Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Avaliação dos efeitos neuroprotetores das vitaminas E e C, da guanosina e do exercício físico sobre o metabolismo energético, o sistema glutamatérgico e a memória em ratos submetidos à hiperprolinemia

Andréa Gisiane Kurek Ferreira

Orientadora: Prof[a]. Dr[a]. Angela Terezinha de Souza Wyse

Porto Alegre, 2011

Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Avaliação dos efeitos neuroprotetores das vitaminas E e C, da guanosina e do exercício físico sobre o metabolismo energético, o sistema glutamatérgico e a memória em ratos submetidos à hiperprolinemia

Andréa Gisiane Kurek Ferreira

Orientadora: Prof^a. Dr^a. Angela Terezinha de Souza Wyse

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas: Bioquímica

Porto Alegre, 2011

*Ao meu esposo **Adenilton**, pelo amor e carinho; e pelo incentivo, companheirismo e entusiasmo durante toda a realização desse trabalho.*

AGRADECIMENTOS

"A maravilhosa disposição e harmonia do universo só pode ter tido

origem segundo o plano de um Ser que tudo sabe e tudo pode.

Isso fica sendo a minha última e mais elevada descoberta"

Isaac Newton (1642-1727)

SUMÁRIO

RESUMO

Hiperprolinemias são erros inatos do metabolismo causados pela deficiência de enzimas envolvidas no catabolismo da prolina, o que resulta em acúmulo tecidual de desse aminoácido. Clinicamente, os pacientes podem apresentar manifestações neurológicas graves como retardo mental e convulsões, cuja fisiopatologia não está totalmente elucidada. O objetivo desse trabalho foi investigar o efeito da prolina sobre parâmetros bioquímicos (metabolismo energético, estresse oxidativo e sistema glutamatérgico) em cérebro e fígado, bem como sobre parâmetros comportamentais de ratos submetidos ao modelo experimental de hiperprolinemia e investigar possíveis efeitos neuroprotetores das vitaminas E e C, da guanosina e do exercício físico sobre as alterações causadas pela prolina. Os resultados mostraram que a administração aguda e crônica de prolina induz peroxidação lipídica, compromete o metabolismo energético cerebral, diminui os níveis de ATP intracelular, inibe a atividade da Na^+,K^+-ATPase sem alterar a expressão de suas subunidades catalíticas, e prejudica a captação de glutamato, embora não diminua o conteúdo de seus transportadores. Todas essas alterações podem estar intimamente relacionadas ao déficit cognitivo causado pela hiperprolinemia, o qual também está relacionado à diminuição dos níveis de BDNF e da atividade colinérgica. A hiperprolinemia também altera a homeostase hepática pela indução de um leve grau de estresse oxidativo e de alterações metabólicas, as quais provavelmente não implicam em dano significativo ao tecido hepático, mas sugerem uma adaptação tecidual ao estresse oxidativo. Além disso, demonstramos que a utilização das vitaminas antioxidantes, da guanosina e/ou do exercício físico pode contribuir para a contenção do estresse oxidativo, da toxicidade glutamatérgica e dos prejuízos na cognição induzidos pela hiperprolinemia. Acreditamos que esses achados possam ser relevantes para o entendimento das alterações neurológicas presentes na hiperprolinemia, além de sugerir possibilidades de neuroproteção, como por exemplo, o exercício físico que poderia ser usado como um adjuvante terapêutico no tratamento dos pacientes hiperprolinêmicos. A magnitude dos efeitos biológicos das vitaminas antioxidantes e especialmente da guanosina requerem estudos adicionais para uma melhor compreensão das suas ações neuroprotetoras na hiperprolinemia.

ABSTRACT

Hyperprolinemias are inborn errors of metabolism of proline caused by genetic defects in the L-proline catabolic pathway, resulting in tissue accumulation of this amino acid. Clinically, patients may present severe neurological manifestations, such as mental retardation and seizures. However, the mechanisms underlying these alterations are not fully understood. The aim of this study was to investigate the effect of proline on biochemical parameters (energy metabolism, oxidative stress and glutamatergic system) in brain and liver, as well as on behavior of rats submitted to experimental hiperprolinemia. In addition, we investigated some neuroprotective effects of vitamins E and C, guanosine and physical exercise on the harmful effects induced by proline. The results showed that acute and chronic proline administration induce lipid peroxidation, impair energy metabolism by inhibiting key enzymes in the brain, decrease of intracellular ATP levels and inhibit the Na^+,K^+-ATPase activity, but not the expression of their catalytic subunits. Moreover, this amino acid impairs glutamate uptake, but not decrease the content of their carriers. All these changes caused by proline may be closely related to cognitive impairment caused by hyperprolinemia, which also seem to involve a reduction in the BDNF levels and cholinergic activity. The hyperprolinemia also alters hepatic homeostasis by inducing a mild degree of oxidative stress and metabolic changes, which probably do not involve significant damage to liver tissue, but show a process of tissue adaptation to oxidative stress. Also, we showed that the antioxidant vitamins, the nucleoside guanosine and physical exercise may contribute to the counteracting of oxidative stress, excitotoxicity and cognitive deficit induced by hyperprolinemia. These findings may be relevant to the comprehension of neurological disorders in hyperprolinemia and suggest possibilities for neuroprotection to hyperprolinemic patients, such as physical exercise, which may be used as an adjuvant therapy for these patients. The magnitude of benefical effects of antioxidant vitamins and especially guanosine on hyperprolinemia requires additional studies to better understand its neuroprotective actions.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ALT: alanina aminotransferase

AMPA: ácido α-amino-3-hidróxi-5-metil-4-isoxazol-epropionato

AST: aspartato aminotransferase

ATP: adenosina 5'-trifosfato

BDNF: fator neurotrófico derivado do encéfalo

CAT: catalase

COX: citocromo *c* oxidase

EIM: erros inatos do metabolismo

EAAC: carreador de aminoácidos excitatórios

EAAT: transportador de aminoácidos excitatórios

$FADH_2$: flavina adenina dinucleotídeo (forma reduzida)

GLAST: transportador de glutamato-aspartato

GLT-1: transportador de glutamato

GSH: glutationa (forma reduzida)

GSH-Px: glutationa peroxidase

H_2O_2: peróxido de hidrogênio

NAD^+: nicotinamida adenina dinucleotídeo (forma oxidada)

NADH: nicotinamida adenina dinucleotídeo (forma reduzida)

$NADP^+$: nicotinamida adenina dinucleotídeo fosfato (forma oxidada)

NADPH: nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

NMDA: N-metil-D-aspartato

O_2^-: ânion superóxido

OH^-: ânion hidroxila

$ONOO^-$: ânion peroxinitrito

P5C: pirrolino-5-carboxilato

RNAm: ácido ribonucléico mensageiro

RNS: espécies reativas do nitrogênio

ROS: espécies reativas do oxigênio

SDH: succinato desidrogenase

SOD: superóxido dismutase

TBARS: substâncias reativas ao ácido tiobarbitúrico

TRAP: potencial antioxidante total

I. INTRODUÇÃO

1.1. Erros Inatos do Metabolismo

Erros inatos do metabolismo (EIM) são doenças hereditárias causadas por mutações em genes que codificam proteínas, geralmente enzimas envolvidas em vias metabólicas. Tais alterações podem causar deficiência parcial ou total dessa proteína e ocasionar bloqueio da rota metabólica onde ela está inserida, causando acúmulo de metabólitos tóxicos e/ou falta de produtos essenciais (Scriver et al., 2001).

Aproximadamente 500 EIM diferentes foram identificados até o momento, correspondendo a cerca de 10% de todas as doenças genéticas (Scriver et al., 2001). Individualmente, as doenças decorrentes dos EIM são consideradas raras. Porém, em conjunto apresentam alta frequência, acometendo aproximadamente 1:1000 recém-nascidos vivos (Giugliani e Coelho, 1997).

As manifestações clínicas dessas doenças são muito variadas e geralmente inespecíficas, dentre as quais podem ser citadas: vômitos, odores peculiares, letargia, atraso no desenvolvimento psicomotor, acidose metabólica, convulsões e coma, sendo que tais sintomas podem aparecer desde as primeiras horas de vida até alguns meses após o nascimento (Kasper et al., 2010). Por outro lado, alguns EIM podem ser absolutamente assintomáticos (Phang et al., 2001). Essa variabilidade na sintomatologia ocorre principalmente em função de diferenças no grau da deficiência enzimática, área do metabolismo e/ou tecido afetados.

A classificação mais utilizada para os EIM é realizada de acordo com a área do metabolismo afetada podendo ser EIM de: aminoácidos, ácidos orgânicos, carboidratos, lipídios, glicosaminoglicanos, glicoproteínas, entre outros (Scriver et al., 2001). Dentre os mais frequentes, estão os EIM de aminoácidos que incluem a hiperprolinemia, objeto de estudo desta tese.

1.2. L-Prolina

A L-prolina (prolina) é um aminoácido não essencial em crianças e adultos, porém condicionalmente essencial em prematuros (Phang et al., 2001). Quanto à sua estrutura química, a prolina é classificada como um aminoácido apolar de cadeia lateral alifática, onde está inserido um grupamento pirrolidina cíclico (Figura 1).
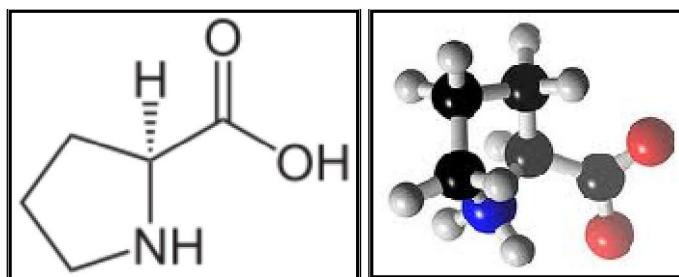
Figura 1. Estrutura química da prolina.

Essa estrutura em anel confere à prolina certa rigidez conformacional, o que promove estabilidade física às proteínas constituídas por esse aminoácido (p. ex. colágeno). Outro aspecto químico relevante é a presença de um grupo amino secundário (-NH) no anel pirrolidina que impede que a prolina seja metabolizada pelas enzimas que usualmente catabolizam os aminoácidos primários, ou seja, aqueles que contêm grupo amino primário (-NH_2) em sua estrutura. Em função dessas peculiaridades químicas da prolina, tem sido descrito uma família de enzimas envolvidas especificamente em seu metabolismo (Phang et al., 2001) (Figura 2).

O metabolismo da prolina envolve outros dois aminoácidos, o glutamato e a ornitina, cujas interconversões conectam a rota metabólica da prolina com outros três sistemas metabólicos importantes, denominados ciclo de Krebs, ciclo da uréia e via das pentoses (Hu et al., 2008).
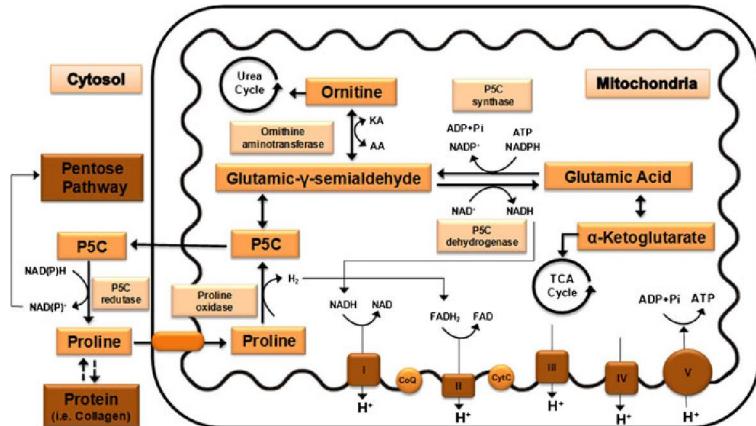
Figura 2. Rota metabólica da prolina (Wyse e Netto, 2011).

Na via de síntese da prolina, as enzimas mitocondriais Δ^1-pirrolino-5-carboxilato (P5C) sintase e ornitina aminotransferase (OAT) atuam sobre o glutamato e a ornitina respectivamente, formando glutamato-γ-semialdeído, um tautômero acíclico com o qual o P5C está em equilíbrio espontâneo. O P5C por sua vez, é transportado para o citosol e reduzido à prolina pela P5C redutase que tem como cofator NADH ou NADPH (Flynn et al., 2002; Phang et al., 2001).

Em sua rota de degradação, a prolina é primeiramente convertida em P5C pela ação da prolina oxidase, uma enzima ligada à membrana mitocondrial interna e presente em órgãos como fígado, rim e cérebro. Essa reação envolve a transferência dos elétrons da prolina a uma flavoproteina (FAD, flavina adenina dinucleotídeo), gerando FADH$_2$. A segunda etapa é não-enzimática e envolve a conversão de P5C à glutamato-γ-semialdeído, que por sua vez pode formar ornitina na reação reversível catalisada pela OAT ou glutamato pela P5C desidrogenase, enzimas localizadas na matriz mitocondrial e amplamente distribuídas nos tecidos. A P5C desidrogenase utiliza NAD$^+$ (nicotinamida adenina dinucleotídeo) como aceptor de elétrons, formando NADH. As coenzimas reduzidas NADH e FADH$_2$ produzidas pelo catabolismo da prolina podem entregar seus elétrons na cadeia respiratória mitocondrial, com subsequente geração de

4

ATP no processo de fosforilação oxidativa (Adams e Frank, 1980; Hagedorn e Phang, 1983; Phang et al., 2001). Com exceção da OAT que catalisa uma reação reversível, as outras quatro enzimas envolvidas nas vias de síntese e degradação da prolina são unidirecionais, o que sugere que o metabolismo da prolina é rigorosamente regulado (Phang et al., 2001).

A função fisiológica básica da rota metabólica da prolina é fornecer prolina para a síntese de proteínas, gliconeogênese hepática e síntese de ornitina e arginina (Phang et al., 2001). Porém, as interconversões da prolina em sua rota metabólica apresentam funções adicionais como, por exemplo, regulação celular por mecanismos redox (Phang et al., 2001). Além disso, estudos mostram um importante papel desse aminoácido no reconhecimento proteína-proteína e na sinalização molecular (Lu et al., 2003). Nesse contexto, o metabolismo da prolina parece desempenhar um papel regulatório em diversas situações fisiológicas ou fisiopatológicas, porém ainda não há um completo entendimento acerca dessas funções (Phang, 1985; Phang et al., 2010; Wyse e Netto, 2011).

1.3. Hiperprolinemias

Os níveis de prolina no plasma e no fluido cérebro espinhal em indivíduos normais oscilam aproximadamente entre 50 - 270 μM e 1 - 4 μM, respectivamente (Phang, 1985). No entanto, níveis elevados desse aminoácido são encontrados em EIM da prolina denominados hiperprolinemias, decorrentes da deficiência nas atividades de enzimas envolvidas no catabolismo da prolina. Atualmente, são descritas na literatura dois tipos de hiperprolinemias: a hiperprolinemia tipo I (HP I) causada pela deficiência da enzima prolina oxidase, que catalisa a conversão

de prolina em P5C; e a hiperprolinemia tipo II (HP II) causada pela deficiência da enzima P5C desidrogenase que converte P5C a glutamato (Figura 3).
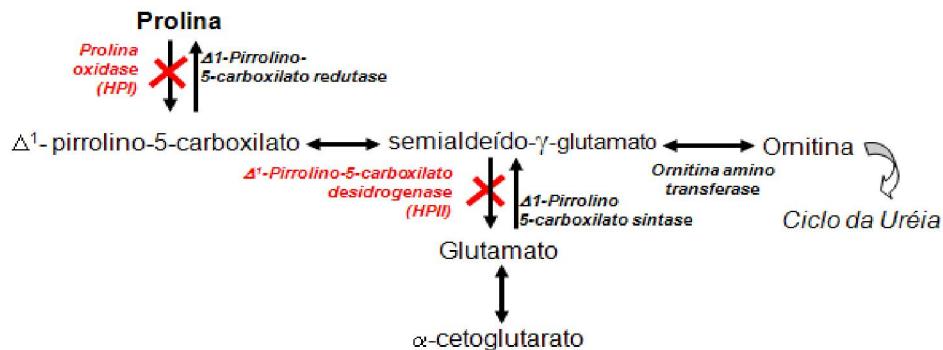
Figura 3: Representação esquemática da rota metabólica da prolina, indicando as deficiências enzimáticas que ocorrem na hiperprolinemia tipo I e tipo II (adaptado de Phang et. al 2001).

Pacientes afetados pela HP I apresentam níveis plasmáticos de prolina elevados (500 - 2600 μM), em relação aos indivíduos normais. A HP II é caracterizada por apresentar níveis plasmáticos e liquóricos de prolina ainda mais elevados do que aqueles encontrados nos pacientes afetados pela HP I, os quais oscilam entre 500 - 3700 μM e 20 - 50 μM, respectivamente (Phang et al., 2001).

As manifestações clínicas das hiperprolinemias ainda não estão bem caracterizadas; enquanto alguns pacientes são assintomáticos, outros podem apresentar alterações graves. O fenótipo dos pacientes com HP I pode incluir doenças renais, defeitos auditivos, alterações oculares, retardo mental e outras alterações neurológicas. Porém, ainda não foi estabelecida uma relação causal entre essas manifestações clínicas e a deficiência da prolina oxidase. Dessa forma, alguns autores têm considerado a HP I como uma condição benigna (Mitsubuchi et al., 2008; Phang et al., 2001; Wyse e Netto, 2011). Por outro lado, tem sido reportada uma correlação direta entre altos níveis de prolina e manifestações neurológicas presentes na HP II, como convulsões e retardo mental (Flynn et al., 1989; Phang et al., 2001; Wyse e Netto, 2011). No entanto,

os mecanismos pelos quais os sintomas da hiperprolinemia ocorrem são pouco compreendidos.

A detecção da HPI é baseada nos níveis elevados de prolina sérica, sem a presença de P5C na urina; porém o diagnóstico é feito por exclusão, pois a determinação direta da atividade da prolina oxidase não é realizada, uma vez que essa enzima não é expressa em leucócitos ou em cultura de fibroblastos, que seriam as amostras biológicas adequadas para a realização dos exames. A presença de P5C na urina associada aos elevados níveis plasmáticos de prolina é indicativa de HPII, sendo que a confirmação do diagnóstico é realizada pela avaliação da atividade enzimática da P5C desidrogenase em leucócitos ou cultura de fibroblastos (Phang et al., 2001).

Até o presente momento, não há um tratamento específico para as hiperprolinemias. As terapias alimentares visando a restrição de prolina são extremamente difíceis, pois a maioria das proteínas contém resíduos desse aminoácido. Além disso, por se tratar de um aminoácido não essencial, a prolina pode ser sintetizada endogenamente a partir de outros precursores. Dessa forma, a restrição alimentar de prolina reduz modestamente os níveis de prolina e parece não influenciar o fenótipo clínico desses pacientes (Mitsubuchi et al., 2008; Phang et al., 2001; Wyse e Netto, 2011).

1.4. Modelo animal exerimental de hiperprolinemia

Modelos animais são amplamente utilizados para a compreensão da fisiopatologia de doenças humanas. No presente estudo utilizamos um modelo experimental quimicamente induzido de hiperprolinemia, a fim de investigar os mecanismos envolvidos nos sintomas dessa doença, os quais são pouco

compreendidos. Nesse modelo, os ratos são submetidos a administrações de prolina a fim de induzir níveis plasmáticos desse aminoácido semelhantes àqueles encontrados em pacientes hiperprolinêmicos, principalmente com HP II (Moreira et al., 1989; Pontes et al., 1999).

1.5. Espécies reativas e estresse oxidativo

Radical livre é definido como uma espécie química que contém um ou mais elétrons desemparelhados, o que os torna altamente reativos (Halliwell e Gutteridge, 2007). Espécies reativas (ER) é um termo coletivo utilizado para designar tanto as espécies químicas radicais, como as não radicais que são agentes oxidantes e/ou são facilmente convertidas em radicais. São exemplos de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) os radicais superóxido ($O_2^{\bullet-}$), hidroxila ($OH^{\bullet}$) e óxido nítrico ($NO^{\bullet}$); e os não radicais peróxido de hidrogênio (H_2O_2), oxigênio singlet ($^1O_2$) e peroxinitrito ($ONOO^-$) (Halliwell e Whiteman, 2004).

As ER são constantemente produzidas no organismo em níveis basais, principalmente durante o processo de respiração celular, através da redução incompleta do oxigênio molecular (que ocorre de 2 a 5%). Essas ER desempenham funções fisiológicas importantes como fagocitose, sinalização celular, regulação de proteínas e plasticidade sináptica (Halliwell e Gutteridge, 2007). Porém, quando em excesso as ER induzem o estresse oxidativo, o qual é definido como um desequilíbrio entre os níveis de ER e das defesas antioxidantes, que pode ser resultado tanto do aumento da produção dessas espécies, como da diminuição dos níveis de antioxidantes ou da combinação de ambos (Halliwell e Gutteridge, 2007). Como consequência do estresse oxidativo

pode ocorrer: (1) adaptação celular por aumento da síntese e da atividade das defesas antioxidantes, protegendo a célula do dano de forma parcial ou completa; (2) lesão celular (dano oxidativo) dos alvos moleculares tais como lipídios, DNA, proteínas, carboidratos; ou (3) morte celular, uma vez que o dano oxidativo, principalmente ao DNA, pode desencadear morte celular por apoptose ou necrose (Halliwell e Whiteman, 2004).

As defesas antioxidantes enzimáticas e não enzimáticas constituem mecanismos essenciais para a detoxificação desses agentes oxidantes a fim de evitar os efeitos danosos das ER. As principais enzimas antioxidantes são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GSH-Px). As defesas antioxidantes não enzimáticas incluem substâncias como GSH, ácido ascórbico (vitamina C), α-tocoferol (vitamina E), melatonina, urato e outras (Halliwell e Gutteridge, 2007).

Evidências sugerem o envolvimento do estresse oxidativo na fisiopatologia de doenças neurodegenerativas como Huntington, Alzheimer e Parkinson (Mancuso et al., 2010; Petrozzi et al., 2007). Além disso, trabalhos realizados em nosso laboratório demonstraram substancial indução de estresse oxidativo cerebral em modelos animais de EIM como hiperprolinemia (Delwing et al., 2003a), hiperargininemia (Wyse et al., 2001b) e homocistinúria (Matte et al., 2004). De fato, o cérebro é altamente suscetível ao dano oxidativo, uma vez que esse órgão apresenta altos níveis de ferro e de neurotransmissores auto-oxidáveis, membrana neuronal rica em ácidos graxos poliinsaturados, elevado consumo de oxigênio, e por fim, modesta capacidade de defesa antioxidante (Halliwell, 2006).

O fígado é um órgão que apresenta alta resistência ao dano oxidativo, pois dispõe de elevada capacidade antioxidante e pode se adaptar facilmente às alterações metabólicas (Genet et al., 2002). No entanto, o estresse oxidativo tem sido reconhecido como um fator importante na fisiopatologia de doenças hepáticas como a hepatite aguda, a cirrose hepática e o carcinoma hepatocelular (Ha et al., 2010; Mantena et al., 2008). Nesse contexto, estudos realizados em nosso laboratório demonstraram que a hipermetioninemia (Stefanello et al., 2009) e a hiper-homocisteinemia (Matte et al., 2009) induzem estresse oxidativo e dano ao tecido hepático de ratos. Interessantemente, uma moderada hiperprolinemia pode ser observada em pacientes com distúrbios hepáticos, tais como cirrose (Shaw et al., 1984) e doença hepática alcoólica (Vargas-Tank et al., 1988).

1.6. Metabolismo energético

O cérebro possui pouca reserva energética, comparada à sua elevada atividade metabólica; portanto, necessita de um suprimento contínuo de glicose, seu principal substrato energético. A glicólise em conjunto com o ciclo de Krebs e a fosforilação oxidativa são as rotas essenciais para a produção de energia cerebral (Erecinska e Silver, 1994; Erecinska et al., 2004).

A glicólise é composta por uma sequência de reações que ocorrem no citosol e que formam como produto final o piruvato. O ciclo de Krebs ocorre na matriz mitocondrial e consiste de uma sequência de reações onde, em cada volta do ciclo, são formadas três moléculas de NADH, uma de $FADH_2$, duas de CO_2 e uma de GTP. O NADH e $FADH_2$ produzidos no ciclo de Krebs são carreadores de elétrons e são utilizados na cadeia respiratória para a produção de ATP na fosforilação oxidativa (Nelson e Cox, 2011).

A cadeia respiratória é composta por quatro complexos enzimáticos e dois transportadores móveis de elétrons (coenzima Q e citocromo c) que realizam a transferência dos elétrons desde as coenzimas reduzidas até o aceptor final, o oxigênio. Os elétrons provenientes do NADH são entregues ao complexo I (ou NADH desidrogenase ou NADH: ubiquinona oxirredutase) que transfere esses elétrons para a ubiquinona, formando ubiquinol. O complexo II (ou succinato: ubiquinona oxirredutase) é formado pela enzima succinato desidrogenase (SDH) e três subunidades hidrofóbicas. Esse complexo reduz a ubiquinona a ubiquinol com elétrons do $FADH_2$, proveniente da oxidação do succinato a fumarato no ciclo de Krebs. O complexo III (citocromo bc_1 ou ubiquinona-citocromo c oxirredutase) transfere elétrons do ubiquinol para o citocromo c. O complexo IV, também denominado citocromo c oxidase (COX), catalisa a transferência dos elétrons do citocromo c reduzido para o O_2, reduzindo-o a H_2O. O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, pelos complexos I, III e IV (Figura 4). Com isso, cria-se um gradiente eletroquímico transmembrana que é utilizado como força próton-motriz pelo complexo V (ATP sintase) para síntese de ATP partir de ADP e Pi, processo denominado fosforilação oxidativa (Nelson e Cox, 2011).

Deficiências no funcionamento normal da cadeia respiratória mitocondrial levam à diminuição da síntese de ATP. Nesse contexto, dados da literatura sugerem que uma diminuição do metabolismo energético pode estar implicada na fisiopatologia de alguns distúrbios neurológicos, como demência, isquemia cerebral e as doenças de Alzheimer e Parkinson (Beal, 2007; Gibson et al., 2009; Kowaltowski et al., 2009; Schurr, 2002).
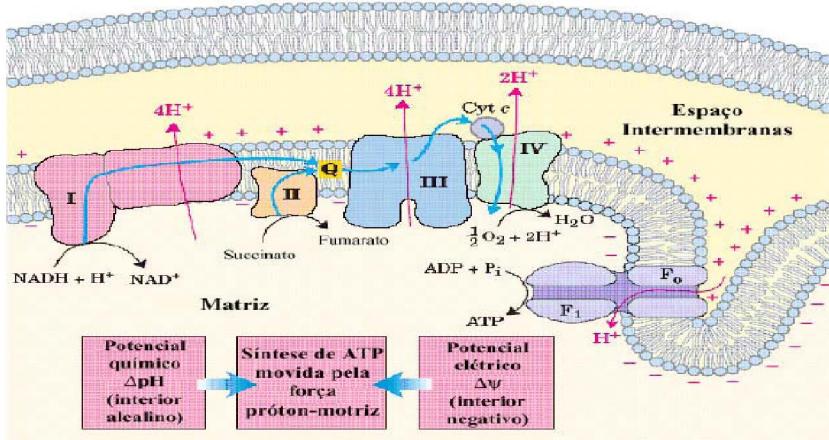
Figura 4. Fosforilação oxidativa (Nelson e Cox, 2006).

1.7. Na$^+$,K$^+$-ATPase

A Na$^+$,K$^+$-ATPase (EC 3.6.3.9) ou bomba de sódio é uma proteína integral de membrana responsável pela manutenção do gradiente iônico neuronal através do transporte ativo de três íons Na$^+$ para o meio extracelular e de dois íons K$^+$ para o meio intracelular. Esse processo ocorre as expensas de ATP, consumindo cerca de 50% do ATP produzido no cérebro (Erecinska et al., 2004). O gradiente iônico gerado pela Na$^+$,K$^+$-ATPase é utilizado para a manutenção da excitabilidade neuronal, regulação do volume celular e transporte de moléculas ligadas ao co-transporte de Na$^+$, como aminoácidos, glicose e neurotransmissores (Jorgensen et al. 2003; Kaplan 2002; Mobasheri et al. 2000).

Quanto a sua estrutura, a Na$^+$,K$^+$-ATPase é formada por duas subunidades catalíticas α, que contêm os sítios de ligação para Na$^+$, K$^+$, ATP e ouabaína (glicosídeo cardíaco e inibidor específico da enzima); duas subunidades β regulatórias, e uma subunidade γ com ação moduladora (Dempski et al., 2009) (Figura 5). Genes que codificam quatro isoformas da subunidade α da Na$^+$,K$^+$-ATPase (α$_1$, α$_2$, α$_3$ e α$_4$) e pelo menos três isoformas da subunidade β (β$_1$, β$_2$ e β$_3$)

foram identificados em mamíferos (Kaplan, 2002; Serluca et al., 2001). As isoformas combinam entre si, formando diversas isoenzimas que apresentam diferentes padrões de distribuição nos tecidos. No cérebro, principalmente três isoformas α (α_1, α_2, α_3) e duas β (β_1 e β_2) são expressas (Richards et al., 2007).
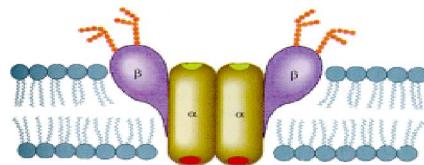
Figura 5. Estrutura da Na^+,K^+-ATPase (adaptado de Voet e Voet 2006).

Dados da literatura sugerem que a expressão e a atividade da Na^+,K^+-ATPase é modulada por múltiplos mecanismos, em resposta às necessidades celulares (Therien e Blostein, 2000). Nesse contexto, a atividade dessa enzima pode ser inibida por radicais livres, produtos de lipoperoxidação e alterações na fluidez da membrana (Dencher et al., 2007).

Aterações na atividade da Na^+,K^+-ATPase parecem estar implicadas na fisiopatologia de doenças neurodegenerativas como Alzheimer, esclerose múltipla, doença de Parkinson e epilepsia (Aperia, 2007; Benarroch, 2011; Hattori et al., 1998). Estudos pré-clínicos também têm demonstrado que as alterações na atividade dessa enzima estão envolvidas em condições que afetam o SNC, tais como isquemia (Wyse et al., 2000), depressão (de Vasconcellos et al., 2005; Gamaro et al., 2003) e EIM como a hiperprolinemia, a hiperargininemia e a homocistinúria (Pontes et al., 2001; Streck et al., 2002; Wyse et al., 2001a).

1.8. Sistema Glutamatérgico

O aminoácido L-glutamato é principal neurotransmissor excitatório no SNC de mamíferos, envolvido em diversas funções fisiológicas importantes tais como

aprendizado, memória, desenvolvimento e envelhecimento (Danbolt, 2001; Segovia et al., 2001). O glutamato é sintetizado nos neurônios pré-sinápticos a partir da glutamina proveniente das células gliais, em uma reação catalisada pela enzima glutaminase, e estocado em vesículas (Meldrum, 2000).

A liberação de glutamato na fenda sináptica ocorre por exocitose dependente de Ca^{2+}, decorrente da despolarização do terminal pré-sináptico (Attwell, 2000) (Figura 6). No meio extracelular, o glutamato exerce suas funções biológicas ao se ligar a receptores glutamatérgicos localizados na superfície das células neurais, principalmente nos neurônios pré e pós-sinápticos. Esses receptores são classificados como ionotrópicos ou metabotrópicos, conforme suas características moleculares. Os receptores ionotrópicos possuem um canal iônico em sua estrutura e, quando ativos, se tornam permeáveis a íons como Na^+, Ca^{2+} e K^+; esses receptores são subdivididos em NMDA, AMPA e cainato. Os receptores metabotrópicos são moléculas acopladas a proteínas G, que atuam modulando a atividade de enzimas como adenilato ciclase e fosfolipase C; esses receptores são subdivididos em receptores do grupo I, II e III (Danbolt, 2001).
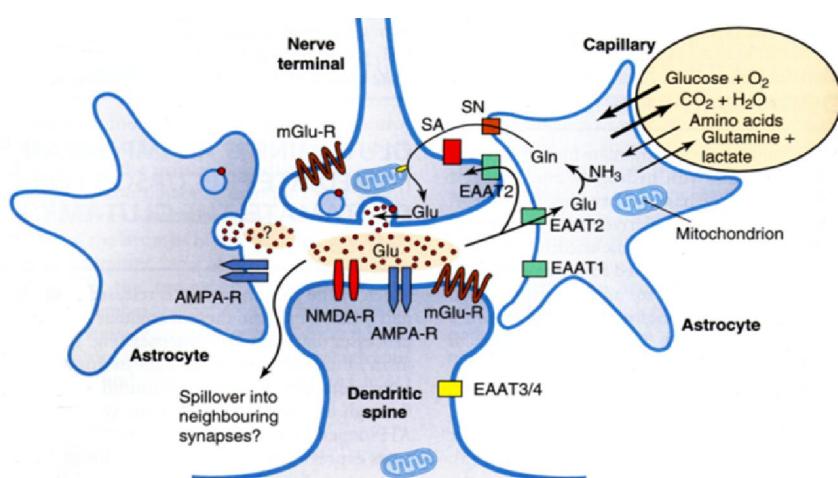
Figura 6. Sinapse glutamatérgica (Siegel et al., 2006).

EAAT1-4: transportador de aminoácidos excitatórios; Gln: glutamina; Glu: glutamato; NMDA-R e AMPA-R: receptores ionotrópicos de glutamato NMDA e AMPA, respecitvamente; mGlu-R: receptor metabotrópico de glutamato; SA e SN: sistema A e sistema N de transporte de glutamina.

Após o glutamato exercer sua ação nos receptores glutamatérgicos, a sinalização glutamatérgica é finalizada por um processo denominado captação de glutamato, onde o glutamato é removido da fenda sináptica por transportadores especializados, dependentes de Na$^+$, localizados principalmente nas membranas gliais (Anderson e Swanson, 2000). Há cinco diferentes isoformas de transportadores de glutamato, identificadas até o momento: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5, sendo que GLAST e GLT-1 são quantitativamente, os principais transportadores de glutamato em todas as regiões cerebrais, responsáveis por aproximadamente 90% da captação desse aminoácido (Danbolt, 2001).

O processo de captação de glutamato é realizado contra o gradiente de concentração, uma vez que a concentração desse aminoácido na fenda sináptica é aproximadamente cem vezes menor que a sua concentração intracelular e mil vezes menor que a sua concentração intravesicular (Nicholls, 2008). Para isso, os transportadores usam o gradiente eletroquímico gerado pela enzima Na$^+$,K$^+$-ATPase para o co-transporte de 3 Na$^+$: 1 H$^+$: 1 glutamato para o meio intracelular, enquanto que 1 íon K$^+$ é lançado para o meio extracelular, com gasto de 1 ATP (Kanner, 2006).

A amplitude da sinalização glutamatérgica depende basicamente da concentração de glutamato no fluido extracelular e do tempo de permanência do glutamato na fenda sináptica (Danbolt, 2001). Desse modo, cabe ressaltar a importância do funcionamento adequado dos transportadores para uma efetiva captação de glutamato, uma vez que a superestimulação dos receptores glutamatérgicos pode induzir excitotoxicidade, um processo que envolve influxo

de íons Na^+, Ca^{2+}, estresse oxidativo, déficit energético, e que pode culminar em morte neuronal (Maragakis e Rothstein, 2001; 2004; Nicholls, 2008).

Nesse contexto, a excitotoxicidade tem sido implicada na fisiopatologia de várias doenças agudas e crônicas do SNC incluindo epilepsia, isquemia e doença de Alzheimer (Danbolt, 2001; Maragakis e Rothstein, 2004). Estudos realizados em nosso laboratório mostraram que hiperprolinemia aguda prejudicou a captação de glutamato em cérebro de ratos (Delwing et al., 2007b).

1.9. Memória

A capacidade de aprender e recordar são funções essenciais do SNC. O aprendizado consiste na aquisição de novas informações ou novos conhecimentos através da experiência, enquanto a memória consiste na capacidade de armazenar e evocar essas informações (Squire e Kandel, 2003). Desse modo, a memória compreende pelo menos três fases, a aquisição, a consolidação e a evocação das informações (Izquierdo, 2002; Squire e Kandel, 2003).

As memórias podem ser classificadas de acordo com diversos critérios, por exemplo quanto ao conteúdo (declarativa ou não-declarativa), função (memória espacial de referência ou de trabalho), duração (curta ou longa duração), natureza (associativa ou não associativa) ou motivação (de recompensa ou aversiva) (Quillfeldt, 2010).

A memória declarativa é referente à informação que é transmitida ou expressa, enquanto a não-declarativa, também denominada de memória de procedimento, representa informações sobre habilidades motoras ou sensoriais que não podem ser transmitidas oralmente (Izquierdo, 2002).

A memória espacial envolve aspectos da memória declarativa e da não-declarativa, bem como de curta e longa duração (Moscovitch et al., 2006). É responsável pelo conhecimento, codificação, armazenamento e recuperação de informações sobre o arranjo espacial dos objetos ou rotas específicas (Kessels et al., 2001). A memória espacial pode ser dividida em memória espacial de referência ou de trabalho. A memória espacial de trabalho é transitória e precede as memórias de curta e longa duração. Ela serve para manter por alguns segundos, no máximo poucos minutos, a informação que está sendo processada no momento. A memória espacial de referência exibe uma maior capacidade de armazenamento, duração e resistência aos interferentes do que a memória espacial de trabalho (Izquierdo, 2002; Quillfeldt, 2010).

Um dos métodos amplamente utilizados para a avaliação da memória espacial em roedores é o labirinto aquático de Morris (Morris et al., 1982; Netto et al., 1993; Quillfeldt, 2010). Lesões no hipocampo, córtex cerebral e estriado parecem prejudicar o desempenho dos animais nessa tarefa (D'Hooge e De Deyn, 2001). Estudos realizados em nosso laboratório mostraram que ratos submetidos à hiperprolinemia crônica durante uma importante etapa do desenvolvimento do SNC (6º ao 28º dia de vida), apresentam déficit na memória espacial quando testados no labirinto aquático de Morris na idade adulta (a partir dos 60º dia de vida) (Bavaresco et al., 2005; Delwing et al., 2006).

Dados da literatura apontam para a participação dos sistemas glutamatérgico e gabaérgico, bem como proteínas cinases e mecanismos hormonais nos processos de memória (Cammarota et al., 2005; Izquierdo e Medina, 1997). Além disso, tem sido proposto o envolvimento da Na^+,K^+-ATPase, acetilcolinesterase (AChE) e do fator neurotrófico derivado do encéfalo (BDNF) na

modulação da cognição (Ballard et al., 2005; Lu et al., 2005; Sato et al., 2004; Wyse et al., 2004).

1.10. Acetilcolinesterase

O sistema colinérgico é uma das mais importantes vias de modulação do SNC, desempenhando um papel fundamental em várias funções vitais, como aprendizado, memória, motivação, recompensa, fluxo sanguíneo cerebral e processamento sensorial e motor (Sofuoglu e Mooney, 2009).

A acetilcolina (ACh) é um neurotransmissor clássico, sintetizado pela enzima colina acetiltransferase a partir de acetato e colina, e armazenado em vesículas no neurônio pré-sináptico. A atividade colinérgica é controlada principalmente pela enzima AChE que hidrolisa rapidamente a ACh nas sinapses colinérgicas e junção neuromuscular finalizando a transmissão colinérgica (Zimmerman e Soreq, 2006).

A AChE (EC 3.1.1.7) é uma serina hidrolase, que pertence à família α/β hidrolase e apresenta alta especificidade para o neurotransmissor ACh (Soreq e Seidman, 2001). O sítio ativo da AChE é formado por um sítio esterásico, que contém uma tríade catalítica composta pelos aminoácidos serina, histidina e glutamato e por um sítio de ligação da colina ou aniônico (Lane et al., 2006; Soreq e Seidman, 2001; Zimmerman e Soreq, 2006).

Além do seu papel clássico na transmissão colinérgica, a AChE apresenta outras funções não colinérgicas, como o crescimento de neuritos (Layer et al., 1993), diferenciação pós-sináptica (Chacon et al., 2003), hematopoiese, osteogênese (Grisaru et al., 1999), adesão celular (Silman e Sussman, 2005) e regulação de funções imunes (Kawashima e Fujii, 2000).

A hipofunção colinérgica parece estar associada a prejuízos cognitivos característicos de algumas doenças neurodegenerativas. Nesse contexto, o tratamento com inibidores da AChE aumenta os níveis de ACh, o que parece melhorar o aprendizado e a memória em pacientes com a doença de Alzheimer (Ballard et al., 2005).

1.11. Fator Neurotrófico Derivado do Encéfalo

O fator neurotrófico derivado do encéfalo (BDNF, do inglês - *brain-derived neurotrophic factor*), a neurotrofina mais abundante do SNC, apresenta funções relacionadas a importantes eventos, tais como neurogênese, um processo intimamente relacionado à plasticidade sináptica, sobrevivência neuronal e a formação/modulação de sinapses glutamatérgicas e gabaérgicas (Bekinschtein et al., 2008; Berchtold et al., 2010; Gottmann et al., 2009).

O BDNF é sintetizado no retículo endoplasmático como um pró-peptídeo (pró-BDNF), estocado em vesículas no complexo de Golgi, podendo ser proteoliticamente transformado na sua forma madura (mBDNF). O pró-BDNF e o mBDNF são secretados de maneira dependente da atividade sináptica (Pang e Lu, 2004) e ligam-se aos receptores de quinase relacionados à tropomiosina (TrkB) e ao receptor pró-apoptótico p75[NTR] (Huang e Reichardt, 2003). Porém, a maioria dos efeitos biológicos conhecidos do BDNF são causados pela ativação do receptor TrkB que leva à ativação das vias de sinalização da proteína quinase ativada por mitógeno (MAPK), fosfolipase C e fosfoinositídeo 3-quinase (PI3K) (Kaplan e Miller, 2000). Nesse contexto, foi demonstrado que as vias de sinalização PI3K/Akt são importantes para a formação da memória espacial (Bekinschtein et al., 2008; Mizuno et al., 2000).

Diversas evidências sugerem que o BDNF desempenha um importante papel no processamento das diferentes fases da memória. Estudos mostram que o BDNF facilita a liberação de glutamato e aumenta a fosforilação e expressão dos receptores AMPA e NMDA (Carvalho et al., 2008; Tyler et al., 2002). Além disso, camundongos mutantes BDNF-deficientes, apresentam déficit na retenção da memória em tarefas de avaliação da memória espacial (Linnarsson et al., 1997), reforçando a hipótese do envolvimento dessa neurotrofina na memória.

1.12 Vitaminas E e C

O α-tocoferol (vitamina E) é um potente antioxidante lipossolúvel que possui a propriedade de finalizar a propagação de reações dos radicais livres nas membranas lipídicas. Tem sido relatado que os radicais peroxil (ROO$^\bullet$) formados durante a lipoperoxidação reagem cerca de mil vezes mais rápido com a vitamina E do que com os ácidos graxos poliinsaturados (PUFA) (Buettner, 1993). O grupo hidroxila do tocoferol reage com o radical peroxil para formar o correspondente hidroperóxido lipídico e o radical α-tocoferil, que é pouco reativo e não ataca as biomoléculas (Halliwell e Gutteridge, 2007; Traber e Stevens, 2011).

O ácido ascórbico (vitamina C), é uma vitamina hidrossolúvel conhecida por sua importante ação antioxidante nos sistemas biológicos. Fisiologicamente o ácido ascórbico encontra-se na forma do ânion ascorbato, que em função do seu baixo potencial de redução, caracteriza-se por ser um varredor de radicais livres de amplo espectro, eficaz contra radicais peroxil, hidroxil, superóxido, oxigênio *singlet* e peroxinitrito (Rice, 2000). Além disso, a vitamina C tem a importante função de regenerar a vitamina E através da redução do radical α-tocoferil (forma oxidada da vitamina E), o que prolonga seu efeito antioxidante e permite que a

vitamina E desempenhe seu papel como inibidor da peroxidação lipídica nas membranas célulares (Traber e Stevens, 2011).

Os possíveis efeitos benéficos da suplementação com vitaminas E e C são frequentemente investigados em doenças neurodegenerativas, nas quais o estresse oxidativo parece ter um papel importante (Beal, 2007; Halliwell et al., 1992; Kowaltowski et al., 2009). Nesse contexto, estudos mostram que a vitamina E é capaz de retardar a progressão do déficit cognitivo na doença de Alzheimer (Mecocci, 2004; Sano et al., 1997). Estudos realizados no nosso laboratório demonstraram que a administração das vitaminas E e C é capaz de prevenir o déficit cognitivo de ratos submetidos à hiperprolinemia (Delwing et al., 2006), bem como a inibição das atividades de enzimas cerebrais como a AChE e a COX causadas pela administração de prolina (Delwing et al., 2005b; 2007a).

1.13. Guanosina

A guanosina é um nucleosídeo descrito como um potencial neuroprotetor em modelos experimentais *in vitro* e *in vivo* de doenças cerebrais associadas à excitotoxicidade glutamatérgica (Chang et al., 2008; Lara et al., 2001; Schmidt et al., 2000; 2009). Nesse contexto, estudos mostram que a administração de guanosina é capaz de prevenir convulsões induzidas pelo ácido quinolínico, um agonista glutamatérgico (Lara et al., 2001; Schmidt et al., 2000). Além disso, ratos submetidos ao modelo de hipóxia e isquemia apresentaram prejuízo na captação de glutamato, cujo efeito foi prevenido pela administração de guanosina (Moretto et al., 2009). Recentemente, foi reportado um efeito antioxidante indireto da guanosina, possivelmente relacionado a sua capacidade de estimular a captação de glutamato, reduzindo os níveis de glutamato extracelular e consequentemente

o estresse oxidativo que acompanha a hiperestimulação dos receptores glutamatérgicos (Roos et al., 2009).

Os mecanismos moleculares pelos quais a guanosina exerce seus efeitos neuroprotetores não são conhecidos. No entanto, dados sugerem a existência de um sítio de ligação específico para a guanosina na membrana plasmática, uma vez que os efeitos da guanosina parecem ser mediados através da via de sinalização dependente de proteína G envolvendo as rotas da MAPK e da PI3K/Akt/PKB (Di Iorio et al., 2004; Rathbone et al., 2008). Além disso, a guanosina parece estimular a liberação de fatores tróficos como o BDNF e o fator de crescimento neural (NGF) (Rathbone et al., 2008).

1.14. Exercício Físico

Diversas evidências na literatura sugerem que o exercício físico pode apresentar benefícios para a saúde especialmente sobre a função cognitiva, diminuindo o declínio mental associado com o avanço da idade e o risco para doenças neurodegenerativas (Cotman e Berchtold, 2002; Kramer et al., 2005).

Estudos recentes utilizando modelos animais têm contribuído para a compreensão das bases neurobiológicas dos efeitos benéficos do exercício físico, os quais sugerem que o exercício voluntário pode induzir a plasticidade sináptica (Christie et al., 2008; Farmer et al., 2004; Vaynman et al., 2004b), promover a vascularização cerebral (Cotman e Berchtold, 2002; Pereira et al., 2007) e facilitar a recuperação após injúria cerebral (Griesbach et al., 2007).

O efeito neuroprotetor do exercício físico, bem como sua influência sobre o aprendizado e a memória, têm sido atribuídos à sua capacidade de induzir neurogênese (Farmer et al., 2004; van Praag et al., 1999; 2005; Vaynman et al.,

2004b). Ernst e colaboradores (2006) observaram o aumento da neurogênese em ratos adultos após três dias de exercício físico, embora esse aumento tenha sido mais expressivo após uma semana de atividade física. Em adultos, a neurogênese ocorre no giro denteado do hipocampo e na zona subventricular adjacente ao ventrículo lateral, podendo ocorrer também no neocórtex (Ernst et al., 2006; Takemura, 2005; von Bohlen Und Halbach, 2011).

Um dos principais mecanismos propostos para os efeitos benéficos do exercício sobre a cognição é o aumento nos níveis de BDNF, o qual parece estar relacionado à sobrevivência neuronal, diferenciação, alteração da plasticidade sináptica e memória (Berchtold et al., 2001; Cotman e Berchtold, 2002; Griesbach et al., 2007; Sim et al., 2004; Yamada et al., 2002). Estudos mostram que o exercício pode aumentar os níveis de RNAm e a proteína BDNF e que esse aumento pode se manter por várias semanas após a realização do exercício (Cotman e Berchtold, 2002; Radak et al., 2006; Vaynman et al., 2003; 2004a).

Os benefícios do exercício físico também estão relacionados ao seu efeito protetor contra a ação dos radicais livres (Radak et al., 2006). Nesse sentido, estudos mostram que o exercício diminui a incidência de doenças associadas ao estresse oxidativo (Radak et al., 2008; Radak et al., 2010). Por outro lado, tem sido demonstrado que há geração de ERO durante o exercício (Powers e Jackson, 2008; Ristow e Schmeisser, 2011), o que promove a ativação do sistema antioxidante e consequente adaptação do organismo ao estresse oxidativo (Radak et al., 2008).

II. OBJETIVOS

2.1. Objetivo geral

A fim de melhor compreender os mecanismos envolvidos nas alterações neurológicas presentes na hiperprolinemia, o objetivo geral desse trabalho foi investigar o efeito da prolina sobre parâmetros neuroquímicos e comportamentais em ratos submetidos ao modelo animal experimental de hiperprolinemia, tais como metabolismo energético, estresse oxidativo, sistema glutamatérgico e à memória. Além disso, investigamos os possíveis efeitos neuroprotetores das vitaminas E e C, da guanosina e do exercício físico sobre as alterações bioquímicas e comportamentais causadas pela prolina. Considerando que o fígado é um dos principais órgãos envolvidos no catabolismo da prolina, também avaliamos alguns parâmetros de estresse oxidativo, bem como o perfil metabólico e morfológico no tecido hepático de ratos hiperprolinêmicos.

2.2. Objetivos específicos:

Os objetivos específicos estão subdivididos em cinco capítulos, que serão apresentados na forma de artigos científicos, como segue:

➢ **Capítulo I**

• Investigar o efeito da administração aguda de prolina sobre a atividade das enzimas SDH e COX, a produção CO_2 a partir de [U-^{14}C]glicose e [1-^{14}C]acetato, bem como sobre os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) em córtex cerebral de ratos no período neonatal e de ratos jovens;

- Verificar a influência do pré-tratamento com vitaminas E e C sobre o efeito causado pela administração aguda de prolina sobre as atividades da SDH e COX, bem como sobre os níveis de TBARS em córtex cerebral de ratos no período neonatal e de ratos jovens.

➤ Capítulo II

- Investigar o efeito da hiperprolinemia crônica e a influência da co-administração das vitaminas E e C sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica e a expressão gênica das subunidades catalíticas (isoformas α1, α2 e α3) dessa enzima, bem como sobre os níveis de TBARS em córtex cerebral de ratos jovens.

➤ Capítulo III

- Verificar o efeito da administração aguda e crônica de prolina sobre a captação do glutamato e o imunoconteúdo dos transportadores de glutamato GLAST e GLT-1 em córtex cerebral de ratos jovens;

- Verificar o efeito da administração aguda e crônica de prolina sobre a atividade da Na^+,K^+-ATPase (em homogenato) e os níveis de ATP em córtex cerebral de ratos jovens;

- Avaliar a influência do pré-tratamento com guanosina sobre o efeito causado pela administração aguda de prolina sobre a captação de glutamato, imunoconteúdo de GLAST e GLT-1, Na^+,K^+-ATPase, níveis de ATP e TBARS em córtex cerebral de ratos jovens;

• Analisar o efeito do tratamento concomitante com guanosina à administração crônica de prolina sobre a captação de glutamato, imunoconteúdo de GLAST e GLT-1, Na$^+$,K$^+$-ATPase e os níveis de ATP e TBARS em córtex cerebral de ratos jovens.

➢ **Capítulo IV**

• Investigar o efeito do exercício físico, realizado em ratos do 30º ao 60º dia de vida, sobre o aprendizado e a memória espacial na tarefa do labirinto aquático de Morris em ratos adultos submetidos à hiperprolinemia crônica (6º ao 29º dia de vida). Esse protocolo experimental de hiperprolinemia associada ao exercício físico foi denominado *protocolo para hiperprolinemia de tempo parcial*;

• Investigar o efeito do exercício físico, realizado do 30º ao 60º dia de vida dos ratos, sobre o aprendizado e a memória espacial na tarefa do labirinto aquático de Morris em ratos adultos submetidos à hiperprolinemia crônica do 6º ao 60º dia de vida. Esse protocolo experimental de hiperprolinemia associada ao exercício físico foi denominado *protocolo para hiperprolinemia de tempo total*;

• Verificar o efeito da hiperprolinemia crônica e do exercício físico sobre o imunoconteúdo de BDNF e a atividade da enzima AChE em hipocampo e córtex cerebral de ratos adultos submetidos aos *protocolos para hiperprolinemia de tempo parcial e total*.

➢ **Capítulo V**

• Investigar o efeito da administração crônica de prolina sobre parâmetros de estresse oxidativo, denominados potencial antioxidante total não enzimático

(TRAP), TBARS, oxidação do DCFH, níveis de GSH, sulfidrilas e carbonilas, bem como sobre a atividade das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH-Px) em fígado de ratos jovens;

- Analisar a histologia do fígado em relação à morfologia, presença de células inflamatórias e ao conteúdo de glicogênio/glicoproteínas no tecido hepático de ratos jovens submetidos à administração crônica de prolina;

- Avaliar o efeito da administração crônica de prolina sobre o metabolismo hepático através da determinação da taxa de oxidação da glicose a CO_2, e da síntese e conteúdo de glicogênio e lipídios;

- Determinar a glicemia e as atividades de enzimas marcadoras de dano hepático tais como alanina aminotransferase (ALT) e aspartato aminotransferase (AST) em soro de ratos submetidos à administração crônica de prolina.

III. METODOLOGIA E RESULTADOS

3.1. Modelos experimentais

Os capítulos I, II, III, IV e V serão apresentados na forma de artigos científicos, os quais apresentam desenhos experimentais semelhantes entre si no que se refere aos modelos de hiperprolinemia e aos tratamentos utilizados como possibilidades de neuroproteção (vitaminas, guanosina e exercício físico).

3.1.1. *Modelo experimental de hiperprolinemia aguda:* Os ratos foram submetidos a uma única administração subcutânea de prolina e são sacrificados 1 h depois. Nesse modelo, o ***pré-tratamento com vitaminas E e C*** ou ***guanosina*** é realizado durante 7 dias através de uma administração intraperitoneal diária da substância. No oitavo dia, os animais foram submetidos a uma única administração aguda de prolina.

a) Pré-tratamento com vitaminas E e C ou guanosina, seguido de hiperprolinemia aguda (ratos de 12 dias):
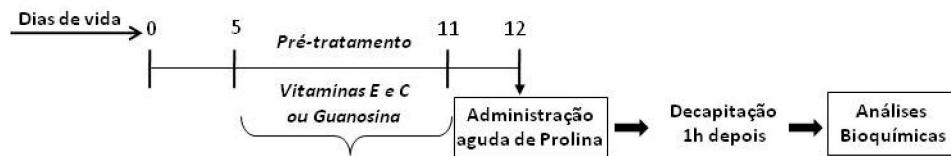
b) Pré-tratamento com vitaminas E e C ou guanosina, seguido de hiperprolinemia aguda (ratos de 29 dias):
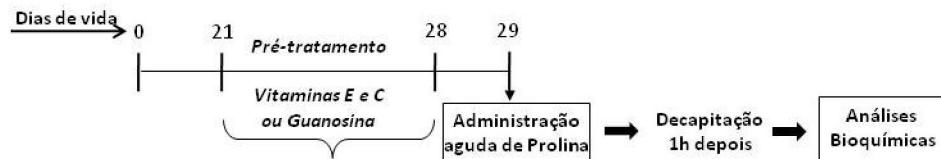
3.1.2. Modelo experimental de hiperprolinemia crônica: Os ratos foram submetidos a duas injeções subcutâneas diárias de prolina do 6º ao 28º dia de vida. O tratamento concomitante **com vitaminas E e C** ou **guanosina** é realizado durante todo o período de tratamento com prolina (6º ao 28º dia) através de uma administração

intraperitoneal diária da substância. Os animais foram sacrificados 12 h após as últimas administrações.

Modelo experimental de hiperprolinemia crônica com ou sem administração concomitante de vitaminas E e C ou guanosina:
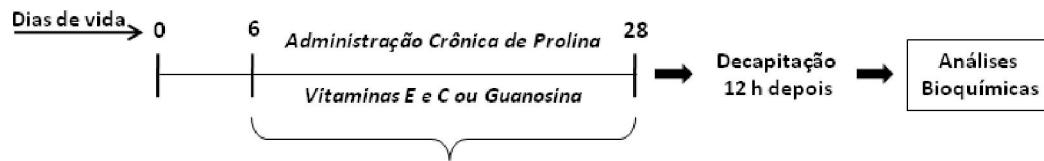
3.1.3. Modelo experimental de hiperprolinemia crônica associada ao exercício físico: Os ratos foram submetidos a duas injeções subcutâneas diárias de prolina do 6º ao 28º dia de vida (no protocolo para *hiperprolinemia de tempo parcial*) ou do 6º ao 60º dia de vida (no protocolo para *hiperprolinemia de tempo total*). Em ambos os protocolos experimentais o exercício físico em esteira foi realizado do 30º ao 60º dia de vida. Após o término do período de exercícios, os animais foram submetidos aos testes comportamentais no labirinto aquático de Morris.

a) Protocolo experimental para hiperprolinemia de tempo parcial:
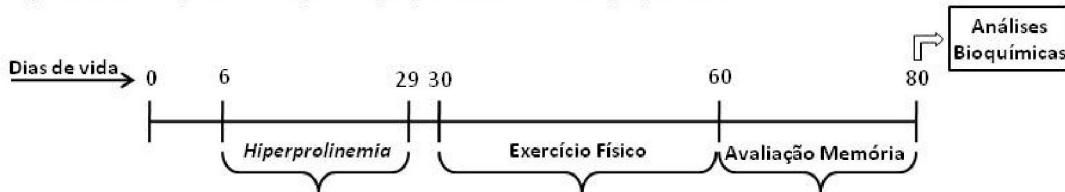
b) Protocolo experimental para hiperprolinemia de tempo total:

Capítulo I

ORIGINAL PAPER

Proline impairs energy metabolism in cerebral cortex of young rats

Andréa G. K. Ferreira · Daniela D. Lima · Débora Delwing · Vanize Mackedanz ·
Bárbara Tagliari · Janaína Kolling · Patrícia F. Schuck · Moacir Wajner ·
Angela T. S. Wyse

Periódico: Metabolic Brain Disease

Status: Publicado

ORIGINAL PAPER

Proline impairs energy metabolism in cerebral cortex of young rats

Andréa G. K. Ferreira · Daniela D. Lima · Débora Delwing · Vanize Mackedanz · Bárbara Tagliari · Janaína Kolling · Patrícia F. Schuck · Moacir Wajner · Angela T. S. Wyse

Abstract In the present study we investigated the effect of acute hyperprolinemia on some parameters of energy metabolism, including the activities of succinate dehydrogenase and cytocrome c oxidase and $^{14}CO_2$ production from glucose and acetate in cerebral cortex of young rats. Lipid peroxidation determined by the levels of thiobarbituric acid-reactive substances, as well as the influence of the antioxidants α-tocopherol plus ascorbic acid on the effects elicited by Pro on enzyme activities and on the lipid peroxidation were also evaluated. Wistar rats of 12 and 29 days of life received one subcutaneous injection of saline or proline (12.8 or 18.2 μmol/g body weight, respectively) and were sacrificed 1 h later. In another set of experiments, 5- and 22-day-old rats were pretreated for a week with daily intraperitoneal administration of α-tocopherol (40 mg/kg) plus ascorbic acid (100 mg/kg) or saline. Twelve hours after the last injection, rats received one injection of proline or saline and were sacrificed 1 h later. Results showed that acute administration of proline significantly reduced cytochrome c oxidase activity and increased succinate dehydrogenase activity and $^{14}CO_2$ production in cerebral cortex, suggesting that Pro might disrupt energy metabolism in brain of young rats. In addition, proline administration increased the thiobarbituric acid-reactive substances levels, which were prevented by antioxidants. These findings suggest that mitochondrial dysfunction and oxidative stress may be important contributors to the neurological dysfunction observed in some hyperprolinemic patients and that treatment with antioxidants may be beneficial in this pathology.

A. G. K. Ferreira · V. Mackedanz · B. Tagliari · J. Kolling ·
P. F. Schuck · M. Wajner · A. T. S. Wyse
Laboratório de Neuroproteção e Doença Metabólica,
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde,
Universidade Federal do Rio Grande do Sul,
Rua Ramiro Barcelos, 2600-Anexo,
Porto Alegre, RS, Brazil

D. D. Lima
Centro de Ciências da Saúde,
Universidade Comunitária Regional de Chapecó,
Avenida Senador Attílio Fontana, 591-E,
CEP 89809-000 Chapecó, SC, Brazil

D. D. Lima
Departamento de Ciências da Saúde,
Universidade Regional Integrada do Alto Uruguai e das Missões -
Campus de Frederico Westphalen,
Rua Assis Brasil, 709,
CEP 98400-000 Frederico Westphalen, RS, Brazil

D. Delwing
Departamento de Ciências Naturais,
Centro de Ciências Exatas e Naturais,
Universidade Regional de Blumenau,
Rua Antônio da Veiga, 140,
CEP 89012-900 Blumenau, SC, Brazil

A. T. S. Wyse (✉)
Departamento de Bioquímica, ICBS,
Universidade Federal do Rio Grande do Sul,
Rua Ramiro Barcelos, 2600-Anexo,
CEP 90035-003 Porto Alegre, RS, Brazil
e-mail: wyse@ufrgs.br

Introduction

Hyperprolinemia type II (HP II) is an inborn error of amino acid metabolism characterized by tissue accumulation of proline (Pro) due to deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity. A considerable number of

affected patients present neurological manifestations including seizures and mental retardation (Phang et al. 2001). Furthermore, a relationship between high concentration of Pro and neurological symptoms has been demonstrated (Flynn et al. 1989; Phang et al. 2001).

Abnormalities of energy metabolism and oxidative stress seem to underlie the pathophysiology of conditions affecting the central nervous system (CNS) such as seizures, dementia, cerebral ischemia, Alzheimer's disease and Parkinson's disease (Heales et al. 1999; Blass 2001; Schurr 2002; Beal 2005; Petrozzi et al. 2007). In this context, it has been reported that high Pro levels may cause alterations in the cell redox, giving rise to a decrease in the oxygen consumption necessary to oxidize the NADH formed by the cell, leading to a decrease of energy production (Phang et al. 2001).

Although the mechanisms underlying the symptoms in HP II are still obscure, there are some evidence suggesting the involvement of oxidative stress and energy deficit in this disorder. In this respect, we have previously reported that rats subjected to a chemical experimental model of hyperprolinemia type II (Pontes et al. 1999) increase lipid peroxidation and decrease the brain antioxidant defenses (Delwing et al. 2003a, b). Additionally, these alterations caused by hyperprolinemia were prevented by the antioxidants α-tocopherol plus ascorbic acid, suggesting that free radical formation was probably involved in such effects (Delwing et al. 2005). We also demonstrated that Pro administration alters the activities of enzymes considered critical for normal CNS function, such as Na^+,K^+-ATPase, creatine kinase and citocrome c oxidase (Pontes et al. 1999, 2001; Kessler et al. 2003; Delwing et al. 2007a).

The tricarboxylic-acid (TCA) cycle and the electron transport chain are essential routes for the production of cellular energy. Impairment of energy metabolism has been associated to brain damage as a consequence of the reduction of ATP production and of the generation of reactive oxygen species (ROS) (Beal 2005; Bubber et al. 2005). In this scenario, it has been demonstrated that the CNS is especially vulnerable to oxidative damage (Nunomura et al. 2006) and particularly sensitive to disturbances of energy generation (Erecinska et al. 2004).

In the present study we investigated the effect of acute Pro administration on some parameters of energy metabolism and oxidative stress. We determined the activities of succinate dehydrogenase (SDH), an enzyme involved in both the TCA and the electron transport chain and cytochrome c oxidase, a crucial complex of the respiratory chain, as well as $^{14}CO_2$ production from [U-^{14}C] glucose and [1-^{14}C] acetate in cerebral cortex of rats. Thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, as well as the influence of the antioxidants α-tocopherol plus ascorbic acid on the effects elicited by Pro on enzymes activities and on the lipid peroxidation

were also tested in order to evaluate the involvement of free radicals in these effects.

Materials and methods

Animals and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. The animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22±1°C), with free access to water and commercial protein chow. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology. Our study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Proline and α-tocopherol plus ascorbic acid administration

We performed two sets of experiments. First, rats received a single subcutaneous injection of saline (control) or Pro. Rats of 12 days of life received 12.8 μmol of Pro/g body weight, while 29-day-old rats received 18.2 μmol of Pro/g body weight. Pro was dissolved in 0.9% NaCl and the pH was adjusted to 7.2–7.4 with 0.1 N NaOH. The animals were killed 1 h after injection by decapitation without anaesthesia.

For the second set of experiments, 5- and 22-day-old rats were pretreated for a week with daily intraperitoneal administration of saline (control) or α-tocopherol (40 mg/kg) plus ascorbic acid (100 mg/kg), according to protocols previously described (Fighera et al. 1999; Wyse et al. 2002; Delwing et al. 2006). Twelve hours after the last injection, animals received one injection of saline or Pro as described above, and were killed 1 h later.

Tissue and homogenate preparation

After decapitation, the brain was immediately removed and the cerebral cortex was dissected and kept chilled until homogenization. For the determination of enzymatic activities, cerebral cortex was homogenized (1:20, w/v) in SETH (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI mL^{-1} heparin) buffer, pH 7.4. The homogenates were centrifuged at 800 x g for 10 min and the supernatants kept at−70°C until used for enzyme activity determination. The maximum period between homogenate preparation and enzyme analysis was always less than 5 days. For $^{14}CO_2$ production, the animals were killed on the day of the experiments, the brain was rapidly removed and the

cerebral cortex was homogenized (1:10, w/v) in Krebs-Ringer bicarbonate buffer pH 7.4. For TBARS assays, 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. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

Succinate dehydrogenase activity

The activity of SDH was determined according to Fischer et al. (1985). Immediately before the assay, the samples were frozen and thawn three times to break mitochondrial membranes. The enzymatic activity was measured following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1$ mM^{-1}cm^{-1}) in the presence of phenazine methasulfate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 μM DCIP was preincubated with 40–80 μg homogenate protein at 30°C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone, and 40 μM DCIP were added. The reaction was initiated by the addition of 1 mM PMS and monitored for 5 min.

Cytochrome c oxidase activity

The activity of cytochrome c oxidase was determined according to Rustin et al. (1994). Enzymatic activity was measured by following the decrease in absorbance due to oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$ mM^{-1}cm^{-1}). The reaction buffer contained 10 mM potassium phosphate pH 7.0, 0.6 mM n-dodecyl-β-D-maltoside and 2–4 μg homogenate protein. The reaction was initiated by the addition of 0.7 μg reduced cytochrome c and monitored for 10 min.

$^{14}CO_2$ production

$^{14}CO_2$ production was evaluated in homogenates added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated in a metabolic shaker at 35°C for 15 min (90 oscillations/min) with 625 μM n-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. Thereafter, one of the following substrates was added: [U-^{14}C] glucose (0.055 μCi) plus 5.0 mmol/L of unlabelled glucose or [1-^{14}C] acetate (0.055 μCi) plus 1.0 mmol/L of unlabelled acetate. The flasks were gassed with an O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and parafilm M. Glass centre wells containing a folded 65 mm x 5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation, 0.2 ml of 50% trichloroacetic acid was added to the medium and 0.1 ml of benzethonium

hydroxide was added to the centre of the wells via needles introduced through the rubber stopper. The flasks were left to stand for a further 30 min to complete $^{14}CO_2$ trapping and then openned. The filter papers were removed and added to vials containing Opti Phase "Hi-safe" 3 scintillation fluid, and radioactivity was counted (Dutra-Filho et al. 1995). Results are expressed as nmol CO_2/h/g tissue.

Thiobarbituric Acid Reactive Species (TBARS)

TBARS levels were determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid were added to 500 μl of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at $1,000 \times g$ for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The results were reported as nmol of TBARS per mg protein.

Protein determination

The protein content of cerebral cortex homogenates was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's t-test or by one-way ANOVA followed by the Duncan multiple range test when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $p < 0.05$ were considered to be significant.

Results

We first investigated the effect of acute administration of Pro on cytochrome c oxidase and SDH in rat cerebral cortex. Figure 1 shows that SDH activity (A) was significantly increased in cerebral cortex of 12-day-old rats [$t(6) = 4.26$; $p < 0.01$] and 29-day-old rats [$t(6) = 3.37$; $p < 0.05$] subjected to acute Pro administration. Conversely, administration of Pro significantly decreased cytochrome c oxidase activity (B) in the cerebral cortex of 12-day-old [$t(8) = 6.37$; $p < 0.001$] and 29-day-old rats [$t(8) = 3.84$; $p < 0.01$], as compared to the control group.

Next, we evaluated the effects of Pro administration on $^{14}CO_2$ production from glucose and acetate in cerebral cortex of rats. Figure 2 shows that a single Pro injection
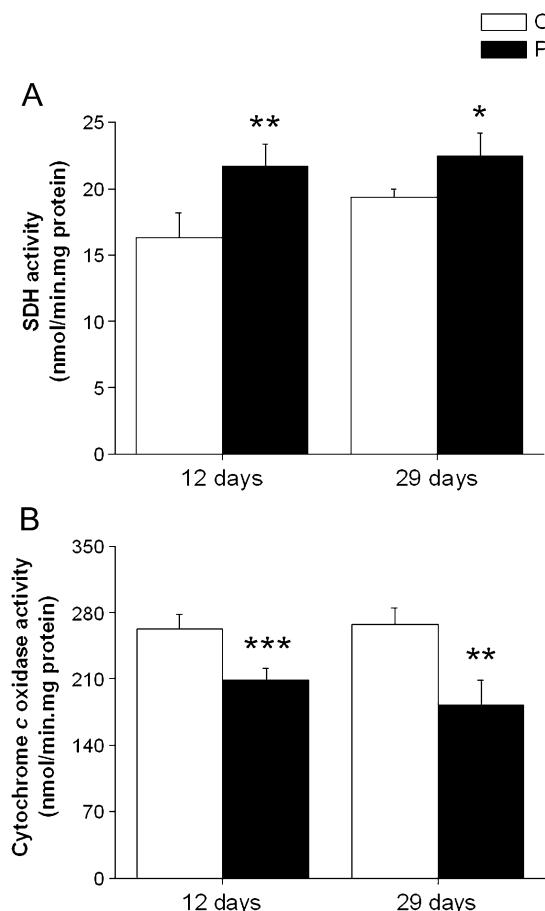
Fig. 1 Effect of acute administration of proline on the activities of succinate dehydrogenase (a) and cytochrome c oxidase (b) in cerebral cortex of 12- and 29-day-old rats. Data are mean ± S.D. for 4–5 animals in each group. The results are expressed in nmol/min mg protein. Different from control, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (Student's t-test for unpaired samples)

Fig. 2 Effect of acute administration of proline on $^{14}CO_2$ production from [U-^{14}C] glucose (a) and [1-^{14}C] acetate (b) in cerebral cortex of 12- and 29-day-old rats. Data are mean ± S.D. for 5–7 animals in each group. Different from control, * $p<0.05$ (Student's t-test for unpaired samples)

significantly increased $^{14}CO_2$ production from glucose (A) in cerebral cortex of 12 [$t(9)=3.25$; $p<0.05$] and 29-day-old rats [$t(11)=2.54$; $p<0.05$]. Similarly, acute administration of Pro significantly increased $^{14}CO_2$ production from acetate (B) in cerebral cortex of 12 [$t(12)=2.29$; $p<0.05$] and 29-day-old rats [$t(11)=2.33$; $p<0.05$], as compared to the control group.

We also measured TBARS levels in cerebral cortex of rats submitted to Pro administration. Figure 3 shows that Pro administration increased TBARS in cerebral cortex of 12-day-old [$t(8)=3.11$; $p<0.05$] and 29-day-old rats [$t(10)=5.25$; $p<0.001$].

Finally, we evaluated the effect of pretreatment for a week with antioxidants (α-tocopherol plus ascorbic acid) on the effects elicited by Pro on SDH and cytochrome c oxidase activities, as well as on TBARS levels. As can be seen in Figs. 4 and 5, the antioxidants *per se* did not alter the enzyme activities, but prevented the increase of SDH activity (12-day-old: [$F(3, 13)=4.46$; $p<0.05$]; 29-day-old:

Fig. 3 Effect of acute administration of proline on thiobarbituric acid-reactive substances in cerebral cortex of 12- and 29-day-old rats. Data are mean ± S.D. for 5 animals in each group. Different from control, * $p<0.05$, *** $p<0.001$ (Student's t-test for unpaired samples)
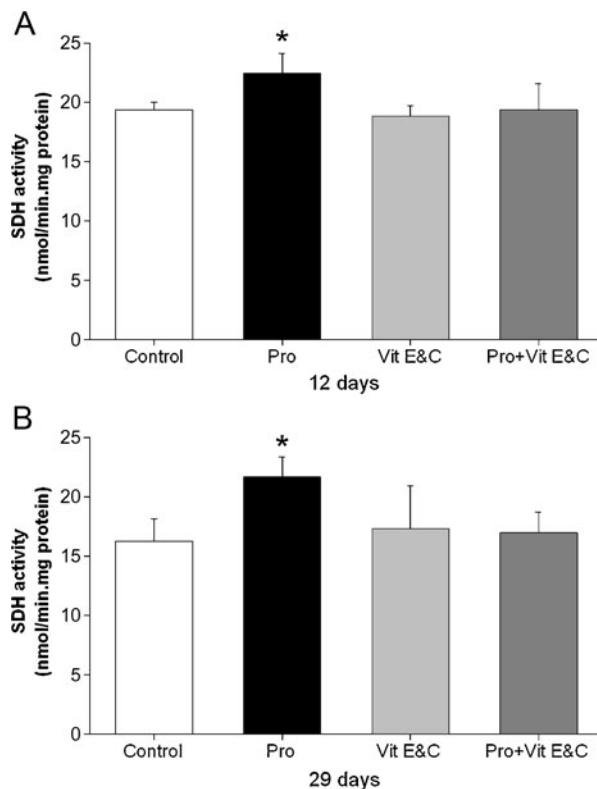
Fig. 4 Effect of acute administration of proline, vitamins E and C and proline plus vitamins E and C on succinate dehydrogenase activity in cerebral cortex of 12-day-old rats (**a**) and 29-day-old rats (**b**). Data are mean ± S.D. of 4–5 animals in each group. The results are expressed as nmol/min mg protein. * Different from other groups, $p < 0.05$ (Duncan multiple range test). Pro, proline; Vit, vitamin E and C

$[F_{(3, 13)} = 4.48; p < 0.05]$) and the reduction on cytochrome c oxidase activity caused by Pro [12-day-old: $F_{(3, 17)} = 11.34; p < 0.001$]; 29-day-old: $[F_{(3, 16)} = 11.96; p < 0.01]$).

Furthermore, Fig. 6 shows that pretreatment with α-tocopherol plus ascorbic acid *per se* caused a marked reduction of TBARS levels in cerebral cortex of 12-day-old rats (A) that received saline and decreased this parameter below the control levels in the animals subjected to acute hyperprolinemia $[F_{(3, 16)} = 27.76; p < 0.001]$. In contrast, these vitamins *per se* did not alter this parameter in 29-day-old rats (B) treated with saline, but partially prevented the increase of TBARS levels caused by Pro $[F_{(3, 16)} = 11.30; p < 0.001]$.

Discussion

Patients with hyperprolinemia type II usually present neurological dysfunction manifested by seizures and mental retardation, whose pathophysiology is not yet known (Phang et al. 2001).

Since animal models are useful to better understand the pathophysiology of diseases, we have developed a chemical
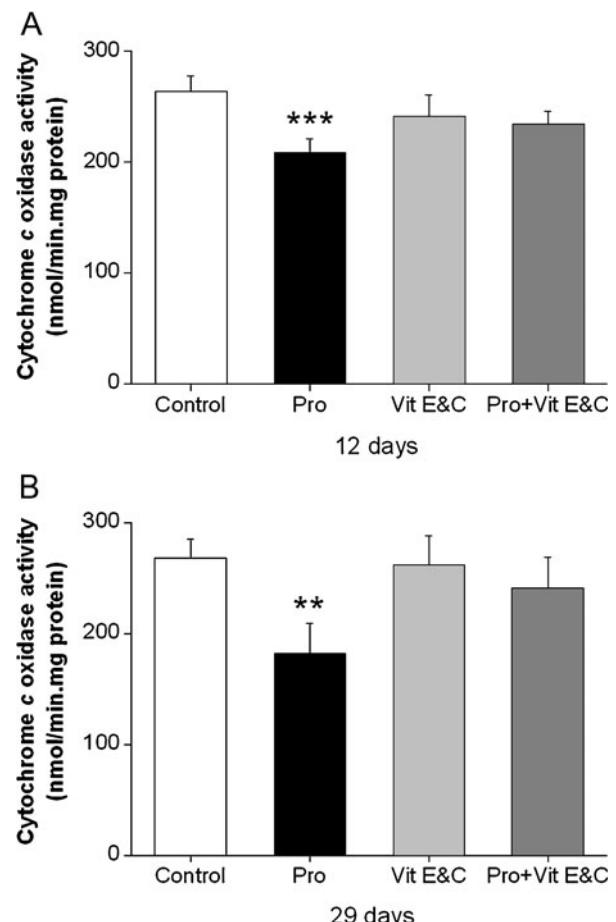
Fig. 5 Effect of acute administration of proline, vitamins E and C and proline plus vitamins E and C on cytochrome c oxidase activity in the cerebral cortex of 12-day-old rats (**a**) and 29-day-old rats (**b**). Data are mean ± S.D. of 5–6 animals in each group. The results are expressed in nmol/min mg protein. Different from other groups, ** $p < 0.01$, *** $p < 0.001$ (Duncan multiple range test). Pro, proline; Vit, vitamins E and C

experimental model of hyperprolinemia in rats (Pontes et al. 1999) that mimic the tissue levels of Pro found in human HP II (Phang et al. 2001) in order to investigate the effect of Pro on several biochemical and behavioral parameters. By using this model, we showed that Pro provokes memory deficit (Bavaresco et al. 2005; Delwing et al. 2006), decreases the activities of acetylcholinesterase (Delwing et al. 2003c, 2005), Na$^+$,K$^+$-ATPase (Pontes et al. 1999, 2001), cytochrome c oxidase (Delwing et al. 2007a) and creatine kinase (Kessler et al. 2003), as well as glutamate uptake in brain of rats (Delwing et al. 2007b).

In the present study we extended our investigation initially evaluating the activity of SDH, an enzyme of the TCA cycle located in the inner mitochondrial membrane where it can directly transfer electrons into the electron transport chain (Nelson and Cox 2008). We observed that SDH activity was significantly increased in cerebral cortex of 12- and 29-day-old rats subjected to acute hyperprolinemia.
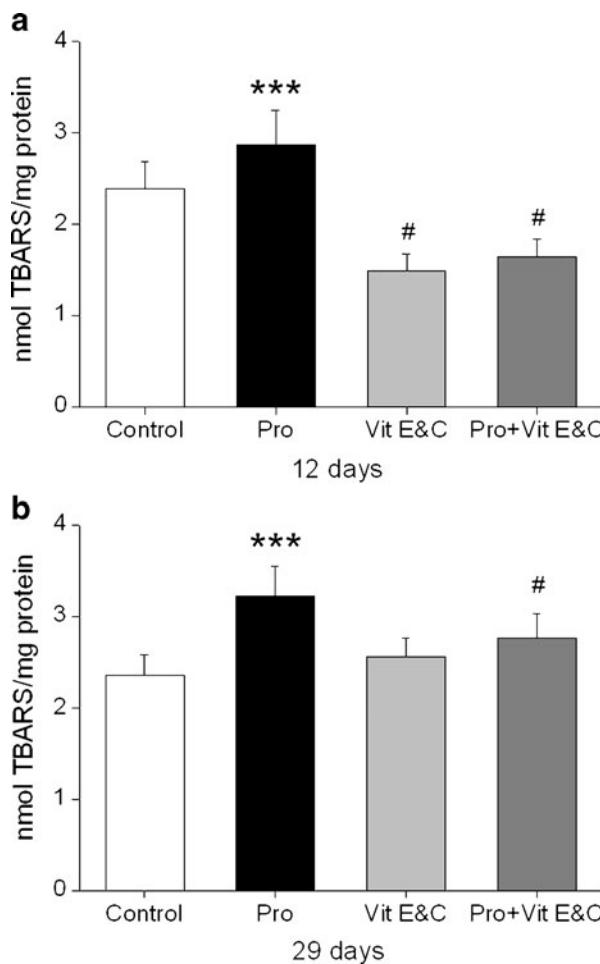
Fig. 6 Effect of acute administration of proline, vitamins E and C and proline plus vitamins E and C on thiobarbituric acid-reactive substances in cerebral cortex of 12-day-old rats (**a**) and 29-day-old rats (**b**). Data are mean ± S.D. for 5 animals in each group. *** Different from control group, # different from both control and proline groups, $p<0.001$ (Duncan multiple range test). Pro, proline; Vit, vitamins

We also evaluated the effects of Pro administration on cytochrome c oxidase activity. The activity of this enzyme was significantly reduced, suggesting that Pro compromised the respiratory chain in brain of young rats. Our findings are similar to those found in preclinical and clinical studies showing that SDH activity was increased whereas cytochrome c oxidase activity was decreased in cerebral cortex of mice exposed to chronic hypoxia (Cáceda et al. 2001) and in brain of patients with Alzheimer's disease (Bubber et al. 2005; Parker et al. 1994).

Furthermore, some studies have shown that when oxidative phosforilation is impaired, the increased SDH activity is associated with activation of anaerobic pathways coupled with the reverse operation of the TCA cycle in towards the reduction of oxalacetate to succinate (Gronow and Cohen 1984; Pisarenko 1996; Weinberg et al. 2000). Utilization of this anaerobic pathway may provide strategies

to limit mitochondrial dysfunction and allow cellular repair before the onset of irreversible injury (Weinberg et al. 2000). Although we cannot explain precisely the mechanisms underlying the increase on SDH activity following Pro acute administration, it is reasonable to propose that could be an early compensatory response to overcome the energy gap secondary to the reduced activity of cytochrome c oxidase.

The effect of acute hyperprolinemia on $^{14}CO_2$ production from glucose and acetate was also evaluated in order to investigate the effect of Pro on glycolysis and TCA activity, respectively. We found that $^{14}CO_2$ production from both substrates was significantly increased after Pro administration. The higher rate of $^{14}CO_2$ production from glucose suggests a stimulation of glycolysis. Likewise, the increase of $^{14}CO_2$ production from acetate indicates an activation of the TCA, since acetate enters the cycle directly via acetyl-CoA. These results are consistent with the increased SDH activity observed after Pro administration.

At the present, we cannot establish whether the degree of inhibition of cytochrome c oxidase and consequently of the electron transfer chain caused by Pro would alter ATP biosynthesis. However, if this is the case, an increase in ADP/ATP ratio would stimulate the pathways of fuel oxidation, such as the TCA cycle, in order to supply more NADH and FAD(2H) to the electron transport chain in an attempt to increase ATP levels (Nelson and Cox 2008). Accordingly, the increase in SDH activity observed in our study could be a consequence of stimulation TCA cycle stimulation.

On the other hand, the rate of the TCA cycle is also regulated the rate of the electron transport chain (Nelson and Cox 2008). As we found that Pro impairs the electron transport chain decreasing cytochrome c oxidase activity, it is possible that the stimulation of the TCA cycle may lead to the accumulation of NADH, since that electron transport chain is not working sufficiently to oxidize NADH.

Furthermore, considering that cytochrome c oxidase is the terminal and rate-limiting enzyme of the mitochondrial respiratory chain, catalyzing the transfer of electrons from cytochrome c to molecular oxygen (Capaldi 1995), an inhibition of this enzyme can potentially lead to incomplete reduction of oxygen and consequently increase free radical formation and reduce ATP synthesis (Bose et al. 1992; Milatovic et al. 2001; Gupta et al. 2002). On the other hand, increased levels of free radicals can damage mitochondrial components and inhibit cytochrome c oxidase activity (Cadenas and Davies 2000; Sharpe and Cooper 1998). Besides, it is well established that 4-hydroxynonenal and malondialdehyde, products of lipid peroxidation, inhibit the activity of this purified enzyme by forming adducts (Chen et al. 2000, 2001).

Indeed, it has been reported that high Pro levels can leads to alterations in the cell redox, giving rise to a decrease in the oxygen consumption necessary to oxidize the NADH formed by the cell, decreasing energy production (Phang et al. 2001).

In order to investigate the involvement of oxidative stress on the effects elicited by Pro, we evaluated whether pretreatment with the antioxidants α-tocopherol plus ascorbic acid could prevent these effects. Our results revealed that these substances prevented both the decrease of cytochrome c oxidase activity and the increase of SDH activity, suggesting that free radicals scavenged by α-tocopherol and ascorbic acid were involved in the impairment of energy metabolism caused by Pro. Our results are in agreement with others studies showing that the antioxidant α-tocopherol protection is crucial to maintain normal oxidative phosphorylation (Vatassery 1998; Vatassery et al. 2004; Navarro et al. 2005; Delwing et al. 2007a). We therefore measured the TBARS levels in cerebral cortex of 12- and 29-day-old rats. Results revealed that TBARS were significantly increased, indicating that Pro induced lipid peroxidation. Besides, α-tocopherol plus ascorbic acid pretreatment prevented this effect. Interestingly, we also observed that these vitamins *per se* reduced TBARS levels in cerebral cortex of 12-day-old rats but not in 29-day-old rats, suggesting that very young rats are more sensitive to these antioxidants. This possibly occurred due to the immaturity of the blood brain barrier that may be more permeable to these substances at this age group (Erecinska et al. 2004).

In summary, in the present study we demonstrated that high levels of Pro similar to those found in HP II, significantly altered important parameters of energy metabolism in brain of rats, by increasing succinate dehydrogenase activity and $^{14}CO_2$ production and decreasing cytochrome c oxidase activity. These data suggest that Pro might disrupt energy metabolism in brain of young rats. In addition, we showed that TBARS levels were increased in Pro-treated rats and that pretreatment with the antioxidants α-tocopherol plus ascorbic acid prevented the harmful effects elicited by Pro, supporting the hypothesis that oxidative stress has a central role in the cerebral dysfunction promoted by Pro. The mechanisms by which Pro administration elicited these alterations are not clear, however we suggest that Pro induces oxidative stress, impairs the respiratory chain at the level of cytochrome c oxidase, which probably limits ATP production and increases ROS production in a vicious cycle. These findings suggest that mitochondrial dysfunction and oxidative stress may be important contributors to the brain damage in HP II and further strengthens the possibility that treatment with antioxidants and/or energetic substrates may be beneficial in this pathology.

References

Bavaresco CS, Streck EL, Netto CA, Wyse ATS (2005) Chronic hyperprolinemia provokes a memory deficit in the Morris water maze task. Metab Brain Dis 20:73–80

Beal MF (2005) Mitochondria take center stage in aging and neurodegeneration. Ann Neurol 58:495–505

Blass JP (2001) Brain metabolism and brain disease is metabolic deficiency the proximate cause of Alzheimer dementia. J Neurosci Res 66:851–856

Bose R, Schnell CP, Pinsky C, Zitko V (1992) Effects of excitotoxins on free radical indices in mouse brain. Toxicol Lett 60:211–219

Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE (2005) Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. Ann Neurol 57:695–703

Cáceda R, Gamboa JL, Boero JA, Monge-C C, Arregui A (2001) Energetic metabolism in mouse cerebral cortex during chronic hypoxia. Neurosci Lett 301:171–174

Cadenas E, Davies KJA (2000) Mitochondrial free radical generation, oxidative stress and aging. Free Radic Biol Med 29:222–230

Capaldi RA (1995) Structure and function of cytochrome c oxidase. Ann Rev Biochem 59:418–427

Chen J, Petersen DR, Schenker S, Henderson GI (2000) Formation of malondialdehyde adducts in liver of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase. Alcohol Clin Exp Res 24:544–552

Chen J, Henderson GI, Freeman GL (2001) Role of 4-hydroxynonenal in modification of cytochrome c oxidase in ischemia/reperfused rat heart. J Mol Cell Cardiol 33:1919–1927

Delwing D, Bavaresco CS, Chiarani F, Wannmacher CMD, Wajner M, Dutra-Filho CS, Wyse ATS (2003a) In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. Brain Res 991:180–186

Delwing D, Bavaresco CS, Wannmacher CMD, Wajner M, Dutra-Filho CS, Wyse ATS (2003b) Proline induces oxidative stress cortex of rats. Int J Dev Neurosci 21:105–110

Delwing D, Chiarani F, Delwing D, Bavaresco CS, Wannmacher CMD, Wajner M, Wyse ATS (2003c) Proline reduces acetylcholinesterase activity in cerebral cortex of rats. Metab Brain Dis 18:79–86

Delwing D, Chiarani F, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT (2005) Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci Res 52:69–74

Delwing D, Bavaresco CS, Monteiro SC, Matté C, Netto CA, Wyse AT (2006) Alpha-tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res 168:185–189

Delwing D, Delwing D, Chiarani F, Kurek AG, Wyse AT (2007a) Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci 25:17–22

Delwing D, Delwing D, Sanna RJ, Wofchuck S, Wyse ATS (2007b) Proline promotes decrease in glutamate uptake in slices of cerebral cortex and hippocampus of rats. Life Sci 81:1645–1650

Dutra-Filho CS, Wajner M, Wannmacher CM, Gassen E, Candiago RH, Wilhelms AM, de Malfussi HF (1995) 2-Hydroxybutyrate

and 4-hydroxybutyrate inhibit CO_2 formation from labeled substrates by rat cerebral cortex. Biochem Soc Trans 23:228S

Erecinska M, Cherian S, Silver IA (2004) Energy metabolism in mammalian brain during development. Prog Neurobiol 73:397–445

Fighera MR, Queiros CM, Stracke MP, Brauer MC, Gonzales-Rodrigues LL, Frussa-Filho R, Wajner M, Mello CF (1999) Ascorbic acid and alpha-tocopherol attenuate methylmalonic acid induced convulsions. Neuroreport 10:2039–2043

Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, Janssen AJ (1985) Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin Chim Acta 153:23–26

Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM (1989) Type II hyperprolinemia in a pedigree of Irish travelers (nomads). Arch Dis Child 64:1699–1707

Gronow GHJ, Cohen JJ (1984) Substrate support for renal functions during hypoxia in the perfused rat kidney. Am J Physiol 247: F618–F631

Gupta RC, Milatovic D, Dettbarn WD (2002) Depletion of energy metabolites following acetylcholinesterase inhibitor-induced status epilepticus: protection by antioxidants. Neurotoxicology 22:271–282

Heales SJ, Bolaños JP, Stewart VC, Brookes PS, Land JM, Clark JB (1999) Nitric oxide, mitochondria and neurological disease. Biochim Biophys Acta 1410:215–28

Kessler A, Costabeber E, Dutra-Filho CS, Wyse ATS, Wajner M, Wannmacher CMD (2003) Proline reduces creatine kinase activity in the brain cortex of rats. Neurochem Res 28:1175–1180

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–267

Milatovic D, Zivin M, Gupta RC, Dettbarn WD (2001) Alterations in cytochrome c oxidase activity and energy metabolites in response to kainic acid-induced status epilepticus. Brain Res 31:67–78

Navarro A, Gómez C, Sánchez-Pino MJ, González H, Bández MJ, Boveris AD, Boveris A (2005) Vitamin E at high doses improves survival, neurological performance and brain mitochondrial function in aging male mice. Am J Physiol Regul Integr Comp Physiol 289:R1392–R1399

Nelson DL, Cox MM (2008) Lehninger principles of biochemistry. W. H Freeman and Company, New York

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA (2006) Involvement of oxidative stress in Alzheimer disease. J Neuropathol Exp Neurol 65:631–641

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Parker WD Jr, Parks J, Filley CM, Kleinschmidt-DeMasters BK (1994) Electron transport chain defects in Alzheimer's disease brain. Neurobiology 44:1090–1096

Petrozzi L, Ricci G, Giglioli NJ, Siciliano G, Mancuso M (2007) Mitochondria and Neurodegeneration. Biosci Rep 27:87–104

Phang JM, Hu CA, Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1821–1838

Pisarenko OI (1996) Mechanisms of myocardial protection by amino acids: facts and hypotheses. Clin Exp Pharmacol Physiol 23:627–633

Pontes ZL, Oliveira LS, Bavaresco CS, Streck EL, Dutra-Filho CS, Wajner M, Wannmacher CMD, Wyse ATS (1999) Proline administration decreases Na$^+$, K$^+$-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab Brain Dis 14:265–272

Pontes ZL, Oliveira LS, Franzon R, Wajner M, Wannmacher CMD, Wyse ATS (2001) Inhibition of Na$^+$, K$^+$-ATPase activity from rat hippocampus by proline. Neurochem Res 26:1321–1326

Rustin P, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, Munnich A (1994) Biochemical and molecular investigations in respiratory chain efficiencies. Clin Chim Acta 228:35–51

Schurr A (2002) Energy metabolism, stress hormones and neural recovery from cerebral ischemia/hypoxia. Neurochem Int 41:1–8

Sharpe MA, Cooper CE (1998) Interactions of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. J Biol Chem 273:30961–30972

Vatassery GT (1998) D. Geriatrics 53(Suppl. 1):S25–S27

Vatassery GT, DeMaster EG, Lai JCK, Smith WE, Quach HT (2004) Iron uncouples oxidative phosphorylation in brain mitochondria isolated from Vitamin E-deficient rats. Biochim Biophys Acta 1688:265–273

Weinberg JM, Venkatachalam MA, Roeser NF, Nissim I (2000) Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. Proc Natl Acad Sci USA 97:2826–2831

Wyse ATS, Zugno AI, Streck EL, Matte C, Calcagnotto T, Wannmacher CMD, Wajner M (2002) Inhibition of Na$^+$, K$^+$-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by Vitamins E and C treatment. Neurochem Res 27:1685–1689

Capítulo II

ORIGINAL PAPER

Role of antioxidants on Na$^+$,K$^+$-ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats

Andréa G. K. Ferreira · Francieli M. Stefanello · Aline A. Cunha ·
Maira J. da Cunha · Talita C. B. Pereira · Carla D. Bonan · Maurício R. Bogo ·
Carlos A. Netto · Angela T. S. Wyse

Periódico: Metabolic Brain Disease

Status: Publicado

ORIGINAL PAPER

Role of antioxidants on Na⁺,K⁺-ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats

Andréa G. K. Ferreira · Francieli M. Stefanello · Aline A. Cunha ·
Maira J. da Cunha · Talita C. B. Pereira · Carla D. Bonan · Maurício R. Bogo ·
Carlos A. Netto · Angela T. S. Wyse

Abstract Considering that Na⁺,K⁺-ATPase is an embedded-membrane enzyme and that experimental chronic hyperprolinemia decreases the activity of this enzyme in brain synaptic plasma membranes, the present study investigated the effect of chronic proline administration on thiobarbituric acid-reactive substances, as well as the influence of antioxidant vitamins E plus C on the effects mediated by proline on Na⁺,K⁺-ATPase activity in cerebral cortex of rats. The expression of Na⁺,K⁺-ATPase catalytic subunits was also evaluated. Results showed that proline increased thiobarbituric acid-reactive substances, suggesting an increase of lipid peroxidation. Furthermore, concomitant administration of vitamins E plus C significantly prevented the increase of lipid peroxidation, as well as the inhibition of Na⁺,K⁺-ATPase activity caused by proline. We did not observe any change in levels of Na⁺,K⁺-ATPase mRNA transcripts after chronic exposure to proline and vitamins E plus C. These findings provide insights into the mechanisms through which proline exerts its effects on brain function and suggest that treatment with antioxidants may be beneficial to treat neurological dysfunctions present in hyperprolinemic patients.

Keywords hyperprolinemia · Na⁺,K⁺-ATPase · Catalytic subunits · Lipid peroxidation · Vitamin E and C · Antioxidant

A. G. K. Ferreira · A. A. Cunha · M. J. da Cunha · C. A. Netto ·
A. T. S. Wyse (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da
Saúde, Universidade Federal do Rio Grande do Sul,
Rua Ramiro Barcelos, 2600-Anexo,
CEP 90035–003, Porto Alegre, RS, Brazil
e-mail: wyse@ufrgs.br

F. M. Stefanello
Departamento de Bioquímica, Instituto de Química e Geociências,
Universidade Federal de Pelotas,
Campus Universitário Capão do Leão,
Pelotas, RS, Brazil

T. C. B. Pereira · C. D. Bonan · M. R. Bogo
Departamento de Biologia Celular e Molecular,
Faculdade de Biociências, Pontifícia Universidade
Católica do Rio Grande do Sul,
Avenida Ipiranga,
6681, Porto Alegre, RS, Brazil

Introduction

Na⁺,K⁺-ATPase (EC 3.6.1.3) is a membrane-bound enzyme that plays an essential role in controlling neuronal excitability by maintaining electrochemical gradients, through active transport of Na⁺ and K⁺ ions across the cell membrane, at the expense of ATP (Kaplan 2002; Mobasheri et al. 2000). Structurally, the enzyme is composed of α- and β-subunits. The α-subunit is catalytic and contains the binding sites for the cations, ATP and ouabain (a cardiac glycoside and specific inhibitor of the enzyme); the β-subunit is regulatory and is responsible for proper trafficking of the complex and its correct insertion into the plasma membrane (Blanco and Mercer 1998; Geering 2001). Individual genes of four α-subunit isoforms (α_1, α_2, α_3 and α_4) and at least three β-subunit isoforms (β_1, β_2 and β_3) of Na⁺,K⁺-ATPase have been identified in mammals (Kaplan 2002; Serluca et al. 2001). A third subunit, named γ-subunit, has also been described in some tissues; it apparently modulates the enzyme activity (Dempski et al. 2009). The isoforms combine to form several Na⁺,K⁺-ATPase isozymes which show different tissue distribution patterns. In the brain, mainly three α isoforms (α_1, α_2, α_3)

and two β isoforms (β_1 and β_2) are expressed; β_3 is expressed but at very low level (Wetzel et al. 1999).

Considering that Na$^+$,K$^+$-ATPase is one of the most abundant brain enzyme, consuming about 40–50% of the ATP generated (Kaplan 2002), it is not surprising that alterations in its activity may cause a variety of abnormalities. Indeed, Na$^+$,K$^+$-ATPase has been suggested to play a central role in the pathogenesis of neurodegenerative diseases, such as Alzheimer, multiple sclerosis, Parkinson's disease and epilepsy (Grisar et al. 1992; Hattori et al. 1998; Rose and Valdes 1994). Mutations in catalytic subunits expressed in the brain can also give rise to diseases such as epilepsy and parkinsonism (Aperia 2007). Preclinical studies have also demonstrated that alterations of this enzyme activity is implicated in several conditions affecting the CNS, such as ischemia (Wyse et al. 2000), depression (de Vasconcellos et al. 2005; Gamaro et al. 2003), ovariectomy (Ben et al. 2009; Monteiro et al. 2005; 2007) and metabolic diseases (Pontes et al. 1999; 2001; Streck et al. 2002; Wyse et al. 2001).

Hyperprolinemia type II (HP II) is a metabolic disease caused by deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity characterized by tissue accumulation of proline (Pro). Affected patients usually present neurological symptomatology, including seizures and mental retardation; a relationship between high concentration of Pro and neurological symptoms has been established (Farrant et al. 2001; Flynn et al. 1989; Phang et al. 2001). Since the mechanisms underlying the HP II pathophysiology are still obscure, we have developed a chemical experimental model of hyperprolinemia in rats that mimics the tissue levels of Pro found in human HP II (Moreira et al. 1989; Phang et al. 2001). It has been reported that rats subjected to experimental hyperprolinemia present significant inhibition of Na$^+$,K$^+$-ATPase activity in cortical synaptic plasma membranes (Pontes et al. 1999) and that Pro elicits oxidative stress and decreases antioxidant defenses in rat brain (Delwing et al. 2003). Moreover, some biochemical and behavioral effects caused by experimental hyperprolinemia were prevented by the antioxidants vitamins E plus C, suggesting that free radicals formation may be involved in such effects (Delwing et al. 2005).

Considering that a variety of regulatory mechanisms assure appropriate Na$^+$,K$^+$-ATPase expression and activity adapted to changing physiological demands (Blanco and Mercer 1998; Geering 2001; Jorgensen et al. 2003; Kaplan 2002; Mobasheri et al. 2000), in the present study we investigated the possible mechanisms involved in the inhibition of Na$^+$,K$^+$-ATPase activity elicited by hyperprolinemia. A parameter of lipid peroxidation, namely thiobarbituric acid-reactive substances (TBARS) was studied, as well as the influence of antioxidant vitamins E plus C on the Pro-mediated effects on Na$^+$,K$^+$-ATPase activity.

The alterations on the Na$^+$,K$^+$-ATPase activity after chronic exposure to Pro and/or antioxidant vitamins were also investigated in association with changes in expression of Na$^+$,K$^+$-ATPase catalytic subunits (isoforms α_1, α_2 and α_3) in cerebral cortex of rats.

Experimental procedures

Animals

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry at the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant room temperature (22±1°C), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80–23, revised 1996) and was approved by the University Ethics Committee.

Proline and vitamins E plus C administration

Experimental chronic hyperprolinemia was chemically induced by daily subcutaneous administration of proline from the 6th to the 28th day of life as described by Pontes et al. (1999). Proline (Sigma Chemical Co., USA) was dissolved in 0.9% NaCl, buffered to pH 7.4 with NaOH and administered twice a day. During the first 8 days of treatment (6th–13th day of life) rats received 12.8 μmol Pro/g body weight, from the 14th to 17th day they received 14.6 μmol Pro/g body weight, from the 18th to 21th day they received 16.4 μmol Pro/g body weight and from the 22th to 28th day of life they received 18.2 μmol Pro/g body weight. Rats subjected to this treatment achieved plasma Pro levels between 1.0 and 2.0 mM, similar to those found in hyperprolinemic type II patients (Moreira et al. 1989; Phang et al. 2001). Control animals received saline injections in the same volumes as those applied to Pro-treated rats. Animals were killed 12 h after the last injection by decapitation without anesthesia; by that time, blood and brain levels of Pro had returned to normal (Moreira et al. 1989). Besides saline or Pro administration, rats received a single daily intraperitoneal injection of vitamins E (40 mg/kg) plus C (100 mg/kg) or vehicle (saline), according to the protocols previously described (Delwing et al. 2006; Fighera et al. 1999; Wyse et al. 2002).

Tissue and homogenate preparation

After decapitation, the brain was removed and cerebral cortex was dissected out and immediately frozen in liquid

nitrogen. To allow for the preparation of synaptic plasma membrane, cortical tissue was homogenized in 10 vol. 0.32 mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared for subsequent determination of Na^+,K^+-ATPase activity. For TBARS assays, tissue 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 $750 \times g$ for 10 min at 4°C, the pellet was discarded and the supernatant was immediately separated and used for biochemical measurements.

Preparation of synaptic plasma membrane

Synaptic plasma membrane fraction from cerebral cortex was prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al. 1995). They were isolated using a discontinuous sucrose density gradient, consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at $69,000 \times g$ for 2 h, the fraction between 0.8 and 1.0 sucrose interface was taken as the membrane enzyme preparation.

Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 200 μl. After 10 min of pre-incubation at 37°C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 5 min. Controls were carried out under the same conditions with addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos 1984; Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986) and enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

Thiobarbituric Acid Reactive Species (TBARS)

TBARS levels were determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μl of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% thiobarbituric acid were added to 500 μL of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at $1,000 \times g$ for 10 min, the supernatant was taken and the resulting pink-stained TBARS were determined spectrophotometrically at 535 nm; the results were reported as nmol of TBARS per mg protein.

Analysis of gene expression by semi-quantitative RT-PCR

The analysis of Na^+,K^+-ATPase catalytic subunits expression were carried out by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. All chemicals for these experiments were purchased from Invitrogen, USA.

The cerebral cortex frozen in liquid nitrogen was submitted to total RNA extraction with TRIzol reagent in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR from 2 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42°C. cDNA (0.1 mL) was used as a template for PCR with the specific primers for Na^+,K^+-ATPase catalytic subunits (Table 1). β-actin-PCR was carried out as an internal standard. PCR reactions were performed with a total volume of 25 μL using a final concentration of 0.08 μM of each primer indicated below, 1.6 mM of $MgCl_2$ and 1 U Taq Platinum Polymerase in the supplied reaction buffer. Conditions for Na^+, K^+-ATPase catalytic subunits PCR were as follows: initial 2 min denaturation step at 94°C; 1 min at 94°C, 1 min annealing step at 62°C, 1 min extension step at 72°C for 30 cycles and a final 10 min extension at 72°C. Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step

Table 1 Primer sequences and PCR amplification conditions

Na^+,K^+-ATPase catalytic subunits	GenBank accession number	Primers (5′–3′)	PCR product	T (°C)	Cycles
alpha 1	NM_012504	F-TCTATGGACGACCATAAACTCAGCCTGG R-AGCAGACAGCACGACCCCGAGGTAC	297	62	30
alpha 2	NM_012505	F-ACCAAGTGGATCTGTCCAAGGGCCTC R-GCTTCCTGGTAGTAGGAGAAGCAGCCAG	292	62	30
alpha 3	NM_012506	F-AAAGATGACAAGAGCTCGCCCAAGAAG R-TGATCTCCACCAGGTCCCCGACCAC	538	62	30
β-actin	NP_742006	F-TATGCCAACACAGTGCTGCTGG R-TACTCCTGCTTCCTGATCCACAT	210	54	35
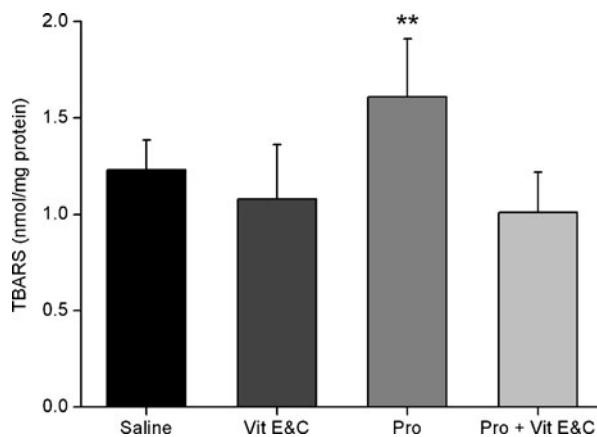
Fig. 1 Effect of proline, vitamins E&C, and proline plus vitamins E&C administration on thiobarbituric acid-reactive substances in cerebral cortex of rats. Data are mean ± S.D. for 7 animals per group. * Different from control group, $p<0.01$ (Duncan multiple range test). *Pro, proline; Vit, vitamin*

at 54°C, 1 min extension step at 72°C for 35 cycles and a final 10 min extension at 72°C. PCR products were submitted to electrophoresis using a 1% agarose gel with GelRed®. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder. The relative abundance of each mRNA versus β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Protein determination

The protein content of tissue samples was determined using bovine serum albumin as standard, according to Lowry et al. (1951) or Bradford (1976).

Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan's multiple range test when appropriate. All analy-

ses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $p<0.05$ were considered to be significant.

Results

Initially, the lipid peroxidation was measured by TBARS assay. Figure 1 shows that chronic Pro administration significantly increased TBARS (30%) in rat cerebral cortex, when compared to controls (saline-treated). Interestingly, the concomitant treatment with antioxidant vitamins E plus C prevented this effect [$F(3,24)=6.47$; $p<0.01$].

Next, the influence of antioxidant vitamins E plus C administration on Na^+,K^+-ATPase activity in cortical synaptic plasma membranes of rats submitted to chronic hyperprolinemia was investigated. *Post hoc* analysis showed that treatment with vitamins *per se* did not alter enzyme activity, but significantly prevented the inhibition of Na^+,K^+-ATPase activity caused by chronic Pro administration [$F(3,12)=23.55$; $p<0.001$] (Fig. 2).

Finally, we analyzed the relative expression of cortical Na^+, K^+-ATPase catalytic subunits after chronic exposure to Pro and vitamins E plus C by semi-quantitative RT-PCR. As shown in Fig. 3, the relative expressions of isoforms α_1, α_2 and α_3 of the Na^+,K^+-ATPase were not altered by Pro and/or vitamins treatment in cerebral cortex of rats ([$F(3,13)=1.06$; $p>0.05$]; [$F(3,13)=0.654$; $p>0.05$]; [$F(3,13)=0.26$; $p>0.05$], respectively).

Discussion

It has been reported that experimental chronic hyperprolinemia significantly decreases Na^+,K^+-ATPase activity

Fig. 2 Effect of proline, vitamins E&C, and proline plus vitamins E&C administration on Na^+,K^+-ATPase activity in synaptic plasma membranes from cerebral cortex of rats. Data are mean ± S.D. for 4–5 animals per group. Results are expressed in nmol Pi/min mg protein. * Different from control group, # different from both control and proline groups, $p<0.001$ (Duncan multiple range test). *Pro, proline; Vit, vitamin*

Fig. 3 Gene expression of α_1 (**a**), α_2 (**b**) and α_3 (**c**) subunits of Na$^+$, K$^+$-ATPase and β-actin after proline, vitamins E&C and proline plus vitamins E&C administration in cerebral cortex of rats. Electrophoresis data are representative of three individual experiments. Results are expressed as optical densitometry (O.D.) of the Na$^+$,K$^+$-ATPase subunits-related genes versus β-actin expression (mean $\pm$ S.D) of three independent replicates of RT-PCR experiments. *Pro, proline; Vit, vitamin*

in synaptic plasma membranes from rat cerebral cortex (Pontes et al. 1999). In the present study, we extended this investigation in an attempt to identify the possible mechanisms involved in enzyme inhibition. Since Na$^+$,K$^+$-ATPase is a membrane-bound enzyme, we first investigated the effect of Pro administration on a classic parameter of lipid peroxidation. Results showed an increase in TBARS levels in cerebral cortex from rats chronically treated with Pro. We also observed that concomitant administration of vitamin E plus C significantly prevented the increase on TBARS levels, as well as the inhibition of Na$^+$,K$^+$-ATPase activity promoted by this amino acid. Together, these findings support the involvement of reactive species and/or lipid peroxidation in the Pro-elicited effects.

It has been demonstrated that Na$^+$,K$^+$-ATPase is particularly susceptible to free radical attack since its inhibition has been associated with changes in plasma membrane lipid composition (Dencher et al. 2007), in the redox state of regulatory sulphydryl groups (Pari and Murugavel 2007) and in other amino acid residues caused by free radicals or lipid peroxidation (Potts et al. 2006; Siems et al. 1996). In addition, we have shown that Na$^+$,K$^+$-ATPase activity is inhibited in other animal models of inborn errors of metabolism and that oxidative stress was present in these conditions (Stefanello et al. 2005; Wyse et al. 2001; Wyse et al. 2002). Interestingly, in the present study we found that vitamins E and C partially prevented the inhibition of Na$^+$,K$^+$-ATPase activity promoted by Pro.

Considering that Na$^+$,K$^+$-ATPase is the target of multiple regulatory mechanisms activated in response to changing cellular requirements which modulate its activity and expression (Therien and Blostein 2000), we asked whether changes on Na$^+$,K$^+$-ATPase activity after chronic hyperprolinemia and antioxidants supplementation could be a consequence of alterations in the transcriptional control of enzyme. The relative expression of catalytic subunits, isoforms α_1, α_2 and α_3 of the Na$^+$,K$^+$-ATPase after chronic exposure to Pro and vitamins E plus C was then evaluated, however no changes in the levels of Na$^+$,K$^+$-ATPase mRNA transcripts were observed.

There is considerable evidence that regulatory post-translational mechanisms such as protein phosphorylation are essential to assure activity adapted to physiological demands of Na$^+$,K$^+$-ATPase (Blanco and Mercer 1998; Geering 2001; Jorgensen et al. 2003; Kaplan 2002; Mobasheri et al. 2000). In this sense, we performed computational analysis in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool, which revealed that rat Na$^+$,K$^+$-ATPase presents a high score of possible PKC phosphorylation sites of the α_1 subunit (Ser23) and PKA phosphorylation sites of the α_2 and α_3 subunits (Ser940 and Ser933, respectively). Therefore, the decrease on Na$^+$,K$^+$-ATPase activity observed in present

work could be possibly attributed to changes in phosphorylation state of enzyme. In this context, it has been suggested that phosphorylation may occur either directly or as a result of secondary modulators such as nitric oxide (NO), which is also a potent mediator of oxidative stress (Therien and Blostein 2000). In fact, we cannot rule out that Pro elicits NO generation, since a previous work demonstrated that its administration inhibited acetylcholinesterase activity in rat brain and that this effect was prevented by administration of N^ω-nitro-L-arginine methyl ester (L-NAME), a potent nitric oxide sinthase inhibitor, suggesting that Pro affects this parameter through NO production and/or oxidative stress (Delwing et al. 2005). Altogether, these observations prompt us to hypothesize that Na^+,K^+-ATPase inhibition mediated by Pro may be attributed, at least in part, to changes in the enzyme phosphorylation state.

Another important modulator of Na^+,K^+-ATPase activity is the level of ATP into the cell. It has been reported that a reduction of intracellular ATP inhibits the activity of this enzyme (Therien and Blostein 2000) and it was recently shown that Pro disrupts energy metabolism such as creatine kinase, cytocrome c oxidase and succinate dehydrogenase possibly leading to limitation of enzymatic activities related to ATP production (Delwing et al. 2007; Ferreira et al. 2010; Kessler et al. 2003).

In summary, the present study demonstrated that hyperprolinemia increases TBARS levels and decreases the Na^+,K^+-ATPase activity; both effects are prevented by concomitant treatment with vitamins E plus C, thus suggesting the involvement of reactive oxygen species and/or lipid peroxidation in Pro-elicited effects. Therefore, it is reasonable to propose that mechanisms like lipid peroxidation, energy metabolism deficit and/or post-translational modifications may be interacting to promote Pro neurotoxicity. Additional studies in order to evaluate whether the administration of antioxidants may be beneficial in this pathology.

Conflict of interest The authors declare that they have no conflict of interest.

References

Aperia A (2007) New roles for an old enzyme: Na^+, K^+ -ATPase emerges as an interesting drug target. J Intern Med 261:44–52

Ben J, Soares FM, Cechetti F, Vuaden FC, Bonan CD, Netto CA, Wyse ATS (2009) Exercise effects on activities of Na^+, K^+ -ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302:248–255

Blanco G, Mercer RW (1998) Isozymes of the Na^+, K^+ -ATPase: heterogeneity in structure, diversity in function. Am J Physiol 275:F633–F650

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

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

de Vasconcellos AP, Zugno AI, dos Santos AH, Nietto FB, Crema LM, Goncalves M, Franzon R, Wyse ATS, da Rocha ER, Dalmaz C (2005) Na^+, K^+-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: effect of chronic lithium treatment and possible involvement in learning deficits. Neurobiol Learn Mem 84:102–110

Delwing D, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse ATS (2003) Proline induces oxidative stress in cerebral cortex of rats. Int J Dev Neurosci 21:105–110

Delwing D, Chiarani F, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT (2005) Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci Res 52:69–74

Delwing D, Tagliari B, Chiarani F, Wannmacher CM, Wajner M, Wyse AT (2006) Alpha-tocopherol and ascorbic acid administration prevents the impairment of brain energy metabolism of hyperargininemic rats. Cell Mol Neurobiol 26:177–189

Delwing D, Chiarani F, Kurek AG, Wyse AT (2007) Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci 25:17–22

Dempski RE, Friedrich T, Bamberg E (2009) Voltage clamp fluorometry: combining fluorescence and electrophysiological methods to examine the structure-function of the Na^+, K^+-ATPase. Biochim Biophys Acta 1787:714–720

Dencher NA, Frenzel M, Reifschneider NH, Sugawa M, Krause F (2007) Proteome alterations in rat mitochondria caused by aging. Ann NY Acad Sci 1100:291–298

Farrant RD, Walker V, Mills GA, Mellor JM, Langley GJ (2001) Pyridoxal phosphate de-activation by pyrroline-5-carboxylic acid. Increased risk of vitamin B6 deficiency and seizures in hyperprolinemia type II. J Biol Chem 276:15107–15116

Ferreira AGK, Lima DD, Delwing D, Mackedanz V, Tagliari B, Kolling J, Schuck PF, Wajner M, Wyse ATS (2010) Proline impairs energy metabolism in cerebral cortex of young rats. Metab Brain Dis 25:161–168

Fighera MR, Queiroz CM, Stracke MP, Brauer MC, Gonzalez-Rodriguez LL, Frussa-Filho R, Wajner M, de Mello CF (1999) Ascorbic acid and alpha-tocopherol attenuate methylmalonic acid-induced convulsions. Neuroreport 10:2039–2043

Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM (1989) Type II hyperprolinaemia in a pedigree of Irish travellers (nomads). Arch Dis Child 64:1699–1707

Gamaro GD, Streck EL, Matte C, Prediger ME, Wyse ATS, Dalmaz C (2003) Reduction of hippocampal Na^+, K^+-ATPase activity in rats subjected to an experimental model of depression. Neurochem Res 28:1339–1344

Geering K (2001) The functional role of beta subunits in oligomeric P-type ATPases. J Bioenerg Biomembr 33:425–438

Grisar T, Guillaume D, Delgado-Escueta AV (1992) Contribution of Na^+, K^+-ATPase to focal epilepsy: a brief review. Epilepsy Res 12:141–149

Hattori N, Kitagawa K, Higashida T, Yagyu K, Shimohama S, Wataya T, Perry G, Smith MA, Inagaki C (1998) CI-ATPase and Na^+, K^+-ATPase activities in Alzheimer's disease brains. Neurosci Lett 254:141–144

Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287

Jorgensen PL, Hakansson KO, Karlish SJ (2003) Structure and mechanism of Na$^+$, K$^+$-ATPase: functional sites and their interactions. Annu Rev Physiol 65:817–849

Kaplan JH (2002) Biochemistry of Na$^+$, K$^+$-ATPase. Annu Rev Biochem 71:511–535

Kessler A, Costabeber E, Dutra-Filho CS, Wyse AT, Wajner M, Wannmacher CM (2003) Proline reduces creatine kinase activity in the brain cortex of rats. Neurochem Res 28:1175–1180

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

Mobasheri A, Avila J, Cozar-Castellano I, Brownleader MD, Trevan M, Francis MJ, Lamb JF, Martin-Vasallo P (2000) Na$^+$, K$^+$-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. Biosci Rep 20:51–91

Monteiro SC, Matte C, Delwing D, Wyse ATS (2005) Ovariectomy increases Na$^+$, K$^+$-ATPase, acetylcholine sterase and catalase in rat hippocampus. Mol Cell Endocrinol 236:9–16

Monteiro SC, Mattos CB, Scherer EB, Wyse ATS (2007) Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of Na$^+$, K$^+$-ATPase and cholinesterases. Metab Brain Dis 22:156–171

Moreira JC, Wannmacher CM, Costa SM, Wajner M (1989) Effect of proline administration on rat behavior in aversive and non-aversive tasks. Pharmacol Biochem Behav 32:885–890

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Pari L, Murugavel P (2007) Diallyl tetrasulfide improves cadmium induced alterations of acetylcholinesterase, ATPases and oxidative stress in brain of rats. Toxicology 234:44–50

Phang JM, Hu CA, Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, vol 3. McGraw-Hill, New York, pp 1821–1838

Pontes ZE, Oliveira LS, Bavaresco CS, Streck EL, Dutra-Filho CS, Wajner M, Wannmacher CM, Wyse AT (1999) Proline administration decreases Na$^+$, K$^+$-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab Brain Dis 14:265–272

Pontes ZL, Oliveira LS, Franzon R, Wajner M, Wannmacher CM, Wyse AT (2001) Inhibition of Na$^+$, K$^+$-ATPase activity from rat hippocampus by proline. Neurochem Res 26:1321–1326

Potts MB, Koh SE, Whetstone WD, Walker BA, Yoneyama T, Claus CP, Manvelyan HM, Noble-Haeusslein LJ (2006) Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. NeuroRx 3:143–153

Rose AM, Valdes R Jr (1994) Understanding the sodium pump and its relevance to disease. Clin Chem 40:1674–1685

Serluca FC, Sidow A, Mably JD, Fishman MC (2001) Partitioning of tissue expression accompanies multiple duplications of the Na$^+$, K$^+$-ATPase alpha subunit gene. Genome Res 11:1625–1631

Siems WG, Hapner SJ, van Kuijk FJ (1996) 4-hydroxynonenal inhibits Na$^+$, K$^+$-ATPase. Free Radic Biol Med 20:215–223

Stefanello FM, Chiarani F, Kurek AG, Wannmacher CM, Wajner M, Wyse ATS (2005) Methionine alters Na$^+$, K$^+$-ATPase activity, lipid peroxidation and nonenzymatic antioxidant defenses in rat hippocampus. Int J Dev Neurosci 23:651–656

Streck EL, Zugno AI, Tagliari B, Sarkis JJ, Wajner M, Wannmacher CM, Wyse AT (2002) On the mechanism of the inhibition of Na$^+$, K$^+$-ATPase activity caused by homocysteine. Int J Dev Neurosci 20:77–81

Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol 279:C541–C566

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

Wetzel RK, Arystarkhova E, Sweadner KJ (1999) Cellular and subcellular specification of Na$^+$, K$^+$-ATPase alpha and beta isoforms in the postnatal development of mouse retina. J Neurosci 19:9878–9889

Wyse ATS, Bolognesi G, Brusque AM, Wajner M, Wannmacher CMD (1995) Na$^+$,K$^+$-ATPase activity in the synaptic plasma membrane from the cerebral cortex of rats subjected to chemically induced phenylketonuria. Med Sci Res 23:261–263

Wyse ATS, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na$^+$, K$^+$-ATPase activity after brain ischemia. Neurochem Res 25:971–975

Wyse AT, Bavaresco CS, Bandinelli C, Streck EL, Franzon R, Dutra-Filho CS, Wajner M (2001) Nitric oxide synthase inhibition by L-NAME prevents the decrease of Na$^+$, K$^+$-ATPase activity in midbrain of rats subjected to arginine administration. Neurochem Res 26:515–520

Wyse AT, Zugno AI, Streck EL, Matte C, Calcagnotto T, Wannmacher CM, Wajner M (2002) Inhibition of Na$^+$, K$^+$-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem Res 27:1685–1689

Capítulo III

Evidence that hyperprolinemia alters glutamatergic homeostasis in rat brain: neuroprotector effect of guanosine

Andréa G. K. Ferreira, Aline A. da Cunha, Emilene B. Scherer, Fernanda R. Machado, Maira J. da Cunha, Andressa Braga, Ben Hur Mussulini, Júlia D. Moreira, Susana Wofchuk, Diogo O. Souza, Angela T. S. Wyse

Evidence that hyperprolinemia alters glutamatergic homeostasis in rat brain: neuroprotector effect of guanosine

Andréa G. K. Ferreira, Aline A. da Cunha, Emilene B. Scherer, Fernanda R. Machado, Maira J. da Cunha, Andressa Braga, Ben Hur Mussulini, Júlia D. Moreira, Susana Wofchuk, Diogo O. Souza, Angela T. S. Wyse

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 -Anexo, Porto Alegre, RS, Brazil.

Address reprint requests to: Dr. Angela T. S. Wyse, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Phone 55 51 3308 5573, Fax 55 51 3308 5535, E-mail: wyse@ufrgs.br

Running head: Hyperprolinemia alters glutamatergic homeostasis in rat brain

Abstract

This study investigated the effects of acute and chronic hyperprolinemia on glutamate uptake, as well as some mechanisms underlying the proline effects on glutamatergic system in rat cerebral cortex. The protective role of guanosine on effects mediated by proline was also evaluated. Results showed that acute and chronic hyperprolinemia reduced glutamate uptake, Na^+,K^+-ATPase activity, ATP levels and increased lipoperoxidation. GLAST and GLT-1 immunocontent were increased in acute, but not in chronic hyperprolinemic rats. Our data suggest that the effects of proline on glutamate uptake may be mediated by lipid peroxidation and disruption of Na^+,K^+-ATPase activity, but not by decreasing in glutamate transporters. This probably induces excitotoxicity and subsequent energy deficit. Guanosine was effective to prevent most of the effects promoted by proline, reinforcing its modulator role in counteracting the glutamate toxicity. However, further studies are needed to assess the modulatory effects of guanosine on experimental hyperprolinemia.

Keywords: Hyperprolinemia; guanosine; glutamate uptake; glutamate transporters; GLAST; GLT-1.

Introduction

Hyperprolinemia is the biochemical hallmark for two inherited metabolic disorders caused by defects in the L-proline catabolic pathway. Enzymatic deficiencies of proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase give rise to hyperprolinemia type I (HPI) and hyperprolinemia type II (HPII), respectively (1, 2). Although both enzymes deficiencies lead to tissue accumulation of proline, HPII causes higher plasma levels, which has been related to neurological manifestations such as seizures and mental retardation (1-3).

The exact mechanism underlying the symptoms of hyperprolinemia are far from being understood; however, alterations in the glutamatergic homeostasis seem to be involved by following reasons: firstly, high levels of glutamate are found in the cerebrospinal fluid of patients with HPII (1, 4, 5). Besides, there are some evidences from experimental data that proline might potentiates glutamate transmission by overstimulation of receptors NMDA and AMPA (6-9). In addition, short-term exposure to proline is able to impair the glutamate uptake in cerebral cortex and hippocampus of rats (10).

As the principal excitatory neurotransmitter, glutamate plays an essential role in brain plastic processes such as learning/memory, development and ageing (11, 12). On the other hand, overstimulation of the glutamatergic system may leads to a process known as excitotoxicity that involves intracellular increase of ions such as Na^+, Ca^{2+}, depletion of ATP levels and ultimately neuronal damage (13). In fact, excitotoxicity has been implicated in the pathogenesis of various acute and chronic CNS disorders including epilepsy, ischemia and

Alzheimer's disease (14-16). In this scenario, the maintenance of glutamate concentrations below neurotoxic levels is essential for normal brain function.

The main mechanism of glutamate clearance from the synaptic cleft is the glutamate uptake, accomplished by transporters that remove the glutamate from the extracellular fluid and therefore, maintain its physiologic levels and avoid excitotoxicity (11, 17, 18). The major glutamate transporters are GLAST and GLT-1, located predominantly in glial cels (11, 15, 19). These transporters are sodium-dependent proteins, using electrochemical sodium gradient generated by Na^+,K^+-ATPase activity to drive the uphill transport of glutamate; then, Na^+,K^+-ATPase may be a key regulator of glutamate transporter activity (20).

Na^+,K^+-ATPase is an inserted-membrane enzyme, whose activity is highly energy dependent, since it consumes about 40–50% of the ATP produced in brain to pumps Na^+ and K^+ across the cell membrane (21, 22). Therefore, disturbance in the plasma membrane composition and fluidity, as well as alterations in the energy availability can compromise the activity of this enzyme (23, 24). It has been reported that Na^+,K^+-ATPase is particularly susceptible to free radical attack and lipid peroxidation (25, 26). We have previously reported that rats subjected to a chemical experimental model of hyperprolinemia present a significant inhibition in activity of cerebral Na^+,K^+-ATPase (27), without alteration of the expression of their catalytic subunits (28). This effect was suggested to be associate to oxidative stress induced by proline (29, 30), since Na^+,K^+-ATPase activity was reestablished by antioxidants treatment (10, 28).

Increasing evidence have suggested that the nucleoside guanosine has relevant neuroprotective role in a variety of *in vitro* and *in vivo* models that involve the over activation of glutamate receptors, by counteracting glutamate

excitotoxicity (31-34). Nevertheless, the exact site of interaction and mechanisms involved in the neuroprotection afforded by guanosine is still unknown (35, 36). To our knowledge, there is no study that investigates the protector effect of guanosine on damage observed in animals subjected to a metabolic disease model.

Based on previous works from our laboratory showing that a single proline administration (acute treatment) decreases the glutamate uptake in slices from cerebral cortex of rats (10), in the present study we extended our investigation initially evaluating the effect of chronic proline administration on this parameter. In order to elucidate some possible mechanisms involved in the alterations of glutamate uptake elicited by acute and chronic hyperprolinemia, we measured the immunocontent of glutamate transporters GLAST and GLT-1 in homogenate of cerebral cortex from rats. Besides, we also evaluated Na^+,K^+-ATPase activity, ATP levels and lipid peroxidation by thiobarbituric acid-reactive substances (TBARS) in homogenate from the same cortical samples. The possible neuroprotective role of guanosine on the proline-mediated effects was also evaluated.

Experimental procedures

Animals

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of

Laboratory Animals" (NIH publication no. 80-23, revised 1996) and was approved by the University Ethics Committee.

Proline and guanosine administration

Proline and guanosine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solutions for injections were prepared in 0.9% NaCl. For acute experiments, 21-day-old Wistar rats were pretreated for one week with daily intraperitoneal administration of guanosine (7.5 mg/Kg) or saline (control). Twelve hours after the last injection, animals received one injection of proline correspondent to 18.2 mmol/g body weight or saline and were killed 1 h after the injection by decapitation without anaesthesia.

For chronic experiments, the rats were subjected to treatment from the 6th to the 28th day of life, where they received one single daily intraperitoneal injection of guanosine (7.5 mg/Kg) or saline plus two daily subcutaneous injections of proline or saline. The guanosine dose was choose according to Schmidt (31) and Jiang et al. (37). The proline doses were according described by Moreira et al. (38) and Pontes et al. (27). During the first 8 days of treatment (6th - 13th day of life) rats received 12.8 µmol Pro/g body weight, from the 14th to 17th day they received 14.6 µmol Pro/g body weight, from the 18th to 21th day they received 16.4 µmol Pro/g body weight and from the 22th to 28th day of life they received 18.2 µmol Pro/g body weight. Rats subjected to this treatment achieved plasma proline levels between 1.0 and 2.0 mM, which are similar to those found in hyperprolinemic patients (1, 38). The animals were killed 12 h after the last injection by decapitation without anesthesia, when the blood levels of Pro had returned to normal (38).

Glutamate uptake activity

Glutamate uptake was performed according to Frizzo et al. (39). Cerebral cortex was cut into 400 μm thick slices with a McIlwain chopper. Briefly, slices were pre-incubated in Hank's balanced salt solution (HBSS) at 35°C for 15 min, followed by adding a solution containing 0.33 mCi/mL L-[2,3-3 H] glutamate (Amersham International, UK) with 100 μM unlabeled glutamate at 35°C. Incubation was stopped after 5 min with two ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5 N NaOH. Sodium-independent uptake was determined on ice (4°C), using HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake and were calculated as nmol of glutamate/min/mg protein. Both the specific and non-specific uptakes were performed in triplicate.

Western blotting for GLAST and GLT-1

Cerebral cortex were dissected out and immediately homogenized in a 25 mM HEPES solution (pH 7.4) with 0.1 % SDS and protease inhibitor cocktail (Sigma, USA). Samples (20 μg protein/well) were separated in an 8 % SDS-PAGE mini-gel and transferred to a nitrocellulose membrane using a Trans-Blot system (Bio-Rad, São Paulo/SP, Brazil). Membranes were processed as follow: (1) blocking with 5 % bovine serum albumin (Sigma, USA) for 2 h; (2) incubation with primary antibody overnight: 1:1000 rabbit anti-GLAST or rabbit anti-GLT-1 glutamate transporters (AlphaDiagnostic International); 1:2000 mouse anti-β-Actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary

antibody for rabbit 1:3000 and mouse 1:3000 (Amersham Pharmacia Biotech) for 2 h; (4) chemiluminescence (ECL, Amersham Pharmacia Biotech, São Paulo/SP, Brazil) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and bands intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). In order to determine the adequate amount of protein to be assayed, different protein concentrations were carried out in the same gel for each antibody tested.

Na$^+$,K$^+$-ATPase activity

The cerebral structure was homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at 1000 × g for 10 min; the supernatants were removed for Na$^+$,K$^+$-ATPase activity determination.

The reaction mixture for Na$^+$,K$^+$-ATPase assay contained 5 mM MgCl$_2$, 80 mM NaCl, 20 mM KCl and 40 mM Tris–HCl, pH 7.4. After 10 min of sample pre-incubation at 37 ºC, the reaction was initiated by addition of ATP to a final concentration of 3 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na$^+$,K$^+$-ATPase activity was calculated by the difference between the two assays according to Wyse et al. (40). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (41). Specific activity of the enzyme was expressed as nmol Pi released/min/mg of protein.

ATP determination

Immediately after the rats had been sacrificed, the cerebral cortex was dissect out and frozen in liquid nitrogen. Each cortical hemisphere was weighed and homogenized in 5 ml of 0.1 M NaOH (to inactivate cellular ATPases activity). Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin Elmer, Waltham, MA, USA), according to Witt et al. (42). Chemiluminescence was measured using the Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet-tissue weights in grams and expressed as µmol/g.

Thiobarbituric Acid Reactive Species (TBARS)

The 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 800 g for 10 min at 4 °C, the pellet was discarded and the supernatant was taken to TBARS assay according to Ohkawa et al. (43). Briefly, to eppendorfs were added in order of appearance: 200 µL of tissue supernatant; 50 µL of SDS 8.1%; 375 µL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 375 µL of 0.8 % thiobarbituric acid. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 hour. The mixture was allowed to cool on water for 5 min and was centrifuged 750 g for 10 min. The resulting pink stained TBARS were determined in spectrophotometer at 535 nm in a Beckman DU$^®$ 800 (Beckman Coulter, Inc., Fullerton, CA, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. Results are expressed as nmol TBARS/mg protein.

Protein determination

The protein content of samples was determined using bovine serum albumin as standard, according to Lowry et al. (44) for TBARS assay, Peterson (45) for glutamate uptake or Bradford (46) for Na^+,K^+-ATPase and GLAST and GLT-1 immunocontent.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Data were analyzed by one-way ANOVA followed by Duncan`s multiple range test when appropriate. Differences were considered statistically significant if p < 0.05.

Results

We first studied the effect of acute and chronic administration of proline on glutamate uptake, as well as the influence of guanosine administration in the effects promoted by proline. Fig.1A shows that acute proline injection significantly decreased glutamate uptake in cortical slices of rats, when compared to controls (saline administration) $[F(3,18) = 4.50; p < 0.05]$. Besides, the pretreatment with guanosine *per se* did not alter glutamate uptake and did not prevent the reduction of this parameter caused by acute proline administration. Similarly, Fig. 1B shows that chronic proline administration promoted a significant decrease in glutamate uptake when compared to control group, but when guanosine was administrated concomitantly to proline, the reduction of glutamate uptake was completely prevented $[F(3,16) = 6.04; p < 0.05]$.

Next, the glutamate transporters immunocontent were determined in animals subjected to acute or chronic proline administration with or without

guanosine treatment. As can be observed in Fig. 2, GLAST (A) [$F_{(3,16)} = 6.28$; p < 0.01] and GLT-1 (B) [$F_{(3,16)} = 14.77$; $p < 0.001$] were increased in groups that received acute proline with or without guanosine pretreatment, as compared to control. Conversely, chronic proline administration and/or guanosine did not alter GLAST (A) [$F_{(3,17)} = 0.58$; $p > 0.05$] and GLT-1 (B) [$F_{(3,16)} = 1.59$; $p > 0.05$] immunocontent in the cerebral structure studied.

Considering that proline inhibits Na^+,K^+-ATPase (27) and that the normal activity of this enzyme is closely related to clearance of glutamate from the extracellular fluid and synaptic cleft, we also evaluated the influence of guanosine on the alterations in the Na^+,K^+-ATPase caused by acute and chronic hyperprolinemia. Fig. 3A shows that rats subjected to acute proline treatment presented a significant inhibition of Na^+,K^+-ATPase activity. The pretreatment with guanosine did not alter *per se* the Na^+,K^+-ATPase activity, but partially prevented the inhibition of this enzyme caused by acute proline administration [$F_{(3,19)} = 11.24$; $p < 0.001$]. In addition, Fig. 3B shows that chronic hyperprolinemia also elicited a significant inhibition of Na^+,K^+-ATPase activity in homogenate of cerebral cortex of rats, but when guanosine was administrated concomitantly to proline, completely prevented the inhibition of Na^+,K^+-ATPase activity [$F_{(3,18)} = 4.31$; $p < 0.05$].

Since the correct functioning of Na^+,K^+-ATPase depends on the availability of ATP and integrity of plasma membrane, we investigated the effect of acute and chronic hyperprolinemia, as well as the influence of guanosine on ATP levels and lipid peroxidation was assessed by TBARS assay. Fig. 4 shows that proline acute (A) [$F_{(3,18)} = 5.22$; $p < 0.01$] and chronic (B) [$F_{(3,18)} = 5.69$; $p < 0.05$] administration significantly decrease the intracellular ATP concentration, when

compared to control group. Moreover, pretreatment with guanosine followed to acute proline administration, as well as concomitant guanosine administration to chronic proline treatment, was unable to prevent the reduction of ATP levels caused by proline.

Fig. 5A depicts that acute proline administration significantly increased TBARS levels in cerebral cortex of rats [$F(3,18) = 10.59$; $p < 0.001$]. *Post hoc* analysis showed that pretreatment with guanosine did not alter *per se* this parameter, but significantly prevented the increase of TBARS caused by proline. Similarly, Fig. 5B shows that chronic proline administration causes a significant increase in TBARS levels when compared to control group, however the concomitant treatment with guanosine prevented this effect [$F(3,22) = 7.72$; $p < 0.01$].

Discussion

Since a previous work from our laboratory demonstrated that a single proline administration decreases the glutamate uptake in cerebral cortex of rats (10), in the present study we investigated the effects of chronic exposure to proline on this parameter, as well as some mechanisms underlying the effects of acute and chronic proline administration on glutamate homeostasis. Considering that guanosine has been related as a possible neuroprotective in conditions of glutamatergic toxicity, the influence of this nucleoside on the proline-mediated effects was also evaluated. Results revealed that both acute and chronic proline administration decreased the glutamate uptake in cortical slices of rats. The pretreatment with guanosine during seven days was insufficient to prevent the decreased glutamate uptake promoted by acute proline administration. However,

when guanosine was administrated concomitantly to proline in the chronic treatment (from 6th to 28th day of life) the proline-mediated effect on glutamate uptake was completely prevented, suggesting that long term exposure to guanosine seem to be necessary to its protective effects on glutamate uptake. However, further studies are needed to elucidate the modulatory effects of guanosine on this parameter.

Since GLAST and GLT-1 are responsible to account for the majority of extracellular glutamate (11) and in order to investigate whether the impairment of glutamate uptake caused by proline had some relationship to these glutamate transporters levels, we evaluated the immunocontent of GLAST and GLT-1 in homogenate from cerebral cortex of rats. Interestingly, results showed that acute proline injection increased the levels of these transporters, which may reflect an early attempt to supply (but without success) the ineffective glutamate uptake activity. In addition, the treatment for a week with guanosine also was able to increase *per se* GLAST and GLT-1 levels. This capacity of guanosine for upregulate GLAST and GLT-1, may be a possible and interesting mechanism of action for this nucleoside that, at least to our knowledge, was not yet reported.

Conversely, chronic administration of proline and/or guanosine did not alter GLAST and GLT-1 content in the rat cerebral cortex, suggesting that successive administrations for a long time make them unresponsive. Since we did not found a direct association between the glutamate uptake activity and level of glutamate transporters, it is reasonable to suggest that the dysfunction of glutamate uptake promoted by proline does not involve a quantitative disorder of transporters protein, but perhaps an alteration on its proper functioning.

One of the mechanisms proposed for driving the glutamate transport is based on the reliance of glutamate uptake to Na^+,K^+-ATPase activity, since this enzyme generates an electrochemical sodium gradient that is used by the transporters to drive the uphill transport of the neurotransmitters (20). In this context, it has been reported that experimental hyperprolinemia inhibits the activity of Na^+,K^+-ATPase, but not the expression of their catalytic their subunits in cerebral cortex of rats (27, 28). Therefore, we evaluated the influence of treatment with guanosine on inhibition of this enzyme activity caused by proline. The results showed that in both acute and chronic models, guanosine administration was able to prevent significantly the inhibitory effect of proline on Na^+,K^+-ATPase activity, although the chronic effect has been more prominent.

According to our data, there seems to be an involvement of inhibition Na^+,K^+-ATPase activity with impairment of glutamate uptake caused by proline. Futhermore, the protective effects of guanosine seem to depend on time of exposure to this nucleoside, since in the acute model, the pretreatment with guanosine for seven days prevented only partially the inhibition of Na^+,K^+-ATPase activity caused by proline, which not appears to be sufficient to normalize glutamate uptake. However, the concomitant guanosine treatment to chronic proline administration completely prevented the inhibition of the enzyme and glutamate uptake.

Activation of ionotropic glutamate receptors has been shown to increase energy consumption by leads to influx of ions, which have to be pumped out again in a process requiring energy (11). Besides that, mitochondrial ATP production is impaired during excitotoxic insults and neuronal ATP levels are

diminished rapidly (47, 48). Therefore, we determined the ATP levels in cerebral cortex of rats subjected to hyperprolinemia, as well as the influence of guanosine on this parameter. Results showed a significant decrease in the ATP levels following acute and chronic proline administration. Guanosine did not affect *per se* the ATP levels, neither was able to avoid the effect promoted by proline on this parameter. In the acute model, the diminished ATP corroborates with hypothesis that proline induces excitotoxicity, since the glutamate uptake is impaired and possibly, the glutamate levels are enhanced in the brain generating excitotoxicity, process that involves energy depletion. In this scenario, guanosine pretreatment did not prevent the reduced glutamate uptake caused by proline and consequently was unable to restore the ATP levels. On the other hand, in the chronic model, guanosine prevented the reduction in glutamate uptake while the ATP levels remains decreased suggesting that other molecular alterations (inaccessible to guanosine action) may be involved in the long term proline exposure. In agreement with this observation, it has been demonstrated that proline inhibits cytochrome *c* oxidase, an enzyme step limiting to ATP synthesis (49).

Some reports have demonstrated that glutamate transport is impaired by 4-hydroxynonenal (HNE), which is an aldehyde generated by peroxidation of membrane lipids (50, 51). Beyond that, Na^+,K^+-ATPase is a membrane-inserted enzyme and its inhibition has been associated with changes in plasma membrane lipid composition (23). Therefore, we also investigated the effects of proline and guanosine administration on TBARS, a classic parameter of lipid peroxidation, in order to verify if the alterations on membrane lipids may be linked to the proline and/or guanosine-elicited effects on Na^+,K^+-ATPase and

consequently, on glutamate uptake. Results showed that both acute and chronic proline administration increased TBARS, in accordance to our previous data (28, 52). Moreover, guanosine treatment was able to prevent both acute and chronic proline-mediated effects on TBARS. Lipid peroxidation and ROS production may be consequences to overstimulation of glutamate receptors (11, 53). Then, the effects of proline and guanosine on TBARS might be resulting from their effects on glutamate uptake. In accordance, it has been described an antioxidant role of guanosine, possibly indirect and related to its ability to stimulate glutamate uptake (54).

In conclusion, this study demonstrates that high proline levels, similar to those found in human hyperprolinemia can impair glutamate uptake probably by disruption in Na^+,K^+-ATPase activity and lipid peroxidation, but not by alterations in glutamate transporters. Therefore, we suggested that the alterations in glutamate homeostasis caused by hyperprolinemia may lead to excitotoxicity and subsequent energy deficit. Interestingly, guanosine was effective to prevent most of the effects promoted by proline, reinforcing its modulator role in counteracting the glutamate toxicity. Additional studies are requested to better elucidate the magnitude of the modulatory effects of guanosine on experimental hyperprolinemia.

Acknowledgments

References

1. Phang JM, Hu CA, Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (ed) The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp 1821–1838

2. Wyse AT, Netto CA (2011) Behavioral and neurochemical effects of proline. Metab Brain Dis, IN PRESS:

3. Flynn MP, Martin MC, Moore PT et al. (1989) Type II hyperprolinaemia in a pedigree of Irish travellers (nomads). Arch Dis Child 64: 1699-707

4. Van Harreveld A, Fifkova E (1973) Effects of amino acids on the isolated chicken retina, and on its response to glutamate stimulation. J Neurochem 20: 947-62

5. Rhoads DE, Peterson NA, Raghupathy E (1983) Selective inhibition of synaptosomal proline uptake by leucine and methionine enkephalins. J Biol Chem 258: 12233-7

6. Cohen SM, Nadler JV (1997) Proline-induced potentiation of glutamate transmission. Brain Res 761: 271-82

7. Nadler JV (1987) Sodium-dependent proline uptake in the rat hippocampal formation: association with ipsilateral-commissural projections of CA3 pyramidal cells. J Neurochem 49: 1155-60

8. Nadler JV, Bray SD, Evenson DA (1992) Autoradiographic localization of proline uptake in excitatory hippocampal pathways. Hippocampus 2: 269-78

9. Fremeau RT, Jr., Caron MG, Blakely RD (1992) Molecular cloning and expression of a high affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. Neuron 8: 915-26

10. Delwing D, Sanna RJ, Wofchuk S et al. (2007) Proline promotes decrease in glutamate uptake in slices of cerebral cortex and hippocampus of rats. Life Sci 81: 1645-50

11. Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65: 1-105

12. Segovia G, Porras A, Del Arco A et al. (2001) Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev 122: 1-29

13. Nicholls DG (2008) Oxidative stress and energy crises in neuronal dysfunction. Ann N Y Acad Sci 1147: 53-60

14. Maragakis NJ, Rothstein JD (2001) Glutamate transporters in neurologic disease. Arch Neurol 58: 365-70

15. Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 15: 461-73

16. Meldrum BS (1994) The role of glutamate in epilepsy and other CNS disorders. Neurology 44: 14-23

17. Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32: 1-14

18. Attwell D (2000) Brain uptake of glutamate: food for thought. J Nutr 130: 1023-5

19. Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51: 333-55

20. Rose EM, Koo JC, Antflick JE et al. (2009) Glutamate transporter coupling to Na,K-ATPase. J Neurosci 29: 8143-55

21. Mobasheri A, Avila J, Cozar-Castellano I et al. (2000) Na^+,K^+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. Biosci Rep 20: 51-91

22. Kaplan JH (2002) Biochemistry of Na^+,K^+-ATPase. Annu Rev Biochem 71: 511-35

23. Dencher NA, Frenzel M, Reifschneider NH et al. (2007) Proteome alterations in rat mitochondria caused by aging. Ann N Y Acad Sci 1100: 291-8

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

25. Siems WG, Hapner SJ, van Kuijk FJ (1996) 4-hydroxynonenal inhibits Na^+,K^+-ATPase. Free Radic Biol Med 20: 215-23

26. Potts MB, Koh SE, Whetstone WD et al. (2006) Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. NeuroRx 3: 143-53

27. Pontes ZE, Oliveira LS, Bavaresco CS et al. (1999) Proline administration decreases Na^+,K^+-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab Brain Dis 14: 265-72

28. Ferreira AG, Stefanello FM, Cunha AA et al. (2011) Role of antioxidants on Na^+,K^+-ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats. Metab Brain Dis 26: 141-7

29. Delwing D, Bavaresco CS, Chiarani F et al. (2003) In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. Brain Res 991: 180-6

30. Delwing D, Bavaresco CS, Wannmacher CM et al. (2003) Proline induces oxidative stress in cerebral cortex of rats. Int J Dev Neurosci 21: 105-10

31. Schmidt AP, Tort AB, Silveira PP et al. (2009) The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: reversal by guanosine. Pharmacol Biochem Behav 91: 549-53

32. Schmidt AP, Lara DR, Maraschin JF et al. (2000) Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res 864: 40-3

33. Lara DR, Schmidt AP, Frizzo ME et al. (2001) Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain Res 912: 176-80

34. Chang R, Algird A, Bau C et al. (2008) Neuroprotective effects of guanosine on stroke models in vitro and in vivo. Neurosci Lett 431: 101-5

35. Frizzo ME, Soares FA, Dall'Onder LP et al. (2003) Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res 972: 84-9

36. Dal-Cim T, Martins WC, Santos AR et al. (2011) Guanosine is neuroprotective against oxygen/glucose deprivation in hippocampal slices via large conductance Ca^{2+}-activated K^+ channels, phosphatidilinositol-3 kinase/protein kinase B pathway activation and glutamate uptake. Neuroscience 183: 212-20

37. Jiang S, Fischione G, Giuliani P et al. (2008) Metabolism and distribution of guanosine given intraperitoneally: implications for spinal cord injury. Nucleosides Nucleotides Nucleic Acids 27: 673-80

38. Moreira JC, Wannmacher CM, Costa SM et al. (1989) Effect of proline administration on rat behavior in aversive and nonaversive tasks. Pharmacol Biochem Behav 32: 885-90

39. Frizzo ME, Lara DR, Prokopiuk Ade S et al. (2002) Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell Mol Neurobiol 22: 353-63

40. Wyse ATS, Streck EL, Worm P et al. (2000) Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem Res 25: 971-5

41. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal Biochem 157: 375-80

42. Witt KA, Mark KS, Hom S et al. (2003) Effects of hypoxia-reoxygenation on rat blood-brain barrier permeability and tight junctional protein expression. Am J Physiol Heart Circ Physiol 285: H2820-31

43. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-8

44. Lowry OH, Rosebrough NJ, Farr AL et al. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-75

45. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83: 346-56

46. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-54

47. Greenwood SM, Connolly CN (2007) Dendritic and mitochondrial changes during glutamate excitotoxicity. Neuropharmacology 53: 891-8

48. Greenwood SM, Mizielinska SM, Frenguelli BG et al. (2007) Mitochondrial dysfunction and dendritic beading during neuronal toxicity. J Biol Chem 282: 26235-44

49. Delwing D, Chiarani F, Kurek AG et al. (2007) Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci 25: 17-22

50. Blanc EM, Keller JN, Fernandez S et al. (1998) 4-hydroxynonenal, a lipid peroxidation product, impairs glutamate transport in cortical astrocytes. Glia 22: 149-60

51. Pedersen WA, Cashman NR, Mattson MP (1999) The lipid peroxidation product 4-hydroxynonenal impairs glutamate and glucose transport and choline acetyltransferase activity in NSC-19 motor neuron cells. Exp Neurol 155: 1-10

52. Ferreira AG, Lima DD, Delwing D et al. (2010) Proline impairs energy metabolism in cerebral cortex of young rats. Metab Brain Dis 25: 161-8

53. Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1: 623-34

54. Roos DH, Puntel RL, Santos MM et al. (2009) Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system. Toxicol In Vitro 23: 302-7

Legend to figures

Fig. 1 Effect of acute (A) and chronic (B) proline, guanosine and proline plus guanosine administration on glutamate uptake in slices from cerebral cortex of rats. Data are mean ± S.D. for 6-7 animals in each group. Different from control, $*p < 0.05$ and $**p < 0.01$ (Duncan multiple range test). Pro, proline; Guo, guanosine.

Fig. 2 Effect of acute and chronic proline, guanosine and proline plus guanosine administration on glutamate transporters immunocontent, GLAST (A) and GLT-1 (B) in homogenates from cerebral cortex of rats. Data are mean ± S.D. for 6-7 animals in each group. Different from control, $**p < 0.01$ and $***p < 0.001$; [#] Different from both control and proline groups (Duncan multiple range test). Pro, proline; Guo, guanosine.

Fig. 3 Effect of acute (A) and chronic (B) proline, guanosine and proline plus guanosine administration on Na^+,K^+-ATPase activity in homogenates from cerebral cortex of rats. Data are mean ± S.D. for 6-7 animals in each group. Different from control, $*p < 0.05$ and $***p < 0.001$; [#] Different from both control and proline groups (Duncan multiple range test). Pro, proline; Guo, guanosine.

Fig. 4 Effect of acute (A) and chronic (B) proline, guanosine and proline plus guanosine administration on ATP levels in homogenates from cerebral cortex of rats. Data are mean ± S.D. for 6-7 animals in each group. Different from control,

*p < 0.05 and **p < 0.01 (Duncan multiple range test). Pro, proline; Guo, guanosine.

Fig. 5 Effect of acute (A) and chronic (B) proline, guanosine and proline plus guanosine administration on thiobarbituric acid-reactive substances (TBARS) in homogenates from cerebral cortex of rats. Data are mean ± S.D. for 6-7 animals in each group. Different from control, **p < 0.01 and ***p < 0.001 (Duncan multiple range test). Pro, proline; Guo, guanosine.

Figure 1

A)

B)
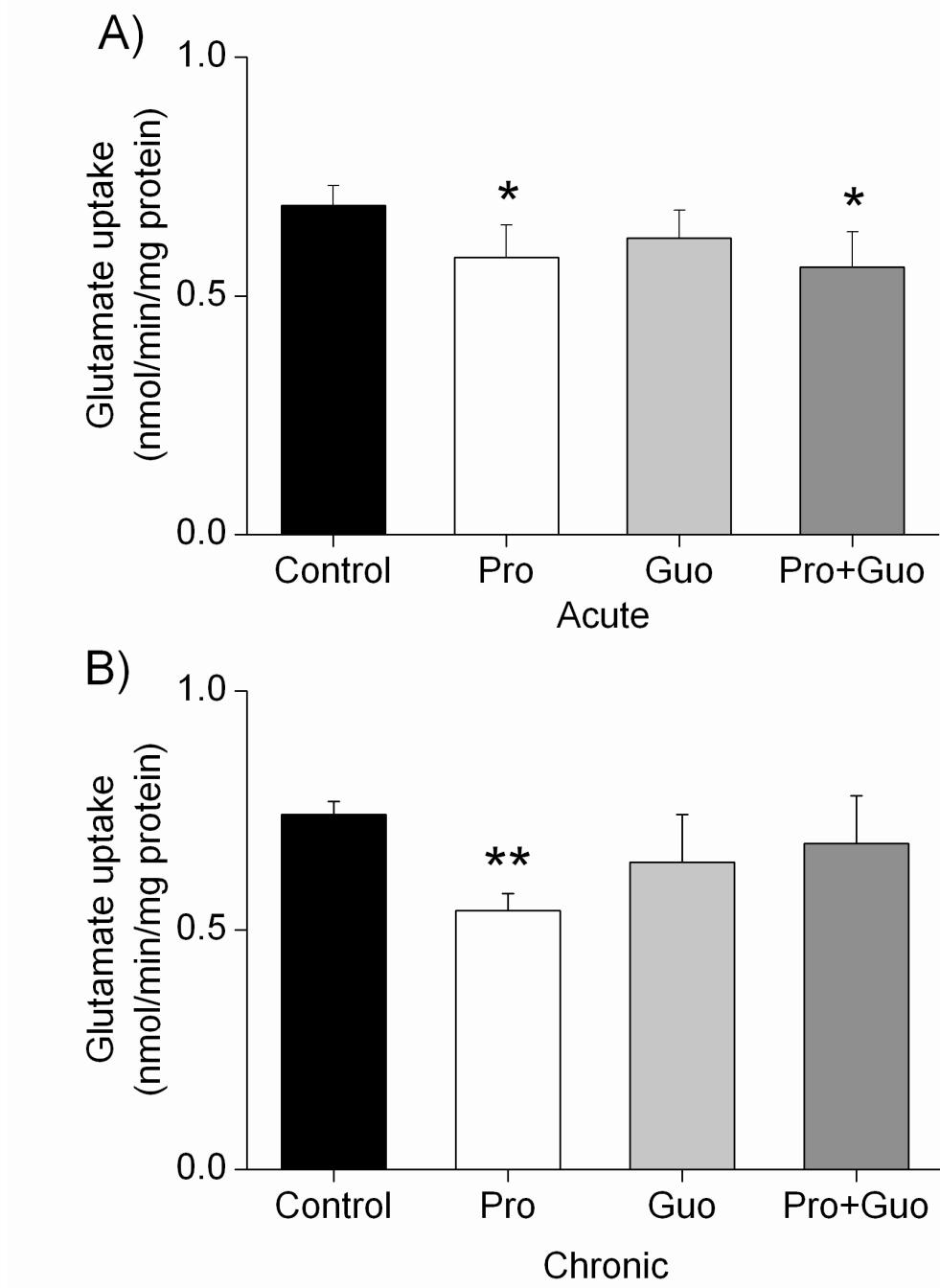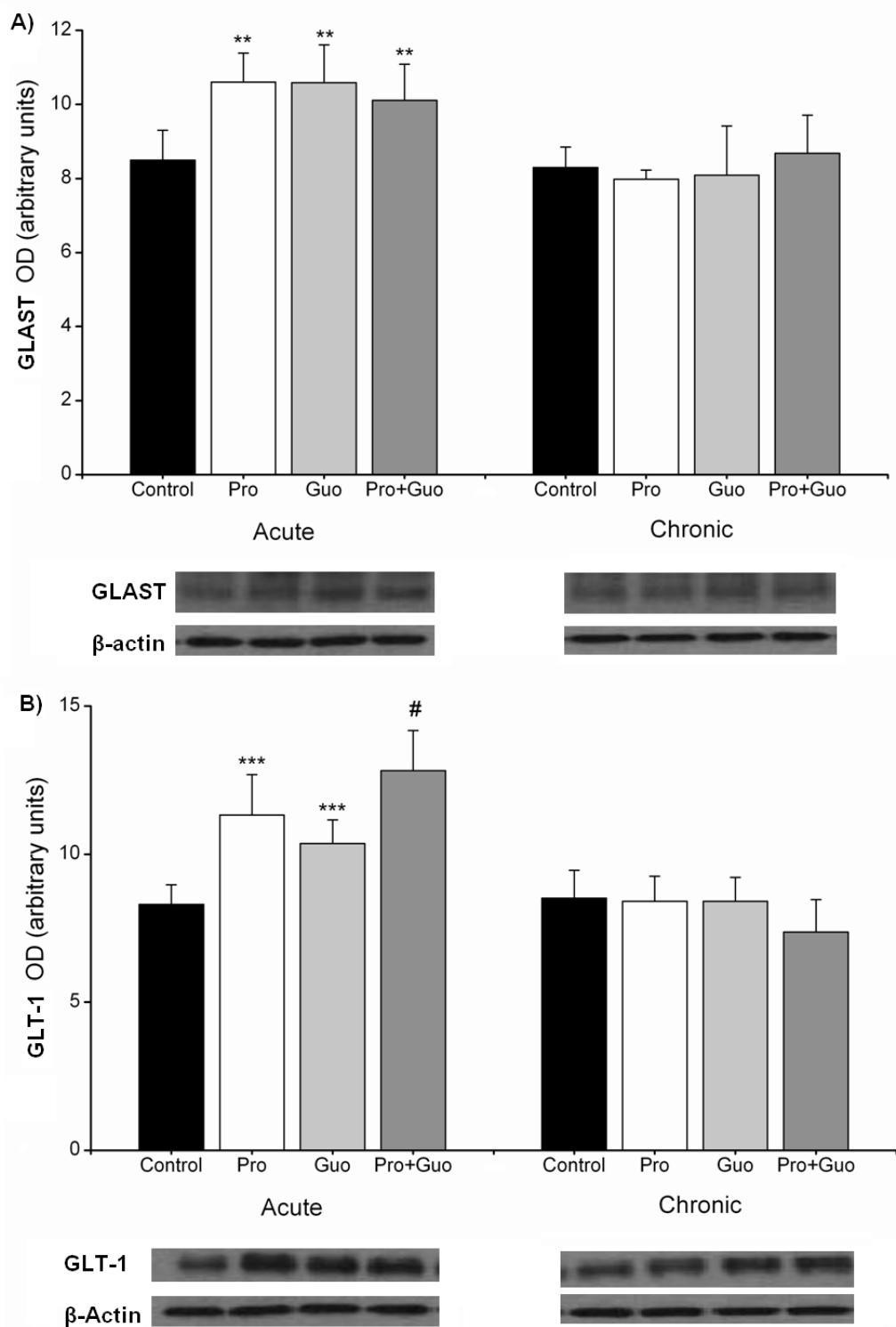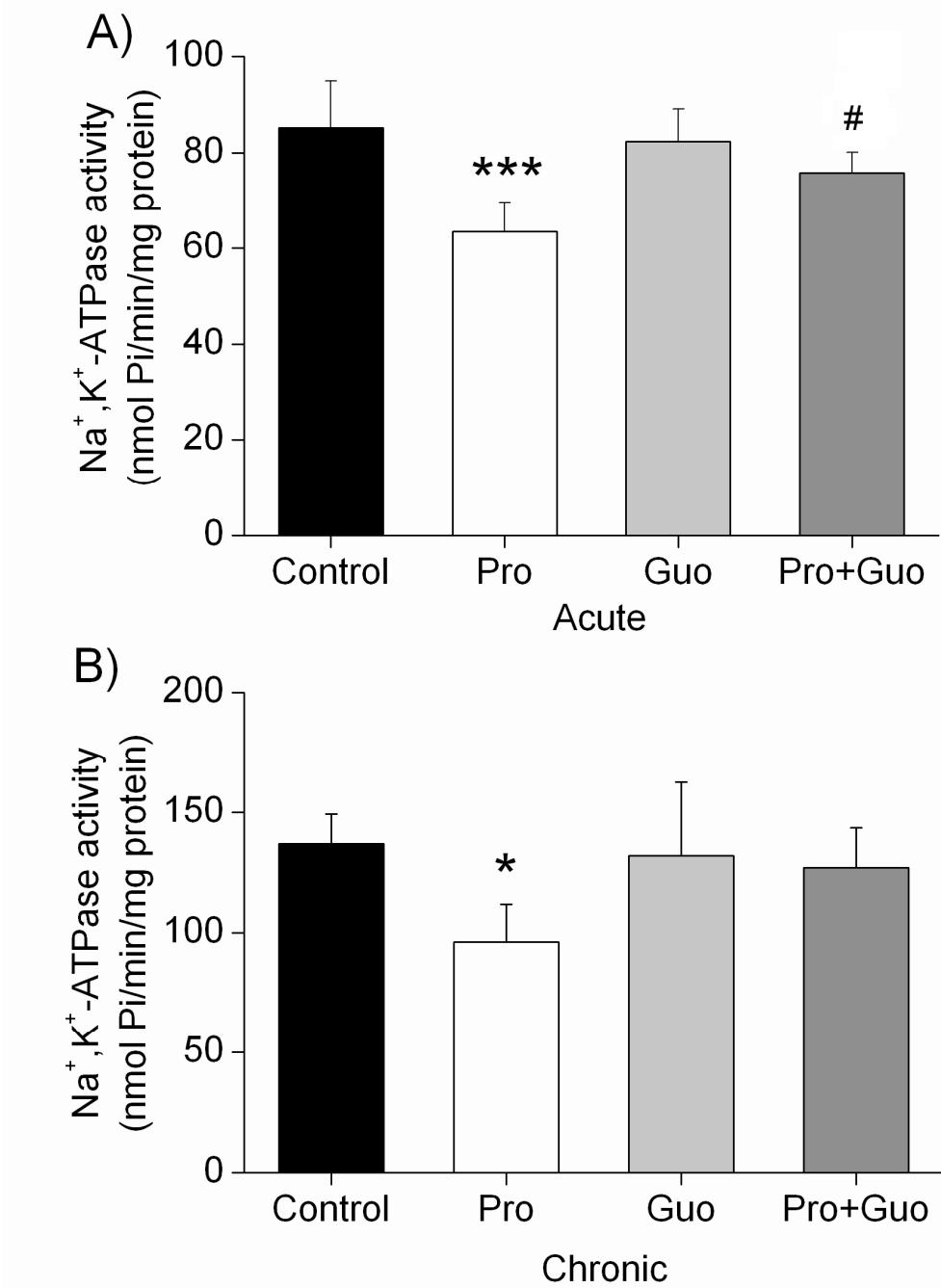
Figure 2
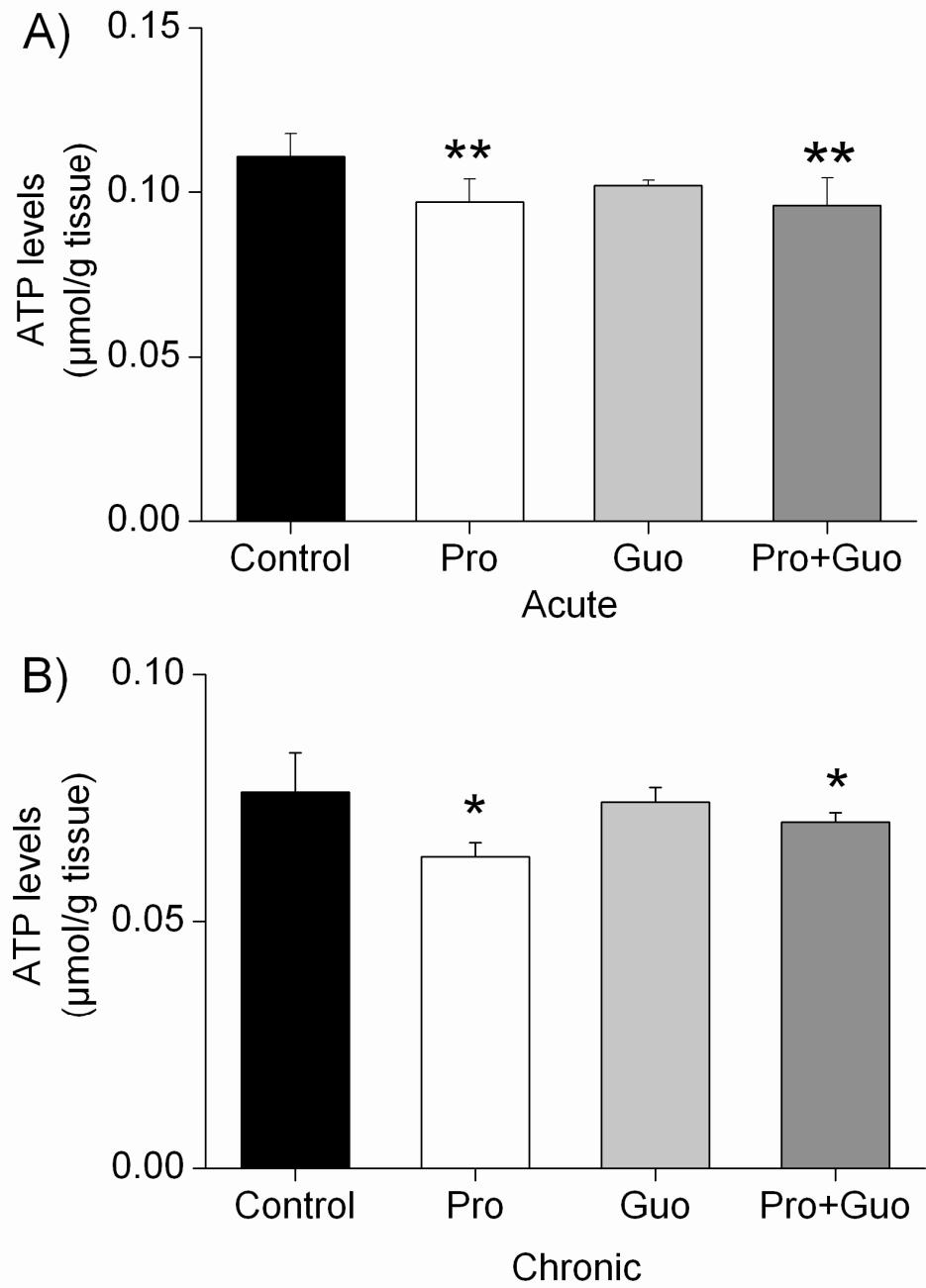
Figure 3

Figure 4

Figure 5
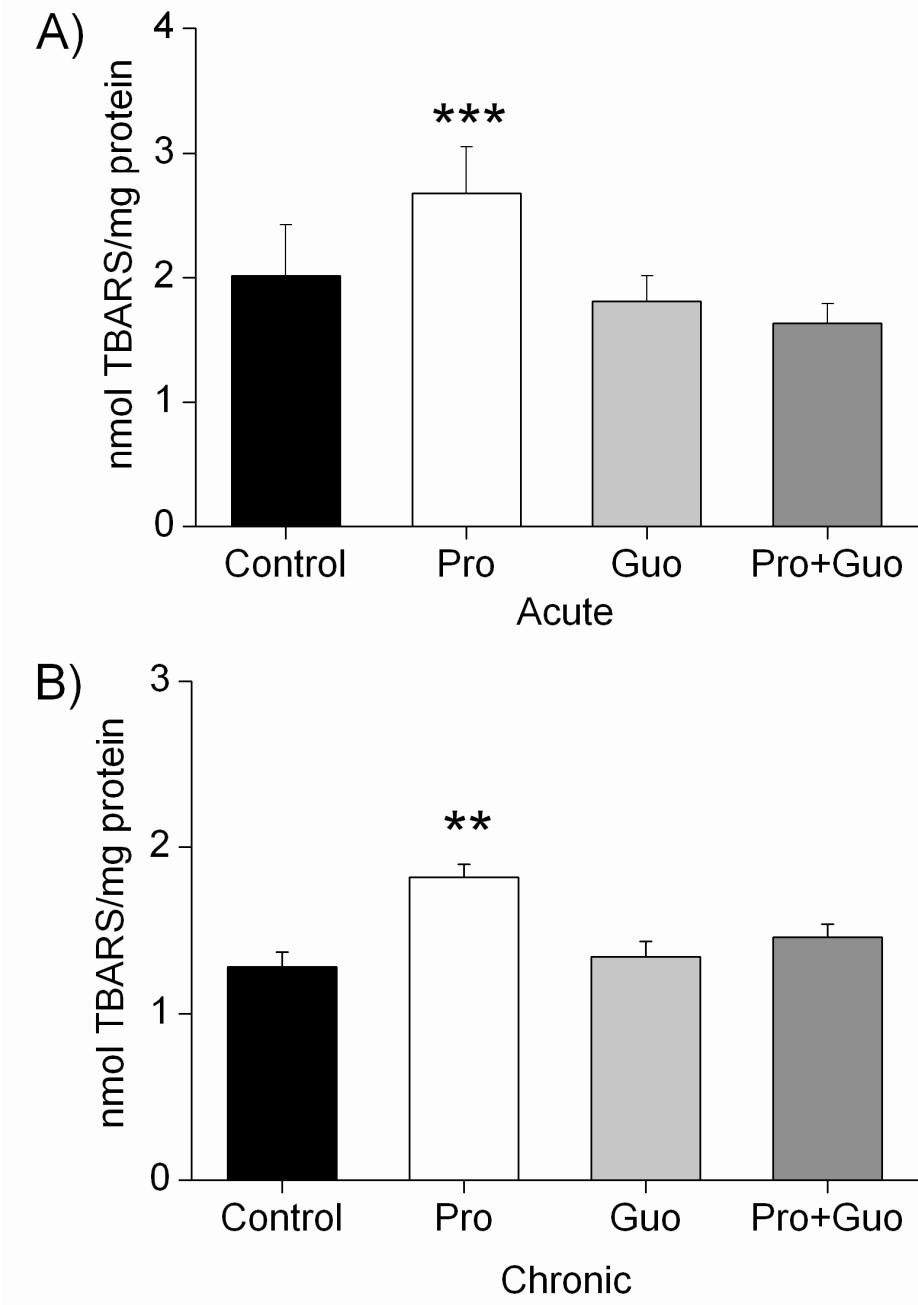
Capítulo IV

Physical exercise reverses cognitive impairment of rats subjected to experimental hyperprolinemia

Andréa G. K. Ferreira, Emilene Scherer, Maira da Cunha, Fernanda Machado, Aline A. da Cunha, Jeferson Graeff, Carlos A. Netto and Angela T. S. Wyse

Physical Exercise Reverses Cognitive Impairment in Rats Subjected to Experimental Hyperprolinemia

Andréa G. K. Ferreira · Emilene B. Scherer · Maira J. da Cunha ·
Fernanda R. Machado · Aline A. da Cunha · Jeferson S. Graeff ·
Carlos A. Netto · Angela T. S. Wyse

Abstract This study investigated whether physical exercise would reverse proline-induced performance deficits in water maze tasks, as well as its effects on brain-derived neurotrophic factor (BDNF) immunocontent and brain acetylcholinesterase (AChE) activity in Wistar rats. Proline administration followed *partial time* (6th–29th day of life) or *full time* (6th–60th day of life) protocols. Treadmill exercise was performed from 30th to 60th day of life, when behavioral testing was started. After that, animals were sacrificed for BDNF and AChE determination. Results show that proline impairs cognitive performance, decreases BDNF in cerebral cortex and hippocampus and increases AChE activity in hippocampus. All reported effects were prevented by exercise. These results suggest that cognitive, spatial learning/memory, deficits caused by hyperprolinemia may be associated, at least in part, to the decrease in BDNF levels and to the increase in AChE activity, as well as support the role of physical exercise as a potential neuroprotective strategy.

Keywords Hyperprolinemia · Exercise · Spatial memory · BDNF · Acetylcholinesterase

A. G. K. Ferreira · E. B. Scherer · M. J. da Cunha ·
F. R. Machado · A. A. Cunha · J. S. Graeff ·
C. A. Netto · A. T. S. Wyse (✉)
Laboratório de Neuroproteção e Doença Metabólica,
Departamento de Bioquímica, Instituto de Ciências Básicas da
Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro
Barcelos, 2600-Anexo, Porto Alegre, RS, Brazil
e-mail: wyse@ufrgs.br

Introduction

Hyperprolinemia is a phenotype common to two inherited metabolic disorders caused by distinct genetic defects in the L-proline catabolic pathway. Hyperprolinemia type I (HPI) is caused by proline oxidase deficiency, the first step in the conversion of proline to glutamate, whereas hyperprolinemia type II (HPII) is due to the absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity. Although both enzymes deficiencies lead to tissue accumulation of proline, HPII causes higher plasma levels and shows a causal relationship with neurological manifestations such as seizures and mental retardation, while a coincidental association between HPI and clinical features has been discussed [1, 2].

Since the mechanisms underlying hyperprolinemia symptoms are still obscure, our group has developed a chemical model for hyperprolinemia in rats mimicking tissue levels of proline found in human HPII [3, 4]. Considering that HP is an inherited disease, the animal model of hyperprolinemia was designed to sustain high levels of proline from the earliest days of life, comprising the period of rapid brain development with intense synaptogenesis and myelination in rats, which finalizes approximately at 21 days of life [5–7]. Rats chronically treated with proline following such experimental model present acquisition and memory deficits, as assessed in the Morris water maze, when they reach adulthood (approximately 30 days after the interruption of proline administration) [8, 9].

Dietary therapies, i.e., restriction of proline, are currently used in HP patients, but only modest control of plasma proline is achieved with no impact in clinical condition [2, 10]. Therefore, the search for new therapeutic strategies it is necessary; one that can be considered is of moderate physical activity, because of its positive effects

on cognitive function both in humans and rodents [11–16]. Clinical and preclinical studies show that aerobic activity improves learning, increases trophic factors release, induces synaptic plasticity and promotes development of new neuronal architecture [17]. One of the mechanisms proposed for exercise effects on cognition is the exercise-induced increase of brain derived neurotrophic factor (BDNF) [12, 18], which has been described as a major player in the mechanisms governing the multiple phases of cognition, including acquisition, memory consolidation/storage, and memory retrieval [11, 19]. BDNF seems to act promoting synaptic facilitation [20] and neurotransmitter release [21–24], and has been strongly implicated in spatial memory modulation [25, 26].

Available evidence demonstrates that the cholinergic system also plays an important role in cognition [27–29], triggering a series of intracellular events related to neuronal plasticity and memory encoding. Cholinergic activity is mainly controlled by acetylcholinesterase (AChE), the enzyme that hydrolyses acetylcholine (ACh), so finishing cholinergic transmission [30]. Reduction of brain ACh appears to be associated to cognitive deficits [28] and a correlation between changes in AChE activity and cognitive deficits in animal models of metabolic diseases has been established [31, 32].

In order to investigate the influence of physical exercise on the cognitive deficit in Morris water maze spatial tasks produced by hyperprolinemia in rats, two different protocols of proline administration were utilized, namely: (a) *partial time hyperprolinemia* and (b) *full time hyperprolinemia*. The first protocol was designed to evaluate whether the exercise is able to recover the cognitive deficit already established by hyperprolinemia during the critical period of brain development [8, 9], while the second protocol was performed to verify whether the putative beneficial cognitive effects of exercise would remain if hyperprolinemia is extended through adulthood. Furthermore, BDNF immunocontent and AChE activity were determined in cerebral cortex and the hippocampus, two cerebral structures known to participate in learning/memory modulation in experimental rats.

Experimental Procedure

Animals

Male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80-23, revised 1996) and the experimental protocol was approved by the Ethics Committee of the Federal University of Rio Grande do Sul.

Proline Administration

Chronic hyperprolinemia was chemically induced by daily subcutaneous administration of proline, following two different protocols regarding the duration of treatment. In the protocol of partial time hyperprolinemia, rats received proline administration from the 6th to the 29th day of life, as described: (a) 12.8 μmol Pro/g body weight from 6th to 13th day of life; (b) 14.6 μmol Pro/g body weight from 14th to 17th day; (c) 16.4 μmol Pro/g body weight from 18th to 21th day and (d) 18.2 μmol Pro/g body weight from 22th to 29th day of life. After treatment discontinuation (30th day), animals started treadmill exercise until the 60th day of life. In the protocol of full time hyperprolinemia, proline was injected from the 6th to the 60th day of life in the dosing regimen above described, except that the higher dose, 18.2 μmol Pro/g body weight, was injected from 22th to 60th day of life. In this protocol, exercise training was performed from 30th to the 60th day of life, concomitant with proline administration. Doses were calculated from pharmacokinetic parameters of proline [4]. Proline solution (Sigma Chemical Co., USA) was prepared in 0.9% NaCl and administered twice a day.

Rats subjected to this treatment achieved plasma proline levels between 1.0 and 2.0 mM, which are similar to those found in hyperprolinemic patients [2]. Control animals received saline injections in the same volumes as those applied to proline-treated rats.

Exercise Training

Twenty five-days-old animals were habituated with the treadmill apparatus to minimize novelty stress and randomly assigned to one of the four experimental groups: (1) control (saline–sedentary); (2) proline (proline–sedentary); (3) exercise (saline–exercise) and (4) proline plus exercise (proline–exercise).

Treadmill exercise started on the 30th day of life and finished on the 60th day. Training consisted of 20 min of running sessions on an adapted motorized rodent treadmill (INBRAMEDTK01, Porto Alegre, Brazil), three times per week [33]. Animals from the control group (sedentary) were transported to the experimental room, handled exactly as the exercised ones and maintained in the turned off treadmill for 20 min [34].

A moderate intensity exercise protocol was used [33–35], i.e., exercise intensity was set at 60% of animal's maximal oxygen uptake [36]. The oxygen uptake (VO_2)

peak was estimated for all animals before training, considering the exhaustion, as follows: each rat ran on a treadmill at a low initial speed followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to continue running); the time to fatigue (in min) and workload (in m/min) were taken as indexes of exercise capacity, that was assigned as VO_2 max [36, 37].

Training in the treadmill involved gradual increase of running speed and time: in the 1st week, at 18 m/min for the first 3 min, 24 m/min for the next 2 min, 36 or 48 m/min for the following 10 min, 24 m/min for the following 2 min and 18 m/min for the last 3 min; in the 2nd week, at 18 m/min for the first 3 min, 24 m/min for the next 1 min, 36 or 48 m/min for the following 12 min, 24 m/min for the following 1 min and 18 m/min for the last 3 min; in 3rd and 4th weeks, at 18 m/min for the first 3 min, 36 or 48 m/min for the next 14 min, and 18 m/min for the last 3 min. Animals that initially refused to run were encouraged by gently tapping their backs [33, 35]. Behavioral testing was started 24 h after the last exercise session, when the rats reached 61 days of life (see Fig. 1).

Behavioral Testing

Behavioral studies were performed in the Morris water maze, a task widely employed for the study of spatial memory [38, 39]; experiments were conducted between 8 and 12 h a.m. The maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water maintained at constant temperature of 23°C; the tank was theoretically divided into four equal quadrants for the purpose of analysis and several distal visual cues were placed on the room walls. Trials were recorded by a video camera mounted above the center of the tank. Videotapes were analyzed using a dedicated software (ANY-maze®).
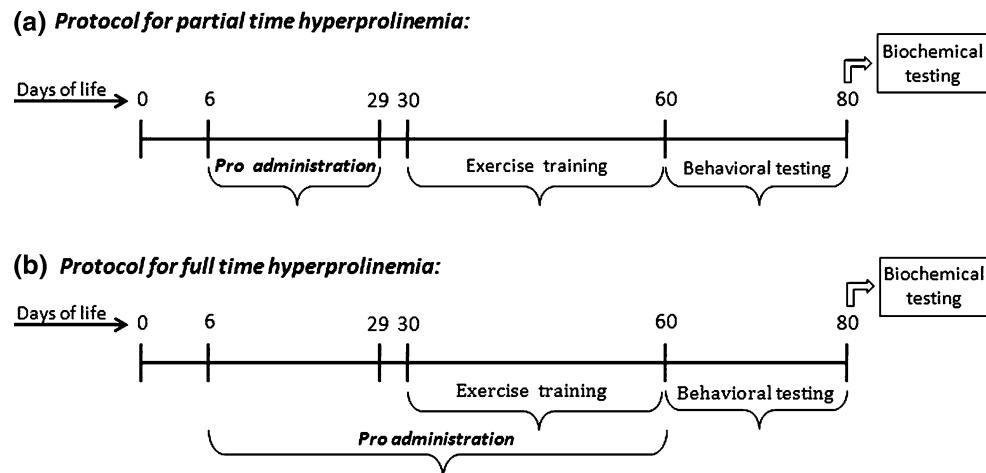
Reference Memory Task

The task consisted of seven training and one test sessions. In the acquisition phase, rats had daily sessions of four trials per day for 7 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the four starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failure, they were gently guided to it and allowed to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was of 15–20 min [38]. One day after the last training trial, animals were subjected to a probe trial in which the platform was removed; four parameters were then recorded, namely latency to cross over the platform location, the number of target crossings and the time spent in target (the quadrant in which the platform was located in training sessions) and opposite quadrants. Such parameters were taken as a measure for spatial memory [38]. In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

Working Memory Task

One week after the reference memory test, the working memory version of Morris water maze was performed. The task consisted of four consecutive trials per day, with intertrial interval of 1–2 min, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day

Fig. 1 Timeline of experimental procedures

(a) *Protocol for partial time hyperprolinemia:*

(b) *Protocol for full time hyperprolinemia:*

during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance [38].

Biochemical Analyses

Animals were sacrificed, after behavioral testing, by decapitation without anesthesia; the brain was removed and the hippocampus and cerebral cortex were dissected out for determination of BDNF immunocontent and AChE activity.

Analysis of BDNF Immunocontent

Mature BDNF protein was assessed using the E-Max ELISA kit (Promega) according to the manufacturer's recommendations and to Scherer and colleagues [40]. Briefly, cerebral cortex and hippocampus were individually homogenized in lysis buffer containing: 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4°C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with Block and Sample buffer. Plates were then incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h; color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL, was performed for each plate.

Acetylcholinesterase Assay

For the AChE activity essay, cerebral cortex and hippocampus were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at $1,000 \times g$. The supernatants were used for AChE activity analysis, according to Ellman and colleagues [41], with some modifications. Hydrolysis rates were measured at acetylthiocholine (AcSCh) concentration of 0.8 mM in 300 μL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25°C. 15 μL of cerebral cortex and hippocampus supernatants were added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) and specific enzyme activity was determined as μmol AcSCh per hour per milligram of protein. All samples were run in triplicate.

Protein Determination

Protein was measured by the method of Lowry et al. [42], using bovine serum albumin as standard.

Statistical Analysis

Statistics was performed using the Statistical Package for the Social Science (SPSS) software in a PC-compatible computer. Data from acquisition phase (training days) in the Morris water maze and from working memory were analyzed by factorial ANOVA for repeated measures considering days and trials as the repeated measure, respectively. Data from probe trial, BDNF immunocontent and AChE activity were analyzed by two-way ANOVA. AChE data were analyzed as original values (μmol AcSCh/h/mg protein), but expressed as percentage of controls. For all statistical tests, the factors were *proline* and *exercise*. Data are expressed as mean ± SEM; $P < 0.05$ is considered significant.

Results

Reference Memory Task

Both chronic hyperprolinemia experimental protocols affect the acquisition phase of Morris water maze reference memory in adult rats. For the partial time hyperprolinemia protocol (from 6th to 29th day of life), repeated measures ANOVA revealed a significant days × proline × exercise interaction ($F = 3.86$, $P < 0.01$). As shown in Fig. 2a, proline-treated rats had a lower ability to find the platform on days 3–7 ($P < 0.05$) when compared to the control group. Exercise per se did not alter control performance; however it reversed the effects of proline from day 3 onwards. Four parameters were evaluated in the reference memory probe trial: latency to cross over the platform location; the number of target crossings and the time spent in target and opposite quadrants (Fig. 2b). Two way ANOVA showed a significant interaction between proline and exercise in latency to cross over the platform location [$F_{(1, 42)} = 15.54$; $P < 0.001$] with a significant increase of this parameter caused by proline treatment ($P < 0.001$); this effect was also reversed by exercise. Proline and/or exercise did not alter the number of crossings over the platform location, neither the time spent in target or opposite quadrants ($P > 0.05$ in all cases).

Although there was no significant interaction of days × proline × exercise ($F = 0.49$, $P > 0.05$), as showed by repeated measures ANOVA, a significant main effect of days [$F_{(6,47)} = 86.79$; $P < 0.001$] and proline [$F_{(1,47)} = 6.94$;

Fig. 2 Effects of exercise and chronic proline administration, following the experimental protocol of partial time hyperprolinemia, on spatial reference memory. In the acquisition phase **a**, data show latencies to find the hidden platform, **b** shows probe trial parameters: latency to cross over the platform location (*B1*), number of crossings over platform position (*B2*), time spent in the target quadrant (*B3*) and time spent in the opposite quadrant (*B4*). Data are expressed as mean ± SEM for 11–13 animals in each group. Different from other groups, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (repeated measures ANOVA). *Pro + Exe* proline plus exercise

Fig. 3 Effects of exercise and chronic proline administration, following the experimental protocol of full time hyperprolinemia, on spatial reference memory. In the acquisition phase **a**, data show latencies to find the hidden platform, **b** shows probe trial parameters: latency to cross over the platform (*B1*), number of crossings over platform position (*B2*), time spent in the target quadrant (*B3*) and time spent in the opposite quadrant (*B4*). Data are expressed as mean ± SEM for 11–16 animals in each group. Different from other groups, $*P < 0.05$; $**P < 0.01$, $***P < 0.01$ (repeated measures ANOVA). *Pro + Exe* proline plus exercise

$P < 0.05$], but not of exercise [$F(1,47) = 2.26$; $P > 0.05$] was revealed. As depicted in Fig. 3a, proline-treated rats had a lower performance to find the platform from day 4 to 7 ($P < 0.05$). Exercise per se did not affect control performance, but reversed the proline cognitive effect. Two way ANOVA of the probe trial (Fig. 3b) showed a significant interaction of proline × exercise in the latency to cross over the platform location [$F = 12.80$; $P < 0.01$]. Proline treatment significantly increased latency to cross over the platform location when compared to control group ($P < 0.05$) and this effect was also reversed by exercise. However, proline and/or exercise did not alter other probe trial variables *($P > 0.05$ in all cases)*.
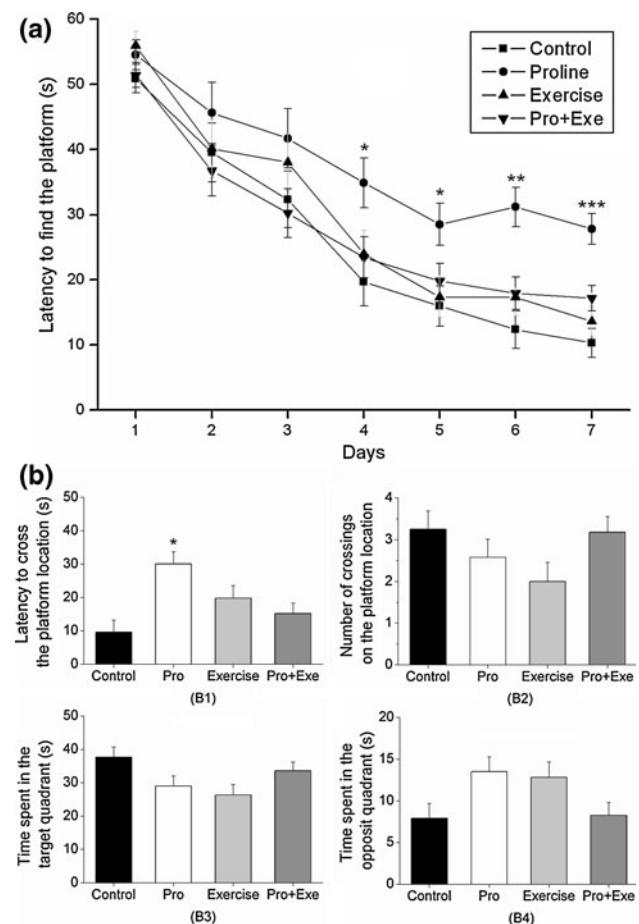
Working Memory Task

Both experimental protocols of chronic proline administration affected working memory in the Morris water maze. Repeated measures ANOVA showed a significant interaction of trials × proline × exercise after partial time hyperprolinemia [$F = 11.91$; $P < 0.001$] and full time hyperprolinemia [$F = 3.89$; $P < 0.05$]. Figure 4a and b show that proline impaired performance of rats in the working memory version of Morris water maze in 3rd and 4th trials ($P < 0.05$). Exercise per se did not alter control latencies to find the platform, but reversed the higher latencies induced by proline.
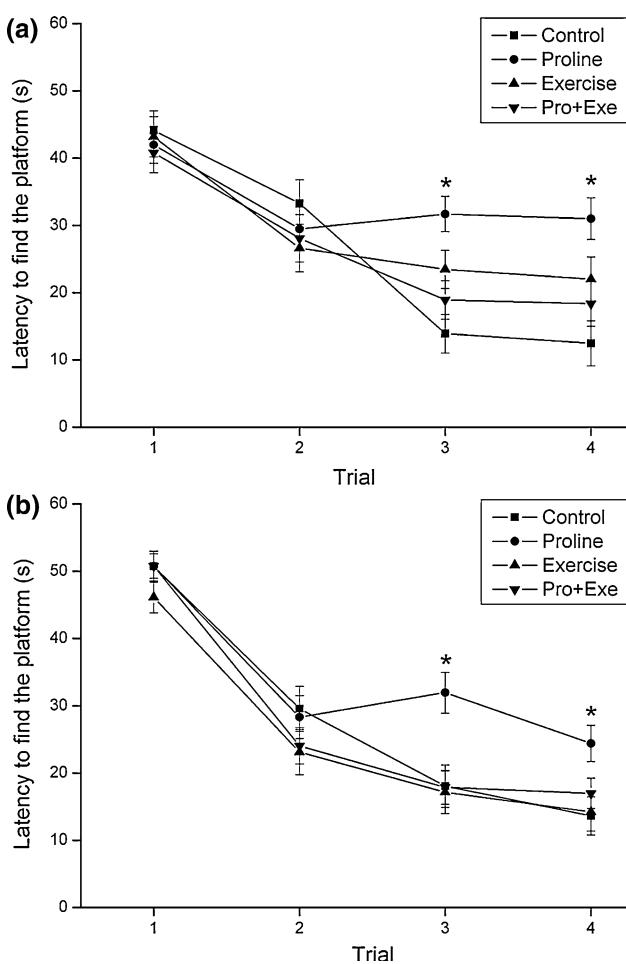
Fig. 4 Effects of exercise and chronic proline administration following experimental protocols of partial time (**a**) and full time (**b**) hyperprolinemia on spatial working memory. Data show latencies to find the platform on each trial during the 4 days and are expressed as mean ± SEM for 11–16 animals per group. Different from other groups, *$P < 0.05$ (repeated measures ANOVA). *Pro + Exe* proline plus exercise

Fig. 5 Effects of exercise and chronic proline administration following experimental protocols of partial time (**a**) and full time (**b**) hyperprolinemia on BDNF immunocontent in cerebral cortex and hippocampus of rats. Results are expressed as mean ± SEM for 5–6 animals in each group. Different from other groups, **$P < 0.01$; ***$P < 0.001$ (two-way ANOVA). *Pro + Exe* proline plus exercise

BDNF Immunocontent

Mature BDNF levels in cerebral cortex and hippocampus of rats were measured at the end of behavioral testing for each experimental protocol. Results for partial time hyperprolinemia analyzed by two way ANOVA, revealed a significant interaction (proline × exercise) in cerebral cortex [$F = 43.07$; $P < 0.001$] and hippocampus [$F = 9.92$; $P < 0.01$]. Figure 5a shows that proline administration significantly reduced BDNF levels in cerebral cortex ($P < 0.01$) and hippocampus of rats ($P < 0.001$). Although exercise per se did not alter this parameter, it was able to reverse the proline effect on BDNF immunocontent in both cerebral structures studies.

Similarly, Fig. 5b shows that proline administration following full time hyperprolinemia also significantly reduces BDNF levels in cerebral cortex ($P < 0.01$) and hippocampus of rats ($P < 0.01$). Two-way ANOVA revealed a significant interaction (proline versus exercise) in cerebral cortex [$F = 8.64$; $P < 0.05$] and hippocampus [$F = 6.79$; $P < 0.05$] of rats, with the exercise reversing the proline effect on BDNF immunocontent.

AChE Activity

Figure 6 shows that AChE activity in cerebral cortex was not affected in partial time (Fig. 6a) [$F(1,19) = 0.01$; $P > 0.05$] nor in full time [$F(1,17) = 0.25$; $P > 0.05$] hyperprolinemia protocols (Fig. 6b). However, a significant interaction (proline × exercise) was observed in hippocampus after partial time [$F = 8.18$; $P < 0.01$] and full time hyperprolinemia [$F = 7.49$; $P < 0.05$]. AChE activity in the hippocampus was significantly increased in both
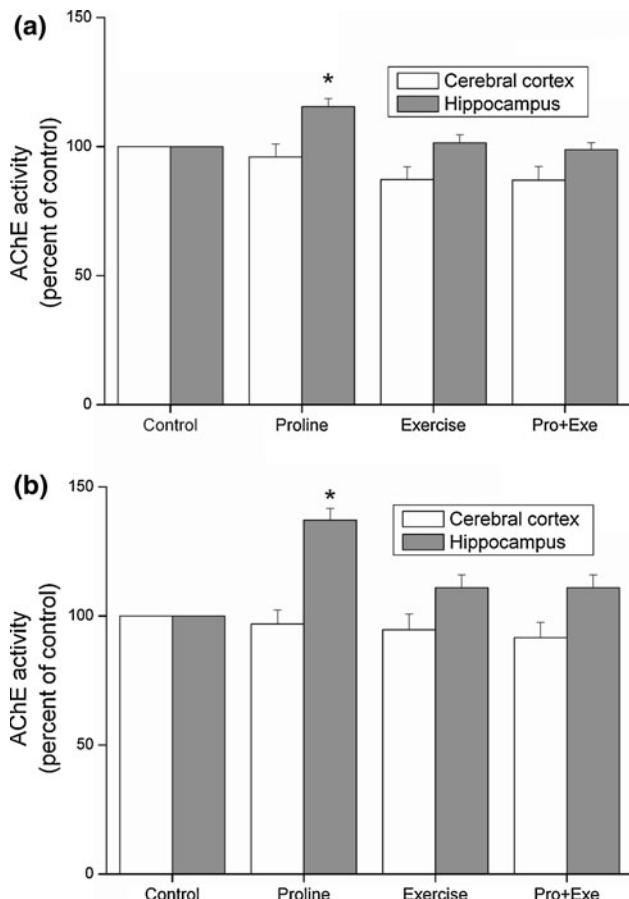
Fig. 6 Effects of exercise and chronic proline administration following experimental protocols of partial time (**a**) and full time (**b**) hyperprolinemia on acetylcholinesterase (AChE) activity in cerebral cortex and hippocampus of rats. Results are expressed as percentage of control ± SEM for 5–7 animals per group. Different from other groups, *$P < 0.05$ (two-way ANOVA). *Pro + Exe* proline plus exercise

protocols ($P < 0.05$) and this effect was reversed by exercise training (Fig. 6a, b).

Discussion

Present study investigates the effects of a moderate-intensity treadmill exercise on the cognitive deficit caused by chronic proline administration. It was previously shown [8, 9] that rats submitted to experimental hyperprolinemia in early life, during the period characterized by rapid brain development [5–7], presented cognitive deficits in adulthood, as assessed in the Morris water maze. We have here demonstrated that both protocols of experimental hyperprolinemia, namely partial time and full time hyperprolinemia, produced reference and working memory deficits, and confirming the working hypothesis, forced treadmill exercise training was able to prevent such deficits.

Proline-treated rats had a significant impairment in acquisition and/or memory consolidation, as can be observed by increased latency to find the platform location in training (acquisition) and test (retention) sessions. Results also showed reduced efficiency to find the platform in the working memory task, suggesting a deficit of spatial navigation [38]. These findings confirm previous studies showing that proline treatment impairs spatial memory in rats [8, 9] and indicate that the impairment on learning/memory was established in early life, independent of proline levels in adulthood. Proline-induced cognitive deficits cannot be attributed to any impairment in locomotor activity since the swim speed of all groups did not differ (general mean ≈ 23 cm/s). Moreover, previous reports showed that proline does not modify locomotor activity in open field [4, 9]. Results also demonstrated that treadmill running exercise, while not affecting per se the water maze performance (in control groups), was able to reverse the cognitive impairment in proline-treated rats from the 6th to the 29th day of life (protocol of partial time hyperprolinemia), and from the 6th to the 60th day of life (protocol of full time hyperprolinemia). This supports a potential neuroprotective effect of physical exercise, even when cognitive deficits are already established and hyperprolinemia remains. In agreement with that, there is extensive evidence that physical exercise can improve cognitive function and exert neuroprotective action in various conditions affecting the central nervous system (CNS) such as Alzheimer's disease [43, 44], cerebral ischemia [33, 45], schizophrenia [46] and metabolic diseases [35, 47].

In order to investigate the possible mechanisms underlying the spatial memory deficit caused by hyperprolinemia, BDNF levels and AChE activity, neurochemical parameters closely related to memory processing [12, 29], were evaluated in the hippocampus and cerebral cortex, two cerebral structures associated with memory modulation [29]. BDNF is a neurotrophin that modulates different aspects of neuronal function during development and in the mature nervous system [48]. It was shown that proline administration significantly reduced BDNF immunocontent in cerebral cortex and hippocampus of rats in both experimental protocols utilized. In addition, exercise was able to reverse such decrease of BDNF. Interestingly, lower brain BDNF levels found in proline-treated rats correlated to worse performance of rats in the water maze, suggesting that proline may cause memory deficit probably by reducing BDNF availability. Also, hyperprolinemic rats subjected to exercise (proline plus exercise group) showed a reversion on variables, BDNF immunocontent and performance in the water maze, thus suggesting that BDNF may be related with cognitive recovery caused by exercise. Given the proposed involvement of BDNF signaling in brain disorders like schizophrenia [49],

traumatic injury [50], dementia [51] and Alzheimer's disease [52], the finding that a short and moderate exercise protocol is sufficient to enhance learning and memory suggests that exercise might be an accessible form of intervention that could be used in conjunction with standard care measures for hyperprolinemic patients [53].

AChE plays a key role in synaptic transmission by hydrolyzing ACh. It has also been associated with brain development, learning and memory [27, 28] and several "nonclassical" AChE activities have been described, as neurite growth [54] and synaptic development and main-tenance [55]. Presented results show that chronic proline administration did not alter AChE in cerebral cortex of rats. In this context, a previous study showed that cortical AChE activity was decreased 1 h after the administration of proline (acute treatment), but not 12 h after the last injec-tion of proline in chronic treatment when the levels of this amino acid returned to normal values [56], raising the hypothesis that the presence of proline is necessary to promote such effect in this cerebral structure. On the other hand, our results also show that the activity of AChE was significantly increased in the hippocampus of rats subjected to both experimental models of chronic proline adminis-tration (partial and full time hyperprolinemia) and sacri-ficed after behavioral studies (adulthood rats). In this scenario, it is conceivable that a decrease in ACh levels will follow, reducing the cholinergic activity in this cere-bral structure, what could be associated with the memory impairment observed. Results also revealed that in exer-cised hyperprolinemic rats (proline plus exercise group) the activity of enzyme returned to normal (similar to control), suggesting that exercise can influence brain cholinergic mechanisms. These findings are supported by studies showing the interaction of hippocampus with cholinergic innervations and their relationship to memory processes. In this context, it has been demonstrated that cholinergic activation of the hippocampus is necessary for both spatial and contextual memory consolidation [57–59] and that extracellular ACh levels in the hippocampus increase during exploration or learning [60–62]. Extensive literature supports the idea that an ACh-mediated mechanism also regulates BDNF gene expression in the hippocampus par-ticularly [63–65], suggesting that ACh-mediated activation of the hippocampus could underlie the regulation of BDNF by exercise [12].

It is worth noting that the exercise protocol used in this study did not influence per se the behavioral performance, BNDF levels neither AChE in control rats. This observa-tion is in agreement with other reports using a similar exercise protocol [47, 66], although other studies have shown that exercise increases BDNF levels [53, 67]. These controversial results might be attributed to many factors, such as distinct exercise duration and intensity, the motivation for physical activity (either forced or voluntary) as well as the age of experimental animals [11, 68].

Summarizing, present study demonstrates through two experimental protocols that the impairment on cognition caused by hyperprolinemia was established in early life and appears not be related whether proline levels continues high throughout life. Indeed, it has been described that any defect causing even a modest increase in proline levels during critical periods of development may have a dramatic effect on CNS functioning [69]. Elucidation of the mechanisms through which proline exerts its effects on spatial learning/memory requires more studies; the reduction in neurotro-phins levels, such as BDNF, and modulation of cholinergic system appear to be involved. These data also reinforce the potential neuroprotective role of physical exercise on learning/memory in metabolic diseases such as hyperproli-nemia, even when cognitive deficits are already established.

References

1. Hu CA, Bart Williams D, Zhaorigetu S et al (2008) Functional genomics and SNP analysis of human genes encoding proline metabolic enzymes. Amino Acids 35:655–664
2. Phang JM, Hu CA, Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inher-ited disease. McGraw-Hill, New York, pp 1821–1838
3. Pontes ZL, Oliveira LS, Franzon R et al (2001) Inhibition of Na^+, K^+-ATPase activity from rat hippocampus by proline. Neuro-chem Res 26:1321–1326
4. Moreira JC, Wannmacher CM, Costa SM et al (1989) Effect of proline administration on rat behavior in aversive and nonaver-sive tasks. Pharmacol Biochem Behav 32:885–890
5. Davis HP, Squire LR (1984) Protein synthesis and memory: a review. Psychol Bull 96:518–559
6. Erecinska M, Cherian S, Silver IA (2004) Energy metabolism in mammalian brain during development. Prog Neurobiol 73: 397–445
7. Aghajanian GK, Bloom FE (1967) The formation of synaptic junctions in developing rat brain: a quantitative electron micro-scopic study. Brain Res 6:716–727
8. Bavaresco CS, Streck EL, Netto CA et al (2005) Chronic hyperprolinemia provokes a memory deficit in the Morris water maze task. Metab Brain Dis 20:73–80
9. Delwing D, Bavaresco CS, Monteiro SC et al (2006) Alpha-tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res 168:185–189
10. Mitsubuchi H, Nakamura K, Matsumoto S et al (2008) Inborn errors of proline metabolism. J Nutr 138:2016–2020
11. Berchtold NC, Castello N, Cotman CW (2010) Exercise and time-dependent benefits to learning and memory. Neuroscience 167:588–597
12. Cotman CW, Berchtold NC (2002) Exercise: a behavioral inter-vention to enhance brain health and plasticity. Trends Neurosci 25:295–301

13. Kramer AF (1999) Ageing, fitness and neurocognitive function. Nature 402:750

14. Sutoo D, Akiyama K (2003) Regulation of brain function by exercise. Neurobiol Dis 13:1–14

15. van Praag H, Shubert T, Zhao CM et al (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680–8685

16. Ang ET, Dawe GS, Wong PT et al (2006) Alterations in spatial learning and memory after forced exercise. Brain Res 1113: 186–193

17. Wu CW, Chen YC, Yu L et al (2007) Treadmill exercise counteracts the suppressive effects of peripheral lipopolysaccharide on hippocampal neurogenesis and learning and memory. J Neurochem 103:2471–2481

18. Griesbach GS, Hovda DA, Gomez-Pinilla F (2009) Exercise-induced improvement in cognitive performance after traumatic brain injury in rats is dependent on BDNF activation. Brain Res 1288:105–115

19. Bekinschtein P, Cammarota M, Izquierdo I et al (2008) BDNF and memory formation and storage. Neuroscientist 14:147–156

20. Tyler WJ, Alonso M, Bramham CR et al (2002) From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn Mem 9: 224–237

21. Albensi BC (2001) Models of brain injury and alterations in synaptic plasticity. J Neurosci Res 65:279–283

22. Levine ES, Crozier RA, Black IB et al (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci USA 95:10235–10239

23. Levine ES, Dreyfus CF, Black IB et al (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci USA 92:8074–8077

24. Takei N, Sasaoka K, Inoue K et al (1997) Brain-derived neurotrophic factor increases the stimulation-evoked release of glutamate and the levels of exocytosis-associated proteins in cultured cortical neurons from embryonic rats. J Neurochem 68:370–375

25. Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci 3:533–535

26. Yamada K, Mizuno M, Nabeshima T (2002) Role for brain-derived neurotrophic factor in learning and memory. Life Sci 70:735–744

27. Zimmerman G, Soreq H (2006) Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res 326:655–669

28. Ballard CG, Greig NH, Guillozet-Bongaarts AL et al (2005) Cholinesterases: roles in the brain during health and disease. Curr Alzheimer Res 2:307–318

29. Deiana S, Platt B, Riedel G (2011) The cholinergic system and spatial learning. Behav Brain Res 221:389–411

30. Amenta F, Tayebati SK (2008) Pathways of acetylcholine synthesis, transport and release as targets for treatment of adult-onset cognitive dysfunction. Curr Med Chem 15:488–498

31. Zugno AI, Pereira LO, Mattos C et al (2008) Guanidinoacetate administration increases acetylcholinesterase activity in striatum of rats and impairs retention of an inhibitory avoidance task. Metab Brain Dis 23:189–198

32. Stefanello FM, Monteiro SC, Matte C et al (2007) Hypermethioninemia increases cerebral acetylcholinesterase activity and impairs memory in rats. Neurochem Res 32:1868–1874

33. Cechetti F, Rhod A, Simão F et al (2007) Effect of treadmill exercise on cell damage in rat hippocampal slices submitted to oxygen and glucose deprivation. Brain Res 1157:121–125

34. Scopel D, Fochesatto C, Cimarosti H et al (2006) Exercise intensity influences cell injury in rat hippocampal slices exposed to oxygen and glucose deprivation. Brain Res Bull 71: 155–159

35. Ben J, Soares FM, Cechetti F et al (2009) Exercise effects on activities of Na$^+$, K$^+$-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302: 248–255

36. Brooks GA, White TP (1978) Determination of metabolic and heart rate responses of rats to treadmill exercise. J Appl Physiol 45:1009–1015

37. Arida RM, Scorza FA, dos Santos NF et al (1999) Effect of physical exercise on seizure occurrence in a model of temporal lobe epilepsy in rats. Epilepsy Res 37:45–52

38. Netto CA, Hodges H, Sinden JD et al (1993) Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. Neuroscience 54:69–92

39. D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. Brain Res Rev 36:60–90

40. Scherer EB, da Cunha MJ, Matte C et al (2010) Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. Neurobiol Learn Mem 94:247–253

41. Ellman GL, Courtney KD, Andres V Jr et al (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

42. Lowry OH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

43. Adlard PA, Perreau VM, Pop V et al (2005) Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. J Neurosci 25:4217–4221

44. Um HS, Kang EB, Leem YH et al (2008) Exercise training acts as a therapeutic strategy for reduction of the pathogenic phenotypes for Alzheimer's disease in an NSE/APPsw-transgenic model. Int J Mol Med 22:529–539

45. Zhang F, Wu Y, Jia J (2011) Exercise preconditioning and brain ischemic tolerance. Neuroscience 177:170–176

46. Pajonk FG, Wobrock T, Gruber O et al (2010) Hippocampal plasticity in response to exercise in schizophrenia. Arch Gen Psychiatry 67:133–143

47. Ben J, Soares FM, Scherer EB et al (2010) Running exercise effects on spatial and avoidance tasks in ovariectomized rats. Neurobiol Learn Mem 94:312–317

48. Gottmann K, Mittmann T, Lessmann V (2009) BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. Exp Brain Res 199:203–234

49. Egan MF, Weinberger DR, Lu B (2003) Schizophrenia, III: brain-derived neurotropic factor and genetic risk. Am J Psychiatry 160:1242

50. Horsfield SA, Rosse RB, Tomasino V et al (2002) Fluoxetine's effects on cognitive performance in patients with traumatic brain injury. Int J Psychiatry Med 32:337–344

51. Ando S, Kobayashi S, Waki H et al (2002) Animal model of dementia induced by entorhinal synaptic damage and partial restoration of cognitive deficits by BDNF and carnitine. J Neurosci Res 70:519–527

52. Tsai SJ, Hong CJ, Liu HC et al (2004) Association analysis of brain-derived neurotrophic factor Val66Met polymorphisms with Alzheimer's disease and age of onset. Neuropsychobiology 49:10–12

53. Vaynman S, Ying Z, Gomez-Pinilla F (2004) Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. Eur J Neurosci 20:2580–2590

54. Layer PG, Weikert T, Alber R (1993) Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res 273:219–226

55. Silman I, Sussman JL (2005) Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Curr Opin Pharmacol 5:293–302

56. Delwing D, Chiarani F, Wannmacher CM et al (2005) Effect of hyperprolinemia on acetylcholinesterase and butyrylcholinesterase activities in rat. Amino Acids 28:305–308

57. Herrera-Morales W, Mar I, Serrano B et al (2007) Activation of hippocampal postsynaptic muscarinic receptors is involved in long-term spatial memory formation. Eur J Neurosci 25:1581–1588

58. Gale GD, Anagnostaras SG, Fanselow MS (2001) Cholinergic modulation of pavlovian fear conditioning: effects of intrahippocampal scopolamine infusion. Hippocampus 11:371–376

59. Wallenstein GV, Vago DR (2001) Intrahippocampal scopolamine impairs both acquisition and consolidation of contextual fear conditioning. Neurobiol Learn Mem 75:245–252

60. Ragozzino ME, Unick KE, Gold PE (1996) Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. Proc Natl Acad Sci USA 93:4693–4698

61. Stancampiano R, Cocco S, Cugusi C et al (1999) Serotonin and acetylcholine release response in the rat hippocampus during a spatial memory task. Neuroscience 89:1135–1143

62. Giovannini MG, Rakovska A, Benton RS et al (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. Neuroscience 106:43–53

63. Knipper M, da Penha Berzaghi M, Blochl A et al (1994) Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. Eur J Neurosci 6:668–671

64. Ferencz I, Kokaia M, Keep M et al (1997) Effects of cholinergic denervation on seizure development and neurotrophin messenger RNA regulation in rapid hippocampal kindling. Neuroscience 80:389–399

65. Lapchak PA, Araujo DM, Hefti F (1993) Cholinergic regulation of hippocampal brain-derived neurotrophic factor mRNA expression: evidence from lesion and chronic cholinergic drug treatment studies. Neuroscience 52:575–585

66. Cechetti F, Fochesatto C, Scopel D et al (2008) Effect of a neuroprotective exercise protocol on oxidative state and BDNF levels in the rat hippocampus. Brain Res 1188:182–188

67. Liu HL, Zhao G, Cai K et al (2011) Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. Behav Brain Res 218:308–314

68. Stranahan AM, Lee K, Martin B et al (2009) Voluntary exercise and caloric restriction enhance hippocampal dendritic spine density and BDNF levels in diabetic mice. Hippocampus 19:951–961

69. Guilmatre A, Legallic S, Steel G et al (2010) Type I hyperprolinemia: genotype/phenotype correlations. Hum Mutat 31:961–965

Capítulo V

Experimental hyperprolinemia induces mild oxidative stress, metabolic changes and tissue adaptation in rat liver

Andréa G. K. Ferreira, Aline A. da Cunha, Fernanda R. Machado, Carolina D. Pederzolli, Giovana R. Dalazen, Adriano M. de Assis, Marcelo L. Lamers, Marinilce F. dos Santos, Carlos S. Dutra-Filho, Angela T. S. Wyse

Experimental hyperprolinemia induces mild oxidative stress, metabolic changes and tissue adaptation in rat liver

Andréa G. K. Ferreira[1], Aline A. da Cunha[1], Fernanda R. Machado[1], Carolina D. Pederzolli[1], Giovana R. Dalazen[1], Adriano M. de Assis[1], Marcelo L. Lamers[2], Marinilce F. dos Santos[3], Carlos S. Dutra-Filho[1], Angela T. S. Wyse[1*]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

[2]Departamento de Ciências Morfológicas, Instituto de Ciências Biomédicas, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2492, CEP 90035-003, Porto Alegre, RS, Brazil.

[3]Departamento de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Prof. Lineu Prestes, 1524, CEP 05508-000São Paulo, SP, Brazil.

*Address reprint requests to: Dr. Angela T. S. Wyse, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Phone 55 51 3308 5573, Fax 55 51 3308 5535, E-mail: wyse@ufrgs.br

Running head: Effect of hyperprolinemia in liver of rats

This manuscript contains 4 figures and 3 tables.

Abstract

The present study investigated the effects of chronic hyperprolinemia on oxidative and metabolic status in liver and serum of rats. Wistar rats received daily subcutaneous injections of proline from their 6th to 28th day of life. Twelve hours after the last injection the rats were sacrificed and liver and serum were collected. Results showed that hyperprolinemia induced a significant reduction in total antioxidant potential and thiobarbituric acid-reactive substances. The activities of the antioxidant enzymes catalase and superoxide dismutase were significantly increased after chronic proline administration, while glutathione peroxidase activity, dichlorofluorescin oxidation, glutathione, sulfhydryl and carbonyl content remained unaltered. Histological analyses of the liver revealed that proline treatment induced changes of the hepatic microarchitecture and increased the number of inflammatory cells and the glycogen content. Biochemical determination also demonstrated an increase in glycogen concentration, as well as a higher synthesis of glycogen in liver of hyperprolinemic rats. Regarding to hepatic metabolism, it was observed an increase on glucose oxidation and a decrease on lipid synthesis from glucose. However, hepatic lipid content and serum glucose levels were not changed. Proline administration did not alter the aminotransferases activities, serum markers of hepatic injury. Our findings suggest that hyperprolinemia alters the liver homeostasis possibly by induction of a mild degree of oxidative stress and metabolic changes. The hepatic alterations caused by proline probably do not implicate in substantial hepatic tissue damage, but rather demonstrate a process of adaptation of this tissue to oxidative stress. However, the biological significance of these findings requires additional investigation.

Introduction

L-Proline is a proteinogenic amino acid, which has been described to play regulatory roles in physiologic or pathophysiologic situations (Phang et al., 2010). Such attribution is primarily based on the particularity of proline has an own metabolic system, that can serves to special functions as redox-regulation, energy source, stress signaling and apoptosis (Phang et al., 2008; Phang et al., 2001).

Hyperprolinemia is a biochemical hallmark present in two inherited metabolic disorders caused by distinct defects in the L-proline catabolic pathway. Hyperprolinemia type I (HPI) and type II (HPII) are caused by deficiencies in the activities of proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase, respectively. Clinically, patients may exhibit diverse phenotypes; whereas some patients have neurological, renal and/or auditory defects, others are asymptomatic (Mitsubuchi et al., 2008). Beyond that, mild hyperprolinemia is also present in some hepatic disorders, such as alcoholic liver disease (Vargas-Tank et al., 1988) and cirrhosis (Shaw et al., 1984). This correlation is pertinent, as far as proline is primarily catabolized in the liver, but also in brain and kidney (Phang et al., 2001).

Studies addressing the proline metabolism in plants, fungi and mammals suggest that this amino acid may have opposing effects on the intracellular redox environment (Krishnan et al., 2008; Phang et al., 2010; Wyse and Netto, 2011). Because the catabolism of this amino acid involves the transfer of electrons from substrate proline to flavine adenine dinucleotide (FAD), proline may be a direct substrate for the generation of ATP (Hagedorn and Phang, 1983). Nevertheless, it has been shown that proline-derived electrons can directly reduce oxygen to produce superoxide autogenously (Liu et al., 2005; White et al., 2007). In this setting, proline acts as a prooxidant in the microenvironment of diseases as

cancer, which may be an important mechanism for reducing carcinogenesis (Phang et al., 2008; Rivera and Maxwell, 2005). Besides, previous works showed that proline can mediate oxidative stress in brain of rats (Delwing et al., 2003a; Delwing et al., 2003b). On the other hand, it has been described that proline is able to perform chemical reaction, producing adducts with hydroxyl radical (Alia et al., 2001; Floyd and Nagy, 1984; Halliwell and Gutteridge, 2007; Rustgi et al., 1977). In this context, studies have shown that proline can modulate the intracellular redox environment and protect mammalian cells against oxidative stress (Krishnan et al., 2008). Therefore, proline may have multiple functions in stress adaptation, exhibiting dual functions as a pro-oxidant and as a ROS scavenger (Krishnan et al., 2008; Phang et al., 2010).

It is known that high concentration of free radicals can elicit oxidative stress and promote cellular injury. Following liver injury, several cell types can secrete inflammatory cytokines that stimulate the recruitment of inflammatory cells which in turn, induce the generation of more reactive oxygen species (ROS) (Decker, 1990; Hernandez-Gea and Friedman, 2011). In this context, *in vitro* and *in vivo* observations suggest that oxidative stress and associated damage may represent a common link between different forms of chronic liver injury (Ha et al., 2010; Nair et al., 2010).

Given the putative role of proline in mechanisms redox and considering that little is known about the effects of hyperprolinemia on liver, either due to inherited or hepatic disorders, in the present study we evaluated the hepatic oxidative and metabolic status in rats subjected to chronic experimental hyperprolinemia. Firstly, we investigated the effect of proline on several oxidative stress parameters, namely total radical-antioxidant potential (TRAP), glutathione (GSH) levels, thiobarbituric acid-reactive substances (TBARS), reactive species production (DCFH oxidation), sulfhydryl and carbonyl content, as well as on antioxidant enzymes activities, superoxide dismutase (SOD), catalase (CAT) and

glutathione peroxidase (GPx) in liver of rats. Besides, histological analyses were performed in order to verify the occurrence of morphological changes and evaluate the glycogen/glycoprotein content in hepatic tissue sections of hyperprolinemic rats. Hepatic metabolic status was assessed by measuring synthesis and concentration of glycogen and lipid, as well as on glucose oxidation to CO_2. Glucose levels and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also determined in serum of rats.

Material and methods

Animals

Male or female Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. Animal care followed the NIH ''Guide for the Care and Use of Laboratory Animals'' (NIH publication no. 80-23, revised 1996) and was approved by the University Ethics Committee.

Proline administration

Chronic hyperprolinemia was chemically induced by daily subcutaneous administration of proline from the 6th to the 28th day of life as described by Pontes et al., (1999) and Moreira et al., (1989). Proline (Sigma Chemical Co., USA) was dissolved in 0.9% NaCl and administered twice a day. During the first 8 days of treatment (6th - 13th day of life) rats received 12.8 μmol Pro/g body weight, from the 14th to 17th day they received 14.6 μmol Pro/g body weight, from the 18th to 21th day they received 16.4 μmol Pro/g body weight and from the 22th to 28th day of life they received 18.2 μmol Pro/g body weight.

Control animals received saline injections in the same volumes as those applied to proline-treated rats. The animals were killed 12 h after the last injection by decapitation without anesthesia, when the blood levels of proline had returned to normal (Moreira et al., 1989). Rats subjected to this treatment achieved plasma proline levels between 1.0 and 2.0 mM, which are similar to those found in hyperprolinemic patients (Moreira et al., 1989; Phang et al., 2001).

Tissue and homogenate preparation

The rats were sacrificed by decapitation without anesthesia 12 h after the last injection of proline. Blood was collected and the serum was separated by centrifugation, at 800 x g for 5 min at 25 ºC. The liver was quickly removed and processed as follow.

For oxidative stress parameters determination, the liver 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 800 x g for 10 min at 4 ºC, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays.

For measurement of hepatic lipids, frozen liver samples were thawed on ice and was homogenized in 20 volumes (1:20, w/v) of deionized water and centrifuged at 800 x g for 10 min at 4 ºC. The pellet was discarded and the supernatant was taken to triglycerides measure and extraction/isolation of lipids to dried lipid extracts according to Folch et al. (1957) to cholesterol determination.

Thiobarbituric Acid Reactive Species (TBARS)

TBARS levels were determined according to the method described by Ohkawa (1979). Briefly, 50 µl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5, 1.5 ml of 0.8% thiobarbituric acid and 700 µl of distilled water were

added to 500 µl of tissue homogenate in a Pyrex tube. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. After cooling with tap water, the mixture was allowed to cool on water for 5 min, centrifuged at 750 × g for 10 min and the resulting pink color was determined in a spectrophotometer at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. The results were reported as nmol of TBARS per mg protein.

2′ 7′ dichlorofluorescin oxidation assay (DCFH)

Reactive species production was measured following a method based on 2′7′-dichlorofluorescin (DCFH) oxidation (LeBel et al., 1992). Samples (60 µL) were incubated for 30 min at 37 °C in the dark with 240 µL of 100 µM 2′7′-dichlorofluorescin diacetate (DCF-DA) solution in a 96 wells plate. DCF-DA is deacetylated by intracellular esterases to form non-fluorescent DCFH, which is rapidly oxidized by some reactive oxygen and/or nitrogen species (ROS and/or RNS) present in samples, producing the highly fluorescent compound de-esterified dichlorofluorescein (DCF), which can be measured at λ_{ex} = 488 nm and λ_{em} = 525 nm. A calibration curve was performed with standard DCF (0.25–10 mM) and the levels of reactive species were calculated as nmol DCF/mg protein.

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) thermolysis in Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) (Evelson et al., 2001; Lissi et al., 1992). Two-hundred and forty microliter of a system containing ABAP (10 mM) dissolved in 50 mM sodium phosphate buffer pH 8.6 plus luminol (5.6 mM), was added to a microplate and the initial chemiluminescence was measured. Ten

microliter of 300 µM Trolox (water-soluble α-tocopherol analogue, used as a standard) or 10 µl of liver supernatant was then added to each plate well, producing a decrease in the initial chemiluminescence value. This value is kept low, until the antioxidants present in the sample are depleted, when chemiluminescence returns to its initial value. The time spent by the sample to keep chemiluminescence return to its low initial value is directly proportional to the antioxidant capacity of the tissue. The results were represented as nmol Trolox/mg protein.

Superoxide dismutase (SOD)

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide (O_2^-) which is a substrate for SOD (Marklund, 1985). Briefly, to 15 µL of each sample were added 215 µL of a mixture containing 50 mM Tris buffer pH 8,2 with 1 mM EDTA and 30 mM catalase. After that, 20 µL of pyrogallol were added and the absorbance was immediately recorded each 30 s for 3 min at 420 nm in SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation and the specific activity is reported as SOD units/mg protein.

Catalase (CAT)

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The method used is

based on the disappearance of H_2O_2 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 0.1 - 0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein.

Glutathione peroxidase (GPx)

GPx activity was measured using tert-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as GPx units/mg protein.

Reduced glutathione content (GSH)

This method is based on the reaction of GSH with the fluorophore *o*-phtalaldeyde (OPT) after deproteinizing the samples and was measured according to Browne and Armstrong (1998). Initially, metaphosphoric acid was used to deproteinize the samples, which were then centrifuged at 1000 x *g* for 10 min. Briefly, to 15 μL of each sample were taken 200 μL of a mixture containing 15 μL of OPT 1 mg/mL (prepared in methanol) plus 185 μL of 100 mM sodium phosphate buffer pH 8.0 with 5 mM EDTA in a 96 wells plate. The assay was allowed to stand in the dark for exactly 15 min. After that, the fluorescence was measured at λ_{ex} = 350 nm and λ_{em} = 420 nm. A calibration curve was also performed with a commercial GSH solution, and the results were calculated as nmol GSH/mg protein.

Total sulfhydryl content

This assay was performed as described by Aksenov and Markesbery (2001), which is based on the reduction of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, 50 µL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 30 µL of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. Results were reported as nmol TNB/mg protein.

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman and Levine, 2003). In this study, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 100 µL of homogenate were added to plastic tubes containing 400 µL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 hour and vortexed each 15 minutes. After that, 500 µL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant was discarded and the pellet re-suspended in 600 µL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at $60^{o}C$ for 15 minutes. After that, it was centrifuged at 20,000 g for 3 minutes and the absorbance was measured at 370 nm (UV) in a quartz cuvette with a Hitachi U-2001 double-beam spectrophotometer with temperature control (Hitachi High Technologies

America, Inc., Life Sciences Division, Pleasanton, CA, USA). Results were represented as protein carbonyl content (nmol/mg protein).

Histological analysis

For each group, six rats were anesthetized with a mixture of ketamine and xylazine (75 and 10 mg/kg, respectively) and submitted to transcardiac perfusion with 0.9% saline followed by fresh 4% formaldehyde solution, pH 7.4. The liver was removed and post-fixed in 4% formaldehyde solution for 6 h at 4ºC. Fragments from the central portion, left and right lobes were processed and embedded in Paraplast®. Semiserial 5 µm-thick sections were placed in glass slides and stained with hematoxylin and eosin for morphological analysis or submitted to periodic acid-Schiff (PAS) staining for the analysis of glycoproteins/glycogen (Bancroft and Stevens, 1990). Pictures were taken using a CCD camera coupled to an Olympus CX-40 microscope. The software Adobe Photoshop was used to construct the panels.

Hepatic glycogen synthesis

For the measurement of hepatic glycogen synthesis, the liver was dissected and cut into 300 µm slices using a McIlwain tissue chopper (100-120 mg). It was incubated in a beaker with a medium containing Krebs Ringer bicarbonate buffer (pH 7.4), 5 mM D-glucose and 0.2 µCi D[U-^{14}C]glucose for glycogen synthesis from glucose or 0.2 mM L-alanine and 0.2 µCi L[U-^{14}C]alanine for glycogen synthesis from alanine. Incubations were carried out in ambient content that was gassed with a 95% O_2:5% CO_2 mixture for 1 h. Liver slices were incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min), according to the method of Dolnikoff et al. (2001). Incubation was stopped by placing the bottles in ice. Afterward, 1 mL of 60% KOH was added to each beaker. After 15 min in a boiling water

bath, 3 mL of 96% ethanol was added to the tubes to precipitate glycogen. After precipitation, glycogen was suspended in 0.2 mL of water, the scintillation liquid (OptiPhase HiSafe3 from PerkinElmer-USA) was added and the samples were assessed in a scintillation counter. Results were reported as pmol of glucose or alanine incorporated into glycogen.

Hepatic glycogen content

The hepatic glycogen concentration was measured using the Krisman method (1962). Briefly, the liver was dissected and approximately 100 mg of tissue was treated with 1 mL of KOH 30%. After 15 min in boiling water bath, was added 3 mL of 96% ethanol to precipitate glycogen. Liver glycogen was measured with the colorimetric method and results were reported as mg glycogen/100 mg tissue.

Glucose oxidation to CO_2 and lipid synthesis from D[U-^{14}C]glucose

For the measurement of CO_2 production from D[U-^{14}C]glucose and lipid synthesis, liver slices (between 100-120 mg) were incubated in 1.0 mL Krebs Ringer bicarbonate buffer pH 7.4, containing 5.0 mM glucose plus 0.2 μCi D[U-^{14}C]glucose. Before incubation, the reaction medium was gassed with a 95% O_2:5% CO_2 mixture for 1 minute. Flasks were sealed with rubber caps and the slices were incubated at 37ºC for 1 h in a metabolic shaker (60 cycles/min), according to the method of Dolnikoff et al. (2001). Incubations were stopped by adding 0.25 mL 50% trichloroacetic acid through the rubber cap. Then 0.2 mL of 1 M sodium hydroxide was injected into the central wells. The flasks were shaken for an additional 30 minutes at 37ºC to trap CO_2. Afterwards, the contents of the central well were transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter. The

flask contents were homogenized and transferred to tubes. After centrifugation, the precipitate was washed three times with 10% trichloroacetic acid and lipids were extracted with chloroform:methanol (2:1). The chloroform/methanol phase was evaporated in vials and radioactivity was measured. All the results were expressed with respect to the initial specific activity of the incubation medium.

Hepatic Lipids content

For measurement of hepatic lipids, frozen liver samples were thawed on ice, homogenized in deionized water and triglycerides were assayed by commercially available diagnostic kits supplied by Labtest[®]. To hepatic cholesterol determination, extraction and isolation of lipids to dried lipid extracts were done according to Folch et al. (1957). Then, cholesterol was enzymatically assayed in lipid extract by colorimetric Labtest[®] kit. Triglycerides and cholesterol levels were expressed as mg/g tissue.

Serum biochemical parameters

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and glucose levels were determined in serum of rats using commercially available diagnostic kits supplied by Labtest® (Labtest, MG, Brazil). ALT and AST activities were expressed as units/L and glucose levels as mg/dL.

Protein determination

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

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Data were analyzed by Mann-Whitney U-test for unpaired sample and values of $p < 0.05$ were considered to be significant. Descriptive data were expressed as mean ± S.D.

Results

Initially, we investigated the effects of chronic proline administration on several parameters of oxidative stress in the liver of rats. Table 1 show that proline-treated rats presented a significant reduction in TRAP ($p < 0.05$) and TBARS levels ($p < 0.05$). In contrast, DCFH oxidation and GSH, sulfhydryl and carbonyl content were not altered ($p > 0.05$). Enzymatic antioxidant defenses were also evaluated by determination of SOD, CAT and GPx activities. As can be seen in table 2, SOD ($p < 0.01$) and CAT ($p < 0.05$) activities were significantly increased after chronic proline administration as compared to control, while GPx activity remained unaltered ($p > 0.05$). Because SOD and CAT are oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water (Halliwell and Gutteridge, 2007), a ratio between SOD and CAT enzyme activities were applied. The hepatic SOD/CAT ratio did not change in proline-treated rats, when compared to control [control = 38.2 ± 7.3 and proline = 41.2 ± 6.7, expressed in arbitrary units; ($p > 0.05$)].

The liver structural integrity was evaluated by morphological analysis in control and proline-treated rats. In the control animals (Fig. 1A and C), we can observe the normal appearance of the hepatic cells. In contrast, hyperprolinemic rats (Fig. 1B and D) exhibited a slight disruption of the hepatic microarchitecture and a higher presence of inflammatory cells as lymphocytes and neutrophils around the hepatic cells and at the portal space. These proline-associated changes were accompanied by a markedly increase in the

glycogen content revealed by the PAS staining (Fig. 2B and D), when compared to the respective control (Fig. 2A and C).

Based on histological evidences, we also performed biochemical analyses in order to measure glycogen/glycoproteins content in liver. Fig. 3 shows that hyperprolinemia significantly increased glycogen synthesis from both D[U-^{14}C]glucose ($p < 0.05$) (A) and L[U-^{14}C]alanine ($p < 0.05$) (B), as well as glycogen concentration ($p < 0.05$) (C). Regarding to hepatic metabolism, Fig. 4 depicts the increased rate of D[U-^{14}C]glucose oxidation to CO_2 (A) ($p < 0.05$) and decreased D[U-^{14}C]glucose conversion to lipid (B) ($p < 0.05$). In addition, hepatic lipid content such as triglycerides [control = 30.2 ± 3.5 and proline = 31.9 ± 2.5; $p > 0.05$)] and cholesterol [control = 1.55 ± 0.17 and proline = 1.53 ± 0.08; $p > 0.05$)] were not changed by proline administration as compared to control.

Serum biochemical parameters, including glucose levels, as well as ALT and AST activities were also evaluated. Table 3 show that proline administration did not alter the glucose levels, ALT and AST activities ($p > 0.05$).

Discussion

The present study investigated the effects of chronic administration of proline on the hepatic oxidative and metabolic status in rats, utilizing a chemical experimental model of hyperprolinemia, which mimic the tissue levels of proline found in human HP II (Phang et al., 2001; Pontes et al., 1999). By using this animal model, previous works showed that proline provokes several neurotoxic effects in rats, such as memory impairment (Delwing et al., 2006), inhibition of enzymes activities as acetylcholinesterase, Na$^+$,K$^+$-ATPase and aminotransferases (Delwing et al., 2005; Pontes et al., 1999; Shanti et al., 2004), energy deficit (Delwing et al., 2007; Ferreira et al., 2010) and induction of oxidative stress (Delwing et al., 2003a; Delwing et al., 2003b).

Herein, initially was investigated the effect of hyperprolinemia on several parameters of oxidative stress in liver tissue. Considering that non-enzymatic antioxidant defenses, act as the first line in the removal of ROS, TRAP was employed to evaluate the total potential of the main antioxidants found in hepatic tissue such as GSH, uric acid, ascorbic acid and α-tocopherol (Evelson et al., 2001; Lissi et al., 1995). Our results showed that proline significantly decreased TRAP, suggesting that high concentrations of this amino acid cause a reduction on quantity of non-enzymatic antioxidants in liver. Based on this finding, we also measured the levels of GSH, which is the antioxidant present at higher concentrations in liver and that most contributes to TRAP values (Evelson et al., 2001). Results showed that this parameter was not altered by chronic hyperprolinemia, indicating that decreased TRAP values by proline did not reflect alteration of the GSH content. However, it is conceivable that other tissue antioxidants could be decreased in liver of hyperprolinemic rats.

Regarding the enzymatic antioxidant defenses, we observed that chronic hyperprolinemia provoked a significant increase of SOD and CAT activity, but not of GPx activity in rat liver. Interestingly, these enzymes activities increased in the same proportion (around 30% each) and there was not an imbalance in the SOD/CAT ratio. There are evidences that the continued presence of low concentrations of ROS is able to induce upregulation of antioxidant enzymes, as a cellular strategy of adaptation to oxidative stress (Halliwell and Gutteridge, 2007; Halliwell and Whiteman, 2004). Therefore, based on data from our and other studies (Liu et al., 2005; White et al., 2007), it is conceivable to suggest that high proline levels could lead to generation of superoxide radical in hepatic tissue. Superoxide is dismutated by SOD with consequent generation of H_2O_2, which is in turn reduced by CAT. Then, the increase in activities these antioxidant enzymes in liver of rats observed in present work may be a consequence from tissue adaptation to sustained

production of ROS, mainly superoxide and hydrogen peroxide, which are substrates scavenged by SOD and CAT, respectively.

We also investigated the effect of hyperprolinemia on two markers of protein oxidation in liver of rats: sulfhydryl content, which is employed to verify protein damage to sulphydryl groups (Aksenov and Markesbery, 2001) and carbonyl content, formed mainly by oxidation of side chains of some amino acid residues (Dalle-Donne et al., 2003). In this study, it was observed that liver of rats subjected to chronic hyperprolinemia did not present oxidative damage to proteins.

RNS and ROS production was assessed by DCFH oxidation assay, which is a probe used to detect peroxyl, alkoxyl, nitrogen dioxide, carbonate, hydroxyl and peroxynitrite (Halliwell and Whiteman, 2004). We showed that proline did not alter the levels of reactive species detected by DCFH assay. Additionally, lipid peroxidation was also evaluated in liver tissue by TBARS assay, which identify mainly malondialdehyde, a final product of fatty acid peroxidation (Ohkawa et al., 1979). Interestingly, TBARS levels were significantly decreased after chronic exposure to proline. We cannot explain the exact mechanism by which proline reduces TBARS levels, but this result is in agreement with works showing that this amino acid can scavenge some free radicals, for example, by physical quenching of oxygen singlet and chemical reaction with hydroxyl radicals (Alia et al., 2001; Floyd and Nagy, 1984; Halliwell and Gutteridge, 2007; Rustgi et al., 1977). In this context, proline accumulation has been observed in many microorganisms and plants exposed to environmental stresses, as a mechanism of protection against ROS produced during stress conditions (Alia et al., 2001; Chen and Dickman, 2005; Chen et al., 2006; Siripornadulsil et al., 2002). Therefore, the unchanged levels of reactive species (assessed by DCFH assay) and reduced incidence on lipid peroxidation (detected by TBARS) in rat liver submitted to proline administration, could be attributed to an effective detoxification of ROS/RNS by non

enzymatic and enzymatic antioxidant defense as demonstrated by decreased TRAP and increased SOD and CAT activities, respectively. Besides, this putative role of proline in decreasing lipid peroxidation in rat liver, as well as the upregulation of antioxidant enzymes could explain at least in part, the hyperprolinemia found in patients affected by hepatic disorders, as a mechanism to protect liver from oxidative injury.

Recently, we have reported that proline elicits oxidative stress in brain of rats (Delwing et al., 2003a; Delwing et al., 2003b). Nevertheless, in rat liver proline did not produced similar effects. Owing to high regenerative capacity of liver, including high antioxidant defenses and adaptability to metabolic alterations regulating body homeostasis, this organ is thought to be more resistant to damage by free radical attack (Genet et al., 2002). In addition, it is known the increased susceptibility of central nervous system to oxidative insult, since there is a high content of polyunsaturated fatty acids (PUFAs) in brain, which are sensitive to oxidative modifications (Halliwell and Gutteridge, 2007). Furthermore, brain has a decreased of antioxidant enzymes activities (especially CAT) when compared to liver, that may favor the onset and maintenance of a pro-oxidative state (Halliwell and Gutteridge, 2007).

The effect of chronic proline exposure on the liver structural integrity was evaluated by histological analysis. Hematoxylin/eosin staining showed that proline promotes disruption of hepatocytes arrangement and increases the number of inflammatory cells around the hepatic cells and at the portal space, when compared to control rats. These histological findings could be explained on the basis that proline administration induced a mild degree of oxidative stress in the liver of rats. Since free radicals are also released in large amounts from inflammatory cells activated (Decker, 1990), their increased number reinforces the evidence of generation of ROS/RNS in liver of rats. Staining for the analysis of glycoproteins/glycogen revealed that changes in the hepatic microarchitecture are similar

(although less severe) to histological phenotype to that observed in liver of patients with glycogen storage disease (Salganik et al., 2009), presenting a marked increase in the glycogen content in liver of proline-treated rats. Biochemical determination reaffirmed this finding, which demonstrate that proline increases glycogen concentration and the synthesis of glycogen from both direct (from D[U-^{14}C]glucose) and indirect (from L[U-^{14}C]alanine) pathways in the liver of rats. In this context, studies show that proline increase hepatic glucose-6-P concentration, suggesting that a proline metabolite inhibits the glucose-6-phosphatase activity and thus directs glucose-6-P away from glucose production and toward glycogen synthesis (Bode et al., 1992). This finding could explain the glycogenic effect of proline found in present work.

Since we observed an increased deposition of glycogen in hepatocytes caused by proline (an amino acid gluconeogenic) administration (Phang et al., 2001), we also verified other possible fates of hepatic glucose after chronic treatment with proline. Results showed that hyperprolinemic rats presented an decreased convertion of D[U-^{14}C]glucose to lipid in liver. Nevertheless, hepatic lipid content such as triglycerides and cholesterol were not changed. Besides, an increased glucose oxidation capability by hepatic tissue was demonstrated when compared to controls, suggesting a stimulation of glycolytic pathway and TCA cycle. However, the glucose levels in serum of rats were not altered by proline administration. These findings might be related to the fact that pyrroline-5-carboxylate, the immediate precursor and degradation product of proline, is the sole intermediate directly connecting the tricarboxylic acid and urea cycles with amino acid metabolism (Phang, 1985).

Finally, we evaluated the activities of aminotransferases that are serum markers of hepatocyte injury. We observed that ALT and AST activities remained unchanged after

chronic proline administration, suggesting that proline did not induce substantial liver damage.

Taken together, these results suggest that hyperprolinemia alters the liver homeostasis through the induction of a mild degree of oxidative stress, which appears be tolerated by liver cells because their sufficient antioxidant defense capacity to remove the reactive species formed. Additionally, proline also induces metabolic changes in liver, observed here by the increase on glycogen content and higher glucose oxidation. Then, it may be reasonable to conceive that the alterations in parameters of oxidative stress detected in present work, probably do not implicate in liver tissue damage, but demonstrate a process of adaptation this tissue to oxidative stress. Based on these findings and other studies from our laboratory, we hypothesized that some tissues are more affected by hyperprolinemia than others: while hyperprolinemia induces oxidative stress and metabolic alterations in brain, the liver appears to be more resistance to damage by ROS caused by proline. To our knowledge, there are no studies showing the effects of hyperprolinemia on oxidative and metabolic status in liver. Our data reinforce the hypothesis that proline and its interconversions function as a unique mechanism for redox balance (Araujo et al., 2001; Krishnan et al., 2008; Phang et al., 2010; Wyse and Netto, 2011), which may be related to increased levels of this amino acid in hepatic diseases. Also, we cannot rule out that liver, as a central organ for metabolism, fulfill the role to counterbalance the oxidative and metabolic effects of systemic hyperprolinemia. However, the mechanisms and biological significance of these findings need further studies to be fully understood.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

References

Aebi H. 1984. Catalase in vitro. Methods Enzymol 105:121-6.

Aksenov MY, Markesbery WR. 2001. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302:141-5.

Alia, Mohanty P, Matysik J. 2001. Effect of proline on the production of singlet oxygen. Amino Acids 21:195-200.

Araujo P, Wassermann GF, Tallini K, Furlanetto V, Vargas CR, Wannmacher CM, Dutra-Filho CS, Wyse AT, Wajner M. 2001. Reduction of large neutral amino acid levels in plasma and brain of hyperleucinemic rats. Neurochem Int 38:529-37.

Bancroft JD, Stevens A. 1990. Theory and Practice of Histological Techniques. Edinburgh: Churchill Livingstone.

Bode AM, Foster JD, Nordlie RC. 1992. Glyconeogenesis from L-proline involves metabolite inhibition of the glucose-6-phosphatase system. J Biol Chem 267:2860-3.

Browne RW, Armstrong D. 1998. Reduced glutathione and glutathione disulfide. Methods Mol Biol 108:347-52.

Chen C, Dickman MB. 2005. Proline suppresses apoptosis in the fungal pathogen Colletotrichum trifolii. Proc Natl Acad Sci U S A 102:3459-64.

Chen C, Wanduragala S, Becker DF, Dickman MB. 2006. Tomato QM-like protein protects Saccharomyces cerevisiae cells against oxidative stress by regulating intracellular proline levels. Appl Environ Microbiol 72:4001-6.

Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. 2003. Protein carbonylation in human diseases. Trends Mol Med 9:169-76.

Decker K. 1990. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur J Biochem 192:245-61.

Delwing D, Bavaresco CS, Chiarani F, Wannmacher CM, Wajner M, Dutra-Filho CS, de Souza Wyse AT. 2003a. In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. Brain Res 991:180-6.

Delwing D, Bavaresco CS, Monteiro SC, Matte C, Netto CA, Wyse AT. 2006. Alpha-tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res 168:185-9.

Delwing D, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT. 2003b. Proline induces oxidative stress in cerebral cortex of rats. Int J Dev Neurosci 21:105-10.

Delwing D, Chiarani F, Kurek AG, Wyse AT. 2007. Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci 25:17-22.

Delwing D, Chiarani F, Wannmacher CM, Wajner M, Wyse AT. 2005. Effect of hyperprolinemia on acetylcholinesterase and butyrylcholinesterase activities in rat. Amino Acids 28:305-8.

Dolnikoff M, Martin-Hidalgo A, Machado UF, Lima FB, Herrera E. 2001. Decreased lipolysis and enhanced glycerol and glucose utilization by adipose tissue prior to development of obesity in monosodium glutamate (MSG) treated-rats. Int J Obes Relat Metab Disord 25:426-33.

Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi EA. 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch Biochem Biophys 388:261-6.

Ferreira AG, Lima DD, Delwing D, Mackedanz V, Tagliari B, Kolling J, Schuck PF, Wajner M, Wyse AT. 2010. Proline impairs energy metabolism in cerebral cortex of young rats. Metab Brain Dis 25:161-8.

Floyd RA, Nagy I. 1984. Formation of long-lived hydroxyl free radical adducts of proline and hydroxyproline in a Fenton reaction. Biochim Biophys Acta 790:94-7.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509.

Genet S, Kale RK, Baquer NZ. 2002. Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (Trigonellafoenum graecum). Mol Cell Biochem 236:7-12.

Ha HL, Shin HJ, Feitelson MA, Yu DY. 2010. Oxidative stress and antioxidants in hepatic pathogenesis. World J Gastroenterol 16:6035-43.

Hagedorn CH, Phang JM. 1983. Transfer of reducing equivalents into mitochondria by the interconversions of proline and delta 1-pyrroline-5-carboxylate. Arch Biochem Biophys 225:95-101.

Halliwell B, Gutteridge JMC. 2007. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. New York: Oxford University Press.

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.

Hernandez-Gea V, Friedman SL. 2011. Pathogenesis of liver fibrosis. Annu Rev Pathol 6:425-56.

Krishnan N, Dickman MB, Becker DF. 2008. Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress. Free Radic Biol Med 44:671-81.

Krisman CR. 1962. A method for the colorimetric estimation of glycogen with iodine. Anal Biochem 4:17-23.

LeBel CP, Ischiropoulos H, Bondy SC. 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5:227-31.

Lissi E, Pascual C, Del Castillo MD. 1992. Luminol luminescence induced by 2,2'-Azo-bis(2-amidinopropane) thermolysis. Free Radic Res Commun 17:299-311.

Lissi E, Salim-Hanna M, Pascual C, del Castillo MD. 1995. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 18:153-8.

Liu Y, Borchert GL, Donald SP, Surazynski A, Hu CA, Weydert CJ, Oberley LW, Phang JM. 2005. MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells. Carcinogenesis 26:1335-42.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-75.

Marklund SL. 1985. Pyrogallol autoxidation. In Greenwald RA, editor. Handbook for Oxygen Radical Research. Boca Raton: CRC Press, p 243–247.

Mitsubuchi H, Nakamura K, Matsumoto S, Endo F. 2008. Inborn errors of proline metabolism. J Nutr 138:2016-2020.

Moreira JC, Wannmacher CM, Costa SM, Wajner M. 1989. Effect of proline administration on rat behavior in aversive and nonaversive tasks. Pharmacol Biochem Behav 32:885-90.

Nair J, Srivatanakul P, Haas C, Jedpiyawongse A, Khuhaprema T, Seitz HK, Bartsch H. 2010. High urinary excretion of lipid peroxidation-derived DNA damage in patients with cancer-prone liver diseases. Mutat Res 683:23-8.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-8.

Phang JM. 1985. The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul 25:91-132.

Phang JM, Donald SP, Pandhare J, Liu Y. 2008. The metabolism of proline, a stress substrate, modulates carcinogenic pathways. Amino Acids 35:681-90.

Phang JM, Hu CA, Valle D. 2001. Disorders of proline and hydroxyproline metabolism. In Scriver CR, Beaudet AL, Sly WS, Valle D, editor. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill, p 1821–1838.

Phang JM, Liu W, Zabirnyk O. 2010. Proline metabolism and microenvironmental stress. Annu Rev Nutr 30:441-63.

Pontes ZE, Oliveira LS, Bavaresco CS, Streck EL, Dutra-Filho CS, Wajner M, Wannmacher CM, Wyse AT. 1999. Proline administration decreases Na^+,K^+-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab Brain Dis 14:265-72.

Reznick AZ, Packer L. 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 233:357-63.

Rivera A, Maxwell SA. 2005. The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway. J Biol Chem 280:29346-54.

Rustgi S, Joshi A, Moss H, Riesz P. 1977. E.s.r. of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydroxyl radical. Int J Radiat Biol Relat Stud Phys Chem Med 31:415-40.

Salganik SV, Weinstein DA, Shupe TD, Salganik M, Pintilie DG, Petersen BE. 2009. A detailed characterization of the adult mouse model of glycogen storage disease Ia. Lab Invest 89:1032-42.

Shanti ND, Shashikumar KC, Desai PV. 2004. Influence of proline on rat brain activities of alanine aminotransferase, aspartate aminotransferase and acid phosphatase. Neurochem Res 29:2197-206.

Shaw S, Worner TM, Lieber CS. 1984. Frequency of hyperprolinemia in alcoholic liver cirrhosis: relationship to blood lactate. Hepatology 4:295-9.

Siripornadulsil S, Traina S, Verma DP, Sayre RT. 2002. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 14:2837-47.

Stadtman ER, Levine RL. 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 25:207-18.

Vargas-Tank L, Wolff C, Castillo T, Carrion Y, Hoppe A, Martinez V, Arman-Merino R. 1988. Serum proline and blood lactate levels in alcoholic patients without hepatic failure: relationship with alcohol ingestion and histological activity. Liver 8:58-62.

Wendel A. 1981. Glutathione peroxidase. Methods Enzymol 77:325-33.

White TA, Krishnan N, Becker DF, Tanner JJ. 2007. Structure and kinetics of monofunctional proline dehydrogenase from Thermus thermophilus. J Biol Chem 282:14316-27.

Wyse AT, Netto CA. 2011. Behavioral and neurochemical effects of proline. Metab Brain Dis. *In Press*.

Table 1. Effect of chronic hyperprolinemia on some parameters of oxidative stress, such as dichlorofluorescin oxidation assay (DCFH), thiobarbituric acid-reactive substances (TBARS), total radical-trapping antioxidant potential (TRAP), glutathione (GSH), sulfhydryl and carbonyl content in the liver of rats.

Parameters	Control	Proline
DCFH oxidation	18.01 ± 2.41	18.08 ± 2.84
TBARS	1.07 ± 0.07	0.92 ± 0.11*
TRAP	141.74 ± 26.58	89.94 ± 34.70*
GSH	5.84 ± 0.85	4.81 ± 1.23
Sulfhydryl content	61.67 ± 5.48	64.47 ± 4.55
Carbonyl content	2.38 ± 0.62	2.47 ± 0.25

Data are expressed as mean ± SD as follow: DCFH (nmol DCF/mg protein) TBARS (nmol TBARS/mg protein), TRAP (nmol Trolox/mg protein), GSH (nmol GSH/mg protein) sulfhydryl (nmol TNB/mg protein) and carbonyl content (nmol carbonyl/mg protein). *$p <$ 0.05, compared to control (Mann-Whitney-U test) for five to six animals in each group.

Table 2. Effect of chronic hyperprolinemia on superoxide dismutase (SOD), catalase (CAT) and gluthatione peroxidase (GPx) activities in the liver of rats.

Enzyme activities (units/mg protein)	Control	Proline
SOD	4.1 ± 0.44	5.6 ± 0.34**
CAT	158.14 ± 42.61	234.05 ± 28.09*
GPx	423.75 ± 55.99	421.50 ± 43.65

Data are mean ± SD for five to six animals in each group, reported as units/mg protein. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. One CAT unit is defined as 1 mmol of hydrogen peroxide consumed per minute. One GPx unit is defined as 1 mmol of NADPH consumed per minute. $*p < 0.05$; $**p < 0.01$, compared to control (Mann-Whitney-U test).

Figure 1
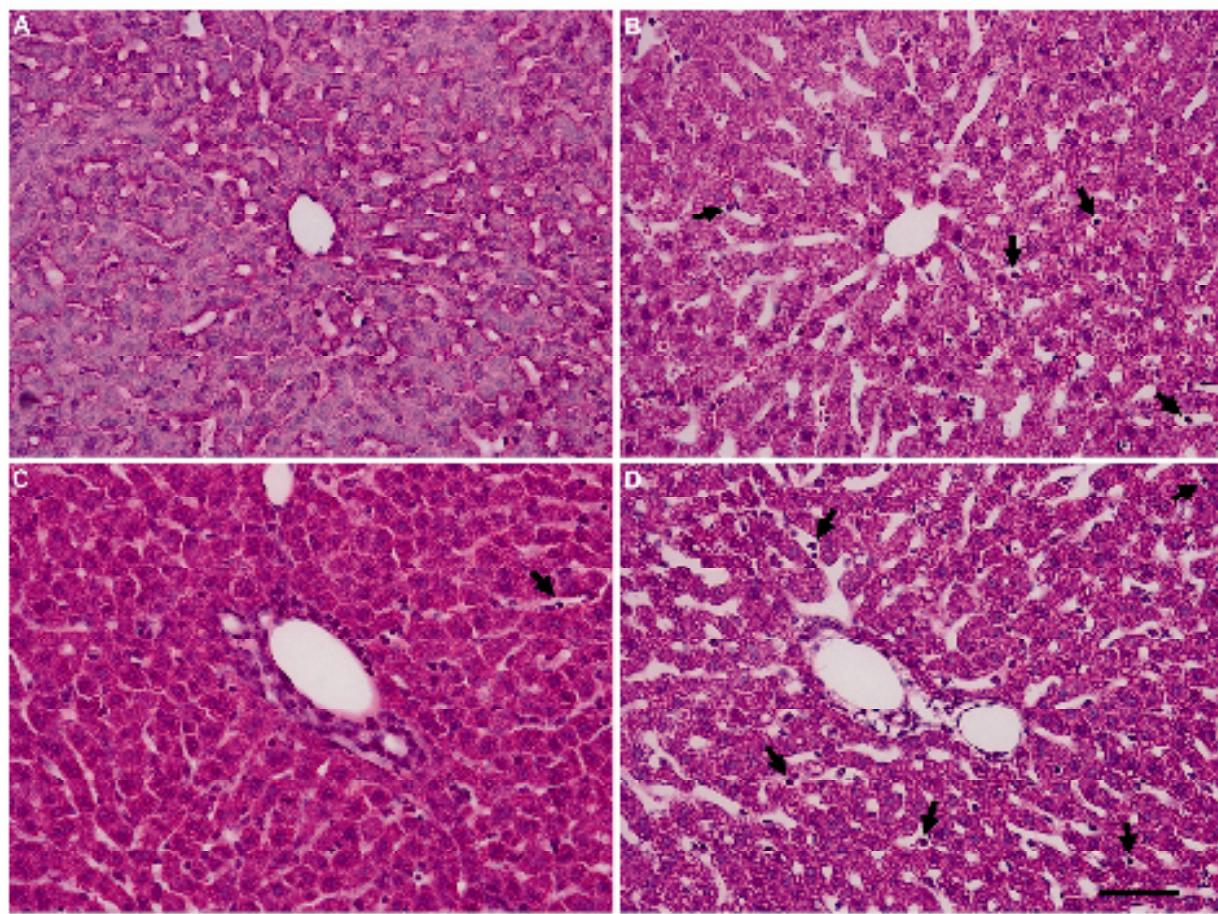
Figure 2
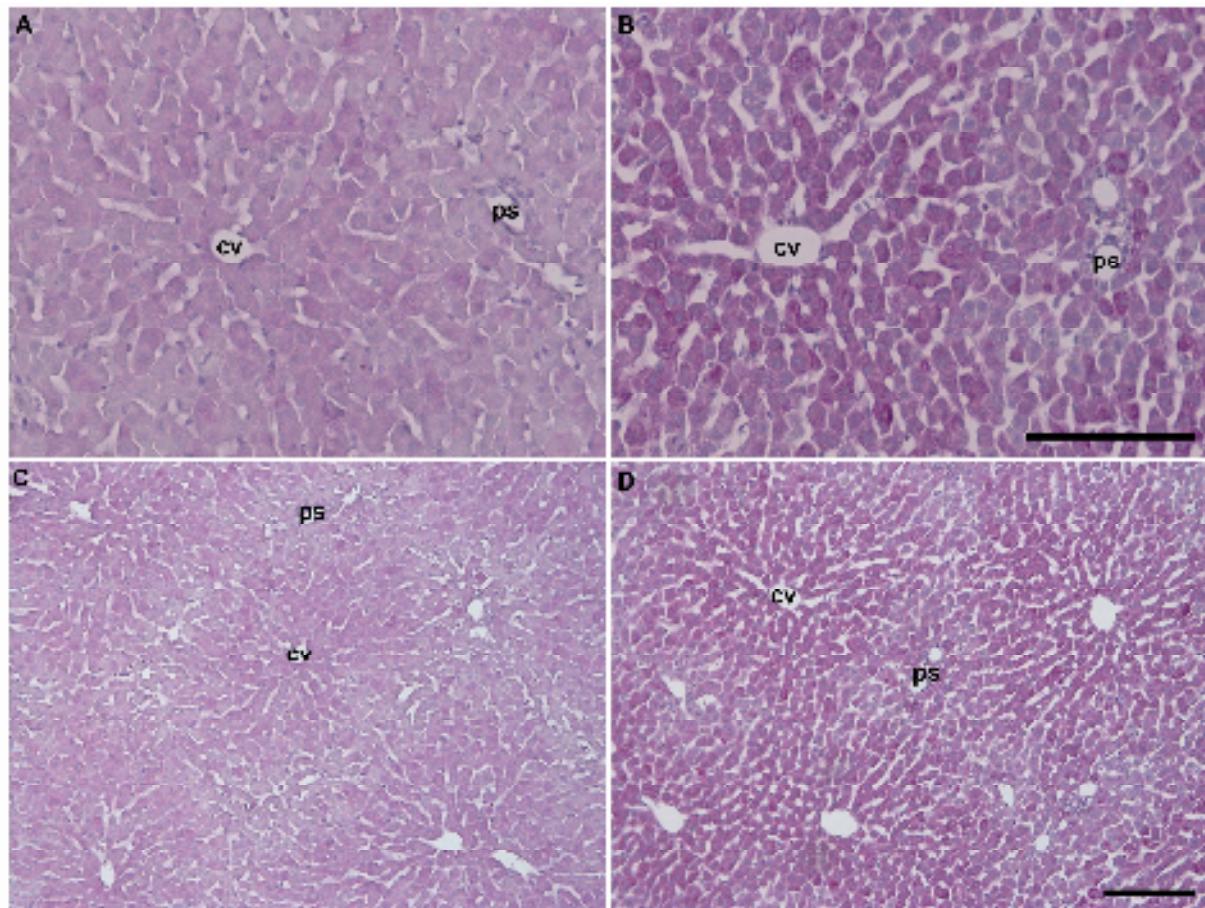
Figure 3
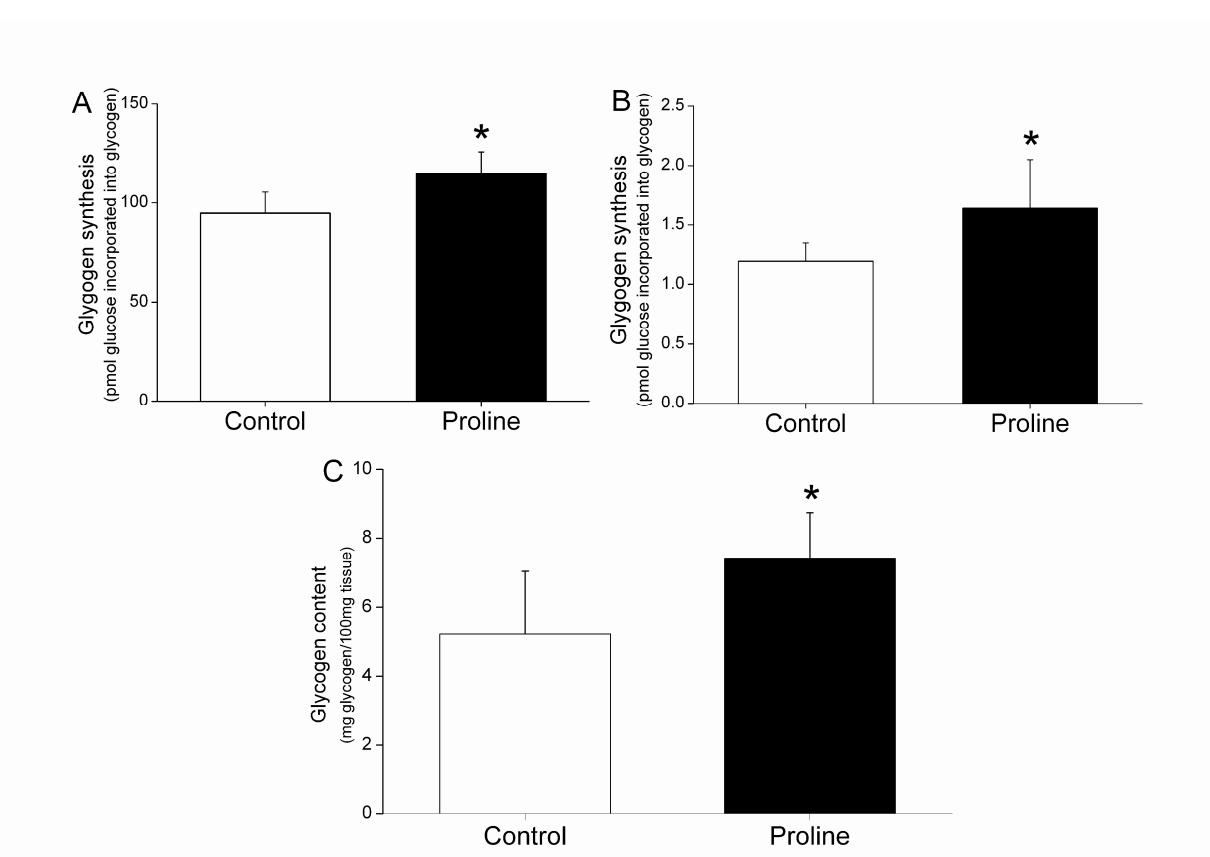
Figure 4
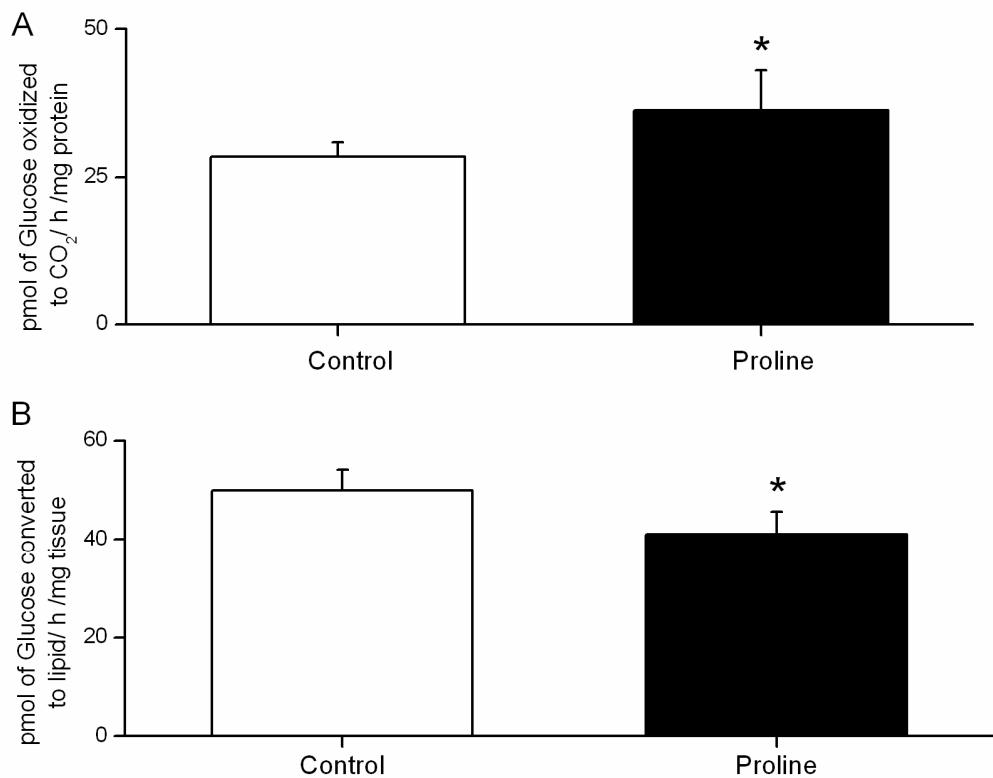
Table 3. Effect of chronic hyperprolinemia on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and on glucose levels in serum of rats.

Parameters	Control	Proline
ALT (U/L)	20.14 ± 2.89	18.80 ± 1.74
AST (U/L)	80.41 ± 6.24	79.60 ± 7.77
Glucose (mg/dL)	86.57 ± 17.07	90.00 ± 2.22

Data are expressed as mean ± SD for seven animals in each group. There is no difference between groups (Mann-Whitney-U test).

IV. DISCUSSÃO

As hiperprolinemias são EIM da prolina causadas por deficiências nas enzimas envolvidas na rota de degradação desse aminoácido, que se caracteriza bioquimicamente por níveis elevados de prolina no plasma e no fluido cerebro-espinhal dos pacientes afetados. As manifestações clínicas das hiperprolinemias podem se apresentar de forma variada; enquanto alguns pacientes são assintomáticos, outros podem apresentar alterações neurológicas graves como retardo mental e convulsões (Mitsubuchi et al., 2008; Phang et al., 2001; Wyse e Netto, 2011).

A neurotoxicidade da prolina tem sido evidenciada através de diversos estudos, porém os mecanismos pelos quais esse aminoácido atua no SNC são pouco conhecidos. Neste contexto, foi desenvolvido em nosso laboratório um modelo animal químico de hiperprolinemia, no qual os ratos são submetidos à administração de prolina a fim de induzir níveis plasmáticos desse aminoácido semelhantes àqueles encontrados em pacientes hiperprolinêmicos (Moreira et al., 1989; Pontes et al., 1999). Através desse modelo, temos mostrado que ratos hiperprolinêmicos apresentam déficit no aprendizado e memória (Bavaresco et al., 2005; Delwing et al., 2006), alterações nas atividades de enzimas do metabolismo energético cerebral, como a Na^+,K^+-ATPase (Pontes et al., 2001) e a creatina cinase (Kessler et al., 2003) e indução de estresse oxidativo em cérebro desses animais (Delwing et al., 2003a; 2003b; 2005a).

Baseado nos estudos prévios do nosso laboratório (citados acima) e considerando que o estresse oxidativo e anormalidades no metabolismo energético parecem estar envolvidos na fisiopatologia de doenças que afetam o SNC (Beal, 2005; Gibson et al., 2009; Kowaltowski et al., 2009; Petrozzi et al., 2007), no presente trabalho primeiramente investigamos o efeito da administração

aguda de prolina sobre as atividades das enzimas SDH e COX em córtex cerebral de ratos.

Os resultados mostraram que a hiperprolinemia aguda aumentou a atividade da SDH e diminuiu a atividade da COX, sugerindo que a prolina pode alterar o funcionamento da cadeia respiratória em cérebro de ratos. Nossos achados estão de acordo com dados prévios do nosso grupo, mostrando que a prolina é capaz de inibir a atividade da COX (Delwing et al., 2007a). Além disso, outros estudos demonstraram uma ativação na atividade da SDH e inibição da COX em córtex cerebral de camundongos submetidos à hipóxia (Caceda et al., 2001) e no cérebro de pacientes com doença de Alzheimer (estudos *postmortem*) (Bubber et al., 2005; Parker et al., 1994). Nesse contexto, tem sido sugerido que o aumento na atividade da SDH está associado à ativação de vias anaeróbias em situações em que a fosfoforilação oxidativa está prejudicada, como uma estratégia para compensar o déficit energético decorrente da disfunção mitocondrial (Weinberg et al., 2000).

A fim de investigar a possível influência da prolina sobre a via glicolítica e o ciclo de Krebs, avaliamos o efeito da hiperprolinemia aguda sobre a produção de $^{14}CO_2$ a partir de glicose e acetato. Os resultados mostraram que a oxidação de ambos os substratos a $^{14}CO_2$ aumentou no córtex cerebral dos ratos após a administração de prolina, sugerindo uma estimulação da glicólise e do ciclo de Krebs. Estes resultados são consistentes com o aumento da atividade da SDH observado após a administração de prolina.

Tem sido descrito que os radicais livres podem danificar os componentes mitocondriais (Cadenas e Davies, 2000; Kowaltowski et al., 2009) e que produtos da peroxidação lipídica como o malondialdeído podem prejudicar a atividade da

COX (Chen et al., 2000; Chen et al., 2001), sendo que inibição desse ou de qualquer outro complexo da cadeia respiratória pode conduzir à incompleta redução do oxigênio e, consequentemente, aumentar a formação de radicais livres e diminuir a síntese de ATP (Gupta et al., 2001; Milatovic et al., 2001; Nicholls, 2008).

Para avaliar o envolvimento do estresse oxidativo sobre os efeitos exercidos pela prolina, determinamos os níveis de TBARS, um parâmetro de peroxidação lipídica, no córtex cerebral de ratos submetidos à administração aguda de prolina e investigamos se o pré-tratamento com as vitaminas E e C poderiam prevenir os efeitos causados por esse aminoacido sobre a SDH, COX e TBARS. Nossos resultados revelaram um aumento nos níveis de TBARS, indicando que a prolina induz peroxidação lipídica. Além disso, o pré-tratamento com as vitaminas antioxidantes preveniu os efeitos da prolina sobre os parâmetros analisados, sugerindo que o estresse oxidativo pode estar envolvido na disfunção do metabolismo energético causado por esse aminoácido. Esses resultados estão de acordo com outros estudos que mostram que a vitamina E é essencial para a manutenção da fosforilação oxidativa (Delwing et al., 2007a; Navarro et al., 2005; Vatassery, 1998).

Embora não possamos explicar precisamente os mecanismos pelos quais a prolina exerce seus efeitos sobre a COX e SDH, é razoável propor que o aumento na atividade da SDH causada pela administração aguda de prolina poderia ser uma resposta inicial para compensar o déficit de energia secundário à inibição da atividade da COX, que provavelmente ocorre via radicais livres. Em conjunto, esses dados sugerem que a prolina induz estresse oxidativo, prejudica a cadeia

respiratória ao nível da COX, o que provavelmente limita a produção de ATP e aumenta a produção de ERO em um ciclo vicioso.

Tendo em vista que a disfunção mitocondrial e o estresse oxidativo parecem contribuir para os efeitos causados pela prolina no cérebro, que a Na^+,K^+-ATPase é uma importante enzima cerebral, cuja atividade é altamente dependente de energia, consumindo cerca de 50% do ATP produzido no cérebro e que a prolina é capaz de inibir a atividade dessa enzima no cérebro de ratos (Pontes et al., 1999; 2001), nosso próximo objetivo foi investigar possíveis mecanismos envolvidos na inibição da Na^+,K^+-ATPase causada pela prolina.

Considerando que a Na^+,K^+-ATPase é uma enzima inserida na membrana, particularmente susceptível ao ataque dos radicais livres e às alterações na composição lipídica da membrana plasmática (Potts et al., 2006; Siems et al., 1996), avaliamos o efeito da administração crônica de prolina e das vitaminas E e C sobre os níveis de TBARS e sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de córtex cerebral de ratos.

Os resultados mostraram um aumento nos níveis de TBARS no córtex cerebral de ratos tratados cronicamente com prolina, cujo efeito foi prevenido pela administração concomitante das vitaminas E e C. Porém, interessantemente a administração dessas vitaminas preveniu apenas parcialmente a inibição da Na^+,K^+-ATPase causada pela prolina, sugerindo que outros mecanismos, além do estresse oxidativo e da peroxidação lipídica, podem estar envolvidos na inibição dessa enzima.

Com o objetivo de identificar esses possíveis mecanismos, verificamos se as alterações na atividade da Na^+,K^+-ATPase causadas pela hiperprolinemia crônica e/ou pela suplementação de antioxidantes poderiam estar relacionadas a

alterações na expressão gênica das subunidades catalíticas dessa enzima. No entanto, nenhuma alteração nos níveis de RNAm das subunidades catalíticas α1, α2 e α3 da Na$^+$,K$^+$-ATPase foi observada.

Evidências na literatura sugerem que diversos mecanismos regulam a atividade da Na$^+$,K$^+$-ATPase de acordo com as demandas fisiológicas (Blanco e Mercer, 1998; Geering, 2001; Jorgensen et al., 2003; Kaplan, 2002; Mobasheri et al., 2000). Nesse contexto, cabe ressaltar que um importante modulador dessa enzima é o nível de ATP intracelular, cuja diminuição tem sido relacionado à inibição da sua atividade (Nicholls, 2008; Therien e Blostein, 2000).

Devido a importância do funcionamento adequado da Na$^+$,K$^+$-ATPase, responsável pela geração do potencial de membrana necessário para a manutenção da excitabilidade neuronal, alterações na sua atividade podem causar uma série de anormalidades neurológicas, as quais podem estar envolvidas na fisiopatologia das disfunções cerebrais presentes em doenças metabólicas como a hiperprolinemia. De fato, alterações na Na$^+$,K$^+$-ATPase tem sido relacionadas a fisiopatologia de doenças neurodegenerativas como Alzheimer, esclerose múltipla, doença de Parkinson e epilepsia (Aperia, 2007; Benarroch, 2011; Hattori et al., 1998).

Outro aspecto relevante do funcionamento da Na$^+$,K$^+$-ATPase é o gradiente eletroquímico gerado pelo fluxo dos íons Na$^+$ e K$^+$ através da membrana celular, que é utilizado para o transporte de moléculas ligadas ao co-transporte de Na$^+$, incluindo neurotransmissores como o glutamato (Jorgensen et al., 2003; Kaplan, 2002; Mobasheri et al., 2000).

O glutamato é o principal neurotransmissor excitatório do SNC, o qual desempenha um papel essencial nos processos de plasticidade sináptica do

cérebro, subjacentes a funções como aprendizado e memória (Danbolt, 2001; Segovia et al., 2001). Quando liberado na fenda sináptica, o glutamato exerce seus efeitos biológicos ao ligar-se nos receptores glutamatérgicos presentes na superfície das células neurais. Uma vez que não existe uma enzima específica no meio extracelular capaz de metabolizar o glutamato (Attwell, 2000; Chen e Swanson, 2003; Danbolt, 2001), o término da sinalização glutamatérgica depende da remoção do glutamato da fenda sináptica (captação de glutamato), realizado por transportadores especializados localizados na membrana celular dos astrócitos e nos terminais neuronais pré-sinápticos. Esses transportadores dependem do gradiente de sódio gerado pela Na^+,K^+-ATPase, para transportar o glutamato para dentro da célula contra o seu gradiente de concentração. Nesse contexto, estudos prévios do nosso grupo mostraram que a administração aguda de prolina prejudica a captação de glutamato em cérebro de ratos (Delwing et al., 2007b).

Dando continuidade a esses estudos, o objetivo dessa etapa do trabalho foi verificar o efeito da administração crônica de prolina sobre a captação do glutamato, bem como investigar alguns mecanismos subjacentes aos efeitos da administração aguda e crônica de prolina sobre a homeostase glutamatérgica em córtex cerebral de ratos jovens. Além disso, tendo em vista que a guanosina tem sido relatada como um possível neuroprotetor em condições de toxicidade glutamatérgica, a influência desse nucleosídeo sobre os efeitos mediados pela prolina também foi avaliada.

Os resultados revelaram que a administração aguda e crônica de prolina diminuiu a captação de glutamato em fatias de córtex cerebral de ratos, de acordo com resultados já demonstrados (Delwing et al., 2007b). O pré-tratamento com

guanosina durante sete dias não foi suficiente para prevenir o prejuízo na captação de glutamato causada pela administração aguda de prolina. No entanto, quando a guanosina foi administrada concomitantemente à prolina no tratamento crônico, a inibição da captação de glutamato causada pela prolina foi completamente prevenida, sugerindo que há a necessidade de um maior tempo de exposição à guanosina para que ocorra seu efeito protetor sobre a captação de glutamato.

Desde que GLAST e GLT-1 são quantitativamente os principais transportadores de glutamato, responsáveis por aproximadamente 90% da captação do glutamato extracelular (Danbolt, 2001), e a fim de investigar se o comprometimento da captação de glutamato causado pela prolina está relacionado aos níveis dos transportadores de glutamato, avaliamos o imunoconteúdo de GLAST e GLT-1 em homogenato de córtex cerebral de ratos.

Interessantemente, os resultados mostraram que a injeção aguda de prolina aumentou os níveis desses transportadores, o que pode refletir uma tentativa de compensar a captação de glutamato prejudicada. Além disso, observamos que o tratamento por uma semana com guanosina foi capaz de aumentar *per se* os níveis de GLAST e GLT-1 em córtex cerebral dos ratos. Este efeito pode ser um possível e interessante mecanismo de ação para esse nucleosídeo na contenção da toxicidade glutamatérgica que, pelo menos para o nosso conhecimento, ainda não havia sido demonstrado.

Por outro lado, a administração crônica de prolina e/ou guanosina não alterou o conteúdo de GLAST e GLT-1 no córtex cerebral de ratos, sugerindo que sucessivas administrações por um longo período de tempo parecem promover uma adaptação do organismo. Desse modo, uma vez que não encontramos uma

relação direta entre a atividade de captação de glutamato e os níveis dos transportadores, é razoável sugerir que a disfunção da captação de glutamato promovida pela prolina não envolve um distúrbio quantitativo desses transportadores, mas possivelmente uma alteração no seu funcionamento.

Conforme descrito anteriormente, o principal mecanismo proposto para o transporte de glutamato é dependente de sódio e, portanto, depende da atividade da Na^+,K^+-ATPase (Rose et al., 2009). Sendo assim, avaliamos a atividade dessa enzima em homogeneizado de córtex cerebral de ratos submetidos ao tratamento agudo e crônico com prolina e/ou guanosina. Cabe ressaltar que nessa etapa do trabalho, utilizamos o homogeneizado para avaliar a atividade da Na^+,K^+-ATPase ao invés da preparação de membrana plasmática sináptica, mais purificada, para que o conteúdo celular contemplasse tanto células gliais (onde os transportadores de glutamato estão presentes em altas concentrações), como células neuronais (onde a Na^+,K^+-ATPase está presente em alta concentração).

Os resultados revelaram uma inibição na atividade da Na^+,K^+-ATPase após a administração aguda e crônica de prolina. Além disso, a administração de guanosina foi capaz de prevenir significativamente a inibição dessa enzima, embora o efeito protetor observado no tratamento crônico tenha sido mais proeminente. Esses dados sugerem mais uma vez que o efeito protetor da guanosina parece depender do tempo, já que no modelo agudo o pré-tratamento com guanosina durante sete dias preveniu apenas parcialmente a inibição da Na^+,K^+-ATPase causada pela prolina, o que aparece ser insuficiente para normalizar a captação de glutamato, a qual não foi prevenida pela administração desse nucleosídeo.

Estudos demonstram que a ativação de receptores glutamatérgicos aumenta o consumo de energia da célula, uma vez que leva ao influxo de íons, os quais têm de ser bombeados novamente para fora, em um processo que requer energia (Danbolt, 2001). Além disso, a produção de ATP mitocondrial é prejudicada durante o insulto excitotóxico e os níveis de ATP neuronal diminuem rapidamente (Greenwood e Connolly, 2007; Greenwood et al., 2007). Baseado no que foi exposto, determinamos os níveis de ATP em córtex cerebral de ratos submetidos à hiperprolinemia aguda e crônica e avaliamos a influência da guanosina sobre esse parâmetro.

Os resultados mostraram que tanto a administração aguda como a administração crônica de prolina, diminuiram os níveis de ATP intracelular. Além disso, vimos que a guanosina não foi capaz de evitar esse efeito promovido pela prolina sobre os níveis de ATP. No modelo agudo, a diminuição dos níveis de ATP corrobora com a hipótese de que a prolina induz excitotoxicidade, uma vez que observamos que a captação de glutamato está prejudicada e, possivelmente, os níveis de glutamato estão aumentados na fenda sináptica gerando excitotoxicidade e gasto energético. Nesse cenário, o pré-tratamento com guanosina não preveniu a redução da captação de glutamato causada pela prolina e também não foi capaz de reestabelecer os níveis de ATP.

Por outro lado, no modelo crônico a guanosina preveniu a redução da captação de glutamato, enquanto os níveis de ATP permaneceram diminuidos, sugerindo que outras alterações, celulares e/ou moleculares (inacessíveis à ação da guanosina) podem estar envolvidas nos efeitos da prolina a longo prazo. De acordo com essa observação, foi demonstrado que a prolina inibe a COX, uma enzima essencial para a síntese de ATP (Delwing et al., 2007a).

Uma vez que a literatura relata que os radicais livres e a peroxidação lipídica podem inibir a captação de glutamato diretamente por alterar a atividade dos transportadores ou indiretamente por prejudicar a atividade da Na^+,K^+-ATPase (Blanc et al., 1998; Danbolt, 2001; Pedersen et al., 1999), também investigamos os efeitos da administração de prolina e de guanosina sobre os níveis de TBARS, a fim de avaliar a peroxidação lipídica. Os resultados mostraram que a administração aguda e crônica de prolina aumentou o TBARS, de acordo com os dados anteriores (Ferreira et al., 2010; 2011). Além disso, o tratamento com guanosina foi capaz de prevenir o aumento dos níveis de TBARS, mediados pela administração aguda e crônica desse aminoácido. De acordo, tem sido descrito um papel antioxidante da guanosina, possivelmente indireto e relacionado à sua capacidade de estimular a captação de glutamato (Roos et al., 2009).

Em resumo, esse capítulo da tese demonstrou que elevados níveis de prolina podem prejudicar a captação de glutamato possivelmente por induzir peroxidação lipídica e prejudicar a atividade da Na^+,K^+-ATPase, mas sem alterar quantitativamente os transportadores de glutamato. Sugerimos que as alterações na homeostase do glutamato causadas pela hiperprolinemia podem levar à excitotoxicidade e subsequente déficit energético.

Tendo em vista que pacientes hiperprolinêmicos usualmente apresentam graus variados de distúrbios neurológicos e cognitivos (Di Rosa et al., 2008; Phang et al., 2001), que a prolina altera parâmetros que estão envolvidos nos processos de formação da memória, e considerando que estudos prévios do nosso grupo mostraram que ratos submetidos à hiperprolinemia experimental na fase inicial da vida (do 6º ao 28º dia de vida), apresentaram déficit cognitivo quando avaliados no labirinto aquático de Morris na vida adulta (60º - 70º dia de

vida), ou seja, aproximadamente 30 dias após a interrupção do tratamento com prolina, investigamos se o exercício físico poderia exercer um efeito benéfico sobre o déficit cognitivo causado por esse aminoácido e buscamos identificar mecanismos neuroquímicos envolvidos nos efeitos da prolina e do exercício sobre a memória.

Para a avaliação da influência do exercício sobre os efeitos causados pela prolina na memória, um protocolo experimental semelhante aos estudos anteriores foi delineado, onde os ratos foram submetidos à administração crônica de prolina do 6º ao 29º dia de vida, quando o tratamento foi descontinuado e os ratos iniciaram o exercício físico em esteira (do 30° ao 60º dia de vida). Após o término do período de exercícios os ratos foram submetidos aos testes comportamentais para a avaliação da memória espacial no labirinto aquático de Morris (D'Hooge e De Deyn, 2001; Netto et al., 1993). Uma vez que o exercício iniciou quando os ratos atingiram 30 dias de vida, ou seja, após o término do tratamento com prolina, este delineamento experimental de hiperprolinemia associada ao exercício físico foi denominado *protocolo para "hiperprolinemia de tempo parcial"*.

Os resultados obtidos confirmaram os estudos prévios mostrando que os ratos submetidos a administração crônica de prolina apresentaram um prejuízo significativo na aquisição e/ou consolidação da memória, observado pela maior latência em encontrar o local da plataforma de escape no decorrer dos dias de treinamento (aquisição) e no dia do teste (retenção). Os ratos hiperprolinêmicos também apresentaram um prejuízo na tarefa de memória de trabalho, a qual avalia a memória de curta duração. Interessantemente, o exercício físico foi capaz

de reverter o prejuízo cognitivo causado pela administração crônica de prolina tanto na memória de referência como na de trabalho.

Não obstante, considerando que a hiperprolinemia é uma doença metabólica hereditária, cujo tratamento muitas vezes é ineficaz em reduzir os níveis plasmáticos de prolina, os quais permanecem elevados durante toda a vida, nos questionamos se o suposto efeito benéfico do exercício sobre o déficit de memória causado pela prolina permaneceria se a hiperprolinemia fosse estendida ao longo da vida. Assim, um segundo protocolo experimental denominado *protocolo para "hiperprolinemia de tempo total"* também foi realizado. Neste protocolo, a prolina foi administrada de forma contínua do 6º ao 60º dia de vida, enquanto o exercício foi realizado do 30º ao 60º dia de vida, ou seja, as injeções de prolina continuaram durante todo o período de treinamento físico.

Os resultados obtidos a partir do *protocolo para "hiperprolinemia de tempo total"* foram muito similares aos observados no *protocolo para "hiperprolinemia de tempo parcial"*, o qual demonstrou que a prolina prejudica a memória de referência e de trabalho. Além disso, o exercício físico também foi capaz de reverter o déficit cognitivo causado pela prolina nesse modelo experimental.

Em conjunto, os resultados provenientes dos dois protocolos experimentais utilizados indicam que a deficiência na aprendizagem/memória decorrente da hiperprolinemia provavelmente é estabelecida na fase inicial da vida dos ratos, um período considerado crítico para o desenvolvimento do cérebro, quando há intensa sinaptogênese e mielinização (Erecinska et al., 2004). De fato, tem sido descrito que mesmo um aumento modesto nos níveis de prolina durante períodos críticos de desenvolvimento pode ter um efeito dramático sobre o funcionamento do SNC (Guilmatre et al., 2010).

Nossos dados também evidenciam o potencial neuroprotetor do exercício físico, o qual se mostrou efetivo mesmo quando o déficit cognitivo já estava estabelecido e quando a hiperprolinemia permaneceu até a vida adulta dos ratos. Nesse contexto, diversos trabalhos têm mostrado que o exercício físico pode melhorar a função cognitiva e exercer efeitos neuroprotetores em várias condições que afetam o SNC, tais como a doença de Alzheimer (Adlard et al., 2005; Um et al., 2008), isquemia cerebral (Cechetti et al., 2007; Zhang et al., 2011), esquizofrenia (Pajonk et al., 2010) e doenças metabólicas (Ben et al., 2009; 2010).

A fim de investigar os possíveis mecanismos envolvidos nos efeitos da prolina e do exercício físico sobre a memória, também avaliamos os níveis de BDNF e a atividade da AChE no hipocampo e córtex cerebral dos ratos ao final dos experimentos comportamentais uma vez que esses parâmetros neuroquímicos parecem estar envolvidos nos mecanismos de formação e modulação da memória (Cotman e Berchtold, 2002; Deiana et al., 2011).

O BDNF é uma neurotrofina que modula os diferentes aspectos da função neuronal durante a fase de desenvolvimento do sistema nervoso, bem como no sistema nervoso maduro (Gottmann et al., 2009). Os resultados mostraram que a prolina reduziu o imunoconteúdo de BDNF no hipocampo e córtex cerebral de ratos em ambos os protocolos experimentais utilizados e que o exercício foi capaz de reverter esse efeito, sugerindo que o déficit de memória causado pela prolina pode estar relacionado a uma diminuição da disponibilidade do BDNF e que o restabelecimento dos níveis dessa neurotrofina pode estar relacionada à melhora na cognição induzida pelo exercício. Considerando que alterações nos níveis de BDNF têm sido reportadas em distúrbios cerebrais como a esquizofrenia

(Egan et al., 2003), lesões traumáticas (Horsfield et al., 2002), demência (Ando et al., 2002) e doença de Alzheimer (Tsai et al., 2004), a constatação de que o exercício moderado durante um período de tempo relativamente curto é suficiente para melhorar a aprendizagem/memória é relevante, uma vez que o exercício representa uma forma acessível de intervenção que pode ser usada em conjunto com o tratamento padrão em diversas patologias (Vaynman et al., 2004b).

A AChE é uma enzima que apresenta um papel indispensável na transmissão sináptica hidrolisando ACh e parece estar associada com os processos de desenvolvimento cerebral, aprendizado e memória (Ballard et al., 2005; Zimmerman e Soreq, 2006). Nossos resultados mostraram que a administração crônica de prolina aumentou a atividade da AChE em hipocampo, mas não em córtex cerebral de ratos. Com base nesses achados, é concebível que a constante estimulação dessa enzima pela prolina promova uma diminuição nos níveis de ACh reduzindo a atividade colinérgica no hipocampo, o que pode estar associado ao prejuízo na memória observada nos ratos hiperprolinêmicos. Os resultados também revelaram que ratos hiperprolinêmicos submetidos ao exercício apresentaram a atividade da AChE semelhante à atividade observada nos ratos controles, sugerindo que o exercício pode influenciar mecanismos colinérgicos no cérebro. Neste contexto, foi demonstrado que a ativação colinérgica no hipocampo é necessária para a consolidação da memória espacial (Gale et al., 2001; Herrera-Morales et al., 2007; Wallenstein e Vago, 2001) e que os níveis extracelulares de ACh aumentam no hipocampo durante atividade de exploração ou aprendizagem (Giovannini et al., 2001; Ragozzino et al., 1996). Extensa literatura suporta a idéia de que um mecanismo mediado por ACh

também regula a expressão gênica do BDNF no hipocampo o que pode ser subjacente à regulação do BDNF pelo exercício (Cotman e Berchtold, 2002).

Cabe ressaltar que o protocolo para a realização do exercício físico utilizado neste estudo não influenciou *per se,* tanto o desempenho no labirinto aquático de Morris, quanto os níveis de BDNF e a atividade da AChE em ratos que receberam administração de salina (controles). Esta observação está de acordo com outros estudos, os quais utilizaram um protocolo de exercício semelhante (Ben et al., 2010; Cechetti et al., 2008), embora outros estudos tenham mostrado que o exercício aumenta os níveis de BDNF (Liu et al., 2011; Vaynman et al., 2004a). Esses dados controversos em relação ao BDNF podem ser atribuídos a diferentes fatores, tais como a duração e intensidade do exercício realizado, a motivação para a atividade física (forçado ou voluntário), bem como a idade dos animais experimentais (Berchtold et al., 2010; Stranahan et al., 2009).

Além das alterações neurológicas presentes em pacientes hiperprolinêmicos (Phang et al., 2001; Wyse e Netto, 2011), dados da literatura mostram que a hiperprolinemia também pode estar presente em certas doenças hepáticas, tais como doença hepática alcoólica (Vargas-Tank et al., 1988) e cirrose (Shaw et al., 1984). Porém, ainda não foi estabelecido se o elevado nível desse aminoácido é a causa ou a consequência dos danos hepáticos. Portanto, considerando que o fígado é um dos principais órgãos envolvidos no catabolismo da prolina, além do cérebro e dos rins (Phang et al., 2001), também avaliamos alguns parâmetros de estresse oxidativo, bem como o perfil morfológico e metabólico em fígado de ratos submetidos ao modelo de hiperprolinemia crônica.

Nessa etapa do trabalho, nós inicialmente demonstramos que a prolina diminuiu o TRAP, sugerindo que altas concentrações desse aminoácido podem

causar uma redução na quantidade de antioxidantes não enzimáticos no fígado. Com base nesta descoberta, também determinamos os níveis de GSH, que é o antioxidante presente em concentrações mais elevadas nesse órgão e que mais contribui para os valores do TRAP (Evelson et al., 2001). Porém, os resultados mostraram que os níveis de GSH não foram alterados pela hiperprolinemia crônica, indicando que a diminuição nos valores do TRAP causados pela prolina não refletem a alteração do conteúdo de GSH. No entanto, é possível que outros antioxidantes possam estar diminuídos no fígado de ratos hiperprolinêmicos.

Em relação às defesas antioxidantes enzimáticas, observou-se que a hiperprolinemia crônica aumentou as atividades das enzimas SOD e CAT, mas não alterou a atividade da GSH-Px no fígado de ratos. Nesse contexto, tem sido reportado que o catabolismo da prolina pode reduzir diretamente o oxigênio e produzir superóxido (Liu et al., 2005; White et al., 2007). Adicionalmente, trabalhos do nosso grupo têm mostrado que a prolina pode induzir o estresse oxidativo no cérebro de ratos (Delwing et al., 2003a; Delwing et al., 2003b), portanto, é concebível que os elevados níveis de prolina no tecido hepático podem gerar radical superóxido, o qual é dismutado pela SOD formando H_2O_2, que por sua vez é reduzido pela CAT. Uma vez que evidências sugerem que o aumento da expressão e da atividade de enzimas antioxidantes pode decorrer da contínua presença de ERO como uma estratégia de adaptação celular ao estresse oxidativo (Halliwell e Whiteman, 2004; Halliwell e Gutteridge, 2007), é razoável propor que o aumento nas atividades da SOD e CAT no fígado de ratos pode ser uma consequência da adaptação do tecido hepático devido a produção de ERO pela prolina, principalmente superóxido e peróxido de hidrogênio. A produção de ERO e ERN foi avaliada pelo ensaio de oxidação do DCFH, o qual detecta radicais como peroxil, alcoxil, dióxido de nitrogênio, hidroxila e

peroxinitrito (Halliwell e Whiteman, 2004). No entanto, a prolina não alterou os níveis de ER detectadas por esse ensaio. Porém, não podemos descartar que outras ER não detectadas pela oxidação do DCFH (como por exemplo o superóxido) possam estar presentes em concentrações elevadas no tecido hepático.

Uma das consequências do aumento da produção de ERO é o dano a biomoléculas, tais como proteínas e lipídios. No entanto, neste estudo nós observamos que o fígado de ratos submetidos à hiperprolinemia crônica não apresentou dano oxidativo às proteínas como mostrado pelo inalterado conteúdo de carbonilas e sulfidrilas, e os níveis de TBARS foram ainda diminuídos pela a exposição crônica à prolina. Até o momento, não podemos explicar o mecanismo exato pelo qual a prolina reduz os níveis de TBARS, mas esse resultado está de acordo com estudos que mostram que em algumas situações esse aminoácido pode exercer propriedades antioxidantes atuando sobre alguns radicais livres, por exemplo oxigênio *singlet* e radicais hidroxila (Alia et al., 2001; Floyd e Nagy, 1984; Halliwell e Gutteridge, 2007; Rustgi et al., 1977). Nesse contexto, o acúmulo de prolina tem sido observado em muitos microrganismos e plantas expostas ao estresse ambiental, como um mecanismo de proteção contra as ERO produzidas durante condições de estresse (Alia et al., 2001; Chen e Dickman, 2005; Chen et al., 2006; Siripornadulsil et al., 2002).

Portanto, os níveis inalterados de espécies reativas (avaliada pelo ensaio DCFH) e a diminuição da peroxidação lipídica (detectada por TBARS) no fígado de ratos submetidos à administração de prolina, poderiam ser atribuídas a uma eficaz detoxificação das ERO/ERN pelas defesas antioxidantes enzimáticas e não enzimáticas como demonstrado pelo aumento das atividades da SOD e CAT e

pela diminuição do TRAP. Além disso, esse efeito da prolina na diminuição da peroxidação lipídica em fígado de ratos, bem como o aumento de enzimas antioxidantes poderia explicar pelo menos em parte, a hiperprolinemia encontrada em pacientes afetados por distúrbios hepáticos, como um mecanismo de proteção do fígado contra o dano oxidativo. De fato, as atividades das aminotransferases ALT e AST, as quais são marcadores séricos de lesão hepática, permaneceram inalteradas após a administração crônica de prolina, sugerindo que a prolina não induz dano substancial ao tecido hepático.

A próxima etapa do nosso estudo foi avaliar o efeito da exposição crônica de prolina sobre a integridade estrutural do fígado por análise histológica. A coloração com hematoxilina/eosina demonstrou que a prolina promoveu um certo desarranjo na estrutura dos hepatócitos e aumentou o número de células inflamatórias quando comparado ao grupo controle. Esses achados histológicos podem estar relacionados ao estresse oxidativo induzido pela prolina no fígado desses ratos uma vez que as células inflamatórias podem gerar grandes quantidades de ER (Decker, 1990). Adicionalmente, realizamos uma coloração para a análise do conteúdo de glicoproteínas/glicogênio, a qual revelou um aumento acentuado no conteúdo de glicogênio hepático nos ratos tratados com prolina. Análises bioquímicas confirmaram essa constatação, demonstrando que a prolina aumenta a síntese e a concentração de glicogênio. Nesse contexto, um estudo demonstrou que a prolina aumenta a concentração hepática de glicose-6-P, sugerindo que um metabólito da prolina inibe a atividade de glicose-6-fosfatase e, portanto, direciona a glicose-6-P para a síntese de glicogênio (Bode et al., 1992).

Baseados nos resultados relativos ao aumento do glicogênio nos hepatócitos causado pela administração de prolina (Phang et al., 2001), também avaliamos outros possíveis destinos da glicose hepática após o tratamento crônico com prolina. Os resultados mostraram que os ratos hiperprolinêmicos apresentaram uma diminuição da conversão [U-^{14}C]glicose para lipídios no fígado. Porém, as concentrações de lipídeos hepáticos (triglicerídeos e colesterol) não foram alteradas. Além disso, um aumento na oxidação de glicose pelo tecido hepático foi demonstrado quando comparado aos controles, sugerindo uma estimulação da via glicolítica e do ciclo de Krebs. No entanto, os níveis de glicose no soro de ratos não foram alterados pela administração de prolina.

Esses resultados sugerem que a hiperprolinemia altera a homeostase do fígado através da indução de um leve grau de estresse oxidativo, que parece ser tolerado pelas células do fígado devido à modulação de sua capacidade de defesa antioxidante suficiente para remover as espécies reativas formadas. Além disso, a prolina também induz alterações metabólicas no fígado, observadas pelo aumento no conteúdo de glicogênio e na oxidação de glicose. Baseados nesses resultados, podemos sugerir que as alterações nos parâmetros de estresse oxidativo detectadas no presente trabalho provavelmente não causam dano no tecido hepático, mas demonstram um processo de adaptação tecidual ao estresse oxidativo. Nossos dados reforçam a hipótese de que a prolina e suas interconversões na rota metabólica atuam na manutenção de equilíbrio redox celular (Krishnan et al., 2008; Phang et al., 2010; Wyse e Netto, 2011), que pode estar relacionado ao aumento dos níveis desse aminoácido ácido em doenças hepáticas. No entanto, os mecanismos e significado biológico desses achados necessitam de mais estudos para ser totalmente compreendidos.

Demonstramos ao longo desse trabalho que a hiperprolinemia induz peroxidação lipídica, compromete o metabolismo energético cerebral através da inibição de enzimas cerebrais e diminui os níveis de ATP intracelular, inibe a atividade da Na^+,K^+-ATPase sem alterar a expressão de suas subunidades catalíticas e prejudica a captação de glutamato, mas não diminui o conteúdo de seus transportadores. Todas essas alterações podem estar intimamente relacionadas ao déficit cognitivo causado pela hiperprolinemia, os quais também demonstramos estar relacionados a diminuição do níveis de BDNF e da atividade colinérgica. Esses achados podem ser relevantes para o entendimento das alterações neurológicas presentes na hiperprolinemia. Além disso, demonstramos algumas possibilidades de neuroproteção para tais efeitos causados pela prolina, como a utilização de vitaminas antioxidantes, do nucleosídeo guanosina e do exercício físico, os quais podem contribuir para a contenção do estresse oxidativo, da toxicidade glutamatérgica e dos prejuízos na cognição induzidos pela hiperprolinemia. A hiperprolinemia também altera a homeostase hepática através da indução de um leve grau de estresse oxidativo e de alterações metabólicas as quais, provavelmente não implicam em dano significativo ao tecido hepático, mas demonstram um processo de adaptação desse tecido ao estresse oxidativo.

V. CONCLUSÕES

• A administração aguda de prolina prejudicou o metabolismo energético cerebral observado pela inibição da atividade da COX. Além disso, a prolina aumentou a atividade da SDH e a oxidação de glicose e acetato, provavelmente como uma tentativa de compensar o déficit energético decorrente da inibição da COX. Esses efeitos foram significativamente prevenidos pelo pré-tratamento com vitaminas E e C.

• A administração crônica de prolina induziu lipoperoxidação e inibiu a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de córtex cerebral de ratos, mas não alterou a expressão das subunidades catalíticas da enzima. O tratamento concomitante com vitaminas E e C foi capaz de prevenir parcialmente a inibição da atividade da Na^+,K^+-ATPase e de prevenir completamente a peroxidação lipídica causadas pela hiperprolinemia crônica.

• O déficit cognitivo causado pela administração crônica de prolina provavelmente envolve uma diminuição dos níveis de BDNF no córtex cerebral e hipocampo e um aumento na atividade da AChE no hipocampo de ratos. O exercício físico foi capaz de reverter o prejuízo cognitivo causado pela hiperprolinemia crônica, bem como os efeitos causados pela prolina sobre o conteúdo de BDNF e sobre a atividade da AChE.

• A administração crônica de prolina induziu um leve grau de estresse oxidativo no fígado de ratos observados através da diminuição do TRAP e aumento nas atividades da SOD e CAT. A análise histológica revelou um aumentou no número de células inflamatórias e no conteúdo de glicoproteínas/glicogênio. Análises bioquímicas mostraram que a prolina alterou o metabolismo hepático, promovendo um aumento na síntese e concentração de glicogênio e na oxidação de glicose e diminuição na síntese de lipídios a partir de

glicose. A concentração de lipídeos hepáticos (triglicerídeos e colesterol), bem como a glicemia e as atividades das enzimas marcadoras de dano hepático ALT e AST não foram alteradas em soro de ratos.

CONCLUSÃO GERAL

Os resultados do presente estudo mostram em conjunto, que a hiperprolinemia causa uma série de alterações bioquímicas, tais como indução de estresse oxidativo, déficit energético e alterações no sistema glutamatérgico, as quais podem contribuir para as disfunções neurológicas características dessa patologia. Além disso, nossos resultados sugerem algumas possibilidades de neuroproteção que podem ser acessíveis ao pacientes hiperprolinêmicos, como por exemplo, o exercício físico. A magnitude dos efeitos biológicos das vitaminas antioxidantes e especialmente da guanosina requerem estudos adicionais para uma melhor compreensão das suas ações neuroprotetoras na hiperprolinemia experimental e humana.

VI. PERSPECTIVAS

- Avaliar as atividades das enzimas da cadeia respiratória: complexo I, complexo II e SDH, bem como a função mitocondrial em cérebro e fígado de ratos submetidos à administração crônica de prolina.

- Avaliar se a hiperprolinemia induz morte neuronal, caracterizando o tipo de morte e os mecanismos envolvidos.

- Investigar mecanismos de sinalização mediados por cálcio como influxo celular e mitocondrial.

- Investigar possíveis mecanismos envolvidos nos efeitos da administração de guanosina e do exercício físico sobre as alterações bioquímicas e/ou comportamentais em ratos causados pela administração de prolina.

VII. REFERÊNCIAS BIBLIOGRÁFICAS

Adams E, Frank L (1980) Metabolism of proline and the hydroxyprolines. Annu Rev Biochem 49, 1005-61

Adlard PA, Perreau VM, Pop V, Cotman CW (2005) Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. J Neurosci 25, 4217-21

Alia, Mohanty P, Matysik J (2001) Effect of proline on the production of singlet oxygen. Amino Acids 21, 195-200

Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation and physiological functions. Glia 32, 1-14

Ando S, Kobayashi S, Waki H, Kon K, Fukui F, Tadenuma T, Iwamoto M, Takeda Y, Izumiyama N, Watanabe K, Nakamura H (2002) Animal model of dementia induced by entorhinal synaptic damage and partial restoration of cognitive deficits by BDNF and carnitine. J Neurosci Res 70, 519-27

Aperia A (2007) New roles for an old enzyme: Na^+,K^+-ATPase emerges as an interesting drug target. J Intern Med 261, 44-52

Attwell D (2000) Brain uptake of glutamate: food for thought. J Nutr 130, 1023-5

Ballard CG, Greig NH, Guillozet-Bongaarts AL, Enz A, Darvesh S (2005) Cholinesterases: roles in the brain during health and disease. Curr Alzheimer Res 2, 307-18

Bavaresco CS, Streck EL, Netto CA, Wyse AT (2005) Chronic hyperprolinemia provokes a memory deficit in the Morris water maze task. Metab Brain Dis 20, 73-80

Beal MF (2005) Mitochondria take center stage in aging and neurodegeneration. Ann Neurol 58, 495-505

Beal MF (2007) Mitochondria and neurodegeneration. Novartis Found Symp 287, 183-92; discussion 192-6

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH (2008) BDNF and memory formation and storage. Neuroscientist 14, 147-56

Ben J, Soares FM, Cechetti F, Vuaden FC, Bonan CD, Netto CA, Wyse AT (2009) Exercise effects on activities of Na$^+$,K$^+$-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302, 248-255

Ben J, Soares FM, Scherer EB, Cechetti F, Netto CA, Wyse AT (2010) Running exercise effects on spatial and avoidance tasks in ovariectomized rats. Neurobiol Learn Mem 94, 312-7

Benarroch EE (2011) Na$^+$,K$^+$-ATPase: functions in the nervous system and involvement in neurologic disease. Neurology 76, 287-93

Berchtold NC, Kesslak JP, Pike CJ, Adlard PA, Cotman CW (2001) Estrogen and exercise interact to regulate brain-derived neurotrophic factor mRNA and protein expression in the hippocampus. European Journal of Neuroscience 14, 1992-2002

Berchtold NC, Castello N, Cotman CW (2010) Exercise and time-dependent benefits to learning and memory. Neuroscience 167, 588-97

Blanc EM, Keller JN, Fernandez S, Mattson MP (1998) 4-hydroxynonenal, a lipid peroxidation product, impairs glutamate transport in cortical astrocytes. Glia 22, 149-60

Blanco G, Mercer RW (1998) Isozymes of the Na$^+$,K$^+$-ATPase: heterogeneity in structure, diversity in function. Am J Physiol 275, F633-50

Bode AM, Foster JD, Nordlie RC (1992) Glyconeogenesis from L-proline involves metabolite inhibition of the glucose-6-phosphatase system. J Biol Chem 267, 2860-3

Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE (2005) Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. Ann Neurol 57, 695-703

Buettner GR (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol and ascorbate. Arch Biochem Biophys 300, 535-43

Caceda R, Gamboa JL, Boero JA, Monge CC, Arregui A (2001) Energetic metabolism in mouse cerebral cortex during chronic hypoxia. Neurosci Lett 301, 171-4

Cadenas E, Davies KJ (2000) Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 29, 222-30

Cammarota M, Bevilaqua LR, Rossato JI, Ramirez M, Medina JH, Izquierdo I (2005) Relationship between short- and long-term memory and short- and long-term extinction. Neurobiol Learn Mem 84, 25-32

Carvalho AL, Caldeira MV, Santos SD, Duarte CB (2008) Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br J Pharmacol 153 Suppl 1, S310-24

Cechetti F, Rhod A, Simão F, Santin K, Salbego C, Netto CA, Siqueira IR (2007) Effect of treadmill exercise on cell damage in rat hippocampal slices submitted to oxygen and glucose deprivation. Brain Res 1157, 121-5

Cechetti F, Fochesatto C, Scopel D, Nardin P, Goncalves CA, Netto CA, Siqueira IR (2008) Effect of a neuroprotective exercise protocol on oxidative state and BDNF levels in the rat hippocampus. Brain Res 1188, 182-8

Chacon MA, Reyes AE, Inestrosa NC (2003) Acetylcholinesterase induces neuronal cell loss, astrocyte hypertrophy and behavioral deficits in mammalian hippocampus. J Neurochem 87, 195-204

Chang R, Algird A, Bau C, Rathbone MP, Jiang S (2008) Neuroprotective effects of guanosine on stroke models in vitro and in vivo. Neurosci Lett 431, 101-5

Chen C, Dickman MB (2005) Proline suppresses apoptosis in the fungal pathogen Colletotrichum trifolii. Proc Natl Acad Sci U S A 102, 3459-64

Chen C, Wanduragala S, Becker DF, Dickman MB (2006) Tomato QM-like protein protects Saccharomyces cerevisiae cells against oxidative stress by regulating intracellular proline levels. Appl Environ Microbiol 72, 4001-6

Chen J, Petersen DR, Schenker S, Henderson GI (2000) Formation of malondialdehyde adducts in livers of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase. Alcohol Clin Exp Res 24, 544-52

Chen J, Henderson GI, Freeman GL (2001) Role of 4-hydroxynonenal in modification of cytochrome c oxidase in ischemia/reperfused rat heart. J Mol Cell Cardiol 33, 1919-27

Chen Y, Swanson RA (2003) The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. J Neurochem 84, 1332-9

Christie BR, Eadie BD, Kannangara TS, Robillard JM, Shin J, Titterness AK (2008) Exercising our brains: how physical activity impacts synaptic plasticity in the dentate gyrus. Neuromolecular Med 10, 47-58

Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. Trends Neurosci 25, 295-301

D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. Brain Res Rev 36, 60-90

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65, 1-105

de Vasconcellos AP, Zugno AI, dos Santos AH, Nietto FB, Crema LM, Goncalves M, Franzon R, Wyse ATS, da Rocha ER, Dalmaz C (2005) Na$^+$,K$^+$-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: effect of chronic lithium treatment and possible involvement in learning deficits. Neurobiol Learn Mem 84, 102-10

Decker K (1990) Biologically active products of stimulated liver macrophages (Kupffer cells). Eur J Biochem 192, 245-61

Deiana S, Platt B, Riedel G (2011) The cholinergic system and spatial learning. Behav Brain Res 221, 389-411

Delwing D, Bavaresco CS, Chiarani F, Wannmacher CM, Wajner M, Dutra-Filho CS, de Souza Wyse AT (2003a) In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. Brain Res 991, 180-6

Delwing D, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT (2003b) Proline induces oxidative stress in cerebral cortex of rats. Int J Dev Neurosci 21, 105-10

Delwing D, Chiarani F, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT (2005a) Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci Res 52, 69-74

Delwing D, Chiarani F, Wannmacher CM, Wajner M, Wyse AT (2005b) Effect of hyperprolinemia on acetylcholinesterase and butyrylcholinesterase activities in rat. Amino Acids 28, 305-8

Delwing D, Bavaresco CS, Monteiro SC, Matte C, Netto CA, Wyse AT (2006) Alpha-tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res 168, 185-9

Delwing D, Chiarani F, Kurek AG, Wyse AT (2007a) Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci 25, 17-22

Delwing D, Sanna RJ, Wofchuk S, Wyse AT (2007b) Proline promotes decrease in glutamate uptake in slices of cerebral cortex and hippocampus of rats. Life Sci 81, 1645-50

Dempski RE, Friedrich T, Bamberg E (2009) Voltage clamp fluorometry: combining fluorescence and electrophysiological methods to examine the structure-function of the Na^+,K^+-ATPase. Biochim Biophys Acta 1787, 714-20

Dencher NA, Frenzel M, Reifschneider NH, Sugawa M, Krause F (2007) Proteome alterations in rat mitochondria caused by aging. Ann N Y Acad Sci 1100, 291-8

Di Iorio P, Ballerini P, Traversa U, Nicoletti F, D'Alimonte I, Kleywegt S, Werstiuk ES, Rathbone MP, Caciagli F, Ciccarelli R (2004) The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. Glia 46, 356-68

Di Rosa G, Pustorino G, Spano M, Campion D, Calabro M, Aguennouz M, Caccamo D, Legallic S, Sgro DL, Bonsignore M, Tortorella G (2008) Type I hyperprolinemia and proline dehydrogenase (PRODH) mutations in four Italian children with epilepsy and mental retardation. Psychiatr Genet 18, 40-2

Egan MF, Weinberger DR, Lu B (2003) Schizophrenia, III: brain-derived neurotropic factor and genetic risk. Am J Psychiatry 160, 1242

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

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

Ernst C, Olson AK, Pinel JP, Lam RW, Christie BR (2006) Antidepressant effects of exercise: evidence for an adult-neurogenesis hypothesis? J Psychiatry Neurosci 31, 84-92

Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi EA (2001) Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch Biochem Biophys 388, 261-6

Farmer J, Zhao X, van Praag H, Wodtke K, Gage FH, Christie BR (2004) Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. Neuroscience 124, 71-79

Ferreira AG, Lima DD, Delwing D, Mackedanz V, Tagliari B, Kolling J, Schuck PF, Wajner M, Wyse AT (2010) Proline impairs energy metabolism in cerebral cortex of young rats. Metab Brain Dis 25, 161-8

Ferreira AG, Stefanello FM, Cunha AA, da Cunha MJ, Pereira TC, Bonan CD, Bogo MR, Netto CA, Wyse AT (2011) Role of antioxidants on Na$^+$,K$^+$ -ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats. Metab Brain Dis 26, 141-7

Floyd RA, Nagy I (1984) Formation of long-lived hydroxyl free radical adducts of proline and hydroxyproline in a Fenton reaction. Biochim Biophys Acta 790, 94-7

Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM (1989) Type II hyperprolinaemia in a pedigree of Irish travellers (nomads). Arch Dis Child 64, 1699-707

Flynn NE, Meininger CJ, Haynes TE, Wu G (2002) The metabolic basis of arginine nutrition and pharmacotherapy. Biomed Pharmacother 56, 427-38

Gale GD, Anagnostaras SG, Fanselow MS (2001) Cholinergic modulation of pavlovian fear conditioning: effects of intrahippocampal scopolamine infusion. Hippocampus 11, 371-6

Gamaro GD, Streck EL, Matte C, Prediger ME, Wyse ATS, Dalmaz C (2003) Reduction of hippocampal Na^+,K^+-ATPase activity in rats subjected to an experimental model of depression. Neurochem Res 28, 1339-44

Geering K (2001) The functional role of beta subunits in oligomeric P-type ATPases. J Bioenerg Biomembr 33, 425-38

Genet S, Kale RK, Baquer NZ (2002) Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (Trigonellafoenum graecum). Mol Cell Biochem 236, 7-12

Gibson GE, Starkov A, Blass JP, Ratan RR, Beal MF (2009) Cause and consequence: mitochondrial dysfunction initiates and propagates neuronal dysfunction, neuronal death and behavioral abnormalities in age-associated neurodegenerative diseases. Biochim Biophys Acta 1802, 122-34

Giovannini MG, Rakovska A, Benton RS, Pazzagli M, Bianchi L, Pepeu G (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. Neuroscience 106, 43-53

Giugliani R, Coelho J (1997) Diagnóstico de erros inatos do metabolismo da América Latina. J. C. Braz J Gen 20, p. 147-54

Gottmann K, Mittmann T, Lessmann V (2009) BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. Exp Brain Res 199, 203-34

Greenwood SM, Connolly CN (2007) Dendritic and mitochondrial changes during glutamate excitotoxicity. Neuropharmacology 53, 891-8

Greenwood SM, Mizielinska SM, Frenguelli BG, Harvey J, Connolly CN (2007) Mitochondrial dysfunction and dendritic beading during neuronal toxicity. J Biol Chem 282, 26235-44

Griesbach GS, Gomez-Pinilla F, Hovda DA (2007) Time window for voluntary exercise-induced increases in hippocampal neuroplasticity molecules after traumatic brain injury is severity dependent. J Neurotrauma 24, 1161-71

Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H (1999) Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 264, 672-86

Guilmatre A, Legallic S, Steel G, Willis A, Di Rosa G, Goldenberg A, Drouin-Garraud V, Guet A, Mignot C, Des Portes V, Valayannopoulos V, Van Maldergem L, Hoffman JD, Izzi C, Espil-Taris C, Orcesi S, Bonafe L, Le Galloudec E, Maurey H, Ioos C, Afenjar A, Blanchet P, Echenne B, Roubertie A, Frebourg T, Valle D, Campion D (2010) Type I hyperprolinemia: genotype/phenotype correlations. Hum Mutat 31, 961-5

Gupta RC, Milatovic D, Dettbarn WD (2001) Depletion of energy metabolites following acetylcholinesterase inhibitor-induced status epilepticus: protection by antioxidants. Neurotoxicology 22, 271-82

Ha HL, Shin HJ, Feitelson MA, Yu DY (2010) Oxidative stress and antioxidants in hepatic pathogenesis. World J Gastroenterol 16, 6035-43

Hagedorn CH, Phang JM (1983) Transfer of reducing equivalents into mitochondria by the interconversions of proline and delta 1-pyrroline-5-carboxylate. Arch Biochem Biophys 225, 95-101

Halliwell B, Gutteridge JM, Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 119, 598-620

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) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97, 1634-58

Halliwell B, Gutteridge JMC, 2007. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. Oxford University Press, New York.

Hattori N, Kitagawa K, Higashida T, Yagyu K, Shimohama S, Wataya T, Perry G, Smith MA, Inagaki C (1998) Cl-ATPase and Na^+,K^+-ATPase activities in Alzheimer's disease brains. Neurosci Lett 254, 141-4

Herrera-Morales W, Mar I, Serrano B, Bermudez-Rattoni F (2007) Activation of hippocampal postsynaptic muscarinic receptors is involved in long-term spatial memory formation. Eur J Neurosci 25, 1581-8

Horsfield SA, Rosse RB, Tomasino V, Schwartz BL, Mastropaolo J, Deutsch SI (2002) Fluoxetine's effects on cognitive performance in patients with traumatic brain injury. Int J Psychiatry Med 32, 337-44

Hu CA, Phang JM, Valle D (2008) Proline metabolism in health and disease. Preface. Amino Acids 35, 651-2

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72, 609-42

Izquierdo I, Medina JH (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol Learn Mem 68, 285-316

Izquierdo I, 2002. Memória. Artmed Editora SA, Porto Alegre.

Jorgensen PL, Hakansson KO, Karlish SJ (2003) Structure and mechanism of Na$^+$,K$^+$-ATPase: functional sites and their interactions. Annu Rev Physiol 65, 817-49

Kanner BI (2006) Structure and function of sodium-coupled GABA and glutamate transporters. J Membr Biol 213, 89-100

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10, 381-91

Kaplan JH (2002) Biochemistry of Na$^+$,K$^+$-ATPase. Annu Rev Biochem 71, 511-35

Kasper DC, Ratschmann R, Metz TF, Mechtler TP, Moslinger D, Konstantopoulou V, Item CB, Pollak A, Herkner KR (2010) The national Austrian newborn screening program - eight years experience with mass spectrometry. past, present, and future goals. Wien Klin Wochenschr 122, 607-13

Kawashima K, Fujii T (2000) Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 86, 29-48

Kessels RP, de Haan EH, Kappelle LJ, Postma A (2001) Varieties of human spatial memory: a meta-analysis on the effects of hippocampal lesions. Brain Res Brain Res Rev 35, 295-303

Kessler A, Costabeber E, Dutra-Filho CS, Wyse AT, Wajner M, Wannmacher CM (2003) Proline reduces creatine kinase activity in the brain cortex of rats. Neurochem Res 28, 1175-80

Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE (2009) Mitochondria and reactive oxygen species. Free Radic Biol Med 47, 333-43

Kramer AF, Colcombe SJ, McAuley E, Scalf PE, Erickson KI (2005) Fitness, aging and neurocognitive function. Neurobiol Aging 26 Suppl 1, 124-7

Krishnan N, Dickman MB, Becker DF (2008) Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress. Free Radic Biol Med 44, 671-81

Lane RM, Potkin SG, Enz A (2006) Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol 9, 101-24

Lara DR, Schmidt AP, Frizzo ME, Burgos JS, Ramirez G, Souza DO (2001) Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain Res 912, 176-80

Layer PG, Weikert T, Alber R (1993) Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res 273, 219-26

Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9, 2581-7

Liu HL, Zhao G, Cai K, Zhao HH, Shi LD (2011) Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. Behav Brain Res 218, 308-14

Liu Y, Borchert GL, Donald SP, Surazynski A, Hu CA, Weydert CJ, Oberley LW, Phang JM (2005) MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells. Carcinogenesis 26, 1335-42

Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. Nat Rev Neurosci 6, 603-14

Lu KP, Liou YC, Vincent I (2003) Proline-directed phosphorylation and isomerization in mitotic regulation and in Alzheimer's Disease. Bioessays 25, 174-81

Mancuso M, Orsucci D, LoGerfo A, Calsolaro V, Siciliano G (2010) Clinical features and pathogenesis of Alzheimer's disease: involvement of mitochondria and mitochondrial DNA. Adv Exp Med Biol 685, 34-44

Mantena SK, King AL, Andringa KK, Eccleston HB, Bailey SM (2008) Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases. Free Radic Biol Med 44, 1259-72

Maragakis NJ, Rothstein JD (2001) Glutamate transporters in neurologic disease. Arch Neurol 58, 365-70

Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 15, 461-73

Matte C, Monteiro SC, Calcagnotto T, Bavaresco CS, Netto CA, Wyse AT (2004) In vivo and in vitro effects of homocysteine on Na^+,K^+-ATPase activity in parietal, prefrontal and cingulate cortex of young rats. Int J Dev Neurosci 22, 185-90

Matte C, Stefanello FM, Mackedanz V, Pederzolli CD, Lamers ML, Dutra-Filho CS, Dos Santos MF, Wyse AT (2009) Homocysteine induces oxidative stress, inflammatory infiltration, fibrosis and reduces glycogen/glycoprotein content in liver of rats. Int J Dev Neurosci 27, 337-44

Mecocci P (2004) Oxidative stress in mild cognitive impairment and Alzheimer disease: a continuum. J Alzheimers Dis 6, 159-63

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130, 1007S-15S

Milatovic D, Zivin M, Gupta RC, Dettbarn WD (2001) Alterations in cytochrome c oxidase activity and energy metabolites in response to kainic acid-induced status epilepticus. Brain Res 912, 67-78

Mitsubuchi H, Nakamura K, Matsumoto S, Endo F (2008) Inborn errors of proline metabolism. J Nutr 138, 2016-2020

Mizuno M, Yamada K, Olariu A, Nawa H, Nabeshima T (2000) Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. J Neurosci 20, 7116-21

Mobasheri A, Avila J, Cozar-Castellano I, Brownleader MD, Trevan M, Francis MJ, Lamb JF, Martin-Vasallo P (2000) Na^+,K^+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. Biosci Rep 20, 51-91

Moreira JC, Wannmacher CM, Costa SM, Wajner M (1989) Effect of proline administration on rat behavior in aversive and nonaversive tasks. Pharmacol Biochem Behav 32, 885-90

Moretto MB, Boff B, Lavinsky D, Netto CA, Rocha JB, Souza DO, Wofchuk ST (2009) Importance of schedule of administration in the therapeutic efficacy of guanosine: early intervention after injury enhances glutamate uptake in model of hypoxia-ischemia. J Mol Neurosci 38, 216-9

Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297, 681-3

Moscovitch M, Nadel L, Winocur G, Gilboa A, Rosenbaum RS (2006) The cognitive neuroscience of remote episodic, semantic and spatial memory. Curr Opin Neurobiol 16, 179-90

Navarro A, Gomez C, Sanchez-Pino MJ, Gonzalez H, Bandez MJ, Boveris AD, Boveris A (2005) Vitamin E at high doses improves survival, neurological performance, and brain mitochondrial function in aging male mice. Am J Physiol Regul Integr Comp Physiol 289, R1392-9

Nelson DL, Cox MM, 2011. Princípios de bioquímica de Lehninger Artmed Editora SA, Porto Alegre.

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

Nicholls DG (2008) Oxidative stress and energy crises in neuronal dysfunction. Ann N Y Acad Sci 1147, 53-60

Pajonk FG, Wobrock T, Gruber O, Scherk H, Berner D, Kaizl I, Kierer A, Muller S, Oest M, Meyer T, Backens M, Schneider-Axmann T, Thornton AE, Honer WG, Falkai P (2010) Hippocampal plasticity in response to exercise in schizophrenia. Arch Gen Psychiatry 67, 133-43

Pang PT, Lu B (2004) Regulation of late-phase UP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res Rev 3, 407-430

Parker WDJ, Parks J, Filley CM, Kleinschmidt-DeMasters BK (1994) Electron transport chain defects in Alzheimer's disease brain. Neurology 44, 1090-6

Pedersen WA, Cashman NR, Mattson MP (1999) The lipid peroxidation product 4-hydroxynonenal impairs glutamate and glucose transport and choline acetyltransferase activity in NSC-19 motor neuron cells. Exp Neurol 155, 1-10

Pereira AC, Huddleston DE, Brickman AM, Sosunov AA, Hen R, McKhann GM, Sloan R, Gage FH, Brown TR, Small SA (2007) An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A 104, 5638-43

Petrozzi L, Ricci G, Giglioli NJ, Siciliano G, Mancuso M (2007) Mitochondria and neurodegeneration. Biosci Rep 27, 87-104

Phang JM (1985) The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul 25, 91-132

Phang JM, Hu CA, Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (ed) The Metabolic and Molecular Bases of Inherited Disease. 3. McGraw-Hill, New York

Phang JM, Liu W, Zabirnyk O (2010) Proline metabolism and microenvironmental stress. Annu Rev Nutr 30, 441-63

Pontes ZE, Oliveira LS, Bavaresco CS, Streck EL, Dutra-Filho CS, Wajner M, Wannmacher CM, Wyse AT (1999) Proline administration decreases Na^+,K^+-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab Brain Dis 14, 265-72

Pontes ZL, Oliveira LS, Franzon R, Wajner M, Wannmacher CM, Wyse AT (2001) Inhibition of Na^+,K^+-ATPase activity from rat hippocampus by proline. Neurochem Res 26, 1321-6

Potts MB, Koh SE, Whetstone WD, Walker BA, Yoneyama T, Claus CP, Manvelyan HM, Noble-Haeusslein LJ (2006) Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. NeuroRx 3, 143-53

Powers SK, Jackson MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88, 1243-76

Quillfeldt JA (2010) Behavioral Methods to Study Learning and Memory in Rats. In: Andersen ML, Tufik S (ed) Animal Models as Tools in Ethical Biomedical Research. Unifesp, São Paulo

Radak Z, Toldy A, Szabo Z, Siamilis S, Nyakas C, Silye G, Jakus J, Goto S (2006) The effects of training and detraining on memory, neurotrophins and oxidative stress markers in rat brain. Neurochem Int 49, 387-392

Radak Z, Chung HY, Goto S (2008) Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med 44, 153-9

Radak Z, Hart N, Sarga L, Koltai E, Atalay M, Ohno H, Boldogh I (2010) Exercise plays a preventive role against Alzheimer's disease. J Alzheimers Dis 20, 777-83

Ragozzino ME, Unick KE, Gold PE (1996) Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. Proc Natl Acad Sci USA 93, 4693-8

Rathbone M, Pilutti L, Caciagli F, Jiang S (2008) Neurotrophic effects of extracellular guanosine. Nucleosides Nucleotides Nucleic Acids 27, 666-72

Rice ME (2000) Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci 23, 209-16

Richards KS, Bommert K, Szabo G, Miles R (2007) Differential expression of Na^+,K^+-ATPase alpha-subunits in mouse hippocampal interneurones and pyramidal cells. J Physiol 585, 491-505

Ristow M, Schmeisser S (2011) Extending life span by increasing oxidative stress. Free Radic Biol Med 51, 327-36

Roos DH, Puntel RL, Santos MM, Souza DO, Farina M, Nogueira CW, Aschner M, Burger ME, Barbosa NB, Rocha JB (2009) Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative

stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system. Toxicol In Vitro 23, 302-7

Rose EM, Koo JC, Antflick JE, Ahmed SM, Angers S, Hampson DR (2009) Glutamate transporter coupling to Na$^+$,K$^+$-ATPase. J Neurosci 29, 8143-55

Rustgi S, Joshi A, Moss H, Riesz P (1977) E.s.r. of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydroxyl radical. Int J Radiat Biol Relat Stud Phys Chem Med 31, 415-40

Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. N Engl J Med 336, 1216-22

Sato T, Tanaka K, Ohnishi Y, Teramoto T, Irifune M, Nishikawa T (2004) Effects of steroid hormones on Na$^+$,K$^+$-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. Pharmacol Res 49, 151-9

Schmidt AP, Lara DR, de Faria Maraschin J, da Silveira Perla A, Onofre Souza D (2000) Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res 864, 40-3

Schmidt AP, Tort AB, Silveira PP, Bohmer AE, Hansel G, Knorr L, Schallenberger C, Dalmaz C, Elisabetsky E, Crestana RH, Lara DR, Souza DO (2009) The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: reversal by guanosine. Pharmacol Biochem Behav 91, 549-53

Schurr A (2002) Energy metabolism, stress hormones and neural recovery from cerebral ischemia/hypoxia. Neurochem Int 41, 1-8

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

Segovia G, Porras A, Del Arco A, Mora F (2001) Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev 122, 1-29

Serluca FC, Sidow A, Mably JD, Fishman MC (2001) Partitioning of tissue expression accompanies multiple duplications of the Na^+,K^+-ATPase alpha subunit gene. Genome Res 11, 1625-31

Shaw S, Worner TM, Lieber CS (1984) Frequency of hyperprolinemia in alcoholic liver cirrhosis: relationship to blood lactate. Hepatology 4, 295-9

Siegel GJ, Albers RW, Brady ST, Price DL, 2006. Basic neurochemistry. Academic Press, California, USA.

Siems WG, Hapner SJ, van Kuijk FJ (1996) 4-hydroxynonenal inhibits Na^+,K^+-ATPase. Free Radic Biol Med 20, 215-23

Silman I, Sussman JL (2005) Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Curr Opin Pharmacol 5, 293-302

Sim YJ, Kim SS, Kim JY, Shin MS, Kim CJ (2004) Treadmill exercise improves short-term memory by suppressing ischemia-induced apoptosis of neuronal cells in gerbils. Neurosci Lett 372, 256-61

Siripornadulsil S, Traina S, Verma DP, Sayre RT (2002) Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 14, 2837-47

Sofuoglu M, Mooney M (2009) Cholinergic functioning in stimulant addiction: implications for medications development. CNS Drugs 23, 939-52

Soreq H, Seidman S (2001) Acetylcholinesterase: new roles for an old actor. Nat Rev Neurosci 2, 294-302

Squire LR, Kandel ER, 2003. Memória: da mente às moléculas. Artmed, Porto Alegre.

Stefanello FM, Matte C, Pederzolli CD, Kolling J, Mescka CP, Lamers ML, de Assis AM, Perry ML, dos Santos MF, Dutra-Filho CS, Wyse AT (2009) Hypermethioninemia provokes oxidative damage and histological changes in liver of rats. Biochimie 91, 961-8

Stranahan AM, Lee K, Martin B, Maudsley S, Golden E, Cutler RG, Mattson MP (2009) Voluntary exercise and caloric restriction enhance hippocampal dendritic spine density and BDNF levels in diabetic mice. Hippocampus 19, 951-61

Streck EL, Zugno AI, Tagliari B, Sarkis JJ, Wajner M, Wannmacher CM, Wyse AT (2002) On the mechanism of the inhibition of Na^+,K^+-ATPase activity caused by homocysteine. Int J Dev Neurosci 20, 77-81

Takemura NU (2005) Evidence for neurogenesis within the white matter beneath the temporal neocortex of the adult rat brain. Neuroscience 134, 121-32

Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol 279, C541-66

Traber MG, Stevens JF (2011) Vitamins C and E: Beneficial effects from a mechanistic perspective. Free Radic Biol Med

Tsai SJ, Hong CJ, Liu HC, Liu TY, Hsu LE, Lin CH (2004) Association analysis of brain-derived neurotrophic factor Val66Met polymorphisms with Alzheimer's disease and age of onset. Neuropsychobiology 49, 10-2

Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD (2002) From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn Mem 9, 224-37

Um HS, Kang EB, Leem YH, Cho IH, Yang CH, Chae KR, Hwang DY, Cho JY (2008) Exercise training acts as a therapeutic strategy for reduction of the pathogenic phenotypes for Alzheimer's disease in an NSE/APPsw-transgenic model. Int J Mol Med 22, 529-39

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999) Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96, 13427-13431

van Praag H, Shubert T, Zhao CM, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25, 8680-8685

Vargas-Tank L, Wolff C, Castillo T, Carrion Y, Hoppe A, Martinez V, Arman-Merino R (1988) Serum proline and blood lactate levels in alcoholic patients without hepatic failure: relationship with alcohol ingestion and histological activity. Liver 8, 58-62

Vatassery GT (1998) Vitamin E and other endogenous antioxidants in the central nervous system. Geriatrics 53 Suppl 1, S25-7

Vaynman S, Ying Z, Gomez-Pinilla F (2003) Interplay between brain-derived neurotrophic factor and signal transduction modulators in the regulation of the effects of exercise on synaptic-plasticity. Neuroscience 122, 647-57

Vaynman S, Ying Z, Gomez-Pinilla F (2004a) Exercise induces BDNF and synapsin I to specific hippocampal subfields. J Neurosci Res 76, 356-62

Vaynman S, Ying Z, Gomez-Pinilla F (2004b) Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. Eur J Neurosci 20, 2580-90

von Bohlen Und Halbach O (2011) Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. Cell Tissue Res 345, 1-19

Wallenstein GV, Vago DR (2001) Intrahippocampal scopolamine impairs both acquisition and consolidation of contextual fear conditioning. Neurobiol Learn Mem 75, 245-52

Weinberg JM, Venkatachalam MA, Roeser NF, Nissim I (2000) Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. Proc Natl Acad Sci U S A 97, 2826-31

White TA, Krishnan N, Becker DF, Tanner JJ (2007) Structure and kinetics of monofunctional proline dehydrogenase from Thermus thermophilus. J Biol Chem 282, 14316-27

Wyse AT, Bavaresco CS, Bandinelli C, Streck EL, Franzon R, Dutra-Filho CS, Wajner M (2001a) Nitric oxide synthase inhibition by L-NAME prevents the decrease of Na^+,K^+-ATPase activity in midbrain of rats subjected to arginine administration. Neurochem Res 26, 515-20

Wyse AT, Bavaresco CS, Hagen ME, Delwing D, Wannmacher CM, Severo Dutra-Filho C, Wajner M (2001b) In vitro stimulation of oxidative stress in cerebral cortex of rats by the guanidino compounds accumulating in hyperargininemia. Brain Res 923, 50-7

Wyse AT, Netto CA (2011) Behavioral and neurochemical effects of proline. Metab Brain Dis, IN PRESS

Wyse ATS, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem Res 25, 971-5

Wyse ATS, Bavaresco CS, Reis EA, Zugno AI, Tagliari B, Calcagnotto T, Netto CA (2004) Training in inhibitory avoidance causes a reduction of Na^+,K^+-ATPase activity in rat hippocampus. Physiol Behav 80, 475-479

Yamada K, Mizuno M, Nabeshima T (2002) Role for brain-derived neurotrophic factor in learning and memory. Life Sci 70, 735-44

Zhang F, Wu Y, Jia J (2011) Exercise preconditioning and brain ischemic tolerance. Neuroscience 177, 170-6

Zimmerman G, Soreq H (2006) Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res 326, 655-69