UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - BIOQUÍMICA

Efeitos de metabólitos acumulados na síndrome hiperornitinemia-hiperamonemia-homocitrulinúria sobre a homeostase energética e redox cerebral e o comportamento de ratos

CAROLINA MASO VIEGAS

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“Anyone who has never made a mistake has never tried anything new.”

Albert Einstein

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ÍNDICE

PARTE I

INTRODUÇÃO E OBJETIVOS

RESUMO

A síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH) é uma doença metabólica hereditária bioquimicamente caracterizada pelo acúmulo de ornitina (Orn), homocitrulina (Hcit), amônia e ácido orótico (Oro) no sangue e outros tecidos dos pacientes afetados. Os sintomas neurológicos desse distúrbio são comuns, incluindo retardo mental, convulsões e ataxia, porém os mecanismos que levam ao dano cerebral são praticamente desconhecidos. O presente estudo teve o objetivo de avaliar o efeito dos metabólitos acumulados nesta doença sobre a homeostase energética e redox em cérebro de ratos, bem como sobre o desempenho de animais submetidos à hiperornitinemia em tarefas comportamentais. Inicialmente avaliamos o efeito *in vitro* da Orn, Hcit e Oro sobre parâmetros do metabolismo energético em cérebro de ratos jovens. Verificamos que a Orn e a Hcit inibem o ciclo dos ácidos tricarboxílicos, (inibição da produção de CO_2 a partir de acetato e das enzimas aconitase e α-cetoglutarato desidrogenase), bem como a via glicolítica aeróbica (redução na síntese de CO_2 a partir de glicose), além de comprometer o fluxo de elétrons pela cadeia respiratória (inibição do complexo I-III). A Hcit, mas não a Orn, também foi capaz de inibir a atividade da enzima creatina quinase, sendo que essa inibição foi prevenida por GSH, sugerindo um possível papel de espécies reativas oxidando grupamentos tióis, essenciais para a atividade desta enzima. Em contraste, a atividade das outras enzimas do ciclo dos ácidos tricarboxílicos e da cadeia respiratória, bem como a Na^+,K^+-ATPase não foram alteradas *in vitro* pela Orn e pela Hcit nas doses testadas (0,1 a 5 mM). Do mesmo modo, o Oro não interferiu em nenhum dos parâmetros testados.

O próximo passo deste estudo foi avaliar o efeito *in vivo* da administração intracerebroventricular (ICV) de Orn e Hcit sobre parâmetros de estresse oxidativo e metabolismo energético em córtex cerebral de ratos jovens. A Orn e a Hcit aumentaram os níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e a formação de grupamentos carbonilas, indicativos de peroxidação lipídica e dano oxidativo a proteínas, respectivamente. Além do mais, a N-acetilcisteína e a combinação de ácido ascórbico mais α-tocoferol atenuaram a oxidação lipídica e preveniram totalmente o dano oxidativo protéico provocado pela Orn e pela Hcit, sugerindo que espécies reativas estejam envolvidas nestes efeitos. A administração ICV de Hcit, mas não de Orn, também diminuiu os níveis de glutationa reduzida (GSH), bem como a atividade das enzimas catalase e glutationa peroxidase, indicando que a Hcit provoca uma redução nas defesas antioxidantes cerebrais. Quanto aos parâmetros de metabolismo energético avaliados após a administração ICV de Orn e Hcit, verificamos que a Orn e a Hcit inibem o funcionamento do ciclo dos ácidos tricarboxílicos (inibição da síntese de CO_2 a partir de acetato), a via glicolítica (redução na produção de CO_2 a partir de glicose) e a atividade do complexo I-III da cadeia respiratória. A Hcit *in vivo* também inibiu a atividade da aconitase, uma enzima muito susceptível ao ataque de radicais livres.

Investigamos também os efeitos da administração ICV de Orn e Hcit na presença ou ausência de hiperamonemia, induzida pela administração intraperitoneal de urease, sobre parâmetros de estresse oxidativo em córtex cerebral de ratos jovens. A Orn aumentou os níveis de TBA-RS e a formação de carbonilas, sem alterar o conteúdo de grupamentos sulfidrilas e os níveis de GSH. Além disso, a combinação de hiperamonemia com Orn resultou em uma

diminuição no conteúdo de sulfidrilas e GSH, indicando um efeito sinérgico da Orn com a amônia. Além disso, a Hcit causou um aumento nos valores de TBA-RS e da formação de carbonilas, bem como uma diminuição na concentração de GSH sem alterar o conteúdo de sulfidrilas. Em relação ao tratamento com urease, a indução de hiperamonemia pela urease foi capaz de aumentar os níveis de TBA-RS, indicando que hiperamonemia causa dano oxidativo a lipídeos.

Finalmente, produzimos um modelo animal quimicamente induzido de hiperornitinemia através da administração subcutânea de Orn (2-5 µmol/g de peso corporal). Altas concentrações cerebrais de Orn foram alcançadas nesse modelo, indicando que a Orn é permeável a barreira hemato-encefálica. Investigamos, então, o efeito da administração crônica de Orn do 5° ao 28° dia pós-natal sobre o desenvolvimento físico, motor e sobre o desempenho dos ratos adultos nas tarefas de campo aberto e no labirinto aquático de Morris. A administração crônica de Orn não afetou o aparecimento de pelos, a abertura dos olhos, a erupção dos incisivos e o reflexo de queda livre, sugerindo que o desenvolvimento físico e neuromotor não foram comprometidos pela administração crônica de Orn. Similarmente, o desempenho dos ratos no labirinto aquático de Morris na idade adulta não foi alterado pelo tratamento crônico dos mesmos com Orn, indicando que a memória espacial não foi afetada. Entretanto, os animais tratados com Orn não apresentaram habituação ao campo aberto, sugerindo um déficit de aprendizado/memória nesta tarefa. A atividade motora, avaliada pelo número de cruzamentos na tarefa do campo aberto, e pela velocidade de natação no labirinto aquático de Morris foram similares para os animais injetados com Orn e salina, indicando que os animais com hiperornitinemia não apresentaram déficit na atividade locomotora que pudesse atrapalhar seu desempenho nos testes de comportamento. De maneira similar, o número de bolos fecais, o número de *grooming* e o tempo gasto na área central na tarefa de campo aberto foram iguais em ambos os grupos, indicando que não houve alteração na ansiedade dos animais com o tratamento com Orn.

A presente investigação demonstrou pela primeira vez que a Orn e especialmente a Hcit comprometem a homeostase energética e redox celular e que hiperornitinemia crônica durante o período pós-natal prejudica o desempenho de animais adultos na tarefa de campo aberto. É provável, portanto, que as concentrações cerebrais aumentadas de Orn tenham provocado um dano cerebral, possivelmente através do comprometimento do metabolismo energético e da indução de dano oxidativo, alterando vias do metabolismo necessárias para um aprendizado/memória normais. Concluindo, postulamos que alterações na bioenergética e no estado redox cerebral induzido pelos metabólitos acumulados na síndrome HHH, como demonstrado *in vitro* e *in vivo* na presente investigação, possam representar mecanismos patogênicos, contribuindo, ao menos em parte, para os sintomas neurológicos dos pacientes afetados por este transtorno.

ABSTRACT

Tissue accumulation of ornithine (Orn), homocitrulline (Hcit), ammonia and orotic acid (Oro) is the biochemical hallmark of patients affected by hyperornithinemia–hyperammonemia– homocitrullinuria (HHH) syndrome, a disorder clinically characterized by neurological symptoms, whose pathophysiology is practically unknown. The aim of this study was to evaluate the effect of the accumulating metabolite in the HHH syndrome on energetic and redox homeostasis in brain of rats, as well as the rat performance in behavioural tasks when submitted to hiperornithinemia. We first investigated the *in vitro* effect of Orn, Hcit and Oro on parameters of energy metabolism in brain of young rats. We verified that Orn and Hcit inhibited the citric acid cycle (inhibition of CO_2 synthesis from acetate, as well as aconitase and α-ketoglutarate dehydrogenase activities), the aerobic glycolytic pathway (reduced CO_2 production from glucose) and moderately the electron transfer flow (inhibitory effect on complex I–III). Hcit, but not Orn, was also able to inhibit the mitochondrial creatine kinase activity. Furthermore, this inhibition was prevented by glutathione, suggesting a possible role of reactive species oxidizing critical thiol groups of the enzyme. In contrast, the other enzyme activities of the citric acid cycle and of the electron transfer chain, as well as synaptic Na^+,K^+-ATPase were not altered by either Orn or Hcit at concentrations as high as 5.0 mM. Similarly, Oro did not interfere with any of the tested parameters.

The next step of this study was to investigate the *in vivo* effects of intracerebroventricular (ICV) administration of Orn and Hcit on parameters of oxidative stress and energy metabolism in cerebral cortex from young rats. Orn and Hcit increased thiobarbituric acid-reactive substances values and carbonyl formation, indicators of lipid and protein oxidative damage, respectively. Furthermore, N-acetylcysteine and the combination of ascorbic acid plus α-tocopherol attenuated the lipid oxidation and totally prevented the protein oxidative damage provoked by Orn and Hcit, suggesting that reactive species were involved in these effects. The ICV Hcit administration, but not Orn administration, also decreased reduced glutathione (GSH) concentrations, as well as the activity of catalase and glutathione peroxidase, indicating that Hcit provokes a reduction of brain antioxidant defenses. As regards to the parameters of energy metabolism, we verified that Orn and Hcit inhibited the citric acid cycle function (inhibition of CO_2 synthesis from acetate), the aerobic glycolytic pathway (reduced CO_2 production from glucose) and complex I–III activity of the respiratory chain. Hcit also inhibited the activity of aconitase, an enzyme very susceptible to free radical attack.

We also investigated the *in vivo* effects of ICV administration of Orn and Hcit in the presence or absence of hyperammonemia induced by intraperitoneal urease treatment on important parameters of oxidative stress in cerebral cortex from young. Orn increased thiobarbituric acid-reactive substances levels and carbonyl formation, without altering sulfhydryl content and GSH levels. We also observed that the combination of hyperammonemia with Orn resulted in a decrease of sulfhydryl levels and GSH concentrations, highlighting a synergistic effect of ornithine and ammonia. Furthermore, homocitrulline caused increases of thiobarbituric acid-reactive substances values and carbonyl formation, as well as a decrease of GSH concentrations without altering sulfhydryl content. We also observed that urease treatment *per se* was able to enhance thiobarbituric

acid-reactive substances levels indicating that hiperamonemia induce lipid peroxidation.

Finally, we produced a chemical animal model of hiperornithinemia induced by a subcutaneous injection of saline-buffered Orn (2-5 µmol/g body weight) to rats. High brain Orn concentrations were achieved, indicating that Orn is permeable to the blood brain barrier. We then investigated the effect of early chronic postnatal administration of Orn (from the 5th to the 28th day of life) on physical and motor development and on the performance of adult rats in the open field and in the Morris water maze tasks. Chronic postnatal Orn treatment had no effect on the appearance of coat, eye opening or upper incisor eruption, nor on the free-fall righting task, suggesting that physical and motor development were not changed by Orn. However, Orn-treated rats did not habituate to the open field apparatus, implying a deficit of learning/memory. Motor activity was the same for Orn- and saline- injected animals. Motor activity, evaluated by the number of crossings in the open field and by the swimming speed in the Morris water maze, was the same for Orn- and saline- injected animals, indicating no deficit of locomotor activity in rats injected with Orn. Similarly, the number of fecal boli and grooming and the time spent in the central area in the open field task were the same in both groups, implying no alteration of emotionality.

The current investigation shows for the first time that Orn and especially Hcit compromise energetic and redox homeostasis and that chronic hyperornithinemia during postnatal period impairs the adult rat performance in the open field task, where the animals did not habituate to the apparatus. It is possible, however, that high sustained cerebral Orn level could provoke a brain damage, possibly through induction of oxidative stress and/or compromising energy metabolism, altering some metabolic pathways essential for normal learning/memory. In conclusion, we postulate that alterations in the cerebral bioenergetic and redox state induced by metabolites accumulated in HHH syndrome, as demonstrated *in vitro* and *in vivo* in the present study, may represent pathogenic mechanisms contributing, at least in part, to the neurological symptoms of patients affected by this disorder.

LISTA DE ABREVIATURAS

CAT – catalase

CK – creatina quinase

CLp - depuração plasmática

Cr – creatina

EIM – erros inatos do metabolismo

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

GPx – glutationa peroxidase

GSH – glutationa reduzida

H_2O_2 – peróxido de hidrogênio

Hcit – homocitrulina

HHH – hiperornitinemia-hiperamonemia-homocitrulinúria

ICV – intracerebroventricular

i.p – intraperitoneal

L-NAME – N^{ω}-nitro-L-arginina metil éster

NAC – N-acetilcisteína

$NO^{\bullet}$ – óxido nítrico

$ONOO^{-}$ – peroxinitrito

Orn – ornitina

SNC – sistema nervoso central

SOD – superóxido dismutase

TBA-RS – substâncias reativas ao ácido tiobarbitúrico

I.1 INTRODUÇÃO

I.1.1 Erros inatos do metabolismo

O termo erros inatos do metabolismo (EIM) foi utilizado pela primeira vez por Archibald Garrod em 1908 durante estudos realizados em pacientes com alcaptonúria, doença em que os pacientes afetados excretam grandes quantidades de ácido homogentísico na urina. O pesquisador observou que um ou mais indivíduos da mesma família eram afetados sem que seus pais apresentassem a doença. Baseado também na observação da maior incidência de consanguinidade entre os pais dos pacientes e nas leis de Mendel, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da determinação do ácido homogentísico na urina de pacientes com alcaptonúria e da observação de que esta substância era um metabólito normal da degradação da tirosina, Garrod relacionou este acúmulo a um bloqueio no metabolismo do ácido homogentísico. Verificou-se mais tarde que tais alterações resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, usualmente uma enzima (Scriver *et al.*, 2001). Presumiu-se então que, em consequência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores tóxicos da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987).

Até o momento foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver *et al*., 2001). Embora individualmente raras, essas doenças em seu conjunto afetam aproximadamente 1 a cada 500 a 1.000 nascidos vivos (Baric *et al*, 2001).

I.1.2 Doenças do metabolismo da ornitina

A ornitina (Orn) é um aminoácido não protéico que exerce um papel fundamental no metabolismo da ureia, da creatinina e das poliaminas. As vias metabólicas da Orn interagem com o ciclo da ureia e o ciclo dos ácidos tricarboxílicos. A Orn é substrato ou produto de cinco enzimas e é transportada por um proteína carreadora mitocondrial. O metabolismo deste aminoácido ocorre no ciclo da ureia, na biossíntese de poliaminas e creatina e na reação da ornitina amino transferase (OAT) (Valle e Simell, 2001).

No que se refere ao ciclo da ureia, três enzimas são citosólicas (argininosuccinato sintetase, argininosuccinato liase e arginase), enquanto duas estão presentes na matriz mitocondrial (ornitina transcarbamilase e carbamoil fosfato sintetase). Portanto, a realização do ciclo da ureia requer o transporte de Orn para a matriz mitocondrial e citrulina para fora da mitocôndria (Klingenberg, 1970). O transporte de Orn para dentro da mitocôndria no tecido hepático (principal tecido ureogênico) é mediado por um carreador específico, ORNT1 e em menor extensão pelo ORNT2 (Gamble e Lehninger, 1973; Fiermonte *et al*., 2003). A captação de Orn para dentro da mitocôndria em células extra-hepáticas parece envolver diferentes transportadores, incluindo o ORNT2 (Passarella *et al*., 1990; Fiermonte *et al*., 2003). Estas observações são consistentes com a idéia de que o transporte de Orn para dentro da mitocôndria tem diferentes propósitos em diferentes tecidos; a captação de Orn nas células hepáticas serve, primariamente, para a síntese de ureia, enquanto que em outros tecidos serve para a degradação da Orn e a biossíntese de arginina e creatina (Valle e Simell, 2001).

As desordens do metabolismo da Orn são caracterizadas pelo acúmulo de Orn, o que leva a hiperornitinemia. Até o momento foram descritos dois erros inatos do metabolismo que causam hiperornitinemia: a atrofia girata da coróide e da retina (OMIM 258870), com sintomas limitados principalmente aos olhos, e a síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH) com sintomas e sinais predominantemente neurológicos (OMIM 238970).

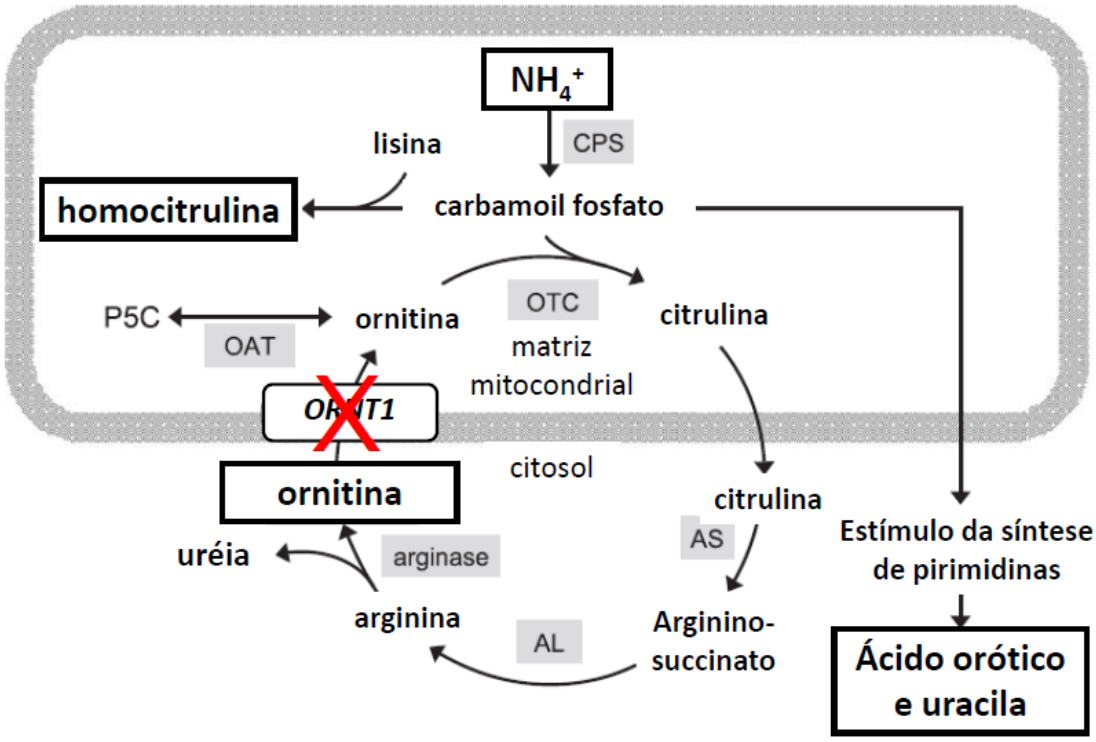
I.1.3 Síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH)

Em 1969 Shih e colaboradores descreveram o primeiro paciente com aumentos no plasma de Orn (hiperornitinemia) e amônia (hiperamonemia), bem como elevada excreção de homocitrulina na urina (homocitrulinúria) (Shih *et al*., 1969).

A síndrome HHH é um transtorno metabólico de herança autossômica recessiva (Palmieri, 2008; Tessa *et al*., 2009) na qual o aumento da concentração de Orn plasmática diferencia esta síndrome de outras desordens do ciclo da ureia, e a hiperamonemia e a homocitrulinúria distingue da atrofia girata, em que os pacientes afetados também apresentam hiperornitinemia (Valle e Simell, 2001).

Esta síndrome é considerada um transtorno do ciclo da ureia e da via de degradação da Orn causada por uma mutação no ORNT1 (SLC25A15), gene que codifica um transportador mitocondrial de Orn (figura 1) (Fell *et al*., 1974; Camacho *et al*., 1999). A inabilidade de transportar Orn do citosol para dentro da mitocôndria resulta em uma deficiência intramitocondrial de Orn e consequentemente da função do ciclo da ureia ao nível da enzima ornitina trascarbamilase, levando à hiperamonemia. Como a rota normal do

catabolismo da Orn ocorre através da enzima intramitocondrial ornitina aminotrasferase, a Orn citosólica se acumula, sendo secretada pela célula, resultando em hiperornitinemia. Na ausência de Orn dentro da mitocôndria, o carbamoil fosfato acumulado provavelmente condensa com lisina e forma homocitrulina (Hcit), através de um mecanismo ainda não esclarecido, levando a homocitrulinúria. Alternativamente, o carbamoil fosfato pode ser direcionado para a biossíntese citosólica das pirimidinas, levando ao aumento da excreção de ácido orótico (Oro) e uracila na urina (Valle e Simell, 2001).



(Figura adaptada de Korman *et al*., 2004)

Figura 1. Rotas metabólicas envolvidas na síndrome HHH devido à deficiência do transportador mitocondrial de ornitina ORNT1 (SLC25A15), levando ao acúmulo dos metabólitos marcados nos retângulos pretos. AL= argininosuccinato liase; AS = argininosuccinato sintetase; CPS = carbamoil fosfato sintetase; OAT = ornitina aminotransferase; OTC = ornitine transcarbamilase; P5C = D1 pirrolina-5-carboxilato.

I.1.3.1 Achados clínicos

Os sintomas da síndrome HHH normalmente aparecem durante o período neonatal ou na infância. No entanto, alguns casos não diagnosticados até a idade adulta já foram descritos (Gatfield *et al*., 1975). Na fase aguda, a doença é caracterizada por episódios intermitentes de hiperamonemia acompanhados por vômitos, ataxia, letargia, confusão mental e coma, similar aos achados clínicos dos pacientes afetados por doenças do ciclo da ureia (Valle e Simell, 2001). Alguns pacientes com a síndrome HHH apresentam doença hepática fulminante, em que o transplante hepático pode ser considerado (Fecarotta *et al*., 2006).

O curso crônico e progressivo da síndrome inclui uma recusa espontânea a alimentos ricos em proteínas, anormalidades de coagulação sanguínea, hipotonia, atraso no desenvolvimento, encefalopatia progressiva com retardo mental e sinais de disfunção motora (Debray *et al*., 2008; Tessa *et al*., 2009).

I.1.3.2 Achados bioquímicos e diagnóstico

Os pacientes acometidos pela síndrome HHH apresentam um aumento plasmático de Orn e amônia, juntamente com elevada excreção urinária de Hcit, sendo esses achados bioquímicos as características principais deste distúrbio e que dão o nome e levam ao diagnóstico deste EIM (Valle e Simell, 2001). A concentração plasmática de Orn varia de 200 a 1080 µM (valores normais: 20 a 100 µM) e a excreção urinária de Orn livre de 20 a 8160 µmol/g de creatinina (valores normais: 9 a 120 µmol/g de creatinina) (Korman *et al*., 2004; Debray *et al*., 2008; Tessa *et al*., 2009). Em relação à Hcit, a concentração urinária varia de 20 a 2380 µmol/g de creatinina (valores

normais: 0 a 30 µmol/g de creatinina), excedendo em muito os valores normais. Embora a síntese e a excreção de Hcit continuam não bem compreendidas, acredita-se que lisina condensa-se com carbamoil fosfato formando homocitrulina (Fell *et al*., 1974; Gjessing *et al*., 1986; Gordon *et al.*, 1987). Os níveis plasmáticos de amônia durante o período de jejum estão levemente mais elevados nos pacientes do que em indivíduos normais. Os valores de amônia posprandial aumentam com a ingestão aumentada de proteínas e pacientes com dieta hiperproteica possuem hiperamonemia crônica (Valle e Simell, 2001).

Em relação a outros aminoácidos, a concentração plasmática de arginina é normal, enquanto a lisina está moderadamente mais baixa e a glutamina e a alanina estão elevadas. Outro metabólito que aparece aumentado no plasma dos pacientes com a síndrome HHH é o lactato, bem como a razão lactato/aspartato (Korman *et al*., 2004).

A síndrome HHH pode ser diferenciada de outras síndromes que levam a hiperamonemia, incluindo as desordens do ciclo da ureia, através dos achados laboratoriais. A tríade hiperornitinemia, hiperamonemia e homocitrulinúria é um achado patognomônico desta doença. O defeito metabólico pode ser confirmado pelo ensaio de incorporação de ^{14}C-L-ornitina, em fibroblastos (Shih *et al*., 1982). A compartimentalização da Orn nos fibroblastos previne a conversão de Orn em prolina e glutamato resultando em uma baixa incorporação de radiatividade em macromoléculas.

I.1.3.3 Tratamento

O objetivo principal do tratamento tem sido prevenir a toxicidade da amônia seguindo os princípios delineados para as desordens do ciclo da ureia.

De maneira geral, os pacientes devem evitar situações de jejum e ingestão excessiva de proteínas. Restrição protéica para menos do que 1,2 g/kg/dia previne a hiperamonemia e resulta também em uma diminuição nas concentrações de Orn plasmática (Valle e Simell, 2001). Além disso, a suplementação com arginina e citrulina também tem sido instituída para diminuir a hiperamonemia e em alguns estudos mostrou-se efetiva para tanto. A utilização de citrulina parece ser mais efetiva, pois é capaz de incorporar na molécula dois grupamentos amino (Gordon *et al*., 1987).

I.1.3.4 Fisiopatogenia

Até o momento os mecanismos que levam aos achados clínicos, especialmente ao dano neurológico, nos pacientes com a síndrome HHH não estão bem esclarecidos. Acredita-se que a o aumento nas concentrações plasmáticas de amônia, que é neurotóxica (Felipo e Butterworth, 2002; Walker, 2009), seja o mecanismo mais importante na fisiopatologia da síndrome HHH. No entanto, alguns pacientes afetados pela síndrome HHH apresentam níveis normais de amônia sanguínea (Valle e Simell, 2001). Sendo assim, o acúmulo de Orn, Hcit e Oro, não podem ser descartados em exercer um papel importante na fisiopatologia desta síndrome. Neste contexto, um estudo recente demonstrou que a Orn e a Hcit induzem *in vitro* estresse oxidativo em córtex cerebral de ratos jovens (Amaral *et al*., 2009). Os pacientes afetados pela síndrome HHH frequentemente apresentam aumento dos níveis plasmáticos de ácido lático e da razão lactato/piruvato, sugerindo a possibilidade de possuírem alterações na homeostase mitocondrial.

I.1.4 Metabolismo energético no cérebro de mamíferos

O cérebro possui uma intensa atividade metabólica, porém suas reservas energéticas são extremamente pequenas em relação à sua demanda. Assim, há necessidade contínua de substratos energéticos para o cérebro de mamíferos, sendo a glicose o principal deles onde, em contraste com outros tecidos, não necessita de insulina para ser captada e oxidada (Dickinson, 1996). O padrão de utilização de glicose varia conforme a etapa de desenvolvimento do SNC, o estado nutricional do indivíduo e o destino de sua cadeia de átomos de carbono (Erecinska *et al.*, 2004; Lieberman e Marks, 2008). Situações de jejum prolongado fazem com que o SNC passe a utilizar corpos cetônicos para a obtenção de energia, a fim de poupar o organismo de um catabolismo protéico exacerbado resultante da necessidade da manutenção da glicemia via gliconeogênese (Lieberman e Marks, 2008). A glicose captada pelo cérebro é, também, fonte de carbono para a síntese de diversas outras biomoléculas (por exemplo, neurotransmissores), o que reforça a idéia de que a utilização de glicose não está atrelada somente à produção de energia.

Mitocôndrias de mamíferos são organelas intracelulares ubíquas, responsáveis pela produção de ATP pelo metabolismo aeróbico, mas também desempenham outras funções intracelulares além da produção de ATP, tendo um papel crítico no processo de apoptose e servindo como um tampão de cálcio. Tecidos com alta atividade oxidativa tais como cérebro, músculos esquelético e cardíaco, apresentam altas concentrações de mitocôndrias (Orth e Schapira, 2001).

I.1.4.1 Ciclo dos ácidos tricarboxílicos

O ciclo dos ácidos tricarboxílicos que ocorre nas mitocôndrias é a via comum de oxidação dos carboidratos, aminoácidos e ácidos graxos. O metabolismo energético cerebral se mostra essencialmente oxidativo, sendo a glicose o principal substrato utilizado (Clark *et al.*, 1993), entrando no ciclo sob a forma de acetil-CoA, que é então oxidada completamente a CO_2 e a energia é conservada na forma de coenzimas reduzidas (NADH e $FADH_2$) e GTP (Nelson e Cox, 2008).

I.1.4.2 Fosforilação oxidativa

A fosforilação oxidativa é o processo principal da produção de energia celular. Todos os passos oxidativos na degradação de carboidratos, lipídios e aminoácidos convergem a esse estágio final da respiração celular, em que a energia da oxidação, provida pelo fluxo de elétrons através das enzimas da cadeia transportadora de elétrons, promove a síntese de ATP (Nelson e Cox, 2008). Quando não há hipóxia, a fosforilação oxidativa é dependente da concentração de ATP, ADP e fosfato inorgânico (Pi) e da razão mitocondrial de $NADH/NAD^{+,}$ que é determinada pela atividade da cadeia transportadora de elétrons e pela transferência de elétrons provenientes de reações catalisadas por enzimas mitocondriais (Erecinska e Silver, 1994).

A cadeia transportadora de elétrons, composta por quatro complexos enzimáticos (complexos I-IV) além da ATPase (complexo V), recebe elétrons das coenzimas NADH e $FADH_2$, e os transfere através de uma série de reações de oxidação-redução até o oxigênio molecular e simultaneamente acopla essa reação exergônica à translocação de prótons através da membrana mitocondrial interna (Wallace, 1999). O fluxo de prótons (gradiente

eletroquímico de prótons) gerado durante o transporte de elétrons na cadeia transportadora de elétrons leva à formação de ATP a partir de ADP e Pi pelo complexo V (ATP sintase) (Babcock e Wikstrom, 1992; Wallace, 1999).

Os complexos da cadeia transportadora de elétrons são formados por várias cadeias polipeptídicas associadas a grupos transportadores de elétrons: NADH desidrogenase (complexo I), succinato: ubiquininona oxirredutase (complexo II), complexo citocromo b-c_1 (complexo III) e citocromo c oxidase (complexo IV), além de elementos móveis que se localizam entre os complexos, são eles a coenzima Q, um componente não protéico lipossolúvel que carreia elétrons entre os complexos I e III, e o citocromo *c*, uma proteína localizada na face externa da membrana que transfere os elétrons do complexo III para o complexo IV (Lieberman e Marks, 2008).

I.1.4.3 Creatina quinase

A creatina quinase (CK) consiste de um grupo de isoenzimas com um papel central no metabolismo energético, principalmente para tecidos com alta demanda energética, como cérebro, músculo cardíaco e esquelético, onde funciona como um efetivo sistema de tampão e transferência intracelular de energia. A CK catalisa a transfosforilação reversível entre ATP e creatina a ADP e fosfocreatina [$MgATP^-$ + creatina $\leftrightarrow$ $(fosfocreatina)^-$ + $MgADP^-$ + H^+], ajudando a manter os níveis dos substratos fosforilados. Sabe-se que durante a excitação nervosa e neuromuscular ocorre um aumento de dez vezes no *turnover* celular de ATP, e que durante essas mudanças rápidas, o sistema creatina/fosfocreatina é necessário tanto como um tampão energético quanto como um sistema de transporte entre os locais de produção e consumo de ATP pelas ATPases para evitar grandes flutuações nos níveis de ATP/ADP

celulares nesses tecidos excitáveis (Bessman e Carpenter, 1985; Schnyder *et al.*, 1991; Wallimann *et al.*, 1992).

As isoformas da CK estão localizadas em sítios de demanda e produção energética. A isoforma citosólica (Ci-CK) consiste de dímeros e é expressa de uma maneira tecido-específica, isto é, cérebro específica (BB-CK), músculo esquelético específica (MM-CK) e um heterodímero músculo cardíaco-específico (MB-CK) (O'Gorman *et al.*, 1996; Schnyder *et al.*, 1991; Wallimann *et al.*, 1992). As formas mitocondriais da CK (Mi-CK) são dispostas em octâmeros e são compostas da isoforma sarcomérica músculo específica Mib-CK e da forma ubíqua Mia-CK, que é encontrada nas mitocôndrias do tecido cerebral (Gross *et al.*, 1996; Saks *et al.*, 1985; Schlegel *et al.*, 1988; Wallimann *et al.*, 1992).

Devido à sua localização próxima a sítios onde ocorrem a geração de energia e o transporte de íons através de membranas, o sistema CK/fosfocreatina desempenha um papel fundamental na homeostase energética neuromuscular. Assim, é presumível que alterações na função da CK levem ao desenvolvimento de vários estados patológicos envolvendo o cérebro, músculo esquelético e músculo cardíaco (Aksenov *et al.*, 2000; Aksenov *et al.*, 1997; Aksenova *et al.*, 1999; David *et al.*, 1998; Hamman *et al.*, 1995).

I.1.5 Radicais livres

Um radical livre é qualquer espécie química capaz de existir de forma independente e que contenha um ou mais elétrons desemparelhados (Southorn e Powis, 1988; Halliwell, 2001; Halliwell, 2006; Halliwell e Gutteridge, 2007a).

Em condições fisiológicas do metabolismo celular aeróbico, o oxigênio molecular (O_2) sofre redução tetravalente, com incorporação de quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 5% do oxigênio utilizado na cadeia respiratória mitocondrial não é completamente reduzido à água, podendo ser convertido a intermediários reativos como o radical superóxido ($O_2^{\bullet-}$) e hidroxila ($OH^{\bullet}$), e também a peróxido de hidrogênio (H_2O_2), processo esse que pode ser exacerbado em condições patológicas (Boveris, 1998).

O termo genérico “espécies reativas de oxigênio” (ERO) é usado para incluir não só os radicais formados pela redução do O_2, como por exemplo, os radicais superóxido ($O_2^{\bullet-}$) e hidroxila ($OH^{\bullet}$), mas também algumas substâncias reativas não radicais derivados do oxigênio, como o H_2O_2 (Halliwell e Gutteridge, 2007a). Além dessas, existem ainda as espécies reativas de nitrogênio (ERN), sendo o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) os principais representantes.

As ERO e ERN ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente, essas espécies reativas são importantes para a função celular (Bergendi *et al*., 1999). Assim, um aumento eventual da liberação local de radicais livres pode ser benéfico, como é o caso da liberação de espécies tóxicas oxidantes pelos neutrófilos que atuam na defesa do hospedeiro contra uma infecção (Delanty e Dichter, 1998; Halliwell e Gutteridge, 2007b). As espécies ativas ainda participam de processos de sinalização celular e também estão envolvidos na síntese e regulação de algumas proteínas (Halliwell e Gutteridge, 2007b).

Por outro lado, quando formadas em excesso, essas espécies altamente reativas têm o potencial de oxidar moléculas (Maxwell, 1995). Com relação aos efeitos prejudiciais das reações oxidantes ao organismo, os radicais livres podem promover lipoperoxidação (oxidação lipídica), causar a oxidação de lipoproteínas de baixa densidade, reagir com proteínas, levando à sua inativação e consequente alteração de sua função, além de reagir com o DNA e RNA, levando a mutações somáticas e a alterações na transcrição gênica (Delanty e Dichter, 1998; Halliwell e Whiteman, 2004), dentre outros efeitos.

I.1.5.1 Defesas antioxidantes

Para evitar os efeitos danosos das espécies reativas, existem mecanismos eficientes para sua eliminação, como a produção endógena de enzimas antioxidantes e alguns antioxidantes não-enzimáticos. Embora diferindo na sua composição, as defesas antioxidantes estão amplamente distribuídas no organismo (Halliwell e Gutteridege, 2007c) e compreendem:

- Agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx);
- Proteínas que diminuem a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo), ao se ligarem aos mesmos, como as transferrinas;
- Proteínas que protegem biomoléculas de dano oxidativo por outros mecanismos, como as chaperonas;

- Agentes de baixo peso molecular que sequestram espécies reativas de oxigênio e nitrogênio, como glutationa (GSH), α-tocoferol, ácido ascórbico e a bilirrubina.

I.1.5.2 Estresse oxidativo

Organismos saudáveis em condições normais produzem espécies reativas, que em sua maior parte são controladas pelos sistemas de defesa antioxidante. No entanto, em determinadas condições patológicas pode haver um desequilíbrio entre a produção de oxidantes e as defesas antioxidantes, favorecendo a ocorrência do estresse oxidativo.

Assim, o termo estresse oxidativo é usado para se referir à situação na qual a geração de espécies reativas ultrapassa a capacidade das defesas antioxidantes disponíveis. Pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição que aceleram a produção de algumas espécies reativas, ou então da combinação de quaisquer desses fatores (Halliwell, 2006).

O estresse oxidativo pode promover adaptação, dano ou morte celular:

- Adaptação: as células podem tolerar um estresse oxidativo moderado, que geralmente resulta em um aumento da síntese de sistemas de defesa antioxidante a fim de restaurar o balanço pró-oxidante / antioxidante.

- Dano celular: o estresse oxidativo pode provocar dano a alvos moleculares (DNA, proteínas, carboidratos e lipídios) (Halliwell e Gutteridge, 2007b). Nesses casos, a resposta à injúria tecidual pode ser

reversível: a célula entra em um estado de homeostase alterado temporário ou prolongado, que não leva à morte celular.

- Morte celular: pode ocorrer tanto por necrose quanto por apoptose. Na morte celular por necrose, a célula incha e se rompe, liberando seu conteúdo para o meio extracelular. Pode haver a liberação de antioxidantes, como a CAT e GSH, e também de pró-oxidantes, como o íon cobre e ferro e proteínas do grupo heme, agente esses que podem afetar as células adjacentes, podendo até mesmo induzi-las a um estresse oxidativo. Já na apoptose, o mecanismo intrínseco de morte celular programada é ativado e não há a liberação do conteúdo celular. A apoptose pode estar acelerada em certas doenças, tais como as desordens neurodegenerativas, havendo envolvimento do estresse oxidativo (Halliwell e Gutteridge, 2007c).

I.1.5.3 Estresse oxidativo e doenças neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a neurodegeneração das mais comuns doenças neurodegenerativas, as doenças de Alzheimer, Huntington e Parkinson (Alexi *et al*., 2000; Mendéz-Álvarez *et al*., 2001; Behl *et al*., 2002; Halliwell, 2006), sem, entretanto, obter até o momento uma explicação completamente satisfatória para explicar o dano cerebral nessas patologias. No entanto, acredita-se que possíveis mecanismos envolvam deficiência no metabolismo energético, estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA (excitotoxicidade), ou, possivelmente, um somatório desses fatores (Rose e Henneberry, 1994).

Estudos prévios demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Schapira *et al*., 1990a). Essa inibição do complexo I pode acarretar um aumento na geração de espécies reativas, tais como ânion superóxido, radicais hidroxila e peroxinitrito, as quais poderiam inibir um ou mais passos da cadeia transportadora de elétrons. Dessa forma, é possível que o estresse oxidativo e a disfunção mitocondrial formem um "ciclo vicioso" na doença de Parkinson (Schapira *et al*., 1989, 1990a,b; Janetzky *et al*., 1994; Gu *et al*., 1998).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é possível que o estresse oxidativo tenha um papel chave na morte neuronal. Tem sido proposto que o peptídeo β-amilóide, o formador das placas senis, tenha a capacidade de gerar radicais livres espontaneamente. Estudos *in vivo* também evidenciaram um dano oxidativo em cérebros humanos *postmortem* com doença de Alzheimer, através da observação de aumento de 8-hidroxi-2'-deoxiguanosina (8-OHdGA), produtos de oxidação de outras bases e de RNA, carbonilas de proteínas, nitrotirosina e marcadores de peroxidação lipídica (Smith *et al*., 1991; Markesbery e Carney, 1999; Nourooz-Zadeh *et al*., 1999; Lovell *et al*., 2000).

Por outro lado, verificou-se um dano oxidativo importante em pacientes portadores da doença de Huntington, particularmente representado pela formação de 3-nitrotirosina nas áreas afetadas (Alexi *et al*., 2000). Entretanto, o dano oxidativo observado nessa doença aparentemente tem menor importância do que nas doenças de Parkinson e Alzheimer.

Nos últimos anos, foi também verificado que vários metabólitos acumulados em alguns EIM com comprometimento severo do SNC induzem estresse oxidativo no cérebro de animais experimentais (Latini *et al*., 2007; Ribeiro *et al*., 2007; Zugno *et al*., 2008; Fernandes *et al*., 2011) e em seres humanos (Sitta *et al*., 2006; Deon *et al*., 2007; Barschak *et al*., 2008a,b; Deon *et al*., 2008; Ribas *et al*., 2010; Vargas *et al*., 2011), indicando que os compostos acumulados nestas doenças possam causar dano oxidativo.

I.1.6 Estudos comportamentais

Uma abordagem importante para se avaliar dano funcional causado ao SNC por neurotoxinas é feita através do estudo do desenvolvimento físico e comportamental em modelos animais. Neste particular, o agente potencialmente neurotóxico a ser testado pode causar déficit no desempenho dos animais em tarefas comportamentais. Assim, a realização e a análise do desempenho de animais submetidos a um tratamento com o agente potencialmente tóxico em tarefas comportamentais são importantes para avaliar as possíveis consequências funcionais de um dano neuronal (Olton e Markowska, 1994).

Aprendizado e memória são funções básicas do SNC, 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, com o armazenamento de informações (Izquierdo, 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.

I.1.6.1 Campo aberto

A atividade exploratória de animais de laboratório pode ser analisada pelo modelo de campo aberto que permite observar como o animal se comporta em um ambiente amplo (Ho *et al*., 2002).

O teste é realizado em uma caixa de madeira com dimensões estabelecidas, em que o fundo é dividido por linhas pretas em 12 quadrados iguais, servindo para se analisar aprendizado/memória e outras formas de comportamento. Neste experimento pode-se avaliar a movimentação espontânea entre as divisões (número de cruzamentos) da caixa que indica a atividade exploratória dos animais. Também se observa o número de *rearings* (quando o animal se apóia somente com as patas traseiras), *groomings* e bolos fecais para avaliar habituação e emocionalidade (Vianna, 2000).

I.1.6.2 Labirinto aquático de Morris

A tarefa do labirinto aquático de Morris é adequada para se avaliar aprendizado/memória espaciais 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 procuraram escapar da mesma. Enfatize-se que a tarefa do labirinto aquático de Morris é adequada para a avaliação da integridade funcional de algumas estruturas do sistema nervosas centrais envolvidas com os processos relacionados com aprendizado e memória e particularmente com o raciocínio espacial, como, por exemplo, o hipocampo e o estriado (Olton e Markowska, 1994; Save e Poucet, 2000).

I.2 OBJETIVOS

I.2.1 Objetivo geral

O objetivo deste trabalho foi investigar os efeitos *in vitro* e *in vivo* dos metabolitos acumulados na síndrome HHH (ornitina, homocitrulina, amônia e ácido orótico) sobre a homeostase energética e redox celular cerebral em ratos jovens. Objetivamos também avaliar os efeitos de um modelo de hiperornitinemia, através da administração crônica de ornitina a ratos durante o desenvolvimento cerebral, sobre tarefas comportamentais.

I.2.2 Objetivos específicos

- Determinar os efeitos *in vitro* da ornitina (Orn), da homocitrulina (Hcit) e do ácido orótico (Oro) em homogeneizado córtex cerebral de ratos de 30 dias de vida sobre:

a) a produção de CO_2 a partir de glicose e acetato marcados radioativamente (atividade da via glicolítica e do ciclo dos ácidos tricarboxílicos) e sobre as atividades dos complexos da cadeia respiratória I-IV (transporte de elétrons), bem como sobre a atividade das enzimas creatina quinase (transferência intracelular de energia) e Na^+,K^+-ATPase (neurotransmissão)

b) a atividade das enzimas do ciclo dos ácidos tricarboxílicos, citrato sintase, aconitase, isocitrato desidrogenase, α-cetoglutarato desidrogenase, succinato desidrogenase, fumarase e malato desidrogenase;

- Determinar os efeitos *in vivo* da Orn e da Hcit através da administração intracerebroventricular em córtex cerebral de ratos de 30 dias de vida sobre:

a) a peroxidação lipídica, medida através das substâncias reativas ao ácido tiobarbitúrico (TBA-RS), sobre a oxidação de proteínas, determinada através da formação de carbonilas e da medida de grupamentos sulfidrila e sobre a geração de espécies reativas do nitrogênio, avaliada através da produção de nitratos e nitritos;

b) as defesas antioxidantes não enzimáticas, medidas pelo conteúdo de glutationa reduzida (GSH) bem como as atividades das enzimas antioxidantes superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT);

c) a produção de CO_2, a partir de glicose e acetato marcados radioativamente (atividade da via glicolítica e do ciclo dos ácidos tricarboxílicos), bem como a atividade das enzimas do ciclo dos ácidos tricarboxílicos, citrato sintase, aconitase, isocitrato desidrogenase, α-cetoglutarato desidrogenase, succinato desidrogenase e malato desidrogenase;

d) as atividades dos complexos da cadeia transportadora de elétrons I-IV e das enzimas creatina quinase e Na^+,K^+-ATPase;

- Determinar o efeito de níveis sistêmicos elevados de amônia, induzidos por injeção intraperitoneal de urease, combinado com a administração intracerebroventricular da Orn ou da Hcit em córtex cerebral de ratos de 30 dias de vida sobre:

a) a peroxidação lipídica, determinada através da medida de TBA-RS, sobre a oxidação de proteínas, medida através da formação de carbonilas e da medida de grupamentos sulfidrila;

b) as defesas antioxidantes não enzimáticas, medidas pelo conteúdo de GSH;

- Estabelecer um modelo de hiperornitinemia quimicamente induzido pela administração subcutânea de Orn em ratos, mimetizando um dos achados bioquímicos desta doença;

- Investigar os efeitos do modelo de hiperornitinemia através administração crônica subcutânea da Orn em ratos jovens (5° ao 28° dia de vida) sobre o desenvolvimento físico e motor, bem como sobre o desempenho dos animais na fase adulta nas tarefas de campo aberto e labirinto aquático de Morris;

PARTE II

ARTIGOS CIENTÍFICOS

Capítulo I

Experimental evidence that ornithine and homocitrulline disrupt energy metabolism in brain of young rats

Carolina Maso Viegas, Ângela Zanatta, Lisiane Aurélio Knebel, Patrícia Fernanda Schuck, Anelise Miotti Tonin, Gustavo da Costa Ferreira, Alexandre Umpierrez Amaral, Carlos Severo Dutra Filho, Clovis Milton Duval Wannmacher e Moacir Wajner

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Research Report

Experimental evidence that ornithine and homocitrulline disrupt energy metabolism in brain of young rats

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Brain

ABSTRACT

Tissue accumulation of ornithine (Orn), homocitrulline (Hcit), ammonia and orotic acid (Oro) is the biochemical hallmark of patients affected by hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome, a disorder clinically characterized by neurological symptoms, whose pathophysiology is practically unknown. In the present study, we investigated the in vitro effect of Orn, Hcit and Oro on important parameters of energy metabolism in brain of 30-day-old Wistar rats since mitochondrial abnormalities have been observed in the affected patients. We first verified that Orn and Hcit significantly inhibited the citric acid cycle (inhibition of CO_2 synthesis from [1-^{14}C] acetate, as well as aconitase and α-ketoglutarate dehydrogenase activities), the aerobic glycolytic pathway (reduced CO_2 production from [U-^{14}C] glucose) and moderately the electron transfer flow (inhibitory effect on complex I–III). Hcit, but not Orn, was also able to significantly inhibit the mitochondrial creatine kinase activity. Furthermore, this inhibition was prevented by GSH, suggesting a possible role of reactive species oxidizing critical thiol groups of the enzyme. In contrast, the other enzyme activities of the citric acid cycle and of the electron transfer chain, as well as synaptic Na^+,K^+-ATPase were not altered by either Orn or Hcit at concentrations as high as 5.0 mM. Similarly, Oro did not interfere with any of the tested parameters. Taken together, these data strongly indicate that Orn and Hcit compromise brain energy metabolism homeostasis and Hcit also interferes with cellular ATP transfer and buffering. It is therefore suggested that Orn and especially Hcit may be involved in the neurological damage found in patients affected by HHH syndrome.

1. Introduction

Hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome (OMIM 238970) is an autosomal recessive disorder of urea cycle and ornithine (Orn) degradation pathway caused by a mutation in *ORNT1* (SLC25A15), gene that encodes a mitochondrial ornithine transporter (Fell et al., 1974; Camacho et al., 1999). The inability to import Orn from

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the cytosol into the mitochondria results in intramitochondrial Orn deficiency and a functional impairment of the urea cycle at level of ornithine transcarbamylase, with consequent hyperammonemia. Because the normal pathway for Orn catabolism proceeds via the intramitochondrial enzyme ornithine-δ-aminotransferase, cytosolic ornithine accumulates resulting in hyperornithinemia. In the absence of intramitochondrial Orn, accumulating carbamoyl phosphate may condense with lysine to form homocitrulline (Hcit) leading to homocitrullinuria by a mechanism not yet clarified. Alternatively, carbamoyl phosphate can be shunted through the cytosolic pyrimidine biosynthetic pathway leading to increased excretion of orotic acid (Oro) and uracil in the urine (Valle and Simell, 2001).

Ammonia, a waste product of protein breakdown in the body, is toxic when its levels become high. The main pathway of ammonia detoxification is the urea cycle, which is only completely expressed in the liver, although other tissues, including brain, may express some of the constituent enzymes. In HHH syndrome, the lack of ornithine in the mitochondrial matrix causes a blockage of the urea cycle therefore accumulating ammonia and causing hyperammonemia. Regarding specifically to the brain, which is especially sensitive to the effects of excess of ammonia, it lacks carbamoyl phosphate synthase I and ornithine transcarbamylase, making this organ unable to remove ammonia in the form of urea. Consequently, brain ammonia is metabolized almost exclusively to glutamine via the glutamine synthetase reaction (Cooper and Plum, 1987). In this scenario, astrocytes are the site of ammonia detoxification in brain, due to the predominant localization of glutamine synthetase in these cells (Martinez-Hernandez et al., 1977). Glutamine is released from glial cells to neurons where it is converted back to glutamate releasing ammonia into neurons. Glutamine is also released from the brain to the circulation where it reaches the liver and is converted to glutamate.

On the other hand, ammonia is also formed in the brain mainly through glutamate dehydrogenase, which catalyses the reversible oxidative deamination of glutamate, particularly in astrocytes (Cooper and Plum, 1987). L-Glutaminase, particularly abundant in nerve endings of glutamatergic neurons, also releases ammonia and constitutes an integral part of the glutamate–glutamine cycle, in which a molecule of ammonia is transferred from the astrocyte to the neighboring neuron. Finally, enzymes of the purine nucleotide cycle may also be responsible for generating a significant fraction of brain ammonia (Schultz and Lowenstein, 1978).

The HHH syndrome has a wide spectrum of clinical presentations. Protein intolerance, growth failure, liver dysfunction, coagulopathy, and variable neurological features such as lethargy, seizures, coma, progressive spastic paraparesis, mental retardation and learning disability have been observed in patients with this disease (Debray et al., 2008; Koike et al., 1987; Lemay et al., 1992). Neuropathological findings include multiple, nonspecific T2 hyperintense foci in occipital, parietal and frontal white matter, typically seen in demyelinating diseases, with subcortical and cortical atrophy associated with swelling (Al-Hassnan et al., 2008).

The mechanisms of central nervous system (CNS) impairment in the HHH syndrome are poorly known (Palmieri, 2008; Salvi et al., 2001), although it has been hypothesized that the neurologic damage presented by the patients could likely be secondary to the episodic hyperammonemia. However, chronic accumulation of Orn, HCit, Oro and other metabolic factors cannot be ruled out as contributing causes of the neurological symptoms and brain abnormalities seen in patients affected by HHH syndrome, so that the investigation of the role of these accumulating metabolites on the CNS function will eventually lead to a better understanding of the relationship between the clinical features and the biochemical abnormalities of this disorder.

Considering that mitochondrial abnormalities and lactate accumulation and excretion are found in HHH syndrome (Valle and Simell, 2001), the present study was undertaken to investigate the in vitro influence of Orn, Hcit and Oro on important parameters of cellular energy metabolism, such as CO_2 production from [U-^{14}C] glucose (aerobic glycolysis) or [1-^{14}C] acetate and the enzyme activities of citric acid cycle (CAC) (CAC activity), respiratory chain complexes I–IV (respiratory chain function), creatine kinase (cellular energy transfer) and Na^+,K^+-ATPase in brain preparations from young rats.

2. Results

2.1. *Orn and Hcit inhibit CO_2 production from glucose and acetate in rat cerebral cortex*

First, we investigated the effect of Orn, Hcit and Oro on CO_2 production from labeled substrates in cortical homogenates. Fig. 1A shows that CO_2 production from [U-^{14}C] glucose was significantly inhibited by Orn at 0.5 mM and higher concentrations [$F_{(4,21)}=3.175$; $p<0.05$] in a dose-dependent manner [$p<0.05$]. CO_2 formation from [1-^{14}C] acetate was also markedly inhibited by Orn in cortical homogenates [$F_{(4,25)}=6.058$; $p<0.001$] in a dose-dependent manner at 1 mM and higher concentrations [$p<0.001$]. In addition, Hcit inhibited CO_2 production from [U-^{14}C] glucose (1 mM and higher) [$F_{(4,24)}=7.471$; $p<0.001$] in a dose-dependent manner [$p<0.001$] and [1-^{14}C] acetate (0.1 mM and higher) [$F_{(4,23)}=6.489$; $p<0.001$] (Fig. 1B). In contrast, Oro did not alter CO_2 generation from [U-^{14}C] glucose or [1-^{14}C] acetate (Table 1). These results strongly suggest that the aerobic glycolytic pathway and the CAC activity were compromised by Orn and Hcit.

2.2. *Orn and Hcit inhibit complex I–III activity of the respiratory chain in rat cerebral cortex*

The next set of experiments was performed to evaluate the effect of Orn, Hcit and Oro on the activities of the respiratory chain complexes I–IV. We found that complex I–III activity was significantly inhibited by Orn [$F_{(4,35)}=3.446$, $p<0.05$] (Fig. 2) and Hcit [$F_{(4,20)}=4.524$, $p<0.01$] (Fig. 3), with no significant alteration of the activities of the respiratory chain complexes II, II–III and IV. Furthermore, Oro did not affect any of these complex activities (Table 1).

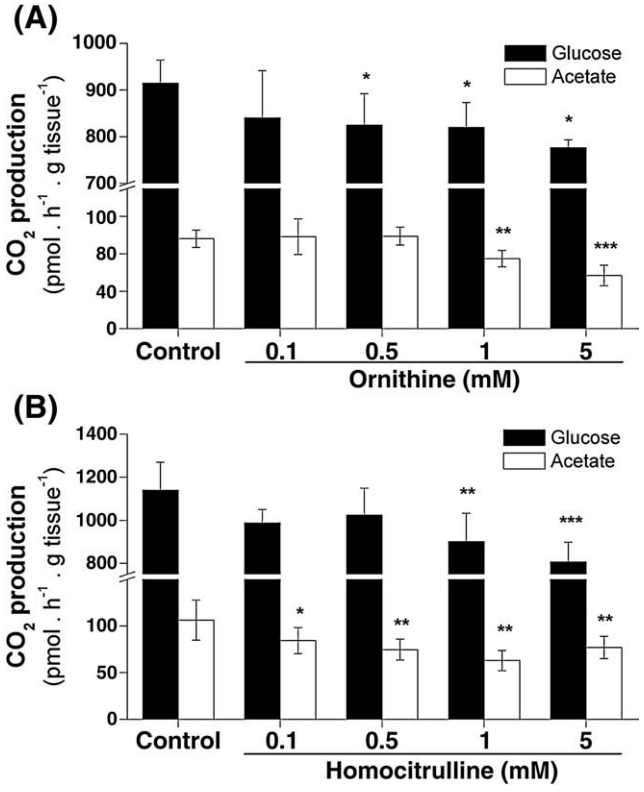

Fig. 1 – Effect of ornithine (A) and homocitrulline (B) on CO_2 formation from [U-^{14}C] glucose and [1-^{14}C] acetate in rat cerebral cortex homogenates. Values are means ± standard deviation for five to six independent experiments (animals) per group and expressed as pmol CO_2 h^{-1} g tissue^{-1}. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, compared to controls (Duncan multiple range test).

2.3. *Orn and Hcit inhibit aconitase and α-ketoglutarate dehydrogenase activities in mitochondrial enriched fractions from cerebrum*

Inhibition of complex I–III might be partly responsible for the inhibitory action of Orn and Hcit on the CAC (lower CO_2 production), but it does not rule out a possible inhibitory effect of these amino acids on one or more enzyme steps of the cycle. Therefore, we also evaluated the effect of Orn and Hcit on CAC enzyme activities. We found that Orn and Hcit significantly inhibited aconitase and α-ketoglutarate dehydrogenase activities (Fig. 4; Orn: aconitase [$F_{(3,20)} = 5.984$, $p < 0.01$]; α-ketoglutarate dehydrogenase [$F_{(3,18)} = 4.716$, $p < 0.05$]; Hcit: aconitase [$F_{(3,16)} = 17.745$, $p < 0.001$]; α-ketoglutarate dehydrogenase [$F_{(3,20)} = 3.586$, $p < 0.05$]) in mitochondrial enriched fractions from cerebrum. In contrast, these compounds did not alter citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase activities (Table 2).

2.4. *Hcit inhibits mitochondrial creatine kinase activity from rat cerebral cortex*

We also examined the effect of Orn, Hcit and Oro on total creatine kinase (tCK) activity in homogenates from rat cerebral cortex. Hcit significantly inhibited tCK activity [$F_{(4,20)} = 2.892$, $p < 0.05$] (Fig. 5A), whereas Orn and Oro did not alter this activity (Table 3). We then investigated the effect of Hcit (5.0 mM) on CK activity on mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions from rat cerebral cortex. It can be seen that Hcit had no effect on Cy-CK activity (Fig. 5B), but significantly inhibited Mi-CK activity [$t\ (5) = 4.873$, $p < 0.01$] (Fig. 5C).

We also pre-incubated total homogenates from cerebral cortex in the presence of the antioxidant GSH, the free radical scavengers SOD plus CAT, trolox (α-tocopherol) or the nitric oxide synthase inhibitor N-ω-nitro-L-arginine methyl ester (L-NAME) in order to test whether the significant reduction of cerebral cortex CK activity caused by Hcit could be mediated by oxidative attack. We initially verified that these antioxidants per se did not alter tCK activity (data not shown). It was then noted that GSH fully prevented, whereas SOD plus CAT, trolox and L-NAME did not affect the Hcit-induced inhibitory effect on tCK activity [$F_{(5,30)} = 4.273$; $p < 0.01$] (Fig. 6).

2.5. *Orn, Hcit and Oro do not alter Na^+, K^+-ATPase activity from synaptic plasma membranes of rat cerebral cortex*

The next set of experiments was carried out to test the influence of Orn (5.0 mM), Hcit (5.0 mM) and Oro (1.0 mM) on Na^+, K^+-ATPase activity in synaptic plasma membranes prepared from cortical homogenates. Cortical homogenates were pre-incubated with the metabolites, the synaptic plasma membranes were isolated and Na^+, K^+-ATPase activity was determined afterwards. We also investigated the effect of Orn,

Table 1 – Effect of orotic acid (Oro) on CO_2 production from labeled substrates and on the activities of respiratory chain complexes in rat cerebral cortex.

	CO_2 production		Respiratory chain complexes			
	[U-^{14}C] glucose	[1-^{14}C] acetate	Complex I–III	Complex II	Complex II–III	Complex IV
Control	888 ± 96.5	64.8 ± 19.0	7.33 ± 0.70	5.90 ± 0.28	16.6 ± 3.41	173 ± 17.3
Oro 0.1 mM	848 ± 127	55.5 ± 17.4	6.94 ± 1.98	6.68 ± 1.01	18.2 ± 3.53	172 ± 11.2
Oro 0.5 mM	833 ± 88.7	72.8 ± 31.3	6.01 ± 1.86	6.56 ± 0.91	16.2 ± 4.52	175 ± 16.8
Oro 1 mM	765 ± 95.8	58.6 ± 9.70	5.65 ± 1.34	7.60 ± 2.07	17.4 ± 2.46	175 ± 20.3

Values are mean ± standard deviation for four to six independent (animals) experiments per group. CO_2 production is expressed as pmol CO_2 h^{-1} g tissue^{-1} and the activities of complexes I–III, II, II–III and IV are expressed as nmol min^{-1} mg protein^{-1}. No significant differences between groups were detected (one-way ANOVA).

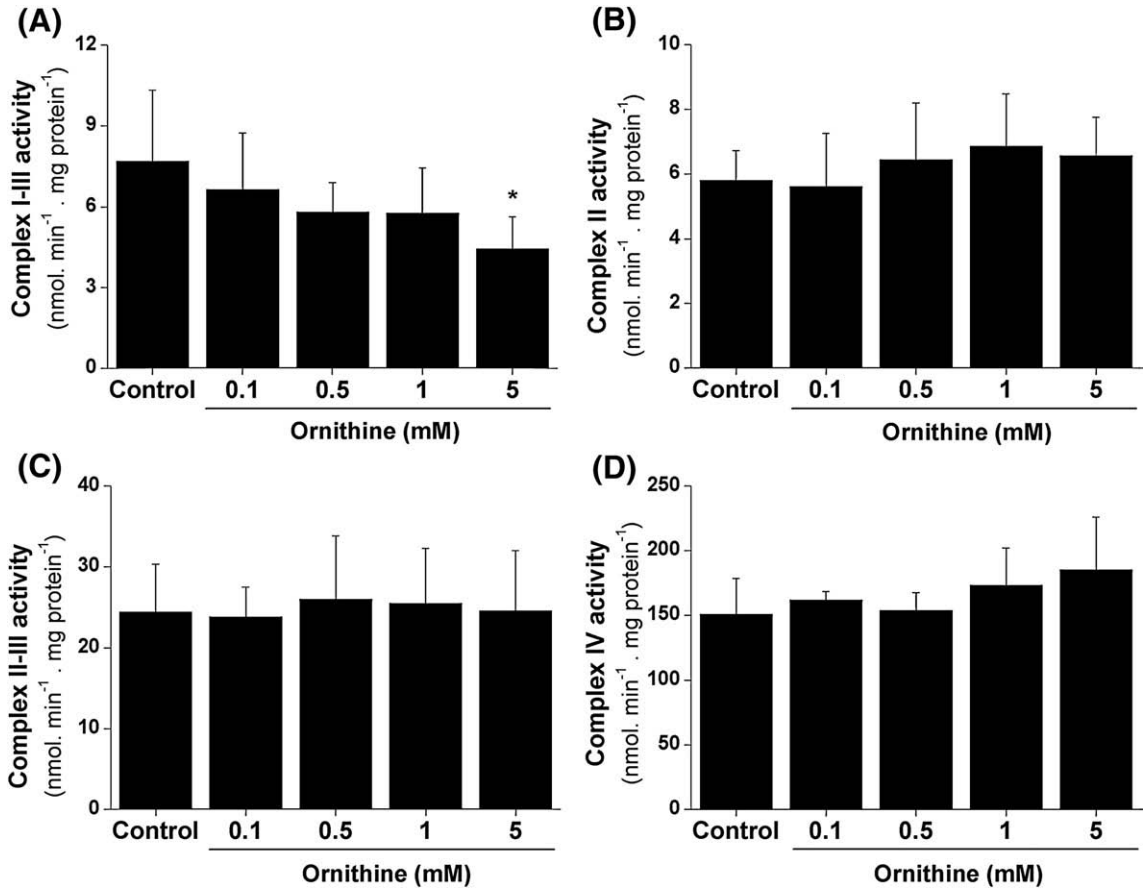

Fig. 2 – Effect of ornithine on the activities of respiratory chain complex in rat cerebral cortical homogenates. Values are means ± standard deviation for four to six independent experiments (animals) per group. The activity of complex I–III (A) is expressed as nmol cytochrome c reduced min^{-1} mg $protein^{-1}$. The activities of complex II (B), II–III (C) and IV (D) are expressed, respectively, as nmol DCIP reduced min^{-1} mg $protein^{-1}$, nmol cytochrome c reduced min^{-1} mg $protein^{-1}$ and nmol cytochrome c oxidized min^{-1} mg $protein^{-1}$. *$p < 0.05$, compared to control (Duncan multiple range test).

Hcit and Oro on Na^+, K^+-ATPase activity from purified synaptic membrane preparations by directly exposing these membranes to the metabolites. We observed that under both conditions (with or without pre-incubation) these metabolites did not change Na^+, K^+-ATPase activity. Table 4 shows the results of the first set of experiments, in which cortical homogenates were pre-incubated with the compounds. Similar results were obtained with direct exposure of purified synaptic membranes to these metabolites (results not shown).

3. Discussion

Hyperornithinemia, homocitrullinuria and orotic aciduria occurs in HHH syndrome, a genetic disorder caused by a defect in the gene that encodes the mitochondrial ornithine transporter that imports Orn from the cytosol into the mitochondrion (Camacho et al., 1999; Valle and Simell, 2001). The disease was described for the first time in 1969 in a patient with various neurologic symptoms, including periodic crises with lethargy, vomiting, ataxia, choreoathetosis, developmental delay, severe muscle spasticity and mental retardation (Shih et al., 1969; Valle and Simell, 2001). Although hyperammonemia has been implicated in its neuropathology, some patients with severe CNS damage present normal blood ammonia levels and worsening of the neurologic signs does not relate to relapses of hyperammonemia observed in patients with HHH syndrome (Salvi et al., 2001; Korman et al., 2004). Therefore, it is conceivable that other factors such as the accumulation of Orn, Hcit and/or orotic acid may play a role in the pathogenesis of this disorder. Increased plasma Orn concentrations differentiate HHH syndrome from other urea cycle disorders, and both hyperammonemia (especially postprandial) and homocitrullinuria distinguish this disease from gyrate atrophy in which major symptoms of progressive chorioretinal degeneration is the hallmark (Fukuda et al., 1983; Javadzadeh and Gharabaghi, 2007).

Interestingly, lactic acid, ketone bodies and citric acid cycle intermediates accumulation and excretion are also increased in HHH syndrome and mitochondrial abnormalities are detected in these patients (Gatfield et al., 1975; Haust et al., 1981; Metoki et al., 1984; Salvi et al., 2001), indicating an impaired mitochondrial function. Therefore, in the present study we tested whether Orn, Hcit and Oro at concentrations similar to those found in blood of patients affected by HHH syndrome could compromise brain bioenergetics in young rats.

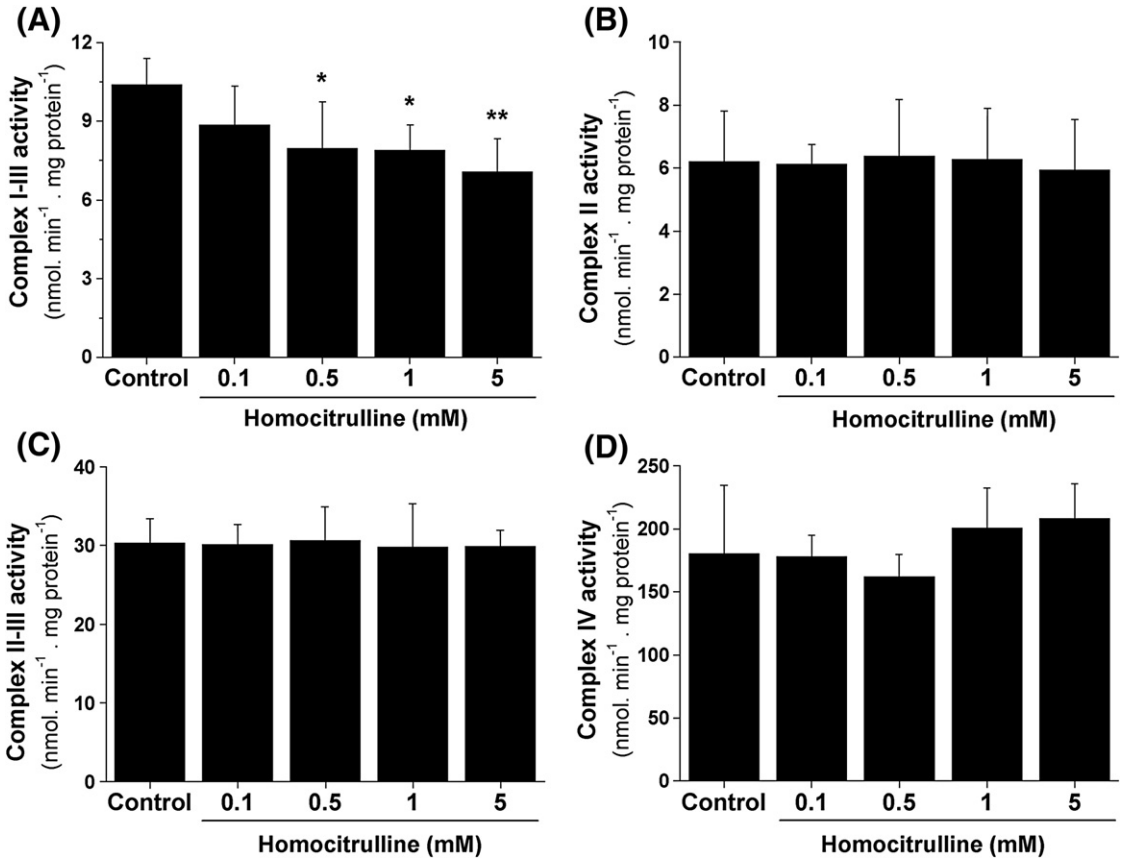

Fig. 3 – Effect of homocitrulline on the activities of respiratory chain complex in rat cerebral cortical homogenates. Values are means ± standard deviation for four to six independent experiments (animals) per group. The activity of complex I–III (A) is expressed as nmol cytochrome c reduced min^{-1} mg $protein^{-1}$. The activities of complex II (B), II–III (C) and IV (D) are expressed, respectively, as nmol DCIP reduced min^{-1} mg $protein^{-1}$, nmol cytochrome c reduced min^{-1} mg $protein^{-1}$ and nmol cytochrome c oxidized min^{-1} mg $protein^{-1}$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control (Duncan multiple range test).

We initially verified that Orn and Hcit significantly decreased CO_2 formation from labeled glucose and acetate. The lower rate of CO_2 production from glucose indicates deficient aerobic glycolysis, which could result from inhibition of one or more glycolytic enzymes or from an inhibition of the Krebs cycle and/or the respiratory chain. We also observed that Orn and Hcit significantly reduced the rate of CO_2 production from acetate, indicating a blockage of the Krebs cycle since acetate enters the cycle via acetyl-CoA. In contrast, Oro had no effect on this parameter.

The next step of our investigation was to explain why the CAC activity was inhibited by these substances. We therefore investigated the effects of Orn and Hcit on the respiratory chain function by measuring the activities of complexes I–IV and also on various enzymes of the CAC. We first observed that Orn and Hcit moderately reduced the electron transport chain flow (complex I–III pathway). Both amino acids were also able to significantly inhibit α-ketoglutarate dehydrogenase and aconitase activities, without altering the activities of citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase and fumarase. Therefore, it seems reasonable to attribute the reduced function of the Krebs cycle (lower CO_2 formation) mainly due to the inhibitory effects of Orn and Hcit on the important enzymes α-ketoglutarate dehydrogenase and aconitase. In contrast, it is difficult to envisage that the small reduction of complex I–III activity may have contributed to the inhibition of the CAC.

We then tested the effects of Orn, Hcit and Oro on the activities of creatine kinase (CK) and Na^+, K^+-ATPase from synaptic plasma membranes of cerebral cortex of rats. We found that these metabolites did not change the activity of Na^+, K^+-ATPase, a critical enzyme activity necessary for neurotransmission. However, Hcit, but not Orn or Oro, provoked a significant reduction of total and mitochondrial CK activities, suggesting that Hcit also impairs brain cellular energy buffering and transfer.

Considering that CK activity decreases after brain exposure to agents promoting generation of free radicals probably by oxidation of essential cysteine residues of the catalytic center (Arstall et al., 1998; Burmistrov et al., 1992; Konorev et al., 1998; Stachowiak et al., 1998; Wolosker et al., 1996), we next investigated the effect of various antioxidants on the inhibitory effect of Hcit on CK activity in order to test whether this inhibition was mediated by oxidation of thiol groups of the enzyme. We verified that GSH that acts as a naturally-occurring thiol-reducing and protecting agent (Meister and Anderson, 1983) fully prevented the inhibitory role of Hcit on

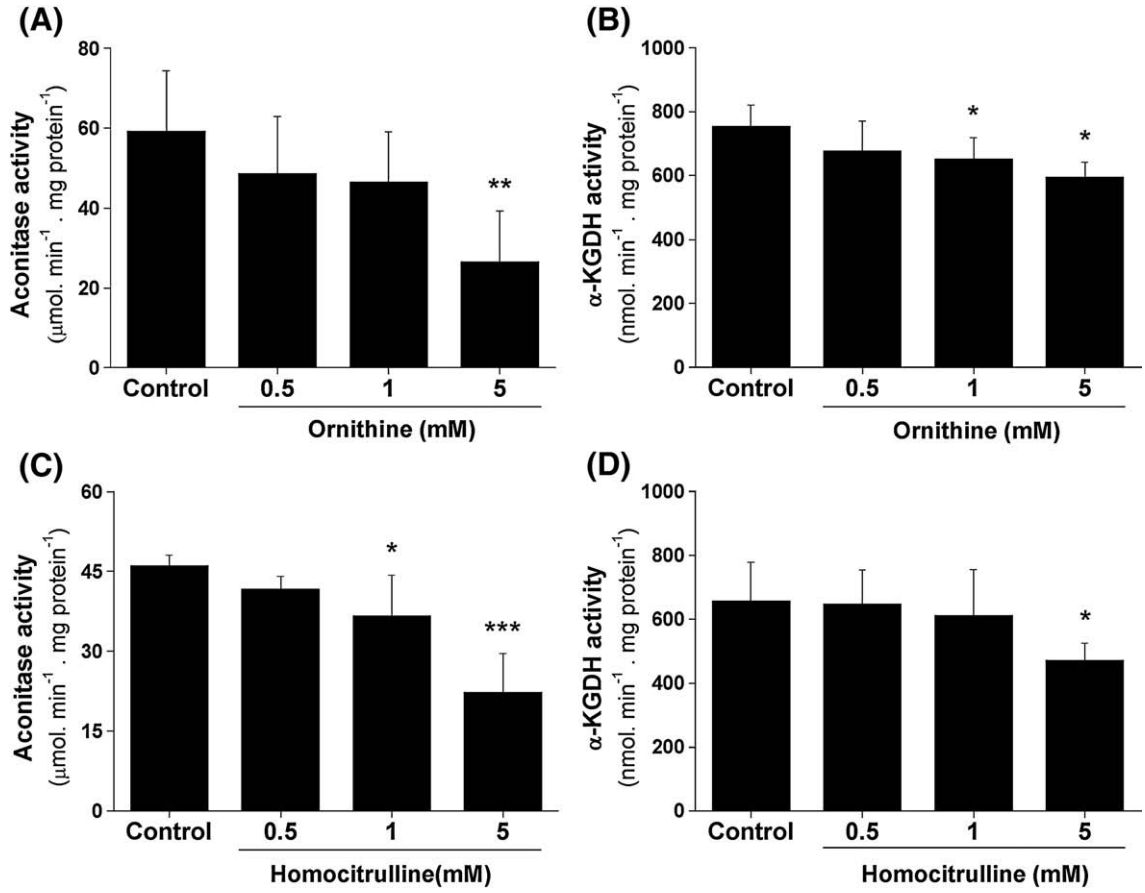

Fig. 4 – Effect of ornithine (A, B) and homocitrulline (C, D) on aconitase and α-ketoglutarate dehydrogenase (α-KGDH) activities in mitochondrial enriched fractions from rat brain. Values are means ± standard deviation for five to six independent experiments (animals) per group. The activities of aconitase and α-KGDH are expressed as μmol NADPH min^{-1} mg $protein^{-1}$ and μmol NADH min^{-1} mg $protein^{-1}$, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, compared to control (Duncan multiple range test).

tCK activity from cerebral cortex, indicating that Hcit probably inhibited CK activity by inactivating critical sulfhydryl groups of the enzyme. On the other hand, α-tocopherol, which is an excellent trapping agent for lipid peroxyl radicals ($ROO^{\cdot}$) (Arstall et al., 1998) the nitric oxide synthase inhibitor L-NAME and the combination of SOD plus CAT that scavenge the radicals superoxide and hydrogen peroxide did not prevent Hcit inhibitory activity, indicating that the reactive species peroxyl, superoxide and nitric oxide or peroxynitrite were probably not involved in this inhibition.

It is difficult to determine the pathophysiological relevance of our data since to our knowledge brain concentrations of Orn and Hcit are not yet established in HHH syndrome (Palmieri, 2008). It should be however stressed that Hcit exerted the most significant effects at lower concentrations (0.1–0.5 mM) on energy production, as compared to Orn, and, besides, Hcit also markedly reduced mitochondrial CK activity, an enzyme necessary for cellular energy transfer and buffering. Furthermore, the effects elicited by Orn and Hcit on the biochemical parameters of energy metabolism were evident at similar

Table 2 – Effect of ornithine (Orn) and homocitrulline (Hcit) on the activities of the citric acid cycle enzymes in enriched mitochondrial fractions from rat cerebrum.

	Citrate synthase	Isocitrate dehydrogenase	Succinate dehydrogenase	Fumarase	Malate dehydrogenase
Control	1049 ± 110	77.9 ± 25.5	35.0 ± 4.63	322.8 ± 49.5	20.6 ± 0.32
Orn 5 mM	1032 ± 127	70.6 ± 22.8	35.1 ± 7.94	302.1 ± 44.4	20.2 ± 1.07
Hcit 5 mM	1083 ± 138	60.2 ± 12.3	24.6 ± 7.59	317.3 ± 38.1	21.2 ± 1.21

Values are mean ± standard deviation for four to six independent (animals) experiments per group. The activity of citrate synthase is expressed as nmol TNB min^{-1} mg $protein^{-1}$. The activities of isocitrate dehydrogenase and malate dehydrogenase are expressed as nmol NADH min^{-1} mg $protein^{-1}$, whereas succinate dehydrogenase and fumarase are expressed as nmol DCIP min^{-1} mg $protein^{-1}$ and nmol fumarate min^{-1} mg $protein^{-1}$, respectively. No significant differences were detected (one-way ANOVA).

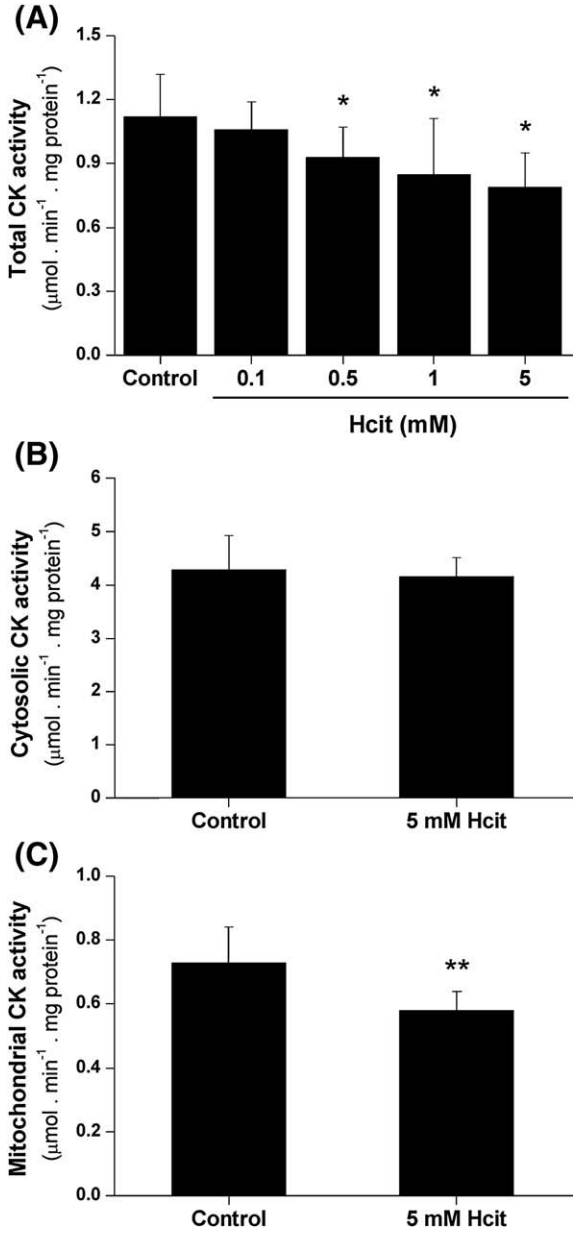

Fig. 5 – Effect of homocitrulline (Hcit) on total (A), cytosolic (B) and mitochondrial (C) creatine kinase (CK) activities in cerebral cortex preparations from rats. Values are means ± standard deviation of four to six independent experiments (animals) performed in triplicate and are expressed as μmol creatine min^{-1} mg $protein^{-1}$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control (Duncan multiple range test or Student's t-test for paired samples).

concentrations found in plasma of patients affected by this disorder. It is also feasible that higher concentrations of these amino acids may take place in stress situations, such as occurs during episodes of metabolic decompensation characterized by intense catabolism and proteolysis in which the levels of the accumulating metabolites may dramatically increase (Camacho et al., 1999). Regarding Orn, it is difficult to envisage how Orn-induced disruption of bioenergetics could be responsible for the neurodegeneration of HHH syndrome since this amino acid also accumulates in ornithine aminotransferase deficiency, which is characterized by gyrate atrophy of the choroids and retina, with no alteration of the CNS (Javadzadeh and Gharabaghi, 2007; Kaiser-Kupfer et al., 1983; Simell and Takki, 1973).

Table 3 – Effect of ornithine and orotic acid on total creatine kinase activity in rat cerebral cortex.

	Concentrations (mM)				
	0	0.1	0.5	1.0	5.0
Ornithine	1.13 ± 0.20	1.13 ± 0.29	1.06 ± 0.19	0.99 ± 0.33	0.92 ± 0.13
Orotic acid	1.98 ± 0.36	1.40 ± 0.30	1.63 ± 0.14	1.47 ± 0.32	–

Values are mean ± standard deviation for five to six independent (animals) experiments per group performed in triplicate. Creatine kinase activity is expressed as μmol creatine min^{-1} mg $protein^{-1}$. No significant differences between groups were detected (one-way ANOVA).

In conclusion, this is the first report showing that the metabolites accumulating in HHH syndrome, especially Hcit, impair bioenergetics in brain cortex, by reducing the velocity of the citric acid cycle and creatine kinase activity, and consequently decreased energy production and transfer. The present data indicate that the pathogenesis of the brain damage occurring in this disorder cannot be exclusively attributed to hyperammonemia. Furthermore, it may explain the mitochondrial abnormalities and the increased urinary excretion of lactate, 2-hydroxyglutyrate, various Krebs cycle intermediates and glutaric acid observed in patients with HHH syndrome. In case our in vitro data are confirmed in vivo in animal experiments and also in tissues from patients affected

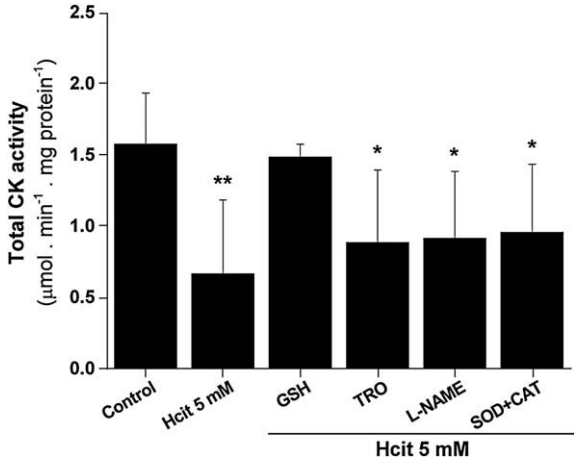

Fig. 6 – Effect of the antioxidants reduced glutathione (GSH; 0.2 mM), trolox (TRO; 1.5 μM), N-ω-nitro-L-arginine methyl ester (L-NAME; 0.5 mM) and the combination of superoxide dismutase (SOD; 50 mU mL^{-1}) plus catalase (CAT; 50 mU mL^{-1}) on the inhibition induced by 5 mM homocitrulline (Hcit) on total creatine kinase activity from rat cerebral cortex. Data are means ± standard deviation of six independent experiments (animals) performed in triplicate and are expressed as μmol creatine min^{-1} mg $protein^{-1}$. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control (Duncan multiple range test).

Table 4 – Effect of ornithine (Orn), homocitrulline (Hcit) and orotic acid (Oro) on Na^+, K^+-ATPase activity on synaptic plasma membranes isolated from rat cerebral cortex.

	Control	Orn 5 mM	Hcit 5 mM	Oro 1 mM
Na^+K^+-ATPase activity	2267±676	2212±483	2437±550	2301±530

Metabolites were pre-incubated at 37 °C for 1 h with rat cerebral cortex homogenates, synaptic plasma membranes were isolated and the activity measured afterwards. Values are mean±standard deviation for four to six independent (animals) experiments per group performed in duplicate and expressed as nmol Pi min^{-1} mg $protein^{-1}$. No significant differences between groups were detected (one-way ANOVA).

by HHH syndrome, it is tempting to speculate that energy buffering and transfer impairment may contribute to the neurological damage found in this disorder. In this scenario, besides a diet poor in proteins that has to be used chronically, the prompt and aggressive treatment of infections and management of fever during acute metabolic decompensation reducing the risk of increased catabolism with elevation of brain Orn and Hcit concentrations seems justified and may prevent irreversible brain injury in these patients.

4. Experimental procedures

4.1. *Reagents*

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [U-^{14}C] glucose and [1-^{14}C] acetate, which were purchased from Amersham International plc, UK and homocitrulline, which was obtained from MP Biomedicals, LLC Solon, Ohio, USA.

4.2. *Animals*

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used in the assays. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

4.3. *Cerebral preparations*

The animals were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated. For CO_2 production, the cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). The homogenates were centrifuged at 800 ×*g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For the determination of the activities of citric acid cycle enzymes, mitochondrial enriched fractions from cerebrum were prepared according to Rosenthal et al. (1987), with slight modifications. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. For the preparation of mitochondrial and cytosolic fractions, the homogenates were centrifuged at 800 ×*g* for 10 min at 4 °C and the pellet discarded (Ramirez and Jimenez, 2000). The supernatant was then centrifuged at 27,000 ×*g* for 30 min at 4 °C in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase enzymatic assay. The supernatants were further centrifuged at 125,000 ×*g* for 60 min at 4 °C in an OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for determination of cytosolic creatine kinase activity. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for mitochondrial and cytosolic creatine kinase, citric acid cycle enzymatic and CO_2 production assays, which were performed in the same day of the preparations.

The biochemical parameters were determined in the presence of various concentrations of Orn (0.1–5.0 mM), Hcit (0.1–5.0 mM) or Oro (0.1–1.0 mM) whereas control groups did not contain the metabolite in the incubation medium. Orn, Hcit and Oro were dissolved on the day of the experiments in the buffer used for each technique with pH adjusted to 7.4. Some experiments were performed in the presence of glutathione (GSH; 0.2 mM), soluble α-tocoferol (trolox; 1.5 μM), the nitric oxide synthase inhibitor N-ω-nitro-L-arginine methyl ester (L-NAME; 0.5 mM) or the combination of superoxide dismutase (SOD; 50 mU mL^{-1}) plus catalase (CAT; 50 mU mL^{-1}).

4.4. *Preparation of synaptic plasma membrane from rat cerebral cortex*

Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. The homogenate was pre-incubated at 37 °C for 1 h in the presence or absence of 5.0 mM Orn, 5.0 mM Hcit or 1.0 mM Oro. Membranes were prepare afterwards according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 ×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation. In some experiments, Orn (0.1–5.0 mm), Hcit (0.1–5.0 mM) and Oro (0.1–1.0 mM) were incubated at 37 °C for 30 min with purified synaptic membranes.

4.5. *CO_2 production*

Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume

of 0.45 mL. Flasks were pre-incubated at 35 °C for 30 min in the presence of Orn (0.1–5.0 mM), Hcit (0.1–5.0 mM) or Oro (0.1–1.0 mM) in a metabolic shaker (90 oscillations min^{-1}) with 625 μM *n*-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. Controls did not contain the metabolites in the incubation medium. After pre-incubation, [U-^{14}C] glucose (0.055 μCi) plus 5.0 mM of unlabeled glucose or 0.055 μCi [1-^{14}C] acetate plus 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 60 nm/4 nm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Reis de Assis et al., 2004). Results were calculated as ρmol CO_2 h^{-1} g $tissue^{-1}$.

4.6. *Spectrophotometric analysis of the respiratory chain complexes I–IV activities*

The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) were determined in homogenates from cerebral cortex according to Fischer et al. (1985). The activity of NADH:cytochrome *c* oxidoreductase (complex I–III) was assayed in cerebral cortex homogenates according to the method described by Schapira et al. (1990) and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). Orn (0.1–5.0 mM), Hcit (0.1–5.0 mM) or Oro (0.1–1.0 mM) were added to the reaction medium at the beginning of the assays, while no metabolite was added to controls. The activities of the respiratory chain complexes were calculated as nmol min^{-1} mg $protein^{-1}$ or mmol min^{-1} mg $protein^{-1}$.

4.7. *Spectrophotometric analyses of the activities of citric acid cycle enzymes*

The activities of the enzymes of the citric acid cycle (CAC) were determined using enriched mitochondrial fractions from cerebrum. Orn and Hcit were supplemented to the medium and submitted to a pre-incubation at 37 °C for 30 min.

Citrate synthase activity was measured according to Srere (1969), by determining DTNB reduction at $\lambda = 412$ nm. The activity of the enzyme aconitase was measuring according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. Isocitrate dehydrogenase activity was accessed by the method of Plaut (1969), by following NAD reduction at wavelengths of excitation and emission of 340 and 466 nm, respectively.

The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004), with modifications. The incubation medium contained 50 mM K_2PO_4, 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.3 mM DTT, 100 μM EGTA, 50 μM coenzyme A-SH, 250 μM α-ketoglutarate and 2 mM NAD^+, pH adjusted to 7.35. The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively.

The activity of succinate dehydrogenase was determined as described by Fischer et al. (1985). Fumarase activity was measured according to O'Hare and Doonan (1985), measuring the increase of absorbance at $\lambda = 250$ nm. Malate dehydrogenase activity was according to Kitto (1969) by following the reduction of NADH at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activities of the citric acid cycle enzymes were calculated as nmol min^{-1} mg $protein^{-1}$, mmol min^{-1} mg $protein^{-1}$ or μmol min^{-1} mg $protein^{-1}$.

4.8. *Spectrophotometric analysis of creatine kinase (CK) activity*

CK activity was measured in total homogenates as well as in the cytosolic and mitochondrial preparations according to Hughes (1962) with slight modifications (Schuck et al., 2002). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. Orn (0.1–5.0 mM), Hcit (0.1–5.0 mM) or Oro (0.1–1.0 mM) were supplemented to the medium and submitted to a pre-incubation at 37 °C for 30 min. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at $\lambda = 540$ nm. Results were calculated as μmol of creatine min^{-1} mg $protein^{-1}$.

4.9. *Spectrophotometric analysis of Na^+, K^+-ATPase activity*

The reaction mixture for the Na^+, K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by

the method of Chan et al. (1986). Enzyme-specific activities were calculated as nmol Pi released^{-1} min^{-1} mg protein.

4.10. *Protein determination*

Protein was measured by the methods of Lowry et al. (1951) using bovine serum albumin as standard.

4.11. *Statistical analysis*

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. For analysis of dose-dependent effects, linear regression was used. The Student t-test for paired samples was used for comparison of two means. Only significant F and t values are shown in the text. Differences between groups were rated significant at $p < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

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REFERENCES

Al-Hassnan, Z.N., Rashed, M.S., Al-Dirbashi, O.Y., Patay, Z., Rahbeeni, Z., Abu-Amero, K.K., 2008. Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome with stroke-like imaging presentation: clinical, biochemical and molecular analysis. J. Neurol. Sci. 15 (264), 187–194.

Arstall, M.A., Bailey, C., Gross, W.L., Bak, M., Balligand, J.L., Kelly, R.A., 1998. Reversible S-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J. Mol. Cell. Cardiol. 30, 979–988.

Burmistrov, S.O., Mashek, O.P., Kotin, A.M., 1992. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp. Klin. Farmakol. 55, 54–56.

Camacho, J.A., Obie, C., Biery, B., Goodman, B.K., Hu, C.A., Almashanu, S., Steel, G., Casey, R., Lambert, M., Mitchell, G.A., Valle, D., 1999. Hyperornithinaemia–hyperammonaemia–homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. Nat. Genet. 22, 151–158.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Cooper, A.J.L., Plum, P., 1987. Biochemistry and physiology of brain ammonia. Physiol. Rev. 67, 40–519.

da Silva, C.G., Ribeiro, C.A., Leipnitz, G., Dutra-Filho, C.S., Wyse, A.A., Wannmacher, C.M., Sarkis, J.J., Jakobs, C., Wajner, M., 2002. Inhibition of cytochrome c oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim. Biophys. Acta 1586, 81–91.

Debray, F.G., Lambert, M., Lemieux, B., Soucy, J.F., Drouin, R., Fenyves, D., Dube, J., Maranda, B., Laframboise, R., Mitchell, G.A., 2008. Phenotypic variability among patients with hyperornithinaemia–hyperammonaemia–homocitrullinuria syndrome homozygous for the delF188 mutation in SLC25A15. J. Med. Genet. 45, 759–764.

Fischer, J.C., Ruitenbeek, W., Berden, J.A., Trijbels, J.M., Veerkamp, J.H., Stadhouders, A.M., Sengers, R.C., Janssen, A.J., 1985. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin. Chim. Acta 153, 23–36.

Fell, V., Pollitt, R.J., Sampson, G.A., Wright, T., 1974. Ornithinemia, hyperammonemia and homocitrullinuria. A disease associated with mental retardation and possibly caused by defective mitochondrial transport. Am. J. Dis. Child. 127, 752–756.

Fukuda, K., Nishi, Y., Usui, T., Mishima, H., Hirata, H., Baba, S., Choshi, K., Tanaka, Y., Akiya, S., 1983. Free amino acid concentrations in blood cells of two brothers with gyrate atrophy of the choroid and retina with hyperornithinaemia. J. Inherit. Metab. Dis. 6, 137–142.

Gatfield, P.D., Taller, E., Wolfe, D.M., Haust, M.D., 1975. Hyperornithinemia, hyperammonemia, and homocitrullinuria associated with decreased carbamyl phosphate synthetase I activity. Pediatr. Res. 9, 7–488.

Haust, M.D., Gatfield, P.D., Gordon, B.A., 1981. Ultrastructure of hepatic mitochondria in a child with hyperornithinemia, hyperammonemia, and homocitrullinuria. Hum. Pathol. 12, 212–222.

Hughes, B.P., 1962. A method for the estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7, 597–603.

Javadzadeh, A., Gharabaghi, D., 2007. Gyrate atrophy of the choroid and retina with hyper-ornithinemia responsive to vitamin B6: a case report. J. Med. Case Reports 1, 27.

Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim. Biophys. Acta 356, 276–287.

Kaiser-Kupfer, M., Kuwabara, T., Uga, S., Takki, K., Valle, D., 1983. Cataract in gyrate atrophy: clinical and morphologic studies. Invest. Ophthalmol. Vis. Sci. 24, 432–436.

Kitto, G.B., 1969. Intra- and extramitochondrial malate dehydrogenase from chicken and tuna heart. Methods Enzymology 13, 106–116.

Koike, R., Fujimori, K., Yuasa, T., Miyatake, T., 1987. [Hyperornithinemia, hyperammonemia and homocitrullinuria syndrome in a family]. Rinsho Shinkeigaku 27, 465–471.

Konorev, E.A., Hogg, N., Kalyanaraman, B., 1998. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett. 427, 171–174.

Korman, S.H., Kanazawa, N., Abu-Libdeh, B., Gutman, A., Tsujino, S., 2004. Hyperornithinemia, hyperammonemia, and homocitrullinuria syndrome with evidence of mitochondrial dysfunction due to a novel SLC25A15 (ORNT1) gene mutation in a Palestinian family. J. Neurol. Sci. 218, 53–58.

Lai, J.C., Cooper, A.J., 1986. Brain alpha-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. J. Neurochem. 47, 1376–1386.

Lemay, J.F., Lambert, M.A., Mitchell, G.A., Vanasse, M., Valle, D., Arbour, J.F., Dube, J., Flessas, J., Laberge, M., Lafleur, L., 1992. Hyperammonemia–hyperornithinemia–homocitrullinuria syndrome: neurologic, ophthalmologic, and neuropsychologic examination of six patients. J. Pediatr. 121, 725–730.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Martinez-Hernandez, A., Bell, K.P., Norenberg, M.D., 1977. Glutamine synthetase: glial localization in brain. Science 195, 1356–1358.

Meister, A., Anderson, M.E., 1983. Glutathione. Annu. Rev. Biochem. 52, 711–760.
Metoki, K., Hommes, F.A., Dyken, P., Kelloes, C., Trefz, J., 1984. Ultrastructural changes in fibroblast mitochondria of a patient with HHH-syndrome. J. Inherit. Metab. Dis. 7, 147–150.
Morrison, J.F., 1954. The activation of aconitase by ferrous ions and reducing agents. Biochem. J. 58, 685–692.
O'Hare, M.C., Doonan, S., 1985. Purification and structural comparisons of the cytosolic and mitochondrial isoenzymes of fumarase from pig liver. Biochim. Biophys. Acta 827, 127–134.
Palmieri, F., 2008. Diseases caused by defects of mitochondrial carriers: a review. Biochim. Biophys. Acta 1777, 564–578.
Plaut, G.W.E., 1969. Isocitrate dehydrogenase from bovine heart. In: Lowenstein, J.M. (Ed.), Methods in Enzymology, Vol. 13. Academic Press, New York, pp. 34–42.
Ramirez, O., Jimenez, E., 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18, 815–823.
Reis de Assis, D., Maria Rde, C., Borba Rosa, R., Schuck, P.F., Ribeiro, C.A., da Costa Ferreira, G., Dutra-Filho, C.S., Terezinha de Souza Wyse, A., Duval Wannmacher, C.M., Santos Perry, M.L., Wajner, M., 2004. Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res. 1030, 141–151.
Rosenthal, R.E., Hamud, F., Fiskum, G., Varghese, P.J., Sharpe, S., 1987. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. J. Cereb. Blood Flow Metab. 7, 752–758.
Rustin, P., Chretien, D., Bourgeron, T., Gerard, B., Rotig, A., Saudubray, J.M., Munnich, A., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35–51.
Salvi, S., Santorelli, F.M., Bertini, E., Boldrini, R., Meli, C., Donati, A., Burlina, A.B., Rizzo, C., Di Capua, M., Fariello, G., Dionisi-Vici, C., 2001. Clinical and molecular findings in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome. Neurology 57, 911–914.
Schapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–2145.
Schuck, P.F., Leipnitz, G., Ribeiro, C.A., Dalcin, K.B., Assis, D.R., Barschak, A.G., Pulrolnik, V., Wannmacher, C.M., Wyse, A.T., Wajner, M., 2002. Inhibition of creatine kinase activity in vitro by ethylmalonic acid in cerebral cortex of young rats. Neurochem. Res. 27, 1633–1639.
Schultz, V., Lowenstein, J.M., 1978. The purine nucleotide cycle. Studies of ammonia production and interconversions of adenine and hypoxanthine nucleotides and nucleosides by rat brain in situ. J. Biol.Chem. 253, 1938–1943.
Shih, V.E., Efron, M.L., Moser, H.W., 1969. Hyperornithinemia, hyperammonemia, and homocitrullinuria. A new disorder of amino acid metabolism associated with myoclonic seizures and mental retardation. Am. J. Dis. Child. 117, 83–92.
Simell, O., Takki, K., 1973. Raised plasma-ornithine and gyrate atrophy of the choroid and retina. Lancet 1, 1031–1033.
Srere, P.A., 1969. Citrate synthase. Methods Enzymology 13, 3–11.
Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273, 16694–16699.
Tretter, L., Adam-Vizi, V., 2004. Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase. J. Neurosci. 24, 7771–7778.
Tsakiris, S., Deliconstantinos, G., 1984. Influence of phosphatidylserine on (Na++K+)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. Biochem. J. 220, 301–307.
Valle, D., Simell, O., 2001. The hyperornithinemias. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The metabolic and Molecular Basis of Inherited Diseases, Vol. 2. McGraw-Hill, New York, pp. 1857–1896.
Wolosker, H., Panizzutti, R., Engelender, S., 1996. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett. 392, 274–276.

Capítulo II

Dual mechanism of brain damage induced in vivo by the major metabolites accumulating in hyperornithinemia–hyperammonemia-homocitrullinuria syndrome

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Research Report

Dual mechanism of brain damage induced *in vivo* by the major metabolites accumulating in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome

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ABSTRACT

Hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome is an autosomal recessive disorder caused by a defect in the mitochondrial ornithine transporter, leading to accumulation of ornithine (Orn), homocitrulline (Hcit) and ammonia. Progressive neurological regression whose pathogenesis is not well established is common in this disease. The present work investigated the *in vivo* effects of intracerebroventricular administration of Orn and Hcit on important parameters of oxidative stress and energy metabolism in cerebral cortex from young rats. Orn and Hcit significantly increased thiobarbituric acid-reactive substances values and carbonyl formation, indicators of lipid and protein oxidative damage, respectively. Furthermore, *N*-acetylcysteine and the combination of the free radical scavengers ascorbic acid plus α-tocopherol attenuated the lipid oxidation and totally prevented the protein oxidative damage provoked by Orn and Hcit, suggesting that reactive species were involved in these effects. Hcit, but not Orn administration, also decreased glutathione concentrations, as well as the activity of catalase and glutathione peroxidase, indicating that Hcit provokes a reduction of brain antioxidant defenses. As regards to the parameters of energy metabolism, we verified that Orn and Hcit significantly inhibited the citric acid cycle function (inhibition of CO_2 synthesis from [1-^{14}C] acetate), the aerobic glycolytic pathway (reduced CO_2 production from [U-^{14}C] glucose) and complex I–III activity of the respiratory chain. Hcit also inhibited the activity of aconitase, an enzyme very susceptible to free radical attack. Taken together, our data indicate that mitochondrial homeostasis is disturbed by Orn and especially by Hcit. It is presumed that

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Abbreviations: HHH, hyperornithinemia–hyperammonemia–homocitrullinuria; Orn, ornithine; Hcit, homocitrulline; ICV, intracerebroventricular

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the impairment of brain bioenergetics and the oxidative damage induced by these metabolites may possibly contribute to the brain deterioration and neurological symptoms affecting patients with HHH syndrome.

1. Introduction

Hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome (OMIM 238970) is an autosomal recessive disorder due to mutation in the gene that encodes the mitochondrial ornithine (Orn) transporter *ORNT1* (SLC25A15) (Camacho et al., 1999; Fell et al., 1974; Korman et al., 2004; Tessa et al., 2009; Valle and Simell, 2001). The inability to import Orn from the cytosol into the mitochondria results in intramitochondrial Orn deficiency and a functional impairment of the urea cycle at the level of ornithine transcarbamylase, with consequent hyperammonemia. The defect also gives rise to cytoplasmatic accumulation of Orn resulting in hyperornithinemia. In the absence of intramitochondrial Orn, accumulating carbamoyl phosphate may condense with lysine to form homocitrulline (Hcit) leading to homocitrullinuria (Valle and Simell, 2001).

The clinical features of neurological symptoms in HHH syndrome are very peculiar since, besides some unspecific signs similar to the others urea cycle defects (hypotonia, seizures, ataxia, coma, etc.), patients exhibit a pyramidal syndrome with progressive spastic paraplegia. Neuropathological findings include multiple, nonspecific T2 hyperintense foci in occipital, parietal and frontal white matter, with subcortical and cortical atrophy associated with swelling typically seen in demyelinating diseases (Al-Hassnan et al., 2008). It should be stressed that among the urea cycle defects, pyramidal dysfunction is also present in argininemia and therefore both disorders share a common characteristic clinical picture (Valle and Simell, 2001).

The mechanisms of central nervous system (CNS) impairment in HHH syndrome are poorly known (Palmieri, 2008; Salvi et al., 2001), although it has been hypothesized that the neurologic damage presented by the patients are probably secondary to the episodic hyperammonemia. However, chronic accumulation of Orn, Hcit and other metabolic factors cannot be ruled out as contributing causes of the neurological symptoms and brain abnormalities seen in these patients, especially during crises of metabolic decompensation, in which the concentrations of these metabolites dramatically increase. It seems therefore justifiable to investigate the role of these accumulating metabolites on important systems necessary for normal CNS function that may lead to a better understanding of the relationship between the clinical features and the biochemical abnormalities in this disorder.

In this scenario, we have recently demonstrated that Orn and Hcit elicit *in vitro* lipid peroxidation, protein oxidative damage and decrease glutathione (GSH) levels and disrupt energy metabolism in brain of young rats (Amaral et al., 2009; Viegas et al., 2009).

In the present study we investigated whether *in vivo* intracerebroventricular (ICV) administration of Orn and Hcit to rats could induce lipid (thiobarbituric acid-reactive substances) and protein (sulfhydryl content and carbonyl formation) oxidative damage, as well as affect the antioxidant defenses (reduced glutathione levels and the activities of the antioxidant enzymes glutathione peroxidase, catalase and superoxide dismutase) and nitrates and nitrites production. We also tested the influence of *in vivo* ICV administration of these amino acids on parameters of aerobic glycolysis (CO_2 production from [U-^{14}C] glucose), citric acid cycle (CAC) activity (CO_2 production from [1-^{14}C] acetate and the enzyme activities of the CAC), electron transfer flow through the respiratory chain (complex I–IV activities), as well as on intracellular ATP transfer (creatine kinase activity) and the activity of Na^+, K^+-ATPase, an important enzyme necessary for normal neurotransmission, in cerebral cortex from young rats.

2. Results

2.1. Oxidative stress parameters

2.1.1. Orn and Hcit intracerebroventricular administration induces lipid peroxidation in cerebral cortex

Initially we studied the effect of intracerebroventricular (ICV) injection of Orn and Hcit on TBA-RS levels in cerebral cortex. Fig. 1A shows that Orn (37%) and Hcit (43%) induced lipid peroxidation (TBA-RS increase) in cerebral cortex 30 min after drug infusion [$F(2,16)=6.671$; $p<0.01$]. Next, we examined the effect of i.p. daily injections of *N*-acetylcysteine (NAC: 150 mg/kg), α-tocopherol (40 mg/kg) plus ascorbic acid (100 mg/kg), or saline (0.9% NaCl) for 3 days (pre-treatment), on Orn and Hcit-induced lipid oxidative damage. As shown in the figure, pre-treatment with NAC fully prevented the lipoperoxidation induced by Hcit, but only attenuated the lipid peroxidation caused by Orn. It can be also seen that pre-treatment with α-tocopherol plus ascorbic acid partially prevented the lipid peroxidation elicited by Orn and Hcit (Fig. 1B and C) (Orn: [$F(3,20)=3.183$; $p<0.05$]; Hcit: [$F(3,18)=4.278$; $p<0.05$]).

2.1.2. Orn and Hcit intracerebroventricular administration induces protein oxidative damage in cerebral cortex

We also investigated whether oxidation of tissue proteins was affected by ICV administration of Orn or Hcit, by measuring carbonyl and sulfhydryl content. Fig. 2A shows that carbonyl content was significantly enhanced by Orn (90%) and Hcit (140%) in cerebral cortex [$F(2,14)=8.292$; $p<0.01$], indicating that these compounds cause protein oxidative damage. However, ICV administration of Orn or Hcit was not able to affect the sulfhydryl content (nmol/mg protein: $n=7$; control: 86.26±7.97; Orn: 92.08±5.64; Hcit: 90.89±11.57). It was also tested the effects of pre-treatment with NAC (150 mg/kg) or α-tocopherol (40 mg/kg) plus ascorbic acid (100 mg/kg) on Orn-

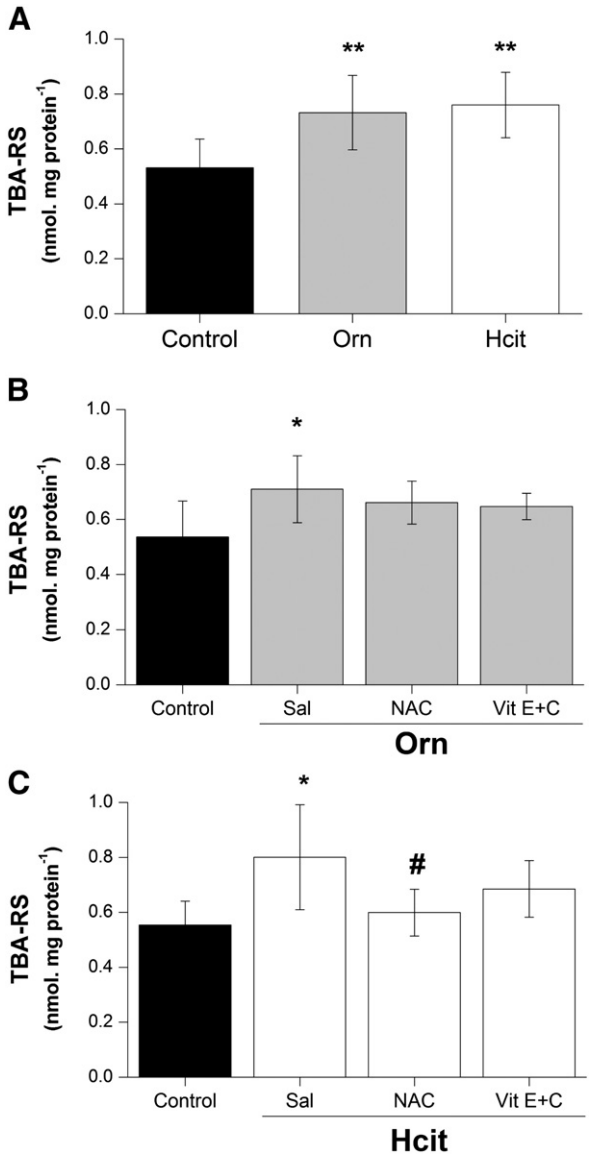

Fig. 1 – Effect of intracerebroventricular administration of ornithine (Orn) and homocitrulline (Hcit) on thiobarbituric acid reactive substances (TBA-RS) levels in rat cerebral cortex. The animals received a single ICV injection of NaCl (control group), Orn (5 μmol) or Hcit (1.6 μmol) and were sacrificed 30 min after injection (panel A). In some experiments the animals were pre-treated daily i.p. for 3 days with saline (0.9% NaCl), N-acetylcysteine (NAC, 150 mg/kg) or α-tocopherol (Vit E, 40 mg/kg) plus ascorbic acid (Vit C, 100 mg/kg), received a single ICV injection of NaCl, Orn (Panel B) or Hcit (panel C) and were sacrificed 30 min later. Data are represented as means ± standard deviation for five to seven independent experiments (animals) per group and expressed as nmol mg protein^{-1}. $^{**}p<0.01$ compared to control (panel A). $^{*}p<0.05$ compared to control (animals pre-treated with saline and injected ICV with NaCl); $^{\#}p<0.05$, compared to Sal (animals pre-treated with saline and injected ICV with Orn or Hcit) (panels B and C), ANOVA followed by Duncan's multiple range test.

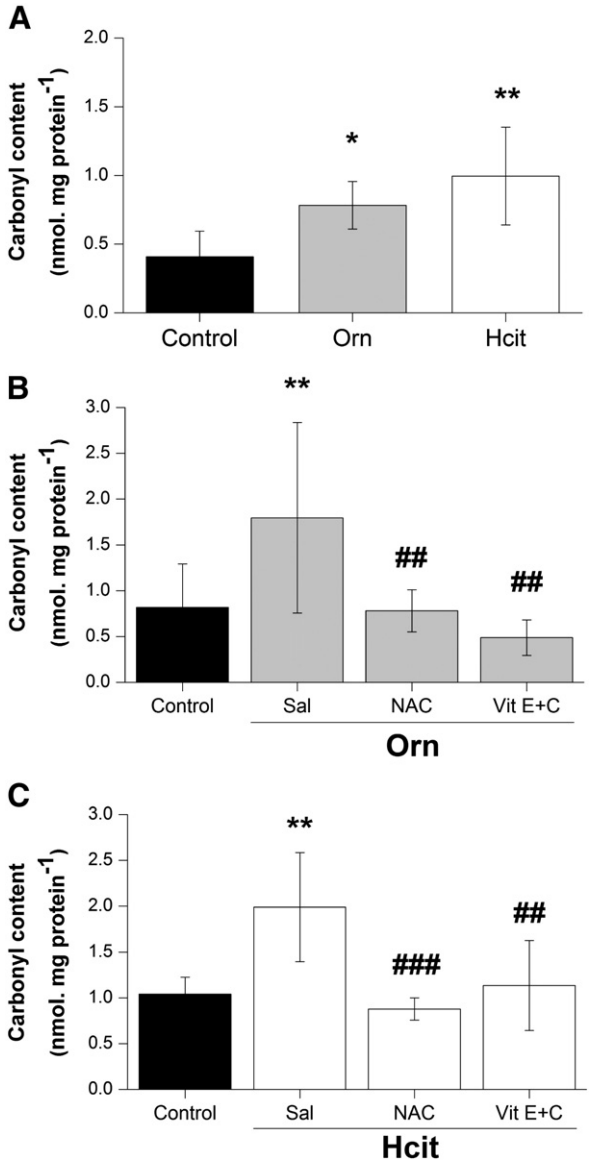

Fig. 2 – Effect of intracerebroventricular administration of ornithine (Orn) and homocitrulline (Hcit) on carbonyl content in rat cerebral cortex. The animals received a single ICV injection of NaCl (control group), Orn (5 μmol) or Hcit (1.6 μmol) and were sacrificed 30 min after injection (panel A). In some experiments the animals were pre-treated daily i.p. for 3 days with saline (0.9% NaCl), N-acetylcysteine (NAC, 150 mg/kg) or α-tocopherol (Vit E, 40 mg/kg) plus ascorbic acid (Vit C, 100 mg/kg), received a single ICV injection of NaCl, Orn (Panel B) or Hcit (panel C) and were sacrificed 30 min later. Data are represented as means ± standard deviation for five to seven independent experiments (animals) per group and expressed as nmol mg protein^{-1}. $^{*}p<0.05$ and $^{**}p<0.01$ compared to control; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$, compared to Sal (rats pre-treated with saline and injected ICV with Orn or Hcit) (panel B and C) (ANOVA followed by Duncan's multiple range test).

and Hcit-induced increase of carbonyl formation. The increase in carbonyl formation caused by Orn and Hcit was fully prevented by this pre-treatment, as shown in Fig. 2B and C (Orn: [$F(3,19)=5.114$; $p<0.01$]; Hcit: [$F(3,18)=8.666$; $p<0.01$]).

2.1.3. *Hcit intracerebroventricular administration decreases glutathione (GSH) concentrations in cerebral cortex*

GSH concentrations measured in cerebral cortex 30 min after Orn and Hcit ICV administration revealed that Hcit moderately reduced (15%) the concentrations of GSH after Hcit injection, whereas Orn did not alter this parameter [$F(2,16)=6.608$; $p<0.01$] (nmol/mg protein: $n=6$; control: 4.25±0.45; Orn: 3.95 ±0.17; Hcit: 3.66±0.14).

2.1.4. *Hcit intracerebroventricular administration inhibits enzymatic antioxidant defenses in cerebral cortex*

The next set of experiments was carried out to investigate the effect of ICV administration of Orn and Hcit on the activities of the antioxidant enzymes SOD, CAT and GPx. Fig. 3 shows that only Hcit was able to reduce the activities of GPx [$F(2,17)=3.786$; $p<0.05$] and CAT [$F(2,18)=8.328$; $p<0.01$], without affecting SOD activity. We also verified that Orn was not able to change any of these activities.

2.1.5. *Orn and Hcit intracerebroventricular administration does not affect nitrite and nitrate generation in cerebral cortex*

The effect of Orn and Hcit on reactive nitrogen species generation was assessed by measuring nitrate and nitrite production. We observed that this parameter was not altered by Orn and Hcit ICV administration (nmol/mg protein: $n=5$; control: 2.88±1.23; Orn: 2.43±0.89; Hcit: 2.15±0.87).

2.2. *Parameters of energy metabolism*

2.2.1. *Intracerebroventricular injection of Orn and Hcit inhibits CO_2 production from glucose and acetate in rat cerebral cortex*

We investigated the effect of ICV injection of Orn and Hcit on CO_2 production from labeled substrates in cortical homogenates. Fig. 4 shows that CO_2 production from [U-^{14}C] glucose was significantly inhibited by Orn (35%) and Hcit (32%) [$F(2,12)=5.515$; $p<0.05$] 30 min after ICV treatment. CO_2 formation from [1-^{14}C] acetate was also inhibited by Orn (32%) and Hcit (25%) administration [$F(2,12)=11.048$; $p<0.01$]. These results suggest that the aerobic glycolytic pathway and the CAC activity were compromised by Orn and Hcit.

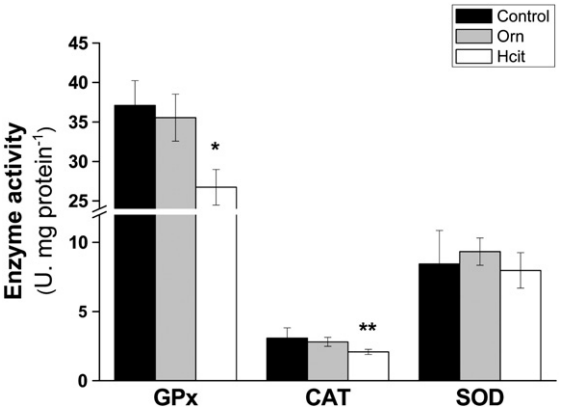

Fig. 3 – Effect of intracerebroventricular administration of ornithine (Orn) and homocitrulline (Hcit) on the activities of glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) in rat cerebral cortex. Data are expressed as means±standard deviation for six to seven independent experiments (animals). *$p<0.05$ and **$p<0.01$, compared to control (ANOVA followed by Duncan's multiple range test).

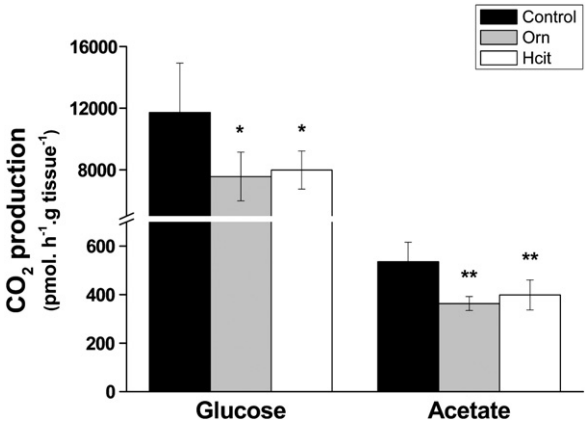

Fig. 4 – Effect of intracerebroventricular administration of ornithine (Orn) and homocitrulline (Hcit) on CO_2 production from [U-^{14}C] glucose and [1-^{14}C] acetate in rat cerebral cortex. Values are means±standard deviation for five independent experiments (animals) per group and expressed as pmol CO_2 h^{-1} g tissue^{-1}. *$p<0.05$ and **$p<0.01$, compared to control (ANOVA followed by Duncan's multiple range test).

2.2.2. *Hcit intracerebroventricular administration inhibits aconitase activity in cerebral cortex*

We also evaluated the effect of Orn and Hcit ICV administration on CAC enzyme activities. We found that Hcit, significantly inhibited (20%) aconitase activity (μmol NADPH min^{-1} mg protein^{-1}: $n=6$; control: 1339.4±82.9; Orn: 1208.4±135.6; Hcit: 1070.4±96.9), [$F(2,14)=8.450$, $p<0.01$], whereas Orn did not alter this activity. Furthermore, citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase activities were not changed by Orn and Hcit administration (results not shown).

2.2.3. *Intracerebroventricular injection of Orn and Hcit inhibits complex I–III activity of the respiratory chain in cerebral cortex*

The next set of experiments was performed to evaluate the effect of ICV injection of Orn and Hcit on the activities of the respiratory chain complexes I–III, II, II–III and IV. We found that complex I–III activity was significantly inhibited by Orn (20%) and Hcit (26%) [$F(2,15)=10.274$; $p<0.01$], with no significant alteration of the other tested activities of the respiratory chain (Table 1).

2.2.4. *Orn and Hcit intracerebroventricular administration does not alter creatine kinase and Na^+, K^+-ATPase activities in cerebral cortex*

We also examined the effect of Orn and Hcit ICV administration on the activities of creatine kinase (CK) and synaptic membrane Na^+, K^+-ATPase prepared from cerebral cortex. We observed that these metabolites did not change these activities (CK: μmol creatine min^{-1} mg protein^{-1}: $n=7$; control: 4.33±0.81; Orn: 5.31±0.97; Hcit: 4.48±0.67; NaK: nmol Pi min^{-1}

Table 1 – Effect of intracerebroventricular injection of ornithine (Orn) and homocitrulline (Hcit) on the activities of respiratory chain complexes in cerebral cortex from young rats.

	Complex I–III	Complex II	Complex II–III	Complex IV
Control	15.6±1.60	1.99±0.28	14.7±1.71	159±43.4
Orn	12.5±1.07 **	2.05±0.59	15.7±3.82	168±56.2
Hcit	11.6±2.03 **	2.22±0.57	16.1±3.78	176±26.9

Values are means±standard deviation for six to seven independent (animals) experiments per group performed in triplicate. The activities of complexes I–III, II, II–III and IV are expressed as nmol. min^{-1} mg $protein^{-1}$.
** $p<0.01$, compared to control (Duncan's multiple range test)

mg $protein^{-1}$: $n=4$; control: 209.8±71.7; Orn: 207.5±42.2; Hcit: 258.3±28.2).

3. Discussion

Patients affected by this HHH syndrome commonly have neurological dysfunction with acute encephalopathy, ataxia, choreoathetosis, developmental delay, severe muscle spasticity and mental retardation, whose neuropathology is poorly known (Shih et al., 1969; Valle and Simell, 2001).

Interestingly, patients with HHH syndrome and argininemia present similarities in clinical features, with progressive neurological deterioration and pyramidal signs that are usually not associated with hyperammonemic decompensation (Korman et al., 2004; Marescau et al., 1990; Salvi et al., 2001; Valle and Simell, 2001). Furthermore, it has been suggested that the lower limb dysfunction observed in HHH syndrome, and also in argininemia, may be related to an altered polyamine metabolism (Shimizu et al., 1990).

On the other hand, many individuals with HHH syndrome present mitochondrial abnormalities, as well as accumulation and excretion of lactic acid, ketone bodies and CAC intermediates (Gatfield et al., 1975; Haust et al., 1981; Metoki et al., 1984; Salvi et al., 2001), indicating an impaired mitochondrial function. Therefore, in the current study we evaluated the *in vivo* effects of these amino acids accumulating in HHH syndrome on important biochemical parameters of mitochondrial homeostasis, particularly those related to bioenergetics and biological oxidations in cerebral cortex of young rats in order to provide mechanistic insights for HHH syndrome neuropathology.

We first verified that Orn and Hcit *in vivo* administration to rats increased TBA-RS levels, as compared with control animals. These results corroborate our previous *in vitro* findings (Amaral et al., 2009). Since TBA-RS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007), the increased values of this parameter elicited by Orn and Hcit strongly indicates that these amino acids caused lipid peroxidation *in vivo*.

Orn, and also Hcit to a higher degree, enhanced carbonyl formation, implying that they caused protein oxidation. In this scenario, carbonyl groups (aldehydes and ketones) are mainly produced by oxidation of protein side chains (especially Pro, Arg, Lys, and Thr), by oxidative cleavage of proteins, or by the reaction of reducing sugars or their oxidation products with lysine protein residues (Dalle-Done et al., 2003). However, we cannot also exclude the possibility that aldehydes resulting from lipid peroxidation may also induce carbonyl generation (Dalle-Done et al., 2003).

It was also observed that the antioxidants *N*-acetylcysteine (NAC), that forms glutathione intracellularly, and the combination of the free radical scavengers ascorbic acid plus α-tocopherol attenuated the lipid oxidation and totally prevented the protein oxidative damage provoked by Hcit, suggesting that ROS generation was involved in its effects. As regards to the reactive species involved in Orn and Hcit pro-oxidant effects, it is feasible that the peroxyl radical, which is scavenged by α-tocopherol whose active form is regenerated (reduced) by ascorbic acid, may underlie at least in part these oxidative effects. However, considering that NAC also prevented these effects, we cannot exclude the possibility that a shortage of GSH could be responsible for lipid and especially protein oxidative damage provoked by Hcit and Orn. In fact, we found that Hcit ICV administration gave rise to a decrease of GSH concentrations, besides significantly inhibiting the activity of the antioxidant enzymes CAT and GPx with no effect on SOD. In contrast, Orn did not significantly affect any of these antioxidant defenses. Furthermore, it is unlikely that reactive nitrogen species participated in the pro-oxidant effects of Orn and Hcit since these compounds did not elicit nitrate and nitrite synthesis. Considering that endogenous GSH is considered the major naturally occurring brain antioxidant and that GPx and CAT activities are important enzymatic antioxidant defenses (Halliwell and Gutteridge, 2007), we presume that the rat cortical antioxidant defenses were compromised by *in vivo* administration of Hcit. Furthermore, it is also conceivable that the reduction of GSH levels may reflect increased reactive species generation elicited by Hcit. In this context, it may be presumed that Orn did not reduce GSH levels probably because it induced less reactive species formation compared to Hcit, reflected by its lower oxidative effects.

Our present data strongly indicate that *in vivo* administration of the major amino acids accumulating in HHH syndrome induces oxidative stress in rat cerebral cortex since this deleterious cell condition results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue (Halliwell and Gutteridge, 2007). It should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 1996), a fact that makes this tissue more vulnerable to increased reactive species.

With respect to the parameters of energy metabolism, Orn and Hcit compromised the aerobic glycolytic pathway and the CAC activity since they significantly decreased CO_2 formation from labeled glucose and acetate, respectively. It is therefore possible that Orn and Hcit may have inhibited the activity of one or more glycolytic enzymes, one or more reactions of the CAC, and/or the respiratory chain.

We did not determine the activities of the glycolytic pathway, but measured CAC enzyme activities and the activities of complexes I–IV of the respiratory chain following Orn and Hcit *in vivo* administration. Hcit significantly inhibited aconitase activity, without altering the other enzymes of the

CAC, whereas Orn did not affect any of these activities. Considering that aconitase is highly vulnerable to oxidative damage (Gardner, 1997) and that Hcit provoked a higher degree of protein oxidative damage compared to Orn, it is possible that aconitase inhibition may have a result of Hcit-induced free radical attack to essential groups of the enzyme. Furthermore, Orn and Hcit significantly reduced the electron transport chain flow by inhibiting the activity of complex I–III. Thus, it is feasible that the inhibition of complex I–III activity by these metabolites and of aconitase by Hcit contributed to the inhibition of the CAC. Altogether, these findings indicate that brain bioenergetics associated to energy production is compromised by Hcit and Orn.

On the other hand, *in vivo* administration of Hcit and Orn did not change the activities of creatine kinase (CK) and synaptic Na^+, K^+-ATPase from cerebral cortex of rats, which are important for cell energy buffering and transfer and to keep the neuronal membrane potential necessary for normal neurotransmission, respectively.

Altogether, our present findings indicate that Hcit exerted more significant effects than Orn on most parameters of oxidative stress and bioenergetics here examined, even though it was administered at a lower dose (1.6 μmol) as compared to Orn (5 μmol), reinforcing that Hcit is relatively a more potent neurotoxin. On the other hand, it seems that the mild to moderate disruption of bioenergetics and oxidative damage induced by Orn could hardly be associated with the neurodegeneration of HHH syndrome since this amino acid also accumulates at high amounts in ornithine aminotransferase deficiency, which is characterized by gyrate atrophy of the choroids and retina, with no alteration of the CNS (Javadzadeh and Gharabaghi, 2007; Kaiser-Kupfer et al., 1983; Simell and Takki, 1973).

At the present we cannot determine the pathophysiological relevance of the present data since to our knowledge brain concentrations of Orn and Hcit are not yet established in HHH syndrome, although blood Orn concentrations may achieve 1 mM during metabolic decompensation in affected patients (Palmieri, 2008; Valle and Simell, 2001). However, considering that the present *in vivo* results are in accordance with previous *in vitro* findings, showing that Orn and particularly Hcit disturb brain bioenergetics (Viegas et al., 2009) and induce oxidative stress (Amaral et al., 2009), it is presumed that a dual mechanism, energy deprivation and oxidative damage with reduction of tissue antioxidant defenses, secondary to acute accumulation of Hcit and Orn, may contribute to the neurological dysfunction characteristic of HHH syndrome. It should be also noted that during stress situations (for example, episodes of metabolic decompensation) characterized by encephalopathy and intense catabolism and proteolysis, much higher concentrations of these amino acids take place therefore facilitating CNS injury (Camacho et al., 1999).

Our findings showing bioenergetics impairment and oxidative stress caused by the major compounds accumulating in HHH syndrome may be interrelated since mitochondrial dysfunction is often associated with large increase of reactive species generation because oxidative phosphorylation is the major source of free radicals, which are byproducts of the cell respiratory cycle (Lemasters et al., 1999). Furthermore, low energy and oxidative damage are key events facilitating the pathogenic cascade leading to necrotic or apoptotic cell death especially in neurons, whose viability highly depends on large amounts of energy to preserve the resting membrane potential (Kroemer and Reed, 2000; Martin et al., 1994). We cannot also exclude the possibility that creatine deficiency, that occurs in OAT deficiency, may also play a role in the neuropathology of HHH syndrome, but this should be further investigated (Dionisi Vici et al., 1987; Valayannopoulos et al., 2009).

In summary, the current findings provide insight into possible mechanisms of brain damage in HHH syndrome caused *in vivo* by Hcit and Orn and indicate that the pathogenesis of this disorder cannot be exclusively attributed to hyperammonemia. Furthermore, the bioenergetics dysfunction caused by Hcit and Orn may explain the mitochondrial abnormalities and the increased urinary excretion of lactate, 2-hydroxyglutyrate, various CAC intermediates and glutaric acid that may be observed in patients with HHH syndrome. Therefore, it is conceivable that, besides a diet poor in proteins that is chronically used, prompt and aggressive treatment of infections with high caloric intake (to reduce the risk of increased catabolism with elevation of brain Orn and Hcit concentrations) and possibly with antioxidants seems justified to avoid aggravation of the brain injury in these patients, especially during acute metabolic decompensation.

4. Experimental procedures

4.1. *Reagents*

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [U-^{14}C] glucose and [1-^{14}C] acetate, which were purchased from Amersham International plc, UK and homocitrulline, which was obtained from MP Biomedicals, LLC Solon, Ohio, USA. Ornithine, homocitrulline, N-acetylcysteine, ascorbic acid (vitamin C) and α-tocopherol (vitamin E) were dissolved in saline solution (NaCl 0.9%).

4.2. *Animals*

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used in the assays. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12-h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

4.3. *Intracerebroventricular (ICV) administration*

The rats were deeply anesthetized with ketamine plus xilazine (75 and 10 mg/kg, i.p., respectively) and placed on a stereotaxic apparatus. Two small holes were drilled in the skull for microinjection, and 2 μL of a 2.5 M ornithine solution (5 μmol)

(pH 7.4 adjusted with NaOH), 0.8 M homocitrulline solution (1.6 μmol) (pH 7.4 adjusted with NaOH) or NaCl (controls) at the same volume and concentration, was slowly injected bilaterally over 4 min into the lateral ventricles via needles connected by a polyethylene tube to a 10-μL Hamilton syringe. The needles (one in each ventricle) were left in place for another 1 min before being softly removed. The coordinates for injections were as follows: 0.6 mm posterior to bregma, 1.1 mm lateral to midline and 3.2 mm ventral from dura (Paxinos and Watson, 1986). The correct position of the needle was tested by injecting 0.5 μL of methylene blue injection (4% in saline solution) and carrying out histological analysis. In some experiments, the effect of antioxidants on Orn and Hcit-induced oxidative damage was also evaluated by preinjecting the animals daily with N-acetylcysteine (NAC, 150 mg/kg, i.p.), or the combination of α-tocopherol (vitamin E, 40 mg/kg, i.p.) plus ascorbic acid (vitamin C, 100 mg/kg, i.p.), or saline (NaCl 0.9%, i.p.) for 3 days, after which the animals received an acute ICV injection of Orn, Hcit or NaCl.

4.4. *Cerebral cortex preparation*

Animals (male rats) were killed by decapitation 30 min after ICV injection of Orn, Hcit or NaCl, and the brain was immediately removed, the vessels and blood removed, and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. For the determination of oxidative stress parameters, cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750×*g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant containing mitochondria was immediately separated and used for the measurements.

For CO_2 production, the cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV and the CAC enzymes, cerebral cortex was homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). The homogenate was centrifuged at 800×*g* for 10 min and the supernatant was kept at −70 °C until being used for enzymatic activity determination. For creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for CO_2 production assays, whose experiments were performed on the same day of the preparations.

4.5. *Preparation of synaptic plasma membrane from rat cerebral cortex*

Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Membranes were prepared according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

4.6. *Oxidative stress parameters*

4.6.1. *Determination of thiobarbituric acid-reactive substances (TBA-RS)*

TBA-RS levels were measured according to the method described by Yagi (1998) with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatant and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein.

4.6.2. *Determination of sulfhydryl (thiol) content*

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of cerebral cortex supernatants. This was followed by 30-min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Results are reported as nmol TNB/mg protein.

4.6.3. *Determination of protein carbonyl formation*

Protein carbonyl content formation, a marker of oxidized proteins, was measured spectrophotometrically according to Levine et al. (1994) and Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation was treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10,000×*g*. The pellet was then washed with 1 mL ethanol/ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl. Then, the tubes were incubated at 37 °C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyls groups/mg of protein, using the extinction coefficient of 22,000×106 nmol/mL for aliphatic hydrazones.

4.6.4. *Determination of nitrate and nitrite content*

Nitrate and nitrite concentrations were determined according to Miranda et al. (2001). Briefly, 12 μL of 20% trichloroacetic acid was added to 300 μL of cerebral cortex supernatants and centrifuged at 12,000×*g* for 10 min. Two hundred microliters of the supernatant was transferred to an eppendorf tube and

incubated with 200 μL of 0.8% VCl_3 in 1 M HCl and 200 μL of the Griess reagent (2% sulfanilamide in 5% HCl and 0.1% N-1-(naphtyl)ethylenediamine in H_2O) at 37 °C for 30 min in a dark room. Absorbance was then determined at 540 nm by spectrophotometry. A calibration curve was performed using sodium nitrate. Each curve point was subjected to the same treatment as supernatants and the concentrations were calculated as mmol/mg protein.

4.6.5. *Determination of reduced glutathione (GSH) levels*
GSH levels were evaluated according to Browne and Armstrong (1998). Tissue supernatants were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein.

4.6.6. *Determination of glutathione peroxidase (GPx) activity*
GPx activity was measured according to Wendel (1981) using *tert*-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide, 0.1 mM NADPH, and the supernatant containing 0.2–0.4 mg protein/mL. One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein.

4.6.7. *Determination of catalase (CAT) activity*
CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.05–0.1 mg protein/mL. One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein.

4.6.8. *Determination of superoxide dismutase (SOD) activity*
SOD activity was assayed according to Marklund (1985) and is based on the capacity of pyrogallol to autoxidize, a process highly dependent on $O_2^{\bullet-}$, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and supernatants containing 0.1–0.2 mg protein/mL. A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results are reported as U/mg protein.

4.7. *Energy metabolism parameters*

4.7.1. *Determination of CO_2 production*
Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated at 35 °C for 10 min in a metabolic shaker (90 oscillations min^{-1}) with 625 μM *n*-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. After pre-incubation, [U-^{14}C] glucose (0.055 μCi) plus 5.0 mM of unlabeled glucose or 0.055 μCi [1-^{14}C] acetate plus 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 60 nm/4 nm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Assis et al., 2004). Results were calculated as pmol CO_2 h^{-1} g $tissue^{-1}$.

4.7.2. *Spectrophotometric analyses of the activities of citric acid cycle (CAC) enzymes*
Citrate synthase activity was measured according to Srere (1969), by determining DTNB reduction at λ=412 nm. The activity of the enzyme aconitase was measured according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. Isocitrate dehydrogenase activity was accessed by the method of Plaut (1969), by following NAD^+ reduction at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Viegas et al. (2009). The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of succinate dehydrogenase was determined as described by Fischer et al. (1985). Fumarase activity was measured according to O'Hare and Doonan (1985), measuring the increase of absorbance at λ=250 nm. Malate dehydrogenase activity was measured according to Kitto (1969) by following the reduction of NADH at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activities of the CAC enzymes were calculated as nmol min^{-1} mg $protein^{-1}$, mmol min^{-1} mg $protein^{-1}$ or μmol min^{-1} mg $protein^{-1}$.

4.7.3. *Spectrophotometric analysis of the respiratory chain complex I–IV activities*
The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate/cytochrome c oxidoreductase (complex II–III) were determined according to Fischer et al. (1985). The activity of NADH/cytochrome c oxidoreductase (complex I–III) was assayed according to the method described by Schapira et al. (1990) and that of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (Silva et al., 2002). The activities of the respiratory chain complexes were calculated as nmol min^{-1} mg $protein^{-1}$ or mmol min^{-1} mg $protein^{-1}$.

4.7.4. *Spectrophotometric analysis of creatine kinase (CK) activity*

CK activity was measured according to Hughes (1962) with slight modifications (Schuck et al., 2002). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM *p*-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ=540 nm. Results were calculated as μmol of creatine min^{-1} mg $protein^{-1}$.

4.7.5. *Spectrophotometric analysis of Na^+, K^+-ATPase activity*

The reaction mixture for the Na^+, K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activities were calculated as nmol Pi $released^{-1}$ min^{-1} mg protein.

4.8. *Protein determination*

Protein was measured by the methods of Lowry et al. (1951) using bovine serum albumin as standard.

4.9. *Statistical analysis*

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *F* was significant. Only significant *F* values are shown in the text. Differences between groups were rated significant at $p<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

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REFERENCES

Aebi, H., 1984. Catalase, in vitro. Methods Enzymol 105, 121–126.

Aksenov, M.Y., Markesbery, W.R., 2001. Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer's disease. Neurosci. Lett. 302, 141–145.

Al-Hassnan, Z.N., Rashed, M.S., Al-Dirbashi, O.Y., Patay, Z., Rahbeeni, Z., Abu-Amero, K.K., 2008. Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome with stroke-like imaging presentation: clinical, biochemical and molecular analysis. J. Neurol. Sci. 15, 187–194.

Amaral, A.U., Leipnitz, G., Fernandes, C.G., Seminotti, B., Zanatta, A., Viegas, C.M., Dutra-Filho, C.S., Wajner, M., 2009. Evidence that the major metabolites accumulating in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome induce oxidative stress in brain of young rats. Int. J. Dev. Neurosci. 27, 635–641.

Assis, D.R., Maria, R.C., Rosa, R.B., Schuck, P.F., Ribeiro, C.A.J., Ferreira, G.C., Dutra-Filho, C.S., Wyse, A.T.S., Wannmacher, C.M.D., Perry, M.L.S., Wajner, M., 2004. Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res. 1030, 141–151.

Browne, R.W., Armstrong, D., 1998. Reduced glutathione and glutathione disulfide. Methods. Mol. Biol. 108, 347–352.

Camacho, J.A., Obie, C., Biery, B., Goodman, B.K., Hu, C.A., Almashanu, S., Steel, G., Casey, R., Lambert, M., Mitchell, G.A., Valle, D., 1999. Hyperornithinaemia–hyperammonaemia–homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. Nat. Genet. 22, 151–158.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Dalle-Done, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.

Dionisi Vici, C., Bachmann, C., Gambarara, M., Colombo, J.P., Sabetta, G., 1987. Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome: low creatine excretion and effect of citrulline, arginine, or ornithine supplement. Pediatr. Res. 22, 364–367.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388, 261–266.

Fell, V., Pollitt, R.J., Sampson, G.A., Wright, T., 1974. Ornithinemia, hyperammonemia and homocitrullinuria. A disease associated with mental retardation and possibly caused by defective mitochondrial transport. Am. J. Dis. Child. 127, 752–756.

Fischer, J.C., Ruitenbeek, W., Berden, J.A., Trijbels, J.M., Veerkamp, J.H., Stadhouders, A.M., Sengers, R.C., Janssen, A.J., 1985. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin. Chim. Acta 153, 23–36.

Gardner, P.R., 1997. Superoxide-driven aconitase FE-S center cycling. Biosci. Rep. Rev. 17, 33–42.

Gatfield, P.D., Taller, E., Wolfe, D.M., Haust, M.D., 1975. Hyperornithinemia, hyperammonemia, and homocitrullinuria associated with decreased carbamyl phosphate synthetase I activity. Pediatr. Res. 9, 487–488.

Halliwell, B., Gutteridge, J.M.C., 2007. Measurement of reactive species. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 268–340.

Halliwell, B., Gutteridge, J.M.C., 1996. Oxygen radicals and nervous system. Trends Neurosci. 8, 22–26.

Haust, M.D., Gatfield, P.D., Gordon, B.A., 1981. Ultrastructure of hepatic mitochondria in a child with hyperornithinemia, hyperammonemia, and homocitrullinuria. Hum. Pathol. 12, 212–222.

Hughes, B.P., 1962. A method for the estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7, 597–603.

Javadzadeh, A., Gharabaghi, D., 2007. Gyrate atrophy of the choroid and retina with hyper-ornithinemia responsive to vitamin B6: a case report. J. Med. Case. Rep. 1, 27.

Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation–sedimentation density gradient centrifugation. Biochim. Biophys. Acta 356, 276–287.

Kaiser-Kupfer, M., Kuwabara, T., Uga, S., Takki, K., Valle, D., 1983. Cataract in gyrate atrophy: clinical and morphologic studies. Invest. Ophthalmol. Vis. Sci. 24, 432–436.

Kitto, G.B., 1969. Intra- and extramitochondrial malate dehydrogenase from chicken and tuna heart. Methods Enzymol. 13, 106–116.

Korman, S.H., Kanazawa, N., Abu-Libdeh, B., Gutman, A., Tsujino, S., 2004. Hyperornithinemia, hyperammonemia, and homocitrullinuria syndrome with evidence of mitochondrial dysfunction due to a novel SLC25A15 (ORNT1) gene mutation in a Palestinian family. J. Neurol. Sci. 218, 53–58.

Kroemer, G., Reed, J.C., 2000. Mitochondrial control of cell death. Nat. Med. Rev. 6, 513–519.

Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, A.L., Herman, B., 1999. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J. Bioenerg. Biomembr. Rev. 31, 305–319.

Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Meth. Enzymol. 233, 346–357.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marescau, B., De Deyn, P.P., Lowenthal, A., Qureshi, I.A., Antonozzi, I., Bachmann, C., Cederbaum, S.D., Cerone, R., Chamoles, N., Colombo, J.P., 1990. Guanidino compound analysis as a complementary diagnostic parameter for hyperargininemia: follow-up of guanidino compound levels during therapy. Pediatr. Res. 27, 297–303.

Marklund, S.L., 1985. Pyrogallol autoxidation. Handbook for Oxygen Radical Research. CRC Press, Boca Raton. pp. 243–247.

Martin, R.L., Lloyd, H.G., Cowan, A.I., 1994. The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neuroscience 17, 251–257.

Metoki, K., Hommes, F.A., Dyken, P., Kelloes, C., Trefz, J., 1984. Ultrastructural changes in fibroblast mitochondria of a patient with HHH-syndrome. J. Inherit. Metab. Dis. 7, 147–150.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 1, 62–71.

Morrison, J.F., 1954. The activation of aconitase by ferrous ions and reducing agents. Biochem. J. 58, 685–692.

O'Hare, M.C., Doonan, S., 1985. Purification and structural comparisons of the cytosolic and mitochondrial isoenzymes of fumarase from pig liver. Biochim. Biophys. Acta 827, 127–134.

Palmieri, F., 2008. Diseases caused by defects of mitochondrial carriers: a review. Biochim. Biophys. Acta 1777, 564–578.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego.

Plaut, G.W.E., 1969. Isocitrate dehydrogenase from bovine heart. In: Lowenstein, J.M. (Ed.), Methods in Enzymology. Academic Press, New York, pp. 34–42.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.

Rustin, P., Chretien, D., Bourgeron, T., Gerard, B., Rotig, A., Saudubray, J.M., Munnich, A., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35–51.

Salvi, S., Santorelli, F.M., Bertini, E., Boldrini, R., Meli, C., Donati, A., Burlina, A.B., Rizzo, C., Di Capua, M., Fariello, G., Dionisi-Vici, C., 2001. Clinical and molecular findings in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome. Neurology 57, 911–914.

Schapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–2145.

Shimizu, H., Maekawa, K., Eto, Y., 1990. Abnormal urinary excretion of polyamines in HHH syndrome (hyperornithinemia associated with hyperammonemia and homocitrullinuria). Brain Dev. 12, 533–535.

Schuck, P.F., Leipnitz, G., Ribeiro, C.A., Dalcin, K.B., Assis, D.R., Barschak, A.G., Pulrolnik, V., Wannmacher, C.M., Wyse, A.T., Wajner, M., 2002. Inhibition of creatine kinase activity *in vitro* by ethylmalonic acid in cerebral cortex of young rats. Neurochem. Res. 27, 1633–1639.

Shih, V.E., Efron, M.L., Moser, H.W., 1969. Hyperornithinemia, hyperammonemia, and homocitrullinuria. A new disorder of amino acid metabolism associated with myoclonic seizures and mental retardation. Am. J. Dis. Child. 117, 83–92.

Silva, C.G., Ribeiro, C.A.J., Leipnitz, G., Dutra-Filho, C.S., Wyse, A.T.S., Wannmacher, C.M.D., Sarkis, J.J., Jakobs, C., Wajner, M., 2002. Inhibition of cytochrome c oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid *in vitro*. Biochim. Biophys. Acta 1586, 81–91.

Simell, O., Takki, K., 1973. Raised plasma-ornithine and gyrate atrophy of the choroid and retina. Lancet 1, 1031–1033.

Srere, P.A., 1969. Citrate synthase. Methods Enzymol. 13, 3–11.

Tessa, A., Fiermonte, G., Onisi-Vici, C., Paradies, E., Baumgartner, M.R., Chien, Y.H., Loguercio, C., de Baulny, H.O., Nassogne, M.C., Schiff, M., Deodato, F., Parenti, G., Rutledge, S.L., Vilaseca, M.A., Melone, M.A., Scarano, G., Aldamiz-Echevarría, L., Besley, G., Walter, J., Martinez-Hernandez, E., Hernandez, J.M., Pierri, C.L., Palmieri, F., Santorelli, F.M., 2009. Identification of novel mutations in the SLC25A15 gene in hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome: a clinical, molecular, and functional study. Hum. Mutat. 30, 741–748.

Tsakiris, S., Deliconstantinos, G., 1984. Influence of phosphatidylserine on ($Na^+ + K^+$)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. Biochem. J. 220, 301–307.

Valayannopoulos, V., Boddaert, N., Mention, K., Touati, G., Barbier, V., Chabli, A., Sedel, F., Kaplan, J., Dufier, J.L., Seidenwurm, D., Rabier, D., Saudubray, J.M., de Lonlay, P., 2009. Secondary creatine deficiency in ornithine delta-aminotransferase deficiency. Mol. Genet. Metab. 97, 109–113.

Valle, D., Simell, O., 2001. The hyperornithinemias. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic and Molecular Basis of Inherited Diseases. McGraw-Hill, New York, pp. 1857–1896.

Viegas, C.M., Zanatta, A., Knebel, L.A., Schuck, P.F., Tonin, A.M., Ferreira, C.G., Amaral, A.U., Dutra Filho, C.S., Wannmacher, C.M., Wajner, M., 2009. Experimental evidence that ornithine and homocitrulline disrupt energy metabolism in brain of young rats. Brain Res. 1291, 102–112.

Wendel, A., 1981. Glutathione peroxidase. Methods Enzymol. 77, 325–332.

Yagi, K., 1998. Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. Methods. Mol. Biol. 108, 107–110.

Capítulo III

Impairment of brain redox homeostasis caused by the major metabolites accumulating in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome *in vivo*

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Abstract:	Ornithine, ammonia and homocitrulline are the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, a genetic disorder characterized by neurological regression whose pathogenesis is still not understood. The present work investigated the in vivo effects of intracerebroventricular administration of ornithine and homocitrulline in the presence or absence of hyperammonemia induced by intraperitoneal urease treatment on important parameters of oxidative stress in cerebral cortex from young rats in order to better understand the role of these metabolites on brain damage. Ornithine increased thiobarbituric acid-reactive substances levels and carbonyl formation, without altering sulfhydryl content and reduced glutathione (GSH) levels. We also observed that the combination of hyperammonemia with ornithine resulted in a significant decrease of sulfhydryl levels and GSH concentrations, highlighting a synergistic effect of ornithine and ammonia. Furthermore, homocitrulline caused increases of thiobarbituric acid-reactive substances values and carbonyl formation, as well as a decrease of GSH concentrations without altering sulfhydryl content. Finally, we observed that urease treatment per se was able to enhance thiobarbituric acid-reactive substances levels. Our data indicate that the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome provoke lipid and protein oxidative damage and a reduction of the antioxidant defenses in the brain. Therefore, it is presumed that oxidative stress may represent a relevant pathomechanism involved in the brain damage found in patients affected by this disease.

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Impairment of brain redox homeostasis caused by the major metabolites accumulating in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome *in vivo*

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Abstract

Ornithine, ammonia and homocitrulline are the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, a genetic disorder characterized by neurological regression whose pathogenesis is still not understood. The present work investigated the *in vivo* effects of intracerebroventricular administration of ornithine and homocitrulline in the presence or absence of hyperammonemia induced by intraperitoneal urease treatment on important parameters of oxidative stress in cerebral cortex from young rats in order to better understand the role of these metabolites on brain damage. Ornithine increased thiobarbituric acid-reactive substances levels and carbonyl formation, without altering sulfhydryl content and reduced glutathione (GSH) levels. We also observed that the combination of hyperammonemia with ornithine resulted in a significant decrease of sulfhydryl levels and GSH concentrations, highlighting a synergistic effect of ornithine and ammonia. Furthermore, homocitrulline caused increases of thiobarbituric acid-reactive substances values and carbonyl formation, as well as a decrease of GSH concentrations without altering sulfhydryl content. Finally, we observed that urease treatment *per se* was able to enhance thiobarbituric acid-reactive substances levels. Our data indicate that the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome provoke lipid and protein oxidative damage and a reduction of the antioxidant defenses in the brain. Therefore, it is presumed that oxidative stress may represent a relevant pathomechanism involved in the brain damage found in patients affected by this disease.

Keywords: Ornithine; homocitrulline; ammonia; hyperornithinemia-hyperammonemia-homocitrullinuria syndrome; oxidative stress; cerebral cortex.

Introduction

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM # 238970) is an autosomal recessive disorder caused by a mutation in *ORNT1* (SLC25A15) gene encoding a mitochondrial Orn transporter (Valle and Simell 2001). As a consequence, cytosolic accumulation and intramitochondrial depletion of Orn occurs, leading to derangement of urea cycle and hyperammonemia. In addition, carbamoyl phosphate condenses with lysine to form homocitrulline (Hcit) (Camacho et al. 1999).

Ammonia, a waste product of protein breakdown in the body, is toxic when its levels become high. The main pathway of ammonia detoxification is the urea cycle, which is only completely expressed in the liver, although other tissues, including brain, express some of the constituent enzymes. In HHH syndrome, the lack of ornithine in the mitochondrial matrix causes a blockage of the urea cycle therefore accumulating ammonia and causing hyperammonemia. Regarding specifically to the brain, which is especially sensitive to the effects of excess of ammonia, it lacks carbamoyl-phosphate synthase I and ornithine transcarbamylase, making this organ unable to remove ammonia in the form of urea. Consequently, brain ammonia is metabolized almost exclusively to glutamine via the glutamine synthetase reaction (Cooper and Plum 1987). In this scenario, astrocytes are the site of ammonia detoxification in brain, due to the predominant localization of glutamine synthetase in these cells (Martinez et al. 1977). Glutamine is released from glial cells to neurons where it is converted back to glutamate releasing ammonia into neurons. Glutamine is also released from the brain to the circulation where it reaches the liver and is converted to glutamate.

Ammonia can be also formed in the brain mainly through glutamate dehydrogenase, which catalyses the reversible oxidative deamination of glutamate,

particularly in astrocytes (Cooper and Plum 1987). L-Glutaminase, particularly abundant in nerve endings of glutamatergic neurons, also releases ammonia and constitutes an integral part of the glutamate–glutamine cycle, in which a molecule of ammonia is transferred from the astrocyte to the neighboring neuron. Finally, enzymes of the purine nucleotide cycle may also be responsible for generating a significant fraction of brain ammonia (Schultz and Lowenstein 1978).

HHH syndrome has a wide spectrum of clinical presentation, including chronic progressive neurological findings, such as mental retardation, hypotonia, peripheral neuropathy, spastic paraplegia, convulsions and cerebellar ataxia, as well as acute episodes of coma or liver disease with coagulopathy (Tessa et al. 2009; Valle and Simell 2001). Neuropathological findings include multiple, nonspecific T2 hyperintense foci in occipital, parietal and frontal white matter, typically seen in demyelinating diseases, with subcortical and cortical atrophy associated with swelling (Al-Hassnan et al. 2008; Haust et al. 1981; Metoki et al. 1984).

The biochemical variability among patients with distinct clinical presentation has not been yet reported and the pathophysiology of the brain damage of HHH syndrome is practically unknown. Furthermore, molecular analysis did not reveal a clear genotype/phenotype correlation nor it clarified the pathogenesis of this variability. Although high levels of ammonia could potentially be responsible for the neurological symptoms, it was observed that worsening of neurologic signs does not commonly relate to relapses of hyperammonemia observed in patients with HHH syndrome (Salvi et al. 2001). It may be therefore concluded that other neurotoxins may be acting in this syndrome.

In this scenario, we have recently demonstrated that Orn and Hcit elicit *in vitro* and *in vivo* lipid peroxidation, protein oxidative damage and decrease glutathione

(GSH) levels and also disrupt energy metabolism in brain of young rats (Amaral et al. 2009; Viegas et al. 2009; Viegas et al. 2011).

In the present study we investigated whether systemic high levels of ammonia induced by urease intraperitoneal (i.p) administration, combined or not with *in vivo* intracerebroventricular (ICV) administration of Orn or Hcit to rats, could provoke lipid (thiobarbituric acid-reactive substances) and protein (sulfhydryl content and carbonyl formation) oxidative damage, as well as affect the antioxidant defenses (reduced glutathione) in cerebral cortex from young rats.

Methods

Animals and reagents

Wistar male rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS – Brazil, were used. The animals were maintained on a 12:12 h light / dark cycle (lights on 07.00 - 19.00 h) in air conditioned constant temperature (22°C ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the "Principles of Laboratory Animal Care (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for homocitrulline, which was obtained from MP Biomedicals, LLC Solon, Ohio, USA. Ornithine, homocitrulline and urease were dissolved in saline solution (NaCl 0.9%) and pH was adjusted at 7.4.

Urease treatment

To induce systemic high levels of ammonia we used an animal model of hyperammonemia according to Diemer and Laursen (1977). The animals received a daily intraperitoneal injection of urease (33U/Kg) solution for 4 days from the 26th till the 29th day of postnatal life. The chosen dose of urease did not cause hyperexitability, convulsions or death (Diemer and Laursen 1977). Control rats received saline at the same volumes.

Determination of plasma ammonia levels

The animals were anesthetized with ketamine plus xilazine (75 and 10 mg/kg i.p, respectively) and had their blood collected by cardiac puncture. The blood was transferred to heparinized tubes and processed for plasma separation.

Ammonia levels were measured 24 hours after the last urease injection using a commercial kit (Sigma, St. Louis, MO). Ammonia levels were assessed based on the reaction with a-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate in the presence of L-glutamate dehydrogenase. Oxidation rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to the manufacturer's protocol.

Intracerebroventricular (ICV) administration

One day after the last injection of saline or urease the rats were deeply anesthetized with ketamine plus xilazine (75 and 10 mg/kg i.p, respectively) and placed on a stereotaxic apparatus. Two small holes were drilled in the skull for microinjection, and 2 μL of a 2.5 M Orn solution (5 μmol) (pH 7.4 adjusted with NaOH), 0.8 M Hcit

solution (1.6 μmol) (pH 7.4 adjusted with NaOH) or saline (controls) at the same volume and concentration, was slowly injected bilaterally over 3 min into the lateral ventricles via needles connected by a polyethylene tube to a 10 μL Hamilton syringe. The needles (one in each ventricle) were left in place for another 1 min before being softly removed. The coordinates for injections were as follows: 0.6 mm posterior to bregma, 1.1 mm lateral to midline and 3.2 mm ventral from dura (Paxinos and Watson 1986). The correct position of the needle was tested by injecting 0.5 μL of methylene blue injection (4% in saline solution) and carrying out histological analysis.

We had six experimental groups: 1) Saline + Saline (Control); 2) Saline + Orn (Orn); 3) Saline + Hci (Hcit); 4) Urease + Saline (Urease); 5) Urease + Orn; and 6) Urease + Hcit.

Cerebral cortex preparation

Animals were killed by decapitation 30 min after ICV injection of Orn, Hcit or saline, and the brain was immediately removed, the vessels and blood removed, and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 x g for 10 min at 4°C to discard nuclei and cell debris (Evelson et al. 2001). The pellet was discarded and the supernatant containing mitochondria was immediately separated and used for the measurements.

Determination of thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS levels were measured according to the method described by Yagi

(1998) with slight modifications. Briefly, 200μL of 10% trichloroacetic acid and 300μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatant and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein and represented as percentage of control.

Determination of protein carbonyl formation

Protein carbonyl content formation, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation were treated with 400 mL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 mL 20% TCA and centrifuged for 5 min at 10,000 x g. The pellet was then washed with 1 mL ethanol: ethyl acetate (1:1, v/v) and re-dissolved in 550 mL 6 M guanidine prepared in 2.5 N HCl. Then, the tubes were incubated at 37°C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyls groups/mg of protein, using the extinction coefficient of 22,000 x 106 nmol/mL for aliphatic hydrazones. The data were expressed as percentage of control.

Determination of sulfhydryl (thiol) content

This assay is based on the reduction of 5,5'-dithio-bis (2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of cerebral cortex supernatants. This was followed by 30-min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Results are reported as nmol TNB/mg protein and represented as percentage of control.

Determination of reduced glutathione (GSH) levels

GSH levels were evaluated according to Browne and Armstrong (1998). Tissue supernatants were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001-0.1 mM), and GSH concentrations were calculated as nmol/mg protein and expressed as percentage of control.

Protein determination

Protein was measured by the methods of Lowry *et al* (1951) using bovine serum albumin as standard.

Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *F* was significant. Differences between groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Urease treatment induces hyperammonemia in young rats

We observed that 24 hours after urease i.p administration plasma ammonia levels increased 7.4-fold (559.0 ± 42.29 μM, N=6), as compared to the basal levels in animals injected with saline (81.5 ± 7.10, N=5).

Lipid peroxidation is induced in vivo by Orn, Hcit and urease treatment (hyperammonemia) in rat cerebral cortex

We initially tested the *in vivo* influence of Orn, Hcit and urease treatment on TBA-RS levels in cerebral cortex. Figure 1 shows that Orn ($P < 0.01$) (A) and Hcit ($P < 0.05$) (B) ICV injection significantly increased TBA-RS levels relative to the control group. We can also observe in the figure that urease treatment *per se* was able to significantly increase TBA-RS values but did not amplify the effects elicited by Orn and Hcit.

Protein oxidative damage is induced in vivo by Orn and Hcit treatment in rat cerebral cortex

We then evaluated the *in vivo* effect of Orn, Hcit and urease treatment on carbonyl formation and sulfhydryl oxidation in cerebral cortex in order to evaluate protein oxidation. We found that Orn ($P < 0.05$) and Hcit ($P < 0.05$) significantly increased protein carbonyl content in cerebral cortex relatively to controls, whereas urease treatment *per se* did not change this parameter and its combination with Orn or Hcit did not enhance the observed effects elicited by these amino acids (Figure 2). Furthermore, Orn, Hcit and urease treatment did not affect sulfhydryl group oxidation

(Figure 3), but the combination of urease, that induces hyperammonemia, and Orn treatment resulted in a significant decrease of sulfhydryl content (Figure 3A).

Reduced glutathione (GSH) concentrations are decreased in vivo by Hcit in rat cerebral cortex

The effect of Orn, Hcit and urease treatment on the brain antioxidant defenses was then investigated by measuring GSH levels. It can be observed in Figure 4 that Hcit significantly diminished the concentrations of GSH ($P < 0.05$), whereas Orn did not alter this parameter. We can also verify in the figure that the reduction of GSH concentrations were achieved by the combination of urease and Orn treatment

Discussion

Current hypotheses postulate that hyperammonemia is mainly responsible for the brain clinical manifestations of patients affected by HHH syndrome (Valle and Simell 2001). However, we have previously found that Orn and Hcit induce oxidative stress (Amaral et al. 2009; Viegas et al. 2011) and disrupt energy metabolism (Viegas et al. 2009; Viegas et al. 2011) in cerebral cortex of young rats *in vitro* and *in vivo*. These data, allied to observations that symptoms of patients with this disorder deteriorate in the absence of hyperammonemia, indicate that Orn and Hcit are neurotoxic and may possibly act synergistically with ammonia leading to brain damage in this disorder. Thus, the aim of the current study was to evaluate the *in vivo* effects of urease treatment that causes hyperammonemia, alone or in combination with Orn and Hcit on cellular redox homeostasis in cerebral cortex of young rats in order to provide mechanistic insights for HHH syndrome neuropathology.

We used the model of hyperammonemia induced by urease treatment as described by Diemer and Laursen (1977). This approach has been widely used as a model of hepatic encephalopathy to evaluate the role of high levels of ammonia on the brain (Butterworth et al. 2009). In our laboratory, ammonia levels increased over 7-fold the basal levels.

We first verified that Orn and Hcit ICV administration increased TBA-RS levels, as compared with animals receiving saline. We also demonstrated that hyperammonemia *per se* induced by urease i.p administration was able to significantly raise TBA-RS levels but did not amplify the effects provoked by Orn and Hcit on this measurement. Since TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge 2007), the increased TBA-RS values elicited by Orn, Hcit and high levels of ammonia strongly

indicate that these metabolites caused lipid peroxidation *in vivo*.

Orn and Hcit also induced carbonyl formation, an important indicator of protein oxidative damage, indicating that these amino acids provoke protein oxidation. However, carbonyl formation was not induced by urease treatment, suggesting that high levels of ammonia are more prone to cause lipid rather than protein oxidative damage. It is emphasized that carbonyl groups (aldehydes and ketones) are mainly produced by oxidation of protein side chains (especially Pro, Arg, Lys and Thr), by oxidative cleavage of proteins, or from the reaction of reducing sugars or their oxidation products with lysine protein residues (Dalle-Done et al. 2003).

As regard to sulfhydryl oxidation, Hcit, Orn and ammonia *per se* did not alter this parameter, although the combination of urease treatment with Orn injection was able to decrease the sulfhydryl content, suggesting a synergistic effect of these metabolites. In this context, protein sulfhydryl groups from cysteine residues can be oxidized to form disulfide, potentially altering the redox state of proteins and leading to their inactivation (Kuhn et al., 1999). Taken together, we presume protein oxidation might represent a pathomechanism causing neuronal damage in HHH syndrome. Although the exact mechanisms by which Orn and Hcit caused protein oxidative damage are at the present unknown, it is reasonable that Orn- and Hcit-induced reactive species may interact with protein groups leading to protein oxidation. Ammonia may enhance these effects, by synergistically acting with Orn.

Finally we found that Hcit ICV administration *per se* gave rise to a decrease of GSH concentrations. In contrast, Orn and urease administration did not significantly affect this antioxidant defense, when administered separately, but Orn plus urease treatment decreased GSH levels showing a synergistic effect of these compounds. Considering that endogenous GSH is considered the major naturally-occurring brain

(Halliwell and Gutteridge, 2007), we presume that the rat cortical antioxidant defenses were compromised by *in vivo* administration of Hcit and Orn with high levels of ammonia. Furthermore, it is also conceivable that the reduction of GSH levels may reflect increased reactive species generation elicited by Hcit and also by Orn plus urease treatment. In this context, it is feasible that Orn did not reduce GSH levels itself probably because it induced less reactive species formation compared to Hcit as previously observed (Viegas et al. 2011), but Orn together with high levels of ammonia may better reflect the *in vivo* condition found in HHH syndrome.

At this point we stress that high tissue levels of ammonia has been shown to enhance the production of free radicals in a dose-dependent manner in primary cultures of astrocytes exposed to ammonia (Kosenko et al. 2003; Murthy et al. 2001; Reinehr et al. 2007). Furthermore, increase of superoxide production and decreased activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase were observed in brain of rats injected with ammonia-acetate (Kosenko et al. 1997; Kosenko et al. 2003).

Since oxidative stress results from an imbalance between formation of free radicals and their neutralization by antioxidants in a tissue, our present data strongly indicate that the major metabolites accumulating in HHH syndrome induce oxidative stress *in vivo* in rat cerebral cortex, a deleterious condition that may lead to cell death (Halliwell and Gutteridge, 2007). At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 1996), a fact that makes this tissue more vulnerable to increased reactive species.

It is difficult to determine the pathophysiological relevance of our data since to our knowledge Hcit, Orn and ammonia brain concentrations are not yet established in

HHH syndrome. However, considering that the levels found in plasma for Orn in patients affected by this disorder are around 0.2-1.4 mM (Valle and Simell 2001), our present results may be of relevance to suggest that high concentrations of Orn in the brain are implicated in cerebral damage by altering the cellular redox homeostasis. As regarded to Hcit, there is no report on Hcit levels in plasma, whereas Hcit concentrations in urine from these patients out of crises are around 0.2 mmol per mol creatinine (Korman et al. 2004). Furthermore, it is feasible that higher tissue Hcit and Orn concentrations may take place in stress situations, such occurs during episodes of metabolic decompensation characterized by intense catabolism and proteolysis in which the levels of the accumulating metabolites within neural cells predominate over those found in plasma and cerebrospinal fluid (Hoffmann et al. 1993).

The most interesting findings of the present work were the observations that high levels of ammonia and Orn act synergistically inducing protein oxidative damage and reducing the antioxidant defenses in the brain. Furthermore, hyperammonemia itself was able to cause lipid peroxidation but did not induce protein oxidative damage or decrease GSH levels. It is therefore presumed lipid oxidation seems to be more susceptible to high levels of ammonia at the concentrations achieved in our model.

In conclusion, we demonstrate in this report that hyperammonemia contributes together with Orn inducing protein oxidation and impairing the antioxidant defenses in the brain *in vivo*. We also confirmed previous results from our laboratory showing that Orn and Hcit *per se* disturb brain redox homeostasis. These data may be relevant to explain worsening of neurologic symptoms in some patients with HHH syndrome particularly during episodic crises of metabolic stress in which hyperammonemia is commonly observed.

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Legends to Figures

Figure 1. Effect of intracerebroventricular administration of ornithine (Orn, A) or homocitrulline (Hcit, B) on thiobarbituric acid reactive substances (TBA-RS) levels in the presence or absence of hyperammonemia (urease treatment) in rat cerebral cortex. Data are represented as means ± standard deviation for six to seven independent experiments (animals) per group and expressed as percentage of control (Controls [nmol. mg protein^{-1}] A: 0.562 ± 0.106; B: 0.592 ± 0.035). *P<0.05 and **P<0.01 compared to control (ANOVA followed by Duncan's multiple range test).

Figure 2. Effect of intracerebroventricular administration of ornithine (Orn, A) or homocitrulline (Hcit, B) on carbonyl content in the presence or absence of hyperammonemia (urease treatment) in rat cerebral cortex. Data are represented as means ± standard deviation for five to seven independent experiments (animals) per group and expressed as percentage of control (Controls [nmol. mg protein^{-1}] A: 2.96 ± 0.291; B: 4.61 ± 1.03). *p<0.05 compared to control (ANOVA followed by Duncan's multiple range test).

Figure 3. Effect of intracerebroventricular administration of ornithine (Orn, A) or homocitrulline (Hcit, B) on sulfhydryl oxidation in the presence or absence of hyperammonemia (urease treatment) in rat cerebral cortex. Data are represented as means ± standard deviation for five to seven independent experiments (animals) per group and expressed as percentage of control (Controls [nmol. mg protein^{-1}] A: 110.4 ± 20.9; B: 68.7 ± 12.8). **p<0.01 compared to control (ANOVA followed by Duncan's multiple range test).

Figure 4. Effect of intracerebroventricular administration of ornithine (Orn, A) or homocitrulline (Hcit, B) on glutathione (GSH) levels in the presence or absence of hyperammonemia (urease treatment) in rat cerebral cortex. Data are represented as means ± standard deviation for five to seven independent experiments (animals) per group and expressed as percentage of control. (Controls [nmol. mg protein^{-1}] A: 6.54 ± 1.47; B: 4.19 ± 0.36). *$p<0.05$ compared to control (ANOVA followed by Duncan's multiple range test).

References

Aksenov MY, Markesbery WR (2001) Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302: 141–145. doi: 10.1016/S0304-3940(01)01636-6

Al-Hassnan ZN, Rashed MS, Al-Dirbashi OY, Patay Z, Rahbeeni Z, Abu-Amero KK (2008) Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome with stroke-like imaging presentation: clinical, biochemical and molecular analysis. J Neurol Sci 15 (264): 187-194. doi: 10.1016/j.jns.2007.08.003

Amaral AU, Leipnitz G, Fernandes CG, Seminotti B, Zanatta A, Viegas CM, Dutra-Filho CS, Wajner M (2009) Evidence that the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome induce oxidative stress in brain of young rats. Int J Dev Neurosci 27 635-641. doi: 10.1016/j.ijdevneu.2009.08.004

Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. Methods Mol Biol 108: 347–352.

Butterworth RF, Norenberg MD, Felipo V, Ferenci P, Albrecht J, Blei AT (2009) Experimental models of hepatic encephalopathy: ISHEN guidelines. Liver Int 29(6):783-788. doi: 10.1111/j.1478-3231.2009.02034.x

Camacho JA, Obie C, Biery B, Goodman BK, Hu CA, Almashanu S, Steel G, Casey

R, Lambert M, Mitchell GA, Valle D (1999) Hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. Nat Genet 22: 151-158. doi: 10.1038/9658

Cooper AJ, Plum F (1987) Biochemistry and physiology of brain ammonia. Physiol Rev 67: 440–519.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta. 329: 23–38. doi: 10.1016/S0009-8981(03)00003-2

Diemer NH, Laursen H (1977) Glial cell reactions in rats with hyperammoniemia induced by urease or porto-caval anastomosis. Acta Neurol Scand. 55(6):425-442.

Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi E (2001) Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch Biochem Biophys. 388:261–266. doi:10.1006/abbi.2001.2292

Halliwell B, Gutteridge JMC (1996) Oxygen radicals and nervous system, Trends Neurosci 8: 22–26.

Halliwell B, Gutteridge JMC (2007) Measurement of reactive species. In: Halliwell B, Gutteridge JMC (eds) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 268–340.

Haust MD, Gatfield PD, Gordon BA (1981) Ultrastructure of hepatic mitochondria in a child with hyperornithinemia, hyperammonemia, and homocitrullinuria. Hum Pathol 12: 212-222.

Hoffmann GF, Meier-Augenstein W, Stöckler S, Surtees R, Rating D, Nyhan WL (1993) Physiology and pathophysiology of organic acids in cerebrospinal fluid. J Inherit Metab Dis 16(4):648-669.

Korman SH, Kanazawa N, Abu-Libdeh B, Gutman A, Tsujino S (2004) Hyperornithinemia, hyperammonemia, and homocitrullinuria syndrome with evidence of mitochondrial dysfunction due to a novel SLC25A15 (ORNT1) gene mutation in a Palestinian family. J Neurol Sci 218: 53-58. doi: 10.1016/j.jns.2003.10.017

Kosenko E, Kaminsky Y, Kaminsky A, Valencia M, Lee L, Hermenegildo C, Felipo V (1997) Superoxide production and antioxidant enzymes in ammonia intoxication in rats. Free Radic Res 27(6):637-644.

Kosenko E, Venediktova N, Kaminsky Y, Montoliu C, Felipo V (2003) Sources of oxygen radicals in brain in acute ammonia intoxication in vivo. Brain Res 981(1-2):193-200. doi: 10.1016 /S0006-8993(03)03035-X

Kuhn DM, Aretha CW, Geddes TJ (1999) Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. The Journal of Neuroscience 19: 10289–10294.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent, J Biol Chem 193: 265-275.

Martinez-Hernandez A, Bell KP, Norenberg MD (1977) Glutamine synthetase: Glial localization in brain. Science 195:1356–1358.

Metoki K, Hommes FA, Dyken P, Kelloes C, Trefz J (1984) Ultrastructural changes in fibroblast mitochondria of a patient with HHH-syndrome. J Inherit Metab Dis 7: 147-150.

Murthy CR, Rama Rao KV, Bai G, Norenberg MD (2001) Ammonia-induced production of free radicals in primary cultures of rat astrocytes. J Neurosci Res 66(2):282-288. doi: 10.1002/jnr.1222

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Academic Press.

Reinehr R, Görg B, Becker S, Qvartskhava N, Bidmon HJ, Selbach O, Haas HL, Schliess F, Häussinger D (2007) Hypoosmotic swelling and ammonia increase oxidative stress by NADPH oxidase in cultured astrocytes and vital brain slices. Glia 55(7):758-771. doi: 10.1002/glia.20504

Reznick AZ, Packer L (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods. Enzymol 233: 357–363.

Salvi S, Santorelli FM, Bertini E, Boldrini R, Meli C, Donati A, Burlina AB, Rizzo C, Di Capua M, Fariello G, Dionisi-Vici C (2001) Clinical and molecular findings in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome. Neurology 57: 911-914.

Schultz V, Lowenstein JM (1978) The purine nucleotide cycle. Studies of ammonia production and interconversions of adenine and hypoxanthine nucleotides and nucleosides by rat brain in situ. J Biol Chem 253: 1938–1943.

Tessa A, Fiermonte G, Dionisi-Vici C, Paradies E, Baumgartner MR, Chien YH, Loguercio C, de Baulny HO, Nassogne MC, Schiff M, Deodato F, Parenti G, Rutledge SL, Vilaseca MA, Melone MA, Scarano G, Aldamiz-Echevarría L, Besley G, Walter J, Martinez-Hernandez E, Hernandez JM, Pierri CL, Palmieri F, Santorelli FM (2009) Identification of novel mutations in the SLC25A15 gene in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome: a clinical, molecular, and functional study. Hum Mutat 30(5): 741–748. doi: 10.1002/humu.20930

Valle D, Simell O (2001) The hyperornithinemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds.) The metabolic and Molecular Basis of Inherited Diseases, McGraw-Hill, New York, pp 1857-1896.

Viegas CM, Zanatta A, Knebel LA, Schuck PF, Tonin AM, Ferreira Gda C, Amaral AU, Dutra Filho CS, Wannmacher CM, Wajner M (2009) Experimental evidence that ornithine and homocitrulline disrupt energy metabolism in brain of young rats. Brain Res 1291:102-112. doi: 10.1016/j.brainres.2009.07.021

Viegas CM, Busanello EN, Tonin AM, de Moura AP, Grings M, Ritter L, Schuck PF, Ferreira Gda C, Sitta A, Vargas CR, Wajner M (2011) Dual mechanism of brain damage induced in vivo by the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome. Brain Res 19(1369):235-244. doi: 10.1016/j.brainres.2010.10.112

Yagi K (1998) Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. Methods Mol Biol 108:107–110.

Figure 1

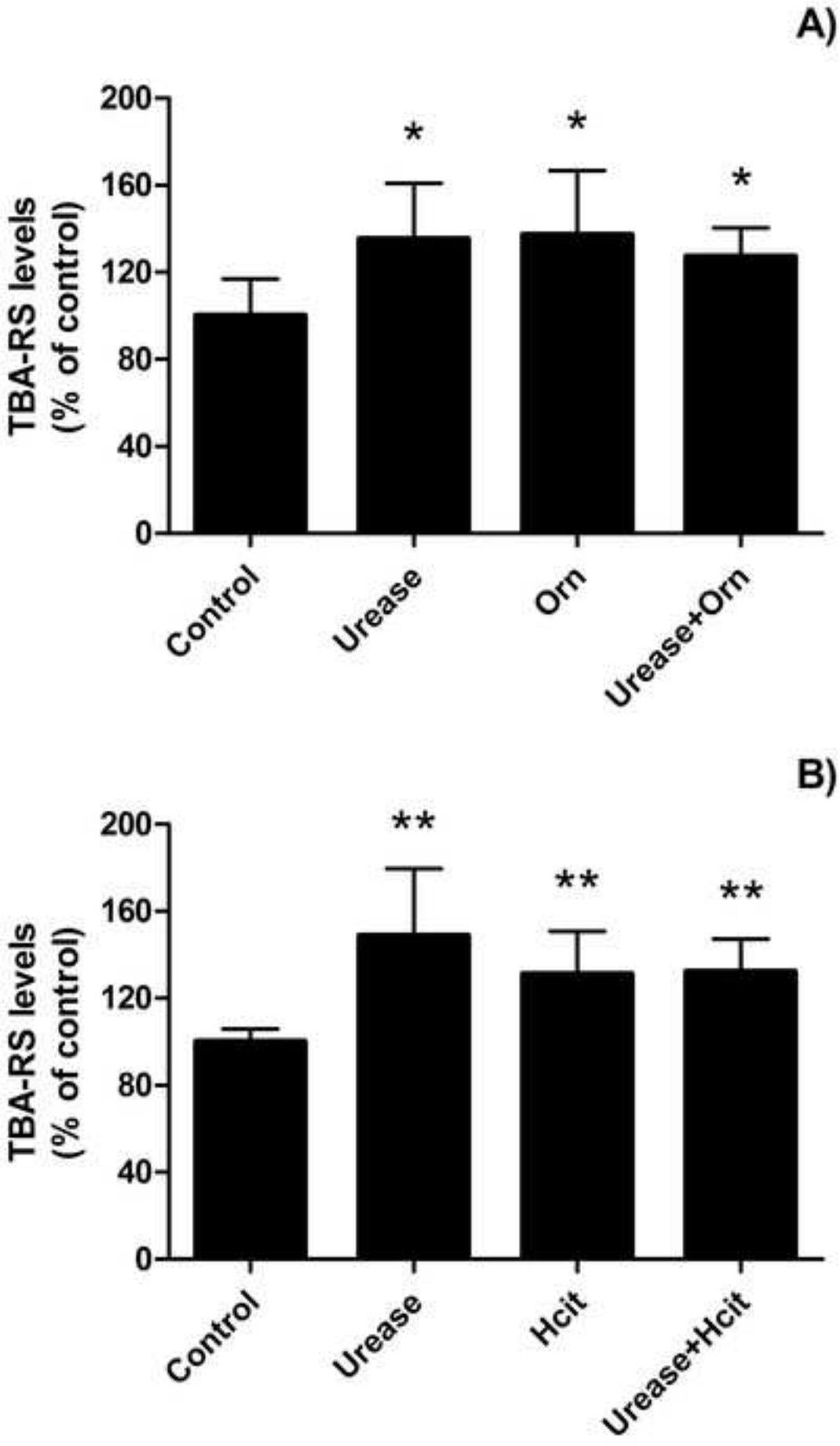
A)
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Figure 2

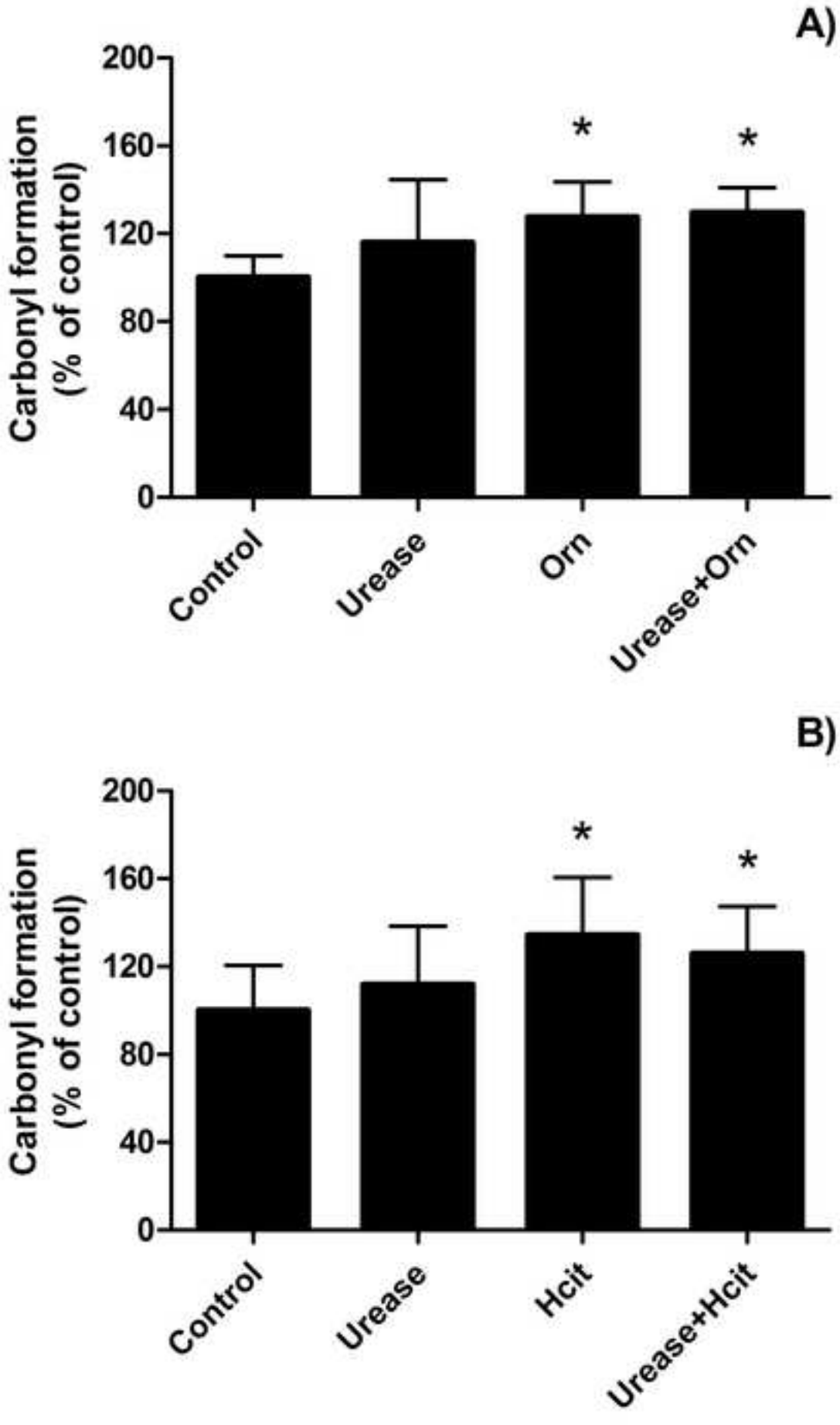
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Figure 3

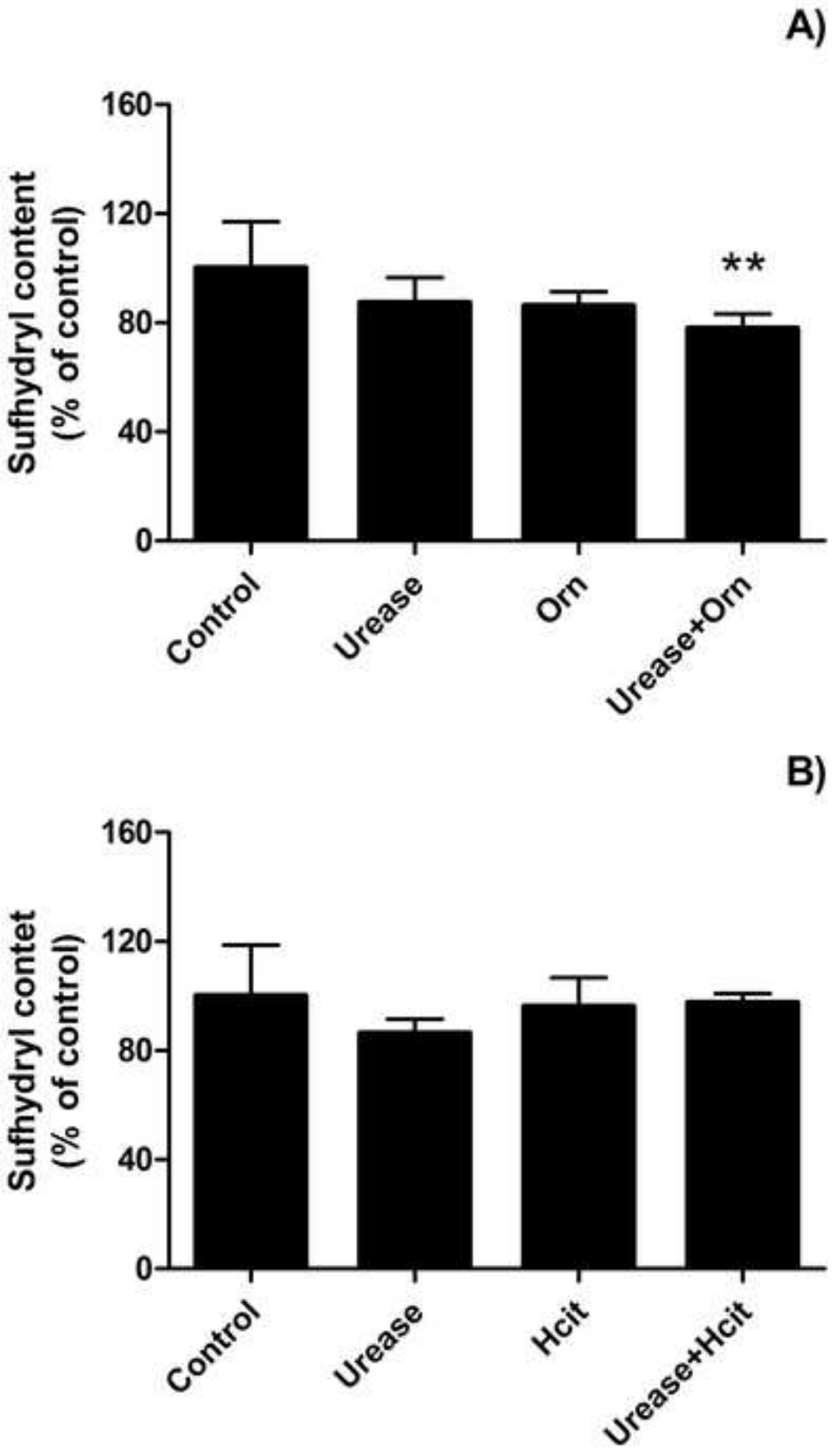
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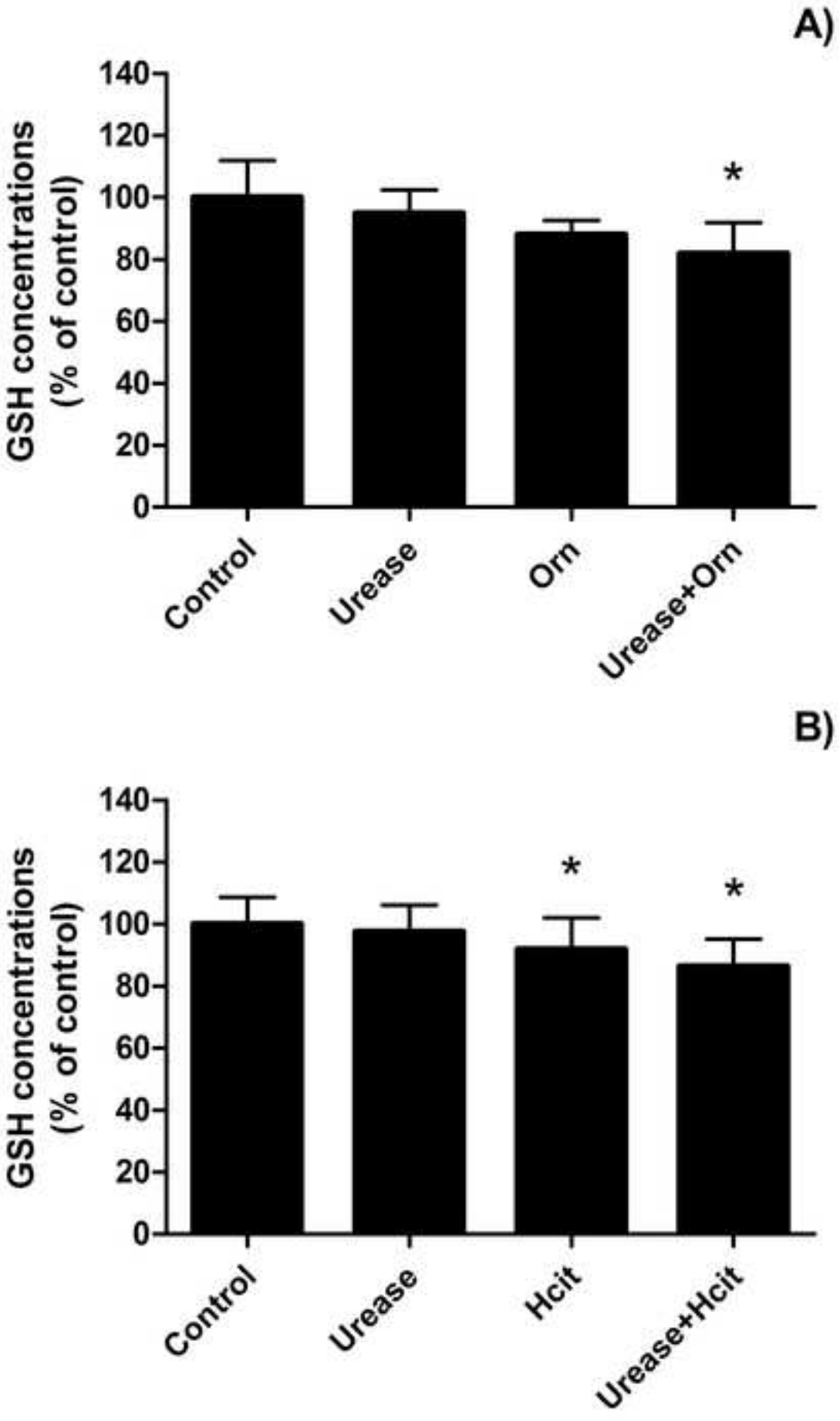
Figure 4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GSH concentrations (% of control)
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Control
Urease
Hcit
Urease+Hcit

Capítulo IV

Chronic postnatal ornithine administration to rats provokes learning deficit in the open field task

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Metabolic Brain Disease

Metabolic Brain Disease

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Chronic postnatal ornithine administration to rats provokes learning deficit in the open field task

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Tables: 4

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Abstract

Hyperornithinemia is the biochemical hallmark of hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome, an inherited metabolic disease clinically characterized by mental retardation whose pathogenesis is still poorly known. In the present work, we produced a chemical animal model of hiperornithinemia induced by a subcutaneous injection of saline-buffered Orn (2-5 μmol/g body weight) to rats. High brain Orn concentrations were achieved, indicating that Orn is permeable to the blood brain barrier. We then investigated the effect of early chronic postnatal administration of Orn on physical development and on the performance of adult rats in the open field and in the Morris water maze tasks. Chronic Orn treatment had no effect on the appearance of coat, eye opening or upper incisor eruption, nor on the free-fall righting reflex and on the adult rat performance in the Morris water maze task, suggesting that physical development and spatial localization were not changed by Orn. However, Orn-treated rats did not habituate to the open field apparatus, implying a deficit of learning/memory. Motor activity was the same for Orn- and saline- injected animals. These data indicate that early chronic Orn administration compromises brain functioning, causing deficit of performance in the open field task that may be possibly associated to the mental retardation observed in patients with HHH syndrome.

Keywords: Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome; ornithine; animal model; pharmacokinetic parameters; open field task

Introduction

High ornithine (Orn) tissue concentrations is the biochemical hallmark of hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM 238970), an autosomal recessive disorder characterized by plasma levels of Orn over 1 mM, besides homocitrullinuria and hyperammonemia (Debray et al. 2008). Although heterogeneous, HHH syndrome is clinically characterized by liver dysfunction and prominent chronic neurological features including psychomotor/mental retardation, hypotonia, peripheral neuropathy and spastic paraplegia or acute encephalopathy with generalized or myotonic convulsions. The basic defect of this condition is a mutation in the gene that encodes the mitochondrial Orn transporter *ORNT1* (SLC25A15), which is highly expressed in the liver (Fiermonte et al. 2003). A common clinical manifestation of patients with the HHH syndrome is cognitive impairment, which may be secondary to the brain damage induced by the accumulating metabolites that include elevated levels of Orn, homocitrulline (Hcit) and ammonia (Valle and Simell 2001). The characteristic neuropathological features include mild cortical atrophy and severe atrophy in the subcortical white matter (Debray et al. 2008).

The mechanisms of central nervous system (CNS) impairment in this disease are still poorly known (Salvi et al. 2001; Palmieri 2008). Although it has been hypothesized that the neurologic damage presented by the patients may be secondary to episodic hyperammonemia during crises, a large cohort study with 16 affected patients revealed that hyperammonemia is mild and asymptomatic (Debray et al. 2008). We have previously found that Orn elicits *in vitro* (Amaral et al. 2009) and *in vivo* (Viegas et al. 2011) lipid peroxidation, protein oxidative damage and a decrease of reduced glutathione (GSH) levels, apart from disrupting energy metabolism in brain of young rats (Viegas et al. 2009). These data support the hypothesis that Orn provokes brain

damage that may contribute to the neurological symptoms and brain abnormalities seen in these patients, especially during crises of metabolic decompensation, in which the concentrations of this metabolite dramatically increase. Considering that deficit of memory is associated with mitochondrial dysfunction and oxidative stress (Bickford et al. 1999; Serrano and Klann 2004; Silva et al. 2004) it seems therefore justifiable to investigate the role of high brain concentrations of Orn on animal performance in behavioral tasks that evaluate learning/memory.

Therefore, the objective of the present study was to develop a chemically-induced model of hyperornithinemia in rats and to investigate the effect of sustained high concentrations of Orn on rat development and behavior. It was not the purpose of this investigation to mimic the genetic HHH syndrome because other metabolites accumulate in this disease. This model was applied during early postnatal development (5^{th} to the 28^{th} day of life) and the cognitive performance in the open field and in the Morris water maze tasks was measured in adult rats 30 days after the last injection in order to test whether chronic Orn administration to infantile rats could provoke developmental delay and long-standing or permanent brain damage involved in learning/ memory.

Experimental procedures

Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ornithine was dissolved in saline solution (NaCl 0.9%) pH 7.4.

Animals

Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used in the experiments. A total of 63 Wistar rats from our stock were used. Pregnant rats were housed in individual cages and left undisturbed throughout gestation. Twenty-four hours after delivery the litters were culled to eight pups, consisting of only males. Half of them were assigned to the experimental condition and the other half served as controls. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Acute ornithine (Orn) treatment and quantification

Seven-, 14- and 22-day-old rats were subcutaneously administered with a single injection of Orn (2, 4 and 5 μmol/g body weight, respectively) buffered with HCl to pH 7.4 and killed by decapitation 30, 60, 120 or 240 min after injection. Pharmacokinetic parameters, such as apparent volume of distribution (V_d), plasma half-time ($t_{1/2}$) and

plasma clearance (CLp), were calculated from plasma Orn levels. The animals were anesthetized with halothane and had their blood collected by cardiac puncture. The blood was transferred to heparinized tubes and processed for plasma separation. Immediately thereafter, the animals were sacrificed by decapitation and had their brain rapidly removed. Cerebrum was dissected on an inverted Petri dish placed on ice and homogenized in 5 volumes (1:5, w/v) of saline solution (0.9% NaCl). Plasma samples and brain homogenates were centrifuged at 400 x g for 3 min and 500 x g for 10 min, respectively. The obtained supernatants were carefully removed for Orn determination. Orn content was determined by high performance liquid chromatography (HPLC) using homocysteic acid as the internal standard (Joseph and Marsden 1986).

Chronic ornithine (Orn) treatment

Saline-buffered Orn, pH 7.4 (2-5 µmol.g of body weight^{-1}), was administered subcutaneously, twice a day, from the 5th to the 28th day of life. Two µmol.g of body weight^{-1} were administered to rats aged 5-12 days, 4 µmol.g of body weight^{-1} to animals of 13-18 days of life and 5 µmol.g of body weight^{-1} to rat aged 19-28 days. Control animals received saline subcutaneously in the same volumes and frequency. Solutions were freshly prepared and each animal received 10 µL solution. g of body weight^{-1}.

Physical development

Eighteen male rats were used for the physical and reflex development studies. Maturation of physical characteristics was determined daily at the appropriate ages by one experimenter that was not aware of the subject condition. Litters were inspected between 8 a.m. and 9 a.m., and progress of physical development was followed

throughout the experiment. The date of appearance of coat, eruption of upper incisors and eye opening, as well as the free fall righting reflex were recorded using previously reported criteria (da Silva et al. 1990; Mello et al. 1994) described in detail by Smart and Dobbing (1971).

Free-fall righting task

On postnatal day 14, we observed the animal ability to turn in midair to land on all four paws after being dropped back downwards from 35 cm onto a cotton wool pad. Each animal was tested in three consecutive trials with 15 s intervals, and scored one point if it landed ventrally, with all legs distant from the body in each trial (da Silva et al. 1990). Therefore, a maximum of three points could be assigned to each animal.

Behavioral studies

After chronic administration of saline and Orn was completed, a subset of male animals was left undisturbed in their cages up to 60 days of age. These animals were consecutively subjected to the open-field and Morris water maze tasks.

Open field task

The apparatus consisted of a wooden box measuring 60 cm × 40 cm × 50 cm with a glass front wall, whose floor was divided by black lines into 12 equal squares. The animals were gently placed facing the rear left corner of the arena and the number of rearings, crossings (squares crossed with the four paws) and grooming responses, as well as the number of fecal boli and the time spent in the central area of the apparatus were recorded for 5 min to evaluate habituation, emotionality and motor activity (Walsh et al. 1976). The testing room was dimly illuminated with indirect white lighting. The

rats' ability to habituate to a new environment was assessed by subjecting the animals to two consecutive 5-min sessions (training and testing) spaced 24 hours (Ferreira et al. 2008).

Morris water maze task

The experimental procedure was carried out as previously described (Pettenuzzo et al. 2002). Spatial learning/memory was tested in the Morris water maze (Morris et al. 1982; Netto et al. 1993), 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). A transparent escape platform (10 cm in diameter) was placed 2 cm below the water surface located in the centre of one of quadrants and remained there throughout the seven days of testing. 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, on each trial. 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 hanged on the walls to provide extra-maze clues so allow rats to develop a spatial map strategy.

Reference memory test

Rats had daily sessions of 4 trials per day for 7 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 to search for the platform during 60 s. 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 it 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 (Warren and Juraska 2000). One day after the last training session (8^{th} day), each rat was subjected to a probe trial in which there was no platform present. The time spent in the quadrant of the former platform position, the time spent in the opposite quadrant, and the latency to cross over the platform place for the first time were taken as a measure for spatial memory. The swimming speed was also evaluated as a measure of motor performance. Each animal has 60 seconds to swim in the pool without platform. The test results were analyzed using the Any Maze software.

Statistical analysis

The Morris water maze results were analyzed by repeated measures ANOVA considering the factors: Orn-treatment x days of training and by the Student *t* test in the probe trial on the test day. The open field obtained data were analyzed by repeated measures ANOVA considering the factors: Orn-treatment x session day or the Student *t* test as described in the results session. Only *F* and *t* significant values are shown in the text. Data are expressed as mean ± S.E.M. $P< 0.05$ was considered to be significant.

Results

Pharmacokinetic parameters

Table I shows plasma Orn levels measured in Wistar rats aged 7, 14 and 22 days after a single injection of the metabolite. It can be seen that the maximal concentrations of the amino acid achieved in blood were approximately 1.4 mM depending on animal age and time after injection. These levels, similar to those found in the human condition (around 1 mM), were approximately 15- to 30-fold higher than the basal levels and were achieved 30 min after injection, decreasing afterwards to nearly normal levels after 4 hours. We then calculated plasma Orn half-life ($t_{1/2}$), apparent distribution volume (V_d) and plasmatic clearance (CLp) (Table I). It is apparent from the table that plasma $t_{1/2}$ of Orn decreased, whereas V_d and CLp increased with age, indicating an increased metabolism (utilization) and/or excretion of this amino acid in older animals.

Cerebral Orn concentrations obtained after a single systemic injection of the metabolite are depicted in Table II. Brain Orn levels in treated animals were approximately 4- to 6-fold higher than the basal levels. The highest brain Orn concentration was achieved 30-60 min after injection (278 µM), indicating that Orn is able to cross the blood--brain barrier (BBB) possibly through the y^+ system that transports cationic amino acid (lysine, arginine and Orn) across the BBB (O'Kane et al. 2006). We cannot rule out that Orn also penetrates into the brain via the y^+L-transport system, a broad scope amino acid transporter (Devés et al. 1998).

Neurodevelopmental and behavioral studies

We initially observed that body weight of rats submitted to chronic Orn-treatment was similar to that of control rats (data not shown) indicating that chronic postnatal administration of Orn does not alter their appetite or provoke malnutrition. We

also assessed the effect of chronic Orn administration on neuromotor development by assessing the day of appearance of some physical landmarks and reflexes. In this context, we did not observe any delay in the day of appearance of coat, eye opening and upper incisor eruption, neither impairment of the free-fall righting reflex in Orn-treated rats (data not shown). These data indicate that chronic Orn administration does not cause delay in the onset of some physical parameters and impairment of the free-fall righting reflex, reflecting a normal physical and neuromotor development.

Figure 1 shows the effect of Orn treatment on the number of rearing responses in the open field task. Repeated measures ANOVA analysis of rearing scores revealed an interaction between group x session day ($F_{(1,16)}$= 9.83; $P < 0.05$), which indicates a deficit in the habituation to a novel environment in rats injected with Orn since this group did not reduce the number of rearing responses at testing session.

The effect of chronic early Orn administration on the number of crossing responses (motor activity), *fecal boli*, grooming responses and time spent in the central area in the open field task during the training is depicted in table III. Statistical analysis showed no significant differences between saline- and Orn-treated animals in these parameters at training and testing sessions, indicating, that Orn did not influence motor activity and emotionality of the animals.

Regarding to Morris water maze task, we observed a decrease in the latency to find the platform during acquisition phase [$F_{(6,84)}$= 7.5; $p<0.01$] in both animal groups (saline and Orn). Furthermore, there was no interaction effect between days of treatment in both saline and Orn treatment and in the learning curve between groups (Figure 2). In addition, by analyzing the probe trial on day 8, with the platform removed, we observed that Orn-treated rats were able to remember the location of the platform, since they had the same latency to find the platform, spent equivalent time in the platform quadrant and

in the opposite quadrant, presented similar number of entries in the platform quadrant. Orn also did not provoke motor deficits, since Orn-treated animals had the same swimming speed than saline-injected animals (Table IV).

Discussion

Patients affected by HHH syndrome commonly have neurological dysfunction, including mental retardation, whose neuropathology is poorly known (Valle and Simell 2001; Shih et al.1969). In this context, we have recently reported that Orn, the metabolite that most accumulates in HHH syndrome, provokes *in vitro* and *in vivo* mitochondrial dysfunction and elicits oxidative stress in rat brain (Amaral et al. 2009; Viegas et al. 2009; Viegas et al. 2011). However, to our knowledge, nothing has been done to investigate whether Orn could provoke alterations in animal behavioral. Therefore, in the present study we first produced high levels of Orn in the brain and plasma of young rats, comparable to those found in patients affected by HHH syndrome by subcutaneous injection of Orn (Valle and Simell 2001). Considering that the two main complications commonly observed in HHH syndrome despite apparently good metabolic control are cognitive impairment and progressive motor disabilities, we used this chemically-induced model of hyperornithinemia to investigate whether high persistent levels of Orn during a period (5^{th} to 28^{th} day of life) of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory (Roisen et al. 1981; Dreyfus et al. 1984; Dutra et al. 1993) could alter the physical and neuromotor development, as well as the performance of adult (60-day-old) rats in the open field and in the Morris water maze tasks.

The highest plasma Orn level achieved in our *in vivo* model (around 1400 µM) occurred 30 min after Orn acute administration, gradually decreasing along time.

Maximal brain Orn concentrations (280 µM) occurred 30-60 min after injection and decreased afterwards to basal values 240 min after Orn administration. These brain concentrations were 4 to 5-fold higher than basal levels, indicating that Orn is able to cross the BBB, which probably occurred through the y^+ system (O'Kane et al. 2006). It was also found that Orn $t_{1/2}$ decreased and CLp and V_d increased with animal age. Therefore, it might be presumed that Orn produced in the liver penetrates into the brain by the y^+ system and possibly exerts its deleterious actions especially during early stages of development when the brain is more vulnerable to hyperornithinemia. Although this model does not exactly mimic HHH syndrome, it reproduces an important biochemical feature of this disease, *i.e.*, elevated tissue levels of Orn.

We thereafter injected Orn twice a day in the animas from 5^{th} to the 28^{th} day of life. The physical development and the free-fall righting reflex of the animals were analyzed during treatment. They were then allowed to recover for 30-40 days before being submitted to behavioral tasks since behavior analysis performed during or shortly after chronic treatment are difficult to interpret (Andersen et al. 1972).

Chronic Orn administration had no effect on body weight, suggesting that Orn injection did not cause malnutrition in the animals. This observation is important since malnourished animals may behave differently in neurobehavioral tests (Smart and Dobbing 1971; Davis and Squire 1984). Therefore, this undesirable effect can be ruled out as for the interpretation of behavioral alterations observed in rats chronically treated with Orn. Orn administration did not also change the date of appearance of coat, eye opening or upper incisor eruption, nor the free-fall righting task. It is concluded that physical development of animals and the free-fall righting reflex were not modified by high tissue sustained levels of Orn.

Regarding to the animal performance in the behavioral studies, we observed that animals treated with Orn presented no habituation as revealed by the lack of reduction of rearing responses along the two sessions of the open field task, whereas the controls (saline injected animals) showed normal habituation. In the open field task, rats present an exploratory behavior consisting of ambulation and rearing responses when first exposed to the box (training session). Memory retention or habituation to a novel environmental can be measured by reduction of rearing responses along sessions in this task, in which the hippocampus and amygdala play a crucial role (Denenberg 1969; Netto et al.1986; Rodrigues et al. 1996). Since a reduction of number of rearing responses along sessions in the open field habituation can be interpreted as the animals recognize the environment and remember the previous exposure to this apparatus (Denenberg 1969; Rodrigues et al. 1996; Izquierdo 1994), and since in the present study Orn-treated animal did not show this pattern, it is assumed that this reflects a learning deficit caused by this amino acid high levels.

Alterations of motor activity or emotionality probably did not contribute to the deficit of habituation caused by Orn since both groups of animals behaved similarly as regards to the number of crossing responses (locomotor activity), as well as the *fecal boli* and grooming responses and the time spent in the central area, which seems to be primarily related to the dimension of emotional reactivity (Bolles 1960; Denenberg 1969; Kametani 1988). These data therefore indicate that Orn treatment is not anxiogenic, neither alters motor activity.

We also observed that saline- and Orn-treated rats had the same performance in the acquisition phase of the water maze task, i.e., decreased the latency to find the platform from the first to the last day of training. Furthermore, both groups stayed for the same time in the quadrant where the platform was formerly located and in the

opposite quadrant along the test session. Orn-treated animals also had a similar number of annulus crossings and presented the same latency to cross over the platform position and swimming speed than saline-treated animals. These data suggest that early Orn treatment did not alter adult rat spatial learning. The findings on swimming speed reinforce the conclusion that Orn treatment does not alter motor activity.

The exact mechanisms through which Orn impairs rat habituation (memory) are still unknown. However, it is possible that the brain Orn concentrations achieved in our chemical model may be high enough to induce brain damage during a phase of rapid CNS maturation and particularly in cerebral structures involved in learning, memory and interaction with the environment. In this context, it should be stressed that oxidative stress is associated with memory deficits (Bickford et al. 1999; Serrano and Klann 2004; da Silva et al. 2004) and energetic substrates such as creatine and succinate are capable to prevent learning/ memory deficit (Vasques et al. 2006). Since Orn has been shown to elicit oxidative stress (Amaral et al. 2009; Viegas et al. 2011) and compromise brain energy metabolism (Viegas et al. 2009; 2011), it could be presumed that Orn may act in one or more of these processes, leading to learning/memory impairment.

In conclusion, the present work shows for the first time that high sustained brain concentrations of Orn during early development impair biochemical pathways involved with long-standing learning/memory processes in habituation to a novel environment. Even though it is difficult to extrapolate our findings to the human condition, the results of the present study indicate that Orn may lead to learning/memory deficits. We cannot also rule out the possibility that homocitrulilne that also accumulates in this disorder may exert neurotoxic effects affecting learning/memory, but this should be investigated in further studies. Our present findings are particularly interesting in view of the psychomotor delay/mental retardation characteristic of patients affected by HHH

syndrome that have been so far mainly attributed to hyperammonemia. The present work, allied to previous studies showing that Orn is able to provoked mitochondrial dysfunction and oxidative stress *in vitro* and *in vivo*, indicate that high brain Orn levels may also be involved in the neurological dysfunction of HHH syndrome.

Legends to Figures

Figure 1. Effect of chronic early postnatal ornithine (Orn) administration on number of rearing. Results are expressed as means ± S.E.M. for 6-8 animals per group (control and Orn). $*P < 0.05$; interaction between group x session day (repeated measures ANOVA); $^{\#}P < 0.05$; compared to Orn group at testing day (Student *t*-test).

Figure 2. Effect of chronic early postnatal ornithine (Orn) administration on acquisition learning in the Morris water maze task. Data represent means ± S.E.M of latency to find the platform of four trials on each day (7 days) for 6 - 8 animals per group (control and Orn). $*P < 0.05$; significant reductions of latency to find the platform was achieved for saline- and Orn-treated animals from day 2 to day 7 of training compared to day 1 (Student *t*-test).

References

Amaral AU, Leipnitz G, Fernandes CG, Seminotti B, Zanatta A, Viegas CM, Dutra-Filho CS, Wajner M (2009) Evidence that the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome induce oxidative stress in brain of young rats. Int J Dev Neurosci 27 635-641. doi: 10.1016/j.ijdevneu.2009.08.004

Andersen AE, Guroff G (1972) Enduring behavioral changes in rats with experimental phenylketonuria. Proc Natl Acad Sci U S A 69(4):863-867.

Bickford PC, Shukitt-Hale B, Joseph J (1999) Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. Mech Ageing Dev 111:141-154.

Bolles RC (1960) Grooming behavior in the rat. J Comp Physiol Psychol 53, 306-310.

da Silva AL, Piato AL, Bardini S, Netto CA, Nunes DS, Elisabetsky E (2004) Memory retrieval improvement by Ptychopetalum olacoides in young and aging mice. J Ethnopharmacol 95(2–3):199-203. doi:10.1016/j.jep.2004.07.019

da Silva VA, Malheiros LR, Bueno FMR (1990) Effect of toluene exposure during gestation on neurobehavioral development of rats and hamsters. Braz J Med Biol Res 23:533-537.

Davis PH, Squire LR (1984) Protein synthesis and memory: a review. Psychol Bull 96:518–559.

Debray FG, Lambert M, Lemieux B, Soucy JF, Drouin R, Fenyves D, Dubé J, Maranda B, Laframboise R, Mitchell GA (2008) Phenotypic variability among patients with hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome homozygous for the delF188 mutation in SLC25A15. J Med Genet 45(11):759-764. doi:10.1136/jmg.2008.059097

Denenberg VH (1969) Open-field bheavior in the rat: what does it mean? Ann N Y Acad Sci 159(3):852-859.

Devés R, Angelo S, Rojas AM (1998) System y L: the broad scope and cation modulated amino acid transporter. Exp Physiol 83: 211-220.

Dreyfus H, Ferret B, Harth S, Giorgio A, Durand M, Freysz L, et al. (1984) Metabolism and function of gangliosides in developing neurons. J Neurosci Res 12:311-322.

Dutra JC, Wajner M, Wannmacher CMD, Dutra-Filho CS, Rache E (1993) Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. J Inherited Metab Dis 16:147-153.

Ferreira GC, Schuck PF, Viegas CM, Tonin A, Ribeiro CA, Pettenuzzo LF, Pereira LO, Netto CA, Wajner M (2008) Chronic early postnatal glutaric acid administration causes

cognitive deficits in the water maze. Behav Brain Res 187(2):411-416. doi:10.1016/j.bbr.2007.10.003

Fiermonte G, Dolce V, David L, Santorelli FM, Dionisi-Vici C, Palmieri F, Walker JE (2003) The mitochondrial ornithine transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. J Biol Chem 278(35):32778-32783.

Izquierdo I. Pharmacological evidence for a role of long-term potentiation in memory. FASEB J 1994;8:1139-1145.

Joseph MH, Marsden CA (1986) Amino acids and small peptides. In: Lim CK (ed) HPLC of small peptides, Oxford: IRL Press, pp 13-27.

Kametani H (1988) Analysis of age related in stress-induced grooming in the rat. Ann N Y Acad Sci 525, 101-113.

Mello CF, Somer JE, Tavaroni V, Graciolli R,Wu V, Torres G, et al. (1994) Effects of postnatal methylmalonate administration on neurobehavioral development of rats. Braz J Med Biol Res 27(3):655-661.

Morris RGM, Garrud P, Rawlins JNP, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681-683.

Netto CA, Dias RD, Izquierdo I (1986) Differential effect of posttraining naloxone, beta-endorphin, leu-enkephalin and electroconvulsive shock administration upon

memory of an open-field habituation and of a water-finding task. Psychoneuroendocrinology 11(4):437-446

Netto CA, Hodges H, Sinden JD, Le Peillet E, Kershaw T, Sowinski P, Meldrum BS, Gray JA (1993) Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. Neuroscience 54:69-92.

O'Kane RL, Viña JR, Simpson I, Zaragozá R, Mokashi A, Hawkins RA (2006) Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y+. Am J Physiol Endocrinol Metab 291(2):412-419. doi:10.1152/ajpendo.00007.2006

Palmieri F (2008) Diseases caused by defects of mitochondrial carriers: a review. Biochim Biophys Acta.1777(7-8):564-78. doi:10.1016/j.bbabio.2008.03.008

Pettenuzzo LF, Schuck PF, Fontella F, Wannmacher CMD, Wyse AT, Dutra- Filho CS, Netto CA, Wajner M (2002) Ascorbic acid prevents cognitive deficits caused by chronic administration of propionic acid to rats in the water maze. Pharmacol Biochem Behav 73:623-629.

Rodrigues AL, Rocha JB, Mello CF, Souza DO (1996) Effect of perinatal lead exposure on rat behaviour in open-field and two-way avoidance tasks. Pharmacol Toxicol 79:150-156.

Roisen FJ, Bartfeld H, Nagele R, Yorke G (1981) Gangliosides stimulation of axonal sprouting in vivo. Science 214:577-578.

Salvi S, Santorelli FM, Bertini E, Boldrini R, Meli C, Donati A, Burlina AB, Rizzo C, Di Capua M, Fariello G, Dionisi-Vici C (2001) Clinical and molecular findings in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome. Neurology 57: 911-914.

Serrano F, Klann E (2004) Reactive oxygen species and synaptic plasticity in the aging hippocampus. Aging Res Ver 3(4):431-443.

Shih VE, Efron ML, Moser HW (1969) Hyperornithinemia, hyperammonemia, and homocitrullinuria. A new disorder of amino acid metabolism associated with myoclonic seizures and mental retardation. Am J Dis Child 117(1):83-92.

Smart JL, Dobbing J (1971) Vulnerability of developing brain. II. Effect of early nutritional deprivation on reflex ontogeny and development of behavior in the rat. Brain Res 28:85-95.

Valle D, Simell O (2001) The hyperornithinemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds.) The metabolic and Molecular Basis of Inherited Diseases, McGraw-Hill, New York, pp 1857-1896.

Vasques V, Brinco F, Viegas CM, Wajner M (2006) Creatine prevents behavioral alterations caused by methylmalonic acid administration into the hippocampus of rats in the open field task. J Neurol Sci 244(1-2):23-29. doi:10.1016/j.jns.2005.12.005

Viegas CM, Busanello EN, Tonin AM, de Moura AP, Grings M, Ritter L, Schuck PF, Ferreira Gda C, Sitta A, Vargas CR, Wajner M (2011) Dual mechanism of brain damage induced in vivo by the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome. Brain Res 19(1369):235-244. doi: 10.1016/j.brainres.2010.10.112

Viegas CM, Zanatta A, Knebel LA, Schuck PF, Tonin AM, Ferreira Gda C, Amaral AU, Dutra Filho CS, Wannmacher CM, Wajner M (2009) Experimental evidence that ornithine and homocitrulline disrupt energy metabolism in brain of young rats. Brain Res 1291:102-112. doi: 10.1016/j.brainres.2009.07.021

Walsh RN, Cummins RA (1976) The open-field test: a critical review. Psychol Bull 83:482-504.

Warren SG, Juraska JM (2000) Sex differences and estropausal phase effects on water maze performance in aged rats. Neurol Learn Mem 74:229-240.

Table I

Table I. Plasma ornithine (Orn) levels (µM) and pharmacokinetic parameters in rats aged 7, 14 and 22 days after a single Orn subcutaneous administration

Age (days)	Dose (µmol.g^{-1})	Time after injection					Pharmacokinetic parameters		
		control	30 min	60 min	120 min	240 min	$t_{1/2}$ (min)	CLp (mL.min^{-1}.Kg^{-1})	V_d (L.Kg^{-1})
7	2	28.3	854.0	544.0	109.2	66.1	46.43	0.033	2.40
14	4	57.7	1428.3	864.5	533.7	86.3	37.85	0.032	2.47
22	5	77.1	1168.5	676.6	99.8	65.5	34.69	0.073	4.03

For plasma levels, data represent median of 3 or 5 independent experiments (animals) and are expressed as µM. For pharmacokinetic parameters, data are expressed as median of 3 or 5 independent experiments (animals). $t_{1/2}$= plasma Orn half-life; V_d= apparent distribution volume; CLp= plasma clearance.

Table II. Cerebral ornitine (Orn) concentrations (µM) in rats aged 7, 14 and 22 days after a single Orn subcutaneous administration

Age (days)	Dose ($\mu mol.g^{-1}$)	Time after injection				
		control	30 min	60 min	120 min	240 min
7	2	52.8	169.6	153.9	127.5	69.3
14	4	45.2	278.0	218.0	69.3	49.3
22	5	26.4	49.5	140.5	91.2	71.5

Data represent median of 3 or 5 independent experiments (animals) and are expressed as µM.

Table III. Effect of postnatal chronic ornithine (Orn) administration on time spent in the central area (seconds), as well as on number of crossings, groomings and *fecal boli* in the open field task in adult rats at the training session

	Saline	Orn
Time spent in the central area (s)	5.71 ± 1.61	4.70 ± 1.65
Number of groomings	2.85 ± 0.89	3.70 ± 0.97
Number of crossing	52.4 ± 6.15	51.4 ± 5.40
Number of *fecal boli*	6.00 ± 0.57	4.90 ± 0.64

Data are reported as mean ± SEM. Rats received Orn subcutaneously from day 5 to day 28 of life. No significant difference was found between groups (Student's t-test for independent samples).

Table IV. Effect of early postnatal chronic ornithine (Orn) administration on the performance of adult rats in the Morris water maze task (probe trial)

Treatment	Saline	Orn
Latency to find the platform (s)	20.5 ± 10.2	21.7 ± 11.6
Number of platform quadrant entries	3.50 ± 0.65	3.40 ± 0.34
Time spent in the platform quadrant (s)	20.87 ± 2.45	19.54 ± 2.92
Time spent in the opposite quadrant (s)	8.70 ± 2.48	9.11 ± 2.22
Swimming speed (m/s)	0.193 ± 0.023	0.184 ± 0.013

Latency to find the platform, number of platform quadrant entries, time spent in the platform quadrant, time spent in the opposite quadrant and swimming speed are shown. Rats received Orn subcutaneously from day 5 to day 28 of life. Data represent means ± S.E.M. for 6-8 animals per group (control and Orn). Results are expressed in seconds (s) or in meters per second (m/s). No significant difference was found between groups (Student's *t*-test for independent samples).

Figure 1

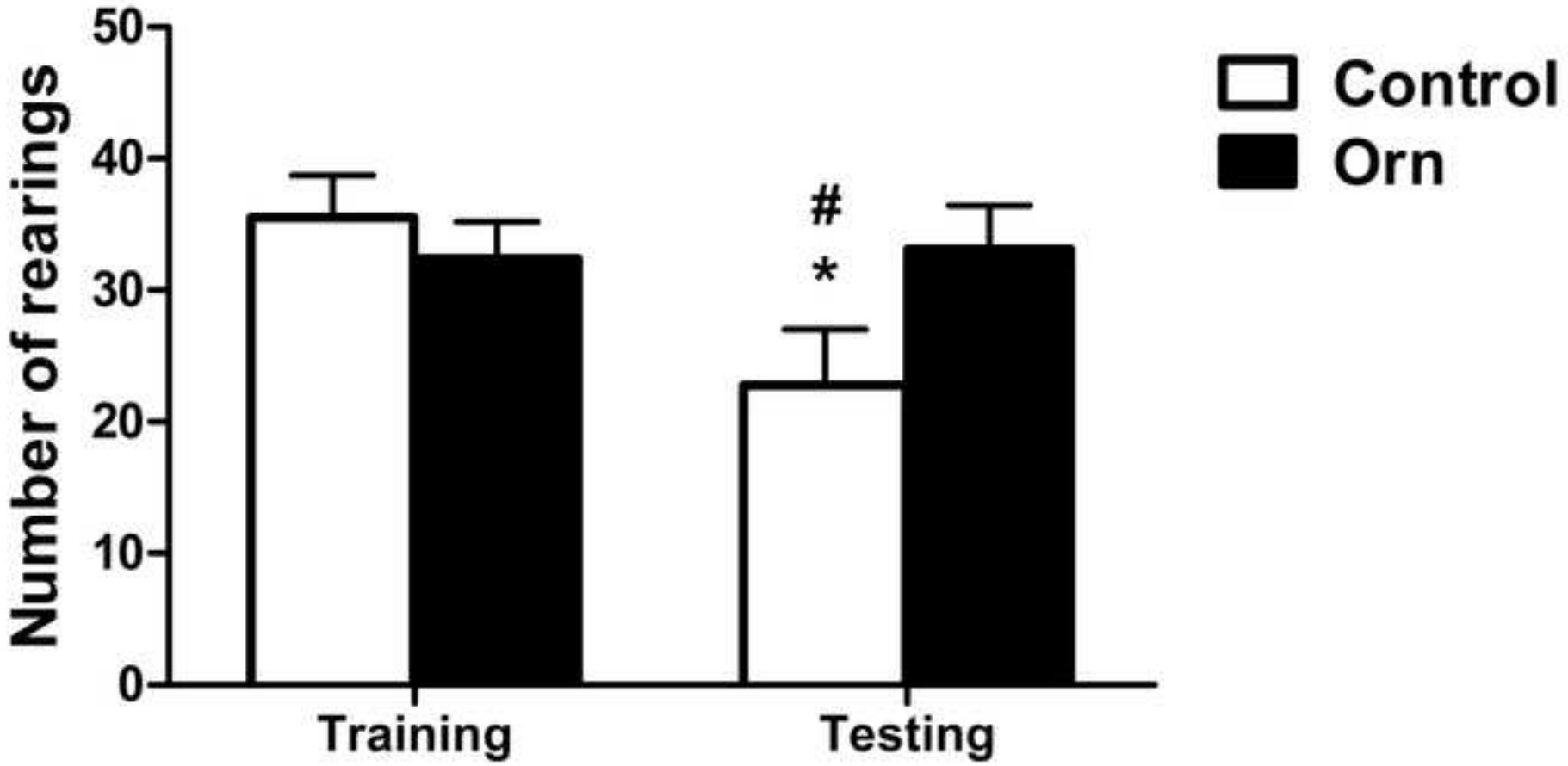
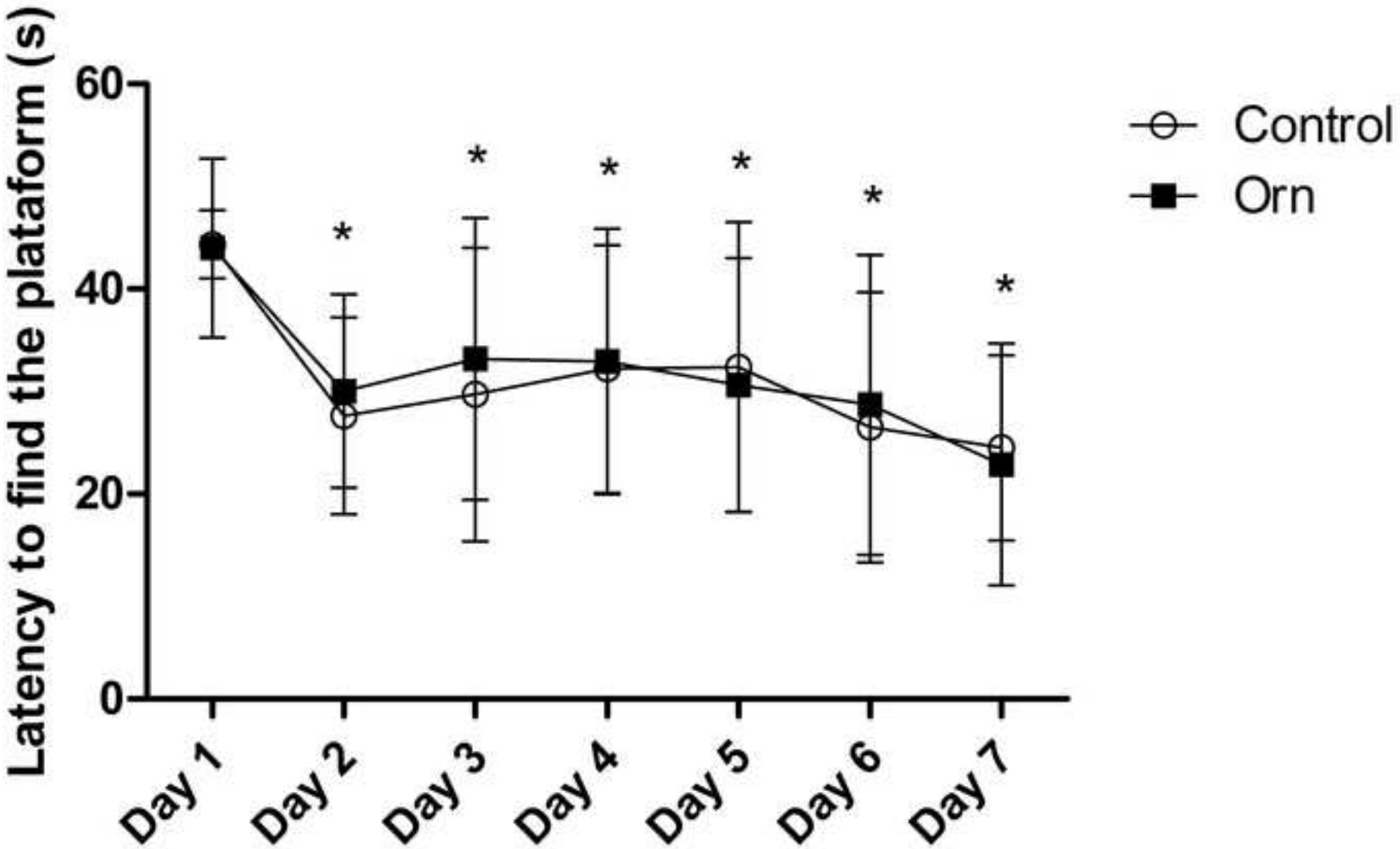

Number of rearings
50
40
30
20
10
0
Training
Testing
#
*
Control
Orn

Figure 2


Latency to find the plataform (s)
60
40
20
0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
*
*
*
*
*
Control
Orn

PARTE III

DISCUSSÃO E CONCLUSÕES

III.1 DISCUSSÃO

Pacientes afetados pela síndrome HHH apresentam disfunção neurológica, caracterizada por encefalopatia aguda, ataxia, coreoatetose, atraso no desenvolvimento, espasticidade muscular severa e retardo mental cuja fisiopatologia é pouco compreendida (Shih *et al*., 1969; Valle e Simell, 2001). Apesar de a hiperamonemia ter sido associada à encefalopatia, alguns pacientes com dano grave no SNC apresentam níveis plasmáticos de amônia normais, de tal forma que a piora dos sintomas neurológicos não parece estar somente associada à hiperamonemia (Salvi *et al*., 2001; Korman *et al*., 2004). Portanto, é possível que outros fatores, como o acúmulo de ornitina (Orn), homocitrulina (Hcit) e/ou ácido orótico (Oro) possam exercer um papel crítico na patogênese desta doença. O aumento plasmático de Orn diferencia a síndrome HHH das desordens clássicas do ciclo da ureia, sendo que a hiperamonemia e a homocitrulinúria distinguem esta doença da atrofia girata cujos pacientes apresentam apenas hiperornitinemia e na qual o principal sinal é a degeneração coreoretinal (Fukuda *et al*., 1983; Javadzadeh e Gharabaghi, 2007).

Muitos indivíduos com a síndrome HHH apresentam anormalidades mitocondriais estruturais, bem como acúmulo e excreção elevada de ácido lático e de intermediários do ciclo dos ácidos tricarboxílicos (Gatfield *et al*., 1975; Haust *et al*., 1981; Metoki *et al*., 1984; Salvi *et al*., 2001), indicando uma função mitocondrial deficiente. Assim, investigamos inicialmente os efeitos *in vitro* da Orn, da Hcit e do Oro sobre parâmetros da homeostasia mitocondrial, particularmente aqueles relacionados à bioenergética celular em córtex

cerebral de ratos jovens (primeiro capítulo do nosso estudo). Analisamos a produção de CO_2 e verificamos que Orn e Hcit diminuem a sua formação a partir dos substratos glicose e acetato. A baixa produção de CO_2 a partir de glicose indica uma deficiência na glicólise, que poderia resultar da inibição de uma ou mais enzimas da via glicolítica ou devido a uma inibição do ciclo dos ácidos tricarboxílicos e/ou da cadeia respiratória. Também observamos que a Orn e a Hcit reduzem a formação de CO_2 a partir de acetato, indicando um bloqueio no ciclo dos ácidos tricarboxílicos já que o acetato entra no ciclo via acetil-CoA (Nelson e Cox, 2008). Em contraste, o Oro não apresentou nenhum efeito neste parâmetro avaliado.

O próximo passo foi investigar de que maneira a atividade do ciclo dos ácidos tricarboxílicos foi inibida por estas substâncias. Para isso avaliamos os efeitos da Orn e da Hcit sobre a função da cadeia respiratória através da medida da atividade dos complexos I-IV e também a atividade das enzimas do ciclo dos ácidos tricarboxílicos. Observamos que a Orn e a Hcit reduziram moderadamente o fluxo de elétrons pela cadeia respiratória (complexo I-III) e que os dois aminoácidos foram capazes de inibir a atividade das enzimas α-cetoglutarato desidrogenase e aconitase, sem alterar a atividade das enzimas citrato sintase, isocitrato desidrogenase, succinato desidrogenase, malato desidrogenase e fumarase. Portanto, é possível que a redução na função do ciclo dos ácidos tricarboxílicos (diminuição na formação de CO_2) possa dever-se aos efeitos inibitórios da Orn e da Hcit sobre as enzimas α-cetoglutarato desidrogenase e aconitase.

Testamos também o efeito da Orn, Hcit e Oro sobre a atividade das enzimas creatina quinase (CK) e Na^+, K^+-ATPase. Observamos que estes

metabólitos não alteraram a atividade da Na^+, K^+-ATPase. Entretanto, a Hcit, mas não a Orn ou o Oro, provocou uma redução na atividade da CK mitocondrial, sugerindo que a Hcit também altera a transferência e tamponamento intracelular de energia nas células neurais. Considerando que a atividade da CK diminui após a exposição cerebral a agentes que promovem a geração de radicais livres, provavelmente pela oxidação dos resíduos de cisteína essenciais, no seu centro catalítico (Burmistrov *et al*., 1992; Wolosker *et al*., 1996; Arstall *et al*., 1998; Stachowiak *et al*., 1998;), testamos o efeito de alguns antioxidantes sobre a inibição da atividade da CK causada pela Hcit para testar se esta inibição foi mediada pela oxidação dos grupamentos tióis ou outros grupamentos da enzima. Verificamos que a GSH, que age como um agente protetor de grupamentos tióis (Meister e Anderson, 1983), preveniu totalmente o papel inibitório da Hcit na atividade da CK em córtex cerebral de ratos, indicando que a Hcit provavelmente inibiu a atividade da CK pela inativação de grupamentos sulfidrilas essenciais da enzima. Por outro lado, o α-tocoferol, que é um agente sequestrador de radicais peroxila (Arstall *et al*., 1998), o L-NAME, que é um inibidor da óxido nítrico sintase e a combinação de SOD mais CAT, que sequestram radicais superóxido e peróxido de hidrogênio, não foram capazes de prevenir o efeito inibitório da Hcit sobre a CK. Estes resultados sugerem que as espécies reativas peroxila, ânion superóxido e óxido nítrico ou peroxinitrito não estariam envolvidas nesta inibição.

É difícil determinar a relevância fisiopatológica dos dados encontrados, já que as concentrações cerebrais de Orn e Hcit não são conhecidas na síndrome HHH até o momento (Palmieri, 2008). Porém, enfatize-se que os efeitos *in vitro* causados pela Orn e pela Hcit sobre os parâmetros bioquímicos

do metabolismo energético ocorreram em concentrações similares às encontradas no plasma dos pacientes afetados pela síndrome HHH. Convém salientar ainda que concentrações mais elevadas desses aminoácidos podem ocorrer em situações de estresse, como as que ocorrem durante os episódios de descompensação metabólica, caracterizada por intenso catabolismo e proteólise, na qual os níveis dos metabólitos acumulados podem aumentar drasticamente (Camacho *et al*., 1999). Tais observações sugerem que nossos resultados possam ser relevantes para os pacientes afetados pela síndrome HHH. Por outro lado, a Hcit exerceu efeitos deletérios mais acentuados e em concentrações mais baixas (0,1–0,5 mM) sobre a obtenção de energia, quando comparadas com a Orn. Similarmente, somente a Hcit reduziu, sugerindo que estes metabólitos causam dano oxidativo a proteínas. a atividade da CK mitocondrial, uma enzima necessária na transferência e tamponamento da energia celular. Presumimos, portanto, que a Hcit parece ser mais neurotóxica do que a Orn.

O próximo passo da nossa investigação (capítulo II) foi avaliar o efeito *in vivo* da Orn e da Hcit através da administração intracerebroventricular (ICV) desses metabólitos em córtex cerebral de ratos jovens sobre parâmetros do metabolismo energético e de estresse oxidativo. Verificamos primeiramente que tanto a administração ICV de Orn como de Hcit em ratos provocou um aumento dos níveis de TBA-RS, quando comparado aos animais controle (injetados com NaCl). Esses resultados corroboram prévios achados *in vitro* do nosso grupo que esses aminoácidos induzem estresse oxidativo (Amaral *et al*., 2009). Considerando que a medida de TBA-RS reflete a quantidade de malondialdeído formado, um produto final da peroxidação de ácidos graxos

(Halliwell e Gutteridge, 2007a), valores aumentados de TBA-RS causados pela Orn e Hcit indicam que estes aminoácidos induzem peroxidação lipídica *in vivo*.

Orn e em maior grau Hcit também induziram um aumento na formação de grupamentos carbonilas (aldeídos e cetonas) que são principalmente produzidos pela oxidação das cadeias laterais de proteínas (especialmente prolina, arginina, lisina e tirosina), e em menor grau pela clivagem oxidativa de proteínas ou pela reação de redução de açucares com resíduos de lisina (Dalle-Donne *et al*., 2003). Aldeídos resultantes da lipoperoxidação também podem induzir a formação de grupamentos carbonilas (Dalle-Donne *et al*., 2003). Tais observações nos levam a concluir que a Orn e a Hcit induzem dano oxidativo proteico.

Foi observado também que os antioxidantes N-acetilcisteína (NAC), que forma GSH intracelularmente, e a combinação dos sequestradores de radicais livres ácido ascórbico e α-tocoferol atenuaram a oxidação lipídica e preveniram totalmente o dano protéico oxidativo provocado pela Orn e pela Hcit, sugerindo que a produção de espécies reativas de oxigênio esteja envolvida nestes efeitos. Em relação às espécies reativas que participaram nos efeitos pro-oxidantes da Orn e da Hcit, é possível que os radicais peroxila, que são sequestrados pelo α- tocoferol, que por sua vez é regenerado para sua forma ativa (reduzida) pelo ácido ascórbico, poderia causar, ao menos em parte, estes efeitos oxidativos. Além disso, considerando que o NAC também foi capaz de prevenir esses efeitos, não podemos excluir a possibilidade de que uma diminuição nos níveis de GSH, formado a partir do NAC, seja responsável pela oxidação lipídica e especialmente protéica, provocado pela Hcit.

Nosso estudo também demonstrou que a administração ICV de Hcit levou a uma diminuição nas concentrações de GSH e a uma inibição na atividade das enzimas antioxidantes CAT e GPx, corroborando com os resultados anteriores que mostraram que o NAC preveniu os efeitos pró-oxidantes da Hcit. Em contraste, a Orn não alterou nenhuma dessas defesas antioxidantes. Por outro lado, como esses compostos não induziram a síntese de nitratos e nitritos, é improvável que espécies reativas do nitrogênio estejam envolvidas nos efeitos pró-oxidantes da Orn e da Hcit. Considerando, portanto, que o GSH é o principal antioxidante cerebral e que a atividade da GPx e a da CAT são importantes defesas antioxidantes enzimáticas (Halliwell e Gutteridge, 2007a), presumimos que as defesas antioxidantes cerebrais foram comprometidas pela administração de Hcit. É possível ainda propor que a redução nos níveis de GSH possa ser devido ao consumo desse importante antioxidante pelo aumento de espécies reativas induzidas pela Hcit. No que se refere à Orn, este aminoácido não reduziu os níveis de GSH provavelmente devido à menor capacidade de induzir a formação de espécies reativas quando comparado com a Hcit, o que se reflete pelos efeitos oxidativos mais brandos exercidos pelo mesmo.

Nossos resultados indicam que a administração *in vivo* dos metabólitos acumulados na síndrome HHH induz estresse oxidativo em córtex cerebral de ratos jovens já que esta condição deletéria para as células resulta de um desequilíbrio entre as defesas antioxidantes e a geração de espécies reativas em um tecido (Halliwell e Gutteridge, 2007a). É importante salientar que o cérebro possui baixa quantidade de defesas antioxidantes quando comparado

com outros tecidos (Halliwell e Gutteridge, 1996), fazendo com que esse tecido seja mais vulnerável ao aumento das concentrações de espécies reativas.

Em relação aos parâmetros de metabolismo energético, a injeção ICV de Orn e de Hcit inibiram a via glicolítica aeróbica e o ciclo dos ácidos tricarboxílicos observado pela diminuição na formação de CO_2 a partir de glicose e acetato, respectivamente. É possível, portanto, que a Orn e a Hcit tenham inibido a atividade de uma ou mais enzimas da via glicolítica, do ciclo dos ácidos tricarboxílicos e/ou da cadeia respiratória.

Nesse particular, demonstramos que a Hcit inibiu a atividade da aconitase, sem alterar a atividade das outras enzimas deste ciclo, enquanto que a Orn não alterou nenhuma das atividades avaliadas. Considerando que a aconitase é altamente vulnerável ao estresse oxidativo (Gardner, 1997) e que a Hcit provocou um dano oxidativo a proteínas em grau maior quando comparado com a Orn, é possível que a inibição da aconitase seja um resultado do ataque de radicais livres a grupamentos essenciais para a atividade da enzima.

Além disso, a administração *in vivo* de Orn e Hcit provocou uma redução no fluxo de elétrons pela cadeia respiratória observado pela inibição da atividade do complexo I-III. Portanto é possível que o bloqueio do transporte de elétrons pela cadeia respiratória (inibição da atividade do complexo I-III por estes metabólitos) com acúmulo de equivalentes reduzidos (NADH e NADPH) possam secundariamente inibir as desidrogenase mitocondriais, bem como a inibição da aconitase pela Hcit contribuiram para a inibição do ciclo dos ácidos tricarboxílicos.

Os experimentos *in vitro* e *in vivo* indicam que a bioenergética cerebral associada à obtenção de energia esta comprometida pela administração de Hcit e Orn.

Por outro lado, a administração *in vivo* de Orn e Hcit não modificou a atividade das enzimas CK e Na^+, K^+-ATPase em córtex cerebral de ratos, que são importantes para a transferência e tamponamento energético cerebral e para a manutenção do potencial de membrana necessário para a neurotransmissão, respectivamente.

Considerando que os resultados *in vivo* (capítulo II) estão de acordo com os obtidos *in vitro*, demonstrando que a Orn e particularmente a Hcit alteram a bioenergética cerebral (capítulo I) e induzem estresse oxidativo (Amaral *et al*., 2009), pode-se inferir que dois mecanismos, redução da obtenção de energia e dano oxidativo com diminuição nas defesas antioxidantes, secundário ao acúmulo agudo de Hcit e Orn, podem contribuir ao menos em parte para a disfunção neurológica característica da síndrome HHH.

Tomados em seu conjunto e considerando que a administração ICV de Hcit ter sido feita em uma dose mais baixa (1,6 μmol) quando comparada com a Orn (5 μmol), nossos resultados sugerem que a Hcit exerce efeitos mais intensos do que a Orn na maioria dos parâmetros de estresse oxidativo e de bioenergética examinados, que a Hcit é uma neurotoxina mais potente.

Avaliamos também o papel da hiperamonemia sobre os efeitos exercidos pela Hcit e Orn sobre parâmetros de estresse oxidativo em córtex cerebral, visto que a hiperamonemia tem sido proposta como o principal fator responsável pelas manifestações clínicas cerebrais dos pacientes com a síndrome HHH (Valle e Simell, 2001). Não afastamos a hipótese de que haja

sinergismo entre as ações provocadas entre os vários principais compostos acumulados na síndrome HHH. Portanto, o objetivo do capítulo III desta tese foi avaliar o efeito *in vivo* do tratamento com urease, que causa hiperamonemia, sozinha ou em combinação com Orn e Hcit, sobre a homeostase redox celular em córtex cerebral de ratos jovens.

Utilizamos neste trabalho o modelo de hiperamonemia induzido pela administração de urease como descrito por Diemer e Laursen (1977). Esta abordagem tem sido usada como um modelo de encefalopatia hepática para avaliar o papel de altos níveis de amônia no cérebro (Butterworth *et al*., 2009). Em nosso laboratório, a aplicação desse modelo levou a um aumento dos níveis plasmáticos de amônia da ordem de sete vezes relativamente aos valores basais.

Verificamos inicialmente que a administração ICV de Orn e a Hcit causaram um aumento nos níveis de TBA-RS, uma medida de peroxidação lipídica, quando comparados com os animais que receberam solução salina. Demonstrarmos também que a hiperamonemia *per se,* induzida pela injeção i.p de urease, foi capaz de aumentar os níveis de TBA-RS, no entanto não amplificou o efeito provocado pela Orn e pela Hcit nesta medida.

Por outro lado, a formação de carbonilas não foi induzida pelo tratamento com urease, sugerindo que lipídios são mais sucetíveis do que proteínas a níveis elevados de amônia. Em relação à oxidação de grupamentos sulfidrilas, a Orn, a Hcit e a amônia *per se* não alteraram este parâmetro, contudo, a combinação de hiperamonemia com a administração ICV de Orn foi capaz de diminuir o conteúdo de sulfidrilas, sugerindo um efeito sinérgico destes metabólitos.

Observamos ainda que a administração ICV de Hcit induziu uma diminuição nas concentrações de GSH, distintamente da Orn administrada isoladamente e do tratamento com uréase. No entanto, a administração in vivo de Orn (ICV) e urease (i.p.) provocaram uma redução dos níveis de GSH, mostrando mais uma vez um efeito sinérgico desses compostos. Neste contexto, é possível que elevadas concentrações cerebrais de Orn juntamente com altos níveis de amônia podem refletir melhor a condição *in vivo* observada na síndrome HHH.

Estudos prévios demonstraram que doses elevadas de amônia induzem a produção de radicais livres (Kosenko *et al*., 2003; Murthy *et al*., 2001; Reinehr *et al*., 2007). Além disso, aumento na produção de superóxido e diminuição da atividade de enzimas antioxidantes, como a GPx, SOD e CAT, foram observadas no cérebro de ratos injetados com acetato de amônia (Kosenko *et al*., 1997; Kosenko *et al*., 2003). Portanto, é possível que a hiperamonemia e o aumento das concentrações cerebrais de Orn tenham provocado um efeito sinérgico na produção de espécies reativas resultando no dano protéico e na redução das defesas antioxidantes no cérebro de ratos, como observado no presente trabalho.

Considerando que o estresse oxidativo resulta do desequilíbrio entre a formação de radicais livres e a sua neutralização por antioxidantes teciduais, nossos resultados indicam que os principais metabólitos acumulados na síndrome HHH induzem estresse oxidativo *in vivo* em córtex cerebral de ratos jovens, uma condição deletéria para o tecido que pode levar à morte celular (Halliwell and Gutteridge, 2007a).

Nossa investigação também teve por objetivo produzir um modelo quimicamente induzido de hiperornitinemia, através da administração de Orn, de forma a mimetizar os níveis plasmáticos de Orn (hiperornitinemia) encontrados nos pacientes afetados pela síndrome HHH (Valle e Simell, 2001). Os níveis plasmáticos de Orn alcançados no nosso modelo animal (aproximadamente 1400 µM) ocorreram 30 min após a administração aguda de Orn e gradualmente diminuíram ao longo do tempo. A concentração máxima de Orn no cérebro (280 µM) ocorreu 30-60 min após a injeção e decaiu aos níveis basais 240 min após a injeção de Orn. Essas concentrações cerebrais foram 4-5 vezes mais elevadas que os valores basais, indicando que a Orn é capaz de atravessar a barreira hemato encefálica provavelmente através do sistema y+ (O`Kane *et al*., 2006). Também foi observado que o tempo de meia-vida ($t_{1/2}$) diminui, a depuração plasmática (CLp) e o volume de distribuição aparente (V_d) da Orn aumentaram com a idade, sugerindo que a metabolização da Orn e a maturidade da função renal aumentam com a idade. Portanto, pode-se presumir que a Orn produzida no fígado penetra no cérebro através do sistema y+ e possivelmente exerce seu efeito deletério durante período precoce do desenvolvimento quando o cérebro está mais vulnerável à hiperornitinemia. Embora este modelo não mimetize exatamente a síndrome HHH, ele reproduz uma importante característica bioquímica desta doença, ou seja, as concentrações teciduais elevadas de Orn.

Nosso próximo passo foi utilizar se este modelo de hiperornitinemia com níveis elevados de Orn durante um período (5° ao 28° dia de vida) de extensa proliferação celular e sinaptogênese em diversas estruturas cerebrais envolvidas no aprendizado/memória (Roisen *et al*., 1981; Dreyfus *et al*., 1984;

Dutra *et al*., 1993) poderia alterar o desenvolvimento físico, neuromotor, bem como o desempenho de animais nas tarefas de campo aberto e labirinto aquático de Morris.

O desenvolvimento físico e a tarefa de queda livre foram analisados durante o tratamento com Orn. Observou-se que a administração crônica de Orn não afetou o peso corporal dos ratos, sugerindo que a Orn não causa má nutrição nos animais. Esta observação é importante já que se sabe que ratos mal nutridos podem agir de maneira diferente em testes comportamentais (Smart e Dobbing, 1971; Davis e Squire, 1984). O aparecimento de pelos, a abertura dos olhos e a erupção dos incisivos foram analisadas (desenvolvimento físico), bem como o comportamento na queda livre (desenvolvimento motor). Nenhuma alteração foi observada nesses parâmetros, indicando que o desenvolvimento físico dos animais não foi alterado pela hiperornitinemia produzida.

Após a indução do modelo de hiperornitinemia, os animais permaneceram 30-40 dias se recuperando, para então serem submetidos às tarefas comportamentais. Em relação ao desempenho dos animais na tarefa de campo aberto, observamos que os animais tratados com Orn não apresentaram habituação, como revelado pela perda na redução do número de *rearings* na sessão de teste realizada 24 horas após o treino (primeira sessão), enquanto os animais controle (injetados com salina) mostraram uma habituação normal, isto é, reduziram o número de *rearings* na sessão de teste. Na tarefa de campo aberto, ratos apresentam um comportamento exploratório que consiste em locomoção e *rearings* quando expostos pela primeira vez a caixa (sessão treino). Retenção da memória ou habituação a um novo

ambiente pode ser medida pela redução no número de *rearings* na sessão teste, quando comparada à sessão treino (Denenberg, 1969; Netto *et al*., 1986; Izquierdo 2001). Já que a redução no número de *rearings* ao longo das sessões na habituação do campo aberto pode ser interpretada como sendo um reconhecimento do ambiente pelo animal indicando que ele se lembra da exposição prévia à caixa (Denenberg, 1969; Izquierdo 2001; Rodrigues *et al.*, 1996), e que neste estudo os animais tratados com Orn não mostraram este padrão de comportamento, pode-se presumir que isto reflete em uma alteração comportamental causada por níveis cerebrais elevados deste aminoácido.

Alterações na atividade motora e na ansiedade provavelmente não contribuíram para o déficit na habituação causada pela Orn já que os dois grupos experimentais agiram de maneira similar quando observados o número de cruzamentos (atividade locomotora), bolos fecais e *grooming* e o tempo gasto na área central, que parecem estar relacionados à ansiedade e emocionalidade (Bolles, 1960; Denenberg, 1969; Kametani, 1988). Estes dados, portanto, sugerem que o tratamento com Orn não foi ansiogênico e não alterou a atividade motora. Tais resultados sugerem que o déficit de habituação na realidade provavelmente refletiu um déficit de aprendizado e memória (Netto et al., 1986).

Já na tarefa do labirinto aquático de Morris, observamos que os animais tratados com Orn ou salina diminuíram a latência para achar a plataforma do primeiro ao último dia do treino, ou seja, foram capazes de lembrar o local da plataforma. Além do mais, os dois grupos permaneceram o mesmo tempo no quadrante onde estava localizada a plataforma e no quadrante oposto à plataforma no dia do teste (dia 8). Os ratos tratados com Orn também tiveram

um número similar de cruzamentos no local da plataforma e apresentaram a mesma latência para cruzar pela primeira vez o local da plataforma, bem como a mesma velocidade de natação quando comparados aos animais controle. Estes resultados sugerem que o tratamento com Orn não alterou o aprendizado espacial em ratos adultos. Os achados de latência para cruzar o local da plataforma e velocidade de natação reforçam que o tratamento crônico com Orn não altera a atividade motora dos animais.

No que se refere aos mecanismos que levam a um déficit na habituação dos animais (memória) causado pela Orn, não podemos precisar com clareza os mesmos. No entanto, é possível que as concentrações cerebrais de Orn verificadas em nosso modelo químico de hiperornitinemia podem ter sido suficientes para induzir um dano cerebral durante a fase de rápida maturação do SNC e particularmente nas estruturas cerebrais envolvidas no aprendizado e memória. Neste contexto, estudos prévios demonstraram que o estresse oxidativo está associado à diminuição de memória (Bickford *et al*., 1999; Serrano *et al*., 2004; Silva *et al*., 2004) e que substratos energéticos como a creatina e o succinato são capazes de prevenir o déficit de aprendizado/memória (Vasques *et al*., 2006). Já que, de acordo com os dados mostrados nos capítulos I e II de que a Orn causa estresse oxidativo *in vivo* e compromete o metabolismo energético cerebral *in vivo* e *in vitro*, poderia se presumir que o déficit na obtenção de energia e o dano oxidativo a componentes celulares possam representar mecanismos patológicos de dano cerebral permanente, levando a uma diminuição no aprendizado/memória dos mesmos.

Em resumo, neste trabalho relatamos pela primeira vez que os metabólitos que se acumulam na síndrome HHH, Orn e Hcit induzem estresse oxidativo e alteram a bioenergética cerebral. No caso dos achados dessa investigação serem confirmados *in vivo* no cérebro em outros estudos e mesmo em tecidos (células sanguíneas, plasma, fibroblastos, músculo esquelético) de pacientes com a síndrome HHH, poderia se presumir que esses mecanismos estejam envolvidos na disfunção neurológica apresentada pelos pacientes com essa doença. Nesse caso, o uso de agentes antioxidantes poderia representar novas abordagens terapêuticas, junto com as medidas usuais no tratamento dos pacientes afetados por essa doença. Salientamos, de acordo com nossos resultados, que a patogênese desta doença não pode ser exclusivamente atribuída a hiperamonemia. Finalmente, os achados comportamentais foram moderados, indicando a necessidade de uma investigação mais profunda do comportamento de animais com altos níveis de Orn, bem como de Hcit e amônia.

III.2 CONCLUSÕES

III.2.1 Inibição *in vitro* pela ornitina (Orn) e homocitrulina (Hcit) sobre o metabolismo energético em córtex cerebral de ratos jovens

- A Orn e a Hcit causaram um déficit na via glicolítica aeróbica e no ciclo dos ácidos tricarboxílicos, observado pela diminuição na produção de CO_2 a partir de glicose e acetato, bem como pela inibição da atividade das enzimas aconitase e α-cetoglutarato desidrogenase (enzimas do ciclo dos ácidos tricarboxílicos) e pela inibição do fluxo de elétrons pela cadeia respiratória (inibição do complexo I-III).
- A Hcit também alterou a transferência e o tamponamento energético, inibindo a atividade da fração mitocondrial da enzima creatina quinase (CK).

III.2.2 Inibição do metabolismo energético e indução de estresse oxidativo *in vivo* pela Orn e Hcit em córtex cerebral de ratos jovens

- A Orn e a Hcit inibiram a via glicolítica e a atividade do ciclo dos ácidos tricarboxílicos observado pela diminuição da produção de CO_2 a partir de glicose e acetato, respectivamente, bem como a atividade da enzima aconitase.
- A Orn e a Hcit induziram lipoperoxidação (dano oxidativo lipídico) determinado através do aumento da medida das substâncias reativas ao ácido tiobarbitúrico e provocaram dano oxidativo protéico, evidenciado pelo aumento da formação de carbonilas.

- O pré-tratamento com antioxidantes (N-acetilcisteína, α-tocoferol e ácido ascórbico) preveniu ou atenuou a lipoperoxidação e dano oxidativo protéico provocado pela Orn e Hcit, indicando que esses efeitos foram mediados por espécies reativas.
- A Hcit alterou as defesas antioxidantes não enzimáticas (diminuição das concentrações de GSH) e enzimáticas (diminuição na atividade das enzimas GPx e CAT) .

III.2.3 Indução de estresse oxidativo *in vivo* pela amônia, Orn e Hcit em córtex cerebral de ratos jovens

- Hiperamonemia *per se* provocada pela injeção de urease induziu lipoperoxidação (aumento da medida das substâncias reativas ao ácido tiobarbitúrico).
- A combinação de hiperamonemia, induzida por urease, com a administração ICV de Orn induziram oxidação protéica (redução na formação de grupamentos carbonilas) e diminuiu as defesas antioxidantes não enzimáticas (diminuição das concentrações de GSH), indicando um efeito sinérgico da Orn e da amônia.

III.2.4 A administração crônica *in vivo* de Orn a ratos jovens prejudicou a habituação de ratos adultos na tarefa de campo aberto

- Estabelecemos um modelo químico experimental de hiperornitinemia através de injeções subcutâneas de Orn
- Verificamos que o modelo químico experimental de hiperornitinemia produzido através de injeções subcutâneas de Orn do 5º ao 28º dia de vida pós-natal não alterou o peso e o desenvolvimento físico e motor

(tempo de aparecimento dos pelos, abertura de olhos e erupção dos incisivos superiores), bem como o reflexo de queda livre dos animais.

- Os ratos tratados cronicamente com Orn apresentaram déficit de aprendizado/memória observada pelo falta de habituação a tarefa de campo aberto (avaliação do número de *rearings*), enquanto a atividade locomotora e a emocionalidade dos animais não variou.
- Os ratos tratados cronicamente com Orn não alteraram a memória espacial na tarefa do labirinto aquático de Morris.

III.3. PERSPECTIVAS

III.3.1 Estudos bioquímicos

a. Investigar efeitos *in vitro* da Hcit e da Orn separadamente e associados sobre parâmetros de estresse oxidativo e metabolismo energético em córtex, cerebelo e fígado de ratos jovens.
b. Investigar o efeito agudo (injeção única intraperitoneal) *in vivo* da Hcit e da Orn separadamente e associados sobre parâmetros de estresse oxidativo e metabolismo energético em córtex cerebral e fígado de ratos jovens.
c. Investigar o efeito crônico (duas injeções subcutâneas diárias do 5º ao 28° dia de vida pós-natal) *in vivo* da Orn sobre parâmetros de estresse oxidativo e metabolismo energético em córtex cerebral e fígado de ratos jovens.
d. Investigar o efeito sinérgico agudo *in vivo* (injeção intracerebroventricular) da Hcit e da Orn sobre parâmetros de estresse oxidativo e metabolismo energético em fígado de ratos jovens.
e. Investigar os efeitos sinérgicos *in vivo* da Orn e Hcit na presença de hiperamonemia provocada por injeções diárias de urease sobre os parâmetros de metabolismo energético em córtex cerebral de ratos jovens.

III.3.2 Parâmetros comportamentais

a. Estudar o efeito de tratamento agudo (injeção intracerebroventricular ou intraperitoneal) de Orn ou Hcit sobre o desempenho de ratos em tarefas comportamentais

b. Estudar o efeito de tratamento crônico de Orn (duas injeções diárias do 5º ao 28° dia de vida pós-natal) com e sem hiperamonemia sobre o desempenho de ratos em tarefas comportamentais.

REFERÊNCIAS

Aksenov M, Aksenova M, Butterfield DA, Markesbery WR, 2000. Oxidative modification of creatine kinase BB in Alzheimer's disease brain. *J Neurochem*. 74**,** 2520-7.

Aksenov MY, Aksenova MV, Payne RM, Smith CD, Markesbery WR, Carney JM, 1997. The expression of creatine kinase isoenzymes in neocortex of patients with neurodegenerative disorders: Alzheimer's and Pick's disease. *Exp Neurol*. 146**,** 458-65.

Aksenova MV, Aksenov MY, Payne RM, Trojanowski JQ, Schmidt ML, Carney JM, Butterfield DA, Markesbery WR, 1999. Oxidation of cytosolic proteins and expression of creatine kinase BB in frontal lobe in different neurodegenerative disorders. *Dement Geriatr Cogn Disord*. 10**,** 158-65.

Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE, 2000. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. *Prog Neurobiol*, 409-70.

Amaral AU, Leipnitz G, Fernandes CG, Seminotti B, Zanatta A, Viegas CM, Dutra-Filho, CS, Wajner M, 2009. Evidence that the major metabolites accumulating in hyperornithinemia–hyperammonemia–homocitrullinuria syndrome induce oxidative stress in brain of young rats. *Int J Dev Neurosci.* 27, 635–41.

Arstall MA, Bailey C, Gross WL, Bak M, Balligand JL, Kelly RA, 1998. Reversible S-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. *J Mol Cell Cardiol.* 30, 979–88.

Babcock GT, Wikstrom M, 1992. Oxygen activation and the conservation of energy in cell respiration. *Nature*. 356**,** 301-9.

Baric I, Fumic K, Hoffmann GF, 2001. Inborn errors of metabolism at the turn of the millennium. *Croat Med J*. 42**,** 379-83.

Barschak AG, Marchesan C, Sitta A, Deon M, Giugliani R, Wajner M, Vargas CR, 2008a. Maple syrup urine disease in treated patients: biochemical and oxidative stress profiles. *Clin Biochem.* 41, 317-24.

Barschak AG, Sitta A, Deon M, Barden AT, Dutra-Filho CS, Wajner M, Vargas CR, 2008b. Oxidative stress in plasma from maple syrup urine disease patients during treatment. *Metab Brain Dis.* 23, 71-80.

Behl C, Moosmann B, 2002. Antioxidant neuroprotection in Alzheimer’s disease as preventive and therapeutic approach. *Free Radic Biol Med*. 33, 182-191.

Bergendi L, Benes L, Duracková Z, Ferencik M, 1999. Chemistry, physiology and pathology of free radicals. *Life Sci*. 65, 1865-74.

Bessman SP, Carpenter CL, 1985. The creatine-creatine phosphate energy shuttle. *Annu Rev Biochem*. 54**,** 831-62.

Bickel H, 1987. Early diagnosis and treatment of inborn errors of metabolism. *Enzyme*. 38**,** 14-26.

Bickford PC, Shukitt-Hale B, Joseph J, 1999. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. *Mech Ageing Dev.* 111, 141-54.

Bolles RC, 1960. Grooming behavior in the rat. *J Comp Physiol Psychol.* 53, 306-10.

Boveris A, 1998. Biochemistry of free radicals: from electrons to tissues. Medicina (B. Aires) 58, 350-56.

Burmistrov SO, Mashek OP, Kotin AM, 1992. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. *Eksp. Klin. Farmakol*. 55, 54–56.

Butterworth RF, Norenberg MD, Felipo V, Ferenci P, Albrecht J, Blei AT, 2009. Experimental models of hepatic encephalopathy: ISHEN guidelines. *Liver Int.* 29(6), 783-8.

Camacho JA, Obie C, Biery B, Goodman BK, Hu CA, Almashanu S, Steel G, Casey R, Lambert M, Mitchell GA, Valle D, 1999. Hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. *Nat Genet.* 22, 151-8.

Clark JB, Bates TE, Cullingford T, Land JM, 1993. Development of enzymes of energy metabolism in the neonatal mammalian brain. *Dev Neurosci*. 15**,** 174-80.

Dalle-Done I, Rossi R, Giustarini D, Milzani A, Colombo R, 2003. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta.* 329, 23–38.

David S, Shoemaker M, Haley BE, 1998. Abnormal properties of creatine kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning. *Brain Res Mol Brain Res*. 54**,** 276-87.

Davis PH, Squire LR, 1984. Protein synthesis and memory: a review. *Psychol Bull.* 96, 518–59.

Debray FG, Lambert M, Lemieux B, Soucy JF, Drouin R, Fenyves D, Dubé J, Maranda B, Laframboise R, Mitchell GA, 2008. Phenotypic variability among patients with hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome homozygous for the delF188 mutation in SLC25A15. *J Med Genet.* 45(11), 759-64.

Delanty N, Dichter MA, 1998. Oxidative injury in the nervous system. *Acta Neurol Scand*. 98, 145-53.

Denenberg VH, 1969. Open-field beheavior in the rat: what does it mean? *Ann N Y Acad Sci.* 159(3), 852-9.

Deon M, Sitta A, Barschak AG, Coelho DM, Pigatto M, Schmitt GO, Jardim LB, Giugliani R, Wajner M, Vargas CR, 2007. Induction of lipid peroxidation and decrease of antioxidant defenses in symptomatic and asymptomatic patients with X-linked adrenoleukodystrophy. *Int J Dev Neurosci.* 25, 441-4.

Deon M, Garcia MP, Sitta A, Barschak AG, Coelho DM, Schimit GO, Pigatto M, Jardim LB, Wajner M, Giugliani R, Vargas CR., 2008. Hexacosanoic

and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-linked adrenoleukodystrophy: Lorenzo's oil effect. *Metab Brain Dis.* 23, 43-9.

Diemer NH, Laursen H, 1977. Glial cell reactions in rats with hyperammoniemia induced by urease or porto-caval anastomosis. *Acta Neurol Scand.* 55(6), 425-42.

Dickinson CJ, 1996. Cerebral oxidative metabolism in hypertension. *Clin Sci (Lond)*. 91**,** 539-50.

Dreyfus H, Ferret B, Harth S, Giorgio A, Durand M, Freysz L, et al. 1984. Metabolism and function of gangliosides in developing neurons. *J Neurosci Res.*12, 311–22.

Dutra JC, Wajner M, Wannmacher CMD, Dutra-Filho CS, Rache E, 1993. Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. *J Inherited Metab Dis.* 16, 147–53.

Erecinska M, Cherian S, Silver IA, 2004. Energy metabolism in mammalian brain during development. *Prog Neurobiol*. 73**,** 397-445.

Erecinska M, Silver IA, 1994. Ions and energy in mammalian brain. *Prog Neurobiol*. 43**,** 37-71.

Fecarotta S, Parenti G, Vajro P, Zuppaldi A, Della Casa R, Carbone MT, Correra A, Torre G, Riva S, Dionisi-Vici C, Santorelli FM, Andria G, 2006. HHH syndrome (hyperornithinaemia, hyperammonaemia, homocitrullinuria), with fulminant hepatitis-like presentation. *J Inherit Metab Dis.* 29(1), 186-9.

Felipo V, Butterworth RF, 2002. Neurobiology of ammonia. *Prog Neurobiol.* 67(4), 259-79.

Fell V, Pollitt RJ, Sampson GA, Wright T, 1974. Ornithinemia, hyperammonemia, and homocitrullinuria. A disease associated with mental retardation and possibly caused by defective mitochondrial transport. *Am J Dis Child.* 127, 752.

Fernandes CG, Borges CG, Seminotti B, Amaral AU, Knebel LA, Eichler P, de Oliveira AB, Leipnitz G, Wajner M, 2011. Experimental evidence that methylmalonic acid provokes oxidative damage and compromises antioxidant defenses in nerve terminal and striatum of young rats. *Cell Mol Neurobiol.* 31(5), 775-85.

Fiermonte G, Dolce V, David L, Santorelli FM, Dionisi-Vici C, Palmieri F, Walker JE, 2003. The mitochondrial ornithine transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. *J Biol Chem.* 278(35), 32778-83.

Fukuda K, Nishi Y, Usui T, Mishima H, Hirata H, Baba S, Choshi K, Tanaka Y, Akiya S, 1983. Free amino acid concentrations in blood cells of two brothers with gyrate atrophy of the choroid and retina with hyperornithinaemia. *J Inherit Metab Dis.* 6, 137-42.

Gamble G, Lehninger AL, 1973. Transport of ornithine and citrulline across the mitochondrial membrane. *J Biol Chem.* 248, 610.

Gardner PR, 1997. Superoxide-driven aconitase FE-S center cycling. *Biosci Rep Rev*. 17, 33-42.

Gatfield PD, Taller E, Wolfe DM, Haust DM, 1975. Hyperornithinemia, hyperammonemia, and homocitrullinuria associated with decreased carbamyl phosphate synthetase I activity. *Pediatr Res.* 9, 488.

Gjessing LR, Lunde HA, Undrum T, Broch H, Alme A, Lie SO, 1986. A new patient with hyperornithinaemia, hyperammonaemia and homocitrullinuria treated early with low-protein diet. *J Inherit Metab Dis.* 9, 186.

Gordon BA, Gatfield DP, Haust DM, 1987. The hyperornithinemia, hyperammonemia, homocitrullinuria syndrome: An ornithine transport defect remediable with ornithine supplements. *Clin Invest Med.* 10, 329.

Gross WL, Bak MI, Ingwall JS, Arstall MA, Smith TW, Balligand JL, Kelly, RA, 1996. Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve. *Proc Natl Acad Sci U S A*. 93**,** 5604-9.

Gu M, Owen AD, Toffa SE, Cooper JM, Dexter DT, Jenner P, Marsden CD, Schapira AH, 1998. Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases. *J Neurol Sci.* 158, 24-29.

Halliwell B, Gutteridge JMC, 1996. Oxygen radicals and nervous system. *Trends Neurosci.* 8, 22-6.

Halliwell B, 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging*. 18, 685-716.

Halliwell B, Whiteman M, 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol.* 142, 231-55.

Halliwell B, 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol*. 141, 312-22.

Halliwell B, Gutteridge JMC, 2007a. Measurement of reactive species. In: Halliwell B, Gutteridge JMC, eds. *Free radicals in biology and medicine.* Oxford: Oxford University Press, p. 268–340.

Halliwell B, Gutteridge JMC, 2007b. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. In: Halliwell B, Gutteridge JMC, eds. *Free radicals in biology and medicine.* Oxford: Oxford University Press, p. 187–267.

Halliwell B, Gutteridge JMC, 2007c. Antioxidant defences. In: Halliwell B, Gutteridge JMC, eds. *Free radicals in biology and medicine.* Oxford: Oxford University Press, p. 79–185.

Hamman BL, Bittl JA, Jacobus WE, Allen PD, Spencer RS, Tian R, Ingwall JS, 1995. Inhibition of the creatine kinase reaction decreases the contractile reserve of isolated rat hearts. *Am J Physiol*. 269**,** H1030-6.

Haust MD, Gatfield PD, Gordon BA, 1981. Ultrastructure of hepatic mitochondria in a child with hyperornithinemia, hyperammonemia, and homocitrullinuria. *Hum Pathol.* 12, 212-22.

Ho YJ, Eichendorff J, Schwarting RK, 2002. Individual response profiles of male Wistar rats in animal models for anxiety and depression. *Behav Brain Res.* 136, 1-12.

Izquierdo I, 1989. Aprendizaje y memoria. In: Cingolani HE, Houssay, AB, eds. *Fisiologia Humana,* de B. Housay, Tomo 4- Neurofisiologia. El Ateneo, Buenos Aires, pp. 311-9.

Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, Zöchling R, Boissl KW, Reichmann H, 1994. Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neurosci Lett.* 169, 126-8.

Javadzadeh A, Gharabaghi D, 2007. Gyrate atrophy of the choroid and retina with hyper-ornithinemia responsive to vitamin B6: a case report. *J Med Case Reports*. 1, 27.

Kametani H, 1988. Analysis of age-related changes in stress-induced grooming in the rat. Differential behavioral profile of adaptation to stress. *Ann N Y Acad Sci.* 525, 101-13.

Klingenberg M, 1970. Metabolic transport in mitochondria. An example for intracellular membrane function. *Essays Biochem.* 6, 119.

Kosenko E, Kaminsky Y, Kaminsky A, Valencia M, Lee L, Hermenegildo C, Felipo V , 1997. Superoxide production and antioxidant enzymes in ammonia intoxication in rats. *Free Radic Res*. 27(6), 637-44.

Kosenko E, Venediktova N, Kaminsky Y, Montoliu C, Felipo V, 2003. Sources of oxygen radicals in brain in acute ammonia intoxication in vivo. *Brain Res*. 981(1-2), 193-200.

Korman SH, Kanazawa N, Abu-Libdeh B, Gutman A, Tsujino S, 2004. Hyperornithinemia, hyperammonemia, and homocitrullinuria syndrome with evidence of mitochondrial dysfunction due to a novel SLC25A15 (ORNT1) gene mutation in a Palestinian family. *J Neurol Sci*. 218, 53-8.

Latini A, Scussiato K, Leipnitz G, Gibson KM, Wajner M, 2007. Evidence for oxidative stress in tissues derived from succinate semialdehyde dehydrogenase-deficient mice. *J Inherit Metab Dis*. 30, 800-10.

Lieberman MA, Marks AD, 2008. *Marks' Basic Medical Biochemistry: A Clinical Approach*, vol. Lippincott Willians & Wilkins.

Lovell MA, Xie C, Markesbery WR, 2000. Decreased base excision repair and increased helicase activity in Alzheimer's disease brain. *Brain Res*. 855, 116-23.

Markesbery WR, Carney JM, 1999. Oxidative alterations in Alzheimer's disease. *Brain Pathol* 9, 133-46.

Maxwell SR, 1995. Prospects for the use of antioxidant therapies. *Drugs* 49, 345-61.

Méndez-Álvarez E, Soto-Otero R, Hermida-Aeijeiras A, López-Real AM, Labandeira-García JL, 2001. Effects of aluminium and zinc on the oxidative stress caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson’s disease. *Biochim Biophys Acta.* 1586, 155-68.

Metoki K, Hommes FA, Dyken P, Kelloes C, Trefz J, 1984. Ultrastructural changes in fibroblast mitochondria of a patient with HHH-syndrome. *J Inherit Metab Dis.* 7, 147-50.

Meister A, Anderson ME, 1983. Glutathione. *Annu Rev Biochem.* 52, 711-60.

Murthy CR, Rama Rao KV, Bai G, Norenberg MD, 2001. Ammonia-induced production of free radicals in primary cultures of rat astrocytes. *J Neurosci Res.* 66(2), 282-8.

Nelson DL, Cox MM, 2008. *Lehninger Principles of Biochemistry*, 5a ed. WH. Freeman and Company, New York.

Netto CA, Dias RD, Izquierdo I, 1986. Differential effect of posttraining naloxone, beta-endorphin, leu-enkephalin and electroconvulsive shock administration upon memory of an open-field habituation and of a water-finding task. *Psychoneuroendocrinology.* 11(4), 437-46.

Nourooz-Zadeh J, Liu EH, Yhlen B, Anggard EE, Halliwell B, 1999. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *J Neurochem*. 72, 734-40.

O'Gorman E, Beutner G, Wallimann T, Brdiczka D, 1996. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle, heart, and brain. *Biochim Biophys Acta*. 1276**,** 161-70.

O'Kane RL, Viña JR, Simpson I, Zaragozá R, Mokashi A, Hawkins RA, 2006. Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y+. *Am J Physiol Endocrinol Metab.* 291(2), 412-9.

Olton DS, Markowska AL, 1994. Memory and hipocampal function as targets for neurotoxic substances. *Neurotoxicology.* 15, 439-44.

Orth M, Schapira AH, 2001. Mitochondria and degenerative disorders. *Am J Med Genet*. 106**,** 27-36.

Palmieri F, 2008. Diseases caused by defects of mitochondrial carriers: a review. *Biochim Biophys Acta.* 1777(7-8), 564-78.

Passarella S, Atlante A, Quagliariello E, 1990. Ornithine/phosphate antiport in rat kidney mitochondria. *Eur J Biochem.* 193, 221.

Reinehr R, Görg B, Becker S, Qvartskhava N, Bidmon HJ, Selbach O, Haas HL, Schliess F, Häussinger D, 2007. Hypoosmotic swelling and ammonia increase oxidative stress by NADPH oxidase in cultured astrocytes and vital brain slices. *Glia.* 55(7), 758-71.

Ribas GS, Manfredini V, de Marco MG, Vieira RB, Wayhs CY, Vanzin CS, Biancini GB, Wajner M, Vargas CR, 2010. Prevention by L-carnitine of DNA damage induced by propionic and L-methylmalonic acids in human peripheral leukocytes in vitro. *Mutat Res.* 702(1), 123-8.

Ribeiro CAJ, Balestro F, Grando V, Wajner M, 2007. Isovaleric acid reduces Na+,K+-ATPase activity in synaptic membranes from cerebral cortex of young rats. *Cell and Mol Neurobiol*. 27(4), 529-40.

Rodrigues AL, Rocha JB, Mello CF, Souza DO, 1996. Effect of perinatal lead exposure on rat behaviour in open-field and two-way avoidance tasks. *Pharmacol Toxicol.* 79, 150- 6.

Roisen FJ, Bartfeld H, Nagele R, Yorke G, 1981. Gangliosides stimulation of axonal sprouting in vivo. *Science*. 214 577-8.

Rose CD, Henneberry RC, 1994. Etiology of the neurodegenerative disorders: a critical analysis. *Neurobiol Aging*. 15, 233-4.

Saks VA, Kuznetsov AV, Kupriyanov VV, Miceli MV, Jacobus WE, 1985. Creatine kinase of rat heart mitochondria. The demonstration of functional coupling to oxidative phosphorylation in an inner membrane-matrix preparation. *J Biol Chem*. 260**,** 7757-64.

Salvi S, Santorelli FM, Bertini E, Boldrini R, Meli C, Donati A, Burlina AB, Rizzo C, Di Capua M, Fariello G, Dionisi-Vici C, 2001. Clinical and molecular findings in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome. *Neurology*. 57, 911-4.

Save E, Poucet B, 2000. Involvement of the hippocampus and associative parietal cortex in the use of proximal and distal landmarks for navigation. *Behav Brain Res.* 109, 195-206.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD, 1989. Mitochondrial complex I deficiency in Parkinson's disease. *Lancet.* 3, 1(8649), 1269.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD, 1990a. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem.* 54(3), 823-7.

Schapira AHV, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD, 1990b. Anatomic and disease specificity of NADH CoQ1 reductase (Complex I) deficiency in Parkinson's disease. *J Neurochem*. 55**,** 2142-5.

Schlegel J, Zurbriggen B, Wegmann G, Wyss M, Eppenberger HM, Wallimann T, 1988. Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: characterization, localization, and structure-function relationships. *J Biol Chem*. 263**,** 16942-53.

Schnyder T, Gross H, Winkler H, Eppenberger HM, Wallimann T, 1991. Structure of the mitochondrial creatine kinase octamer: high-resolution shadowing and image averaging of single molecules and formation of linear filaments under specific staining conditions. *J Cell Biol*. 112**,** 95-101.

Scriver CR, Beaudet AL, Sly WS, Valle D, 2001. *The metabolic and molecular basis of inherited disease*, vol. McGraw-Hill Inc, New York.

Serrano F, Klann E, 2004. Reactive oxygen species and synaptic plasticity in the aging hippocampus. *Aging Res Ver.* 3(4), 431-43.

Shih V, Efron ML, Moser HW, 1969. Hyperornithinemia, hyperammonemia, and homocitrullinuria. A new disorder of amino acid metabolism associated with myoclonic seizures and mental retardation. *Am J Dis Child.* 117, 83.

Shih VE, Mandell R, Herzfeld A, 1982. Defective ornithine metabolism in cultured skin fibroblasts from patients with the syndrome of hyperornithinemia, hyperammonemia and homocitrullinuria. *Clin Chim Acta.* 118, 149.

Silva AL, Piato AL, Bardini S, Netto CA, Nunes DS, Elisabetsky E, 2004. Memory retrieval improvement by Ptychopetalum olacoides in young and aging mice. *J Ethnopharmacol*. 95(2–3), 199-203.

Sitta A, Barschak AG, Deon M, Terroso T, Pires R, Giugliani R, Dutra-Filho CS, Wajner M, Vargas CR, 2006. Investigation of oxidative stress parameters in treated phenylketonuric patients. *Metab Brain Dis*. 21, 287-96.

Smart JL, Dobbing J, 1971. Vulnerability of developing brain. II. Effect of early nutritional deprivation on reflex ontogeny and development of behavior in the rat. *Brain Res.* 28, 85–95.

Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR, 1991. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc Natl Acad Sci USA.* 88, 10540-3.

Southorn PA, Powis G, 1988. Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc.* 63, 381-9.

Stachowiak O, Dolder M, Wallimann T, Richter C, 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J Biol Chem* 273, 16694-9.

Tessa A, Fiermonte G, Dionisi-Vici C, Paradies E, Baumgartner MR, Chien YH, Loguercio C, de Baulny HO, Nassogne MC, Schiff M, Deodato F, Parenti G, Rutledge SL, Vilaseca MA, Melone MA, Scarano G, Aldamiz-Echevarría L, Besley G, Walter J, Martinez-Hernandez E, Hernandez JM, Pierri CL, Palmieri F, Santorelli FM, 2009. Identification of novel mutations in the SLC25A15 gene in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome: a clinical, molecular, and functional study. *Hum Mutat.* 30(5), 741-8.

Valle D, Simell O, 2001. The hyperornithinemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Basis of Inherited Diseases.* New York: McGraw-Hill, p. 1857-96.

Vargas CR, Wajner M, Sitta A, 2011. Oxidative stress in phenylketonuric patients. *Mol Genet Metab.* 104(S), 97-9.

Vasques V, Brinco F, Viegas CM, Wajner M, 2006. Creatine prevents behavioral alterations caused by methylmalonic acid administration into the hippocampus of rats in the open field task. *J Neurol Sci.* 244(1-2), 23-9.

Vianna MR, Alonso M, Viola H, Quevedo J, de Paris F, Furman M, de Stein ML, Medina JH, Izquierdo I, 2000. Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learn Mem*. 7, 333-40.

Zugno AI, Stefanello FM, Scherer EB, Mattos C, Pederzolli CD, Andrade, VM, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT, 2008. Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats. *Neurochem Res.* 33, 1804-10.

Walker V, 2009. Ammonia toxicity and its prevention in inherited defects of the urea cycle. *Diabetes Obes Metab.* 11(9), 823-35.

Wallace DC, 1999. Mitochondrial diseases in man and mouse. *Science*. 283**,** 1482-8.

Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM, 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J*. 281**,** 21-40.

Wolosker H, Panizzutti R, Engelender S, 1996. Inhibition of creatine kinase by S-nitrosoglutathione. *FEBS Lett.* 392, 274-6.