose ou as técnicas histológicas, estudos mais específicos serão necessários para esclarecer a relação entre a exposição do MMA e a morte neuronal.

Embora os resultados do presente estudo possam sugerir uma associação entre o déficit de memória e o aumento dos marcadores pró-inflamatória e apoptóticos no cérebro dos animais tratados com MMA, estes

dados não apontam uma associação direta entre esses. As alterações nestes marcadores podem influenciar o comportamento, afetando a neurotransmissão, o sistema endócrino, a plasticidade neuronal e os circuitos cerebrais (Bossu, Cutuli et al. 2012), e a avaliação desses mecanismos e suas funções vão além do escopo deste estudo. Por isso, é interessante ressaltar que mais experimentos serão realizados para esclarecer se o processo de apoptose e neuroinflamatório estão associados à fisiopatologia da acidemia metilmalônica.

Então, considerando todos os dados encontrados neste trabalho, pode-se sugerir que o estresse oxidativo induzido por MMA prejudica o potencial intrínseco da célula, levando a sinais pró-inflamatórios e criando um ciclo vicioso entre estresse oxidativo e neuroinflamação, causando uma ativação de fatores apoptóticos. Além disso, é plausível propor que essas alterações desempenham um papel importante na disfunção neurológica após a administração de MMA, e assim contribuem para a fisiopatologia do prejuízo cognitivo observado nos pacientes com acidemia metilmalônica. Entretanto, estudos clínicos devem ser conduzida na tentativa de se compreender melhor o envolvimento destas vias oxidativa, inflamatória e apoptóticas nestes pacientes.

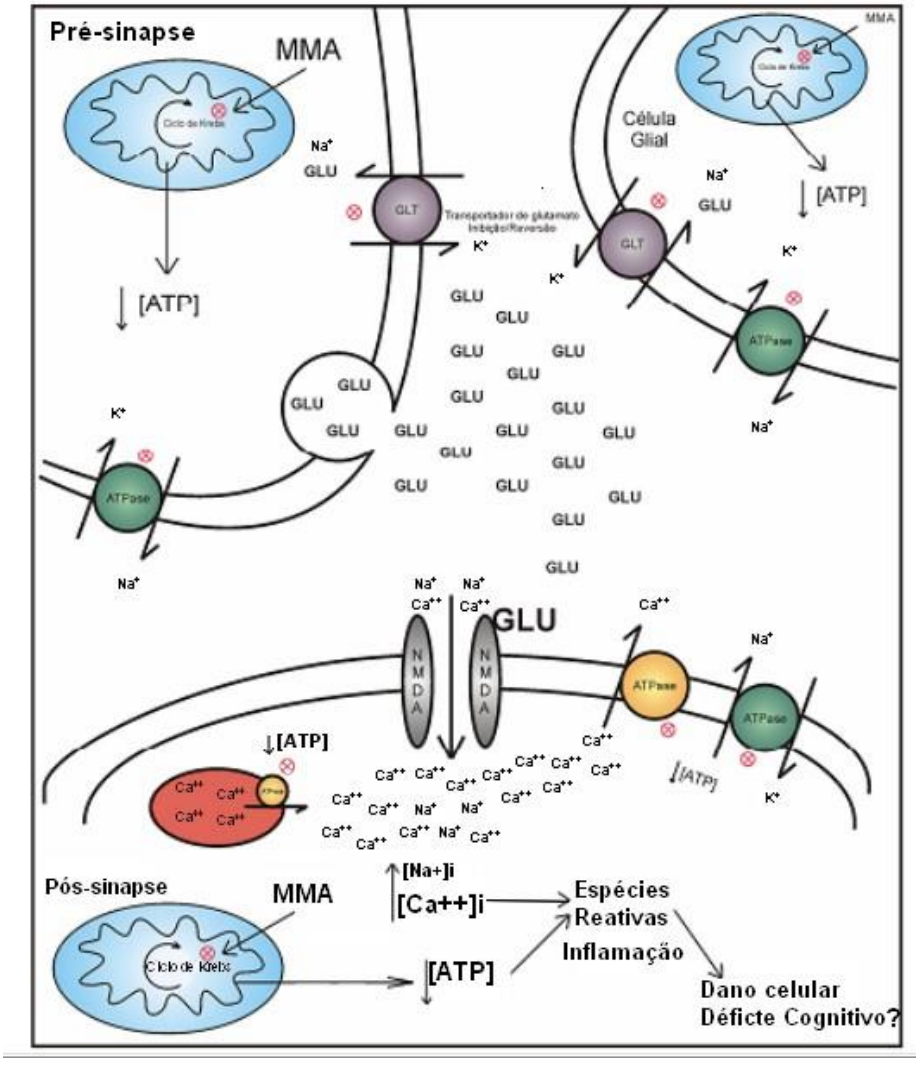
4. CONCLUSÕES PARCIAIS

1. Em relação ao peso corporal dos animais tratados com MMA não observamos desnutrição, sugerindo que as alterações comportamentais e bioquímicas não ocorreram devido a este quadro.
2. O desempenho dos animais no teste de ansiedade e no teste de locomoção e exploração não foi alterado pelo tratamento.
3. MMA potencializou o déficit de memória de trabalho nos animais com 21 e 40 dias de vida, sugerindo que um acúmulo deste ácido orgânico, principalmente nos gânglios da base, pode estar associado ao déficit cognitivo nos pacientes com acidemia metilmalônica.
4. Podemos relatar que o aumento do TNF-α cerebral seja devido à resposta inflamatória induzida pelo MMA, e consequentemente desencadeando o aparecimento das alterações comportamentais.
5. MMA induziu a um aumento do DCFH no córtex cerebral, hipocampo e estriado dos filhotes de camundongos.
6. O aumento da atividade da AchE no corpo estriado, hipocampo e córtex cerebral de filhotes de camundongo contribuiu para o estado pró-inflamatório.
7. Sugere-se que o aumento dos níveis de TNF- α e DCFH induzido pelo MMA, pode ter resultado na ativação da via apoptótica (aumento das caspases 1, 3 e 8) e consequentemente, no déficit cognitivo observado nos animais deste estudo.
8. Nenhum dos tratamentos causou alteração histológica no córtex cerebral, hipocampo e corpo estriatal dos animais, provavelmente porque doses maiores de MMA são necessárias para desencadear estas alterações, ou ainda, seja necessária a presença de um fator de descompensação, como o infeccioso e metabólico, para induzir a morte neuronal.

5. CONSIDERAÇÕES FINAIS

Por fim, conclui-se que o estudo em questão pode inferir que, por meio

de mecanismos ainda não totalmente elucidados, o insulto metabólico transitório com o MMA pode causar e induzir ao aumento de fatores neuroinflamatórios e apoptóticos durante períodos críticos de desenvolvimento, contribuindo para a progressão da disfunção cognitiva em pacientes com acidemia metilmalônica. Dessa forma, podemos concluir também que a administração única de MMA levou ao déficit cognitivo nos animais tratados, demonstrando a importância do diagnóstico precoce, para que a intervenção imediata possa diminuir os efeitos da descompensação metabólica e suas sequelas.



(Patel 2004Patel 2004)

Figura: 2 Figura representativa do mecanismo de ação relacionado ao prejuízo cognitivo induzido pelo MMA. A inibição de SDH neuronal e glial induzida pelo

MMA causa falência energética e inibição das ATPases, causando a despolarização e alterações nos gradientes iônicos. A despolarização provoca a liberação de glutamato armazenado nas vesículas sinápticas e a perda do gradiente leva a inibição dos transportadores de glutamato a nível glial e neuronal. O aumento de glutamato na fenda sináptica e a falência energética, que também atinge a membrana pós-sináptica induz a despolarização, deslocamento de Mg presente no canal do receptor NMDA e influxo de Ca^{2+} para o meio intracelular. O acúmulo de Ca^{2+} proveniente do meio extracelular e da inibição de ATPases presentes no retículo endoplasmático, provavelmente está envolvido na propagação do foco de despolarização e na geração de radicais livres. O estresse oxidativo induzido por MMA prejudica o potencial intrínseco da célula, levando ao aumento de marcadores inflamatórios e criando um ciclo vicioso entre dano oxidativo e neuroinflamação, resultando em uma ativação dos fatores apoptóticos e no prejuízo cognitivo.

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