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

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O PAPEL DA AMÔNIA SOBRE AS ALTERAÇÕES COMPORTAMENTAIS, NEUROINFLAMAÇÃO E APOPTOSE EM UM MODELO EXPERIMENTAL DE ACIDEMIA METILMALÔNICA.

Santa Maria, RS
2017

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Tese de doutorado apresentado ao Programa de Pós-Graduação em Farmacologia da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Doutora em Farmacologia**.

Orientadora: Prof.ªDrª. Michele Rechia Fighera

Co-orientador: Prof. Dr. Luiz Fernando Freire Royes

Santa Maria, RS 2017

Ficha catalográfica elaborada através do Programa de Geração Automática
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Gabbi, Patrícia Gabbi
O PAPEL DA AMÔNIA SOBRE AS ALTERAÇÕES COMPORTAMENTAIS, NEUROINFLAMAÇÃO E APOPTOSE EM UM MODELO EXPERIMENTAL DE ACIDEMIA METILMALÔNICA / Patrícia Gabbi Gabbi.- 2017.
133 p.; 30 cm

Orientador: Michele Rechia Fighera
Coorientador: Luiz Fernando Freire Royes
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós-Graduação em Farmacologia, RS, 2017

1. Ácido Metilmalônico 2. Camundongos 3. Convulões 4. Memória 5. Morte neuronal e glial I. , Michele Rechia Fighera II. , Luiz Fernando Freire Royes III. Título.

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Defesa realizada por videoconferência

Santa Maria, RS

2017

Dedico este trabalho a minha família, pelo incentivo que sempre me deram e aos meus orientadores pela dedicação.

Não é o que acontece com você que importa, mas a maneira como você reage ao que acontece com você.

(Epiteto)

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.

Ao meu esposo, que tanto me orgulha por me fazer, cada dia, uma pessoa mais forte, segura e amada incondicionalmente.

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, Fê tiopental, Ale, Ana Paula, admiro todos, vocês são pessoas incríveis! Obrigada pela ajuda no laboratório, pela parceria, amizade. Agradecimento especial a Viviane por ter me ajudado sempre que precisei, uma pessoa incrível que és! Fernanda também me ajudado sempre que precisei! 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 sempre me apoio! Simoni amiga de todas as horas, sempre me apoiou e me deu força para continuar nessa etapa, 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

Tese de Doutorado

Programa de Pós-Graduação em Farmacologia

Universidade Federal de Santa Maria, RS, Brasil.

O papel da amônia sobre as alterações comportamentais, neuroinflamação e apoptose em um modelo experimental de acidemia metilmalônica.

AUTORA: Patrícia Gabbi

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Co-orientador: Prof. Dr. Luiz Fernando Freire Royes

Local e data de defesa: Santa Maria, 14 de Julho de 2017

Acidemia metilmalônica é um herança autossômica recessiva do metabolismo caracterizada bioquimicamente pela deficiência na atividade da metilmalonil-CoA mutase e pelo acúmulo tecidual de metilmalonato (MMA). Nesta acidemia, também ocorre o acúmulo de propionil-CoA, que causa a inibição do metabolismo da amônia pelo ciclo da uréia, levando a uma hiperamonemia. Clinicamente, esta acidemia caracteriza-se principalmente por alterações neurológicas, incluindo convulsões e déficit cognitivo.Os dados publicados no artigo I revelaram que os camundongos tratados com uma dose intermediária de NH_4Cl (6 mmol/g; i.p.) apresentaram um aumento na duração das convulsões induzidas pelo MMA (0.66 µmol/2 µL; intracerebroventricular (i.c.v). A administração de NH_4Cl (6 mmol/g) também induziu um aumento dos níveis de nitrito/nitrato (NOx) e a produção de ROS mitocondrial, avaliada através da oxidação de 2,7-diacetato de diclorofluoresceína (DCFH-DA) a DCF-RS, assim como, induziu a inibição da atividade da glutamina sintetase (GS) e glutamato descarboxilase (GAD) no córtex cerebral dos animais. A administração de NH_4Cl e MMA não apresentou alteração dos níveis de citocinas, alteração na permeabilidade da barreira hemato-encefálica (BHE) ou dano neuronal nos animais. No entanto, a combinação dos dois tratamentos, aumentou os níveis de DCF-RS e atividade da Na^+,K^+-ATPase, reduziu o potencial mitocondrial ($\Delta\Psi$) e o MTT, assim como, inibiu a atividade da succinato desidrogenase (SDH) no córtex cerebral. A NH_4Cl também alterou o ciclo de GABA caracterizado pela inibição da atividade da GS e GAD, ligação do [^{3}H] flunitrazepam e liberação de GABA após a injeção de MMA. Com base nesses resultados, sugere-se que o aumento das espécies reativas de oxigênio e nitrogênio induzidos pela amônia alteraram o ciclo da glicina/glutamato/GABA e contribuíram para a excitabilidade induzida pelo MMA.Considerando que a amônia modificou a excitabilidade cerebral de animais adultos tratados com MMA, será objetivo desta tese também avaliar o papel da amônia no desenvolvimento cerebral e parâmetros inflamatórios e apoptóticos de animais jovens após a administração de MMA, assim como, a avaliação do desempenho cognitivo desses animais. Os dados apresentados no manuscrito descrevem os resultados da administração de MMA (2.5 µmol/g; intracisterna magna (i.c.m.) ou NaCl (2.5µmol/g; i.c.) no primeiro dia de vida e de NH_4Cl (7.5 mmol/g; i.p.) ou NaCl (0.9%; i.p.) no segundo dia de vida em camundongos. 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 teste de cruz elevado. Os níveis de fator de necrose tumoral-alfa (TNF-α), interleucina 1β e DCF-RS, foram determinados no córtex cerebral, estriado e hipocampo de camundongos com 21 e 40 dias de vida. O teste do labirinto radial mostrou que os animais injetados com o MMA e NH_4Cl 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 com nenhum dos compostos testados. Entretanto, a administração de MMA e NH4Cl aumentou os níveis de TNF- α, DCFH no córtex cerebral, hipocampo e estriado dos camundongos com 21 e 40 dias de vida. Os níveis de interleucina 1β aumentaram no córtex cerebral e estriado, mas não aumentaram no hipocampo tanto em camundongos com 21 e 40 dias de vida.Considerando os dados apresentados, sugere-se que as mudanças no estado oxidativo induzido pela amônia alteram o ciclo glicina/glutamato/GABA, contribuindo com a excitabilidade do MMA e o aparecimento das convulsões. Além disso, a produção aumentada de ROS está associada a um aumento do processo inflamatório cerebral em um período precoce do desenvolvimento, conduzindo ao atraso de aprendizado nos animais tratados com MMA. Dessa forma, é plausível propor que amônia contribui com a disfunção cerebral nos pacientes com acidemia metilmalônica, favorecendo o aparecimento do quadro neurológico.

PALAVRAS CHAVES: Ácido Metilmalônico, camundongos, convulsões, memória, morte neuronal e glial, dano oxidativo e inflamatório.

ABSTRACT

Doctor Tese

Graduating Program in Pharmacology

Federal University of Santa Maria, RS, Brazil

The role of ammonia on behavioral changes, neuroinflammation and apoptosis in an experimental model of methylmalonic acidemia.

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Date and place of defense: Santa Maria, July 14th, 2017

Methylmalonic acidemia is an autosomal recessive metabolism biochemically characterized by a deficiency in the activity of the mutase methylmalonyl-CoA and the tissue accumulation of methylmalonate (MMA). In this acidemia, also occurs the accumulation of propionyl-CoA, which causes the inhibition of the metabolism of ammonia by urea cycle, leading to hyperammonemia. Clinically, this acidemia is characterized mainly by neurological disorders, including seizures and cognitive impairment. The data published in the article revealed that mice with acute treatment of MMA (0.66 mmol/2 uL) intracerebroventricularly (i.c.v) and an intermediate dose of NH 4 Cl (6 mmol/g) showed an increase in the duration of seizures induced by MMA. The administration of NH4Cl (6 mmol/g) also induced an increase of nitrite/nitrate (NOx), as well as the production of mitochondrial ROS over oxidation of 2,7-dichlorofluorescein diacetate (DCFH-DA) DCFH, GS and followed by inhibition GAD. The NH_4Cl plus MMA administration did not alter cytokine levels, plasma fluorescein extravasation, or neuronal damage. However, it potentiated DCF-RS levels, decreased the ΔΨ potential, reduced MTT, inhibited SDH activity, and increased Na^+,K^+-ATPase activity. NH_4Cl also altered the GABA cycle characterized by GS and GAD activity inhibition, [^{3}H] flunitrazepam binding, and GABA release after MMA injection. Since ammonia increased cerebral excitability of animals treated with MMA, it was objective this study to evaluate the ammonia role in development neural and glial of young mice after MMA administration, as well as to verify cognitive performance of these animals. The data presented in the manuscript demonstrated the results of MMA administration (2.5 µmol/g; injected intracisternally) or NaCl (2.5 µmol/g; i.c) on the first day of life and NH4Cl (7.5 mmol/g i.p.) or NaCl (0.9%; i.p.) on the second day of life of mice. From 21° to 33 ° or 40° to 52° days of age, the animals were evaluated for behavioral tasks such as radial maze test and high cross test. The levels of tumor necrosis factor-alpha (TNF-α), interleukin 1-betha, and DCFH were measured in cerebral cortex, striatum and hippocampus of mice with 21 and 40 days of life. The radial maze test showed that the animals injected with MMA and NH_4Cl presented a worse performance in the working memory test, but not in the reference memory test in animals with 21 and 40 days of life. The animals showed no anxiety behavior. In addition, MMA and NH4Cl administration increased levels of TNF-α, DCFH in the cerebral cortex, hippocampus and striatum of mice with 21 and 40 days of life. The Interleukin 1β levels increased in cerebral cortex and striatum but not in hippocampus increased both in mice at 21 and 40 days of life.Considering the data presented, it is suggested that the changes in ROS and reactive nitrogen species (RNS) levels elicited by ammonia alter the glycine/glutamate/GABA cycle and contribute to MMA-induced excitability. Furthermore, increased ROS production is related to an enhanced brain inflammatory process in a precocious period of development, leading to a learning delay in the animals treated with MMA. Thus, it is plausible to propose that ammonia contributes with cerebtral dysfunction in methylmalonic patients, favoring the appereance of neurological symptoms.

Keyword: Methylmalonic acid, mice, seizures, memory, neuronal and glial death, oxidative damage and inflammatory

LISTA DE FIGURAS E TABELAS

LISTA DE ABREVIATURAS E SIGLAS

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	Anteroposterior
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
EEG	Eletroencefalograma
GAD	Glutamato descarboxilase
GFAP	Proteína glial fibrilar ácida
GS	Glutationa sintetase
HOCL	Ácido hipocloroso
H_2O_2	Peróxido de hidrogênio
IL-1β	Interleucina-1 beta
IRC	Insuficiência renal crônica
K^+	Potássio
L	Lateral
LRM	Labirinto radial maze
LCE	Labirinto de cruz elevado
LPO	Peroxidação lipídica
MAP 2	Microtúbulo do tipo 2
MCM	Metilmalonil-CoA mutase
MCD	Memória de curta duração
MLD	Memória de longa duração
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
O_2	Ânion superóxido
O_3	Ozônio
RL	Radical livre
SNC	Sistema nervoso central
TNF-α	Fator de necrose tumoral-alfa
UCDS	Desordens do ciclo da ureia
V	Vertical

SUMÁRIO

APRESENTAÇÃO

Esta Tese de Doutorado segue as recomendações do Manual de Dissertações e Teses (MDT) da Universidade Federal de Santa Maria (UFSM) do ano de 2015. Essa tese compreende dois estudos que investigaram o papel da hiperamonemia nas convulsões e no déficit cognitivo destes animais camundongos e o efeito agudo de MMA e amônia no SNC causem alterações na memória, assim como, nos marcadores de dano oxidativo, inflamatório e de dano celular, sendo que os resultados obtidos geraram dois artigos científicos publicados no periódico amino acids **(ARTIGOS CIENTÍFICOS I e II).**

As seções **MATERIAIS E MÉTODOS, RESULTADOS e DISCUSSÃO** encontram-se nos **ARTIGOS CIENTÍFICOS I e II** os quais representam a íntegra deste estudo. Os itens **DISCUSSÃO** e **CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre os artigos científicos contidos neste trabalho. As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, DISCUSSÃO** e **CONCLUSÕES** desta tese, uma vez que as referências utilizadas para a elaboração dos artigos e manuscrito estão mencionadas nos mesmos.

INTRODUÇÃO

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´ deoxiadenosilcobalamina (AdoCbl), formada a partir da vitamina B_{12} (cobalamina, cbl) (CHANDLER et al., 2009; FENTON, W. A. R., L.E., 1995; ROYES et al., 2007). 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°) na atividade da mesma (CHANDLER; VENDITTI, 2005; MANOLI; VENDITTI, 1993; TANPAIBOON, 2005). A acidemia metilmalônica apresenta uma incidência no Brasil aproximada de 1:50.000 nascidos vivos (Wajner et al., 2009).

Essa acidemia ocorre em neonatos com início agudo apresentando grave descompensação metabólica, diminuição na alimentação, vômitos, desidratação, comprometimento da consciência progressiva podendo chegar ao coma, hipotonia e convulsões, quando ocorre na fase tardia pode apresentar-se crônica e intermitente, podendo desencadear ataques recorrentes de coma cetoacidótico precedido de letargia e ataxia, anorexia, vômitos, hipotonia, hiperamonemia, retardo de crescimento e de desenvolvimento, déficit cognitivo e aprendizagem.

A acidemia metilmalônica ocorre devido à inibição da carbamil fosfato sintetase I, enzima responsável pelo primeiro passo do ciclo da uréia. Um bloqueio na síntese da uréia resulta em um acúmulo de amônia, o que pode explicar a hiperamonemia apresentada por estes pacientes. A degradação de proteínas e utilização de aminoácidos como substratos energéticos, há uma maior liberação de amônia, produto resultante da degradação de aminoácidos (SMITH, 2005). A hiperamonemia é causa primária da neurotoxicidade levando a dificuldades motoras, alterações comportamentais, convulsões, coma e morte. Além disso, os pacientes que possuem níveis séricos elevados de amônia podem apresentar diarréia, vômitos e acidose metabólica entre outras (SMELTZER, BG BARE; SC, 1996).

Os mecanismos de neurotoxicidade da amônia parecem estar relacionados às alterações glutamatérgicas. Entretanto, existem evidências de que os distúrbios provocados pela amônia no SNC sejam decorrentes da combinação de alterações bioquímicas, morfológicas, energéticas e físicoquímicas, tais como alterações no pH

celular e consequente modificações na estrutura celular, principalmente em astrócitos (DOLINSKA; HILGIER; ALBRECHT, 1996; DREWES; LEINO, 1985; ZHOU; NORENBERG, 1999).

Sendo assim, o objetivo do presente estudo foi avaliar se a administração de amônia poderia aumentar as convulsões induzidas pelo MMA, assim como, verificar o efeito desse composto no ciclo glicina/glutamato/GABA, produção de espécies reativas e atividade mitocondrial no cérebro de animais. Considerando que os pacientes com acidemia metilmalônica apresentam atraso no desenvolvimento cognitivo, também será objetivo deste trabalho avaliar se a administração de amônia e MMA no período neonatal poderia causar prejuízo na memória de camundongos jovens e se esta alteração poderia estar relacionada com aumento nos marcadores de dano oxidativo e de inflamatórios, assim como, nos parâmetros de morte neuronal e glial por meio de técnicas de imunohistoquímica em animais com 21 e 40 dias de vida.

2. REVISÃO DE LITERATURA

2.1 Erros inatos do metabolismo

Erros Inatos do Metabolismo (EIM) são chamados de distúrbio hereditário autossômico recessivo. Esse distúrbio resultará na deficiência na atividade de determinadas enzimas, que leva ao bloqueio de uma ou mais rotas metabólicas específicas. Desta forma, além de induzir acúmulo de substâncias tóxicas e/ou a falta de substâncias essenciais, pode gerar distúrbios no desenvolvimento físico e mental de pacientes acometidos por esses EIM (FENTON, 1995; ILLSINGER; DAS, 2010; SCRIVER, 2001).

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; 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 et al., 2012). Essas doenças apresentam-se clinicamente de maneira variável sendo geralmente de sintomatologia grave ou muitas vezes fatal.

2.2 Acidemias Orgânicas

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; SAHOO et al., 2012; SCRIVER, 2001).

A incidência das acidúrias orgânicas na população em geral ainda é pouco conhecida, entretanto, dados europeus mostram que a hiperamonemia acomete aproximadamente 1:35.000 nascidos (SUMMAR et al., 2013). Um estudo realizado na população pediátrica brasileira mostrou que dos 6.866 pacientes analisados com suspeita de EIM, 218 pacientes apresentaram diagnóstico de acidemias orgânicas (WAJNER et al., 2009).

Tabela 1. Acidemias orgânicas diagnosticadas na população pediátrica brasileira durante o período de janeiro de 1994 a julho de 2008.

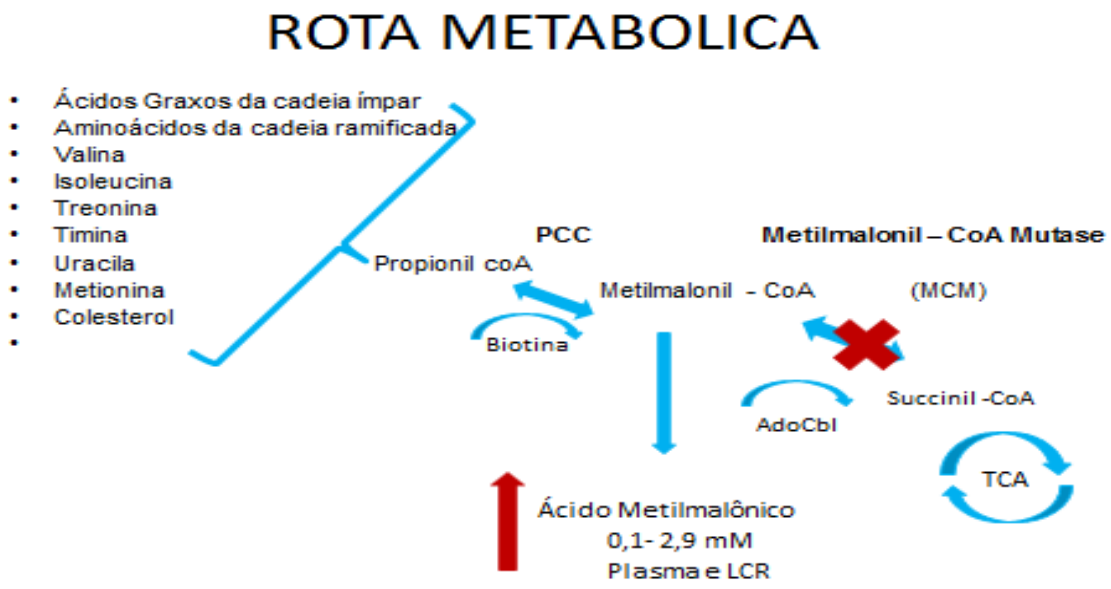
Doenças	Número de pacientes (%)
Número de pacientes analisados	6866
Número de pacientes diagnosticados	218 (3.17%)
Acidemias láticas	57 (26.1%)
Acidemia metilmalônica	34 (15.6%)
Acidemia glutárica tipo I	33 (15.1%)
Acidemia propiônica	18 (8.26%)
Acidúria 3-hidróxi-3-metilglutárica	17 (7.80%)
Acidúria L-2-hidróxi-glutárica	9 (4.13%)
Deficiência de múltiplas carboxilases	9 (4.13%)
Acidemia glutárica tipo II	8 (3.67%)
Acidemia isovalérica	7 (3.21%)
Alcaptonúria	5 (2.29%)
Deficiência de 3-hidróxi-acil-CoA-desidrogenase	5 (2.29%)
Doença de Canavan	4 (1.83%)
Acidúria 3-metilglutacônica	4 (1.83%)
Deficiência de 3-metil-crotonilglicina-CoA	2 (0.92%)
Acidúria D-2-hidróxi-glutárica	1 (0.46%)
Acidúria D-glicérica	1 (0.46%)
Deficiência de glutationa sintetase	1 (0.46%)
Deficiência de 3-cetotiolase	1 (0.46%)
Deficiência de carnitina palmitoil transferase II	1 (0.46%)

Nas últimas décadas, houve um progresso muito grande em diagnosticar recém-nascidos com EIM, como as acidemias orgânicas, evidenciando que essas acidemias são doenças metabólicas frequentes na população (SCRIVER, 1995; WAJNER et al., 2001). Dentre essas acidemias, encontram-se a acidemia isovalérica, acidemias láticas, deficiência da 3-metilcrotonil CoA Carboxilase, deficiência de biotinidase, acidemia 3-metilglutacônica, acidemia 3-hidroxi 3-metilglutárica, acidemia glutárica Tipo I, acidemia propiônica, e acidemia metilmalônica (SAUDUBRAY; CHARPENTIER, 1995; WAJNER et al., 2001).

2.3 Acidemia Metilmalônica

A acidemia metilmalônica é um erro inato do metabolismo autossômico recessivo, caracterizado pelo acúmulo tecidual de MMA e de seus metabólitos como propionato, metilcitrato, β-OH propionato e cetonas da cadeia longas, devido a deficiência da atividade da enzima L-metilmalonil-CoA mutase (MCM) (EC 5.5.99.2) e de seu co-fator, a cianocobalamina (CHANDLER et al., 2009; FENTON, W. A. R., L.E., 1995; ROYES et al., 2007).

Figura 1- A acidemia metilmalônica é caracterizada pela deficiência da enzima metilmalonil coa que converte o metilmalonil coa em sucinil coa o precursor do ciclo de krebs, assim o metilmalonil coa será desviado para rotas alternativas formando ácido metilmalônico e várias biomoleculas são percursoras dessa via ácidos Graxos da cadeia ímpar, Aminoácidos da cadeia ramificada e colesterol.



Fonte: (Criada por Gabbi, 2017)

As incidências na população ocidental variam de 1:48.000 a 1:61.000 nascidos com MMA (BAUMGARTNER et al., 2014), enquanto na Arábia Saudita, onde a taxa de consanguinidade é elevada, é de pelo menos 1 para cada 740 nascimentos (BAUMGARTNER et al., 2014; HOFFMANN, 1994; RAHBEENI et al., 1994). Um estudo realizado na população pediátrica brasileira sobre acidemias orgânicas mostrou que dos 218 pacientes diagnosticados, 34 (15,6%) apresentaram quadro compatível com acidemia metilmalônica (WAJNER et al., 2009), sendo a

incidência desta acidemia de 1:50.000 nascidos vivos (BAUMGARTNER et al., 2014).

As manifestações clínicas desta acidemia 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 da sucção recusa alimentar, vômitos, perda excessiva de peso, distensão abdominal e manifestações predominantemente neurológicas (encefalopatia aguda grave ou crônica), retardo mental, atrofia cerebral, coma, convulsões muitas vezes com um padrão eletroencefalográfico (EEG) de surto-supressão, assim como, déficit cognitivo, hipotonia generalizada e letargia (O'SHEA et al., 2012). Os pacientes que sobrevivem às crises de descompensação metabólica apresentam um grau variável de atraso no desenvolvimento psicomotor e cognitivo. Além das manifestações neurológicas, os pacientes acometidos por esse distúrbio podem apresentar insuficiência renal crônica e hepática (CORNEJO, 2003; DS;FENTON, 2001; LEHNERT et al., 1994; MORATH et al., 2008).

Em relação aos achados patológicos, são observados além da desmielinização, atrofia cortical e dos gânglios da base (DE BAULNY et al., 2005; DEODATO et al., 2006; HARTING et al., 2008; VAN DER MEER et al., 1994). Além disso, após as crises de descompensacão metabólica ou induzida por infecção, são encontrados aumento nos níveis de metabolitos tóxicos nos gânglios da base, principalmente no globo pálido (DEODATO et al., 2006; HARTING et al., 2008). Quando as crises encefalopáticas 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).

Enfatize-se que aproximadamente 70% dos pacientes afetados pela acidemia metilmalônica também apresentam severa hiperamonemia devido ao acúmulo de propionil-CoA, que causa inibição da metabolização da amônia pelo ciclo da ureia (FELIPO; BUTTERWORTH, 2002; O'SHEA et al., 2012). Estudos prévios demonstraram que a hiperamonemia provoca neurotoxicidade em várias situações patológicas (BOSOI; ROSE, 2009; FELIPO; BUTTERWORTH, 2002; WALKER, 2009).

Atualmente o diagnóstico começa a partir da análise de metabólitos excretados na urina ou acumulados no plasma. Esse material é 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 et al., 2006; FOWLER; LEONARD; BAUMGARTNER, 2008).

O quadro laboratorial é caracterizado principalmente por acidose metabólica, hipercetonemia, hiperamonemia, hipoglicemia, anemia, leucopenia, acidose lática, hiperglicinemia, neutropenia e trombocitopenia (IMEN et al., 2012; ZWICKLER 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.

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 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 et al., 2003). No entanto, os recém-nascidos que apresentam acidemia metilmalônica em estágios iniciais apresentam uma concentração variante entre 10 e 20 mmol/mol de creatinina e em estágio mais grave podem atingir até 20.000 mmol/mol (FOWLER et al., 2008), em pacientes com hiperamonemia prolongada ou quando os níveis sanguíneos elevam-se entre 0,2 e 0,5 mM durante os dois primeiros anos de vida, o dano pode ser irreversível (BACHMANN, 2003; ENNS, 2008; UCHINO; ENDO; MATSUDA, 1998).

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

Portanto, o diagnóstico precoce continua sendo crucial para um melhor prognóstico para os pacientes afetados com acidemia metilmalônica e hiperamonemia. Sendo assim, um diagnóstico fetal ou precoce em crianças

assintomáticas, poderia prevenir o desenvolvimento de crises encefalopáticas e as alterações neurológicas nesses pacientes.

2.4 Hiperamonemia na Acidemia Metilmalônica

A amônia é uma substância proveniente do metabolismo dos compostos nitrogenados e degradação das proteínas, tecidos e outros produtos residuais. Dessa forma, a amônia apresenta uma estrutura simples, mas com moléculas biologicamente potentes, que acumulam no organismo em concentrações no sangue de 0,1 a 0,3 mM (MANS; DEJOSEPH; HAWKINS, 1994; ROSE et al., 1998). Em animais com insuficiência hepática aguda, a concentração de amônia no cérebro pode chegar de 1 a 5 mM (SWAIN; BUTTERWORTH; BLEI, 1992), essas concentrações são descritas no estágio de coma (SWAIN et al., 1992) que são extremamente tóxicas levando assim, ao aparecimento de distúrbios funcionais no SNC, podendo conduzir a morte (BOSOI; ROSE, 2009; COOPER; PLUM, 1987).

A hiperamonemia tem efeitos potencialmente prejudiciais para o SNC e principalmente para o cérebro em desenvolvimento (TUCHMAN et al., 2008), o qual é mais suscetível a essa condição (FELIPO; BUTTERWORTH, 2002). Dependendo do tempo e duração da exposição, os efeitos podem incluir desde convulsões, déficits cognitivos, coma, e no caso do cérebro em desenvolvimento uma atrofia cortical, dilatação ventricular, desmielinização, retardo mental e paralisia cerebral (BRAISSANT; MCLIN; CUDALBU, 2013) .

A amônia encontrada no SNC pode se originar tanto de rotas enzimáticas cerebrais, como da atividade das enzimas glutamato desidrogenase (GAD) e da glutamina sintase (GS) quando proveniente da circulação, principal fonte em situações de hiperamonemia (FELIPO; BUTTERWORTH, 2002). O transporte da amônia através da barreira hematoencefálica se deve à combinação da difusão passiva de NH_3 e transporte de NH_4^+ por canais de cátions e pela aquaporina (OTT; CLEMMESEN; LARSEN, 2005; SAPAROV et al., 2007). No SNC, o metabolismo da amônia é realizado pela enzima GS, através da conversão do glutamato a glutamina com a incorporação de NH_4^+. Essa enzima é encontrada apenas nos astrócitos e, em situações de hiperamonemia, a GS pode não realizar a detoxificação completa da amônia, que pode se acumular nos astrócitos, levando

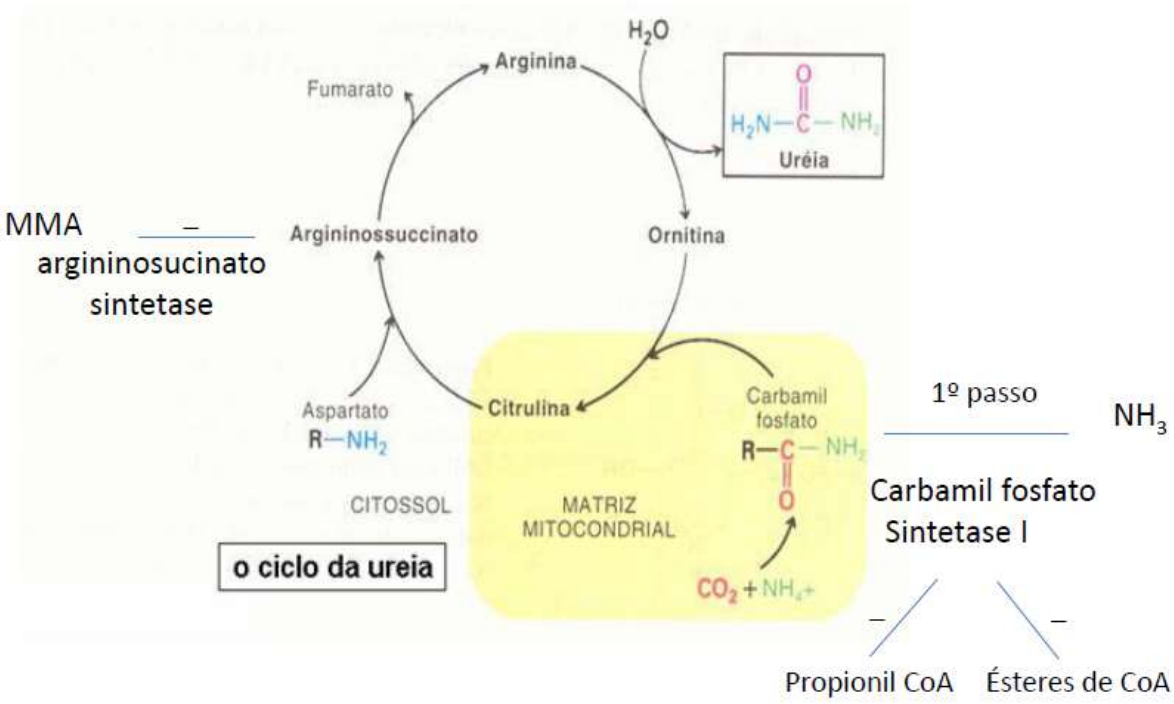
assim, a redução do pH intracelular e alterações na homeostase iônica, e com consequente acúmulo intracelular de água, levando ao inchaço astroglial (MARTINEZ-HERNANDEZ; BELL; NORENBERG, 1977; NORENBERG; RAMA RAO; JAYAKUMAR, 2009). De fato, autores tem mostrado o envolvimento da hiperamonemia nas alterações neurológicas de algumas doenças do SNC, como na acidemia propiônica e metilmalônica (TUCHMAN et al., 2008).

Em crianças, a hiperamonemia pode ser causada por desordens inerentes ou adquirida sendo que a causa mais frequente de hiperamonemia em crianças está relacionada com desordens do ciclo da uréia (UCDs) (CAGNON; BRAISSANT, 2007), a qual tem uma prevalência total estimada de 1:8.200 nos Estados Unidos, (GROPMAN; BATSHAW, 2004). A hiperamonemia também é uma das principais alterações laboratoriais encontradas na acidemia metilmalônica, que ocorre devido ao aumento no *pool* de ésteres de acil-CoA mitocondrial. Estes ésteres apresentam um efeito inibitório sobre a N-acetilglutamato sintetase, reduzindo a conversão de amônia para ureia em 70% dos pacientes com esta acidemia (FENTON, W. A.; ROSENBERG, 1995; MORATH et al., 2008) (figura 2).

Além disso, o propionil CoA, um metabólito que acumula na acidemia, inibe competitivamente a síntese de N acetilglutamato a partir de acetil CoA (COUDE; SWEETMAN; NYHAN, 1979a; GLASGOW; CHASE, 1976). O N-acetilglutamato é um ativador alostérico da carbamil fosfato sintetase, a enzima responsável pelo primeiro passo do ciclo da uréia em crianças (CAGNON; BRAISSANT, 2007; FELIPO et al., 2012). Este bloqueio na síntese da uréia resulta em um acúmulo de amônia, justificando assim a hiperamonemia nos pacientes acometidos por esta patologia. Além desse bloqueio, a própria inibição da carbamil fosfato sintetase I por ésteres de CoA pode causar hiperamonemia .

A hiperamonemia é uma das principais causas da neurotoxicidade na acidemia metilmalônica. Aproximadamente 90% desses pacientes apresentam insuficiência hepática (SMELTZER, BG BARE ; SC, 1996; STEWART; WALSER, 1980; WALKER, 2014) e sintomas como euforia, diarréia, vômitos e acidose metabólica. Além dessas alterações inespecíficas, os pacientes também apresentam um quadro clínico neurológico com perda de consciência, letargia, desorientação, sonolência, dificuldades motoras, convulsões e coma (BAJAJ et al., 2011).

Figura 2 – Efeito do propionil CoA, dos ésteres de CoA e do MMA sobre a síntese da uréia. Na acidemia metilmalônica a hiperamonemia está associada a uma redução no ciclo da uréia. O propionil CoA inibe competitivamente a síntese de N-acetilglutamato, um ativador alostérico da carbamil fosfato sintetase I, comprometendo este passo do ciclo. Os ésteres de CoA inibem diretamente a carbamil fosfato sintetase I. O MMA inibe a argininosucinato sintetase (STEWART; WALSER, 1980)



2.7 Mecanismos de Neurotoxicidade da Amônia

A hiperamonemia é um sintoma de descompensação metabólica que pode resultar em neurotoxicidade crônica. Uma das principais complicações graves da hiperamonemia é a insuficiência hepática aguda que leva ao edema cerebral (CAULI et al., 2009). No entanto, o aumento significativo de glutamina no SNC também tem sido postulado como sendo uma causa do edema cerebral (BLEI et al., 1994; TAKAHASHI et al., 1991). A glutamina acumulando-se pode atuar como um osmólito causando aumento na pressão osmótica nos astrócitos que resulta em inchaço mitocondrial (BRUSILOW et al., 2010; ZIELINSKA; POPEK; ALBRECHT, 2014). Estudos com síndromes hiperamonêmicas estão relacionados

diretamente com alteração astrocíticas consistindo de inchaço celular no caso da hiperamonemia aguda. Além disso, quando o cérebro é exposto a altas concentrações de amônia, pode apresentar a expressão alterada de várias proteínas astrocitárias, como a proteína glial fibrilar ácida (GFAP) (FELIPO; BUTTERWORTH, 2002).

Segundo (ALBRECHT; NORENBERG, 2006) a glutamina e a amônia apresentam um papel importante no desenvolvimento do inchaço dos astrócitos e edema cerebral. A glutamina sintetizada em excesso nos astrócitos é transportada para a mitocôndria onde é metabolizada pela glutaminase ativada por fosfato. O acúmulo de amônia na mitocôndria carregado pela glutamina vai levar ao estresse oxidativo e consequentemente ao inchaço dos astrócitos (SCOTT et al., 2013).

Estudos têm demostrado que os efeitos tóxicos da hiperamonemia, estão relacionados com o inchaço dos astrócitos e com a sinalização glutamatérgica através do receptor de NMDA. A ativação desses receptores pela amônia pode resultar em um influxo de Ca^{2+} e a consequente ativação de diversas enzimas, como a óxido nítrico sintase (NOS) e estresse oxidativo (NORENBERG; RAO; JAYAKUMAR, 2005; SCOTT et al., 2013; SKOWRONSKA; ALBRECHT, 2013). Além disso, já foi demonstrado que a amônia provoca um aumento da concentração extracelular de glutamato, principalmente em decorrência de uma diminuição na sua captação pelas células astrogliais (OHARA et al., 2009; ROSE, 2006).

Sendo assim, tem se sugerido que altas doses de amônia também podem levar à depleção de adenosina trifosfato (ATP) via receptores NMDA (KOSENKO et al., 1994). A hiperamonemia altera também a transdução de sinais associados aos receptores glutamatérgicos ionotrópicos tipo NMDA no cerebelo (ELMLILI et al., 2010) e metabotrópicos (mGluRs) na substância nigra (CAULI et al., 2006).

Em modelos animais de acidemia metilmalônica, tem sido sugerido que a estimulação excessiva do receptor do glutamato, em particular, o receptor de NMDA, induz uma inibição da glutamato descarboxilase (GAD), enzima envolvida na síntese do neurotransmissor GABA (MALFATTI et al., 2007). Desta forma, a inibição da glutamato descarboxilase (GAD), a principal via de síntese de GABA

no SNC, leva a depleção de GABA e redução do tônus GABAérgico e aparecimento de convulsões (SALAZAR et al., 1994). Além disso, estudos anteriores mostraram que receptores glutamatérgicos do tipo NMDA estão envolvidos nas convulsões e no dano oxidativo induzido pelo MMA (DE MELLO, C. F. et al., 1996; ROYES et al., 2007).

Estudos mais recentes descrevem que a causa exata dessas alterações não são completamente compreendidas, tem sido sugerido que as mudanças na glutamina e a expressão da GS no SNC podem refletir alterações astrogliais em situações hiperamonêmicas e os modelos de acidemia metilmalônica são caracterizados por respostas inflamatórias, barreira hemato encefálica e a concentração de glutamato extracelular (EID et al., 2013; SKOWRONSKA; ALBRECHT, 2013).

Além disso, estudos monstram que o processo inflamatório está associado a neurotoxidade da amônia, podendo induzir a liberação de citocinas pró-inflamatórias como TNFα, IL-1β e IL-6 por células astrogliais (BEMEUR; BUTTERWORTH, 2013; BOBERMIN et al., 2012; BUTTERWORTH, 2013). De fato, Ribeiro e colaboradores (2014) mostraram um aumento dos níveis de citocinas inflamatórias no cérebro e no sangue de animais tratados com MMA.

Uma vez que o estresse oxidativo e a resposta inflamatória estão intimamente relacionados, eles podem agir sinergicamente agravando doenças neurodegenerativas e neurometabólicas, incluindo a acidemia metilmalônica (FIGHERA et al., 2003; FIGHERA et al., 1999; RIBEIRO et al., 2013a; ROYES et al., 2003; SALVADORI et al., 2012). Apesar dos mecanismos subjacentes ao dano cerebral nesta acidemia não estarem bem estabelecidos, crescentes evidências sugerem que a excitotoxicidade (BRUSQUE, A. M. et al., 2001; MALFATTI et al., 2007; OKUN et al., 2002), inflamação (RIBEIRO et al., 2013a; SALVADORI et al., 2012), geração de radicais livres (FIGHERA et al., 2003; FIGHERA et al., 1999), e alterações morfológicas e funcionais dos astrócitos (SAUR et al., 2014) desempenham um papel central na neuropatogênese desta acidemia.

3. BIOMARCADORES NA ACIDEMIA METILMALÔNICA

3.1 Estresse oxidativo

O radical livre (RL) é definido como um átomo ou molécula que contém um ou mais elétrons desemparelhados (HALLIWELL, 1989; SOUTHORN; POWIS, 1988). Entretanto o organismo humano está exposto também a vários radicais livres e "espécies reativas derivadas do oxigênio" (EROs) geradas por radiações ionizantes, agentes tóxicos, poluentes ambientais, nos processos patológicos, entre outros.

Além disso, os neurônios cerebrais são também capazes de produzir EROs como ânion superóxido (O_2) e o radical OH, mas também espécies não radicalizadas potencialmente oxidante com o H_2O_2, o ozônio (O_3), o acido hiplocoroso (HOCL), entre outros. Além dessas, existem as espécies reativas de nitrogênio (ERN) tais como: o peróxido de nitrogênio e os radicais superóxido, hidroxila e óxido nítrico (HALLIWELL, 2006;2012). Visto que participam de reações essenciais para o organismo, as EROs são constantemente formadas e podem ser prejudiciais, quando sua produção ultrapassa o controle causando danos às membranas celulares, proteínas e DNA (HALLIWELL, 2012).

As lesões do DNA provocadas por essas espécies reativas, quando não reparadas eficientemente pelos mecanismos de defesa celulares, ela podem levar à morte celular por apoptose ou necrose, erros de transcrição e replicação, indução de vias de sinalização celular e entre outros, além disso, os estágios de iniciação, promoção e/ou progressão tumoral, bem como envelhecimento e neurodegeneração (KLAUNIG et al., 2011).

Alguns estudos *in vitro* e *in vivo* têm sugerido que o estresse oxidativo pode contribuir para o desenvolvimento dos achados neurológicos observado em alguns erros inatos do metabolismo, como, por exemplo, nas acidemias glutárica (LATINI et al., 2005), propiônica (FONTELLA et al., 2000), metilmalônica (FONTELLA et al., 2000; RICHARD et al., 2007), isovalérica (SOLANO et al., 2008) e nas aminoacidopatias homocistinúria (MATTE et al., 2009).

Entretanto, estudo tem demonstrado que ocorre uma estimulação de peroxidação lipídica (LPO) e a oxidação protéica em animais tratados com ácido metilmalônico (FIGHERA et al., 2003; FONTELLA et al., 2000). A peroxidação lipídica é um processo fisiológico e contínuo 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; GUTTERIDGE, 1995). Outros marcadores de dano oxidativo não-enzimático estão

envolvidos como: oxidação de 2,7-diacetato de diclorofluoresceína (DCFH), citocinas pró-inflamatórias e inibição da atividade da enzima Na^+,K^+-ATPase (KREYDIYYEH; ABOU-CHAHINE; HILAL-DANDAN, 2004; KREYDIYYEH; AL-SADI, 2004; LEBEL; ISCHIROPOULOS; BONDY, 1992; LU, L. et al., 2009).

Embora o mecanismo responsável pelo estresse oxidativo nos erros inatos do metabolismo não seja totalmente compreendido, ainda é provável que o acúmulo de metabólitos tóxicos que ocorre nessas doenças induza a formação excessiva de espécies reativas. Além disso, o efeito inibitório de alguns metabólitos sobre os complexos da cadeia respiratória e algumas enzimas do metabolismo energético pode levar a uma maior produção dessas espécies (Wajner *et al*., 2004; Indo *et al*., 2007).

De fato, estudos sugerem o envolvimento das espécies reativas também nas convulsões induzidas pelo MMA, na medida em que as convulsões e o dano oxidativo induzidos pela administração intra-estriatal deste ácido orgânico foram atenuados pela administração de antioxidantes, como o gangliosídeo GM1, vitamina C e o α-tocoferol (FIGHERA et al., 2003; FIGHERA et al., 1999; ROYES et al., 2016b).

Também foi observado um aumento na atividade da MnSOD e uma diminuição de GSH no fígado de camundongos nocaute para o gene *mut* (CHANDLER et al., 2009). Entretanto, em camundongos nocaute para a enzima óxido nítrico sintase induzível foi observado uma redução do estresse oxidativo e das convulsões induzidos pelo MMA, sugerindo assim um envolvimento de espécies reativas do oxigênio e do nitrogênio nos efeitos causados por esse metabólito (RIBEIRO et al., 2009).

2.2 Inflamação e Apoptose

A inflamação ou processo inflamatório compreende um conjunto de cascatas celulares e moleculares ao organismo a uma infecção ou lesão dos tecidos. A resposta inflamatória excessiva pode tornar-se fonte de uma lesão tecidual ainda maior do que a provocada pelo estímulo inicial. Apesar de ser necessária, a inflamação apresenta efeitos colaterais que acabam por danificar células vizinhas saudáveis, exacerbando os danos iniciais, com isso apresentam uma fraca

capacidade de recuperação frente a lesões, tornando-se, portanto extremamente vulneráveis a processos autodestrutivos, tais como os processos imune e inflamatório (GALEA; BROUGH, 2013; GAO et al., 2003).

A neuroinflamação é um mecanismo conhecido na patologia das doenças neurológicas, o que pode contribuir para o dano tecidual, perda de neurônios e disfunção, ou para neuroregeneração e reparo tecidual (CUNNINGHAM, 2013; ROSALES-CORRAL et al., 2010). Este mecanismo é acionado quando ocorre um estimulo ou uma combinação complexa de respostas agudas e crônicas das células do SNC, incluindo neurônios, células da glia (micróglia e astrócitos) e leucócitos infiltrantes, citocinas pro-inflamatórias como IL -1β, IL-6 e o TNF-α e as quimiocinas, óxido nítrico e prostanóides, ROS, eicosanóides e aminoácidos excitatórios (CACQUEVEL et al., 2004; GALEA; BROUGH, 2013; GAO et al., 2002; HEIN; O'BANION, 2009; SKELDON; FARAJ; SALEH, 2014).

Os astrócitos representam o principal elemento celular do sistema homeostático no SNC, sendo responsáveis pela maior parte dos aspectos do suporte metabólico, nutrição, regulação da concentração de íons e neurotransmissores no ambiente, manutenção da barreira hemato encefálica e preservação da integridade dos tecidos após lesão (PARK et al., 2003; PETTY; LO, 2002; THEODOSIS; POULAIN; OLIET, 2008). AS células gliais (astrócitos no SNC e células de Schwann no SNP) controlam o nascimento, a vida e a morte de sinapses, sendo essenciais para processos como aprendizagem e memória (VERKHRATSKY; KIRCHHOFF, 2007).

Um dos acontecimentos centrais da neuroinflamação é a ativação da microglia (LOANE; BYRNES, 2010). As células da microglia expressam um conjunto de receptores que a permitem identificar e reagir a estímulos nocivos e eventos danosos (BLOCK; ZECCA; HONG, 2007). Dessa forma, essas células reagem a estímulos mudando da sua forma quiescente para forma ativa fagocitária e migratória (DAVALOS et al., 2005). Os astrócitos são responsáveis pela homeostase do cérebro, podendo observar profundas alterações nas respostas inflamatórias, bem como nos mediadores pró-inflamatórios (BELANGER; ALLAMAN; MAGISTRETTI, 2011; GAVILLET; ALLAMAN; MAGISTRETTI, 2008; MYER et al., 2006).

Uma vez ativada, a microglia passa então a produzir citocinas e EROs, entre outros fatores inflamatórios (HEIN; O'BANION, 2009; LU, J. et al., 2009), o aumento nos níveis de EROS leva à oxidação de lipídios, proteínas e ácidos nucléicos, aumentando o colapso do potencial de membrana (GREEN; KROEMER, 2004). A resposta da mitocôndria ao dano oxidativo é uma via importante no início da apoptose. Além disso, é sabido que as EROS induzem a ativação das caspases 9 e 3 (GOTTLIEB; VANDER HEIDEN; THOMPSON, 2000; RICCI; GOTTLIEB; GREEN, 2003).

Sendo assim, as citocinas e as caspases constituem o maior grupo de mediadores inflamatórios e de morte celular (CEDERBERG; SIESJO, 2010). As citocinas pró-inflamatórias são as principais responsáveis por iniciar a neuroinflamação, dentre elas estão a interleucina-1alfa (IL-1α), interleucina-1beta (IL-1β) e fator de necrose tumoral-alfa (TNF-α) (CEDERBERG; SIESJO, 2010). Além disso, há evidências de uma maior expressão de TNF-α e IL-1β em animais tratados com MMA, sugerindo assim a presença do processo inflamatório em doenças metabólicas (GOYENECHEA et al., 2012).

Normalmente, as caspases estão no seu estado inativo e necessitam de uma modificação bioquímica para se tornarem ativas e serem capazes de desempenhar as suas funções de regulação (MCILWAIN; BERGER; MAK, 2013).Dependendo os tipos de processos celulares em que estão envolvidas, as caspases são classificadas em apoptóticas (caspases-2, -3, -6, -7, -8, -9 e -10) e inflamatórias (caspases -1, -4, -5, -11, -12 e -13). Por sua vez, as caspases que estão envolvidas no processo apoptótico podem dividir-se em dois grupos: caspases iniciadoras (-2, -8, -9 e -10) e caspases executoras (-3, -6 e -7) (JIMENEZ FERNANDEZ; LAMKANFI, 2015; LAVRIK; GOLKS; KRAMMER, 2005).

Entretanto, na via extrínseca apoptótica, a caspase iniciadora -8 é ativada por dimerização, iniciando o processo de apoptose através da clivagem das caspases executoras -3, -6 e -7 ou através da ativação da via intrínseca apoptótica por outros mecanismos. Na via intrínseca, a caspase iniciadora -9 sofre dimerização, ficando assim ativa para, de forma semelhante à caspase-8, clivar as caspases executoras -3, -6 e -7 (LAVRIK et al., 2005; MCILWAIN et al., 2013)

Pacientes com acidemia metilmalônica e hiperamonemia, principalmente da variante cblB, podem apresentar neutropenia. Dessa forma, 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 e apoptose (CHURCH et al., 1984; GUERRA-MORENO; BARRIOS; SANTIAGO-BORRERO, 2003; JAFARI et al., 2013; MANOLI; VENDITTI, 1993; SEMMLER et al., 2008).

4. ACIDEMIA METILMALÔNICA E CRISES CONVULSIVAS

Uma convulsão pode ser definida como uma manifestação clínica de excessiva e/ou hipersincronia de descargas elétricas, normalmente auto-limitante (ACHARYA; HATTIANGADY; SHETTY, 2008; SCHARFMAN, 2007) podendo ser um evento não específico, súbito, paroxístico, transitório com contrações musculares anormais e excessivas, uni ou bilaterais, que podem ser sustentadas ou descontinuadas (FISHER et al., 2005)**.** A convulsão pode ter uma etiologia neurológica ou não neurológica (GRUENENFELDER, 2008). A descarga convulsiva inicial pode começar numa área focal simples ou podem envolver, sincronicamente, ambos os hemisférios, desde o início (ENGEL, 2001).

A epilepsia é uma doença cerebral crônica que é caracterizada por crises epilépticas recorrentes, e involuntárias, com ou sem perda de consciência, sendo a doença neurológica crônica com uma estimativa na população de 1%, podendo vir a desenvolver algum tipo de epilepsia na vida (LEONARDI; USTUN, 2002).

Entretanto, os EIMs não estão associados frequentemente a epilepsia, pois as convulsões são alterações mais frequentemente desencadeadas durante os episódios de descompensação aguda destas patologias. Dessa forma, se a acidemia metilmalônica for tratada adequadamente as crises convulsivas tornam-se raras, mas podem deixar sequelas cerebrais permanentes se não forem adequadamente controladas (WOLF, N. I.; BAST; SURTEES, 2005).

De fato um estudo realizado com pacientes que, além da acidemia, apresentavam epilepsia, foram observadas convulsões do tipo parcial, tônico-clônica generalizada, tônica, mioclônica, e espasmos epilépticos. Além disso, foram observadas alterações no EEG interictal lentificação da atividade das ondas,

descargas epileptiformes focais ou multifocais, generalizadas, hipsarritmia e um padrão atípico de surto-supressão (MA et al., 2011; PARINI et al., 2013). Achados clínicos mostram que pacientes no período neonatal, também apresentaram convulsões, além de perda de tônus cefálico, distonia do rosto, língua, pescoço, braços e mãos (LIU, Y. et al., 2016; LIU, Y. P. et al., 2012; PARINI et al., 2013).

Em estudos experimentais foram observados que administração de amônia e MMA intraestriatal em ratos adultos apresentaram uma exacerbação do comportamento convulsivo, sugerindo que as convulsões induzidas pelo MMA pode ser uma consequência de excitotoxicidade causada por metabólitos como a amônia (MALFATTI et al., 2007; MALFATTI et al., 2003; MARISCO PDA et al., 2003).

5. Acidemia Metilmalônica e Memória

A Memória pode ser considerada um processo pelo qual há a aquisição, formação, manutenção e recordação de informações, para que posteriormente, quando apropriado, conseguimos evocar a informação aprendida (IZQUIERDO, L. A. et al., 2002). Entre as várias classificações didáticas das memórias, que podem ser relacionadas ao conteúdo (declarativa ou não-declarativa), função (memória de referência ou de trabalho) natureza (associativas ou não) ou motivação (recompensa ou aversiva), a classificação temporal é a que relaciona o período de tempo em que tais memórias encontram-se disponíveis, dividindo-as em memórias de curta ou longa duração (MCD ou MLD) (LEES; JONES; KANDEL, 2000; MCGAUGH; IZQUIERDO, 2000; RANGANATH, 2006).

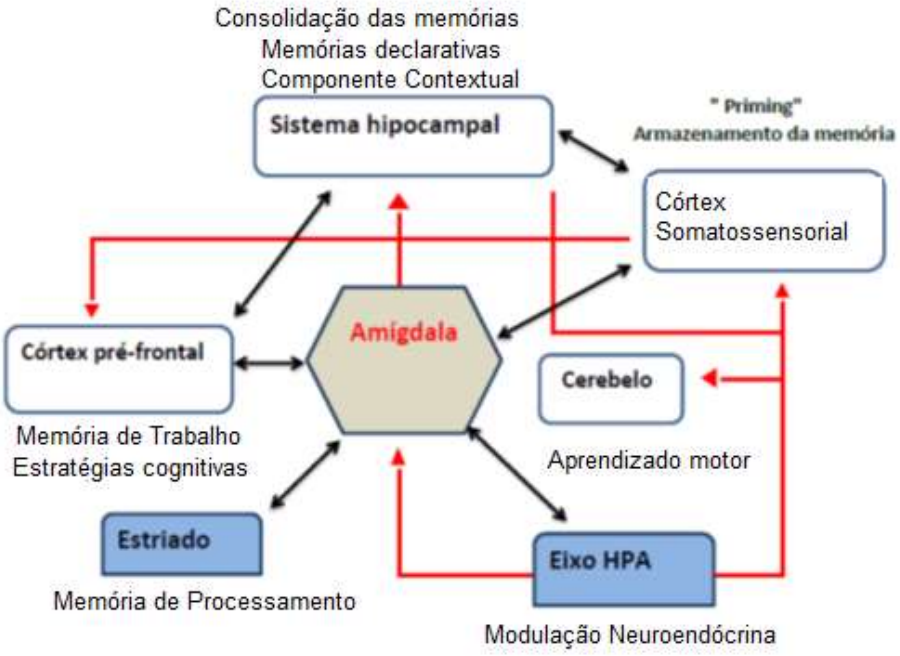
As memórias podem ser classificadas conforme o tempo e duração das mesmas. A memória de curta duração serve para manter uma conversa ou processar uma leitura ou outra atividade que durem alguns minutos ou poucas horas, apresentando como função manter a informação até que esta se consolida. A memória de trabalho muitas vezes é classificada dentro da memória de curta duração, pois ambas são processadas através da ativação sináptica dos neurônios do córtex pré-frontal anterolateral, orbito- frontal (IZQUIERDO, L. A. et al., 2002) e do hipocampo e córtex entorrenal (IZQUIERDO et al., 1998).

A MLD apresenta como propriedade a manutenção da informação estável, utilizando a síntese proteica ou modificação na expressão gênica, perdurando assim,

esta memória por horas, dias ou até anos. Essa memoria compartilha as mesmas estruturas neurais, pois possuem basicamente o mesmo conteúdo de informação que a memória de curta duração, possui mecanismos bioquímicos completamente distintos e ocorre de forma independente e paralela (IZQUIERDO et al., 1998; IZQUIERDO et al., 1999; IZQUIERDO, I. et al., 2002).

Quanto a sua natureza, as memórias podem ser classificadas como explícita ou declarativa. Estas estão relacionadas a fatos ou acontecimentos de nossa vida, fatos históricos marcantes, números de telefones, nomes de pessoas, entre outros, e esta armazenada no lobo temporal e diencéfalo, córtex entorrinal, parahipocampal e pirrinal e vários subnúcleos do complexo da amigdala. A memória implícita ou procedimental se refere á lembrança de procedimentos que está relacionada as habilidades, como dirigir, jogar bola, amarrar o cadarço e aprendizado motor, encontrada no cerebelo e gânglios da base (IZQUIERDO, L. A. et al., 2002; RANGANATH; BLUMENFELD, 2005; SQUIRE; WIXTED; CLARK, 2007).

Figura-3 Esquema da participação diferencial de algumas estruturas cerebrais relacionadas ao processamento de memórias emocionais. Além da participação intrínseca da amígdala, projeções com outros sistemas cerebrais que sustentam diversos componentes cognitivos constituiriam o sistema múltiplo de processamento das memórias. Setas contínuas e conexões diretas ou indiretas, respectivamente. Regiões subcorticais indicadas em azul, regiões corticais indicadas em branco. Eixo HPA: hipotálamo-pituitária-adrenal.



Fonte: (criada por Gabbi, 2017)

O processo de formação de memória no SNC envolve eventos moleculares em diferentes estruturas e lugares (IZQUIERDO, L. A. et al., 2002). Dessa forma, estudos envolvendo pacientes com níveis aumentados de MMA, homocisteína no soro sanguíneo e hiperamonemia, assim como deficiência de vitamina B12, comprovam a existência de uma associação entre o aumento desses marcadores séricos e déficits cognitivos, aprendizagem e memória (JOHANSSON et al., 2015; LEWIS et al., 2005; MCCRACKEN et al., 2006; O'SHEA et al., 2012; TANGNEY et al., 2011).

Estudos comportamentais tem demostrado que hiperamonemia, inflamação e MMA induzem alterações neurológicas como disfunção cognitiva, déficit permanente de aprendizagem e desenvolvimento neuromotor (DUTRA et al., 1991; FELIPO et al., 2012; JOHANSSON et al., 2015). Entretanto, estudos comportamentais, no labirinto aquático de morris e aprendizagem espacial no labirinto radial, mostram um déficit de aquisição de um novo paradigma de localização espacial e de coordenação motora nos animais testados (JOHANSSON et al., 2015; PETTENUZZO; SCHUCK; et al., 2003).

No entanto, pacientes que apresentam algum tipo de EIM, dentre eles a acidemia metilmalônica, apresentam sinais e sintomas neurológicos, muitas vezes relacionados também a neuroinflamação. Este processo inflamatório pode contribuir para o dano tecidual, lesão de neurônios e disfunção motora, aprendizagem, comportamento e memória (ANGLADE; LARABI-GODINOT, 2010; RIVEST, 2009).

Essas alterações levam ao surgimento de respostas agudas e crônicas das células do SNC, incluindo neurônios, células da glia (micróglia e astrócitos) e leucócitos infiltrantes. Isto desencadeia um aumento de proteínas moduladoras do sistema imunológico presentes na superfície das células, e também o aumento na síntese e liberação de mediadores pró-inflamatórios, incluindo citocinas, quimiocinas, óxido nítrico e prostanóides (HEIN; O'BANION, 2009). Dessa forma, estudos mostraram que a administração crônica de MMA induziu déficit de memória e aumento de citocinas inflamatórias no córtex de camundongos (RIBEIRO et al., 2013b).

Nese 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 e hiperamonemia (JAFARI et al., 2013; PETTENUZZO; WYSE; et al., 2003; RIBEIRO et al., 2013b).

Entretanto, o papel da hiperamonemia na disfunção neurológica apresentada pelos pacientes afetados pela acidemia metilmalônica não foi esclarecido, podendo representar um fator fisiopatológico importante na degeneração do sistema nervoso central, quando associada aos níveis elevados dos metabólitos acumulados.

Devido á escassez de evidências clínicas de que a hiperamonemia facilita as crises metabólicas e a disfunção neurológica em pacientes com acidemia metilmalônica, esse estudo tem por objetivo investigar o efeito da administração de MMA e/ou amônia nas convulsões, no teste de memória espacial e ansiedade, assim como, nos parâmetros de estresse oxidativo, inflamatórios e apoptóticos. Além disso, é de interesse dete trabalho avaliar se haveriam alterações gliais e neuronais no cérebro dos animais após a administração de MMA e/ou amônia.

6. HIPÓTESE

Apesar de já existirem estudos evidenciando que a amônia está relacionada com as manifestações neurológicas observadas nos pacientes com acidemia metilmalônica, ainda não foi especificado o papel da hiperamonemia nas convulsões e no déficit cognitivo destes pacientes. Além disso, não há evidências de que o efeito agudo de MMA e amônia no SNC causem alterações na memória, assim como, nos marcadores de dano oxidativo, inflamatórios e de dano celular. Dessa forma, torna-se importante investigar o se esses fatores tem um papel nas alterações neurológicas observadas no modelo animal com acidemia metilmalônica.

7. Objetivos gerais e organização dos trabalhos da qualificação de tese

Este estudo tem por objetivo, primeiramente, investigar e caracterizar as alterações eletroencefalográficas e bioquímicas após a administração de MMA e amônia. E em seguida, verificar se a administração desses compostos poderiam causar atraso de aprendizado, assim como, alterações inflamatórias, apoptóticas e imunohistoquímicas em um período precoce do desenvolvimento cerebral.

7.1. MANUSCRITO I

7.2 Os objetivos específicos deste trabalho foram:

Analisar o efeito da administração de MMA intracerebroventricular e NH_4Cl intraperitoneal em camundongos em relação ao:

1. Aparecimento de crises convulsivas (comportamentais e eletroencefalográficas) com doses intermediárias de MMA e NH_4Cl.;
2. Avaliação dos marcadores de dano oxidativo 2,7-diacetato dichlorodihidrofluoresceina (DCFH), níveis de nitrito e nitrato (NOx) no córtex cerebral;
3. Determinação do $\Delta\Psi$ mitocondrial e redução do metiltetrazol (MTT) no córtex cerebral;
4. Avaliação da atividade da succinato desidrogenase, Na^+,K^+-ATPase, glutamato descarboxilase e glutamina sintetase no córtex cerebral;
5. Análise dos níveis de citocinas (IL-Iβ e TNF-α), ligação [3H] flunitrazepam
6. Avaliação da liberação de neurotransmissores como GABA e glutamato;
7. Análise da permeabilidade da barreira hemato encefálica;
8. Avaliação da concentração da amônia no córtex cerebral e soro;
9. Análise da alteração do volume celular do tecido cerebral.

6.3. MANUSCRITO II

Sabendo que administração de MMA e amônia intensificou aparecimento de alterações comportamentais, o objetivo do estudo do artigo II é analisar o efeito da administração única de MMA no período neonatal (P1), na presença ou não de amônia, em camundongos com 21 dias e 40 dias de vida em relação:

1. Ao peso corporal dos filhotes ao longo do tempo;
2. A atividade locomotora e exploratória dos animais;
3. Ao teste de memória espacial e de trabalho por meio dos testes de ansiedade (teste de cruz elevado) e radial;
4. Os níveis de citocinas (IL- 1β e TNF-α) no hipocampo, córtex e estriado dos animais;
5. Avaliação dos marcadores de dano oxidativo 2,7- diacetato dichlorodihidrofluoresceina (DCFH), no córtex cerebral, hipocampo e estriado dos animais ;
6. A morte neuronal e glial analisadas através de técnicas de imunohistoquímica e western blont.

MANUSCRITO I

Amino Acids
DOI 10.1007/s00726-015-2164-1

ORIGINAL ARTICLE

A neuronal disruption in redox homeostasis elicited by ammonia alters the glycine/glutamate (GABA) cycle and contributes to MMA-induced excitability

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Received: 1 September 2015 / Accepted: 24 December 2015

A neuronal disruption in redox homeostasis elicited by ammonia alters the glycine/glutamate (GABA) cycle and contributes to MMA-induced excitability

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Abstract

Hyperammonemia is a common finding in children with methylmalonic acidemia. However, its contribution to MMA-induced excitotoxicty is poorly understood. The aim of this study was to evaluate the mechanisms by which ammonia influences in the neurotoxicity induced by methylmalonate in mice. The effects of ammonium chloride (NH_4Cl 3, 6, and 12 mmol/kg; s.c.) on electroencephalographic (EEG) and behavioral convulsions induced by MMA (0.3, 0.66, and 1 µmol/2 µL, i.c.v.) were obtained from freely moving mice. Along with EEG recordings, cerebral ammonia levels, TNF-α, IL1β, nitrite/nitrate (NOx) levels, mitochondrial potential (ΔΨ), reactive oxygen species (ROS) generation, Methyl-Tetrazolium (MTT) reduction, succinate dehydrogenase (SDH), and Na^+, K^+-ATPase activity levels were measured in the cerebral cortex. The GABAergic pathway (binding of [^{3}H]flunitrazepam, release of glutamate- GABA; glutamate decarboxylase (GAD) and glutamine synthetase (GS) activity) and neuronal damage (opening of blood brain barrier (BBB) permeability and cellular death volume) were also measured. EEG recordings showed that an intermediate dose of NH_4Cl (6 mmol/kg) increased the duration of convulsive episodes induced by MMA (0.66 µmol/2 µL i.c.v). NH_4Cl (6 mmol/kg) administration also induced neuronal ammonia and NOx increase, as well as mitochondrial ROS generation throughout oxidation of 2,7-dichlorofluorescein diacetate (DCFH-DA) to DCF-RS, followed by GS and GAD inhibition. The NH_4Cl plus MMA administration did not alter cytokine levels, plasma fluorescein extravasation, or neuronal damage. However, it potentiated DCF-RS levels, decreased the ΔΨ potential, reduced MTT, inhibited SDH activity, and increased Na^+,K^+-ATPase activity. NH_4Cl also altered the GABA cycle characterized by GS and GAD activity inhibition, [^{3}H] flunitrazepam binding, and GABA release after MMA injection. On the basis of our findings, the changes in ROS/RNS levels elicited by ammonia alter the glycine/glutamate (GABA) cycle and contribute to MMA-induced excitability.

Keywords: methylmalonate, ammonia, mice, seizures, GABA

1. Introduction

Hereditary methylmalonic acidemia is an inborn, autosomal recessive, genetic disorder characterized by either deficient activity of the mitochondrial enzyme methylmalonil-CoA mutase (MCM, EC 5.4.99.2) or defects in the synthesis of 5'-deoxyadenosylcobalamin, the cofactor of MCM. Methylmalonic acidemia is biochemically characterized by a primary accumulation of methylmalonate (MMA) from L-methylmalonyl-CoA and a secondary accumulation of other metabolites, such as propionate, 3-hydroxypropionate, and 2-methylcitrate (2-MCA) in tissues and bodily fluids (FENTON, W. A.; ROSENBERG, 1995).

Propionic and methylmalonic acidemia are known to be associated with hyperammonemia in approximately 70% of patients (Stewart and Walser, 1980). These patients have an enlarged mitochondrial pool of acyl CoA esters, which leads to an inhibition of the urea circle via decreased synthesis of N-acetylglutamate, an essential activator of carbamylphosphate synthetase (Gebhardt et al 2003). In these metabolic diseases, the hyperammonemia is a symptom of metabolic decompensation that may result in chronic neurotoxicity and an impaired neurological outcome. A critical enzyme for metabolizing ammonia in the Central Nervous System (CNS) is Glutamine Synthase (GS, E.C. 6.3.1.2). Considering that ammonia is initially metabolized to glutamine (Gln), it is not unexpected that hyperammonemia in patients and/or experimental models is usually associated with significant alterations in the glutamine concentration (Albrech et al., 2010). For decades, the formation of Gln was generally viewed as the principal means of ammonia detoxification, as the inhibition or deficiency of this enzyme increased brain excitability and led to the onset of seizures (Suárez et al., 2002; Eid et al., 2012; Braissant et al., 2013). However, the significant increase of glutamine in the CNS has been postulated to be a cause of cerebral edema (Takahashi et al., 1991; Blei et al., 1994) and mitochondrial swelling in hyperammonemic animals (Zieminska et al., 2000, Zielińska et al., 2014). Although the exact causes of these alterations are not fully understood, it has been suggested that changes in glutamine and the expression of GS in the CNS may reflect astroglial alterations in hyperammonemic situations characterized by inflammatory responses and blood–brain barrier (BBB) dysfunction (Eid et al., 2013; Skowronska et al., 2013).

The severity of hyperammonemia can also be observed in other ammonia-metabolism disorders in which the elevated blood ammonia level causes mortality and seizures. The convulsive action induced by toxic concentrations of ammonia in the CNS may involve alterations in amino acids pathways, neurotransmitter systems, cerebral energy, and/or signal transduction (Braissant et al., 2013). Regarding neurotransmission, it has been shown that GABA release and metabolism may be affected by ammonia (Ott & Vilstrup, 2014). The heterogeneous and contradictory findings elicited by this compound in the CNS have resulted in the need for a better understanding of the mechanisms by which it induces excitotoxicity in MMA aciduria and hyperammonemia, especially with regards to the function and regulation of GABAergic and glutamatérgic pathways (Schousboe et al., 2013).

GABAergic function is fine-tuned at multiple levels, including transmitter synthesis by glutamic acid decarboxylase (GAD) (Erlander et al., 1991). GAD catalyzes the decarboxylation of glutamate to GABA and CO_2. It is considered unique among enzymes involved in neurotransmitter synthesis because both its substrate and product are neurotransmitters and exhibit opposite actions. L-glutamate and GABA act as excitatory and inhibitory neurotransmitters, respectively. In the MMA acidemia model, it has been suggested that excessive glutamate receptor stimulation, in particular the NMDA receptor, is involved in MMA-induced glutamate decarboxylase (GAD) inhibition (Malfatti et al., 2007).

With regards to MMA acidemia, Marisco and colleagues (Marisco et al., 2003) showed that rats co-injected with ammonia and MMA presented with an exacerbartion of convulsive behavior, as well as an increase in lipoperoxidation in the cerebral cortex. Furthermore, the significant decrease of glutathione (GSH) and sulfhydryl concentrations in the cerebral cortex and striatum in hyperammonemic rats suggests a synergistic effect of MMA and ammonia on redox homeostasis in the CNS (Viegas et al., 2014). However, only scattered information on the effective participation of ammonia in the excitotoxicity induced by MMA is available (Marisco et al., 2003). Therefore, we decided to investigate whether the convulsive behavior elicited by secondary metabolites (ammonia) in MMA acidemia involves inflammatory processes (interleukins TNF-α and IL-1β), signaling through nitric oxide (NO), free radical damage (Na^+, K^+-ATPase activity), mitochondrial function (membrane potential ΔΨ; ROS production; MTT reduction; SDH activity), the GABAergic pathway

(binding and release of Glutamate/ GABA; GAD and GS activity), changes in blood brain barrier (BBB) permeability, and cell death volume.

2. Experimental procedures

The experiments were conducted using Swiss mice (25-35 g) maintained in a controlled environment (12:12 h light-dark cycle, 24 □ 1 ° C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. All of the experimental protocols were developed with the goals of keeping the number of animals used to a minimum and maintaining these animals' well-being. All of the experiments were conducted in accordance with national and international standards (i.e., the Brazilian School of Animal Experimentation (COBEA) policy and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals) and with the approval of the Ethics Committee of the Federal University of Santa Maria (110/2012).

2.1 Placement of cannula and surgical procedures for electrocorticographic recording

In the present study, the animals were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula and a set of electrodes were implanted for the purpose of EEG recording. The guide cannula was glued to a multipin socket and inserted into the right ventricle through a previously opened skull orifice. Two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP −4.5, L 2.5) along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket and fixed to the skull with dental acrylic cement.

All of the intracerebroventricular (i.c.v.) injections were performed with a needle (30 gauge) protruding 1 mm below a guide cannula. All drugs were injected during a period of 1 min using a Hamilton syringe, and an additional minute was allowed to elapse before the needle was removed to avoid backflow of the drug through the cannula. The procedures for EEG recording were carried out as previously described (Ribeiro et al., 2009). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 cm × 25 cm × 60 cm) for at least 30 min before

beginning the EEG recordings. The animal was then connected to a 100× headstage pre-amplifier (model #8202-DSE3) in a low-torque swivel (Pinnacle Technology Inc, Lawrence, KS, USA), and the EEG was recorded using a PowerLab 16/30 data acquisition system (AD Instruments, Castle Hill, Australia). EEG signals were amplified, filtered (0.1–50.0 Hz, bandpass), digitalized (sampling rate 1 kHz), and stored on a PC for off-line analysis.

2.2 Reagents, drug administration protocol, and seizure evaluation

All of the reagents were purchased from Sigma (St. Louis, USA). To evaluate the effects of MMA and ammonia on electroencephalographic seizures and biochemical parameters, the animals were subcutaneously injected with either sodium chloride (NaCl, 0.9%) or ammonium chloride (NH_4Cl, 3, 6 and 12 mmol/kg; i.p.). After five minutes, either MMA (0.3, 0.66 and 1 µmol/2 µL, i.c.v.) or a vehicle (NaCl, 0.9%) were also injected. With the i.c.v. injection, the drugs were injected over a 2-minute period by using a Hamilton syringe, and an additional minute was allowed to elapse before the needle was removed to avoid backflow of the drug through the cannula. The presence of seizures was monitored in all animals by electroencephalographic recordings. A 10-min baseline recording was obtained to establish an adequate control period. After this baseline recording, the animals were observed for the appearance of generalized tonic-clonic convulsive episodes for 20 min (FERRARO et al., 1999). Tonic-clonic convulsive episodes were defined by generalized whole-body clonus involving all four limbs and the tail, rearing, wild running, or jumping, followed by a sudden loss of upright posture, as well as autonomic signs, such as hypersalivation and defecation. During the 20-min observation period, the latency for generalized tonic-clonic convulsions was measured. The EEG recordings were visually examined for seizure activity, as defined by the occurrence of the following alterations in the recording leads: isolated sharp waves (≥ 1.5x baseline); multiple sharp waves (≥ 2x baseline) in brief spindle episodes (≥ 1 s and ≤ 5 s); multiple sharp waves (≥ 2x baseline) in long spindle episodes (≥ 5 s); spikes (≥ 2x baseline) plus slow waves; multispikes (≥ 2x baseline, ≥ 3 spikes/complex) plus slow waves; and a major seizure (repetitive spikes plus slow waves obliterating the background rhythm, ≥ 5 s).

2.3 Tissue processing for neurochemical analyses of mitochondrial function

Immediately after the behavioral evaluation, the animals were killed by decapitation, and their brains were exposed by removing the parietal bone. The cerebral cortex was dissected on an inverted, ice-cold, Petri dish and homogeneized in cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM EDTA and 320 mM sucrose. The homogeneized tissue was then divided in aliquots for subsequent neurochemical analyses, as described below. In order to determine the effect of MMA and ammonia on mitochondrial function, the mitochondria were isolated as previously described by Bhattacharya et al. (1991), with a few modifications. The cerebral cortex was rapidly removed and immersed in an ice-cold isolation buffer I (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, at pH 7.4). The tissue was homogenized, and the resulting suspension was centrifuged for 3 min at 2000×g in a Hitachi CR21E centrifuge. After centrifugation, the supernatant was recentrifuged for 10 min at 12000×g. The pellet was resuspended in isolation buffer II (100 mM sucrose, 10 mM EDTA, 100 mM Tris–HCl, 46 mM KCl, and 0.5% bovine serum albumin (BSA) free of fatty acids, at pH 7.4) and recentrifuged at 12000×g for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in 125 µl of isolation buffer III (270 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris–HCl, 1 mM K_2HPO_4, at pH 7.4).

The mitochondrial DCF-RS generation was assayed according to Garcia-Ruiz and collaborators. Briefly, the cortical mitochondrial samples (300 µg of protein per mL) were incubated in a medium containing Tris-HCl buffer (20 mM, pH 7.4) EDTA (0.02 mM), sucrose (70 mM), mannitol (230 mM), K_2HPO_4 (1 mM), and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the 2′,7′-dichlorofluorescein diacetate (DCFA-DA, 0,1 µM) addition, and the medium was stirred constantly during the assay period. The fluorescence analysis was performed at 488 nm for excitation and 525 nm for emission, with slit widths of 5 nm.

The mitochondrial ΔΨ determination was assayed according to Akerman and Wikstron (AKERMAN; WIKSTROM, 1976). Briefly, the cortical mitochondrial samples (300 µg protein/mL) were incubated in a medium containing mannitol (230 mM),

sucrose (70 mM), EDTA (0.02 mM), K_2HPO_4 (1 mM), Tris (20 mM, pH 7,4), safranine-O (10 µM), and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the mitochondria addition, and the medium was stirred constantly during the assay period. The fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 5 nm.

Methyl-Tetrazolium (MTT) reduction levels were carried out with a modification (Cohen et, al. 1997) of the method described by Berridge and Tan (1993). The respiration buffer was used as the medium. MTT reduction levels were determined using an index of the dehydrogenase enzyme functions, which are involved in cellular viability. Samples were incubated in a buffer containing glutamate/succinate (5 nM each) and MTT (0,5 mg/ml) for 30 min at 37ºC, and the MTT reduction reaction was stopped by the addition of 1 ml of dimethylsulphoxide (DMSO). The formed formazan levels were determined spectrophotometrically and are reported as the difference in absorbance between 570 and 630 nm. The results were corrected according to the protein content. Individual samples were expressed as a percent of the mean control value in the experiment.

The succinate dehydrogenase activity was assayed as previously described by Green and Narahara (1980), with some modifications, using 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) as the electron acceptor. Briefly, 25 µg of protein was added to a reaction medium containing 50 mM PBS (pH 7.4), 10 mM sodium azide, 0.8 mM 2- (4-Iodophenyl) -3- (4-nitrophenyl) -5-phenyl-2H-tetrazolium chloride (INT), and 1 mM sodium succinate. After incubation for 15 min at 37ºC, the reaction was stopped by adding 1.5 ml of ethanol. The tubes were shaken and then placed on ice for 10 min. Then, the tubes were centrifuged at 800 x g for 10 min at room temperature. The absorbance of 1 ml of supernatant was measured in a spectrophotometer at 458 nm, as a measure of reduced INT. The SDH activity was expressed as nmol of INT / mg of protein/min, and the extinction coefficient of reduced INT was 2.0 x $l0^4$ liters. $mol^{-1}.cm^{-1}$.

2.4 Determination of Ammonia Concentrations in the cerebral cortex

The ammonia concentrations were measured after the behavioral analyses. The ammonia concentration was measured using an Ammonia Assay Kit (AA0100, Sigma-Aldrich Co.) in accordance with the manufacturer's protocol. The

concentration of ammonia was normalized to the protein concentration contained in the samples. The results were expressed in µg/mg of protein.

2.5 Analysis of cytokines

The cerebral cortex was 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). After homogenization, the sample was centrifuged (3000 g for 10 min), and cytokine levels (interleukins' IL1β and TNFα) were measured using a commercially available ELISA Kit from R&D Systems (Minneapolis, MN). The concentration of cytokines was normalized to the protein concentration contained in the samples. The results were expressed in pg/mg of protein.

2.6 BBB permeability assay using fluorescein

In order to evaluate the BBB permeability to small molecular mass compounds, a subset of animals were injected with sodium fluorescein (10 mg in 0.1 ml sterile saline i.p.), according to Olsen and co-workers. In brief, animals were anesthetized with a ketamine HCl (200 mg/kg) i.p. injection 45 min after the sodium fluorescein injection. Transcardial perfusion with PBS was performed, and the brain was removed, weighed, homogenized (1:10) in trichloroacetic acid, and centrifuged 1250 x g for 5 min. The supernatant was then stored at -70ºC until further processing. Blood samples were centrifuged at 3000 rpm for 5 min to obtain serum, which were diluted (1:10; v:v) in trichloroacetic acid. The precipitated serum proteins were separated by centrifugation, and the supernatant was stored at -70ºC until further processing. Samples were analyzed on a fluorometer (Emission: 538; Extinction: 480). The degree of BBB permeability was measured as the percentage of sodium fluorescein in the brain per amount of sodium fluorescein in a milliliter of serum.

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

In order to determine NOx (nitrate and nitrite), an aliquot (200 µL) was homogenized in 200 mM Zn_2SO_4 and acetonitrile (96%, HPLC grade). Afterwards, the homogenate was centrifuged at 16000 x g for 20 min at 4°C, and the supernatant was separated for analysis of the NOx content, as described by Miranda and collaborators (MIRANDA; ESPEY; WINK, 2001). The resulting pellet was suspended in NaOH (6 M) for protein determination.

2.8 Na^+,K^+-ATPase activity measurement

Na^+,K^+-ATPase activity was assayed as previously described. Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 50 µg of protein in the presence or absence of ouabain (1 mM); the final volume of this solution was 350 µL. The reaction was started by adding adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37°C, the reaction was stopped by adding 70 µL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations for these compounds were used, and the reaction was linear with protein and time. The appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by a previously described colorimetric method (FISKE; SUBBAROW, 1927), using KH_2PO_4 as a reference standard. The specific Na^+,K^+-ATPase activity (nmol Pi/mg protein/min) was calculated by subtracting the ouabain-insensitive activity from the overall activity in the absence of ouabain.

2.9 [^{3}H] flunitrazepam binding

The binding of [^{3}H] flunitrazepam to the $GABA_A$ receptor benzodiazepine site was determined by first washing the cell membrane preparation in the following manner: individual aliquots were diluted with five volumes of wash buffer B (50 mM Tris–HCl and 2 mM EDTA, pH 7.4); the aliquots were mixed and centrifuged at 16,000 × g for 10 min at 4 °C; and the samples were incubated for 30 min at 37 °C. This washing procedure was repeated twice, and the final pellet was re-suspended in binding assay buffer C (20 mM HEPES and 1 mM EDTA, pH 7.4). The protein

concentration of each sample was determined by a spectrophotometric protein dye-binding assay based on a previously described method that uses bovine serum albumin as the standard (Bradford, 1976). The incubation was started by adding 1 nM [^{3}H]-flunitrazepam (85.8 Ci/mmol) and was run on ice for 60 min. The reaction was stopped by vacuum filtration, and each filter was washed with 15 ml of cold 10 mM Tris–HCl buffer. The filters were placed individually in polycarbonate tubes, and 1 ml of scintillation liquid was added. The radioactivity of each sample was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. The non-specific binding of each sample was determined by adding 100 µM non-radioactive flunitrazepam to the medium in parallel assays. The specific binding of each sample was considered as the difference between total binding and non-specific binding.

2.10 GAD activity

In order to determinate the glutamic acid decarboxylase (GAD) activity, the cerebral cortex was homogenized in 50 mM potassium phosphate, pH 6.4 (1:10, w/v), using a glass homogenizer. The GAD activity was determined by subtracting the penicillamine-sensitive activity from the overall decarboxylase activity (in the absence of penicillamine), according to Albers and Brady (1959). Aliquots of 500 µl of tissue homogenate (1:10, w/v) were incubated in glass tubes for 60 min at 37 °C in the presence of (concentrations in mM) potassium phosphate buffer (pH: 6.4) 50, 2-mercaptoethanol 10, pyridoxal-P 1, DL-[^{14}C]-glutamic acid (specific activity 58 mCi/mmol) 15 (saturating concentration), in the presence or absence of 10 mM D-penicillamine. Incubations were carried out in flasks after the contents were gassed with 100% O_2 for 1 min and then sealed with rubber caps. The homogenates were incubated at 37 °C for 60 min in a Dubnoff metabolic shaker (60 cycles/min) according to the method of Dunlop et al. (1975). Incubation was stopped by adding 0.25 ml of 50% TCA through the rubber cap. Then, 0.1 ml of 1 M hyamine hydroxide was injected into the central wells. The flasks were shaken for a further 30 min at 37 °C to trap CO_2. After shaking, the contents of the central wells were transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter.

2.11 Glutamine Synthetase activity

The Glutamine Synthetase activity was measured in the cerebral cortex according to Petito et al (1992). The assay was started when 50 µL (150 µg of protein) of homogenate was added to 0.550 ml of a reaction mixture containing 20 mM $MgCl_2$, 100 mM L-glutamate, 200 mM imidazole-HCl buffer (pH 7.4), 20 mM 2-mercaptoethanol, 100 mM hydroxylamine-HCl (previously adjusted to pH 7.4 with 3 M sodium hydroxide), and 20 mM ATP. The reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 0.4 ml of a solution containing 0.74 M ferric chloride, 1.34 M HCl, and 0.4 M trichloroacetic acid. The precipitated protein was removed by centrifugation. After incubation for 15 min at room temperature, the absorbance of the supernatant was read at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate (Sigma) treated with the ferric chloride reagent. The blank samples contained homogenate and the assay mixture but lacked ATP. The glutamine synthetase activity was expressed as nmol/min/mg of protein.

2.12 GABA and Glutamate release assay

In order to determine the effect of MMA and NH_4Cl on GABA and Glutamate release, cortical slices were dissected and quickly incubated for 10 minutes at 37°C in culture plates containing Hank's solution (128 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$, 12 mM Glucose and 20 mM HEPES). GABA and glutamate released from cerebral cortical tissue were measured utilizing reverse phase high-performance liquid chromatography (HPLC) by fluorescent detection, in accordance with Moraes et al. (2012). Briefly, pre-column derivatization was performed with o-phtaldialdehyde. The mobile phase was composed of 50 mM sodium acetate, 5% methanol, and 2-propanol (pH 5.67) as phase A and 70% methanol as phase B. The fluorescent detector was set at 340 nm (excitation wavelength) and 460 nm (emission wavelength), and the amino acid homoserine was used as an internal standard. GABA and glutamate levels were expressed as µg/milligram of protein/minutes.

2.13 Quantification of cell death volume

For quantification of cell death volume, a subset of animals were deeply anesthetised with ketamine HCl (200 mg/kg; i.p.) and euthanized by decapitation. The brains were removed from the skull and sliced into 2-mm-thick coronal sections using a mouse brain matrix (Scienlabor, SP, BR). The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) in PBS for 10 min to visualize the lesion size (Figure 10). The unstained areas were considered to be the injured areas (Schabitz et al., 2003).

2.14 Protein determination

Protein concentration was measured colorimetrically using a Bradford assay (BRADFORD, 1976) and bovine serum albumin (1 mg/ml) as the standard.

2.15 Statistical analysis

Statistical analysis was carried out by a two-way analysis of variance (ANOVA) or a Mann-Whitney test when appropriate. If necessary, a post-hoc Student-Newman-Keuls (SNK) test was carried out. The ammonia concentrations in the plasma and cerebral cortex were analyzed using the unpaired Student's *t* test and expressed as the mean and standard error of the mean. Parametric tests are expressed as the mean +/- S.E.M., and non-parametric tests are expressed as the mean +/- the interquartile range. A probability of $p<0.05$ was considered significant.

3. Results

3.1 Dose response curve of Ammonia and MMA on behavioral and EEG seizures

Figure 1 shows the effect of NH_4Cl (3, 6, and 12 mmol/kg) on behavioral seizures (characterized here by latency and number of myoclonus jerks). Electroencephalographic recordings confirmed the behavioral seizures elicited by NH_4Cl (12 mmol/kg) (Fig. 1F); these seizures were characterized by the occurrence of multispikes plus slow waves and major seizure activity. The multispikes plus slow

waves correlated with myoclonic jerks, which are characteristic of clonic convulsions. The middle dose of NH_4Cl (6 mmol/kg) was selected for further experiments. The behavioral recordings revealed that the MMA injection (0.66 and 1 µmol/2 µL, i.c.v.; Fig. 2) induced behavioral seizures, characterized by the latencies to the first tonic-clonic, generalized convulsions and the duration of the generalized convulsions (Figs. 2G and H). The generalized convulsions appeared in the EEG recordings as major seizure activity and were characterized by 2–3 Hz high-amplitude activity (Figs. 2 E and F). After the ictal discharge, postictal EEG suppression and slow waves were observed, and the present activity was correlated with behavioral catalepsy. In order to investigate the effect of ammonia on MMA-induced seizures, we selected the intermediate concentration of MMA (0.66 µmol/2 µL). Statistical analysis revealed that previous NH_4Cl (6 mmol/kg) administration did not alter the latency [$U(1)=46.50$, $p>0.05$; Fig. 3 C, E], but increased the duration [$U(1)=20.96$; $p<0.01$; Fig. 3 D, F], of convulsive episodes induced by MMA (0.66 µmol/2 µL), indicating that the combination of NH_4Cl and MMA changed convulsive behavior.

3.2 Involvement of inflammatory and BBB breakdown on convulsive behavioral elicited by ammonia in this model of MMA acidemia

In the present study, we showed that the acute administration of NH_4Cl (6 mmol/kg) increased ammonia concentration in plasma [$t(6) = 2.576$; $P < 0.05$ Fig. 4A] and cerebral cortex [$\mathbf{t}(6) = 3.205$; $\mathbf{P} < 0.05$ Fig. 4B] when analyzed 5 min after its injection. On the other hand, the combination of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) did not alter plasma [$F(3,40)=0.69$; $p>0.05$, Fig. 4C] and cerebral ammonia levels [$F(3,40)=0.75$; $p>0.05$, Fig. 4D] after behavioral and EEG recordings (20 min after MMA injection). Our experimental data also revealed that the present protocol of NH_4Cl plus MMA administration did not alter cytokine levels (interleukins IL-1β [$F(3,40)=0.45$; $p>0.05$, Fig. 5A] and TNFα [$F(3,40)=0.78$; $p>0.05$, Fig. 5B]) or plasma fluorescein extravasation [$F(3,40)=0.35$; $p>0.05$, Fig. 5C] (indicator of BBB breakdown), suggesting that the change in convulsive behavioral elicited by ammonia in this model of MMA acidemia is not, at least in part, due to an early inflammatory response and BBB breakdown.

3.3 The ammonia-induced cellular redox alteration had a functional impact on mitochondrial function in this model of MMA acidemia

In the present study, we investigated whether both NH_4Cl and MMA may exert their deleterious effects by mechanisms that cause changes in RNS and ROS. For this propose, we measured the levels of NOx and mitochondrial DCF-RS generation in the cortex of mice. Statistical analysis revealed that NH_4Cl (6 mmol/kg), but not MMA (0.66 µmol/2 µL), injection induced cerebral NOx increase [$F(1,38)=3,37$; $p<0.05$,Fig. 6A] and mitochondrial DCF-RS generation [$F(1,38)=8,01$; $p<0.05$, Fig. 6B]. Statistical analysis also revealed that NH_4Cl plus MMA potentiated mitochondrial DCF-RS levels [$F(3,38)= 6.81$; $p<0.05$; Fig. 6B] when compared with MMA plus saline. The effects of RNS and ROS generation on mitochondrial integrity and function were also characterized by a mitochondrial MTT reduction and ΔΨ assay. Our experimental data revealed that NH_4Cl (6 mmol/kg) plus MMA (0.66 µmol/2 µL) decreased the mitochondrial membrane potential [$F(3,28)=8,41$; $p<0.05$; Figs. 7 A;B] and MTT reduction [$F(3,28)= 9,05$; $p<0.05$; Figs. 7 C]. Statistical analysis also revealed that NH_4Cl (6 mmol/kg) plus MMA (0.66 µmol/2 µL) inhibited SDH activity [$F(3,28)=3,80$; $p<0.05$, Fig. 7 D] and increased Na^+,K^+-ATPase activity [$F(3,32)=2,82$; $p<0.05$, Fig. 7 E] compared to the control group.

3.4 Involvement of GABAergic pathway on convulsive behavioral and neuronal dmage elicited by ammonia in this model of MMA acidemia

Considering that glutamine synthetase (GS) is an enzyme involved in several metabolic pathways in the brain, including the glutamine-glutamate-GABA cycle and the detoxification of ammonia (Eid et al., 2012), we decided to investigate the involvement of this cycle in convulsive behavior. Our experimental data showed that a previous NH_4Cl (6 mmol/kg) injection [$F(1,38)=9,05$; $p<0.05$; Fig. 8 B], as well as NH_4Cl plus MMA treatment, inhibited cerebral glutamine synthetase (GS) activity [$F(3,38)= 4.83$; $p<0.05$; Fig. 8 B] when compared to the control/saline group. Indeed, the NH_4Cl (6 mmol/kg) injection [$F(1,38)=6,16$; $p<0.05$; Fig. 8A], as well as the NH_4Cl plus MMA treatment, inhibited cerebral glutamate decarboxylase (GAD) activity [$F(3,38)= 8.04$; $p<0.05$; Fig. 8A]. The present protocol of NH_4Cl plus MMA treatment also inhibited [^3H] flunitrazepam binding [$F(3,38)= 6.44$; $p<0.05$; Fig. 9 A] when compared to the control/saline group. Interestingly, our experimental data revealed

that MMA (0.66 µmol/2 µL i.c.v) [F(1,38)=6,16; p<0.05; Fig. 9B], NH_4Cl (6 mmol/kg) [F(1,38)=9,05; p<0.05; Fig. 9B], and NH_4Cl plus MMA treatment [F(1,38)=6,16; p<0.05; Fig. 9B] decreased GABA release. On the other hand, Figure 9C show that treatment with MMA (0.66 µmol/2 µL i.c.v) [F(1,38)=6,16; p<0.05; Fig. 9C], but not NH_4Cl (6 mmol/kg) [F(1,38)= 3.07; p<0.05; Fig. 9C], decrease glutamate release. This decrease in glutamate release was also observed in the NH_4Cl plus MMA group [F(1,38)=9,05; p<0.05; Fig. 9C]. The present protocol of acute NH_4Cl and MMA treatment did not induce macroscopic neuronal damage (Figure 10).

4. Discussion

Ammonia has been known to be a neurotoxicant for a long time, and a considerable amount of information regarding the metabolic targets and possible mechanisms of neurotoxicity of this compound has appeared in the literature (Cooper and Lai, 1987; Ott and Vilstrup, 2014). Hyperammonemia is a common finding in children with methylmalonic acidemia (Gebhardt et al., 2003). Like the symptoms found in this organic aciduria, the neuronal increase of this metabolite also induces convulsions (Summar, 2001; Kolker et al., 2003; Deodato et al., 2006; Lee et al., 2008; Yanagawa et al., 2008). In this context, it has been demonstrated that toxic concentrations of ammonia in the CNS suppresses the inhibitory activity of GABA at synaptic levels. This property of ammonia has been proposed as the mechanism by which it induces seizures (Iles and Jack, 1980; Raabe, 1993; Paul, 2003). In an experimental model of MMA aciduria, rats co-injected with ammonia and MMA presented with an exacerbartion of convulsive behavior, suggesting that MMA-induced convulsions may be a consequence of excitotoxicity caused by metabolites, such as ammonia (Marisco et al., 2003). Our data agree with this assumption and contribute novel insights into the EEG recordings involved in ammonia-secondary damage in this model of organic aciduria. The convulsive behavior induced by intermediate doses of NH_4Cl (6 mmol/kg) plus MMA (0.66 µmol/2 µL) and characterized by clonus of the left forelimb and/or left hindlimb and head occurred concomitantly with EEG recordings. Electrographic seizures were defined by the occurrence of episodes consisting of the simultaneous occurrence of at least two of the following alterations in all four recording leads: high-frequency and/or polyspike complexes and/or high-voltage synchronized spike activity.

In inborn errors of metabolism, it has been demonstrated that enzymes of the urea cycle are usually normal (PACKMAN et al., 1978a). However, patients with methylmalonic and propionic acidemia show a reduction in the activity of carbamoylphosphate synthetase and propionyl-CoA accumulation (WOLF, B. et al., 1978a). The accumulation of these metabolites in the liver mitochondria of patients with MMA aciduria leads to the inhibition of the biosynthesis of N-Acetylglutamate, which is an allosteric activator of carbamoylphosphate synthetase (COUDE; SWEETMAN; NYHAN, 1979b). Consequently, the ammonia produced by protein degradation cannot be detoxified in the liver by incorporating it into urea, which leads to high ammonia concentration in the blood. This in turn causes an increased accumulation of ammonia in the CNS (CAULI et al., 2005). In line of this view, our experimental data showed that acute administration of NH_4Cl (6 mmol/kg; i.p) increased the ammonia concentration in the plasma and cerebral cortex 5 min after its injection. Although it is still uncertain which flux is predominant in the CNS, our data agree with the assumption that a cerebral influx of ammonia takes place by passive diffusion of NH_3 and by active transport of NH_4 (Ott and Vilstrup, 2014). It is important to note that ammonia is a normal product in the brain and peripheral tissues. However, in hyperammonemia, the ammonia diffuses from the blood into the CNS and produces a neurotoxic effect (Paul, 2003).

Regarding the role of ammonia in the CNS, the literature related to the effects of elevated plasma ammonia levels on brain metabolism is abundant but heterogeneous in terms of conclusions. In this context, it has been demonstrated that an increased ammonia concentration in the CNS, either directly or in synergy with neuronal injury-derived inflammatory cytokines, causes subtle increases in the transcellular passage of molecules of different size (BBB “leakage”) (Skowrońska and Albrecht, 2012). Infections process, precipitate and exacerbate metabolic crises, which in turn cause neurologic dysfunction, such as seizures (Salvadori et al., 2012; Deodato et al., 2006; Dionisi-Vici et al., 2006). The results presented in this report, however, showed that intermediate doses of NH_4Cl changed MMA-induced convulsive behavior but did not alter cerebral cytokine levels (IL-1β and TNF-α) or plasma fluorescein extravasation (indicator of BBB breakdown). Our experimental data suggest that the convulsive behavior elicited by ammonia is not, at least in part, due to an early inflammatory response and BBB breakdown.

Considering that both NH_4Cl and MMA may exert their deleterious effects by mechanisms that cause changes in RNS and ROS, we investigated the effects of reactive oxygen/nitrogen species generated by ammonia in MMA aciduria. Our experimental data showed that an intermediate dose of NH_4Cl (6 mmol/kg) induced an increase in cerebral NOx and mitochondrial DCF-RS generation. These data agree with the idea that protein oxidation (Gorg et al., 2008), protein tyrosine nitration (Haussinger et al., 2005), and S-nitrosylation of cysteine residues in proteins (Gorg et al., 2008) induced by ammonia accumulation in the CNS may contribute to the excitability of organic aciduria. Our experimental data also revealed that the present protocol of NH_4Cl injection potentiates NOx generation and mitochondrial DCF-RS levels induced by MMA. The ammonia-induced cellular redox alteration had a functional impact on mitochondrial function characterized by a decreased mitochondrial membrane potential ($\Delta\Psi$ assay) and MTT reduction. Our results also revealed that neither intermediate doses of MMA (0,66 µmol/2 µL) nor NH_4Cl (6 mmol/kg i.p.) altered SDH activity. However, its combination induced a significant reduction of SDH activity. These results reinforce the view that in some inborn errors of metabolism, including methylmalonic acidemia, mitochondrial dysfunction and oxidative stress are involved in the seizures induced by MMA. Furthermore, the convulsive behavioral may be a consequence of excitotoxicity secondary to a metabolic primary deficit caused by metabolites, such as ammonia.

In line of this view, we demonstrated that Na^+,K^+-ATPase activity was increased in the NH_4Cl plus MMA-treated group. These data agree with previous data from Malfatti et al (2003), which demonstrated a concomitant activation of Na^+,K^+-ATPase activity in the cerebral cortex with EEG seizures induced by intrastriatal MMA (6 mmol). The increase of cortical Na^+,K^+-ATPase activity evidenced in this report may be secondary to the activation of excitatory afferent pathways to the cortex in the absence of an intervening metabolic derangement. Although the neurochemical mechanisms underlying the effects of RNS and ROS generation are heterogeneous in terms of conclusions, studies have proposed that NO, carbon monoxide, and the metabotropic and N-methyl-D-aspartate (NMDA) glutamate receptors also modulate neuronal Na^+,K^+-ATPase activity by activating protein kinase C and G (Nathansonet al., 1995). This leads to a complex phosphorylation cascade of regulatory proteins in which free radicals play a

modulatory role (Therien and Blostein, 2000). In this sense, it is plausible to propose that a dephosphorylation and subsequent activation of Na^+,K^+-ATPase in the NH_4Cl plus MMA-treated group consumes ATP and increases mitochondrial Ca^{2+}, which leads to the production of free radicals. The RNS and ROS generation inhibits enzymes of the tricarboxylic acid cycle and respiratory chain, such as SDH, leading to the activation of the permeability transition pore, which causes mitochondrial $\Delta\Psi$ and ATP synthesis to collapse.

The mechanisms underlying seizures are complex and vary across the numerous seizure types that have been characterized. A failure is believed to occur in the ability to maintain the balance between brain excitation and inhibition. Thus, neurotransmitters involved in neuronal inhibition, such as gamma aminobutyric acid (GABA), or neuronal excitation, such as glutamate, have attracted the interest of researchers aiming to elucidate the mechanisms involved in the development and/or propagation of seizures (Briggs and Galanopoulou, 2011; Sehar et al., 2015). A role for glutamate (de Mello et al., 1996) has been proposed in the electrophysiological and behavioral effects of MMA. However, little is known about the effects of MMA and ammonia (Marisco et al., 2003) on the other neurotransmitters, such as GABA (Malfatti et al., 2007). In this sense, we decided to investigate whether the convulsive behavior elicited by secondary metabolites (ammonia) in MMAuria alters the glutamine-glutamate- GABA cycle. The results presented in this report showed for the first time that NH_4Cl (6 mmol/kg) administration decreased cerebral Glutamine Synthetase (GS) and glutamate decarboxylase (GAD) activity. Our experimental data also revealed that NH_4Cl plus MMA treatment inhibited cerebral GS and GAD activity when compared to the control/saline group.

The CNS is particularly susceptible to increased concentrations of ammonia and relies heavily on GS in astrocytes to prevent toxicity and neurological dysfunction (Bosoi and Rose, 2009). On the other hand, the elevated concentrations of ammonia observed in liver disease reveal that the capacity of GS to detoxify ammonia in the brain is limited and therefore cannot compensate to maintain ammonia at physiological levels. A decrease in the capacity to clear ammonia in the brain detrimentally leads to sustained elevation in brain ammonia and prolonged neurological dysfunction (Rose and Felipo, 2005). In this sense, our experimental data suggest that changes in ROS/RNS levels elicited by ammonia accumulation

induce the GS activity inhibition (Bidmon et al., 2008) and contribute to the MMA-induced excitability. The potentiation of MMA-induced convulsions elicited by acute ammonia reinforces this assumption and suggests that GS also may play a critical role in supporting inhibitory neurotransmission.

The regulation of GABA-mediated signaling involves several mechanisms, among which the modulation of GABA synthesis or degradation by rate-limiting enzymes, such GAD, play a central role. In addition, GAD is particularly sensitive to reactive species, and GAD inhibition increases $FeCl_3$-induced epileptogenesis (Robitaille et al., 1995). Although no consistent correlation between ictal foci and reduced levels of GABA in intractable non-neoplastic focal epilepsy in humans was found, the activity of GAD is significantly decreased in the epileptic cortex of patients (Lloyd et al., 1986). In the present study, we revealed that NH_4Cl (6 mmol/kg) administration did alter the binding of [^{3}H] flunitrazepam and glutamate release but decreased glutamate decarboxylase (GAD) activity and GABA release. Our experimental data also demonstrated, for the first time, that treatment with intermediate doses of NH_4Cl plus MMA inhibited cerebral GS and GAD activity followed by binding of [^{3}H] flunitrazepam and decreased GABA release. These experimental data reinforce the idea that disturbances of the glycine/glutamate (GABA) cycle elicited by NH_4Cl (6 mmol/kg) lead to selective deficits in GABAergic inhibition and contribute to MMA-induced excitability.

There are lines of evidence suggesting an interaction between the metabolic abnormalities in MMA and neuropathologic changes (Kowaltowski et al., 2008; Morath et al., 2008). Methylmalonic acid at high concentrations in affected individuals with methylmalonic acidemia induces neuronal damage in cultures of embryonic rat striatal cells (McLaughlin et al., 1998). In a 3D model of rat organotypic brain cell cultures, low concentrations of secondary metabolites, such as 2-methylcitrate (2-MCA) and ammonia accumulation, also induce deleterious effects on neurons (limited axonal growth) and glial cells (cell swelling and massive apoptosis) (Jafari et al., 2013). Our experimental data showed, however, that the protocol utilized in this report did not induce neuronal damage. Although ammonium accumulation might be responsible for brain damage in MMAuria patients, not only during metabolic crises but also in a chronic disease course, and even after liver transplantation, it is plausible to propose that a simultaneous increase in ammonia generation and

convulsions does not necessarily imply a cause–effect relationship between these events. However, this explanation remains speculative in nature, and further studies are needed to determine the mechanisms involved in MMA-induced neuronal damage.

During the last few years, neuronal dysfunction has become a major research topic in MMAuria. The understanding of the neuropathological mechanisms is crucial for the development of preventive treatments. In this sense, the results presented in this report demonstrated that ammonia changed MMA-induced EEG seizures, an effect that is not due to an early inflammatory response and BBB breakdown. On the other hand, the ammonia-induced cellular redox alteration had a functional impact on mitochondrial function, altered the glycine/glutamate (GABA) cycle, and contributed to MMA-induced excitability. The demonstration that ammonia and MMA have mutually additive toxicity may be of value in understanding the physiopathology of the neurological signs observed in patients with methylmalonic acidemia and in contributing to the development of new strategies for treatment of these patients.

Acknowledgments

This work was supported by the CNPq (grants: Pronem: 11/2082-4). M.R. Fighera, L.F.F. Royes Furian A.F.; Schneider-Oliveira M are recipients of CNPq fellowships. The authors thank Gustavo Cassol for revision of references. All authors confirm that there is no competing financial conflict of interest.

Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Figure Captions

Figure 1.
The EEG recordings before and after injection NH_4Cl (3, 6 and 12 mmol/kg) in mice is shown in Fig. 1 A to F. The following behavioral repertoire occurred concomitantly with electrographic recorded seizures: the multispikes plus slow waves correlated with myoclonic jerks, which are characteristic of clonic convulsions. The major dose of NH_4Cl (12 mmol/kg) decreased the latency for onset and (G) increased the number of myoclonic jerks (H) in mice. Data are mean + S.E.M. for n = 8–10 in each group

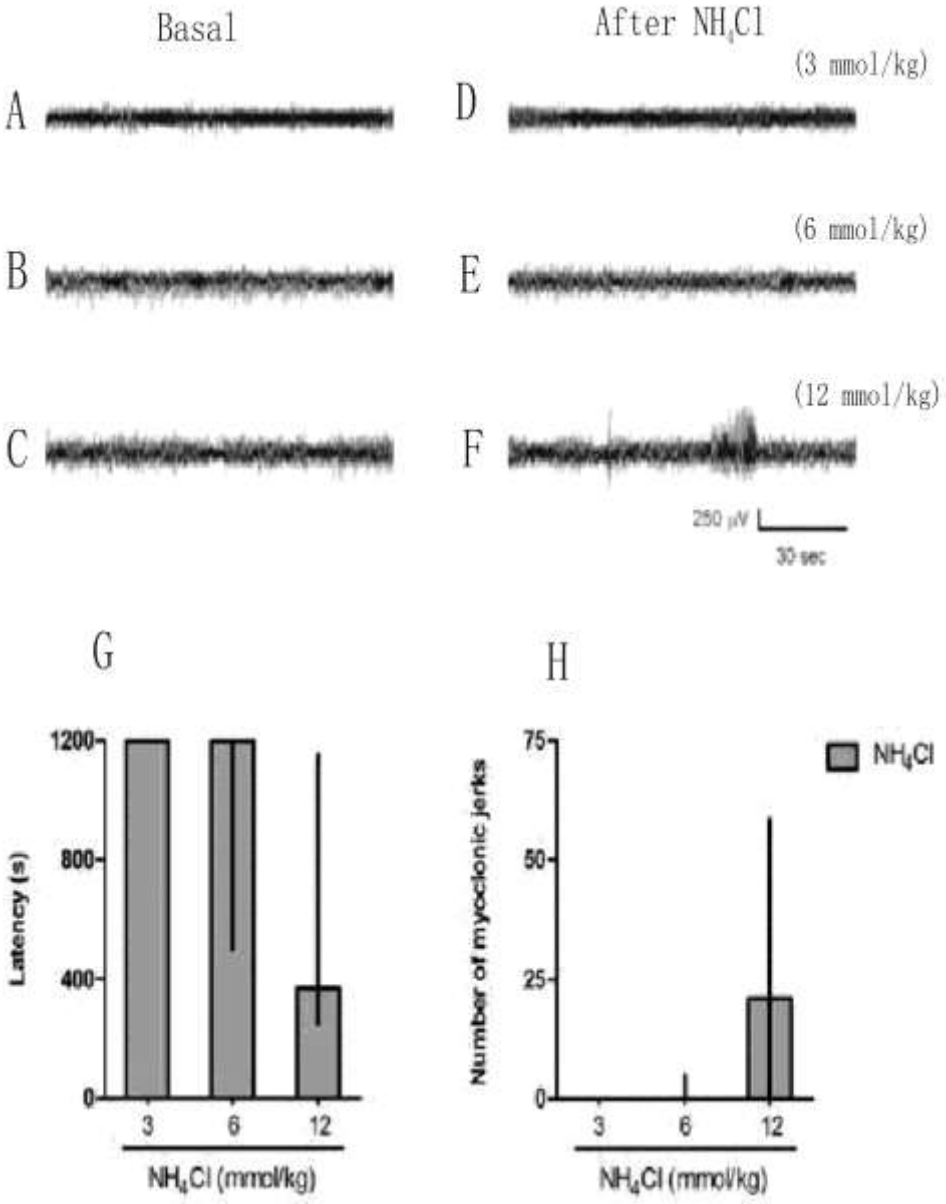


Figure 2
The EEG recordings before and after MMA injection (0.3 to 1.0 μmol/2 μL; i.c.v) is shown in Fig. 2A and F. The following behavioral repertoire observed in mice occurred concomitantly with electrographic recorded seizures: generalized seizures were characterized by the appearance of 2–3 Hz high-amplitude activity. These epileptic discharges (interictal spikes) were defined as abnormal paroxystic in the cerebral cortex and consisted of high-amplitude biphasic sharp transients. The behavioral recordings revealed that the MMA injection (0.66 and 1 μmol/2 μL, i.c.v.) decreased latencies to the first tonic-clonic, generalized convulsions and increased the duration of the generalized convulsions (Figs. 2G and H). Data are mean + S.E.M. for n = 10 in each group.

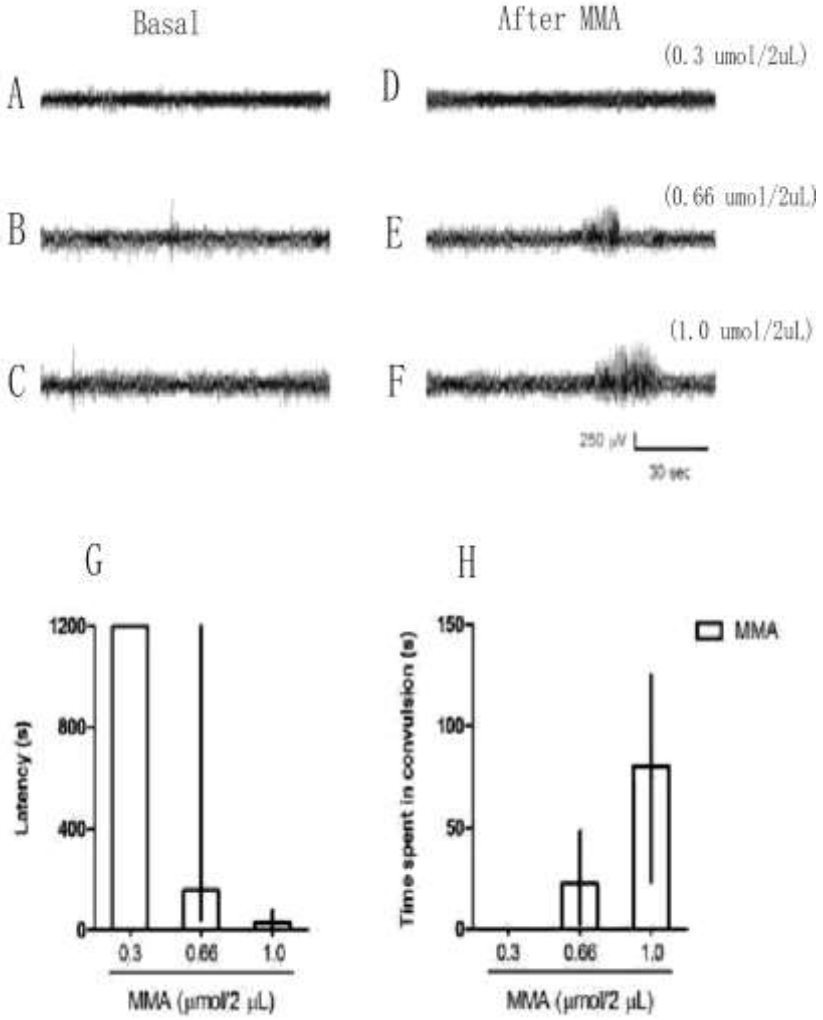


Figure 3
Effect of pre-treatment with NH_4Cl (6 mmol/kg, i.p.) on behavioral and electroencephalographic seizures induced by MMA (0,66 µmol/site i.c.v). Representative electroencephalographic recordings obtained in cortex of animal treated with vehicle/vehicle (A), NH_4Cl /vehicle (B), MMA/vehicle (C), and MMA/ NH_4Cl (D). The latency for onset and (E) total time spent in seizures induced by MMA (F). Data are mean + S.E.M. for n = 10 in each group * P<0.05 compared with MMA/vehicle.

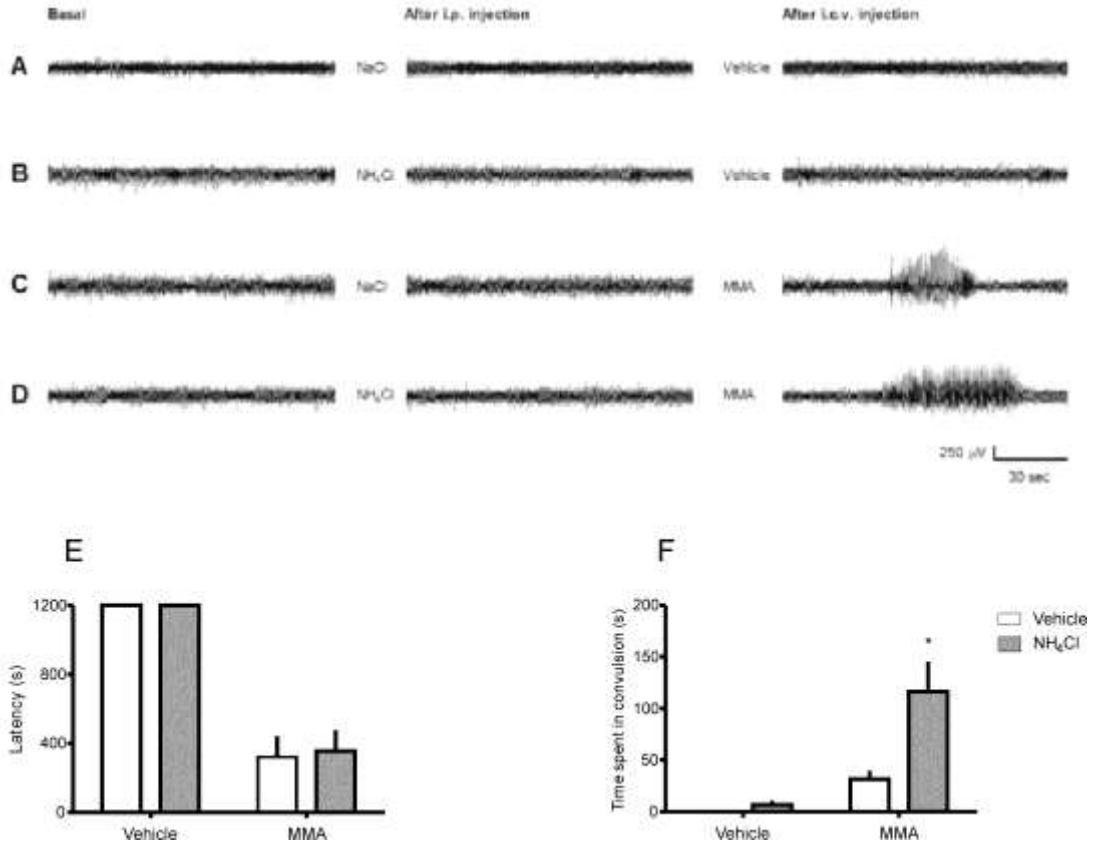


Figure 4
Effect of NH_4Cl (6 and 12 mmol/kg, i.p.) on the latency and duration of tonic clonic seizures induced by MMA (0.3 µmol/site i.c.v). The major dose of NH_4Cl (12 mmol/kg) decreased the latency for onset and (A) increased the duration of tonic clonic seizures (B) in mice. Data are mean + S.E.M. for n = 8–10 in each group.

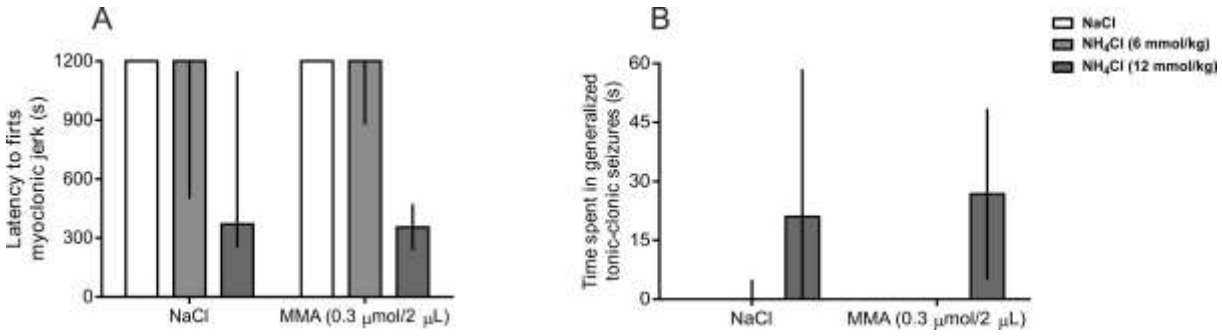


Figure 5
Effect of acute administration of NH_4Cl (6 mmol/kg) on ammonia concentration in plasma (A) and cerebral cortex (B) 5 min after its injection. Plasma (C) and cerebral cortex (D) levels of NH_4Cl levels 20 min after the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL). Data are mean + S.E.M. for n = 8–10 in each group * *P*<0.05 compared with saline treated group.

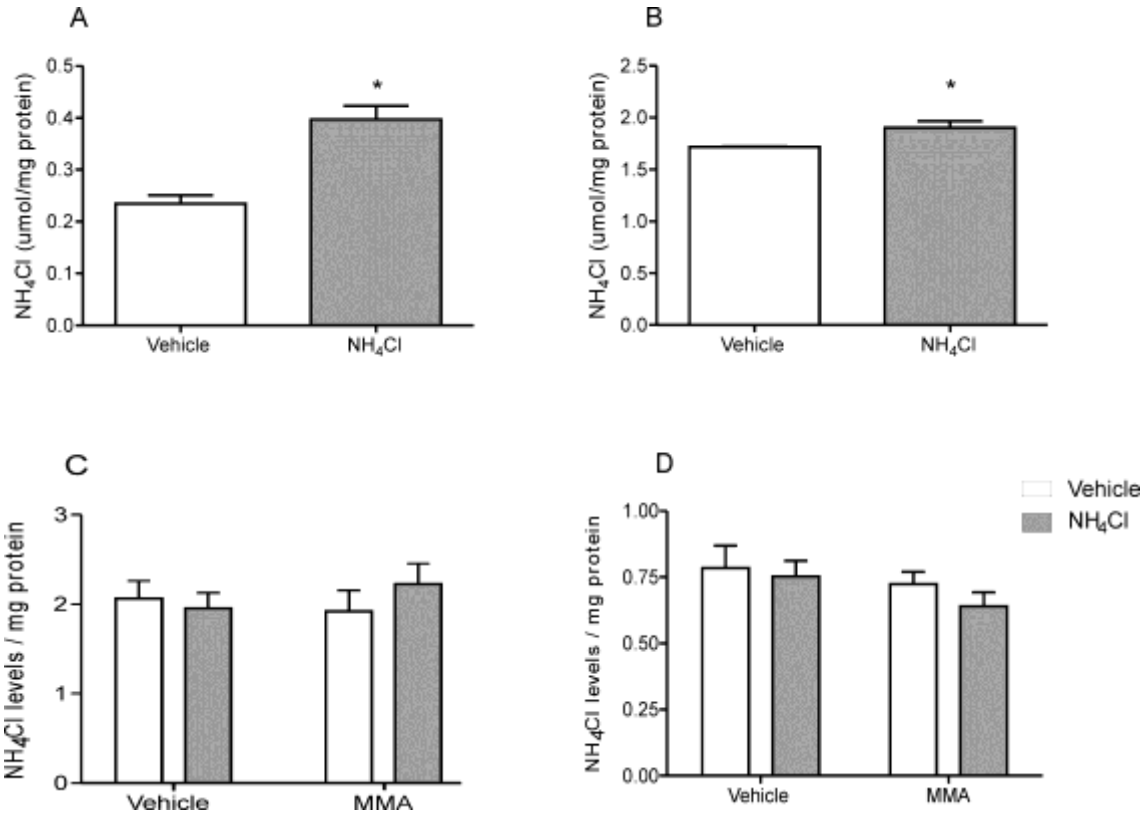


Figure 6
Effect of the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) on IL-1β (A), TNF-a levels (B), in cerebral cortex, BBB permeability (C) and IL-6 levels (D). Data are mean + S.E.M. for n = 8-10 in each group.

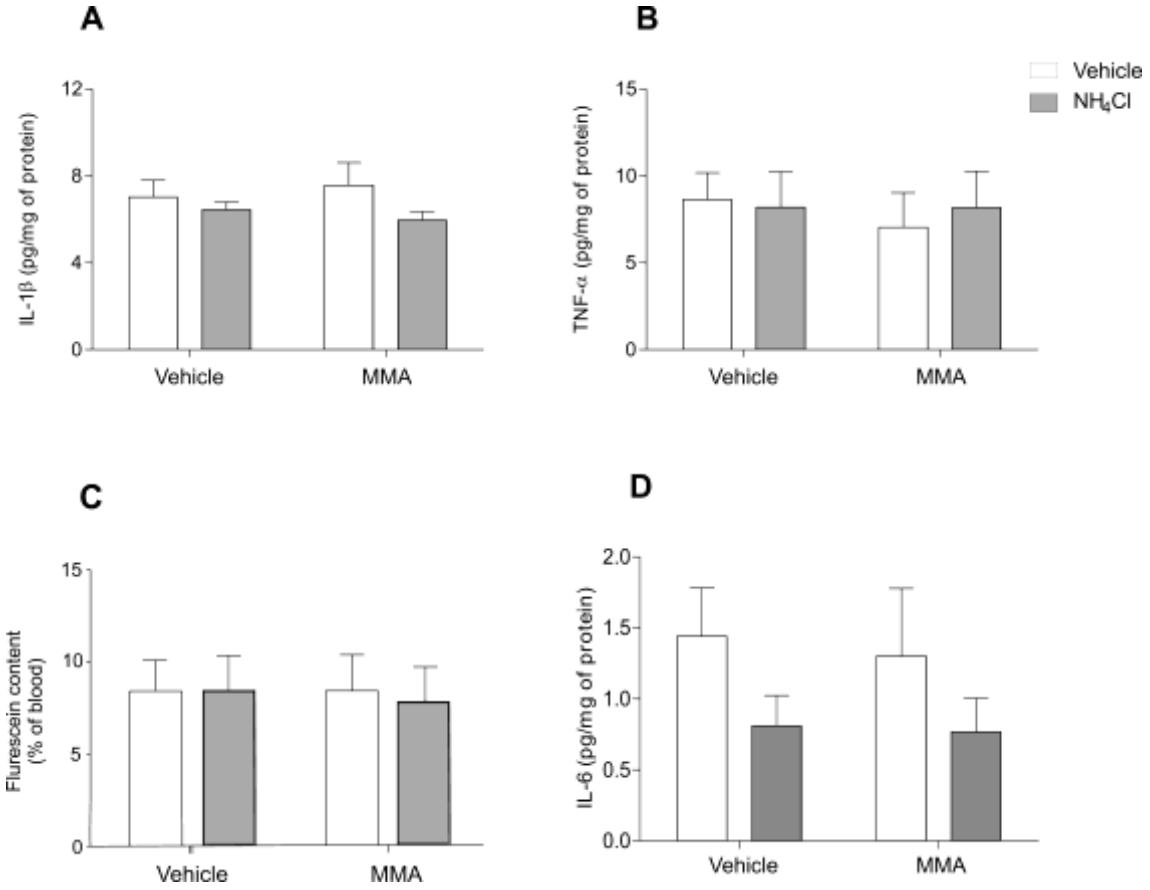


Figure 7

Effect of the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) on NOx content (A), and oxidation of DCFH (B) in cerebral cortex. Data are mean + S.E.M. for n = 8 in each group. * P<0.05 compared with vehicle and $^{\#}$ P<0.05 compared with MMA/vehicle group.

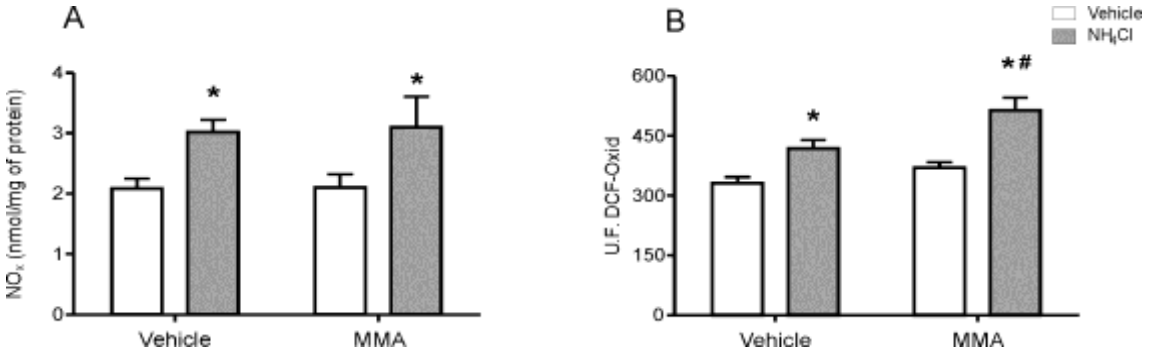


Figure 8

Effect of the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) on mitochondrial membrane potential (A e B), MTT reduction (C), SDH activity (D),

and Na^+, K^+ -ATPase activity (E). Data are mean + S.E.M. for n = 8–10 in each group. * P<0.05 compared with vehicle group.

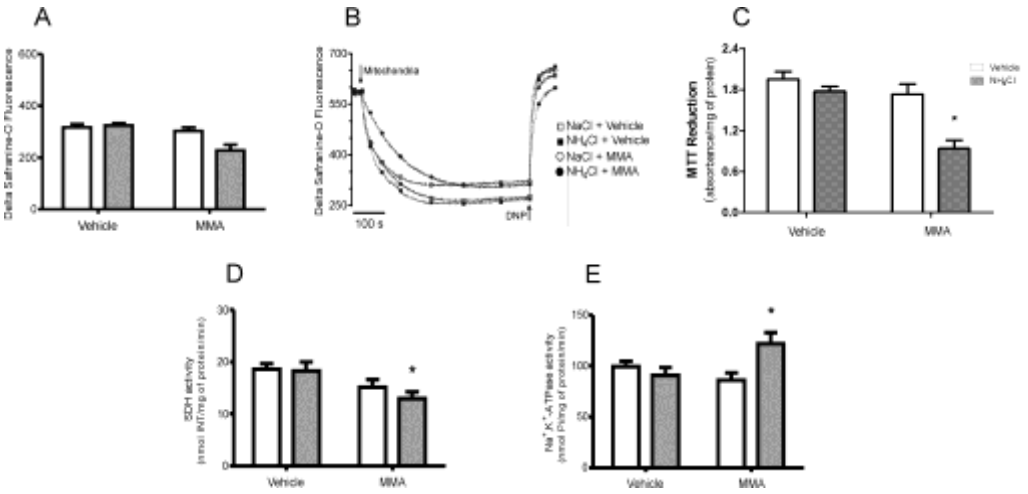


Figure 9

Effect of the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) on [^{3}H] flunitrazepam specific binding (A), GABA release (B), Glutamate release (C) and (D) graphic representation of GABA (unfilled bars) and Glutamate (filled bars) release with all groups. Data are mean + S.E.M. for n = 8–10 in each group. * P<0.05 compared with vehicle group

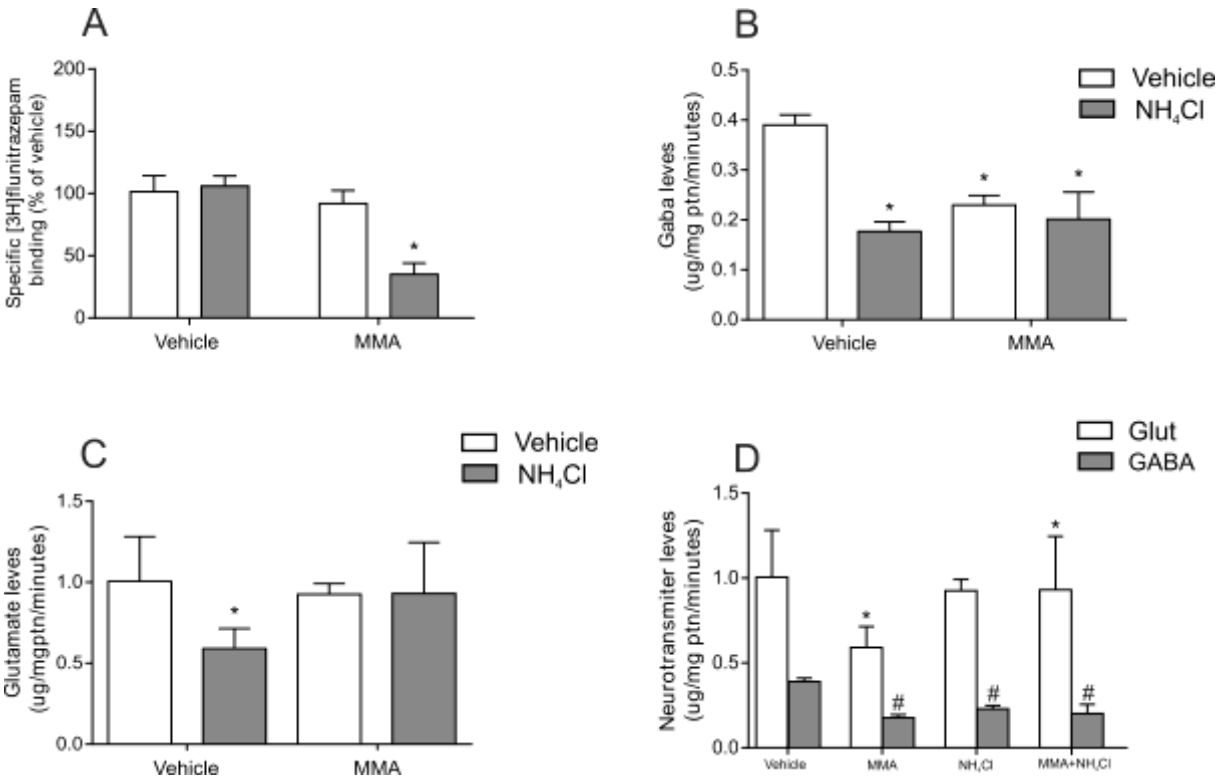


Figure 10
Effect of the acute administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL) on GAD activity (A), and GS activity (B). Data are mean + S.E.M. for n = 8 in each group. * P<0.05 compared with vehicle group.

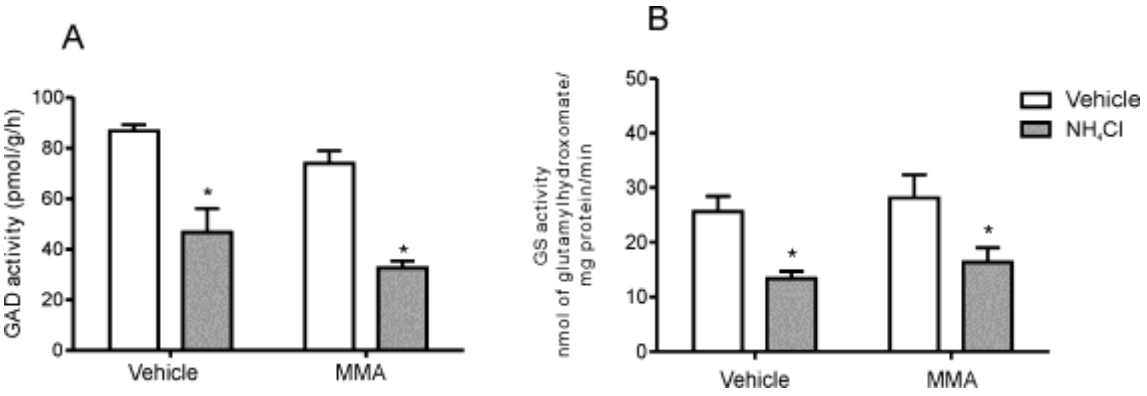
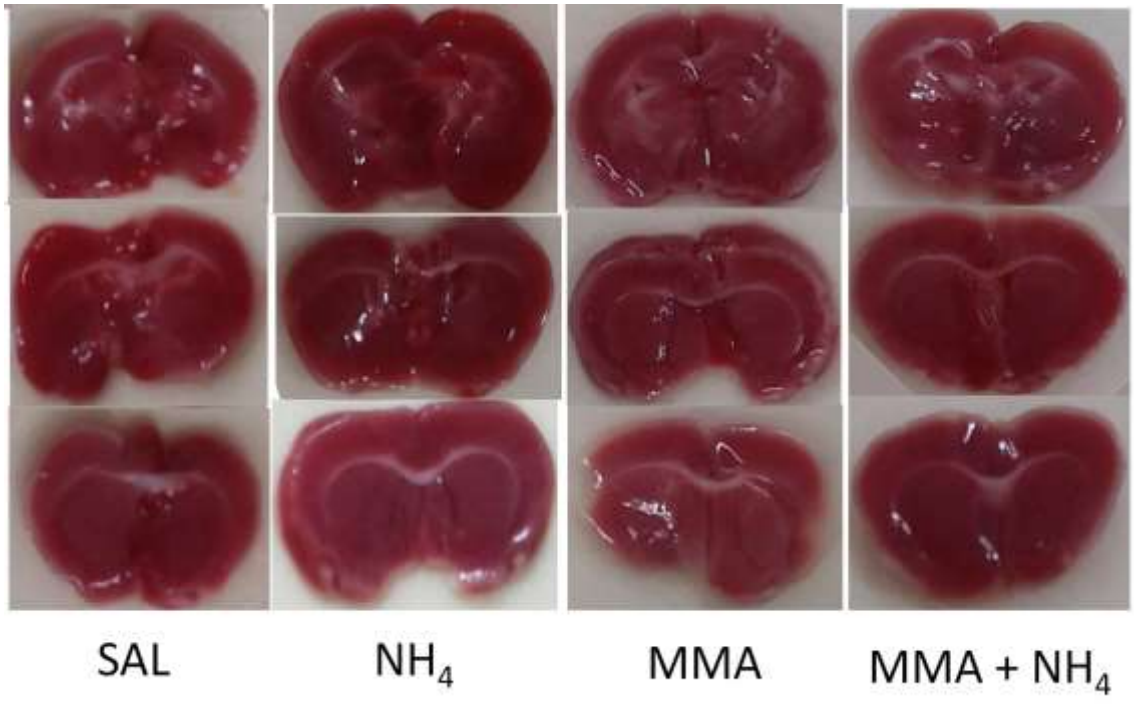


Figure11
Representative picture of absence of macroscopic cellular death after the administration of NH_4Cl (6 mmol/kg) and MMA (0.66 µmol/2 µL).



MAUSCRITO II

Ammonia role in glial dysfunction in methylmalonic academia in mice

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Abstract

Hyperammonemia is a common finding in patients with methylmalonic acidemia. However, its contribution to methylmalonate (MMA)-induced neurotoxicity is poorly understood. The aim of this study was evaluate whether an acute metabolic damage to brain during the neonatal period may disrupt cerebral development, leading to neurodevelopmental disorders, as memory deficit. Mice received a single intracerebroventricular dose of MMA and/or NH_4Cl, administered 12 hs after birth. The maze tests showed that MMA and NH_4Cl injected animals (21 and 40 days old) exhibited deficit in the working memory test, but not in the reference memory test. Furthermore, MMA and NH_4Cl increased the levels of 2',7'-dichlorofluorescein-diacetate (DCF), TNF-α, IL-1β in the cortex, hippocampus and striatum of mice. MMA and NH_4Cl also increased glial proliferation in all structures. Since the treatment of MMA and ammonia increased cytokines levels, we suggested that it might be a consequence of the glial activation induced by the acid and ammonia, leading to delay in the developing brain and contributing to behavioral alterations. However, this hypothesis is speculative in nature and more studies are needed to clarify this possibility.

Keywords
Cerebral cortex, striatum, hippocampus, memory, methylmalonate, glial proliferation.

1. Introduction

The methylmalonic acidemia is a heterogeneous group of autosomal recessive inborn errors of organic acid metabolism caused by tissue and body fluids 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) [1-4]. Most of the patients present an acute life-threatening metabolic crisis in the first months of life, which is usually precipitated by catabolic stress. The biochemical profile is characterized by metabolic acidosis and encephalopathic crises, hyperglycinemia, hypoglycemia, and hyperammonemia [5]. Furthermore, neurological features are also common in this disease, such as hypomyelination, cerebral atrophy, and neurodegeneration [6-9].

Approximately 70% of patients with methylmalonic acidemia are known to present hyperammonemia during the course of the disease [10]. These patients have an enlarged mitochondrial pool of acyl CoA esters, which leads to an inhibition of the urea cycle via decreased synthesis of N-acetylglutamate, an essential activator of carbamylphosphate synthetase [11]. In these metabolic diseases, the hyperammonemia is a symptom of metabolic decompensation that may result in chronic neurotoxicity and an impaired neurological outcome.

The severity of hyperammonemia can also be observed in other ammonia-metabolism disorders in which the elevated blood ammonia level causes mortality and delay of development [12, 13]. The mechanisms underlying ammonia-induced neurotoxicity, although not completely understood, seem to involve ATP depletion, activation of glutamatergic and GABAergic mechanisms, free-radical generation and inflammation [1, 12-15]. In line witt this view, Shawcross et al., [16] proposed that systemic inflammation exacerbates the neuropsychological alterations induced by hyperammonemia. They showed that hyperammonemia disrupted neuropsychological test scores during inflammatory state, but not after its resolution. Hyperammonemia increases also the sensitivity to immune challenges, for example, the injection of lipopolysaccharide (LPS) increases cytokine production similarly in normal or hyperammonemic mice [17]. However, the cognitive deficits induced by LPS were stronger and long-lasting in hyperammonemic mice. These reports support that hyperammonemia and inflammation cooperate in inducing cognitive deficits.

Furthermore, ammonia metabolism plays a pivotal role in astroglial cells [18, 19]. In fact, glutamine synthetase, the enzyme that detoxifies ammonia by condensing it with glutamate to form glutamine, is mainly found in astrocytes [20]. Astroglial dysfunction might, therefore, lead to nerve cell disease [21] and development delay of brain [22]. Many astroglial abnormalities have been reported in hyperammonemia, with astroglial edema among the most prominent [23].

The many changes in cell physiology induced by ammonia might have an effect on the cell cycle, and consequently on astroglial proliferation. In addition, primary or secondary astroglial damage has been implicated in several developmental or perinatal CNS pathologies [24, 25], suggesting that a vulnerability of glia and microglia in early stages of development may critically alter cerebral development and cause neurological abnormalities. However, the effects of ammonia on astroglial proliferation have been little documented.

Then, since studies have reported that methylmalonic acid induces cognitive impairment [26], inflammation [27, 28], and oxidative damage [29], it is plausible to verify if a ammonia administration induces cognitive impairment and glial dysfunction during the neonatal period after MMA injection as well as alters the inflammation and oxidative species production after MMA injection in the cerebral cortex, hippocampus and striatum of mice.

2.Experimental procedures

Ethics Statement

Laboratory experiments were performed in accordance with national and international legislations (Brazilian College of Animal Experimentation [COBEA] 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 Universidade Federal de Santa Maria (UFSM; Permit Number: 2067310115). 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. The mother fed pups 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) with free access to food (Guabi, Santa Maria, Brazil) and water. All efforts were made to minimize the number of animals used and their animals.

MMA and ammonia 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%) [30, 31] and one dose of ammonium acetate is administered intraperitoneally of 7.5 mmol/Kg on the second day of life for animals and 0.9% saline (0.9% SF) as control [32]. 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 (a mixture of local anesthetics (EMLA) cream (2.5% lidocaine/2.5% prilocaine; Ready & Edwards, 1992; Fish et al., 2007) and after injected directly in the 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) [33]. 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 [34]. The testing room was dimly illuminated with indirect white lighting.

Radial arm maze test

The maze consists of a wooden eight radial arms maze (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 [35, 36], 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 mice 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, radial arm maze 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 [37, 38]. 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 (CW, defined as the number of reentries into a baited arm; and “incorrect” working memory errors, defined as the number of reentries into an unabated arm [39, 40].

Elevated plus maze task

Based on the design of File and Gonzalez [41, 42] 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 mainly responsible for the non-enzymatic oxidation of DCF. DCF was prepared in 20 mmol/L sodium phosphate buffer, pH 7.4, containing 140 mmol/L KCI solution and incubated with 100 μL of supernatant for 30 min at 37°C. DCFDA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCF, which is rapidly oxidized and forms 2',7'-dichlorofluorescein (DCF), which is highly fluorescent in the presence of reactive oxygen species. The fluorescence intensity of DCF is proportional to the amount of reactive oxygen species formed. The fluorescence was then measured using excitation and emission wavelengths of 480 and 535 nm, respectively. The calibration curve was constructed using standard DCF concentrations (0.25-10 mmol), and the levels of reactive oxygen species were calculated as pmol DCF formed by mg/protein [43].

Western blot

The technique follows as described in [44], with some modifications. Proteins were then subjected to an 10% SDS-polyacrylamide gel electrophoresis Membranes were stained with 0.1% Ponceau solution served as the transfer control [45], photographed, washed with Tris-buffered saline, containing 0.04% (v/v) Tween 20 (TBS-T) until removal of the Ponceau. Each piece of membrane was blocked with 5% (w/v) BSA in TBS-T for 1 h. Incubated overnight at 4 ^{0}C with a 1:3000 dilution of anti-GFAP antibodies (Santa Cruz, sc-9065) in TBS containing 2.5% bovine serum albumin (BSA) in TBS-T. Before incubated for 1 h with anti-mouse secondary antibody conjugated with streptavidin peroxise (1:10000, Thermo Scientific, 31432) in TBS containing 2.5% BSA. Finally, membranes were developed in fotodocumentador (Carestrem Molecular imaging – Gel Logic 6000 PRO) for capturing luminescence produced by the ECL (pierce).

Immunohistochemistry Method

Animals were deeply anesthetized with sodium thiopental (i.p; 100 mg/kg; Cristalia, Brazil) and lidocaine hydrochloride (i.p; 10 mg/mL; Hypofarma, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Brazil, 20 mL/min) with 200 mL of 0.9% saline solution, followed by 200 mL of a fixative solution 4% paraformaldehyde (Synth, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed from the skulls, post-fixed in the same fixative solution at room temperature for 4 h and cryoprotected by immersion in 15 % and 30 % sucrose solution (Synth, Brazil) in PB at 4 °C until they sank. The brains were then quickly frozen in isopentane (Neon, Brazil), cooled in liquid nitrogen and kept in a freezer (−70 °C) for further analyses [46].

Coronal sections (50 µm thick) of the dorsal hippocampus, primary somatosensory cortex and striatum were obtained using a cryostat (CM1850, Leica, Germany) at −20 °C and collected in a PB saline (PBS), pH 7.4. These areas were identified using [47]. Every fourth section (200 µm apart) was processed for immunostaining.

The free-floating sections were pre-treated with 3% hydrogen peroxide for 30 min, carefully washed in PBS containing 0.5% Triton X-100 (PBS-Tx) for 30 min and incubated with polyclonal GFAP antiserum raised in rabbit (Dako, UK) diluted 1:1000 in 0.5 % of PBS-Tx and with 3 % bovine serum albumin (BSA; Sigma Aldrich, Germany) for 48 h at 4° C. Sections were again washed in PBS-Tx and incubated in anti-rabbit IgG Peroxidase antibody produced in goat (Sigma Aldrich, Germany) diluted 1:500 in PBS-Tx for 2 h at room temperature. The reaction was revealed in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma Aldrich, Germany) dissolved in PBS for 10 min and then 1 µL of 10% H2O2/mL was added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Canada balsam (Chemical Reaction, Brazil) and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS [46, 48].

Optical densitometry

Semi-quantitative densitometric analysis was used to measure the intensity of the GFAP immunoreaction using a Olympus CXN21FS1 microscope (200X, Japan) coupled to a CCD camera (Opton, Brazil) and Image J Software 1.50i (National

Institutes of Health, USA). The digitized images obtained from the selected areas were converted to an 8-bit gray scale. All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares measuring 5422 μm^2 (area of interest, AOI) overlaid on each image. Both left and right sides of each brain were used. For each rat, five images were taken from the hippocampus (CA1, CA3, DG), primary somatosensory cortex and striatum. In each image three AOI's were analyzed in the hippocampus, two in the primary somatosensory cortex and in the striatum. Background correction was done using the rolling ball radius method [49, 50]. The optical density (OD) was calculated using the following formula:

$OD = -\log[INT(x;y)-BL)]/(INC-BL)]$

Where "OD(x,y)" is the optical density at pixel(x,y), "INT(x,y)" or intensity is the intensity at pixel(x,y), "BL" or black is the intensity generated when no light goes through the material and "INC" is the intensity of the incidental light [51].

Protein determination

Protein content was measured colorimetrically by the method of Bradford [52], using bovine serum albumin (1 mg/ml) as standard.

Statistical analysis

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

Results

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 [$F(1,108)= 1.5$; $p>0.05$; Figure 2A) and [$F(1,180)=25.78$; $p>0.05$; 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[F (1,36)=0,56 p>0.05) and crossing [F(1,36)= 0,20; p>0.05; Figure 3A e 3B) and 40 days of life [F(1,34) = 0,66; p>0.05) and [F(1,34) = 0,0001; 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 percentage of time and entries in the open arms in the elevated plus maze did not show a significant drug (Saline, MMA, MMA/NH_4Cl and NH_4Cl) interaction in the 21-day-old (F(1,28) =0.08 and F(1,28) =1.16; respectively) and 40-day-old (F(1,28) = 0.86 and F(1,28) =8.9; respectively) animals. In addition, statistical analysis did not show a significant drug (Saline, MMA, MMA/NH_4Cl and NH_4Cl) interaction for the percentage of time and entries in the enclosed arms in the 21-day-old [F(1,28) =0.0005 and [F(1,28) =2.45; p>0.05; respectively) and 40-day-old [F(1,28) =0.01 and F(1,28) =2.51; p>0.05; respectively) animals, indicating that the treatment had no effect on anxiety-like behavior (Table 1).

Effect of MMA on the RAM Task

To investigate whether MMA and hyperammonemia treatment affects working and reference memory formation, the 21-day-old and 40-day-old pups were evaluated in the RAM task.

Statistical analysis revealed that MMA and hyperammonemia administration did not change the RME in 21-day-old [F(1,38)= 0.92; p>0.05; Figure 4A) and 40-day-old [F(1,38)= 0.36; p>0.05; data not shown) animals compared with their respective control groups. However, statistical analysis showed that MMA and hyperammonemia injection increased the CMWE in group of 21-day-old [F(1,38)= 18.36, p<0.05; Figure 4A) and 40-day-old [F(1,38)= 27.37, p<0.05; Figure 4C) animals. Furthermore, MMA and hyperammonemia injection increased the ICWME in 21-day-old [F(1,38)= 22.19, p<0.05; Figure 4B) and 40-day-old [F(1,38)= 28.51, p<0.05; Figure 4D) animals.

Effect of MMA on DCFH levels

As same studies suggest that oxidative stress may play the important role in brain damage induced by MMA [53-55], 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 and ammonia injection induced an increase on DCFH levels in the cerebral cortex [$F(1,28) = 15.97$, $p<0.01$; Figure 5A); [$F(1,28) = 2,49$, $p<0.01$, Figure 5D), striatum [$F (1,28) = 17.63$, $p<0.01$, Figure 5C); [$F(1,28) = 4.42$, $p<0.05$, Figure 5F) and hippocampus [$F(1,28) =3,61$, $p<0.01$, Figure 5B); [$F (1,28) = 15.64$, $p>0.04$, Figure 5E) in animals with 21 and 40 days of life, respectively.

Effect of MMA on the Inflammatory Biomarkers

The data studies also suggest that inflammatory process may play an important role in the brain damage induced by MMA and NH_4Cl [26, 28], 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 21- and 40-day-old mice.

The statistical analysis revealed that MMA and NH_4Cl injection increased TNF-α levels from the 21- and 40-day-old mice in the cerebral cortex [$F(1,28) =0.45$, $p<0.01$, Figure 6A); [$F(1,28) =0.42$, $p<0.01$, Figure 6D), hippocampus [$F(1,28) =27.56$, $p<0.001$, Figure 6B); [$F(1,28) =6,76$, $p<0.001$, Figure 6E) and striatum [$F(1,28) =53,63$, $p<0.001$, Figure 6C); [$F (1,28) =0.30$, $p<0.01$; Figure 6F), respectively.

Observed that MMA and NH4Cl injection induced an increase on IL-1β levels in the striatum [$F(1,28) = 5.75$, $p<0.01$, Figure 7C); [$F(1,28) =22.27$, $p<0.01$, Figure7F), and hippocampus an increase on IL-1β levels in 21-day-old mice, were not observed in 40-day-old mice in the hippocampus in increased IL-1β levels [$F(1,28) = 5.23$, $p<0.01$, Figure 7B); [$F (1,28) = 2.92$, $p>0.05$, Figure 7E). However, were observed increased IL-1β levels in the cortex in the 21and 40 day-old mice[($F (1,28) = 2.64$, $p>0.05$, Figure 7A) ; [$F(1,28) = 4.39$ $p>0.01$, Figure 7D).

Figure 8 and 9 show Western Blot of the cerebral cortex, striatum and hippocampus of mice with 21 and 40 days after MMA and NH4Cl administration. Representative photomicrographs of the astrocytes (green-GFAP marker) are shown.

GFAP is increased in the cerebral cortex, [F(1,16) =12.91, p<0.05, Figure 8A) ; [F(1,16) = 8.00, p<0.05, Figure 9A), striatum [F(1,16) = 14,22, p<0.01, Figure 8B); [F(1,16) =9,61, p<0.01; Figure 9B), and hippocampus [F(1,16) = 9,82, p<0.05, Figure 8C); [F(1,16) = 16,30, p<0.001; Figure 9C) in animals with 21 and 40 days after MMA and NH4Cl administration.

In the immunohistochemistry technique, MMA plus ammonia treatment increased glial proliferation in CA1 [F(1,16) = 5.414; p< 0.05; Figure 10) and CA3 [F(1,16) = 7.09; p< 0.05; Figure 10) in the animals with 40 days. However, we did not find significant alterations in other structures or in animals with 20 days (data not shown).

Discussion

Patients with methylmalonic acidemia and hyperammonemia, usually present acute clinical features early in life resulting from metabolic decompensation with respiratory distress and neurological symptoms, including psychomotor delay, irritability, lethargy progressing, hypotonia, convulsions, and coma. Most children survive to first acute metabolic crisis, but develop long-term complications including neurological and deficits of memory [56, 57]. 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 MMA is poorly understood.

Notably is known that patients and experimental models of this IEM with hyperammonemia exhibit neuronal damage and changes in several areas of the central nervous system (CNS), as well as in several other neurological diseases [6, 30, 58]. This is often related to oxidative stress and neuronal death, causing cognitive impairment and neuroinflammatory processes [59-61]. The present study shows for the first time evidence that neuroinflammation induced by an acute and transient metabolic insult with MMA and NH_4Cl is sufficient to initiate a pathological process, leading to cerebral structures dysfunction and cognitive impairment. We found that acute MMA and NH_4Cl administration at doses that raised its concentration in the brain of affected patients [30, 31] caused memory deficit and increased levels of DCFH in the cerebral cortex, hippocampus and striatum of 21- and 40-day-old mice. Interesting, MMA and ammonia increased IL-1β levels in the striatum and cerebral cortex as well as TNF- α level in the animals with 21- and 40-day-old mice.

It is important to observe that acute MMA and NH_4Cl administration had no effect on body weight, implying that acute injection did not cause malnutrition in 21- and 40-day-old animals (Figures 1 and 2), as well as the same treatment did not change the performance of mice in the open field (Figure 3) and elevated plus maze tasks, indicating that this organic acid was not anxiogenic in 21- and 40-day-old mice (Table 1).

As regard to the behavior of these mice, the results presented in this report show that the administration of MMA and NH_4Cl during the neonatal period is sufficient to trigger a deficit in the working memory (WM), suggesting that cognitive deficit can be related to the effect of acute MMA and NH_4Cl administration, mainly in the basal ganglia. In agreement with this view, it has been demonstrated that the WM is expressed as a disturbance of executive functions [62, 63] and that this alteration is generally related to disorders in the striatum being interpreted as an inability to inhibit ongoing action or as a failure to initiate a response [62].

Furthermore, it has been shown that the hippocampus is involved in reference memory tasks [64]. Specifically, it is important in establishing allocentric relations [65] and seems to be responsible for correct goal recognition [66]. Together with the hippocampus, prefrontal cortex, ventral striatum, anterior thalamus, and mammillary bodies cooperate in diverse memory processes and learning tasks [67, 68].

Interestingly, our experimental data revealed that present protocol of MMA and NH_4Cl injection had not effect on reference memory. However, how reciprocal connections of the striatum, prefrontal cortex and parietal cortex with the hippocampus are important to WM [69, 70], it is plausible to propose that hippocampal alterations cannot be enough to cause a spatial cognitive deficit, but can collaborate with WM impairment in this experimental model [71]. According, studies coincide with our results showing that methylmalonic acidemia and hyperammonemia patients develop cognitive impairment, mainly after catabolic or infectious events [72-74].

Inflammation and oxidative damage are also features that are found in patients with methylmalonic acidemia and hyperammonemia [28, 75, 76]. In fact, our data showed increased levels of DCFH in the cerebral cortex, hippocampus and striatum as well as increased IL-1β and TNF-α levels in brain of 21- and 40-day-old mice.

Current evidence indicates that cytokines, particularly IL-1β, increase neuronal excitability by activating IL-1 receptors as well as TNF-α by stimulates its receptors (TNFR) [77, 78]. The stimulation of cytokine receptors induces Src kinase-mediated tyrosine phosphorylation of the NR2B subunit in *N*-methyl-D-aspartate (NMDA) receptor. As a consequence, this activation facilitates NMDA receptor-mediated Ca^{2+} influx into neurons, promoting excitotoxicity [79]. Considering that IL-1β can also inhibit glutamate uptake in astrocytes [80] and increase its glial release possibly via TNF-α production [81] it is plausible to propose that increase of pro-inflammatory cytokines result in elevated extracellular glutamate levels and toxicity in this model of organic acidemia. In agreement of this view, previous work (Royes et al., 2016) shows that MMA and ammonia increase cerebral glutamate and DCFH levels, corroborating with our actual data. Furthermore, evidences have demonstrated that excessive glutamate receptor stimulation, in particular the NMDA receptor, has been implicated as a major pathway that leads to MMA-induced toxicity [82, 83].

Another interesting point is that TNF-α and IL-1β acts in their respective receptors and cause activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a transcription factor that migrates to the cell nucleus and promotes an increased production of oxidative species [84, 85]. In the present study we revealed that MMA and ammonia administration induced an increase of oxidative stress in the brain of rats.

In relation to neuroinflammation, experimental models have showed that hyperammonemia and MMA induce activation of astrocytes and microglia, increasing the levels of pro-inflammatory cytokines in the cerebral cortex [26, 86]. The glia is one of the cell types responsible for the production of pro-inflammatory cytokines such as IL-1β and TNF-α, which act as modulators of neurotransmissions within the brain [87]. In line with this view, we showed here that the combined treatment with MMA and ammonia induced glial proliferation, observed by Western blot activation, which coincided with increased TNF-α and IL-1β, proposing a link these events with WM deficit. Interesting, we observed that MMA plus ammonia induced a higher GFAP activation in animals with 40 days, however this was not occurred in 21 days. According to our data, [88] did not also observed astrocyte activation, but found an increased GFAP by Western blot technique in brain of the animals. A possible explanation for this situation may be because there are methodological differences

between Western blot and immunohistochemistry techniques, which may account for the discrepant results. However, this is only a supposition and would require more experiments to verify other glial markers in methylmalonic acidemia models.

Since the combined treatment of MMA and ammonia increased cytokines levels, we suggested that it might be a consequence of the glial activation induced by the acid and ammonia, leading to delay in the developing brain and contributing to behavioral alterations. However, this hypothesis is speculative in nature and more studies are needed to clarify this possibility.

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Table 1. Data are presented as the means ± S.E.M. for n = 10 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. R o percent of number of entries on encloses arms; % No.M o percent of number of entries on means the effects of early postnatal acute MMA e NH_4Cl administration on anxiolytic-like behavior of 21- and 40-day-old mice.

		21life				40life		
Group	Saline	MMA	MMA+ NH_4Cl	NH_4Cl	Saline	MMA	MMA+ NH_4Cl	NH_4Cl
% NO.E	3,657± 1.806	11.427± 6.866	51.046± 16.987	31.233± 12.802	26.745± 5.675	25.907± 7.095	11.958± 0.913	11.807± 3.057
% NO.E.R	71.030± 17.356	40.802± 18.700	63.434± 10.787	79.080± 7.175	92.697± 3.620	71.933± 10.903	89.827± 1.994	89.861± 2.956
% T.O	3,279± 3.069	9.660± 4.657	48.946± 18.153	46.956± 20.045	0.184± 0.184	10.211± 3.261	1.044± 0.178	1.270± 0.307
% T.E	79.497± 6.300	77.368± 5.252	31.046± 15.493	29.430± 13.381	91.466± 2.215	82.166± 3.314	65.900± 6.647	59.261± 11.716
% NO.M	48.276± 9.552	58.178± 6.545	85.804± 6.388	103.701± 11.458	48.276± 9.552	56.139± 5.896	91.496± 11.926	108,404± 4,246
N	7	7	7	7	7	7	7	7

FIGURE and LEGENDS

Figure 1

Representation of the experimental design of intra cisterna magna MMA administration (2.5 µmol/g) or vehicle sodium chloride (NaCl 0.9%) on the day of birth (PO) and ammonium acetate is administered intraperitoneally of 7.5 mmol/Kg on the second day of life for animals and 0.9% saline (0.9% SF) as control. The behavioral and biochemical analysis was conducted in the 21- and 40-day-old mice.

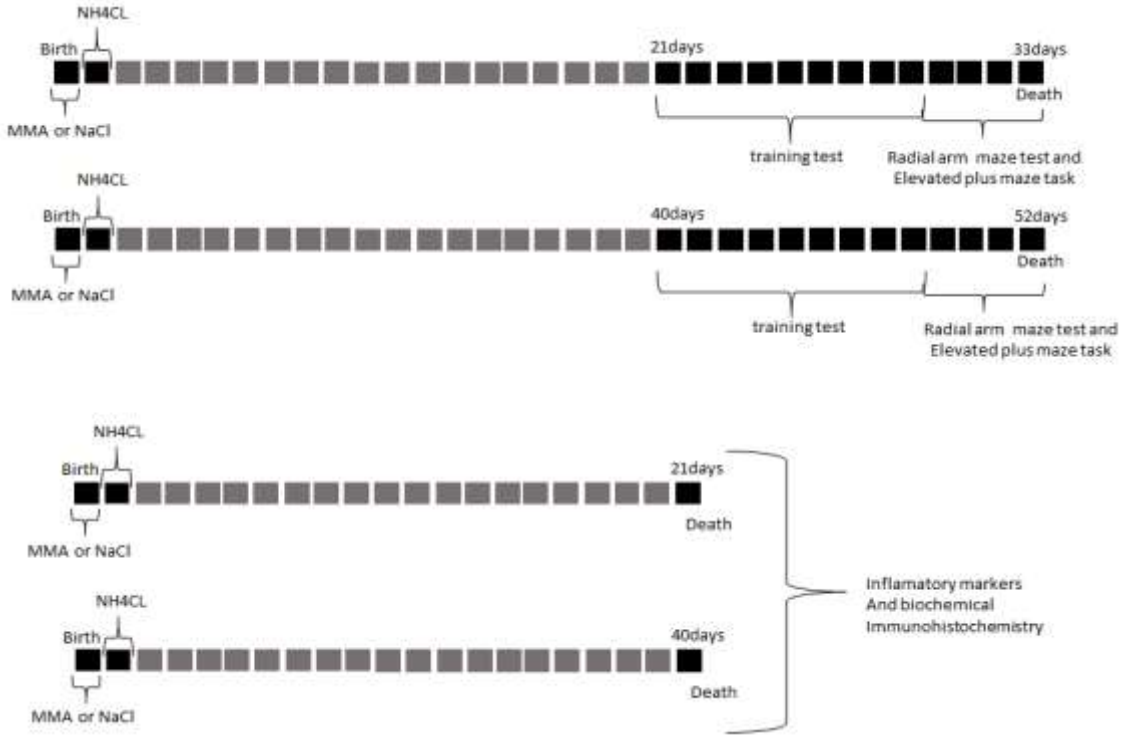


Figure 2.

Effects of postnatal acute MMA e NH_4Cl administration on the physical development of 21- (A) and 40-day-old (B) mice. Data are presented as the means ± S.E.M. for n =10 in each group.

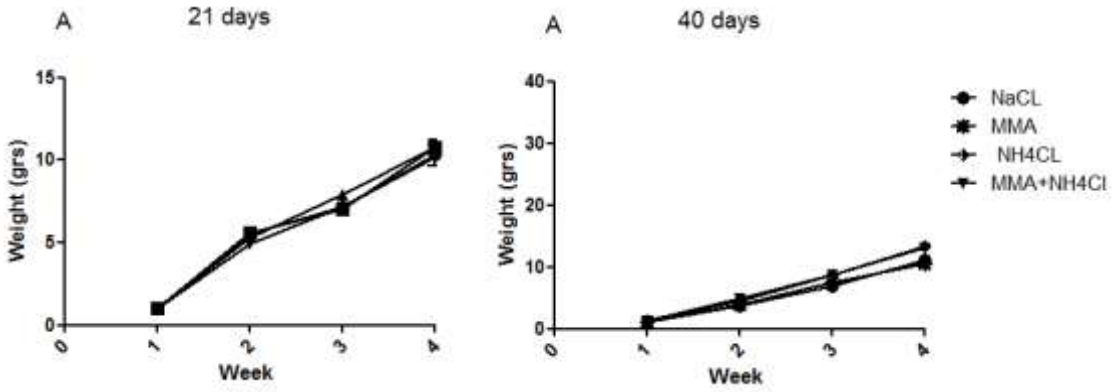


Figure 3.

Effect of postnatal acute MMA e NH_4Cl administration on the numbers of rearing (A, C) and crossings (B, D) of the 21- and 40-day-old mice, respectively. Data are presented as the means ± S.E.M. for n = 10-12 in each group.

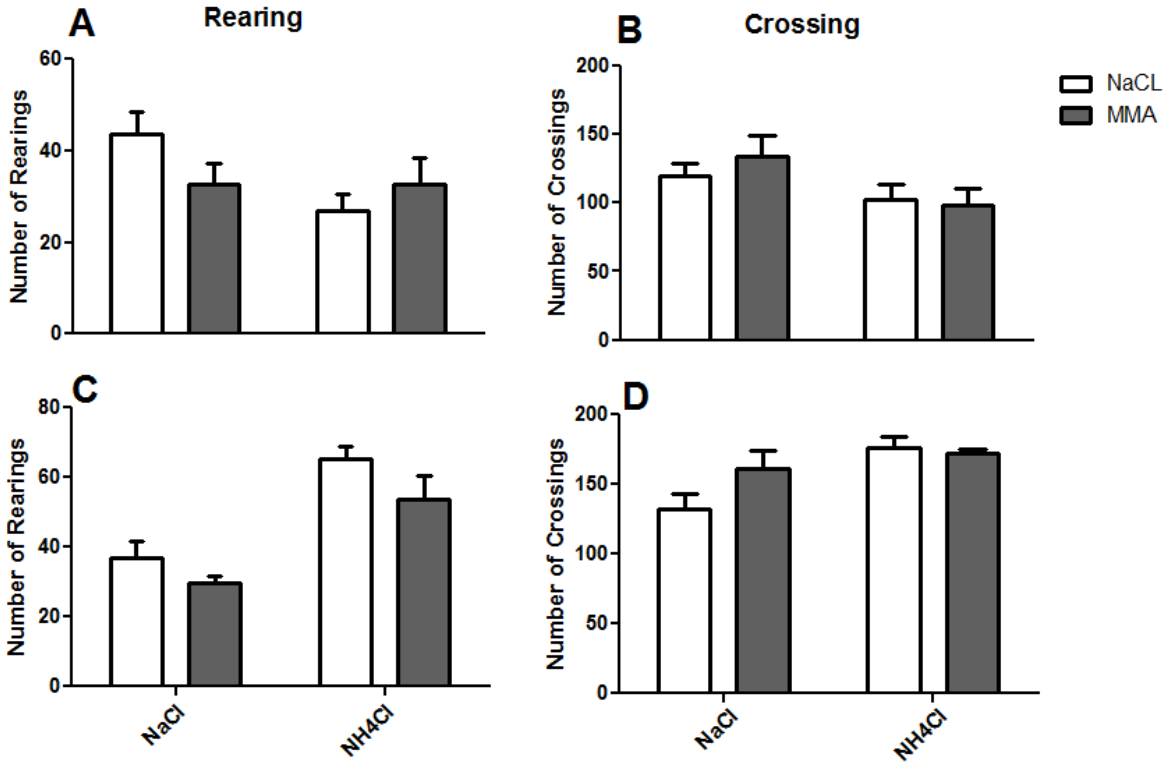


Figure 4.

Acute treatment with MMA e NH_4Cl induced a working memory deficit in 21 and 40-day-old mice. Data are expressed as the means ± S.E.M. for n = 10-12 in each

group. Significance was determined as *P < 0.05 when compared with the control group. (A,C) correct working memory errors (CWME) and (B,D) incorrect working memory errors (ICWME).

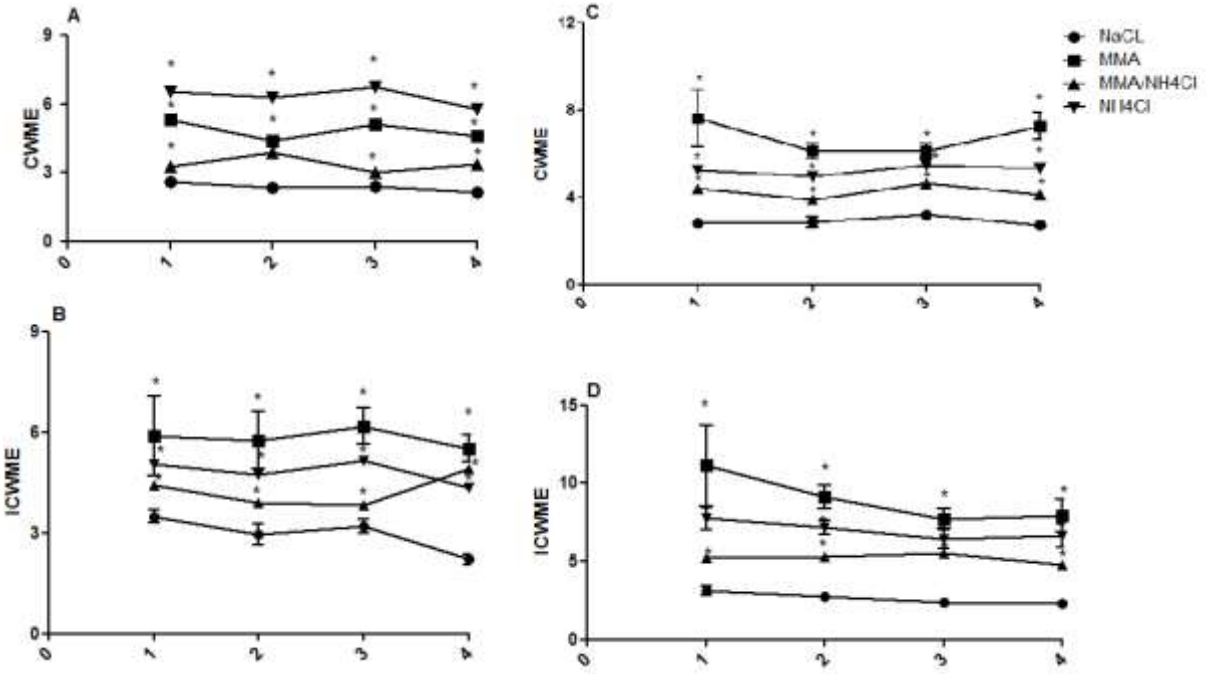


Figure 5.

MMA e NH_4Cl increased the mitochondrial DCFH levels in the cerebral cortex (A and D), hippocampus (B and E) and striatum (C and F) of the mice. Significance was determined as *P< 0.05 compared with the control. Data are the means + S.E.M. for n = 10 in each group.

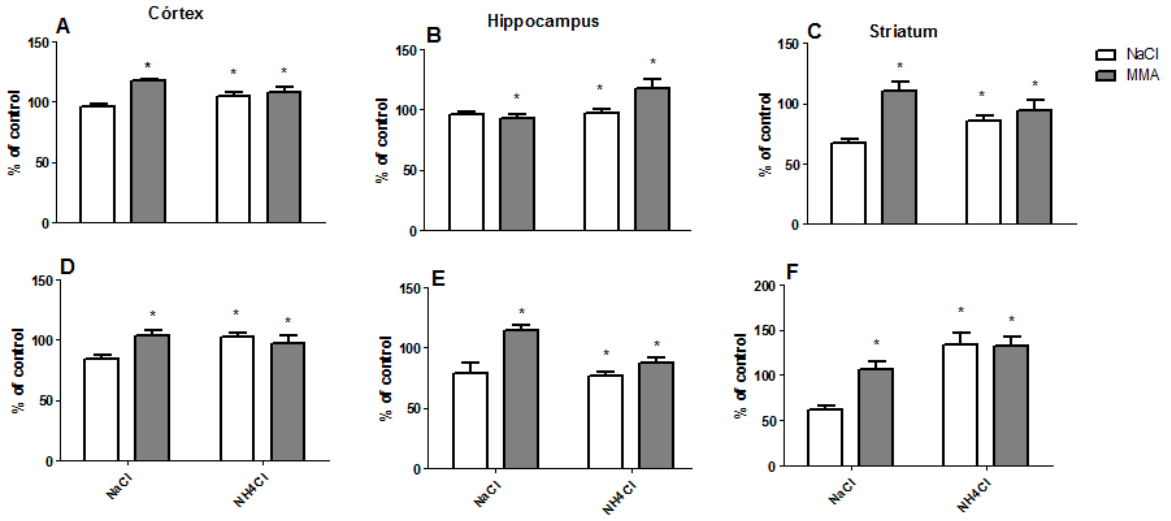


Figure 6.

MMA e NH_4Cl increased TNF-α levels in the cerebral cortex (A and D), hippocampus (B and E) and striatum (C and F) of the mice. Significance was determined as *P< 0.05 compared with the control. Data are the means + S.E.M. for n = 10 in each group.

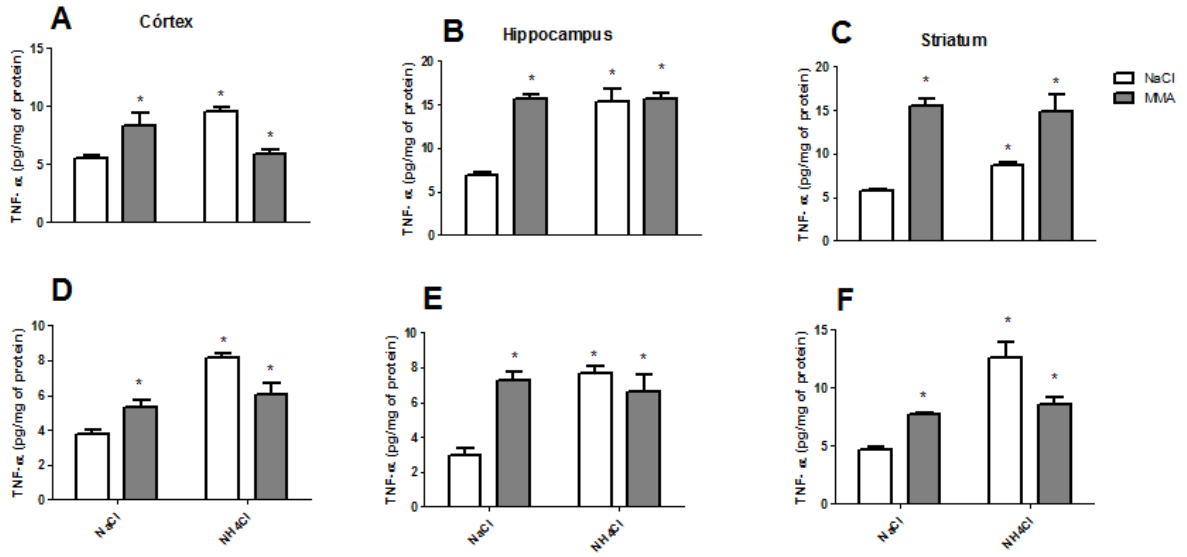


Figure 7.

MMA and *NH_4Cl* increased IL-1β levels in the cerebral cortex (A and D) and striatum (C and F) but not in the hippocampus (B and E) in 21- and 40-day-old mice. Significance was determined as *P< 0.05 compared with the control. Data are the means + S.E.M. for n = 10-15 in each group.

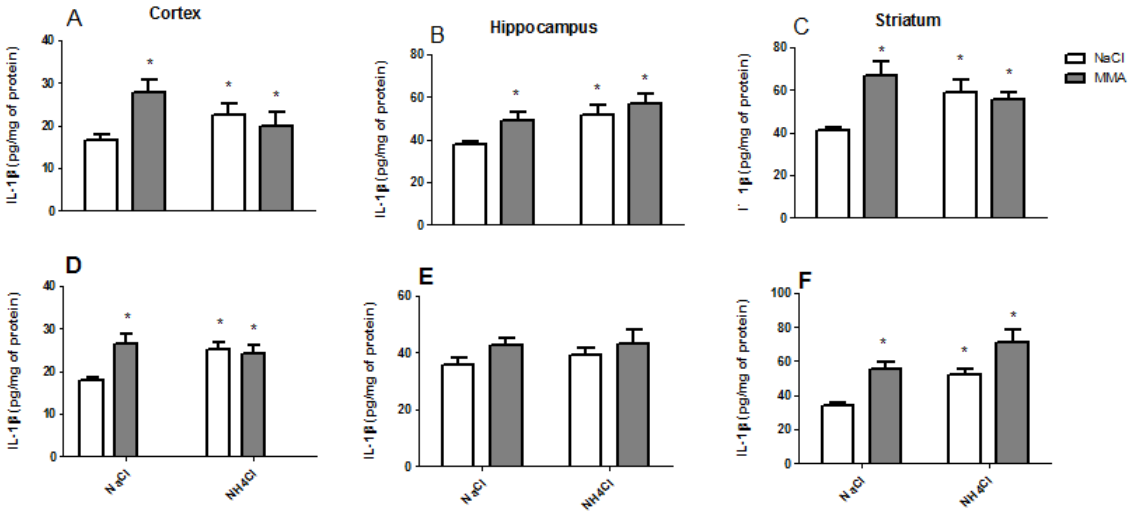


Figure 8.

MMA e NH_4Cl increased GFAP in the cerebral cortex (A), hippocampus (B) and striatum (C) in 21-day-old mice. Significance was determined as *P< 0.05 compared with the control. Data are the means + S.E.M. for n = 3-4 in each group.

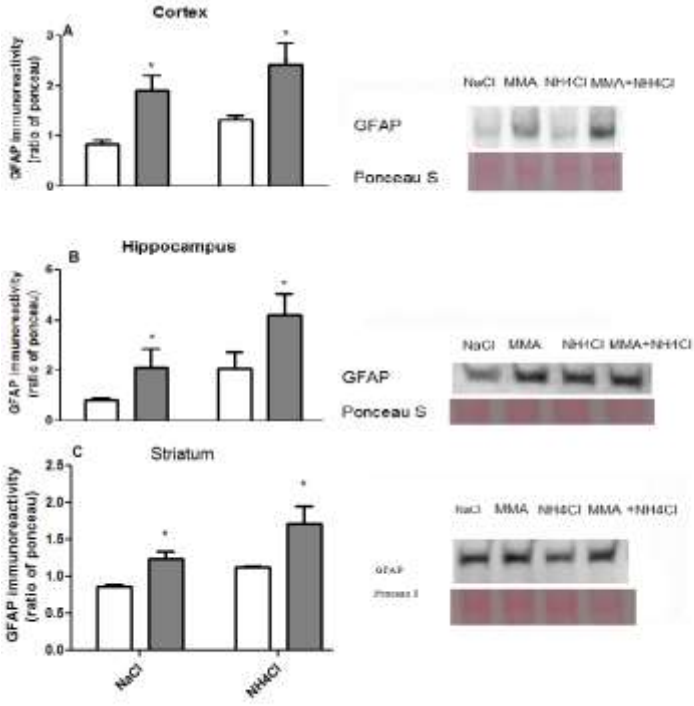
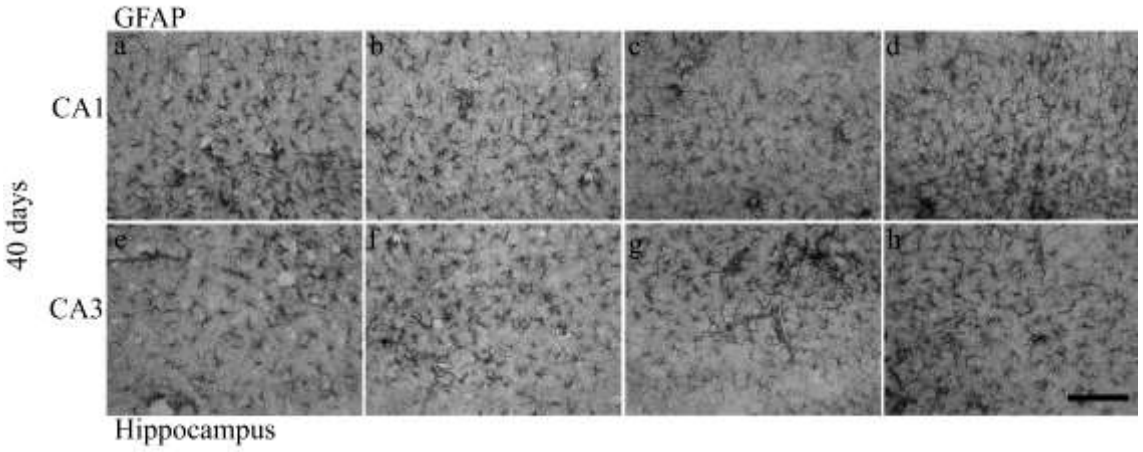


Figure 9.

MMA e NH_4Cl increased GFAP in the cerebral cortex (A), hippocampus (B) and striatum (C) in 40-day-old mice. Significance was determined as *P< 0.05 compared with the control. Data are the means + S.E.M. for n = 3-4 in each group.

Figure 10.

MMA e NH_4Cl *increases* astrocytes (green-GFAP marker) immunohistochemistry showing in the hippocampus 40 days after MMA administration in mice. Representative photomicrographs images (200X) of the CA1 (a, b, c, d), CA3 (e, f, g, h) of the hippocampus from a: saline; b: MMA; c: NH4; d: MMA+NH4 - treated mice. Data are the means + S.E.M. for n = 3-4 in each group. Scale bar: 100 μm



10. DISCUSSÃO

A acidemia metilmalônica é um erro inato do metabolismo que ocorre devido o acúmulo de ácidos orgânicos que levam a intoxicação aguda ou crônica devido à deficiência parcial ou total, na atividade da MCM EC (5.4.99.2) e defeitos na síntese 5´ deoxyadenosylcobalamin (AdoCbl), formada a partir da vitamina B_{12} (cobalamina, cbl) (CHANDLER et al., 2009; FENTON, W. A. R., L.E., 1995; ROYES et al., 2007). Essa acidemia também pode ocorrer devido a defeitos na captação, transporte ou síntese da AdoCbl, o co-fator da enzima, levando ao acúmulo primário de MMA e outros metabólitos nos tecidos e fluídos corporais dos pacientes (FENTON, W. A.; ROSENBERG, 1995; MANOLI; VENDITTI, 1993; SAUDUBRAY; SEDEL; WALTER, 2006).

Os sinais clínicos da acidemia podem aparecer ao nascer, em horas ou até semanas depois do nascimento, iniciando com sintomas gastrintestinais inespecíficos progredindo até manifestações neurológicas como retardo mental, déficit de memória, atrofia cerebral e convulsões, podendo culminar em coma ou morte (DEODATO et al., 2006; HARTING et al., 2008). Em relação aos achados

neuropatológicos, os pacientes podem apresentar lesão cerebral permanente com degeneração seletiva dos núcleos da base e do córtex cerebral (DEODATO et al., 2006; MANOLI; VENDITTI, 1993; O'SHEA et al., 2012).

A hiperamonemia é um dos principais achados na acidemia metilmalônica, e está presente em aproximadamente 70% dos pacientes por ela acometidos (FELIPO; BUTTERWORTH, 2002; O'SHEA et al., 2012). Esta alteração bioquímica parece estar associada à inibição dos ésteres de CoA que estão acumulados na atividade enzimática da N-acetilglutamato sintetase, com isso, diminuindo a conversão de amônia em uréia levando á hiperamonemia e consequentemente induzindo a neurotoxidade (BOSOI; ROSE, 2009; FELIPO; BUTTERWORTH, 2002; WALKER, 2009).

Estes quadros laboratoriais hiperamonêmicos geralmente são acompanhados por alterações clínicas, principalmente neurológicas, como desorientação mental e convulsões. Embora os mecanismos pelos quais a amônia induz neurotoxicidade ainda não estejam completamente esclarecidos, existem evidências de que os mesmos envolvam falência no metabolismo energético e deficiência na produção de ATP para as células gliais, principalmente nos astrócitos (KOSENKO et al., 1995; RAMA RAO; NORENBERG, 2012). Outro mecanismo proposto para a neurotoxicidade do MMA e da amônia seria a modulação de receptores glutamatérgicos e gabaérgicos, assim como, o estresse oxidativo/nitrosativo (MARISCO PDA et al., 2003; SCHOUSBOE; BAK; WAAGEPETERSEN, 2013). Esses metabólitos podem causar alterações na recaptação e liberação de neurotransmissores (FELIPO et al., 1994; ZHOU; NORENBERG, 1999), ou ainda um aumento na geração de espécies reativas ou uma redução na capacidade antioxidante celular (BOBERMIN et al., 2012; GORG et al., 2008; KOSENKO; FELIPO; et al., 1997; KOSENKO; KAMINSKY; et al., 1997; SKOWRONSKA; ZIELINSKA; ALBRECHT, 2010).

Estudos mostram que as concentrações tóxicas de amônia no SNC suprimem a atividade do GABA em níveis sinápticos e aumentam a produção de espécies reativas por modular os receptores NMDA (PAUL, 2003; RAABE, 1993). Nesse contexto, Marisco e colaboradores mostraram que ratos injetados com doses elevadas de MMA (3 µmol/2µL) e amônia (12 mmol/kg) apresentaram uma exacerbação do comportamento convulsivo, sugerindo que as convulsões induzidas

pelo MMA podem ser uma consequência da excitotoxicidade causada pela amônia (MARISCO PDA et al., 2003).

De fato, os dados apresentados nesta tese estão de acordo com a literatura e contribuem com novos conhecimentos a respeito das características EEGráficas e do dano secundário induzido pela amônia neste modelo de acidemia. O comportamento convulsivo induzido por doses intermediárias de amônia (6mmol/kg) e MMA (0,66 µmol/2µL) foi caracterizado por movimentos clônicos de cabeça, assim como, clonia da pata esquerda traseira e/ou dianteira, ocorrendo comcomitantemente com alterações no registro EEGráfico. As crises EEGráficas foram caraterizadas pela ocorrência de pelo menos duas das seguintes alterações: alta frequência e/ou complexos de polipontas e/ou pontas de alta voltagem.

Nos erros inatos do metabolismo, tem sido mostrado que a atividade das enzimas do ciclo da uréia são geralmente normais (PACKMAN et al., 1978b; ROYES et al., 2016a). Entretanto, pacientes com acidemia metilmalônica e propiônica mostram uma redução na atividade da carbamilfosfatase sintase e acúmulo de propionil CoA (ROYES et al., 2016a; WOLF, B. et al., 1978b). O acúmulo desses metabólitos na mitocôndria de células hepáticas de pacientes com acidemia metilmalônica conduz a inibição da biossíntese de N-acetilglutamato, que é um ativador da carbamoil fosfato sintase (COUDE et al., 1979a). Consequentemente, a amônia produzida pela degradação de proteínas não pode ser detoxificada no fígado e formar uréia, levando assim a um acúmulo de amônia no sangue e, possivelmente, no SNC (CAULI et al., 2007). Assim, os dados apresentados nesta tese mostraram que a administração aguda de amônia (6mmol/kg) foi capaz de aumentar a concentração deste metabólito no córtex cerebral e plasma após 5 min da injeção. Embora ainda é incerto como está distribuído o fluxo cerebral de amônia, podemos sugerir que o influxo de amônia cerebral ocorre por difusão passiva de NH_3 e transporte ativo de NH_4, aumentando sua concentração no SNC (OTT; VILSTRUP, 2014). É importante ressaltar que a amônia é um produto normal do metabolismo cerebral e, que em situações de hiperamonemia, ela pode se difundir do sangue para o cérebro e causar seus efeitos neurotóxicos (PAUL, 2003). Entretanto, após 20 minutos da administração combinada de amônia e MMA não se observou um aumento de amônia no plasma e no córtex cerebral de camundongos. Uma possível explicação para este fato seria

que no tempo de 20 minutos, a amônia já foi metabolizada pelo organismo. Pois pode ter ocorrido uma difusão, compartimentalização e metabolização dos compostos de uma maneira mais efetiva, causando uma redução das concentrações sanguíneas e cerebrais após 20 min da administração. Embora, não foi encontrado um aumento nas concentrações de amônia após 20 minutos da administração combinada de amônia e MMA, as alterações bioquímicas descritas nesta tese foram observadas neste período.

De fato, os dados literários a respeito dos efeitos da amônia no metabolismo cerebral são heterogêneos em termos de conclusão. Alguns autores descrevem que amônia poderia agir direta ou sinergicamente com citocinas inflamatórias derivadas de uma lesão neuronal ou processo infeccioso, ocasionando uma "quebra" da BHE e passagem de moléculas de diferentes tamanhos para o SNC (SKOWRONSKA; ALBRECHT, 2012). Isso provavelmente poderia precipitar ou exacerbar crises metabólicas, as quais causariam disfunções neurológicas, como as convulsões (DEODATO et al., 2004; DIONISI-VICI et al., 2006; SALVADORI et al., 2012). Entretanto, neste trabalho a dose intermediária de NH_4Cl não alterou os níveis de citocinas cerebrais (IL-1β, IL-6 e TNF-α) ou o extravasamento de fluoresceína no plasma (quebra da BHE), apenas as convulsões. Esses resultados sugerem que o comportamento convulsivo induzido pela amônia e MMA não é, pelo menos em parte, devido a uma resposta inflamatória precoce e ruptura da BHE.

Considerando que ambos, NH_4Cl e MMA, podem exercer efeitos danosos ao SNC por mecanismos relacionados a produção de ROS/RNS, foi investigado o efeito desses compostos na produção de espécies reativas neste modelo de acidemia orgânica. Os resultados mostraram que a administração combinada de amônia e MMA aumentaram os níveis de NOx cerebral e DCFH mitocondrial, sugerindo que o estresse oxidativo pode contribuir para a neurotoxicidade induzida pela amônia. Além disso, os dados estão de acordo com a idéia de que a oxidação de proteínas, nitração de tirosina e nitrosilação de resíduos de cisteína induzidos pelo acúmulo de amônia no SNC (GORG et al., 2008) podem contribuir para a excitabilidade cerebral nesta acidemia orgânica.

Outro achado interessante, foi a alteração do estado redox celular induzido pela amônia e MMA na função da mitocôndria, caracterizada por um menor potencial de membrana mitocondrial ($\Delta\Psi$), uma redução do MTT e da atividade da SDH. Estes

resultados reforçam a visão de que, em alguns erros inatos do metabolismo, incluindo a acidemia metilmalônica, a disfunção mitocondrial e estresse oxidativo, estão envolvidos nas convulsões induzidas pelo MMA. Além disso, o comportamento convulsivo pode ser uma consequência secundária da excitotoxicidade ou um déficit metabólico primário causado por metabólitos, tais como amônia.

Além disso, foi mostrado um aumento na atividade Na^+, K^+ -ATPase nos grupos tratados com MMA e NH_4Cl. Esses dados concordam com os dados de Malfatti et al. (MALFATTI et al., 2003) que monstraram uma ativação concomitante da atividade da Na^+, K^+ ATPase em córtex cerebral de animais tratados com MMA e crises convulsivas comportamentais e EEGráficas. O aumento da atividade cortical da Na^+, K^+ -ATPase evidenciado neste estudo, pode ser secundário à ativação das vias aferentes excitatórias para o córtex.

Neste sentido, é plausível propor que a combinação de NH_4Cl e MMA induza a uma desfosforilação e ativação da atividade Na^+,K^+ -ATPase aumentando o consumo de ATP e o conteúdo de Ca^{2+} mitocondrial, o que levaria a um aumento de espécies reativas. Essa geração de ERO e ERN pode inibir as enzimas do ciclo do ácido tricarboxílico e da cadeia respiratória, tais como a SDH, conduzindo à abertura do poro de transição, o que faz com que $\Delta\Psi$ mitocondrial e síntese de ATP entrem em colapso.

Outro achado interessante, foi a inibição da atividade da GS e da GAD, assim como, da ligação de $^{[3H]}$ flunitrazepam e da liberação de GABA por doses intermediárias de amônia. Embora as alterações nos níveis de NOx, atividades da GAD e GS são apenas no grupo tratado com amônia, a mesma dose de amônia por si só não teve efeito nas convulsões. Então, provavelmente, as alterações na concentração do glutamato e GABA provocadas pelo NH_4Cl levam a déficits seletivos na inibição GABAérgica e contribuem para a excitabilidade induzida pelo MMA.

Dessa forma, podemos sugerir que os efeitos da amônia e MMA nas atividades enzimáticas, alterações do estado redox e de neurotransmissores estão associadas ao aparecimento das convulsões registradas neste estudo.

Devido ao fato das crises encefalopáticas estarem relacionados com dano cerebral (JAFARI et al., 2013; KOWALTOWSKI et al., 2006; MORATH et al., 2008), foi avaliado se a administração de amônia e MMA poderiam causar alterações no parênquima cerebral dos animais. Interessantemente, os dados mostraram que a administração aguda dos compostos não causou lesão tecidual no cérebro dos animais. Embora o acúmulo de amônia é responsável pelo dano cerebral nos pacientes com acidemia metilmalônica, é plausível propor que a lesão neuronal causada pela amônia e as convulsões não necessariamente implicam em uma relação de causa e efeito entre esses eventos. Entretanto, esta explicação é especulativa e, outros estudos são necessários para determinar o mecanismo de dano neuronal induzido pela amônia e MMA.

Nos últimos anos, um dos maiores objetivos na pesquisa da acidemia metilmalônica foi esclarecer os mecanismos relacionados à disfunção neuronal, pois a compreensão das vias neuropatológicas seria crucial para o desenvolvimento de tratamentos preventivos. Nesse sentido, os resultados apresentados neste estudo demonstraram que a amônia modificou as crises EEGráficas induzidas pelo MMA, um efeito que não parece estar relacionado com uma resposta inflamatória precoce e ruptura da BHE. Por outro lado, a alteração no estado redox celular induzida pela amônia teve um impacto importante na função mitocondrial e no ciclo da glicina/glutamato/GABA, contribuíndo para a excitabilidade cerebral induzida pelo MMA e para o aparecimento das convulsões. Dessa forma, a demonstração de que a amônia e MMA apresentaram uma toxicidade aditiva, pode ser de grande valor na compreensão da fisiopatologia dos sinais neurológicos e contribuir para o desenvolvimento de novas estratégias para o tratamento dos pacientes com acidemia metilmalônica.

Além disso, os dados do manuscrito II também mostram, pela primeira vez, evidências que a inflamação induzida por uma dose aguda e transitória de um insult metabólico com MMA e NH_4Cl é suficiente para iniciar um processo patológico, conduzindo a disfunção de estruturas cerebrais e déficit cognitivo. Nossos dados mostraram que a injeção aguda de MMA e NH_4Cl, nas doses que costumam estar aumentadas no cerébro dos pacientes afetados (BRUSQUE, A. et al., 2001; HOFFMANN et al., 1993), causou déficit de memória e aumentou os níveis de DCFH no córtex cerebral, hipocampo e estriado dos camundongos com 21- e 40

dias. Interessantemente, MMA e amônia também aumentaram os níveis de IL-1β e TNF- α no cérebro dos animais.

Dados adicionais mostraram que a injeção aguda de MMA e NH_4Cl não apresentaram efeito no peso corporal dos animais. Isto é importante, uma vez que animais desnutridos podem apresentar um comportamento diferente em testes neurocomportamentais (DAVIS; LEVY, 1984; SEMINOTTI et al., 2012). Logo, as alterações no teste do labirinto radial, observadas nos animais tratados com MMA e amônia, não foram devido ao efeito nutricional. Da mesma forma, o desempenho dos animais no teste de ansiedade e no teste de locomoção e exploração não foram alterados 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 e amônia avaliando o aprendizado e a memória através do teste do radial, mostrando que a administração destes compostos, 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 (DEVAN et al., 1996; KIRKBY, 1969) 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 atraso para iniciar a próxima resposta (DEVAN 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 gIânglios da base, incluindo a acidemia metilmalônica (FENTON.W.A, 2001). Além disso, tem sido mostrado que o hipocampo está envolvido nas tarefas de memória de referência (D'HOOGE; DE DEYN, 2001), especificamente, em estabelecer relações alostéricas (MORRIS et al., 1982) e tarefas de reconhecimento (HOLLUP et al., 2001). Nesse contexto, junto com o hipocampo, o cortex pré-frontal, estriado ventral, tálamo anterior e corpos mamilares contribuem com diversos processos de memória e aprendizado (IRLE; MARKOWITSCH, 1982; KELLEY et al., 1982). Interessantemente, nossos dados revelaram que o MMA e ammonia não tiveram efeito na memoria de referência. Entretanto, como conexões recíprocas do estriado, cortex pré-frontal e parietal com o hipocampo são importantes para a memoria de trabalho (FARIDI et al., 2014; FUSTER, 2003), é plausível propor que as alterações hipocampais podem não ser

suficientes para causarem deficit na memória espacial, mas podem colaborar com o prejuízo na memória de trabalho. De acordo, outros estudos coincide com nossos dados, mostrando que pacientes com acidemia metilmalônica e a hiperamonemia desenvolvem déficit cognitivo e disfunção estriatal que podem ser observados nos exames de imagem, principalmente após crises catabólicas ou infecções (BINDU et al., 2010; IBANEZ-MICO et al., 2008; JOHANSSON et al., 2015).

A neuroinflamação e o estresse oxidativo também são características encontradas nos pacientes com acidemia metilmalônica e hiperamonemia (FIGHERA et al., 1999; HARTING et al., 2008; SALVADORI et al., 2012). De fato, nossos resultados mostraram níveis aumentados de DCFH, assim como, de IL-1β and TNF-α no cérebro dos animais tratados com MMA e ammonia.

De acordo com esses dados, modelos exprimentais mostraram que a hiperamonemia e MMA induzem ativação de atrócitos e microglia, aumentando os níveis de citocinas inflamatórias no cortex cerebral (HERNANDEZ-RABAZA et al., 2016; RIBEIRO et al., 2013b). A microglia é um dos tipos celulares responseveis pela produção de mediadores inflamatórios, como a IL-1β e TNF-α, que podem agir como moduladores de neurotransmissores no cérebro (MERRILL, 1992). Desde que a amônia e MMA aumentaram os níveis das citocinas, nós podemos sugerir que isto pode ser uma consequência da ativação glial induzida pelo ácido e NH_4Cl, conduzindo a um atraso no desenvolvimento cerebral e contribuindo com as alterações comportamentais. Entretanto, está hipótese é especulativa e mais estudos são necessarios para esclarecer esta possibilidade.

De acordo com esta visão, mostramos aqui que o tratamento combinado com MMA e amônia induziu a proliferação glial, observada pela ativação de Western blot, que coincidiu com o aumento do TNF-α e IL-1β, propondo um link desses eventos com déficit de WM. Interessante, observamos que o MMA mais amônia induziu maior ativação de GFAP em animais com 40 dias, porém isso não ocorreu em 21 dias.

Uma possível explicação para esta situação pode ser porque existem diferenças metodológicas entre Western blot e técnicas de imuno-histoquímica, que podem explicar os resultados discrepantes. No entanto, isso é apenas uma suposição e exigiria mais experiências para verificar outros marcadores gliais em modelos de acidemia metilmalônica.

Dessa forma, a administração única de MMA e amônia, por meio de mecanismos ainda não totalmente elucidados, podem causar e induzir ao aumento de fatores neuroinflamatórios durante períodos críticos de desenvolvimento, contribuindo para o quadro neurológico em pacientes com acidemia metilmalônica. Além disso, podemos concluir também que a administração única de MMA e amônia conduzem ao déficit cognitivo, demonstrando a importância do diagnóstico precoce, para que a intervenção imediata possa diminuir os efeitos da descompensação metabólica e suas sequelas.

10. Conclusão Parciais

1. O comportamento convulsivo induzido por doses intermediárias de amônia (6mmol/kg) e MMA (0,66 µmol/2µL) foi caracterizado por movimentos clônicos de cabeça, assim como, clonia da pata esquerda traseira e/ou dianteira, ocorrendo comcomitantemente com alterações no registro EEGráfico.
2. Caracterização episódios consistindo da ocorrência simultânea de pelo menos duas das seguintes alterações complexos alta frequência e ou poli pontas e ou atividade de ondas pontas sincronizada de alta voltagem.
3. A administração aguda de amônia (6mmol/kg) foi capaz de aumentar a concentração deste metabólito no córtex cerebral e plasma após 5 min da injeção. Entretanto, após 20 minutos da administração combinada de amônia e MMA não se observou um aumento de amônia no plasma e no córtex cerebral de camundongos.
4. A dose intermediária de NH_4Cl não alterou os níveis de citocinas cerebrais (IL-1β, IL-6 e TNF-α) ou o extravasamento de fluoresceína no plasma (quebra da BHE), apenas as convulsões.
5. A administração combinada de amônia e MMA aumentaram os níveis de NOx cerebral e DCFH mitocondrial, sugerindo que o estresse oxidativo pode contribuir para a neurotoxicidade induzida pela amônia.
6. A alteração do estado redox celular induzido pela amônia e MMA na função da mitocôndria, caracterizada por um menor potencial de membrana mitocondrial ($\Delta\Psi$), uma redução do MTT e da atividade da SDH.

7. O aumento da atividade cortical da Na^+, K^+ -ATPase , aumenta o consumo de ATP e o conteúdo de Ca^{2+} mitocondrial, o que levaria a um aumento de espécies reativas.
8. A inibição da atividade da GS e da GAD, assim como, da ligação de $^{[3H]}$ flunitrazepam e da liberação de GABA por doses intermediárias de amônia.
9. A alteração no estado redox celular induzida pela amônia teve um impacto importante na
10. função mitocondrial e no ciclo da glicina/glutamato/GABA, contribuindo para a excitabilidade cerebral induzida pelo MMA e para o aparecimento das convulsões.
11. provavelmente, as alterações na concentração do glutamato e GABA provocadas pelo NH_4Cl levam a déficits seletivos na inibição GABAérgica e contribuem para a excitabilidade induzida pelo MMA.
12. Os dados mostraram que a administração aguda dos compostos não causou lesão tecidual no cérebro dos animais.
13. 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.
14. O desempenho dos animais no teste de ansiedade e no teste de locomoção e exploração não foi alterado pelo tratamento.
15. 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.
16. 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.
17. MMA induziu a um aumento do DCFH no córtex cerebral, hipocampo e estriado dos filhotes de camundongos.
18. O aumento da atividade da AchE no corpo estriado, hipocampo e córtex cerebral de filhotes de camundongo contribuiu para o estado pró-inflamatório.
19. 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

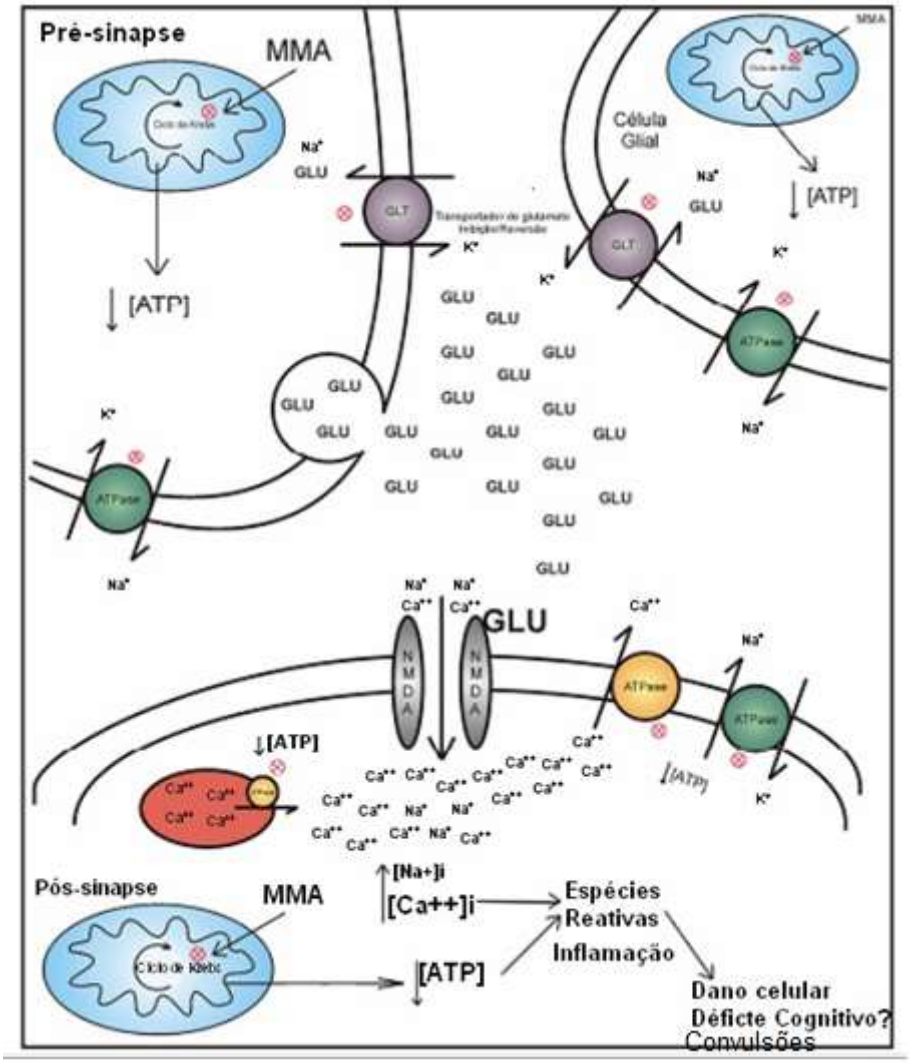
e 8) e consequentemente, no déficit cognitivo observado nos animais deste estudo.

20. 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.

Dessa forma nossos resultados sugerem que a amônia e MMA apresentaram uma toxicidade aditiva e que pode ser de grande valor na compreensão da fisiopatologia dos sinais neurológicos e contribuir para o desenvolvimento de novas estratégias para o tratamento dos pacientes com acidemia metilmalônica, assim como, administração única de MMA e NH_4Cl, por meio de mecanismos ainda não totalmente elucidados, podem causar e induzir ao aumento de fatores neuroinflamatórios durante períodos críticos de desenvolvimento, sendo assim, a importância do diagnóstico precoce, para que a intervenção imediata possa diminuir os efeitos da descompensação metabólica e suas sequelas.

Figura 5 – Esquema representativo da conclusão da presente Tese.

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.



Fonte: (Criado por Gabbi, 2017).

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Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Investigação dos mecanismos de neurotoxicidade do metilmalonato e amônia sobre a memória e nos parâmetros inflamatórios, oxidativos e imunohistoquímicos no cérebro de camundongos jovens.", protocolada sob o CEUA nº 2067310115, sob a responsabilidade de **Michele Rechia Fighera** *e equipe; Alexandra Seide Cardoso; Ana Leticia Fornari Caprara; Fernanda Haupenthal; Marla Perizzi Funghetto; Patricia Gabbi; Luiz Fernando Freire Royes* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 09/04/2015.

We certify that the proposal "Investigation of methylmalonate and ammonia neurotoxicity mechanisms on memory, inflammatory and oxidative parameters and immunohistochemistry damage in brain of young mice.", utilizing 160 Heterogenics mice (160 males), protocol number CEUA 2067310115, under the responsibility of **Michele Rechia Fighera** *and team; Alexandra Seide Cardoso; Ana Leticia Fornari Caprara; Fernanda Haupenthal; Marla Perizzi Funghetto; Patricia Gabbi; Luiz Fernando Freire Royes* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 04/09/2015.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2015 a 04/2017 Área: Neuropsiquiatria/neurologia

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	1 a 1 dias	N:	160
Linhagem:	Swiss			Peso:	1 a 1 g		

Resumo: Acidemia metilmalônica é um doença autossômica recessiva do metabolismo caracterizada bioquimicamente pela deficiência na atividade da metilmalonil-CoA mutase e pelo acúmulo tecidual de metilmalonato (MMA). Nessa acidemia, também ocorre o acúmulo de propionil-CoA, que causa a inibição do metabolismo da amônia pelo ciclo da uréia, levando a uma hiperamonemia. Clinicamente, essa acidemia caracteriza-se principalmente por alterações neurológicas, incluindo convulsões e déficit cognitivo. Além disso, quadros infecciosos e uma ingestão aumentada de proteínas são condições conhecidas por induzir um estado catabólico e precipitar os sintomas citados anteriormente. Em relação aos achados neuropatológicos, observa-se nos pacientes afetados pela acidemia um dano cerebral progressivo, principalmente no córtex cerebral e gânglios de base. Sabendo-se que o MMA causa complicações neurológicas, como o déficit cognitivo e alterações nos parâmetros bioquímicos em modelos experimentais dessa acidemia, o objetivo desse trabalho é investigar se a administração de MMA no período neonatal poderá causar prejuízo na memória de camundongos jovens e se essa alteração pode estar relacionada com aumento nos marcadores inflamatórios, assim como, nos parâmetros de morte neuronal e glial por meio de técnicas de imunohistoquímica. Além disso, considerando que a hiperamonemia está associada ao aparecimento de degeneração neuronal e déficit cognitivo nos pacientes com acidemia metilmalônica, é também importante investigar se a administração de amônia causará alterações nos parâmetros comportamentais, bioquímicos e imunohistoquímicos induzidos pelo MMA em camundongos.

Local do experimento: Laboratório de Bioquímica do Exercício

Santa Maria, 17 de julho de 2017

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Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
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