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EFEITOS IN VIVO DO ÁCIDO METILMALÔNICO SOBRE O COMPORTAMENTO DE RATOS NO LABIRINTO AQUÁTICO DE MORRIS E IN VITRO SOBRE ALGUNS PARÂMETROS DE ESTRESSE OXIDATIVO E SOBRE AS ATIVIDADES DOS COMPLEXOS I-IV DA CADEIA RESPIRATÓRIA EM HOMOGENEIZADO DE TECIDOS DE RATOS

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Nossa busca por descobertas alimenta nossa criatividade em todos os campos, não apenas na ciência. Se chegássemos ao fim da linha o espírito humano definharia e morreria. Mas não creio que um dia sossegaremos: aumentaremos em complexidade, se não em profundidade, e seremos sempre o centro de um horizonte de possibilidades em expansão.

Stephen Hawking

Aos meus pais e avós

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RESUMO

A acidemia metilmalônica é uma desordem metabólica hereditária caracterizada bioquimicamente pelo acúmulo tecidual de ácido metilmalônico (MMA) e clinicamente por deterioração neurológica progressiva e falência renal. Os avanços no tratamento dessa doença alcançados nos últimos anos possibilitaram uma diminuição significativa na mortalidade dos mesmos. Entretanto, a morbidade continua alta, pois a maioria dos pacientes afetados por acidemia metilmalônica, mesmo recebendo o melhor tratamento disponível no momento, apresenta graus variáveis de comprometimento do sistema nervoso central refletido em retardo mental e atraso no desenvolvimento psicomotor. No presente estudo investigamos os efeitos *in vivo* da administração crônica do MMA em ratos Wistar durante o seu desenvolvimento (do 5º ao 28º dia de vida pós-natal) sobre o comportamento dos mesmos na tarefa do labirinto aquático de Morris. A tarefa foi realizada após um período de recuperação dos animais de 30 dias com o intuito de verificar dano neurológico permanente ou de longa duração nos animais. O labirinto aquático de Morris é uma tarefa bastante útil para a avaliação de aprendizado e memória espaciais. O protocolo da tarefa foi ligeiramente modificado para servir ao propósito de nosso trabalho, ou seja, o de avaliar o efeito da administração crônica de drogas sobre o comportamento de ratos.

Verificamos que a administração crônica de MMA não provocou efeito no peso corporal, velocidade de natação e na fase de aquisição da tarefa. Porém, o tratamento prejudicou o desempenho dos animais no treino reverso, o que é condizente com comportamento perseverativo. Também avaliamos o efeito do ácido ascórbico, que foi administrado isoladamente ou em combinação com o MMA para testarmos se o estresse oxidativo poderia estar relacionado com as alterações comportamentais observadas no grupo tratado com MMA. Observamos que este antioxidante preveniu as alterações comportamentais provocadas pelo MMA, indicando que o estresse oxidativo pode estar envolvido com o efeito encontrado.

Passamos então a avaliar o efeito *in vitro* do MMA sobre parâmetros de estresse oxidativo, mais especificamente na técnica de dosagem de substâncias reativas ao ácido tiobarbitúrico (TBA-RS), que é um parâmetro de lipoperoxidação, e sobre o potencial antioxidante total do tecido (TRAP) e a reatividade antioxidante do tecido (TAR), que são parâmetros de defesas antioxidantes teciduais. O MMA na concentração de 2,5 mM aumentou a lipoperoxidação *in vitro* em homogeneizado de estriado e hipocampo de ratos e diminuiu o TRAP e o TAR em homogeneizado de estriado de ratos. Tais resultados indicam fortemente que o MMA induz estresse oxidativo.

Finalmente investigamos o efeito *in vitro* do MMA sobre a atividade enzimática dos complexos da cadeia respiratória em várias estruturas cerebrais e em órgãos periféricos em ratos de 30 dias de vida no sentido de melhor esclarecer os mecanismos fisiopatológicos dos danos teciduais desta doença. Verificamos que o MMA causou uma inibição significativa da atividade do complexo II da cadeia respiratória em estriado e hipocampo quando baixas concentrações de sucinato foram utilizadas no meio de incubação. Além disso, verificamos que este efeito inibitório do MMA sobre o complexo II ocorreu somente após exposição do homogeneizado ao ácido por pelo menos 10 minutos, além do que esta

inibição não foi prevenida pela co-incubação com o inibidor da óxido nítrico sintetase N^{ω}-nitro-L-argininametilester (L-NAME) ou por uma associação de catalase e superóxido dismutase. Estes resultados sugerem que as espécies reativas de oxigênio e nitrogênio mais comuns não estão envolvidas neste efeito, tornando improvável que a inibição do complexo II da cadeia respiratória seja mediada por estresse oxidativo. O MMA também causou uma inibição do complexo II-III em estriado, hipocampo, rim, fígado e coração; e inibiu o complexo I-III em fígado e rim. O complexo IV não foi afetado pela incubação com o ácido em nenhuma das estruturas testadas. Portanto, tomados em seu conjunto, estes resultados indicam que o MMA bloqueia a cadeia respiratória.

Os resultados de nosso trabalho indicam que a administração crônica de MMA em ratos em desenvolvimento provocou alterações comportamentais de longa duração provavelmente mediadas por radicais livres, pois tais alterações foram prevenidas pelo antioxidante ácido ascórbico. O MMA também induziu estresse oxidativo *in vitro* em estriado e hipocampo e inibiu de forma diferenciada os complexos da cadeia respiratória nos tecidos estudados, sendo que as estruturas mais vulneráveis a esta ação foram o estriado e o hipocampo. Finalmente, nossos resultados sugerem que antioxidantes podem ajudar a prevenir, ou pelo menos atenuar, os danos teciduais provocados pelo MMA na acidemia metilmalônica.

ABSTRACT

Methylmalonic acidemia is an inherited metabolic disorder biochemically characterized by tissue accumulation of methylmalonic acid (MMA) and clinically by progressive neurological deterioration and kidney failure. Despite of the improvement of therapy during the last 20 years, the overall outcome of methylmalonic acidemic patients remains disappointing and most of them present a variable degree of psychomotor delay/mental retardation. In the present study we investigated the *in vivo* effect of chronic administration of MMA (from 5th to 28th day of life) on the behavior of rats in the water maze task. In order to verify long term damage, the task was performed after a recover period of 30 days. Water maze is a very useful task to analyze learning and spatial memory. The protocol employed was modified to fit our study.

Chronic postnatal administration of MMA had no effect on body weight, swim speed, and on the acquisition of adult rats in the water maze task. However, MMA treatment provoked a long lasting reversal learning impairment in the rats, which is consistent with perseverative behavior. We also determined the effect of ascorbic acid administered alone or combined with MMA on the water maze task in order to test whether free radicals might be responsible for the behavioral changes observed in MMA-treated animals. Ascorbic acid was able to prevent the behavioral alterations provoked by MMA, indicating that the oxidative stress might be involved in this effect.

Regarding to the oxidative stress *in vitro* experiments, we also verified that MMA (2.5 mM) significantly increased the thiobarbituric acid-reactive substances (TBA-RS), which is a lipid oxidation parameter, in striatal and hippocampal homogenates and reduced total radical trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR), which are a antioxidant defense parameter, in the striatum.

Finally we investigated the in vitro effect of MMA on the activity of the respiratory chain complexes in cerebral and in peripheral structures of 30 day old rats. We verified that MMA caused an inhibition of complex II activity in striatum and hippocampus at low concentrations of succinate in the medium. This inhibitory property of MMA on complex II, only occurred after exposing brain homogenates for at least 10 minutes to the acid; this inhibition was not prevented by co-incubation with L-NAME, or by superoxide dismutase (SOD) plus catalase (CAT). These results suggest that common reactive oxygen and nitrogen species were not involved in this effect. MMA also caused an inhibition of complex II-III in striatum, hippocampus, kidney, liver, and heart, and inhibited complex I-III in liver and kidney. Complex IV was not inhibited in all tested structure. Taken together, these results indicate that MMA blocks the respiratory chain.

Our results indicate that chronic administration of MMA provoked long last behavior alterations, probably mediated by oxidative stress because they were prevented by the antioxidant ascorbic acid. MMA also induced *in vitro* oxidative stress in the striatum and hippocampus, and differentially affected the activity of the respiratory chain on the tissues studied, being the striatum and hippocampus more vulnerable to its effect. Finally, our results suggest that antioxidants may help to prevent or at least attenuate the damage provoked by MMA in methylmalonic acidemia.

Lista de Abreviaturas

AA ácido áscórbico
ATP adenosina trifosfato
CAT catalase
Cbl cobalamina
CNS do inglês, *central nervous system* (sistema nervoso central)
CoA coenzima A
COX citocromo oxidase
CSF do inglês, *cerebrospinal fluid* (líquido cerebroespinhal)
DCIP dicloroindofenol
EDTA ácido etileno-diamino-tetra-acético
EIM erros inatos do metabolismo
ETC do inglês, *eletron transport chain* (cadeia de transporte de elétrons)
$FADH_2$ flavina adenina dinucleotídeo
L-NAME N^{ω}-nitro-L-argininametilester
MA e MMA do inglês, *methylmalonic acid* (ácido metilmalônico)
MA+AA ácido metilmalônico co-administado com ácido ascórbico
NADH nicotinamida adenina dinucleotídio
NMDA n-metil-D-aspartato
3-NP 3 nitro-propiônico
PA do inglês, *propionic acid* (ácido propiônico)
ROS do inglês, *reactive oxygen species* (espécies reativas de oxigênio)
SETH sacarose, EDTA, trizma base, heparina
SMPs do inglês, *submitochondrial particles* (partículas submitocondriais)
SNC sistema nervoso central
SOD superóxido dismutase
TAR do inglês, *total antioxidant reactivity* (reatividade antioxidante total)
TBA-RS do inglês, *thiobarbituric acid reactive species* (substâncias reativas ao ácido tiobarbitúrico)
TRAP do inglês, *total radical-traping antioxidant potential* (potencial antioxidante total)

I INTRODUÇÃO

I.1 Acidemias Orgânicas

I.1.1 Definição

Acidemias orgânicas são distúrbios hereditários do metabolismo de aminoácidos, glicídios ou lipídios, causados por deficiência na atividade de uma enzima e caracterizados bioquimicamente pelo acúmulo de um ou mais ácidos orgânicos (carboxílicos) e/ou derivados em vários tecidos e líquidos biológicos dos indivíduos afetados (Chalmers e Lawson, 1982; Scriver et al., 2001; Cornejo e Raiman, 2003).

I.1.2 Acidemia Metilmalônica

I.1.2.1 Etiologia

A acidemia metilmalônica é causada pela deficiência da enzima L-metilmalonil-CoA mutase ou de seu cofator, a cianocobalamina. Aproximadamente 50% dos casos se devem a um defeito na enzima e podem se apresentar de duas formas: mut (o), quando a enzima não tem qualquer atividade e que não responde a altas doses de vitamina B12, e mut (-), quando a enzima tem uma pequena atividade quando o paciente recebe vitamina B12. Os demais casos se devem a defeitos na síntese e ativação (cblA, cblB, cblC, cblD e cblE) ou transporte da cobalamina (vitamina B12). Todos estes defeitos levam ao acúmulo do ácido metilmalônico e de seus metabólitos (Fenton, Gravel e Rosenblatt, 2001; Cornejo e

Raiman, 2003). A figura I.2 mostra a rota metabólica do ácido metilmalônico. A acidemia metilmalônica é uma das acidemias orgânicas mais freqüentes com incidência de 1: 48.000 nascidos vivos (Scriver et al., 2001).

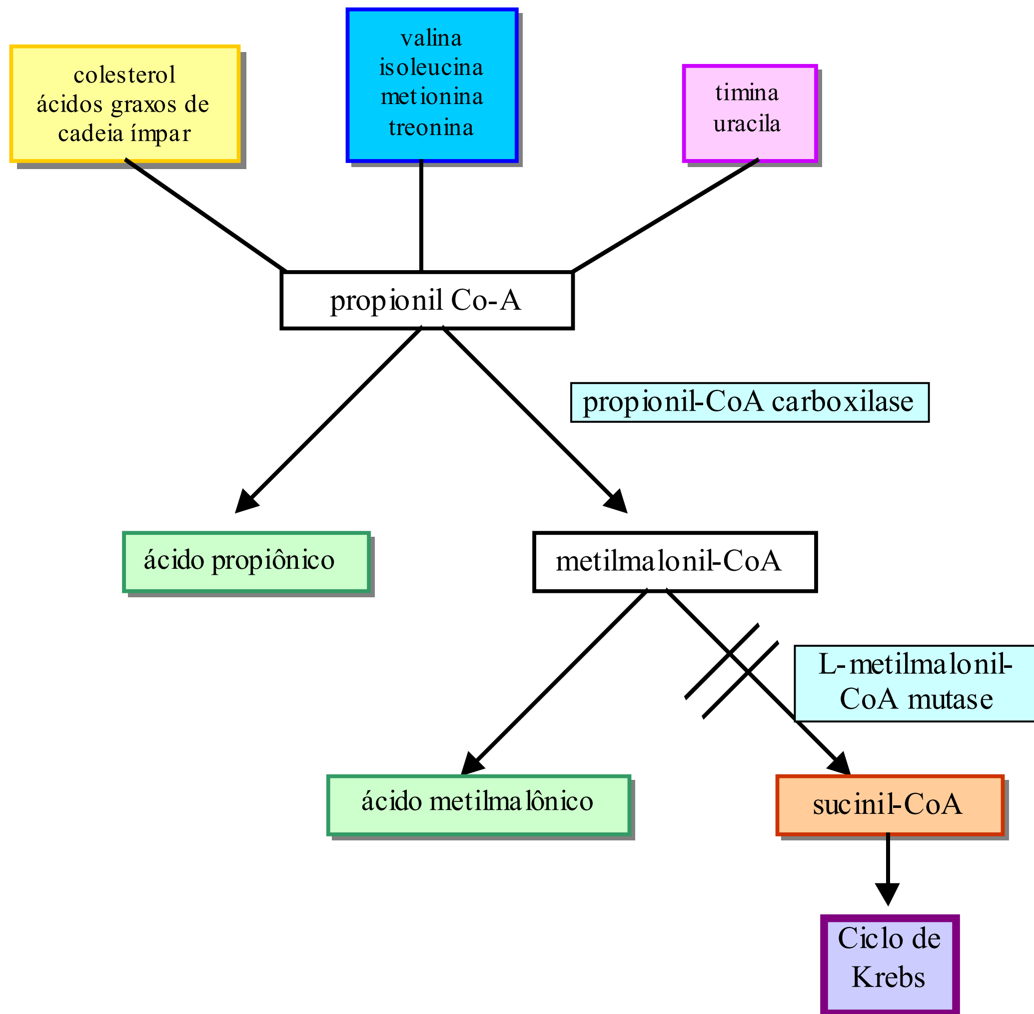

Figura I.2. Rota metabólica do ácido metilmalônico

I.1.2.2 Manifestações Clínicas

As manifestações clínicas da acidemia metilmalônica normalmente ocorrem na primeira semana de vida e são predominantemente neurológicas, com encefalopatia aguda grave ou crônica, letargia, hipotonia, retardo mental, atrofia cerebral, anormalidades no eletroencefalograma, apatia, coma e convulsões. Além das manifestações neurológicas, os pacientes acometidos por acidemia metilmalônica apresentam insuficiência renal crônica, caracterizada por nefrite tubulointersticial com acidose tubular, e diminuição da filtração glomerular (Cornejo e Raimann, 2003). Também ocorre recusa à alimentação, vômitos, desidratação, episódios de cetoacidose, apnéia e taquipnéia (Lehnert et al., 1994; Fenton, Gravel e Rosenblatt, 2001). Os pacientes que sobrevivem às crises apresentam um grau variável de atraso no desenvolvimento psicomotor e no nível de cognição (Van der Meer et al., 1994; Olgier de Baulny et al., 2005).

I.1.2.3 Achados Bioquímicos

A acidemia metilmalônica é caracterizada por grave acidose metabólica, hiperglicinemia e hiperamonemia. Nesta doença, o ácido metilmalônico é o metabólito que se acumula predominantemente, porém podemos encontrar também acúmulo de vários ácidos orgânicos, tais como propiônico, 3-OH-propiônico, β-hidroxibutírico, metilcítrico e também de propionil-CoA (Fenton, Gravel e Rosenblatt, 2001). Nestes pacientes também podemos encontrar: deficiência de carnitina, leucopenia, anemia, trombocitopenia e acidemia láctica (Lehnert et al., 1994).

I.1.2.4 Fisiopatogenia

Vários estudos demonstraram redução na produção de energia cerebral, na síntese de lipídios, bem como alterações na fosforilação de proteínas cerebrais causadas por metabólitos acumulados na acidemia metilmalônica. Foram descritos efeitos *in vivo* e *in vitro* importantes do ácido metilmalônico (MMA) sobre o sistema nervoso central, tais como a diminuição do conteúdo de gangliosídios (NANA/gangliosídios) (Wajner et al., 1988), bem como da utilização de glicose e de corpos cetônicos por prismas de cérebro, provavelmente devido à inibição das atividades da sucinato desidrogenase e da β-hidroxibutirato desidrogenase (Dutra et al., 1991; Dutra et al., 1993), bem como uma formação aumentada de lactato e uma redução na produção de CO_2 em cérebro de ratos jovens (Wajner et al., 1992).

Estudos mais recentes utilizando culturas de células de córtex e estriado de cérebro de embriões de ratos mostraram que a exposição destas células a 10 mM de metilmalonato por 24 horas causou uma mortalidade neuronal superior a 90%, sendo que as células corticais foram as mais vulneráveis (McLaughlin et al., 1998). Um decréscimo na relação ATP/ADP também foi observado após o tratamento das células com MMA, que foi atribuído à produção de malonato, um inibidor clássico da cadeia respiratória e do ciclo de Krebs, a partir de MMA (McLaughlin et al., 1998).Outros estudos também demonstraram a inibição do metabolismo energético pelo MMA (Wajner e Coelho, 1997; Brusque et al., 2002; Marisco et al., 2003; Royes et al., 2003; Fleck et al., 2004; Ostergaard et al., 2005).

Por outro lado, foi verificado que a administração intraestriatal de MMA provoca diminuição no conteúdo de fosfocreatina além de convulsões mediadas pelos receptores glutamatérgicos NMDA. Tais efeitos são prevenidos quando os animais são pré-tratados

por antagonistas destes receptores (Mello et al., 1996; Royes et al., 2003) e atenuadas quando os animais são pré-tratados com ácido ascórbico ou α-tocoferol, o que pode indicar o envolvimento de radicais livres nos episódios convulsivos (Fighera et al., 1999). No entanto outros investigadores não encontraram uma inibição direta do MMA sobre a cadeia respiratória, atribuindo o efeito demonstrado por este ácido ao malonato (Okun et al., 2002; Kolker et al., 2003). Outros estudos mostraram que o MMA altera vários parâmetros do sistema glutamatérgicos bem como estimula a produção de radicais livres *in vitro* (Brusque et al., 2001; Fontella et al., 2000).

Por outro lado, a fisiopatogenia do dano renal característico dos pacientes afetados pela acidemia metilmalônica, tem recebido pouca atenção. Um estudo conduzido por Kashtan e colaboradores (1998) demonstrou que o tratamento crônico com ácido metilmalônico em ratos causou proteinúria e injúria renal tubular, bem como edema mitocondrial e desorganização das cristas no epitélio tubular, sugerindo que uma disfunção mitocondrial pode estar envolvida na injúria renal encontrada nesta doença.

I.1.2.5 Diagnóstico e Tratamento

A acidemia metilmalônica é diagnosticada fundamentalmente pela presença de altas concentrações de ácido metilmalônico na urina. No sangue dos pacientes afetados as concentrações de MMA podem alcançar 2,9 mM, enquanto que em pessoas normais este ácido é praticamente indetectável. Além das altas concentrações de metilmalonato na urina, os pacientes portadores de acidemia metilmalônica apresentam altas concentrações de ácido propiônico e seus metabólitos. Porém, se forem encontradas quantidades excessivas de metilmalonato na urina, deve-se inicialmente descartar deficiência de cobalamina por dosagem da concentração de cobalamina sérica, já que esta vitamina é cofator da enzima

metilmalonil-CoA mutase. Estudos em células cultivadas podem ser realizados para se determinar se o defeito é na metilmalonil-CoA mutase ou no metabolismo da cobalamina (Fenton, Gravel e Rosenblatt, 2001).

O tratamento da acidemia metilmalônica é feito basicamente através da utilização de um leite especial (Propinex ®) pobre nos aminoácidos precursores do MMA em neonatos, bem como através de dieta com restrição protéica (0,5 a 1,5 g/kg/dia) ou preferencialmente por redução da ingestão dos aminoácidos precursores do propionato em infantes (Thomas, 1994). Uma outra estratégia terapêutica é baseada na administração paraenteral de altas quantidades de vitamina B12, o cofator da enzima L-metilmalonil-Coa mutase, visto que alguns pacientes respondem a esta terapia (variantes cblA, cblB, cblC, cblD e cblE). Episódios de cetoacidose devem ser tratados por retirada total de proteínas da dieta e administração parenteral de bicarbonato de sódio, bem como de glicose, que é necessária para evitar o catabolismo. Ataques agudos, particularmente aqueles acompanhados por hiperamonemia, podem ser tratados por diálise peritoneal e/ou hemodiálise. Nutrição parenteral total tem sido usada em pacientes gravemente doentes. A administração de altas doses (100 mg/Kg) de L-carnitina (Boehmer e Bremer, 1968; Olgier de Baulny et al., 2005) e também antibioticoterapia, que elimina a flora intestinal que contribui para a elevação das concentrações plasmáticas de propionato, também podem ser utilizados no tratamento desta doença (Thomas, 1994; Cornejo e Raimann, 2003; Olgier de Baulny et al., 2005).

I.2 Modelos Animais

O uso de modelos animais de doenças, incluindo os erros inatos do metabolismo, que simulem uma patologia no homem facilita o estudo da fisiopatologia das mesmas. Estes

modelos permitem abordagens impossíveis de serem realizadas com seres humanos, além de permitir a separação dos diversos fatores etiopatogênicos da doença (Lo, 1996). Deve-se, entretanto, salientar que nenhum modelo animal pode mimetizar completamente uma doença, mas auxilia muito na compreensão de sua fisiopatogenia (Herchkowitz, 1982).

Um modelo químico agudo para a acidemia metilmalônica foi proposto por Patel e colaboradores (1976), no qual ratos com uma semana de vida recebiam três injeções intraperitoneais de MMA, com duas horas de intervalo entre as aplicações. O grupo controle, mantido sob as mesmas condições, recebia injeções de solução salina (NaCl 0,9%). O modelo, embora satisfatório em mimetizar a doença para os fins propostos, causou a morte de 10% dos animais do grupo experimental, provavelmente devido à administração de altas concentrações de ácido metilmalônico.

Outro modelo experimental crônico de acidemia metilmalônica baseado na restrição de vitamina B_{12} foi descrito e utilizado por vários pesquisadores (Barnes, Young e Nocho, 1963; Williamson, Anderson e Browning, 1969; Orlando, Fiori e Costa, 1973). Este modelo, no entanto, foi abandonado, pois causava anemia megaloblástica nos animais, um sinal ausente nos pacientes com as formas clássicas de acidemia metilmalônica (Orlando, Fiori e Costa, 1973). Um outro modelo químico crônico para estudo da acidemia metilmalônica foi proposto por Dutra e colaboradores (1991), e consistia na administração através de injeções subcutâneas de MMA, duas vezes ao dia, do 5º ao 28º dia de vida, mimetizando os dois principais picos pós-prandiais de MMA encontrados nos pacientes com esta acidemia. As concentrações plasmáticas e cerebrais de MMA provocadas por este modelo foram de, respectivamente, 2,0-2,5 mM e 0,8-1,0 μmol/g (equivalente a 1 mM).

Mais recentemente foi criado um modelo *knockout* de acidemia metilmalônica baseado na homologia do *locus* da mutase humana com o *locus* do camundongo. Neste modelo os autores, utilizando técnicas de genes alvos, interromperam o *locus* da mutase do camundongo no domínio crítico para a ligação a CoA. No entanto, nesse modelo os animais morriam com cerca de 24 horas de vida pós-natal , impossibilitando estudos bioquímicos ou comportamentais (Peters et al., 2003).

I.3 Testes Comportamentais

O uso de tarefas comportamentais em animais para avaliar a neurotoxicidade de uma substância é fundamental, uma vez que, ao verificarmos o desempenho destes animais nestas tarefas, podemos avaliar o dano funcional. No entanto, convém salientar que uma relação direta entre o grau da lesão cerebral e o déficit cognitivo nem sempre ocorre. Extensas lesões podem não causar déficit cognitivo, enquanto que pequenas lesões podem gerar grandes conseqüências funcionais. Vários fatores podem contribuir para a dissociação entre o tamanho da lesão e o déficit funcional. Entre eles podemos citar a localização do dano cerebral, a habilidade prévia do animal, o tempo decorrido desde que ocorreu a lesão e as diferenças individuais. Independentemente destas observações, a realização e análise do desempenho de animais em tarefas comportamentais avaliam de uma forma clara as conseqüências funcionais de um dano neuronal (Olton e Markowska, 1994).

I.3.1 Aprendizado e Memória, Labirinto Aquático de Morris

Aprendizado e memória são funções básicas do SNC (sistema nervoso central) fundamentais para a adaptação de um organismo ao meio ambiente. O aprendizado pode ser

definido como a aquisição de informações através da experiência e a memória como o armazenamento de informações (Izquerdo, 1989). Ao escolhermos uma tarefa para avaliar o aprendizado e a memória de um animal, devemos levar em consideração o interesse e a capacidade do animal de aprender a tarefa e sua capacidade de executá-la. A tarefa do Labirinto Aquático de Morris é adequada para se avaliar cognição em ratos, uma vez que estes animais são bons nadadores e apresentam uma boa capacidade de localização espacial, que é requerida nesta tarefa. Por outro lado, a água é um meio aversivo para estes animais, que ao serem colocados na mesma procuram escapar. A tarefa do labirinto aquático de Morris é, portanto, adequada para a avaliação da integridade funcional de algumas estruturas do sistema nervoso central, como por exemplo, o hipocampo e estriado (Olton e Markowska, 1994; Save e Poucet, 2000).

I.4 Radicais Livres

I.4.1 Definição

Radical livre é qualquer espécie capaz de existência independente que contém um ou mais elétrons não pareados. Radicais livres são produzidos continuamente nas células como subproduto do metabolismo, ou deliberadamente durante alguns processos, como a fagocitose (Halliwell e Gutteridge, 1999). Os radicais livres também são denominados espécies reativas de oxigênio, e este termo abrange os radicais superóxido ($O_2^{\bullet-}$) onde um elétron não pareado é adicionado ao estado basal do O_2, os radicais hidroxila ($OH^{\bullet}$) peroxila ($RO_2^{\bullet}$) alcoxila, ($RO^{\bullet}$) e os derivados não radicais potencialmente oxidantes, como o peróxido de hidrogênio (H_2O_2), o ozônio (O_3), o ácido hipocloroso (HOCl) e os singletos

de oxigênio (Halliwell e Gutteridge, 1999). Além das espécies reativas de oxigênio, as espécies reativas do nitrogênio são potencialmente danosas. Entre elas podemos destacar o radical óxido nítrico, $NO^{\bullet}$ e o peróxido nítrico $ONOO^{-}$ (Beckman e Koppenol, 1996). Uma vez formados, estes compostos geram uma reação de oxidação em cadeia que tem como resultado a destruição, modificação ou inativação de um grande número de moléculas.

A geração de radicais livres é uma conseqüência natural da vida em um ambiente oxidante. As células geram pequenas quantidades de radicais livres enquanto realizam suas funções metabólicas normais. Assim os radicais superóxido e peróxido de hidrogênio são liberados da mitocôndria durante a síntese de ATP e da hemoglobina durante o transporte de oxigênio (Thomas, 2000).

I.4.2 Envolvimento de Radicais Livres em Doenças Neurodegenerativas

O cérebro é especialmente sensível ao estresse oxidativo por várias particularidades intrínsecas deste órgão, como por exemplo:

1) Alto consumo de oxigênio por unidade de massa de tecido;

2) Alto transporte de cálcio através de membranas neuronais, sendo que alterações na homeostase do cálcio podem gerar radicais livres;

3) Presença de aminoácidos excitatórios, como o glutamato, que podem desencadear mecanismos intracelulares de geração de espécies reativas de oxigênio.

4) Várias áreas do cérebro contêm altas concentrações de ferro (por exemplo, a substrancia nigra, o núcleo caudado, o putamen e o *globus palidus*) que estimula a reação de Fenton com produção do radical livre tóxico hidroxila;

5) Ao contrário do plasma, o líquor não possui moléculas com significante capacidade de ligação ao ferro livre;

6) As membranas neuronais contêm alta quantidade de ácidos graxos de cadeia lateral poliinsaturada que sofrem facilmente oxidação (peroxidação) por radicais livres;

7) O metabolismo cerebral é muito ativo gerando peróxido de hidrogênio. Um exemplo disso é a oxidação da dopamina pela monoaminaoxidase (MAO) e

8) O cérebro possui baixos níveis de defesas antioxidantes (Halliwell e Gutteridge, 1999).

Nos últimos anos, muita atenção tem sido focada nos radicais livres como mediadores de dano tecidual em doenças humanas. O estresse oxidativo é considerado um dos mecanismos patológicos do dano cerebral em várias doenças neurodegenerativas, incluindo as doenças de Parkinson, de Alzheimer e de Huntington (Halliwell, 1994; Delanty e Dichter, 1998; Halliwell e Gutteridge, 1999; Matés, Pérez-Gomez e Castro, 1999; Alexi et al., 2000). Estudos conduzidos por Fontella e colaboradores (2000) demonstraram que o ácido metilmalônico (MMA) induz a geração de radicais livres em córtex cerebral de ratos *in vitro*. Adicionalmente, o ácido ascórbico e o α-tocoferol atenuaram as convulsões produzidas pela administração intraestriatal de MMA em ratos (Fighera et al., 1999). Além disso, o tratamento com altas doses de ácido ascórbico resultou em melhora na recuperação de um paciente portador de acidemia metilmalônica após descompensação metabólica, a qual foi acompanhada por diminuição nos níveis de glutationa reduzida (Treacy et al., 1996). Essas observações sugerem, portanto, que os radicais livres estejam envolvidos na patofisiologia da acidemia metilmalônica.

I.5 Fosforilação Oxidativa

I.5.1 Definição

A fosforilação oxidativa é o processo pelo qual o O_2 é reduzido à H_2O, por elétrons doados pelo NADH e $FADH_2$, que fluem através de vários pares redox (cadeia respiratória), gerando ATP a partir de ADP e Pi (Nelson e Cox, 2000). Em eucariotos, a fosforilação oxidativa ocorre nas mitocôndrias, mais especificamente na cadeia respiratória, e é responsável pela maior parte da energia produzida pela célula.

As mitocôndrias são corpúsculos envoltos por uma membrana externa, facilmente permeável a pequenas moléculas e íons, e por uma membrana interna, impermeável à maioria das moléculas e íons, incluindo prótons H^+ (Nelson e Cox, 2000). A membrana interna contém transportadores específicos para a passagem de substâncias como o piruvato, glicerolfosfato, malato, ácidos graxos e outras moléculas essenciais às funções mitocondriais (Abeles, Frey e Jencks, 1992). O fluxo de elétrons do NADH e $FADH_2$ até o O_2 se dá através de complexos enzimáticos ancorados na membrana mitocondrial interna. Essa transferência de elétrons é impulsionada por um crescente potencial redox existente entre o NADH e o $FADH_2$, os outros complexos enzimáticos da cadeia respiratória e o O_2, que é o aceptor final dessa cadeia de reações redox.

A cadeia respiratória é composta por vários complexos enzimáticos e uma coenzima lipossolúvel, a coenzima Q ou ubiquinona (Di Donato, 2000). O complexo I, conhecido como NADH desidrogenase ou NADH: ubiquinona oxidorredutase, transfere os elétrons do NADH para a ubiquinona. O complexo II (sucinato desidrogenase) reduz a ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do sucinato a fumarato no ciclo do ácido

cítrico. O complexo III, citocromo bc_1 ou ubiquinona-citocromo c oxidorredutase, catalisa a redução do citocromo c a partir da ubiquinona reduzida. O complexo IV, conhecido como citocromo oxidase, catalisa a transferência dos elétrons do citocromo c para o O_2, reduzindo-o a H_2O. Todos esses complexos possuem grupamentos prostéticos específicos para desempenharem o papel de aceptores e doadores de elétrons (Abeles, Frey e Jencks, 1992).

O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Com isso, cria-se um gradiente eletroquímico transmembrana utilizado por um quinto complexo proteico, a ATP sintase, para a síntese de ATP. Dessa forma, a oxidação de substratos energéticos está acoplada ao processo de fosforilação do ADP, ou seja, quando o fluxo de prótons volta a favor do gradiente eletroquímico, a energia liberada é utilizada pela ATP sintase, que funciona como uma bomba de prótons ATP dependente (Nelson e Cox, 2000).

I.6 Relação entre Inibição da Cadeia Respiratória, Estresse Oxidativo e Excitotoxicidade

A origem primária dos danos encontrados em doenças neurodegenerativas ainda é controversa. Porém vários estudos demonstram que a inibição da cadeia respiratória, o estresse oxidativo e a excitotoxicidade são eventos relacionados e estão envolvidos na fisiopatogenia destas doenças (Schulz, et al., 1996a; Schulz, et al., 1996b; Swerdlow et al., 1996; Swerdlow et al., 1997). Defeitos na cadeia respiratória podem levar a estresse oxidativo, que pode, por sua vez, danificar os complexos da cadeia respiratória, gerando um ciclo vicioso que pode resultar na morte da célula. Por exemplo, a rotenona, inibidor do complexo I, a azida, inibidor do complexo IV e o malonato, inibidor da sucinato

desidrogenase, aumentam a produção de radicais livres *in vivo* e *in vitro* (Schulz, et al., 1996a; Schulz, et al., 1996b; Cassarino e Bennet Jr, 1999). Por outro lado, grupamentos Fe-S, presentes no complexo I da cadeia respiratória e na sucinato desidrogenase, são especialmente sensíveis ao ataque de radicais superóxido, que são produzidos pela própria cadeia respiratória (Raha e Robinson, 2000). Além disso, substratos energéticos apresentam efeitos neuroprotetores que podem ser aumentados por antioxidantes (Schultz et al., 1996a). Déficit energético pode também resultar em excitotoxicidade por acúmulo de glutamato na fenda sináptica, devido a déficit na captação de glutamato pelas células da glia (excitotoxicidade secundária), ou por liberação de magnésio dos canais ionotrópicos NMDA, levando à entrada de cálcio na célula. O influxo de cálcio na célula, causado por excitotoxicidade primária (superestimulação de receptores glutamatérgicos) ou secundária (redução do metabolismo energético), por sua vez pode causar estresse oxidativo e levar secundariamente à inibição da cadeia respiratória (Nicholls e Budd, 2000; Raha e Robinson, 2000). Portanto, o déficit no metabolismo energético, o estresse oxidativo e a excitotoxicidade são eventos interrelacionados que podem levar à morte celular. Neste sentido é importante definir o evento primordial para que a cascata não seja iniciada e prevenir de forma mais eficaz a morte celular.

II OBJETIVOS

II.1 Objetivo Geral

Apesar da melhora nos últimos anos das terapias utilizadas para o tratamento da acidemia metilmalônica que diminuiu significativamente a mortalidade, a morbidade dos pacientes acometidos por esta enfermidade ainda é alta, com predomínio de disfunção cerebral e renal de grau variável. A maioria deles apresenta variável grau de retardo mental e de desenvolvimento físico, além de insuficiência renal. Acredita-se que isso se deva à falta de conhecimento dos mecanismos responsáveis pelas lesões teciduais características destes pacientes.

O objetivo da presente investigação foi, portanto, o de estudar o efeito crônico *in vivo* da administração do ácido metilmalônico (MMA) a ratos em desenvolvimento sobre o comportamento dos mesmos na tarefa do labirinto aquático de Morris, bem como estudar os possíveis mecanismos das alterações comportamentais apresentadas pelos animais, com especial ênfase para o metabolismo energético e o estresse oxidativo na expectativa de esclarecer a fisiopatogenia dos danos teciduais nessa doença. Também objetivamos investigar o efeito do antioxidante ácido ascórbico sobre as alterações comportamentais possivelmente causadas pelo MMA. Finalmente objetivamos estudar o efeito *in vitro* do MMA sobre alguns parâmetros de estresse oxidativo em estruturas cerebrais e sobre os vários complexos da cadeia respiratória em várias estruturas cerebrais e tecidos periféricos de ratos jovens para avaliar se o MMA tem um efeito seletivo para o sistema nervoso central, ou é capaz de provocar efeitos deletérios em outros tecidos.

II.2 Objetivos Específicos

1. Padronizar uma técnica de tarefa comportamental para avaliar danos de longa duração provocados pela administração crônica de ácido metilmalônico a animais jovens. Para isto, escolhemos a tarefa do labirinto aquático de Morris que é efetiva para a avaliação de aprendizado e memória espaciais.
2. Verificar se o co-tratamento com antioxidantes é capaz de prevenir os efeitos provocados pelo MMA nesta tarefa.
3. Estudar o efeito do MMA sobre parâmetros bioquímico de estresse oxidativo em estruturas cerebrais de ratos na tentativa de melhor explicar os resultados encontrados na tarefa comportamental e testar se o MMA pode induzir lipoperoxidação e alterar as defesas antioxidantes cerebrais *in vitro.*
4. Estudar o efeito *in vitro* do MMA sobre as atividades enzimáticas de vários complexos da cadeia respiratória em estriado e hipocampo de ratos, bem como nos tecidos periféricos rim, fígado e coração de ratos com a finalidade de verificar se o MMA pode comprometer o metabolismo energético nos vários tecidos estudados. Neste particular, um objetivo importante foi o de estudar o efeito do MMA sobre a atividade da cadeia respiratória em rim de ratos com a finalidade de avaliar se os danos renais encontrados nos pacientes podem ser atribuídos a uma inibição do metabolismo energético.

III METODOLOGIA, RESULTADOS E DISCUSSÃO

III.1 Artigo 1

Evaluation of the effect of chronic administration of drugs on rat behavior in the water maze task

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Protocols

Evaluation of the effect of chronic administration of drugs on rat behavior in the water maze task

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Abstract

Tissue accumulation of intermediates of the metabolism occurs in various inherited neurodegenerative disorders, including methylmalonic acidemia (MA). Animal cognition is usually tested by measuring learning/memory of rats in behavioral tasks. A procedure in which rats are chronically injected with the metabolites accumulating in the neurometabolic disorder methylmalonic acidemia from the 5th to the 28th day of life is described. The animals were allowed to recover for approximately 30 days, after which they were submitted to the Morris water maze task. This behavioral task consisted of two steps. The first one is called the acquisition phase, where rats were trained for 5 consecutive days performing four trials per day to find the submerged platform. On each trial, the rat was placed in the water in one of four start locations (N, S, W and E). The animal was then allowed to search for the platform for 60 s. Once the rat located the platform, it was permitted to remain on it for 10 s. The acquisition phase was followed by the probe trial 24 h later, in which the platform is not present. The time spent in the quadrant of the former platform position and the correct annulus crossings were obtained as a measure for spatial memory. The next step was the reversal learning (reversal phase) performed 2 weeks later. Animals were trained for 4 days (four trials per day) to find the hidden platform, which had now been moved to a position diagonally opposite (reversed) from its location in the acquisition phase. On the next day, all animals were submitted to a second probe trial, similar to the first one. We observed that rats chronically injected with methylmalonic acid (MA), although presenting no alterations in the acquisition phase, showed a long lasting reversal learning impairment. Moreover, motor activity, evaluated by the swim speed in the maze, was not altered by MA administration. These results are consistent with perseverative behavior.
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Theme: Neural basis of behavior
Topic: Neurotoxicity

Keywords: Neurodegenerative disorders; Water maze; Perseverative behavior; Methylmalonic acid

1. Type of research

- Drug effect on behavior research
- Study of learning and memory
- Perseverative behavior induced by chronic administration of drugs.

2. Time required

The time required for this task was calculated taking into account standard experiments with 10 animals per group and two groups:

Total duration of protocol: approximately 100 days
Chronic postnatal administration of drugs: 23 days
Time for animal recovery: 30 days
Acquisition phase: 3 h per day, during 5 days. All trials have to be carried out at the same period of day.

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Probe trial: 1 h on the next day (on the 6th day)
Interval of 2 weeks before the reversal training
Reversal learning: 3 h per day during 4 days
Probe trial of reversal learning: 1 h on the next day (on the 5th day)
Analyses of the recorded tape: 10 days.

3. Materials

3.1. Animals

Twenty-four male Wistar rats from our breeding stock were used for the investigation. Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups; rats were weaned at 21 days of life. The animals were divided so that in each cage, there was the same number of rats for each treatment (saline and methylmalonic acid). All animals had free access to commercial chow and water, and were kept on a 12-h light/dark cycle at 24 ± 1 °C. The animals were left to recover for approximately 1 month. On the 60th day of life, spatial learning/memory was tested by the Morris water maze [12,13].

3.2. Reagents

Reagents were purchased from Sigma (St Louis, USA). Methylmalonic acid (MA) was dissolved in saline and the pH adjusted to 7.4 with 1.0 M NaOH on the day of each experiment.

3.3. Special equipment

The Morris water maze apparatus consists of a black circular pool (200 cm in diameter, 100 cm high), theoretically divided into four equal quadrants for the purpose of analysis. The pool was filled to a depth of 50 cm with water (23 ± 1 °C) made opaque by the addition of milk. The escape platform was transparent, had a diameter of 10 cm and was placed 2 cm below the water surface. A video camera was mounted above the center of the tank and all trials were recorded. The room was dimly illuminated. Two black and white large cartoons were hung on the walls in order to provide extra-maze cues, allowing rats to develop a spatial map strategy. Tapes of all trials were recorded in data sheets and analyzed by computerized image analysis systems for the following variables of interest: (1) latency and distance to find the platform during the trials; (2) time spent in the quadrant of the former platform position and correct annulus crossing during the probe trial; and (3) time spent in the target and in the opposite platform quadrant and correct annulus crossings during the reversal probe trial.

4. Detailed procedures

4.1. Drug treatment

Saline-buffered MA, pH 7.4, was administered subcutaneously, twice a day, from the 5th to the 28th day of life to produce chronic chemically induced methylmalonic acidemia. MA doses were calculated to achieve 2.0 to 2.5 mM MA concentration in plasma, and were as follows: 5–12 days of life, 0.72 μmol/g of body weight; 13–19 days of life, 0.89 μmol/g body weight; 20–28 days of life, 1.67 μmol/g of body weight [5]. The brain concentrations of MA were around 1–2 μmol/g brain. Control animals received the same volume of saline subcutaneously twice a day. All solutions were prepared so that each animal received 10 μl solution/g of body weight.

4.2. Morris water maze

4.2.1. Acquisition phase

Rats were submitted to daily sessions of four trials per day for 5 days to find the submerged platform that was located in the center of a quadrant of the tank and remained there throughout training. We observed that all animals of each group were able to swim in a normal way during all trials. On each trial, the rat was placed in the water, facing the edge of the tank, in one of the four standard start locations (N, S, W and E). The order of the start locations varied in a quasi-random sequence so that, for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform (escape latency) and total distance were measured in each trial. Once the rat located the platform, it was permitted to remain on it for 10 s. If the rat did not find the platform within this time, it was guided to it and allowed to remain on it for 10 s. After each trial, the rats were removed, dried in a towel and put back in their home cages. The interval between trials was 15–20 min [13,16]. In order to calculate the swimming speed, we took the distance traveled in the first 15 s of the each trial.

4.2.2. Probe trial

One day after the last training session, each rat was subjected to a probe trial (60 s) in which there was no platform present. The rats were placed in the water maze in the south position (S) because in the acquisition phase, the platform was in the northeast position. The time spent in the quadrant of the former platform position and the correct annulus crossing, i.e., the number of times animals passed through the circular area (10 cm) that formerly contained the submerged platform during acquisition, were taken as measures for spatial memory.

4.2.3. Reversal learning (reversal phase)

Two weeks after the first probe trial, a reversal training phase was run in which animals were trained for 4 days

Table 1
Effect of postnatal chronic methylmalonic acid (MA) administration on body weight (g) of rats

Age of animals (days)					
Group	5	10	15	20	28
Control	11.4 ± 0.41	19.7 ± 0.57	27.6 ± 0.50	35.4 ± 1.79	67.3 ± 3.05
MA	11.3 ± 0.39	19.2 ± 0.53	27.3 ± 0.63	35.8 ± 1.16	69.8 ± 1.85

Data are expressed as mean ± S.E.M. for 11 rats in each group. There was no significant difference between groups (Student's *t*-test).

(four trails per day) to find the hidden platform now located in the diagonally opposite (reversed) quadrant to the location in the acquisition phase. Latency and distance to find the platform and swimming speed were determined in each trial. On day 5, all animals were submitted to a second probe trial. Each rat was placed in the north position (N) in the water maze that did not contain the platform and was allowed to swim for 60 s. The time spent in the target and the opposite platform quadrant and the correct annulus crossings were registered [1].

At all occasions, the experimenter remained at the same location in each trial, corresponding to the adjacent target quadrant, approximately 50 cm from the outside edge of the tank.

4.3. Statistical analysis

Data of acquisition and reversal learning phases were submitted to two-way ANOVA for repeated measures, considering the factors: MA treatment and days of training. Probe trial data were analyzed by the Student's *t*-test. Values of $p < 0.05$ were considered to be significant. All tests were performed on an IBM PC-compatible computer using the Statistical Package for the Social Sciences (SPSS) software.

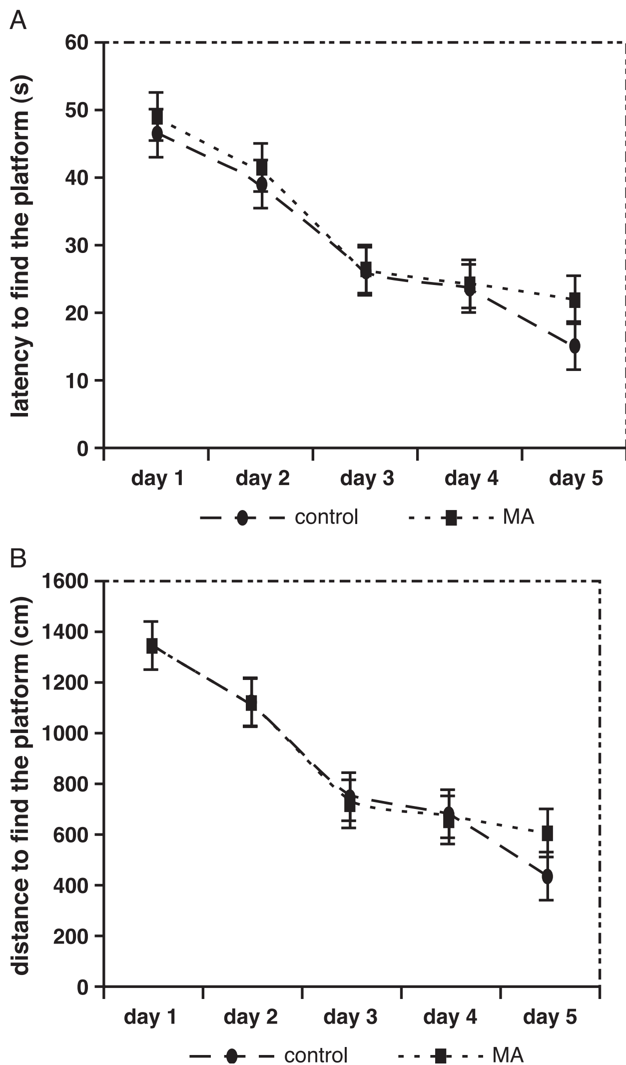

Fig. 1. Effect of methylmalonic acid administration on acquisition learning. Data represent mean ± S.E.M. latency (A) and distance (B) to escape the platform across blocks of four trials on each day ($n = 9-11$ rats/group). MA = methylmalonic acid. There were no differences between groups. Two-way ANOVA analysis is shown in the text.

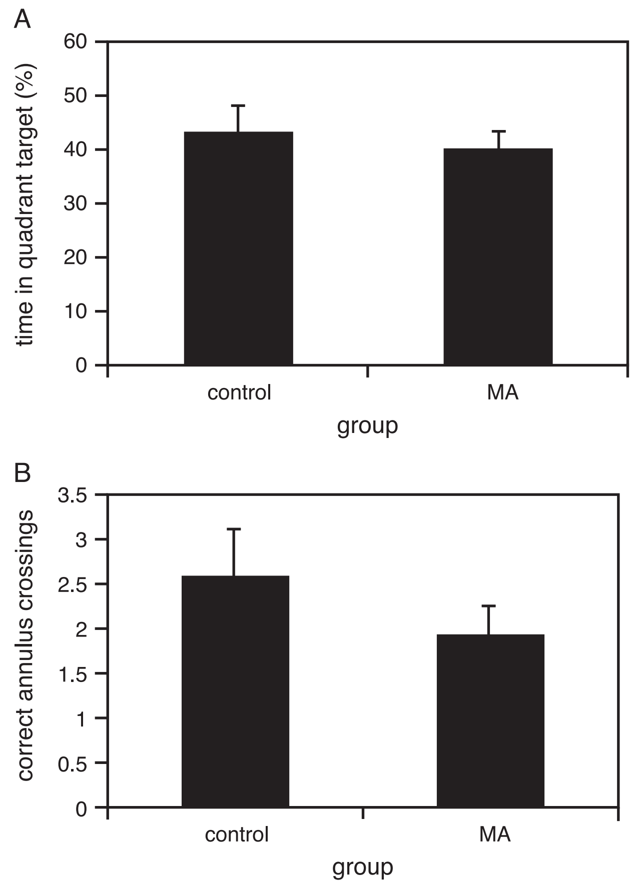

Fig. 2. Probe trial: Time spent in the target quadrant of the former platform position (A) and number of crosses through the former location of the platform (correct annulus crossing) (B) for controls and MA-treated rats ($n = 9-11$). MA = methylmalonic acid. Values are mean ± S.E.M. Student's *t*-test analysis is shown in the text.

5. Results

Animals were daily weighed to determine whether the drug (methylmalonic acid) had any influence on body weight. We found that body weight of rats submitted to chronic MA-treatment did not differ from those of control rats (Table 1), indicating that chronic postnatal administration of MA does not provoke malnutrition.

Fig. 1 shows the performance of animals in the maze task. It can be seen that all animals (saline- and MA-injected rats) improved water maze acquisition performance, i.e., decreased the latency ($F(1,18) = 43.9$ $p < 0.0001$) and distance traveled ($F(1,18) = 44.3$, $p < 0.0001$) to find the platform from the first to the last day of training (day 5). On the first probe trial, with the platform removed, MA-treated rats were able to remember the location of the platform, spending the same period of time in the training quadrant as the controls [$t(18) = 0.32$, $p > 0.05$ (Fig. 2A)]. MA-treated rats also had the same number of correct annulus crossings [$t(18) = 0.988$, $p > 0.05$] as that of controls (Fig. 2B).

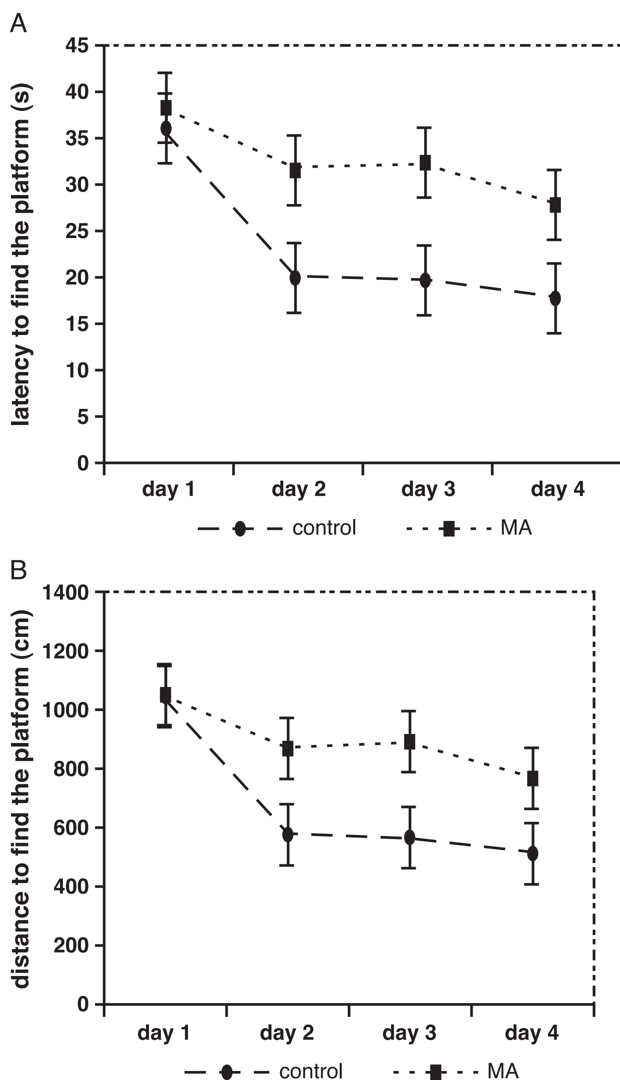

Fig. 3. Effect of methylmalonic acid administration on reversal learning. Data represent mean ± S.E.M. latency (A) and distance (B) to escape the platform across blocks of four trials on each day ($n = 9-11$ rats/group). MA = methylmalonic acid. Two-way ANOVA analysis is shown in the text.

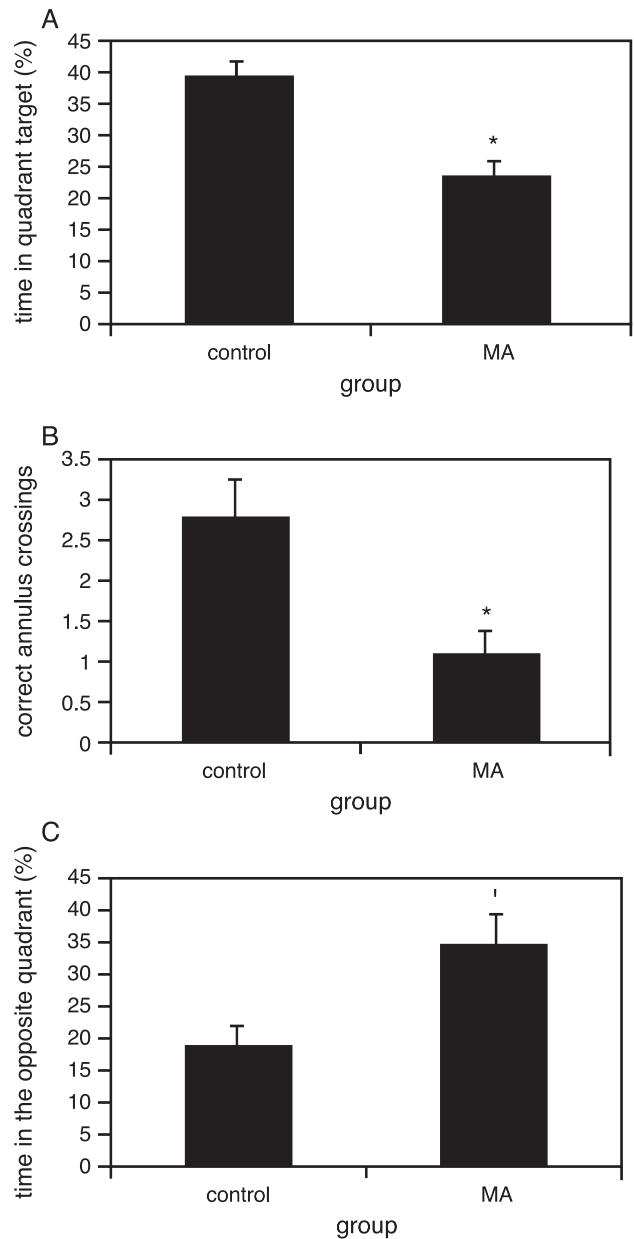

Fig. 4. Second probe trial: Time spent in the target quadrant of the former platform position (A), number of crosses through the former location of the platform (correct annulus crossing) (B) and time spent in the opposite quadrant of the former platform position (C) for controls and MA-treated rats ($n = 9-11$). MA = methylmalonic acid. Values are mean ± S.E.M. *$p < 0.05$, significantly different from the control group. Student's t-test analysis is shown in the text.

Fig. 3 shows that all experimental subjects improved water maze performance in the reversal learning, i.e., the latency ($F(1,18) = 16.00$, $p < 0.0001$) and distance traveled ($F(1,18) = 13.14$, $p < 0.01$) to find the platform decreased

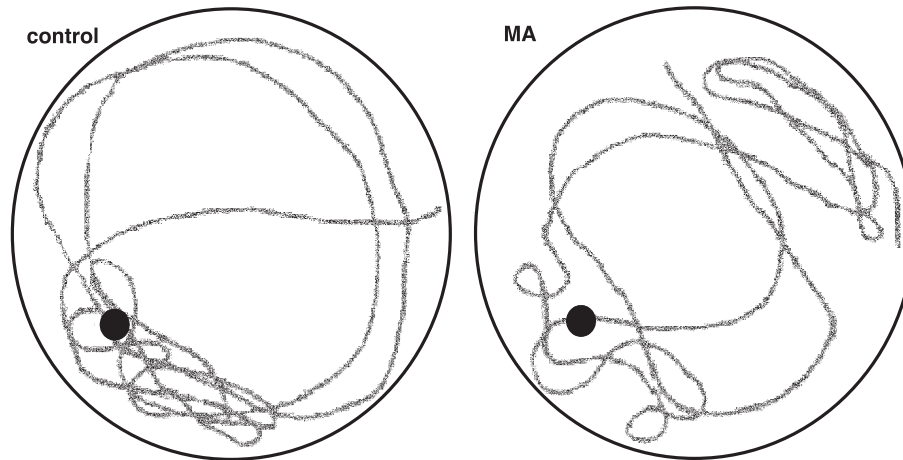

Fig. 5. Swim paths taken by a representative rat in each treated group with respect to time spent in the former location of the platform during the probe trial of reversal learning. MA = methylmalonic acid.

from the first to the last day of training (day 4). Furthermore, group comparisons revealed that MA-treated animals presented a tendency to a higher latency [$F(1,18) = 3.77$, $p = 0.068$] and a higher distance [$F(1,18) = 3.85$, $p = 0.065$] to find the platform, as compared to the saline group along time. On the other hand, swimming speed analysis (expressed in cm/s) showed no difference between MA and rats receiving saline (control: 28.8 ± 1.41; MA: 27.4 ± 1.41 cm/s).

Analysis of the swimming pattern during the second probe trial evidenced that animals submitted to MA administration spent significantly less time in the quadrant of the former platform position [$t(18) = 4.754$, $p < 0.001$] (Fig. 4A) and had an impaired performance in correct annulus crossings [$t(18) = 3.219$, $p < 0.01$] than animals treated with saline (Fig. 4B). Furthermore, MA-treated animals spent significantly more time in the opposite quadrant, i.e., the one where the platform was located in the acquisition phase [$t(18) = -2.928$, $p < 0.05$] (Fig. 4C).

Fig. 5 provides a cartoon with the swim paths of one representative animal from each group in the probe trial. The paths taken by controls were largely confined to the latter training quadrant, whereas the paths taken by MA-treated rats appeared as circular patterns that were more equally distributed within the quadrants where the platform was in the acquisition phase and in the reversal learning.

6. Discussion

6.1. Assessment of the protocol

The Morris water maze apparatus is very useful for the evaluation of spatial learning and memory. The main advantage of this task is that it allows the simultaneous evaluation of learning, spatial memory and working memory as well as motor activity by submitting the same animals to distinct and consecutive phases of training. Therefore, it is a very comprehensive paradigm, which gives reliable results on animal behavior with relatively few subjects.

On the other hand, distinct protocols can be run in the water maze. For example, the platform can be hidden or visible, the cues can be intra or extra-maze, the number of daily trials and the number of training days can vary [4,12,13,16]. The protocol we describe, using a hidden platform, better evaluates the spatial learning and memory since animals must build a spatial map to locate the submerged platform in the pool; that is distinct from what occurs when the platform is visible. We are also able to determine the motor activity of the animals by measuring the swimming speed in the acquisition and in the reversal phase trials. Furthermore, by using two phases (acquisition and reversal), we are able to identify more subtle behavioral alterations in the animals and, importantly, to detect perseverative behavior.

Perseveration is generally seen as a deficit in switching behavior from one mode of responding to another, and thus, apart from motivational or motor deficits, it expresses a disturbance of executive function [4,8]. This behavioral alteration is often related with lesions in the striatum and is interpreted as an inability to inhibit ongoing action or as a failure to initiate a next response [4]. Interestingly, the difficulty in switching behavioral sets associated with perseveration is one of the most frequently observed cognitive deficit in various inherited metabolic diseases involving the basal ganglia, including methylmalonic acidemia [3,7], as well as in other situations associated with basal ganglia lesions [9]. Interestingly, we observed here that the chronic administration of methylmalonic acid to rats during a period of rapid brain development resulted in a normal performance (i.e., learning and reference memory) in the acquisition phase and an impaired performance

in reversal learning, which is consistent with a perseverative behavior [8,9].

6.2. Trouble shooting

Since chronic treatment of animals can lead to a reduction in their appetite and consequently to malnutrition, this factor has to be ruled out before we can evaluate behavioral changes. This is important since malnourished animals may behave differently in neurobehavioral tests, producing a bias to the interpretation of behavioral alterations presented by drug-treated rats.
In order to avoid litter effects, it is desirable to have the same number of animals for each experimental group coming from every home cage (litter).
The position of the experimenter should be the same during all training sessions and probe trials since the animals use the experimenter as a reference point, i.e., an extra-maze cue, and a distinct position of the experimenter along trials would probably alter their spatial map strategy.
The time animals stay on the platform must be strictly regulated to allow the same time for learning the platform position.

6.3. Alternative and support protocols

Other behavioral tasks can be used to test the effect of chronic administration of drugs on learning and memory. The open field, the shuttle avoidance, inhibitory avoidance and T-, four- and eight-arm maze tasks are other alternatives. By using some of these tasks, we have previously verified that various metabolites accumulating in inherited neurodegenerative disorders provoke learning and memory deficits [2,6,10,11,14,15].

The perseverative behavior can be tested either using the water maze with a hidden platform, as well as radial-arm mazes. Generally, two phases are run in this protocol: one for acquisition and the other for reversal learning. The only difference between them is the location of the hidden platform or the target position. The main goal of this procedure is learning a new target place in the same task and environment. Should rats persist looking for the target in the region where the platform was formerly located in the acquisition phase, this behavioral strategy can be interpreted as a perseverative behavior.

7. Quick procedure

7.1. Drug treatment

Saline-buffered MA, pH 7.4, was administered subcutaneously twice a day from the 5th to the 28th day of life. Control animals received the same injection volume of saline twice a day.

7.2. Morris water maze

7.2.1. Acquisition phase

Rats were submitted for 5 days to daily sessions of four trials per day to find the submerged platform that was located in the center of a quadrant of the tank and remained there throughout training. On each trial, the rat was placed in the water in one of the four standard start locations (N, S, W and E). The rat was then allowed 60 s to search for the platform. Latency and distance to find the platform, as well as the swimming speed were measured in each trial.

7.2.2. Probe trial

One day after the last training session, each rat was subjected to a probe trial (60 s) in which there was no platform present. The time spent in the quadrant of the former platform position and the correct annulus crossings were taken as indexes of spatial reference memory.

7.2.3. Reversal learning (reversal phase)

Two weeks after the first probe trial, a reversal learning phase was performed. The only difference between both phases is that platform was located in the quadrant situated diagonally opposite (reversed) to the location in the acquisition phase.

7.2.4. Second probe trial

It was performed a day after the reversal learning, in which all animals were submitted to a second probe trial, similar to the first one.

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III.2 Artigo 2

Ascorbic acid prevents water maze behavioral deficits caused by early postnatal methylmalonic acid administration in the rat

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Research report

Ascorbic acid prevents water maze behavioral deficits caused by early postnatal methylmalonic acid administration in the rat

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Abstract

Methylmalonic acidemia consists of a group of inherited neurometabolic disorders biochemically characterized by accumulation of methylmalonic acid (MA) and clinically by progressive neurological deterioration whose pathophysiology is not yet fully established. In the present study we investigated the effect of chronic administration (from the 5th to the 28th day of life) of methylmalonic acid (MA) on the performance of adult rats in the Morris water maze task. MA doses ranged from 0.72 to 1.67 μmol/g of body weight as a function of animal age; control rats were treated with the same volume of saline. Chronic postnatal MA treatment had no effect on body weight and in the acquisition of adult rats in the water maze task. However, administration of MA provoked long lasting reversal learning impairment in this task. Motor activity, evaluated by the swim speed in the maze, was not altered by MA administration, indicating no deficit of locomotor activity in rats injected with the metabolite. We also determined the effect of ascorbic acid administered alone or combined with MA on the same behavioral parameters in order to test whether free radicals might be responsible for the behavioral changes observed in MA-treated animals. Ascorbic acid was able to prevent the behavioral alterations provoked by MA. Moreover, the in vitro exposure of hippocampal and striatal preparations to MA revealed that the acid significantly reduced total radical-trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR) in the striatum, but not in the hippocampus. Furthermore, MA increased the thiobarbituric acid–reactive substances (TBA–RS) measurement in both structures. These data indicate that oxidative stress might be involved in the neuropathology of methylmalonic acidemia and that early MA administration induces long-lasting behavioral deficits, which are possibly caused by oxygen reactive species generation.
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Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Methylmalonic acid; Methylmalonic acidemia; Water maze; Ascorbic acid

1. Introduction

Methylmalonic acid (MA) is found in large amounts in tissues of patients affected by methylmalonic acidemia, one of the most frequent organic acidemias. This disorder is characterized by severe deficiency of L-methylmalonyl-CoA mutase, caused by seven distinct variants, two corresponding to mutations in the apomutase locus, leading to total (mut°) or nearly total (mut $^{-}$) absence of activity, and five due to defects in various steps of cobalamin synthesis and activation (cblA, cblB, cblC, cblD and cblE) or transport. All these defects lead to increased amounts of methylmalonil-CoA, which is spontaneously converted to MA [12]. The levels of the acid in the blood and cerebrospinal fluid (CSF) are usually as high as 3.0–5.0 mM during crises [12,30], but can be even higher in the brain [18]. Affected individuals present lethargy, coma, vomiting, failure to thrive, muscular hypotonia and developmental retardation. Neuroimaging has revealed a symmetric degeneration of the basal ganglia, particularly the globus pallidus [4]. Histopathology confirmed the necrosis of the globus pallidus [20]. Severe metabolic

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acidosis, convulsions and coma occur during metabolic decompensation, whereas movement disorders develop during or after metabolic crisis. A characteristic neurological abnormality of methylmalonic acidemic patients is choreoathetosis secondary to lesions in the basal ganglia. It has been suggested that these pathological changes are caused by metabolic strokes due to accumulation of the toxic organic acids [17]. However, the mechanisms underlying the pathophysiology of neurological dysfunction in methylmalonic acidemia are poorly known.

Oxidative stress has been demonstrated to be related to the pathophysiologic mechanisms involved in brain injury found in various common neurodegenerative disorders, including Parkinson's, Alzheimer's and Huntington's diseases [25]. Moreover, recent studies demonstrated that chronic antioxidant treatment improves cognitive performance in animal models of Parkinson's [9] and Alzheimer's diseases [28], as well as in aged rats [29]. As regards to methylmalonic acidemia, we recently provided some evidence that MA induces in vitro free radical generation in the rat cerebral cortex [14]. On the other hand, ascorbic acid and α-tocopherol attenuated the convulsions produced by intrastriatal administration of MA to rats [13] and supplementation of high doses of ascorbic acid was shown to significantly improve the recovery of a methylmalonic acidemic patient after a crisis of metabolic decompensation, which was also characterized by decreased levels of reduced glutathione [30].

Therefore, the present investigation was undertaken to determine the influence of early high-sustained levels of MA on the spatial performance of adult rats in the Morris water maze. For this purpose, we used a chemically-induced model of methylmalonic acidemia, in which the plasma and brain MA levels are of the order of 1–2.5 mM and 1–2 μmol/g, respectively [10]. Since previous observations suggest that oxidative stress may be involved in the pathophysiology of the neurological dysfunction of methylmalonic acidemia [14], we also investigated the influence of concomitant administration of the naturally-occurring free radical scavenger ascorbic acid on the behavioral alterations provoked by MA treatment in order to evaluate whether reactive oxygen species could be implicated in these alterations and whether in vivo administration of antioxidants would be able to protect rat performance on this task. Finally, the in vitro effects of MA on various oxidative stress parameters were also investigated in the striatum and hippocampus.

2. Material and methods

2.1. Animals and reagents

For the behavioral studies, a total of 40 male Wistar rats from our breeding stock were used, whereas for the in vitro studies twenty-four male rats of 28 days of age were employed. Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups; rats were weaned at 21 days of life. The animals were divided so that in each cage there was the same number of rats for each treatment (saline, MA, ascorbic acid, MA+ascorbic acid). All animals had free access to commercial chow and water, and were kept on a 12-h light/dark cycle at 24±1 °C. The experimental protocol was approved by the Ethical Committee of the Federal University of Rio Grande do Sul in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Unless otherwise stated, reagents were purchased from Sigma (St Louis, USA).

2.2. In vivo treatment

Saline-buffered MA, pH 7.4, was administered subcutaneously, twice a day, from the 5th to the 28th day of life to produce chemically-induced methylmalonic acidemia. MA doses were calculated to achieve 2.0–2.5 mM plasma MA concentration and were as follows: 5–12 days of life, 0.72 μmol/g of body weight; 13–19 days of life, 0.89 μmol/g body weight; 20–28 days of life, 1.67 μmol/g of body weight [10]. The brain concentrations of MA were around 1–2 μmol/g brain. Control animals received the same volume of saline subcutaneously twice a day. Ascorbic acid (AA) was administered twice a day at a dose of 50 μg/g of body weight alone (AA) or combined with MA (AA+MA). All solutions were prepared so that each animal received 10 μl solution/g of body weight.

2.3. Morris water maze

The animals were left to recover for approximately 1 month. On the 60th day of life, spatial learning/memory was tested in the Morris water maze [26], which consisted of a black circular pool (200 cm in diameter, 100 cm high), theoretically divided into four equal quadrants for the purpose of analysis. The pool was filled to a depth of 50 cm with water (23±1 °C) made opaque by the addition of milk. The escape platform was transparent, had a diameter of 10 cm and was placed 2 cm below the water surface. The experimenter remained at the same location on each trial, corresponding to the adjacent target quadrant, approximately 50 cm from the outside edge of the tank. A video camera was mounted above the center of the tank and all trials were recorded. The room was dimly illuminated. In order to provide extra-maze cues, so allowing rats to develop a spatial map strategy, two black and white large cartoons were hung on the walls.

2.3.1. Acquisition phase

Rats had daily sessions of 4 trials per day for 5 days to find the submerged platform that was located in the center of a quadrant of the tank and remained there throughout

training. We observed that all animals of each group were able to swim in a normal way during all trials. On each trial the rat was placed in the water, facing the edge of the tank, in one of the four standard start locations (N, S, W and E). The order of the start locations was varied in a quasi-random sequence so that, for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform (escape latency) and swimming speed were measured in each trial. Once the rat located the platform, it was permitted to remain on for 10 s. If it did not find the platform within this time, it was guided to it and allowed to remain on it for 10 s. After each trial the rats were removed, dried in a towel and put back in their home cages. The interval between trials was 15–20 min [33].

2.3.2. Probe trial

One day after the last training session, each rat was subjected to a probe trial (60 s) in which there was no platform present. The time spent in the quadrant of the former platform position and the correct annulus crossing, i.e., the number of times animals passed through the circular area that formerly contained the submerged platform during acquisition, were taken as measures for spatial memory.

2.3.3. Reversal learning (reversal phase)

Two weeks after the first probe trial, a reversal training phase was run in which animals were trained to find the hidden platform now located in the diagonally opposite (reversed) quadrant to the location in the acquisition phase, for 4 days (4 trials per day). Latency to find the platform was determined in each trial. On day 5, all animals were submitted to a second probe trial. Each rat was placed in the water maze without the platform and was allowed to swim for 60 s. The time spent in the target and in the opposite platform quadrant and the correct annulus crossings were registered [3].

2.4. Tissue preparation for the in vitro studies

Rats were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The striatum and hippocampus were dissected out, weighed and kept chilled until homogenization. Brain tissue was homogenized in 10 volumes (1:10, w/v) of the appropriate medium (20 mM phosphate buffer containing 140 mM KCl, pH 7.4) for TRAP, TAR and TBA–RS measurements. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris [23,15]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for the analyses. The supernatants were incubated for 60 min at 37 °C in the absence (control group) or in presence of MA (test group, 0.5 or 2.5 mM final concentration, pH 7.4).

2.5. Total radical-trapping antioxidant potential (TRAP)

TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis(2-amidinopropane) (ABAP) [22] at room temperature. Four ml of 10 mM ABAP were added to the vial and the background chemiluminescence was measured. Ten μl of 4 mM luminol were then added and the chemiluminescence was measured. This was considered to be the initial value. Ten μl of 80 μM Trolox or supernanatant were added and chemiluminescence was measured until it reached the initial levels. The addition of Trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time an index that is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of Trolox. The results are reported as nmol of trolox per mg protein.

2.6. Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi et al. [22]. The background chemiluminescence was measured by adding 4 ml of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Fifteen μl of luminol (4 mM) were added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten μl of 10 μM Trolox or tissue supernatant was then added and the chemiluminescence was measured for 60 s. The Trolox or supernatant addition reduces the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of its TAR capacity. TAR measurement was calculated as nmol Trolox per mg protein.

2.7. Thiobarbituric acid–reactive substances (TBA–RS)

TBA–RS was determined according to the method described by Esterbauer and Chesseman [11]. Briefly, 300 μl of cold 10% (w/v) trichloroacetic acid were added to 150 μl of supernatant and centrifuged at 3000 *g* for 10 min. Three hundred μl of the supernatant were transferred to a Pyrex tube and incubated with 300 μl of 0.67% (w/v) thiobarbituric acid in 7.1% (w/v) sodium sulphate in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA–RS was determined in a spectrophotometer at 535 nm. The acid did not produce color when tested without the addition of the supernatant, demonstrating the absence

of a direct reaction to thiobarbituric acid. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the supernatants. TBA–RS was calculated as nmol TBA–RS/mg protein.

2.8. Protein determination

Protein concentrations were determined in supernatants from striatum and hippocampus by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by analysis of variance (ANOVA), followed by post hoc Duncan's multiple range test when appropriate. Values of $P < 0.05$ were considered to be significant. Data acquisition and reversal learning were submitted to three-way ANOVA, considering the factors: MA pretreatment × ascorbic acid treatment × days of training. Data of probe trial followed a one-way (group) analysis, as well as data on the oxidative stress parameters TRAP and TAR. Data from TBA–RS were analyzed by two-way ANOVA, considering the factors: MA × ascorbic acid treatment. All tests were performed on an IBM PC-compatible computer using the SPSS (Statistical Package for the Social Sciences) software.

3. Results

Body weight of rats submitted to chronic MA-treatment did not differ from those of control rats nor from rats receiving ascorbate during treatment (results not shown), indicating that chronic postnatal administration of MA does not provoke malnutrition.

Fig. 1 shows that all groups of animals improved water maze acquisition performance, i.e., decreased the latency to find the platform from the first to the last day of training (day 5) [F (3,36) = 85.83, $P < 0.0001$], however there was no interaction effect between days of training and both MA or AA treatment. On the first probe trial, with the platform removed, MA-treated rats were able to remember the location of the platform, spending the same amount of time in the training quadrant as the control and the other groups [F (3,36) = 1.56, $P > 0.05$] (controls: 42.8% ± 16.4, $n = 9$). MA-treated rats also had the same number of correct annulus crossings [F (3,36) = 0.790, $P > 0.05$] as that of all other groups (control: 2.5 ± 1.8, $n = 9$).

Fig. 2 shows that all experimental groups improved water maze performance in the reversal learning, i.e., the latency to find the platform decreased from the first to the last day of training (day 4) [F (3,36) = 46.31, $P < 0.0001$]. Although individual analysis of MA × vehicle and AA × vehicle, and their interactions with days of training were not significant, the interaction between the three factors showed a trend for significance, [F(3,36) = 3.711, $P =$ 0.062]. After evaluating the linear curves, one-way ANOVA of rat performance on day 4 followed by the Duncan multiple range test revealed that MA treated animals presented a higher latency to find the platform than saline-treated rats [F (3,36) = 2.918, $P < 0.05$]. Furthermore, swim speed analysis (expressed in cm/s) showed no significant difference between all four groups [F(3,36) = 0.257, $P > 0.05$] (control: 28.8 ± 1.41; MA: 27.4 ± 1.41; AA: 28.5 ± 1.48; MA + AA: 29.9 ± 2.00 cm/s).

Analysis of the swimming performance during the second probe trial evidenced that animals submitted to MA administration spent significantly less time in the quadrant of the former platform position [F (3,36) = 7.151, $P <$ 0.001] (Fig. 3A) and had an impaired performance in correct annulus crossings [F (3,36) = 4.654, $P < 0.01$] than all other groups (Fig. 3B). Furthermore, MA-treated animals spent significantly more time in the opposite quadrant, i.e., the one where the platform was located in acquisition phase [F (3,36) = 3.19, $P < 0.05$] (Fig. 3C).

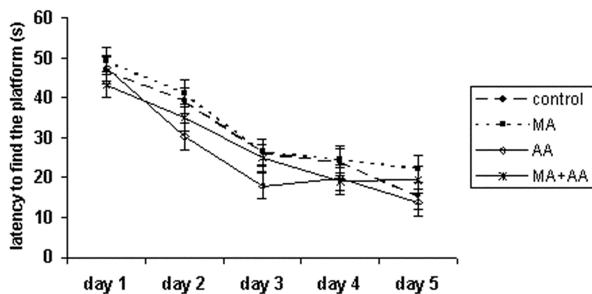

Fig. 1. Effect of methylmalonic acid administration on acquisition learning. Data represent mean ± S.E.M. latency to escape the platform across blocks of four trials on each day ($n = 9–11$ rats/group). MA = methylmalonic acid; AA = ascorbic acid; MA + AA = methylmalonic acid + ascorbic acid. There were no differences between groups. Three-way ANOVA analysis is shown in the text.

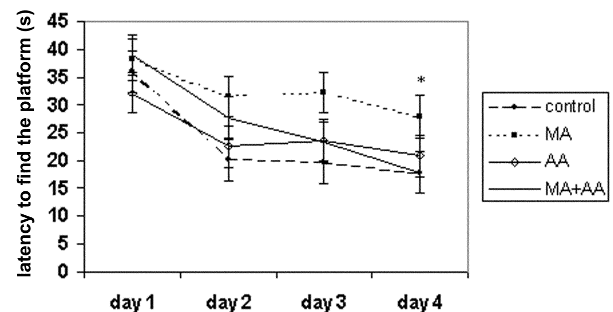

Fig. 2. Effect of methylmalonic acid administration on reversal learning. Data represent mean ± S.E.M. latency to escape the platform across blocks of four trials on each day ($n = 9–11$ rats/group). MA = methylmalonic acid; AA = ascorbic acid; MA + AA = methylmalonic acid + ascorbic acid. * $P < 0.05$, significantly different from the other groups (Duncan multiple range test). Three-way ANOVA analysis is shown in the text.

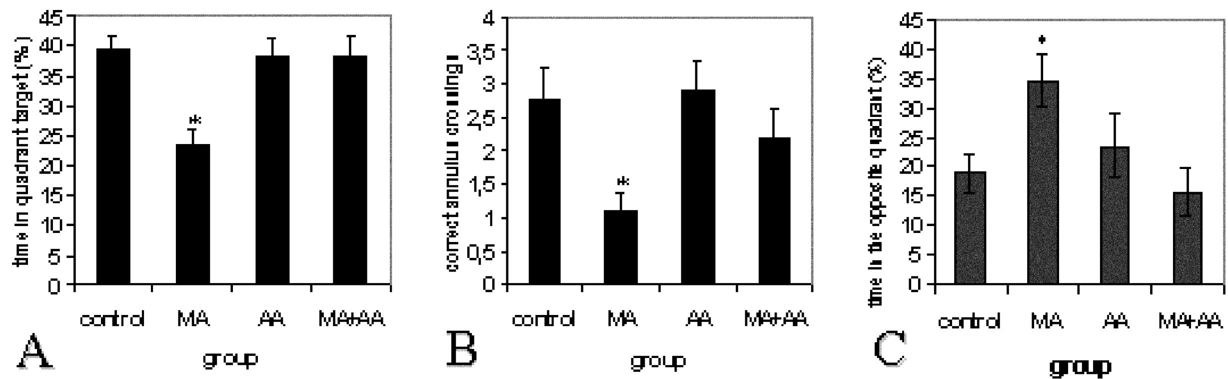

Fig. 3. Second probe trial. Time spent in the target quadrant of the former platform position (A), number of crosses through the former location of the platform (correct annulus crossing) (B) and time spent in the opposite quadrant of the former platform position (C) for controls, MA, AA and MA + AA treated rats ($n = 5$–11). MA = methylmalonic acid; AA = ascorbic acid; MA + AA = methylmalonic acid + ascorbic acid. Values are mean ± S.E.M. * $P < 0.05$, significantly different from the other groups (Duncan multiple range test). One-way ANOVA analysis is shown in the text.

Fig. 4 provides a cartoon with the swim paths of one representative animal from each group in the probe trial. The paths taken by controls, AA and MA + AA were largely confined to the latter training quadrant, whereas the paths taken by MA-treated rats appeared as circular patterns that were more equally distributed within the quadrants where the platform was in the acquisition phase and in the reversal learning.

The in vitro effect of 0.5 and 2.5 mM MA on various parameters of oxidative stress, namely total tissue antioxidant potential (TRAP), total antioxidant reactivity (TAR), and thiobarbituric acid–reactive substances (TBA–RS), was also investigated in striatum and hippocampus of 28-day-old male rats.

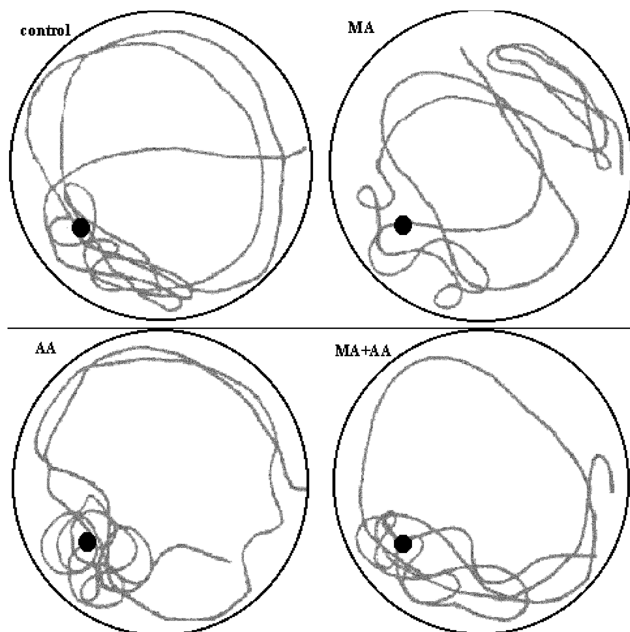

Fig. 4. Swim paths taken by a representative rat in each group with respect to time spent in the former location of the platform during the probe trial of reversal learning. MA = methylmalonic acid; AA = ascorbic acid; MA + AA = methylmalonic acid + ascorbic acid.

The TRAP, which is indicative of the non-enzymatic tissue antioxidant defenses, was significantly reduced by MA in the striatum [F (2,15) = 15.754, $P < 0.0001$] in a concentration-dependent manner ($\beta = -0.703$; $P < 0.01$), but not in the hippocampus [F(2,15) = 1.963. $P > 0.05$] (Fig. 5A and B). The same occurred for the TAR values, which were significantly reduced in the striatum [F (2,15) = 4.653, $P < 0.05$], in a concentration-dependent manner ($\beta = -0.659$; $P < 0.01$), when striatum supernatants were exposed to 0.5–2.5 mM of MA (Fig. 6A). Again, no significant effect of MA occurred in the hippocampus [F (2,15) = 1.0, $P > 0.05$] (Fig. 6B). These findings suggest that the overall content of non-enzymatic antioxidants and the antioxidant reactivity (TAR) in the striatum were significantly reduced by MA, indicating a deficient capacity to modulate the damage associated with an enhanced production of reactive species. These results also indicate that striatum seems to be more vulnerable to MA action than hippocampus.

On the other hand, TBA–RS, which indicates lipid peroxidation, was markedly increased when striatum [F (5,30) = 7.42, $P < 0.0001$] (Fig. 7A) and hippocampus [F(5,30) = 5.055, $P < 0.01$] (Fig. 7B) were exposed to MA, this effect being dose-dependent only for the striatum ($\beta = 0.642$; $P < 0.01$). Furthermore, the two-way 2×3 ANOVA with MA as one factor and AA as the second factor, revealed no interaction between MA and AA both in the striatum [F (3,20) = 0.124, $P > 0.05$] and the hippocampus [F (3,20) = 0.061, $P > 0.05$]. It can be also seen in the figure that when ascorbic acid (200 μM) was incubated with MA, no alteration of TBA–RS values relative to the control was detected, indicating that reactive species were involved in the MA-induced lipid peroxidation.

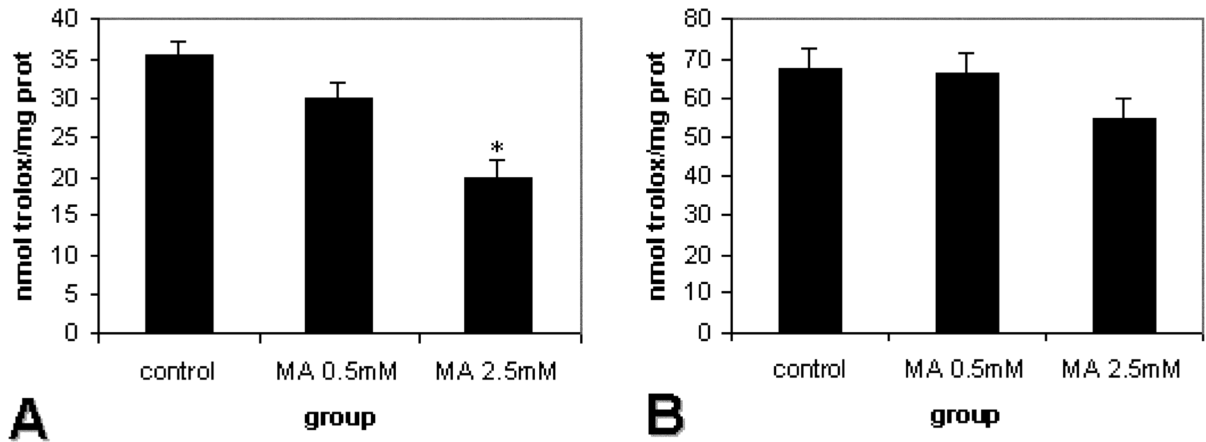

Fig. 5. Effect of methylmalonic acid (MA) on total radical-trapping antioxidant potential (TRAP) in striatum (A) and hippocampus (B) of 28-day-old rats. Results are mean±S.E.M. for six animals per group and are expressed as nmol of trolox per mg protein. * $P<0.05$ compared to control (Duncan multiple range test). One-way ANOVA analysis is shown in the text.

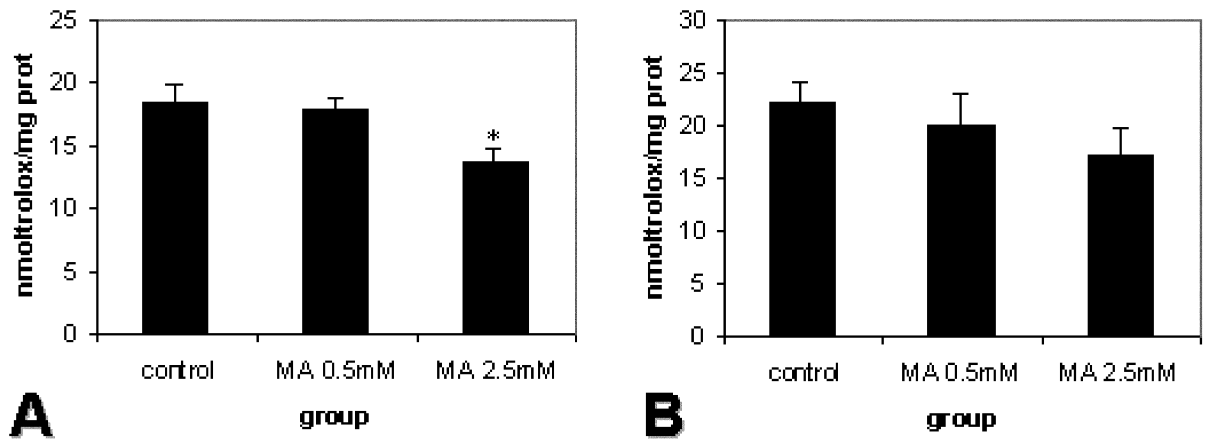

Fig. 6. Effect of methylmalonic acid (MA) on total antioxidant reactivity (TAR) in striatum (A) and hippocampus (B) of 28-day-old rats. Results are mean±S.E.M. for six animals per group and are expressed as nmol of trolox per mg protein. * $P<0.05$ compared to control (Duncan multiple range test). One-way ANOVA analysis is shown in the text.

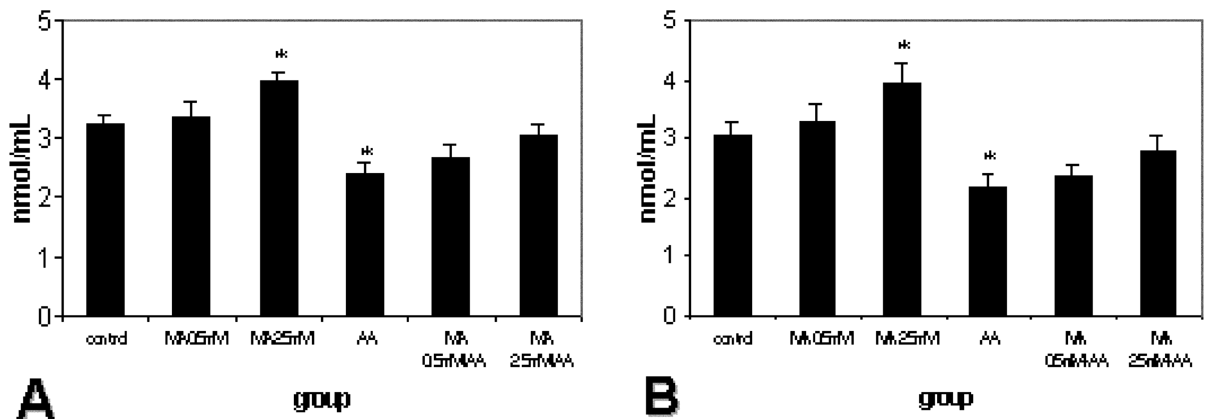

Fig. 7. Effect of methylmalonic acid (MA) on thiobarbituric acid–reactive species (TBA–RS) in striatum (A) and hippocampus (B) of 28-day-old rats. Data represent the mean±S.E.M. for six animals per group and are expressed as nmol/mg protein. Difference from control, * $P<0.05$ (Duncan multiple range test). Two-way ANOVA is shown in the text.

4. Discussion

Methylmalonic acidemic patients usually present a variable degree of neurological dysfunction and lesions in the globus pallidus whose pathophysiology is poorly known. However, it has been recently suggested that increased free radical production caused by methylmalonate may be related to the neurological damage characteristic of methylmalonic acidemia [14].

In the present study, we produced high sustained levels of methylmalonic acid (MA) in the blood and brain of developing rats (5–28th days of life, a period of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory), which are similar to those found in plasma of methylmalonic acidemic patients by using a previously described chemically-induced model for this human condition [10]. Although this model does not exactly mimic human methylmalonic acidemia, it reproduces the main biochemical feature of the disorder, i.e., elevated tissue levels of MA. The animals were then allowed to recover for 30–45 days, since behavioral tasks performed during and shortly after chronic treatment may be difficult to interpret [1]. Thereafter, rat behavior and motor activity were tested in the Morris water maze. This also permitted us to assess long-standing behavioral changes possibly secondary to permanent damage of the central nervous system.

Chronic MA-administration had no effect on body weight, implying that the acid did not cause malnutrition in the animals. This observation is important since malnourished animals may behave differently in neurobehavioral tests so this undesirable effect can be ruled out as for the interpretation of behavioral alterations observed in MA-treated rats.

We first observed that rats receiving MA presented the same acquisition pattern on the water maze task, as compared to all other groups, i.e., they had the same latency to find the platform along time as the other experimental groups. We also verified that rats given chronic MA administration stayed for the same time in the quadrant where the platform was located, as compared to the other groups. These data suggest that MA-treated animals did not have learning/memory deficit in the acquisition phase. However, chronic MA treatment provoked a deficit of performance in the reversal training, i.e., they tended to reach the platform with longer latencies than controls on the last training day. We also observed that MA-treated rats stayed for a significant shorter time (23% of total session time) in the quadrant where the platform was located in the probe of reversal learning, as compared to all other groups (38–40%). It is important to note that the MA-induced deficit to find the platform cannot be attributed to a decreased locomotor activity since the swim speed of all groups was not different.

By looking at the paths that the various animal groups took and also on the time spent on the target and opposite quadrant, we observed that MA-treated animals concentrated on the quadrants where the platform was placed in the acquisition phase. This pattern is consistent with a perseverative behavior, which seems to be often related to response deficits observed in animals with lesions in the striatum and is interpreted as an inability to inhibit ongoing action or as a failure to initiate a next response [7]. Perseveration is generally seen as a deficit in switching behavior from one mode of responding to another, and thus, apart from motivational or motor deficits, it expresses a disturbance of executive function. Interestingly, the difficulty in switching behavioral sets with perseveration as a consequence of mental inflexibility is one of the most frequently observed cognitive deficits in various inherited metabolic diseases involving the basal ganglia [6].

It has been suggested that basal ganglia damage happens because MA inhibits brain energy metabolism by blocking complex **II** of the respiratory chain [32]. Since perseveration involves behavioral responses deficit, it leads to a learning delay to a new response, and this is seen in methylmalonic acidemic patients. Consistent with these findings, 3-nitropropionic acid (3NP), a strong inhibitor of complex **II** of the respiratory chain, also elicits a perseverative behavior in adult rats [24] and causes striatal degeneration characterized by bilateral hypodensities in the putamen and in globus palidus [5], which are neuropathological features of methylmalonic acidemia [6].

More important, the behavioral effects shown by MA-treated animals in the water maze task was prevented by co-administration of ascorbic acid, a free radical scavenger, suggesting that the long-lasting impairment of cognition provoked by MA may be caused by oxidative brain damage and a possible association between free radicals/oxidative damage and cognitive impairment in methylmalonic acidemia. In this context, we also observed that MA provoked in vitro oxidative stress, as determined by the reduced levels of the non-enzymatic antioxidant defenses (TRAP) and that of the antioxidant reactivity (TAR) in the striatum of the animals. An increase of TBA–RS (lipid peroxidation) was seen in both the striatum and hippocampus. These results, allied to previous studies of our laboratory showing that lipid peroxidation is increased by MA in cerebral cortex [14], reinforce the assumption that MA elicits oxidative stress. This is also corroborated by a study demonstrating that supplementation of high doses of ascorbic acid improved the recovery of a methylmalonic acidemic patient after a severe metabolic crisis accompanied by reduction of glutathione levels [30]. Taken together all these observations, we hypothesize that oxidative stress should be considered as an important pathophysiological mechanism underlying tissue damage in methylmalonic acidemia.

This is interesting in view of recent studies demonstrating that increased generation of reactive oxygen species (ROS) is involved in various neurodegenerative disorders such as Parkinson's disease, Huntington's disease and Alzheimer's disease [16].

Regarding the results of our in vitro assays on the

oxidative stress parameters, it was interesting to find that the striatum was more vulnerable than the hippocampus to the MA-induced reactive species. This may possibly be related to the in vivo situation, where this structure is predominantly injured in methylmalonic acidemia. The reason for this finding is obscure, but may be due to the high content of iron in the basal ganglia, as compared to other brain structures [31]. On the other hand, lesions of the striatum have been associated with perseverative behavior [8,19], which occurred in MA-treated rats in the present study.

In a recent report we demonstrated that early chronic postnatal administration of propionic acid (PA) to rats caused a deficit of performance in the acquisition phase and in the probe trial on the water maze task of the adult animals, indicating a deficit in spatial learning and reference memory. Co-treatment of PA with ascorbic acid prevented this learning/memory disability [27], implying a possible association between free radicals/oxidative damage and cognitive impairment. These results differ from those elicited by chronic postnatal administration of MA since we did not observe learning or memory reduction in the present study, but rather perseveration or behavioral inflexibility. This is interesting in view that propionic acidemic patients have a much worse prognosis than methylmalonic acidemic patients and also have a generalized damage of the CNS, as revealed by the severe delayed myelination and cerebral atrophy detected on the cerebral magnetic resonance imaging [2]. Furthermore, even under therapy, a variable degree of mental retardation is characteristically seen in most propionic acidemic children [21].

In conclusion, the present study shows that chronic postnatal administration of MA provokes long-lasting reversal learning deficits in the water maze task and that ascorbic acid treatment prevents this behavioral alteration. Furthermore, MA elicits in vitro oxidative stress predominantly in the striatum, but also in the hippocampus, as measured by three parameters, providing further support for the free radical hypothesis causing brain damage in methylmalonic acidemia. Whether this mechanism or other abnormalities is the main cause of the neurological manifestations of methylmalonic acidemic patients remains to be elucidated. The results of the present and other studies indicating the involvement of oxidative stress in the pathophysiology of methylmalonic acidemic patients [14,13] suggest that antioxidants could represent an adjuvant therapeutic approach in order to prevent the neurological damage and learning disabilities of these patients.

Acknowledgements

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III.3 Artigo 3

Differential inhibitory effects of methylmalonic acid on respiratory chain complex activities in rat tissues

Pettenuzzo, L.F.; Ferreira, G.C.; Schmidt, A.L.; Dutra-Filho, C.S.; Wyse, A.T.S.; e Wajner, M.

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Differential inhibitory effects of methylmalonic acid on respiratory chain complex activities in rat tissues

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Abstract

Methylmalonic acidemia is an inherited metabolic disorder biochemically characterized by tissue accumulation of methylmalonic acid (MMA) and clinically by progressive neurological deterioration and kidney failure, whose pathophysiology is so far poorly established. Previous studies have shown that MMA inhibits complex II of the respiratory chain in rat cerebral cortex, although no inhibition of complexes I–V was found in bovine heart. Therefore, in the present study we investigated the in vitro effect of 2.5 mM MMA on the activity of complexes I–III, II, II–III and IV in striatum, hippocampus, heart, liver and kidney homogenates from young rats. We observed that MMA caused a significant inhibition of complex II activity in striatum and hippocampus (15–20%) at low concentrations of succinate in the medium, but not in the peripheral tissues. We also verified that the inhibitory property of MMA only occurred after exposing brain homogenates for at least 10 min with the acid, suggesting that this inhibition was mediated by indirect mechanisms. Simultaneous preincubation with the nitric oxide synthase inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME) and catalase (CAT) plus superoxide dismutase (SOD) did not prevent MMA-induced inhibition of complex II, suggesting that common reactive oxygen (superoxide, hydrogen peroxide and hydroxyl radical) and nitric (nitric oxide) species were not involved in this effect. In addition, complex II–III (20–35%) was also inhibited by MMA in all tissues tested, and complex I–III only in the kidney (53%) and liver (38%). In contrast, complex IV activity was not changed by MMA in all tissues studied. These results indicate that MMA differentially affects the activity of the respiratory chain pending on the tissues studied, being striatum and hippocampus more vulnerable to its effect. In case our in vitro data are confirmed in vivo in tissues from methylmalonic acidemic patients, it is feasible that that the present findings may be related to the pathophysiology of the tissue damage characteristic of these patients.
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Keywords: Methylmalonic acid; Methylmalonic acidemia; Respiratory chain

1. Introduction

Methylmalonic acid (MMA) is found in large amounts in tissues from patients with methylmalonic acidemia, one of the most frequent organic acidemias. This disorder is caused by severe deficiency of L-methylmalonyl-CoA mutase. There are seven distinct variants, two corresponding to mutations in the apomutase *locus*, leading to total (mut°) or nearly total (mut$^-$) absence of activity, and five due to defects in various steps of cobalamin synthesis, activation or transport (cblA, cblB, cblC, cblD and cblE). These defects lead to increased amounts of methylmalonyl-CoA, which is spontaneously converted to MMA (Fenton et al., 2001). The levels of MMA in the blood and cerebrospinal fluid (CSF) are usually around 2.5 mM during crises (Fenton et al., 2001; Treacy et al., 1996), but may be even higher in the brain (Hoffmann et al., 1993). Affected individuals present lethargy, coma, vomiting, failure to thrive, muscular hypotonia and developmental retardation. Cerebral imaging has revealed a symmetric degeneration of the basal ganglia, particularly the globus pallidus (De Souza et al., 1989; Brismar and Ozand, 1994; Larnaout et al., 1998). It has been suggested that these pathological changes are caused by the accumulation of the toxic organic acids (Heidenreich et al., 1988). Despite the improvement of therapy during the last 20

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years, the overall outcome of methylmalonic acidemic patients, remains disappointing (Van der Meer et al., 1994) and most of them present a variable degree of psychomotor delay/mental retardation. Furthermore, kidney failure is also a serious complication affecting these patients and the mechanisms underlying the pathophysiology of the neurological and particularly the renal dysfunction in methylmalonic acidemia are poorly established. However, chronic administration of MMA to rats causes proteinuria and renal tubular injury, as well as dilated tubuli and mitochondrial swelling and disorganization of cristae in the tubulum epithelium (Kashtan et al., 1998), suggesting that mitochondrial dysfunction may be involved in the kidney injury of methylmalonic acidemia. Accordingly, increased amounts of lactate and reduced *N*-acetylaspartate were observed in the globus pallidus of patients with methylmalonic acidemia, also signaling a deficit of aerobic energy metabolism (Trinh et al., 2001). Furthermore, various in vitro and in vivo animal studies have demonstrated that MMA compromises brain energy production (Marisco et al., 2003; Fleck et al., 2004; Royes et al., 2003; Brusque et al., 2002; Calabresi et al., 1998, 2001; McLaughlin et al., 1998; Wajner and Coelho, 1997; Narasimhan et al., 1996; Toyoshima et al., 1995; Dutra et al., 1993; Wajner et al., 1992). However, other investigators argue that MMA is not the major neurotoxin in methylmalonic acidemia and attribute part of the effects obtained with MMA to its metabolites malonate and methylcitrate (Kolker and Okun, 2005; Kolker et al., 2003; Brusque et al., 2002).

On the other hand, acute intrastriatal injections of MMA and rats chronically treated with MMA present cognitive deficit and convulsions which were totally prevented or partly attenuated by antioxidant therapy (Pettenuzzo et al., 2003; De Mello et al., 1996; Narasimhan et al., 1996). These in vivo studies and a report showing that MMA induces in vitro lipid peroxidation in the brain (Fontella et al., 2000) are indicative that this organic acid also elicits oxidative stress. Finally, it has been shown that MMA alters the glutamatergic neurotransmission in vitro (Brusque et al., 2001; Fontella et al., 2000). Therefore, it is feasible that deficit of energy metabolism, oxidative stress and excitotocity may act synergistically and cooperate to provoke the brain damage characteristic of methylmalonic acidemic patients. In this scenario, MMA has been demonstrated to induce secondary (indirect) excitotoxicity and free radical production (Pettenuzzo et al., 2003; Fontella et al., 2000; Albin and Greenamyre, 1992), similarly to 3-nitropropionate, an irreversible inhibitor of complex II (Binienda et al., 1998; Beal et al., 1993).

In the present study we investigated the in vitro effect of MMA on the respiratory chain complex activities in homogenates from various tissues of young rats, such as striatum, hippocampus, kidney, heart and liver, in the hope to clarify the controversy of whether this organic acid can inhibit some of the activities involved in the mitochondrial electron transport. We finally tested the influence of various antioxidants, namely *N*ω-nitro-L-arginine methyl ester (L-NAME) or catalase (CAT) plus superoxide dismutase (SOD) on the inhibition caused by MMA on complex II activity in the striatum in order to investigate whether oxidative stress might be involved in this effect.

2. Experimental procedures

2.1. Material

2.1.1. Animals and reagents

For this study, we employed Wistar rats of 30 days of age. Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight pups; rats were weaned at 21 days of life. All animals had free access to a commercial chow and water, and were kept on a 12 h light/dark cycle at 24 ± 1 °C. The experimental protocol was approved by the Ethical Committee of the Federal University of Rio Grande do Sul in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Unless otherwise stated, reagents were purchased from Sigma Chemical Co. (St Louis, USA). MMA solution (2.5 mM) was always prepared freshly and had their pH adjusted with 0.1 mM NaOH to 7.4.

2.1.2. Tissue preparation

The animals were sacrificed by decapitation, the brain was rapidly removed and the striatum and hippocampus were isolated and homogenized with a teflon–glass homogenizer (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/ml heparin), pH 7.4. The homogenates were centrifuged at $800 \times g$ for 10 min and the supernatants were immediately kept at −70 °C until used for enzyme activity determination. The liver, heart and kidney were submitted to a perfusion with NaCl 0.9% to remove the blood from these structures. After perfusion, the tissues were similarly prepared as the cerebral structures.

3. Methods

3.1. Respiratory chain enzyme activity determination

Enzymatic analysis of electron transport chain (ETC) activities is the method most commonly used to evaluate suspected defects of mitochondrial energy metabolism. ETC analysis is performed on detergent-solubilized or freeze-thawed specimens of tissue homogenates or isolated mitochondria (Zheng et al., 1990). Most frequently homogenates are employed to measure these activities because the use of mitochondria necessitates large quantities of cells or tissue that are generally not available from newborns or small children.

The activities of the ETC complexes II, II–III, I–III and IV were determined in homogenates according to standard methods previously described in the literature (Fischer et al., 1985; Schapira et al., 1990; Rustin et al., 1994).

The activity of the respiratory chain enzyme complex succinate: DCIP oxiredutase (complex II) was determined according to the method of Fischer et al. (1985) by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm with 700 nm as reference wavelength (ε = 19.1/mM cm). The reaction medium consisting of 40 mM potassium phosphate buffer, pH 7.4, 16.0 mM sodium succinate and 8 μM DCIP was preincubated with 40–80 μg (striatum, hippocampus and liver) or 20–40 μg homogenate protein (heart and kidney) at 30 °C for 20 min. Subsequently, 4 mM sodium azide and 7 μM rotenone were added, and the reaction was initiated by addition of 40 μM DCIP and was monitored for 5 min. We also used in the assays 0.5, 1.0, 2.5 or 5.0 mM succinate (dose–response substrate curve). For the time–response experiments, homogenates were exposed to 2.5 mM MMA for zero, 10 or 20 min, after which the assay to measure complex II activity was carried out. In some experiments, striatal homogenates were preincubated with 0.5 mM L-NAME or 50 mIU CAT plus 50 mIU SOD final concentrations for 20 min alone or in the presence of 2.5 mM MMA, after which the other reagents were supplemented and the reaction carried out. None of the substances supplemented to the assay interfered with the color development or spectrophotometer reading. Results (nmol of reduced DCIP per min per mg protein) were expressed as percentage of controls.

Complex II–III (succinate: cytochrome *c* oxireductase) activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength (ε = 19.1/mM cm), according to the method described by Fischer et al. (1985).

The reaction medium consisting of 40 mM potassium phosphate buffer, pH 7.4 and 16 mM sodium succinate was preincubated with 40–80 μg (striatum, hippocampus and liver) or 20–40 μg homogenate protein (heart and kidney) at 30 °C for 30 min. Subsequently, 4 mM sodium azide and 7 μM rotenone were added, and the reaction was initiated by addition of 0.6 μg/ml cytochrome *c* and monitored for 5 min. We used in this assay 1.0 and 16 mM of succinate.

Complex I–III activity was measured by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$/mM cm), according to Schapira et al. (1990). The reaction mixture contained 20 mM potassium phosphate buffer, pH 8.0, 2 mM KCN, 10 μM EDTA, 50 μM cytochrome *c* and 10–20 μg (striatum, hippocampus and heart) or 2–5 μg (liver and kidney) homogenate protein. The reaction was initiated by addition of 25 μM NADH and was monitored at 25 °C for 3 min before addition of 10 μM rotenone, after which the activity was measured for an additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome *c* reductase activity.

The activity of cytochrome *c* oxidase (complex IV, COX) was measured by the method of Rustin et al. (1994), by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$/mM cm). The reaction mixture contained 10 mM potassium phosphate buffer, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg homogenate protein and the reaction was initiated with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min.

3.1.1. Protein determination

Protein concentrations were determined in homogenates by the method of Lowry et al. (1951), using bovine serum albumin as standard.

3.1.2. Statistical analysis

Data are presented as mean ± S.D. and were analyzed by the Student's *t*-test for paired samples or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when F was significant. Only significant values are shown in the text. Values of $p < 0.05$ were considered to be significant. All tests were performed on an IBM PC-compatible computer using the SPSS (Statistical Package for the Social Sciences) software.

4. Results

We first examined the effect of MMA on the activity of complex II in the striatum, hippocampus, kidney, liver and heart, using various concentrations of succinate (0.5, 1.0, 2.5, 5.0 and 16 mM) as the substrate. It can be seen that preincubation of tissue homogenates with 2.5 mM MMA for 20 min significantly reduced complex II activity in the striatum (succinate 0.5 mM: t (5) = 3.11, $p < 0.05$; succinate 1.0 mM: t (6) = 2.62, $p < 0.05$) and hippocampus when low concentrations of succinate were used in the medium (succinate 0.5 mM: t (5) = 3.12, $p < 0.05$; succinate 0.5 mM: t (5) = 3.97, $p < 0.05$), but not at higher substrate concentrations. In contrast, MMA did not affect complex II activity in the kidney, liver and heart at any succinate concentration, indicating that striatum and hippocampus were more vulnerable to MMA effect (Fig. 1). It can be also seen in the figure that complex II activity was significantly higher in the kidney (D) and heart (E), as compared to the striatum (A) and hippocampus (B) with 1 mM of succinate (F (4, 21) = 16.23; $p < 0.001$), and higher than in the striatum (A) with 16 mM of succinate (F (4, 21) = 49.87; $p < 0.001$).

Taking into consideration that exposition of brain homogenates to 2.5 mM MMA for 20 min inhibited complex II activity, we tested the effect of exposing striatum and hippocampus homogenates for 0, 10 and 20 min with the acid, and using 1.0 mM succinate, which corresponds to the approximate concentration found in the brain (Goldberg et al., 1966). Fig. 2 shows that MMA did not change complex II activity when added at time zero of the assay (no preincubation). In contrast, when brain homogenates were exposed to MMA for 10 or 20 min, the activity of complex II was significantly reduced by 11% (10 min preincubation: t (5) = 5.16, $p < 0.01$) and 15% (20 min preincubation: t (5) = 8.05, $p < 0.01$) in the striatum and 19% (10 min preincubation: t (5) = 4.21, $p < 0.01$) and 20% (20 min preincubation: t (5) = 4.67, $p < 0.01$) in the hippocampus, respectively. In order to test whether the significant reduction of complex II activity caused by MMA was mediated by free radical attack, we preincubated striatum homogenates in the presence of L-NAME or the free radical scavengers CAT and SOD. The enzyme activity was measured in cell homogenates since free radicals can be better produced with the whole cell machinery. We verified that these drugs per se did not affect complex II activity and did not prevent the significant inhibitory effect of MMA (F (5, 18) = 3.415; $p < 0.05$) (Fig. 3). We could not test the influence of reduced glutathione, as well as ascorbic acid plus α-tocopherol on the inhibitory effect elicited by MMA because these antioxidants altered electron transport to DCIP (results not shown).

Fig. 4 shows that 2.5 mM MMA inhibited complex II–III activity in the striatum (35%, t (5) = 14.0, $p < 0.001$), hippocampus (25%, t (5) = 17.2, $p < 0.001$), kidney (20%, t (7) = 9.03, $p < 0.001$), liver (20%, t (6) = 7.0, $p < 0.001$) and heart (20%, t (7) = 8.57, $p < 0.001$), when 1.0 mM of succinate was used in the incubation medium. When tissue homogenates were preincubated with 16 mM of succinate, this complex activity was also inhibited, but to a lesser degree (7–15%), in the striatum (t (5) = 4.02, $p < 0.001$), hippocampus (t (5) = 6.62, $p < 0.001$), kidney (t (6) = 6.61, $p < 0.001$) and liver (t (6) = 4.78, $p < 0.001$). The figure also shows that complex II–III activity was higher in kidney and heart and lower in liver, as compared to the cerebral structures when we used 1 mM (F (4, 24) = 47.28; $p < 0.001$) or 16 mM (F (4, 22) = 74.73; $p < 0.001$) of succinate.

In addition, MMA significantly inhibited the activity of complex I–III in the kidney (53%) (t (6) = 7.06, $p < 0.001$) and liver (38%) (t (6) = 4.30, $p < 0.05$), but not in the striatum, hippocampus and heart (Fig. 5). It can be also observed that this complex activity was higher in the liver and kidney, as compared to the other structures (F (4, 15) = 115.78; $p < 0.001$).

We finally observed that MMA did not affect the activity of complex IV in all tissues studied (Table 1). However, COX activity was much higher in the cerebral structures than in the peripheral tissues (F (4, 22) = 41.72; $p < 0.001$).

We also tested whether malonic acid and methylcitrate were formed from MMA, by incubating striatum and hippocampus homogenates under identical conditions of the assay used to measure complex II activity for up to 1 h with 2.5 mM MMA. The organic acid analyses performed by gas chromatography/mass spectrometry revealed no traces of malonic and methylcitric acids (results not shown).

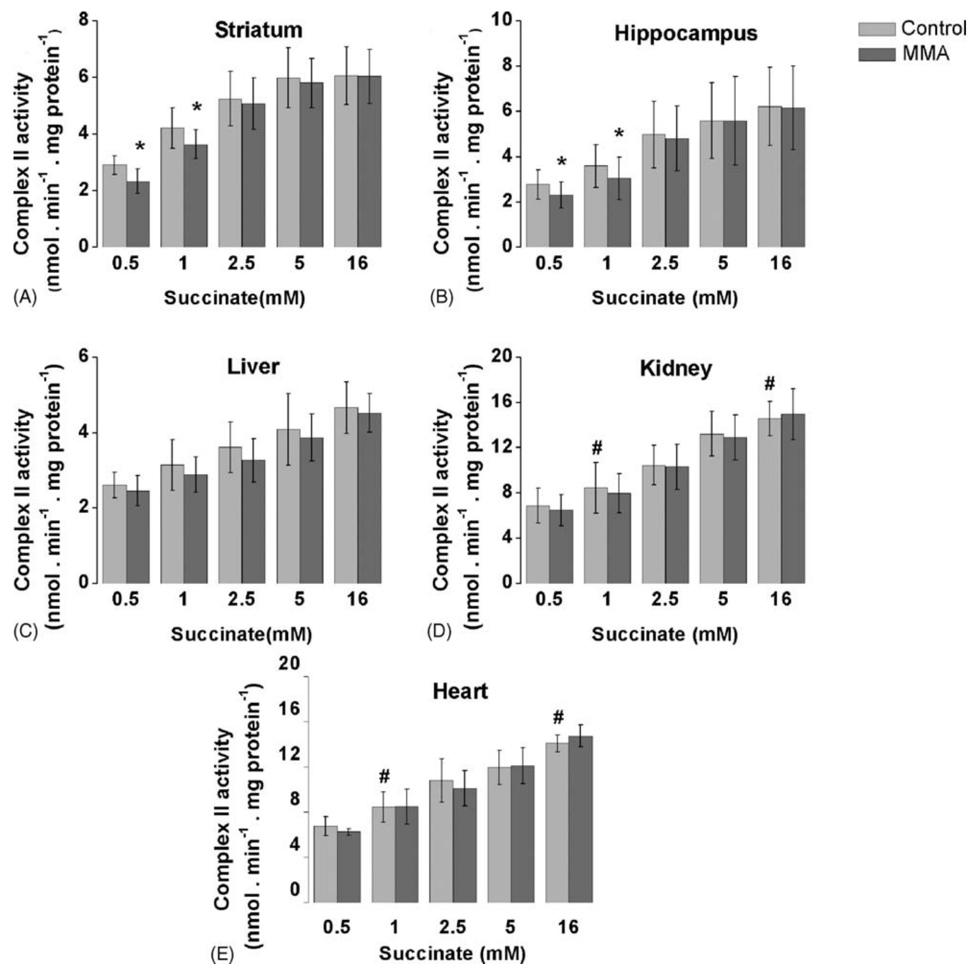

Fig. 1. Effect of substrate (succinate) concentration on the inhibitory action of methylmalonic acid (MMA, 2.5 mM) on succinate: DCIP oxireductase (complex II) activity in striatum (A), hippocampus (B), liver (C), kidney (D), and heart (E) homogenates from young rats. Data are expressed as nmol/min/mg protein (mean ± S.D.) of six experiments (animals) in each group. Differences between control and MMA were calculated by the Student's paired *t*-test, $^{*}p < 0.05$. Differences between controls values of various tissues were calculated by ANOVA followed the Duncan multiple range test, $^{\#}p < 0.05$, compared to striatum and hippocampus.

5. Discussion

In the present work we utilized standard and widespread methods described to measure respiratory chain enzyme activities in tissue homogenates and evaluated the effect of MMA on respiratory chain complexes II, II–III, I–III and IV activities in various cerebral structures, as well as in liver, kidney and heart from young rats in order to verify whether this organic acid could alter such activities. Previous findings from our laboratory and from other laboratories have shown that MMA provokes a significant inhibition of complex II in the brain (Marisco et al., 2003; Fleck et al., 2004; Royes et al., 2003; Brusque et al., 2002; Calabresi et al., 1998, 2001; McLaughlin et al., 1998; Wajner et al., 1992; Wajner and Coelho, 1997; Narasimhan et al., 1996; Toyoshima et al., 1995; Dutra et al., 1993), although other investigators have shown that MMA does not directly inhibit complex II activity in submitochondrial particles (SMPs) from bovine heart, neither the other single electron transferring complexes I–V and the mitochondrial transporters in mice muscle and striatal neuronal cultures from rat embryos (Kolker and Okun, 2005; Kolker et al., 2003; Okun et al., 2002).

We first verified that 2.5 mM MMA significantly inhibited complex II activity in striatum and hippocampus at low concentrations of succinate (0.5 and 1 mM) in the medium, which reinforces previous findings (Fleck et al., 2004; Royes et al., 2003; Brusque et al., 2002; Calabresi et al., 2001; Dutra et al., 1993; McLaughlin et al., 1998; Toyoshima et al., 1995). It should be noted that brain succinate concentrations are about 1 μmol/g (around 1 mM) (Goldberg et al., 1966) and that brain MMA concentrations can be higher than 5 mM (Hoffmann et al., 1993). In contrast, complex II activity was not changed by MMA in the liver, heart and kidney, and this is in agreement with the findings of Okun et al. (2002) and Kolker et al. (2003) verified in submitochondrial membrane particles from bovine heart. It is therefore feasible that MMA-induced inhibitory effect on complex II activity is selective for cerebral structures.

We also observed that the inhibition of complex II activity by MMA depends on the duration of tissue exposition to the acid, indicating an indirect mechanism of inhibition. In this context, we used in our assays tissue homogenates, which contain the whole cell machinery necessary to form other cellular products, rather than isolated mitochondrial

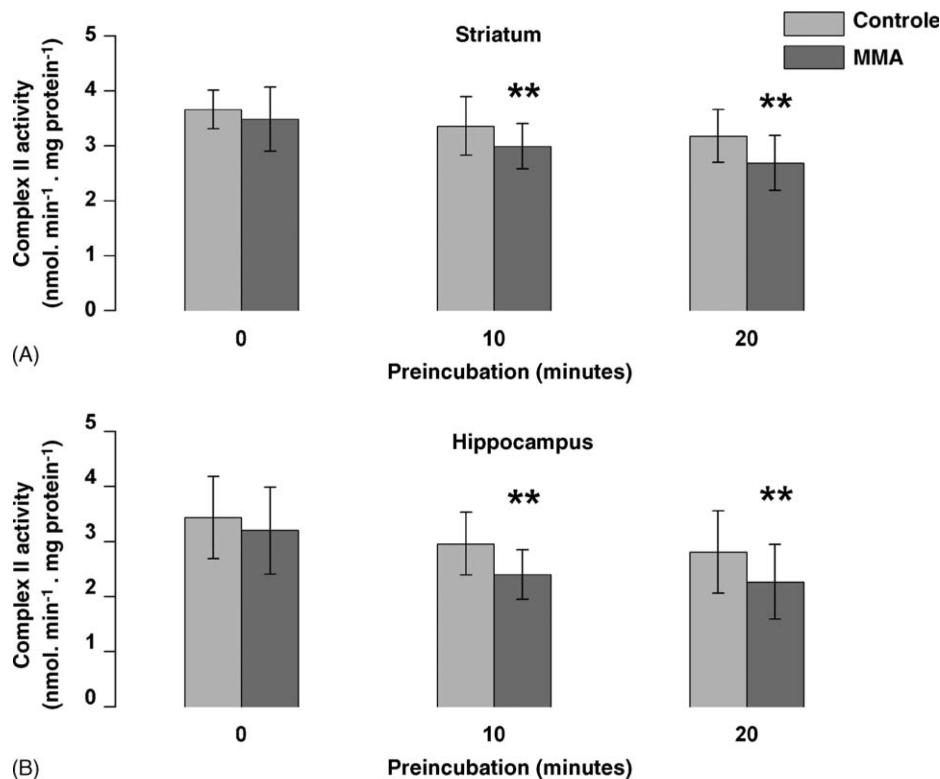

Fig. 2. Effect of time exposure of striatum (A) and hippocampus (B) homogenates from young rats to methylmalonic acid (MMA, 2.5 mM) on its inhibitory action on succinate: DCIP oxireductase (complex II) activity in from. Data are expressed as nmol/min/mg protein (mean ± S.D.) of six experiments (animals) in each group. Differences between control and MMA were calculated by the Student's paired *t*-test, $^{**}p < 0.01$.

membranes. This inhibition could be possibly due to the formation of malonic acid, a classical inhibitor of complex II activity, and/or methylcitrate from MMA, as recently suggested (McLaughlin et al., 1998; Okun et al., 2002). Okun et al. (2002) verified that incubation of primary striatal cell cultures from embryonic rats with 10 mM MMA for up to 8 h gives rise to malonate and methylcitrate formation in nanomolar concentrations after 1–2 h incubation. However, under our experimental conditions we could not detect any of these compounds after incubating brain homogenates with 2.5 mM MMA for up to 1 h, implying that they were not involved in the inhibition caused by MMA. Alternatively, it is feasible the participation of free radicals on the inhibitory property of MMA towards complex II since MMA was previously shown to induce reactive species production in the brain (Pettenuzzo et al., 2003; Fontella et al., 2000; Fighera et al., 1999), methylmalonic acidemic patients have lower levels of reduced glutathione reflecting higher oxidative damage (Treacy et al., 1996) and complex II activity is vulnerable to these radicals (Rafique et al., 2001; Melov et al., 1999; Zhang et al., 1990; Hillered and Ernster, 1983). However, our results showing that the antioxidants L-NAME and catalase plus superoxide dismutase did not influence the inhibitory property of MMA towards complex II activity in the striatum indicate that nitric oxide, superoxide, hydrogen peroxide and possibly hydroxyl radicals were not involved in this effect and makes the reactive species attack hypothesis unlikely.

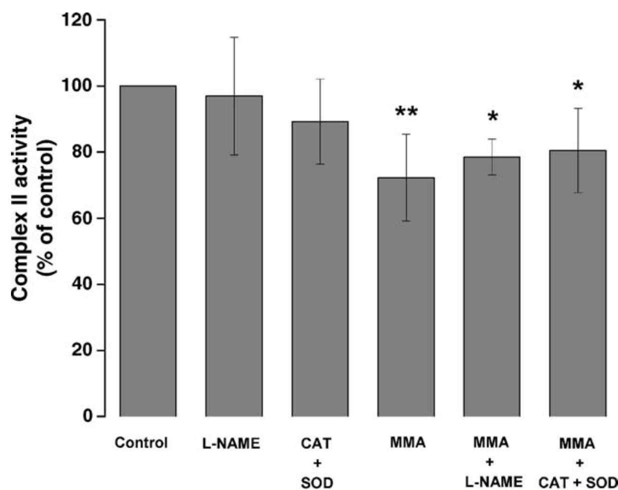

Fig. 3. Effect of preincubation of methylmalonic acid (MMA, 2.5 mM) with antioxidants (L-NAME 0.5 mM or CAT plus SOD 50 mUI each) on its inhibitory action on succinate: DCIP oxireductase (complex II) activity in homogenates from striatum of young rats. Data (nmol/min/mg protein) are expressed as percent of control of 4 experiments (animals) in each group. $^{*}p < 0.05$; $^{**}p < 0.01$, compared to controls (Duncan multiple range test).

Our results are in agreement with previous findings obtained by other investigators using similar experimental conditions and utilizing whole tissues rather than mitochondrial preparations or SMPs (Fleck et al., 2004; Royes et al., 2003; Brusque et al., 2002; Calabresi et al., 2001; McLaughlin et al., 1998; Dutra et al., 1993).

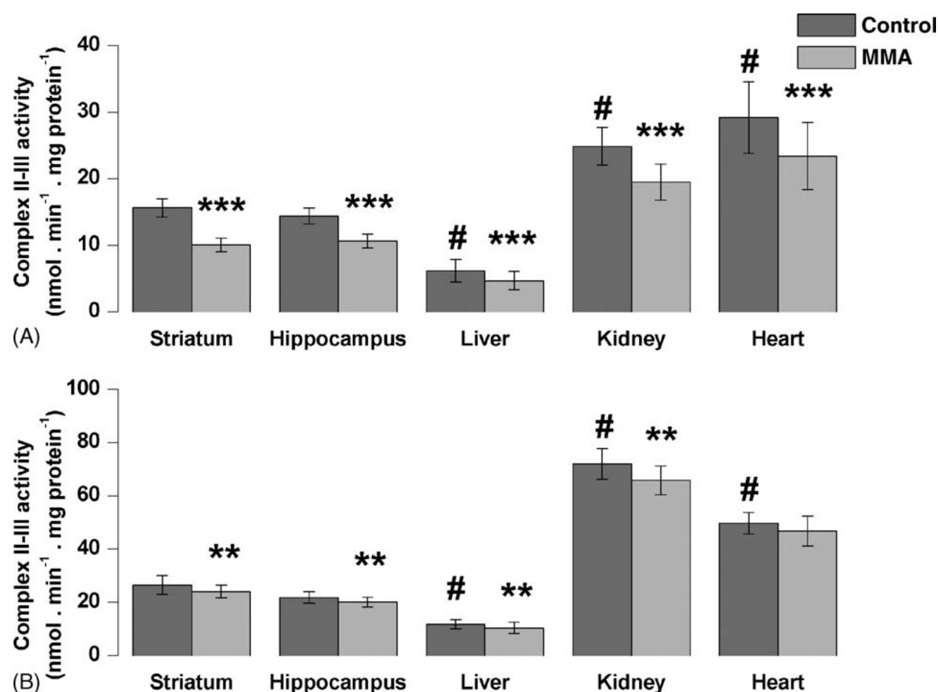

Fig. 4. Effect of methylmalonic acid (MMA, 2.5 mM) on the activity of succinate: cytochrome *c* oxireductase (complex II–III) in homogenates from liver, kidney, heart, striatum and hippocampus of young rats. Tissue homogenates were incubated with 1.0 mM (A) or 16 mM (B) succinate. Data are expressed as nmol/min/mg protein (mean ± S.D.) of six to eight experiments (animals) in each group. Differences between control and MMA were calculated by the Student's paired *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$. Comparison between control values was calculated by one-way ANOVA, followed by the Duncan multiple range test, $^{\#}p < 0.05$, compared to striatum and hippocampus.

MMA also significantly inhibited the respiratory chain complex II–III activity in all tissues and complex I–III activity in the kidney and liver to a higher degree (up to 50%) than that verified in complex II. In contrast, complex IV was not inhibited by MMA in all tested tissues. This is in line with previous findings showing significant inhibitions of complexes I–III and II–III of the respiratory chain to a similar degree caused by MMA in rat cerebral cortex (Brusque et al., 2002). Regarding complex II–III activity, it is interesting to point out that the slowest step in this complex is thought to be the reduction of ubiquinone by complex II, and that inhibitions of complex III as high as 45% may not slow down electron flux through this pathway (Schneider et al., 1982; Taylor et al., 1994). In this context, it may be presumed that MMA inhibition towards complex II–III activity may reflect the inhibitory effect of this metabolite on complex II.

Fig. 5. Effect of methylmalonic acid (MMA, 2.5 mM) on the activity of NADH: cytochrome *c* oxireductase (complex I–III) in homogenates from liver, kidney, heart, striatum and hippocampus of young rats. Data are expressed as nmol/min/mg protein (mean ± S.D.) of four to seven experiments (animals) in each group. Differences between control and MMA were calculated by the Student's paired *t*-test, $^{*}p < 0.05$, $^{***}p < 0.001$. Comparison between control values was calculated by one-way ANOVA, followed by the Duncan multiple range test, $^{\#}p < 0.05$, compared to striatum, hippocampus and heart.

Table 1
Effect of methylmalonic acid (MMA, 2.5 mM) on the activity of cytochrome *c* oxidase (complex IV) (COX) in homogenates from striatum, hippocampus, liver, kidney and heart of young rats

Tissue	Complex IV (nmol/min/mg protein) $n = 7$	
	Control	MMA
Striatum	461.3 ± 78.2	476.1 ± 84.7
Hippocampus	409.5 ± 79.2	415.8 ± 91.7
Liver	47.5 ± 8.7$^{\#}$	44.5 ± 7.2
Kidney	157.7 ± 11.1$^{\#}$	164.7 ± 16.9
Heart	176.6 ± 17.2$^{\#}$	165.6 ± 31.2

Data are expressed as (mean ± S.D.). Differences between control and MMA were calculated by the Student's paired *t*-test. No significant difference was detected. Comparison between control values was calculated by one-way ANOVA (Duncan multiple range test).

$^{\#}$ $p < 0.05$, compared to striatum and hippocampus (Duncan multiple range test).

We also found in the present study that the activities of the distinct complexes of the respiratory chain differed among the various tissues investigated. The distinct activities of respiratory chain complexes observed in the various tissues and the differential MMA-induced tissue inhibition of the various complexes of the respiratory chain may possibly be attributed to distinct cellular milieu, tissue-specific isoforms of various nuclear-encoded subunits of the respiratory chain complexes, as previously emphasized (Rustin et al., 1994; Clay and Ragan, 1988; Merle and Kadenbach, 1980), or alternatively, to organ-specific regulation of electron fluxes of the respiratory chain (Taylor et al., 1993).

Although we cannot totally exclude the possibility that other oxidoreductases may have interfered in our assays, no relevant enzyme activities interfering with the respiratory chain complexes and particularly with complex II have been so far identified by the groups which most contributed to the determination of the various complexes of the respiratory chain and from which we established our methods (Fischer et al., 1985; Rustin et al., 1994; Birch-Machin et al., 1994). However, an NADH-reductase present in the outer mitochondrial membrane has been shown to interfere with rotenone-insensitive NADH-ubiquinone oxidoreductase, but the activity of this complex was not measured in the present study (complex I) (Birch-Machin et al., 1994). Therefore, a possible interference of other artefactual oxidoreductases on the determination of complexes II, II–III, I–III and IV activities in homogenates under the conditions of our assays is unlikely.

As regards to the physiological significance of our findings, we cannot at present establish with certainty whether our in vitro data is related to the neurotoxicity and renal failure of methylmalonic acidemia. However, this may be the case since (1) various respiratory chain complexes were significantly inhibited by 2.5 mM MMA in the cerebral structures and in the kidney; (2) it has been previously shown that concentrations of MMA similar to those used in our study provoked in vitro and/or in vivo decreased ATP (McLaughlin et al., 1998) and creatine phosphate concentrations (Royes et al., 2003) in the brain; (3) the significant inhibitory effects observed here for MMA were achieved with concentrations similar or even lower than those encountered in brain of affected patients (Fenton et al., 2001); (4) a previous work has shown that rats chronically administered with MMA present proteinuria with dilated tubuli and mitochondrial swelling and disorganization of cristae in the tubulum epithelium, indicating mitochondrial dysfunction (Kashtan et al., 1998); and (5) moderate respiratory chain deficiencies as found in the present study may result in a full blockage of a given substrate oxidation in this pathway (Geromel et al., 1997).

In conclusion, to our knowledge the present study reported for the first time a tissue-specific inhibition of respiratory chain activities by MMA. However, although we found significant inhibitions of various respiratory chain complexes in the brain structures and in the kidney, it is difficult at present to establish whether the present in vitro results can be related to the in vivo neuropathology and to the renal failure of patients with methylmalonic acidemia.

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IV DISCUSSÃO

Pacientes afetados por acidemia metilmalônica, mesmo sob protocolos rígidos de tratamento, apresentam graus variáveis de retardo mental/psicomotor, sugerindo que o regime terapêutico disponível atualmente não tem sido plenamente satisfatório e que novas estratégias de tratamento devam ser implementadas. Portanto, considerando que os mecanismos da disfunção neurológica nesta doença são pouco conhecidos, acreditamos que a elucidação da fisiopatogenia do dano cerebral na acidemia metilmalônica poderá contribuir para melhorar a estratégia de tratamento para essa doença. Neste contexto, tem sido sugerido que a produção de radicais livres causada por concentrações elevadas de metilmalonato pode estar relacionada com o dano neurológico característico da acidemia metilmalônica (Fighera et al., 1999; Fontella et al., 2000). Alternativamente, a redução da produção energética causada pelo metilmalonato também pode estar relacionada com o dano neurológico característico destes pacientes (Matsuishi et al., 1991; Wajner et al., 1992; Dutra et al., 1993; Wajner e Coelho, 1997; Wyse et al., 1998; Brusque et al., 2002; Ostergaard et al., 2005). Outra possibilidade é a combinação desses dois fatores, uma vez que um defeito na cadeia respiratória, que tem como conseqüência a redução na produção de energia, pode estimular a produção de radicais livres pelo bloqueio da transferência normal de elétrons através da cadeia. Esta é a hipótese mais aceita para explicar o aumento do estresse oxidativo encontrado em patologias neurodegenerativas como a doença de Parkinson e a doença de Alzheimer (Cassarino e Bennett Jr, 1999). Outro fator potencialmente relacionado com a neuropatologia desta doença é a alteração do sistema de neurotransmissão glutamatérgico causado pelo MMA (Brusque et al., 2001).

No presente estudo empregamos o modelo experimental previamente descrito e desenvolvido em nosso laboratório de acidemia metilmalônica quimicamente induzida (Dutra et al., 1991; Brusque et al., 1999). O ácido foi administrado durante um período em que ocorre rápida proliferação celular e sinaptogênese em várias estruturas cerebrais envolvidas com aprendizado e memória em ratos (Winick and Noble, 1965; Roisen et al., 1981; Dreyfus et al., 1984; Dutra et al., 1993). Este modelo produz níveis elevados e persistentes de ácido metilmalônico (MMA) no sangue e no cérebro de ratos em desenvolvimento (5º ao 28º dia de vida), similares aos encontrados em seres humanos portadores da acidemia metilmalônica.

No presente estudo, inicialmente desenvolvemos um protocolo para avaliar o comportamento de ratos submetidos ao tratamento crônico com o ácido metilmalônico (MMA) como descrito anteriormente (Dutra et al., 1991), utilizando a tarefa do labirinto aquático de Morris. Esta tarefa é bastante útil, pois avalia simultaneamente aprendizado, memória espacial, memória de trabalho e atividade motora. Neste particular, existem vários protocolos aplicáveis a esta tarefa, sendo que o melhor protocolo depende dos objetivos que se deseja alcançar com o experimento. Por exemplo, a plataforma utilizada nesta tarefa comportamental pode ser submersa ou visível, as pistas para o animal podem estar dentro ou fora do aparelho e o número de treinos por dia, bem como o número das sessões de treino também pode variar (Morris et al., 1982; Netto, et al., 1993; Devan, Goad, Petry, 1996; Warren e Juraska, 2000).

O protocolo que desenvolvemos utilizou a plataforma submersa que torna a tarefa mais difícil e apurada, pois, para conseguir localizar a plataforma, o animal precisa construir na sua memória um mapa alocêntrico da sala de experimentação, o que não ocorre quando a plataforma está visível (Morris et al., 1982). A tarefa é dividida em fase de

aquisição e fase reversa, e cada fase é composta por treino e teste. Durante o treino, que geralmente é de vários dias, verificamos o tempo que os animais gastam para encontrar a plataforma submersa, com um teto máximo de um minuto. Os animais usualmente diminuem rapidamente o tempo gasto para encontrar a plataforma ao longo do treino. Esta parte do experimento avalia o aprendizado e a memória espaciais. Na sessão de teste a plataforma é removida e é avaliado o tempo gasto no quadrante onde a plataforma estava localizada, bem como o número de vezes que o animal cruza a correta localização da plataforma. O teste é realizado um dia após o último treino e é importante para avaliar o quão certo o animal estava a respeito da localização da plataforma e a estratégia que utiliza para encontrá-la. Quando o animal fica grande parte do período do teste no quadrante onde a plataforma estava localizada nos treinos, é indicativo de que ele realmente sabia a localização da plataforma, pois construiu um mapa cognitivo utilizando as pistas da sala de experimentação. Pelo contrário, se o animal gasta cerca de 1/4 ou menos do tempo do teste no quadrante onde a plataforma estava, é provável que ele utilizou uma estratégia aleatória, como, por exemplo, percorrer a piscina em círculos até se chocar com a mesma, não sabendo realmente onde a plataforma submersa se localizava durante os treinos. É importante salientar que ambas estratégias levam à localização da plataforma durante os treinos, às vezes com o mesmo tempo de latência. Portanto, é fundamental diferenciar qual a estratégia que está sendo empregada, pois animais com lesões no hipocampo não são capazes de formar um mapa cognitivo com as pistas da sala de experimentação, mas são capazes de encontrar uma plataforma submersa utilizando a estratégia da busca em círculos (esquadrinhamento) (Morris et al., 1982). Também avaliamos a atividade motora dos animais através da medida da velocidade de natação. Esta avaliação é importante, pois é necessário diferenciarmos dificuldade de encontrar a plataforma de dificuldade de nadar.

Além disso, incluímos o treino reverso, que consiste em uma nova sessão de treinos, porém, agora, com a plataforma localizada no quadrante oposto. Esta segunda fase torna a tarefa ainda mais apurada, pois o animal precisa aprender que a plataforma não se encontra mais naquela posição que ele já estava habituado e necessita construir um novo mapa espacial, com a nova posição da plataforma. Esta segunda fase de treinos, chamada fase reversa, tem sido utilizada para detectar comportamento perseverativo.

O comportamento perseverativo é definido como uma dificuldade de alterar uma resposta após o estímulo gerador desta resposta ter sido modificado, o que pode afetar a capacidade de adaptação do animal ao meio. Esta forma de comportamento inflexível geralmente está associada a lesões no estriado (Devan, Goad e Petry, 1996; Massioui et al., 2001), que são encontradas em vários erros inatos do metabolismo tais como a ocorre na acidemia metilmalônica (de Souza et al., 1989; Fenton, Gravel e Rosenblatt, 2001).

Verificamos em nossos experimentos que 30 dias após a sua recuperação (30 dias após a última injeção) o tratamento crônico com MMA não alterou o desempenho dos ratos durante os treinos e o teste da fase de aquisição no labirinto aquático de Morris, isto é, o tratamento crônico com MMA não afetou o aprendizado e a memória espacial dos ratos. Porém, ao avaliarmos o desempenho dos animais no treino reverso, foi observado que o grupo tratado com ácido metilmalônico insistiu em procurar a plataforma no local onde a mesma estava submersa durante a fase de aquisição. Isso se revelou nas latências observadas no último dia de treino da fase reversa, onde os animais tratados com MMA apresentaram uma latência maior para encontrar a plataforma do que os injetados com solução salina (controles). Além disso, ao avaliarmos o desempenho dos animais no teste da fase reversa, observamos que o grupo tratado com MMA permaneceu menos tempo, e cruzou menos vezes pelo local onde a plataforma estava posicionada nesta fase. Ao

analisarmos o tempo gasto pelos grupos no quadrante oposto, isto é, onde a plataforma estava posicionada durante a fase de aquisição observamos que o grupo tratado com MMA gastou mais tempo neste quadrante, cerca de 35% do tempo total do teste. As figuras das trajetórias percorridas pelos diversos grupos revelam claramente que no teste da fase reversa, o grupo tratado com MMA permaneceu procurando a plataforma na posição onde a mesma se localizava na fase de aquisição, ao invés de procurar na nova localização. Esse comportamento não foi verificado nos ratos controles. Todas estas observações são condizentes com um comportamento perseverativo, onde o animal tem dificuldade de extinguir um comportamento que não gera mais a recompensa esperada (resultados apresentados no primeiro artigo).

O próximo passo de nossa investigação foi o de verificar se o co-tratamento com o antioxidante ácido ascórbico poderia alterar o comportamento perseverante dos animais tratados com MMA. Verificamos que esta alteração comportamental foi totalmente prevenida pelo co-tratamento crônico com vitamina C (resultados apresentados no segundo artigo), indicando que o estresse oxidativo poderia estar envolvido com a fisiopatogenia do efeito observado. Assim, investigamos o efeito *in vitro* do MMA sobre a lipoperoxidação, medida através da técnica do TBA-RS, que é uma estimativa da formação de malondialdeído formado durante a oxidação de lipídeos. Os estudos *in vitro* demonstraram que o MMA induz o aumento da lipoperoxidação em homogeneizado de estriado e hipocampo de ratos. Este aumento não foi verificado quando os homogeneizados foram co-incubados com MMA e vitamina C, indicando que o ácido ascórbico, provavelmente por seqüestrar radicais livres, não permitiu a oxidação dos lipídeos provocada pelo MMA. Além do aumento da lipoperoxidação, verificamos que o MMA alterou mais dois parâmetros de estresse oxidativo em homogeneizado de estriado de ratos, diminuindo

significativamente o TAR e o TRAP, que são medidas indicativas das defesas antioxidantes do tecido. O MMA não diminuiu as defesas antioxidantes em homogeneizado de hipocampo de ratos, indicando que apesar de aumentar a lipoperoxidação em hipocampo, o MMA não foi capaz de reduzir significativamente as defesas antioxidantes deste tecido cerebral, como ocorreu no estriado. Em relação a isso, é importante salientar que o estriado é uma estrutura cerebral muito particular em relação ao estresse oxidativo, pois contém alta concentração de ferro, que pode gerar radicais livres através da reação de Fenton, e de dopamina, que, ao ser metabolizada pela monoaminaoxidase (MAO), gera peróxido de hidrogênio (Vymazal et al., 1995; Halliwell e Gutteridge, 1999). Estes achados estão de acordo com observações *in vivo*, onde os animais não apresentaram problemas de aprendizado e memória (fase de aquisição), demonstrando que não houve grandes prejuízos na função hipocampal, mas apresentaram comportamento perseverativo, indicando que o estriado pode ter sido comprometido (Kirkby, 1969; Devan, Goad, Petry, 1996). Estes resultados, associados a estudos prévios de nosso laboratório demonstrando que o MMA aumenta a peroxidação lipídica em homogeneizado de córtex cerebral de ratos (Fontella et al., 2000), reforçam a idéia da participação do estresse oxidativo na fisipatogenia da acidemia metilmalônica. O estudo de Treacy e colaboradores (1996), demonstrando que altas doses de ácido ascórbico ajudaram na recuperação de um paciente portador de acidemia metilmalônica após grave acidose metabólica acompanhada de redução dos níveis de glutationa, também reforça esta idéia. Além disso, é importante salientar que vários estudos implicam o estresse oxidativo na fisiopatogenia de várias doenças neurodegenerativas, como doença de Huntington, doença de Parkinson e doença de Alzheimer (revisado por Halliwell e Gutteridge, 1999).

Trabalhos anteriores demonstraram que o MMA inibe o complexo II da cadeia respiratória no cérebro (Wajner et al., 1992; Dutra et al., 1993; Toyoshima et al., 1995; Narasimhan et al., 1996; Wajner e Coelho, 1997; Brusque et al., 2002; Marisco et al., 2003; Royes et al., 2003; Fleck et al., 2004). Consistente com nossos achados comportamentais com o MMA, o ácido 3-nitropropiônico, um inibidor potente do complexo II da cadeia respiratória, também provoca comportamento perseverante em ratos adultos (Massieu, Del Rio e Montiel, 2001), causando degeneração de estriado, caracterizado por hipodensidade bilateral no putamen e globo pálido (Brouillet et al., 1995), que é um achado neuropatológico também encontrado em pacientes portadores de acidemia metilmalônica (de Souza et al., 1989). Além disso, inibidores do complexo II, como o 3-nitro-propionato e o malonato induzem a geração de espécies reativas de oxigênio (Massieu, Del Rio e Montiel, 2001), bem como excitotoxicidade secundária devido à redução na captação de glutamato da fenda sináptica por astrócitos por déficit energético, levando a lesões no estriado (Beal et al., 1993; Greene et al., 1993). Tais observações indicam que a inibição da cadeia respiratória, o estresse oxidativo e a excitotoxicidade são eventos interrelacionados.

Portanto, também estudamos o efeito do MMA sobre a atividade dos complexos da cadeia respiratória em estriado e hipocampo de ratos (artigo 3), bem como nos tecidos periféricos rins, fígado e coração. É importante salientar que pacientes acometidos pela acidemia metilmalônica apresentam insuficiência renal crônica cuja fisiopatologia não está estabelecida.

Inicialmente verificamos que MMA na concentração de 2,5 mM inibiu significativamente a atividade do complexo II da cadeia respiratória em estriado e hipocampo de ratos, quando foram utilizadas baixas concentrações de sucinato no meio de incubação (0,5 e 1,0 mM), o que está de acordo com resultados prévios de nosso e de

outros laboratórios demonstrando que o MMA inibe o complexo II da cadeia respiratória (Dutra et al., 1993; Toyoshima et al., 1995; McLaughlin et al., 1998; Calabresi et al., 2001; Brusque et al., 2002; Royes et al., 2003; Fleck et al., 2004). É importante salientar que as concentrações cerebrais de sucinato são em torno de 1 μmol/g, cerca de 1mM (Goldberg, Passonneau e Lowry, 1966) e as de MMA podem chegar a 5 mM (Hoffmann et al., 1993). Nas demais estruturas testadas, rim, fígado e coração, o MMA não inibiu a atividade do complexo II da cadeia respiratória, indicando uma especificidade tecidual para esta ação do MMA. Okun e colaboradores (2002) e Kolker e colaboradores (2003) também demonstraram que o MMA não inibia o complexo II da cadeia respiratória em partículas submitocondriais de coração bovino. Tais resultados levaram estes pesquisadores a crer que o MMA não era o principal responsável pelos danos neurológicos apresentados pelos pacientes portadores de acidemias metilmalônica. Nossos resultados indicam que, apesar de não serem conhecidas diferentes isoformas das enzimas do complexo II, o efeito do MMA sobre este complexo parece ser seletivo para tecidos cerebrais. Observamos também que os efeitos provocados pelo MMA neste complexo são dependentes do tempo de incubação com o ácido, indicando que talvez seja necessária a formação de outro composto no meio de incubação para que a inibição ocorra ou mais provavelmente que o efeito do MMA seja indireto. Levando em consideração que nossos experimentos foram realizados utilizando homogeneizado total, apenas centrifugado a baixa rotação para a sedimentação de restos celulares e células não rompidas, não podemos descartar a hipóteses de que outros subprodutos possam estar sendo formados no meio de incubação. Um destes subprodutos poderia ser o malonato, um inibidor clássico do complexo II, como sugerido no estudo conduzido por Okun e colaboradores (2002). Porém não conseguimos detectar a formação

do malonato em nossas condições experimentais, indicando que a inibição que encontramos não pode ser atribuída à formação deste composto.

Outra hipótese que poderia explicar a inibição seletiva do complexo II pelo MMA seria a participação de radicais livres, uma vez que estes complexos enzimáticos são sensíveis ao dano oxidativo (Zhang et al., 1990; Melov et al., 1999; Rafique, Schapira e Cooper, 2001). O cérebro é especialmente vulnerável ao estresse oxidativo devido a vários fatores, tais como o alto consumo de oxigênio por unidade de massa, as altas concentrações de ferro encontradas em regiões como o estriado, o baixo nível de defesas antioxidantes, a presença de aminoácidos excitatórios e o metabolismo da dopamina, que geram peróxido de hidrogênio (Halliwell e Gutteridge, 1999). Além disso, nossos resultados demonstraram que o MMA aumenta a lipoperoxidação em homogeneizado de estriado e hipocampo e diminui as defesas antioxidantes em homogeneizado de estriado de ratos. Porém, a co-incubação do MMA com L-NAME ou a mistura de catalase e superoxido dismutase não preveniu a inibição do complexo II, o que indica que o oxido nítrico, o peróxido de hidrogênio e o superóxido não estão envolvidos na inibição deste complexo enzimático. Não se pode, no entanto, afastar a hipótese de que os radicais livres podem estar sendo gerados através de uma inibição do complexo II pelo MMA (Massieu, Del Rio e Montiel, 2001).

Em nossos estudos também verificamos que o MMA inibiu a atividade dos complexos II-III da cadeia respiratória em todos os tecidos testados. É importante ressaltar que a etapa mais lenta da passagem dos elétrons entre os complexos II-III é a redução da ubiquinona pelo complexo II, e que inibições tão altas quanto 45 % no complexo III podem não tornar mais lenta a passagem dos elétrons através deste caminho. (Schneider, Lemastes e Hackenbrock, 1982; Taylor et al., 1994). Neste contexto, acreditamos que a inibição

provocada pelo MMA no complexo II-III seja mais um reflexo de uma inibição na redução da ubiquinona, (parte do complexo II) do que uma inibição do complexo III propriamente dita. Nossos resultados não demonstraram uma inibição no complexo II em rins, fígado e coração, e a inibição do complexo II-III em estriado foi mais elevada do que a inibição verificada no complexo II isolado. Isto pode ser devido ao fato de que a técnica de medida do complexo II isolado utiliza um aceptor artificial de elétrons, o DCIP, enquanto que na técnica de medida do complexo II-III, é a própria ubiquinona do tecido que recebe os elétrons. Portanto, as diferenças observadas em nossos resultados podem ser atribuídas a uma diferença na afinidade dos aceptores DCIP ou ubiquinona pelos elétrons na presença do MMA.

Além disso, o MMA inibiu mais do que 50% a atividade do complexo I-III no rim e no fígado, sem afetar a atividade deste complexo em estriado, hipocampo e coração. Por outro lado, o MMA não afetou a atividade do complexo IV em nenhuma das estruturas analisadas.

Neste estudo também verificamos que as atividades dos complexos da cadeia respiratória variam entre os tecidos analisados. As distintas atividades dos complexos e as inibições tecido-específicas provocadas pelo MMA poderiam ser atribuídas a: 1) características específicas das células nos diferentes tecidos; 2) isoformas tecido-específicas das várias subunidades das enzimas da cadeia respiratória (Merle e Kadenbach, 1980; Clay e Ragan, 1988; Rustin et al., 1994) e 3) distintas regulações do fluxo de elétrons através da cadeia respiratória que podem variar de um órgão para outro (Taylor et al., 1993).

Inibições da cadeia respiratória têm sido encontradas *postmortem* no cérebro de pacientes afetados por várias doenças neurodegenerativas, em que também foi demonstrado aumento de produção de radicais livres (revisado por Cassarino e Bennet Jr, 1999). O dano

neuronal produzido por toxinas mitocondriais parece envolver uma cascata de efeitos deletérios, onde a depleção energética leva à ativação de receptores NMDA, influxo de cálcio e a geração de radicais livres (Schulz et al., 1996a; Schulz et al., 1996b).

Uma questão interessante é porque a administração sistêmica de um inibidor metabólico como o MMA leva a um comportamento perseverante que indica dano seletivo do estriado. Neste particular, sabe-se que o estriado parece ser mais vulnerável a danos excitotóxicos e oxidativos do que outras estruturas cerebrais por causa da sua alta densidade de terminais nervosos glutamatérgicos e dopaminérgicos (Petersen et al., 2001; Brustovetsky et al., 2003).

Com relação ao significado fisiopatológico de nossos achados, verificamos que as alterações comportamentais foram demonstradas com concentrações in vivo de MMA da ordem de 1-2.5 mM (modelo químico de acidemia metilmalônica utilizado no presente estudo). Além disso, os efeitos in vitro do MMA sobre parâmetros de estresse oxidativo e sobre as atividades de vários complexos da cadeia respiratória foram observados na concentração de 2,5 mM e, portanto, similares as encontradas nos líquidos biológicos dos pacientes afetados por acidemia metilmalônica. Presume-se, portanto, que esses resultados provavelmente são de importância fisiopatológica.

Nossos resultados de alterações comportamentais estão de uma certa forma de acordo com outros resultados observados em nosso laboratório de que a administração crônica de MMA através do mesmo modelo aqui utilizado provoca déficit cognitivo na tarefa não aversiva de campo aberto, mas não em tarefas aversivas, tais como a esquiva inibitória e a esquiva ativa de duas vias (Dutra et al., 1991). Por outro lado, verificamos previamente um atraso no desenvolvimento neuromotor dos animais injetados cronicamente com MMA (Mello et al., 1994).

Em resumo, verificamos em nosso estudo que a administração crônica de MMA provoca comportamento perseverante em ratos que é prevenido pela administração simultânea de ácido ascórbico, sugerindo que a utilização desse antioxidante possa ser empregado, juntamente com outras terapias, no tratamento de pacientes afetados por acidemia metilmalônica. Observamos também que o MMA provoca estresse oxidativo *in vitro* em cérebro de ratos, além de inibir alguns complexos da cadeia respiratória em vários tecidos, indicando uma inibição da fosforilação oxidativa e comprometimento grave do metabolismo energético. Tais resultados estão de acordo com resultados prévios (Schultz et al, 1995; Schulz et al., 1996a; Schultz et al., 1996b; Zeevalk, Bernard e Nickas, 1998; Cassarino e Bennet Jr, 1999; Nicholls e Budd, 2000) e reforçam a idéia de que radicais livres e inibição do metabolismo energético são mecanismos importantes para explicar as lesões teciduais características dos pacientes afetados por acidemia metilmalônica. Tendo em vista os presentes resultados, presume-se que a utilização de substratos energéticos e antioxidantes por via oral que facilmente cruzam a barreira hematoencefálica (creatina e vitamina C) poderiam representar uma terapia adjuvante no tratamento dos pacientes afetados por acidemia metilmalônica.

V Conclusões

1) O tratamento crônico com MMA provocou comportamento perseverativo em ratos na tarefa do labirinto aquático de Morris, sugerindo dano cerebral de longa duração ou permanente.

2) As alterações comportamentais provocadas pelo MMA foram prevenidas pelo tratamento simultâneo com o antioxidante ácido ascórbico, o que sugere a participação do estresse oxidativo nas alterações comportamentais.

3) O MMA provocou aumento da lipoperoxidação *in vitro* em homogeneizado de estriado e hipocampo de ratos, verificado pelo aumento do TBA-RS e diminuiu as defesas antioxidantes teciduais em homogeneizado de estriado de ratos, observado pela diminuição do TRAP e TAR.

4) O MMA (2,5 mM) provocou inibição *in vitro* do complexo II da cadeia respiratória em homogeneizado de estriado e hipocampo de ratos com baixa concentração de sucinato (substrato) no meio de incubação (0,5 e 1,0 mM). Essa inibição somente pode ser detectada após 10 minutos de incubação do homogeneizado com o ácido e não pode ser prevenida pela co-incubação com enzimas antioxidantes, e tampouco com L-NAME, indicando que ela não ocorreu por ação de radicais livres comuns sobre este complexo enzimático.

5) O MMA inibiu também o complexo II-III da cadeia respiratória em homogeneizados de estriado, hipocampo, rim, fígado e coração de ratos, bem como o complexo I-III da cadeia respiratória em homogeneizado de rim e fígado de ratos. Por outro lado, o complexo IV da cadeia respiratória não foi inibido pelo MMA em nenhum tecido testado.

Com base nos resultados apresentados, concluímos que o estriado e o hipocampo são as estruturas mais sensíveis ao ataque do MMA. É importante salientar que lesões no estriado geram comportamento perseverativo, que foi verificado nos ratos tratados cronicamente com MMA, bem como em pacientes portadores de acidemia metilmalônica. Com base nisso, uma estratégia de tratamento seria a utilização de substratos energéticos e antioxidantes para tentar prevenir, ou pelo menos atenuar, os danos causados pela acidemia metilmalônica.

VI PERSPECTIVAS

1) Estudar o efeito da administração crônica simultânea de substratos energéticos (creatina ou sucinato), bem como do antagonista glutamatérgico NMDA MK-801 com ácido metilmalônico para verificar se déficit energético e/ou superestimulação dos canais ionotrópicos NMDA estariam envolvidos com as alterações comportamentais observadas na tarefa do labirinto aquático de Morris provocadas pelo ácido metilmalônico.

2) Testar o efeito do ácido metilmalônico sobre a viabilidade de neurônios cultivados de córtex cerebral, hipocampo e estriado de embriões de ratos para verificar se as células destas estruturas cerebrais são sensíveis aos efeitos tóxicos desse ácido orgânico.

3) Testar o efeito da co-incubação de antioxidantes, substratos energéticos e antagonistas glutamatérgicos com o ácido metilmalônico para esclarecer os mecanismos citotóxicos deste ácido orgânico em neurônios cultivados de embriões de ratos.

4) Estudar o tipo de morte celular (apoptose ou necrose) provocado pelo ácido metilmalônico sobre os neurônios cultivados de embriões de ratos.

5) Estudar os efeitos do ácido metilmalônico sobre vários parâmetros de estresse oxidativo em fígado e rim, para verificar se as inibições na cadeia respiratória provocadas por esse ácido nesses órgãos podem gerar estresse oxidativo.

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