UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

LUCIENE PINHEIRO VIANNA

Efeitos da administração crônica de prolina no conteúdo lipídico de estruturas cerebrais de rato

Porto Alegre, novembro de 2007.

Luciene Pinheiro Vianna

Efeitos da administração crônica de prolina no conteúdo lipídico de estruturas cerebrais de rato

Orientadora: Profª. Dra. Vera Maria Treis Trindade

Co-orientadora: Profª. Dra. Ângela T.S. Wyse

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas-Bioquímica como requisito parcial para obtenção do título de Mestre.

Porto Alegre, novembro de 2007

Agradecimentos

Ao Programa de Pós-Graduação em Ciências Biológicas-Bioquímica.
Ao Departamento de Bioquímica Prof. Tuiskon Dick.
À Pró-Reitora de Pesquisa.
Aos colegas dos laboratórios 21, 25 e 34.

Resumo

Neste trabalho foi investigado o efeito da administração crônica de prolina sobre o conteúdo total de gangliosídios, fosfolipídios e de colesterol, assim como, sobre o perfil de gangliosídios no córtex, no hipocampo, no hipotálamo e no cerebelo de ratos. Também, foi avaliado o conteúdo e o perfil de gangliosídios nas frações solúvel e resistente a detergente obtidas de membranas sinápticas de córtex. Ratos Wistar foram divididos em dois grupos: 1) injetados subcutaneamente com solução 0,9% de NaCl (animais controle) e 2) injetados subcutaneamente com solução de prolina, em concentrações adequadas ao peso corporal (animais hiperprolinêmicos). Tanto a solução de prolina quanto a salina foram administradas do 6° ao 28° dia pós-natal. Doze horas após a última administração, os animais foram sacrificados mediante decapitação sem anestesia. As estruturas cerebrais foram dissecadas e em seguida homogeneizadas em clorofórmio:metanol na proporção 1:1 vpara a extração lipídica. As membranas sinápticas foram obtidas através de centrifugação diferencial e as frações solúvel e resistente a detergente foram isoladas através de tratamento das membranas com Triton X-100 a 4°C para investigação de microdomínios de membrana. Após a realização das análises, os resultados mostraram que os animais submetidos ao tratamento crônico com prolina apresentaram um marcado aumento no conteúdo de gangliosídios no córtex cerebral e no hipocampo, enquanto os conteúdos de fosfolipídios e de colesterol aumentaram somente no hipocampo. Além disso, os conteúdos destes compostos não foram alterados no hipotálamo e no cerebelo de animais hiperprolinêmicos. Por outro lado, o conteúdo de gangliosídios diminuiu nas frações solúvel e resistente a detergente obtidas de membranas sinápticas de córtex de animais hiperprolinêmicos. Embora os perfis de gangliosídios não tenham sido aparentemente modificados, as quantidades absolutas das espécies foram alteradas tanto no extrato total, como nos microdomínios de membrana obtidos do córtex. Estes dados revelam que o tratamento crônico com prolina afeta de forma distinta as diferentes regiões cerebrais quanto à composição lipídica das membranas celulares, refletindo-se sobre a distribuição de lipídios nos microdomínios de membrana do córtex. Entre as conseqüências destes fenômenos poderiam ser sugeridas modulações diferentes nas transmissões sinápticas que contribuiriam para o déficit cognitivo e/ou outras disfunções neurológicas presentes em pacientes com hiperprolinemia tipo II.

Palavras-chave: gangliosídios, fosfolipídios, colesterol, hiperprolinemia tipo II, cérebro, membrana sináptica, microdomínios de membrana enriquecidos em glicoesfingolipídios.

Abstract

In the present work we investigated the effects of chronic proline administration on ganglioside, cholesterol and phospholipid total contents, as well as on ganglioside profile in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats. We also evaluated the ganglioside content and profile in detergent- soluble and resistant fractions isolated from synaptic membranes obtained from cerebral cortex. Wistar rats were divided into two groups: 1) saline (control) and 2) proline injected (hyperprolinemic). Proline solution or saline were administered from 6th to 28th postnatal day, according to body weight. Twelve hours after the last injection, the animals were sacrificed by decapitation without anesthesia. Brain structures were homogenized with chlorophorm:methanol 1:1 for lipid extraction. Synaptic membrane was extracted by differential centrifugation and detergent- soluble and resistant fractions were isolated by cold Triton X-100 treatment. Results showed that rats subjected to chronic proline treatment presented a significant increase of ganglioside content on cortex and hippocampus, while phospholipid and cholesterol contents only increased in hippocampus. However, the content of these components were not altered in hypothalamus and cerebellum of hyperprolinemic rats. On the other hand, ganglioside content decreased in detergent- soluble and resistant fractions isolated from synaptic membrane obtained from hyperprolinemic cortex. Although ganglioside profiles were apparently not modified, the individual absolute quantities were altered in cortex total lipid extract and membrane microdomains obtained from cerebral cortex. Our findings suggest that chronic proline treatment affects, in a distinct manner, different cerebral regions concerning the lipid composition of the cell membranes, reflecting on its distribution in the cortex membrane microdomains. Among these phenomena consequences, different modulations in synaptic transmission may be suggested which may contribute to the impairment in cognition and/or other neurological disfunctions found in hyperprolinemia type II patients.

Keywords: ganglioside, phospholipid, cholesterol, hyperprolinemia, brain, synaptic membrane, membrane rafts.

Lista de abreviaturas

AA - amino acids (aminoácidos)
AMPA - amino-5-methyl propionic acid (ácido alfa-5-metil propiônico)
DRM - detergent-resistent membrane microdomains (microdomínio de membrana resistentes a detergente)
EIM - erros inatos do metabolismo
GABA - γ-aminobutyric acid (ácido gama-aminobutírico)
GEM - glycosphingolipid-enriched membrane microdomains (microdomínios de membrana enriquecidos em glicoesfingolipídios)
HEPES 2-hydroxyethyl piperazine ethanesulfonic acid (ácido etanosulfônico-2-hidroxietilpiperazina)
HPTLC - high performance thin layer chromatography (cromatografia em camada delgada de alta performance)
KA - ketoacid (ceto ácido)
NANA - N-acetyl neuraminic acid (ácido N-acetil neuramínico)
NMDA - N-methyl-D-Aspartic acid (ácido N-metil aspártico)
P5C - Δ^1-pirrolino-5-carboxilato
Pi - fosfato inorgânico
Pro - prolina
SNC - Sistema Nervoso Central
TLC - thin layer chromatography (cromatografia em camada delgada)

Lista de figuras

Lista de tabelas

Sumário

1. INTRODUÇÃO

1.1. Sistema Nervoso Central

O sistema nervoso central (SNC) é responsável pela aquisição de informação ambiental através de receptores sensoriais e transmissão para o cérebro e/ou medula espinhal. Uma vez no cérebro, essa informação é processada e integrada, e uma resposta adequada é iniciada (HAINES, 1997).

O sistema nervoso pode ser visualizado como uma escala de complexidade estrutural. Microscopicamente, a estrutura fundamental funcional é o neurônio, ou célula nervosa. Dispersas entre os neurônios do SNC estão elementos de suporte chamados de células gliais. Na extremidade macroscópica da escala estão as divisões maiores (ou partes) do SNC que são estudadas com o adequado detalhamento anatômico (HAINES, 1997).

O SNC é dividido de acordo com suas funções. Nessa classificação, destacam-se: o córtex, o hipocampo, o hipotálamo e o cerebelo.

O córtex cerebral, responsável por diversas funções nobres, entre elas o processamento da informação sensorial, organização da atividade motora, funções intelectuais superiores - memória, julgamento, planejamento de atividades complexas, processamento da linguagem, cálculo matemático e construção de imagens mentais do ambiente do indivíduo (LYNCH, 1997).

O hipocampo, que processa informações e as converte em memória, sendo, portanto, responsável por funções como aprendizado, interações sociais e consolidação de memórias de longo prazo a partir de memórias imediatas e de

curto prazo, que persistem por segundos e minutos, respectivamente (CHRONISTER e HARDY, 1997).

O hipotálamo, regulador do metabolismo e da temperatura corporal, através do controle central das funções viscerais, pelos sistemas endócrino e visceromotor; também está relacionado ao comportamento afetivo-emocional, pelo sistema límbico; direcionado principalmente à manutenção da homeostase, pela regulação do balanço eletrolítico e de água, ingesta de alimento, temperatura, pressão sangüínea, mecanismo de sono-vigília, ritmo circadiano e metabolismo corporal em geral (MIHAILOFF e HAINES, 1997).

O cerebelo, responsável por funções como aprendizado motor (sem influenciar a função cognitiva), postura e equilíbrio (HAINES, MIHAILOFF e BLOEDEL, 1997).

1.2. Membranas Celulares

A membrana plasmática é atualmente estudada a partir do modelo de bicamada lipídica, com variações nas propriedades físico-químicas de seus componentes de acordo com as necessidades da célula. Estudos demonstram que a membrana das células está estruturada com as várias proteínas presentes sendo solvatadas por camadas de lipídios de diferentes composições (JACOBSON, MOURITSEN e ANDERSON, 2007).

1.2.1. Lipídios de membrana

Embora os lipídios sejam compostos comuns a todas as membranas plasmáticas, diferentes tipos celulares apresentam proporções distintas destes

componentes. Os lipídios de membrana do SNC são representados principalmente por gangliosídios, fosfolipídios e colesterol (AGRANOFF e HAJRA, 1993).

1.2.1.1. Gangliosídios

Os gangliosídios são glicoesfingolipídios contendo ácido siálico na molécula, o que lhes confere certa acidez e, portanto, polaridade em comparação com outros lipídios (Figura 1.1) Estão relacionados com sinalização e interações entre células, crescimento e diferenciação celular e podem estar envolvidos no desenvolvimento neural (sinaptogênese e mielinização), uma vez que as membranas neurais são ricas em gangliosídios (ANDO, 1983; ZELLER e MARCHASE, 1992; NAGAI, 1995).

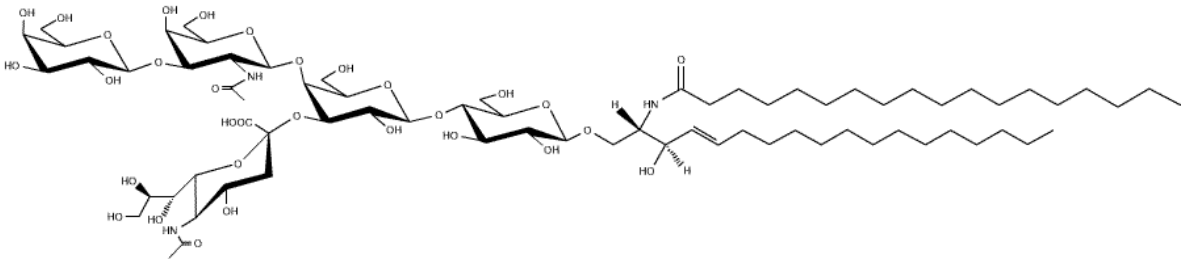


Figura 1.1. Estrutura do gangliosídio GM_1. O gangliosídio mais simples; contém apenas um resíduo de ácido siálico. (Adaptado de WOODS e JACKSON, 2006)

1.2.1.2. Fosfolipídios

Os fosfolipídios são os componentes majoritários das membranas plasmáticas. Sua composição envolve uma molécula de álcool (como glicerol, esfingosinol e inositol) ligada a um fosfato, apresentando grupamentos substituintes (como colina, serina e etanolamina) (NELSON e COX, 2005). Estes lipídios têm função na estruturação da membrana, assim como na sinalização celular (OHVO-REKILÄ et al., 2002).

1.2.1.3. Colesterol

O colesterol é um componente lipídico essencial às membranas biológicas, pois age estruturalmente, modulando propriedades físico-químicas e contribuindo para a formação de microdomínios de membrana ("*rafts*") através de modificações no comportamento e na função de algumas proteínas de membrana (HARDER e SIMONS, 1997; OHVO-REKILÄ et al., 2002; SUZUKI, 2002).

1.2.2. Microdomínios de membrana (*rafts* lipídicos)

Rafts de membrana são domínios pequenos (100-200 nm), heterogêneos, altamente dinâmicos, enriquecidos em esteróis e glicoesfingolipídios que compartimentalizam processos celulares (Figura 1.2) (GIL et al., 2006). Estes domínios também são conhecidos pelas abreviaturas GEM (*glycosphingolipid-enriched membrane microdomains*) e DRM (detergent-resistent *membrane microdomains*) (SIMONS e TOOMRE, 2000). Pequenos *rafts* podem, às vezes, se estabilizar para formar plataformas maiores através de interações proteína-proteína e proteína-lipídio (PIKE, 2006). Acredita-se que esses microdomínios participem em uma variedade de rotas fisiológicas e patológicas importantes à saúde humana através de segregação de proteínas e lipídios no plano da membrana (CHEN et al., 2006). Tais estruturas estão envolvidas com tráfego através da membrana e sinalização, inclusive por neurotransmissores como GABA e glutamato em células neuronais (ALLEN et al., 2007; HANZAL-BAYER e HANCOCK, 2007). Estudos demonstram que a localização e regulação funcional do receptor de glutamato NMDA dependem de neuroregulinas e de receptores tirosina quinase presentes em *rafts* (SCHRATTENHOLZ e SOSKIC, 2006). A

depleção de colesterol ou de esfingolipídios leva à instabilidade de receptores AMPA da superfície celular de neurônios hipocampais em cultura, levando à perda gradual das sinapses (HERING, LIN e SHENG, 2003). O receptor de GABA A e a Na^+, K^+ ATPase, também, estão localizados em microdomínios de membrana em células cerebelares granulares de ratos (DALSKOV et al., 2005).

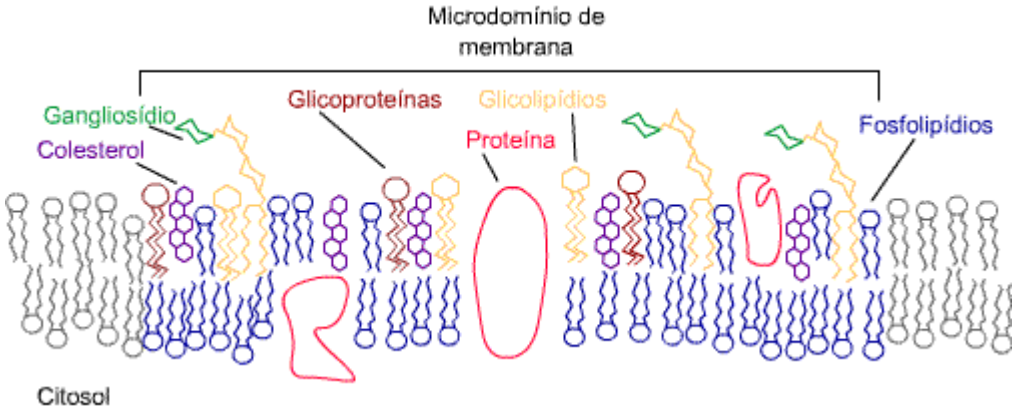


Figura 1.2. Estrutura do microdomínio de membrana. Enriquecidos em esteróis e glicoesfingolipídios, os microdomínios compartimentalizam funções celulares. (Adaptado de ALCHORN, 2007)

1.3. Lipídios de membrana e modelos de danos do sistema nervoso central

Vários estudos têm avaliado as modificações nas membranas biológicas decorrentes de modelos experimentais de danos ao SNC. Num modelo de desnutrição protéica pré- e pós-natal e pós natal em ratos, o conteúdo total de gangliosídios e sialoglicoproteínas em hipotálamo mostrou significativa redução, de maneira mais pronunciada na desnutrição pré- e pós-natal do que apenas na desnutrição pós-natal (MORGAN e NAISMITH, 1982; TRINDADE, PERRY e BERNARD, 1992). A hipóxia-isquemia neonatal reduz o conteúdo total de

gangliosídios e fosfolipídios e altera o padrão de fosfolipídios no hipocampo de ratos (QI e XUE, 1991; RAMIREZ et al., 2003). Em modelos experimentais de acidemia metilmalônica e propiônica, foram demonstradas diminuição do conteúdo total e alteração na distribuição das espécies de gangliosídios em cerebelo de ratos (WAJNER et al., 1988; BRUSQUE et al., 1998; TRINDADE et al., 2002). Numa doença neurodegenerativa (doença de Creutzfield-Jakob), também, foi detectada uma alteração dos lipídios de membrana (OHTANI et al., 1996). Recentemente, demonstramos, num modelo de hipermetioninemia crônica, uma diminuição do conteúdo total de gangliosídios, de fosfolipídios e de colesterol em cérebro de ratos (STEFANELLO et al., 2007).

1.4. Erros Inatos do Metabolismo

Os erros inatos de metabolismo (EIM) são modificações genéticas que causam alterações nas proteínas produzidas a partir do gene modificado. Se estas proteínas alteradas (geralmente enzimas), estiverem relacionadas a rotas metabólicas, podem causar doenças por acúmulo de metabólitos ou pela sua ausência (SCRIVER et al., 2001).

1.4.1. Hiperprolinemias

A prolina (Pro) é um aminoácido pertencente ao grupo dos aminoácidos não essenciais, pois tanto as enzimas relacionadas à sua síntese quanto à sua degradação estão presentes no organismo humano. Classificada como um aminoácido apolar, ela é o único desses compostos com a presença do grupamento amino secundário (imino) na cadeia lateral (Figura 1.3), o que lhe

permite conferir um grau de restrição conformacional à região da proteína em que estiver presente (BERG, TYMOCZKO e STRYER, 2003).

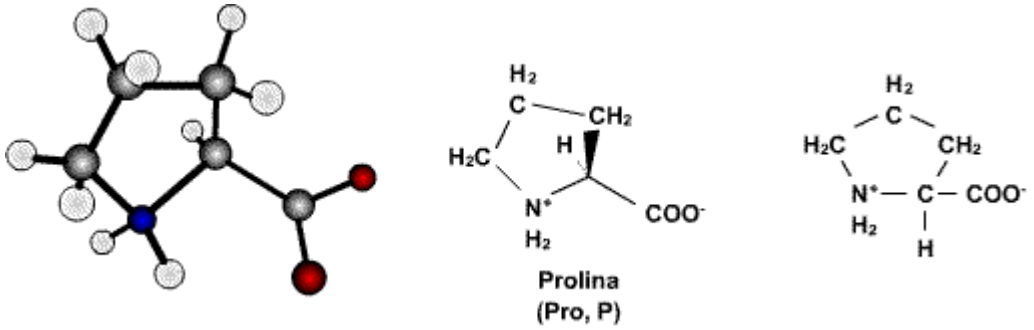


Figura 1.3. Estrutura cíclica da prolina. A cadeia lateral do aminoácido liga o carbono α ao grupamento amino. (Adaptado de BERG, TYMOCZKO e STRYER, 2004)

A estrutura cíclica da prolina é aberta através de oxidação no carbono mais distante do grupo carboxila, pela ação da prolina oxidase, criando uma base de Schiff, Δ^1-pirrolino-5-carboxilato, que é então hidrolisado a um semialdeído linear, glutamato γ-semialdeído. Este intermediário é, então, oxidado no mesmo carbono, produzindo glutamato. A transaminação ou a deaminação do glutamato produz o α-cetoglutarato, que entra no ciclo do ácido cítrico (Figura 1.4) (PHANG, HU e VALLE, 2001).

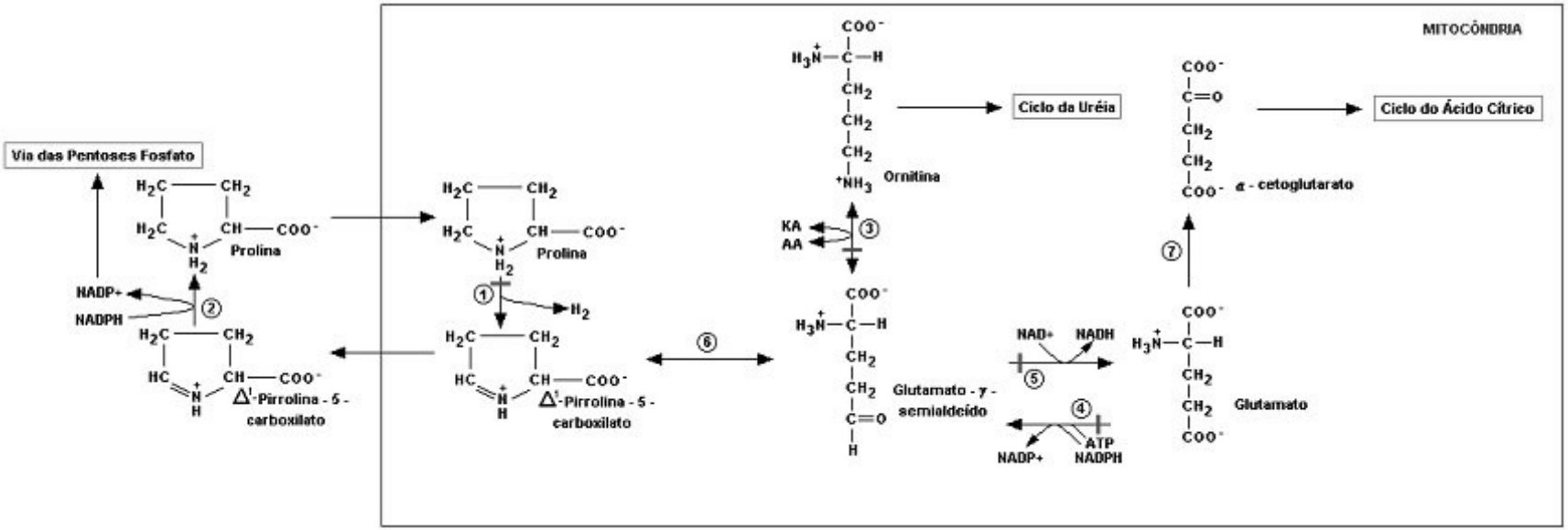


Figura 1.4. Metabolismo da prolina. ① prolina oxidase; ② Δ^1-pirrolino-5-carboxilato redutase; ③ ornitina δ-aminotransferase; ④ Δ^1-pirrolino-5-carboxilato sintase; ⑤ Δ^1-pirrolino-5-carboxilato desidrogenase; ⑥ não-enzimático; ⑦ glutamato desidrogenase. KA - corpos cetônicos; AA – aminoácidos. Barras cinza indicam a enzima deficiente nas hiperprolinemias.

(Adaptado de PHANG, HU e VALLE, 2001).

As deficiências enzimáticas que geram as hiperprolinemias podem se dar em dois pontos, gerando duas doenças distintas: quando a enzima deficiente é a prolina oxidase, há um acúmulo de prolina entre três e cinco vezes os valores normais e denomina-se hiperprolinemia tipo I; quando a enzima deficiente é a Δ^1-pirrolino-5-carboxilato desidrogenase, há um acúmulo de Pro entre dez e quinze vezes mais elevados do que os valores normais, elevação do nível plasmático de Δ^1-pirrolino-5-carboxilato (P5C) e excreção deste intermediário na urina, caracterizando a hiperprolinemia tipo II (Figura 1.4) (PHANG, HU e VALLE, 2001).

1.4.1.1. Hiperprolinemia tipo II

A hiperprolinemia tipo II (HP II) é um erro inato do metabolismo de aminoácidos, causado pela deficiência de atividade da enzima Δ^1-pirrolino-5-carboxilato desidrogenase, que leva a um acúmulo tecidual de prolina. Isto ocorre porque o Δ^1-pirrolino-5-carboxilato além ser o substrato da enzima Δ^1-pirrolino-5-carboxilato desidrogenase mitocondrial é, também, o substrato da Δ^1-pirrolino-5-carboxilato redutase gerando prolina no citosol (Figura 1.4). A prolina pode ser transformada, novamente, em Δ^1-pirrolino-5-carboxilato pela prolina oxidase mitocondrial. Entretanto, no fígado a Δ^1-pirrolino-5-carboxilato redutase tem uma maior atividade que a prolina oxidase, levando, portanto, ao acúmulo de prolina no citosol. Este fato é corroborado pelos valores de Km dos respectivos substratos destas enzimas, favorecendo a síntese de prolina ao invés da sua degradação (PHANG, HU e VALLE, 2001).

Os pacientes são geralmente assintomáticos, mas alguns apresentam sintomas neurológicos, como convulsões e retardo mental (PHANG, HU e VALLE,

2001). Vários estudos mostram uma correlação entre as manifestações clínicas e a hiperprolinemia (FLYNN et al., 1989; PHANG, HU e VALLE, 2001). A prolina em elevadas concentrações (maiores do que 100 mM) ativa os receptores NMDA e AMPA, e é, portanto considerada moduladora da neurotransmissão glutamatérgica (NADLER, 1987; NADLER, BRAY e EVENSON, 1992; FREMEAU, CARON e BLAKELY, 1992; FREMEAU et al., 1995). Esta hipótese é reforçada pelos experimentos que demonstram elevados níveis de prolina no líquido cefalorraquidiano de pacientes hiperprolinêmicos (VAN HARREVELD e FIFKOVA, 1973; RHOADS, PETERSON e RAGHUPATHY, 1983; PHANG, HU e VALLE, 2001).

1.4.1.2. Modelo Experimental de Hiperprolinemia

O modelo utilizado para mimetizar a deficiência enzimática na hiperprolinemia tipo II cronicamente consiste em injetar soluções de prolina com concentrações proporcionais ao peso corporal em ratos do sexto ao vigésimo oitavo dias pós-natal, duas vezes por dia com intervalos de 10 horas. Os níveis plasmáticos de prolina nos ratos submetidos ao tratamento situam-se entre 1,0 e 2,0 mM, similar aos encontrados no plasma de pacientes hiperprolinêmicos (PHANG, HU e VALLE, 2001). As concentrações cerebrais de prolina alcançadas através deste método são 0,25–0,55 μmol/g (aproximadamente 0,25–0,55 mM) (MOREIRA et al., 1989; DELWING et al., 2003a).

1.4.1.3. Estudos previamente realizados com Modelo Experimental de Hiperprolinemia

Como os mecanismos dos sintomas neurológicos da hiperprolinemia ainda não são conhecidos, estudos têm sido feitos para que os mesmos sejam elucidados. Deficiências cognitivas (CHERKIN, ECKARDT e GERBRANDT, 1976; BAVARESCO et al., 2005; DELWING et al., 2006a), diminuição da atividade da enzima Na^+,K^+-ATPase em membrana plasmática sináptica (PONTES et al., 1999; 2001), o aumento da peroxidação lipídica concomitante à diminuição do potencial antioxidante do tecido em cérebro de ratos (DELWING et al., 2003a; 2003b; 2005a; 2006a; 2007a) e alteração na hidrólise de nucleotídeos em sinaptossomos obtidos de córtex cerebral de ratos (DELWING et al., 2007b) têm sido associados à condições de hiperprolinemia. A administração de prolina também altera as atividades de acetilcolinesterase e butirilcolinesterase (DELWING et al., 2003c; 2005b) e a hidrólise de nucleotídeos em soro (DELWING et al., 2006b).

2. OBJETIVOS

Considerando que a administração de prolina causa peroxidação lipídica, reduz a atividade Na^+,K^+ ATPásica, ativa os receptores NMDA e AMPA e que, possivelmente, module a relação entre os lipídios de membrana com estas proteínas, especialmente os lipídios presentes nos microdomínios, os objetivos deste trabalho foram:

- Investigar o conteúdo e o perfil de gangliosídios, assim como da concentração de fosfolipídios e colesterol no córtex cerebral, no hipocampo, no hipotálamo e no cerebelo de ratos submetidos ao modelo crônico de hiperprolinemia tipo II.
- Avaliar quanti- e qualitativamente os gangliosídios nas frações de membranas solúveis e resistentes a detergente, isoladas de membrana sináptica de córtex cerebral.

3. MATERIAIS E MÉTODOS

3.1. Materiais

O aminoácido L-prolina, padrões de gangliosídios (GM1, GD1a, GD1b e GT1b, EDTA, ácido N-acetil-neuramínico, ácido 1-amino, 2-naftol, 4-sulfônico, Triton X-100, DEAE-Sephadex A-50, foram obtidos da Sigma-Aldrich (Saint Louis, MO, USA). Cromatofolhas de Silica-gel 60 em camada delgada (TLC) e cromatofolhas de Silica-gel 60 em camada delgada de alta performance (HPTLC) foram providas pela Merck (Darmstadt, Alemanha). Colunas Sep-Pack C18 foram obtidas da Waters (Milford, MA, USA). O *kit* utilizado para dosagem de colesterol (Colesterol liquiform) foi obtido da Labtest (Lagoa Santa MG, Brasil). Todos os outros produtos químicos e solventes utilizados foram de escala analítica.

3.2. Animais

Ratos Wistar obtidos do biotério do Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, foram separados em grupos de oito no dia do nascimento. Foram mantidos em ciclo claro-escuro de 12 horas (luz das 7 às 19 horas) em ambiente com ar condicionado (22°C de temperatura). Os animais tinham livre acesso a ração comercial com 20% (massa por massa) de proteína e água.

3.3. Tratamento crônico com prolina

A solução de prolina foi feita da seguinte forma: prolina foi dissolvida em solução de NaCl 0,9% e o pH foi ajustado a 7,2-7,4. Esta solução foi administrada subcutaneamente duas vezes ao dia em intervalos de 10 horas do sexto ao vigésimo-oitavo dias de idade. As doses de prolina foram calculadas segundo descrito por MOREIRA e colaboradores (1989). Durante os primeiros oito dias de tratamento, foi administrado 12,8 μmol de prolina por grama de peso corporal; do décimo quarto ao décimo sétimo dias, foi administrado 14,6 μmol de prolina por grama de peso corporal; do décimo oitavo ao vigésimo primeiro dias, foi administrado 16,4 μmol de prolina por grama de peso corporal; e do vigésimo segundo ao vigésimo oitavo dias, foi administrado 18,2 μmol de prolina por grama de peso corporal. Os níveis plasmáticos de prolina alcançados com este tratamento situam-se entre 1,0 e 2,0 mM, similar aos valores encontrados em plasma de pacientes com hiperprolinemia tipo II (PHANG, HU e VALLE, 2001). As concentrações cerebrais de prolina alcançadas por este tratamento situam-se entre 0,25 e 0,55 μmol/g (aproximadamente 0,25–0,55 mM) (DELWING et al., 2003a). Os animais submetidos ao tratamento controle receberam injeções de solução salina nos mesmos volumes aplicados nos animais submetidos ao tratamento com prolina. Os animais foram sacrificados 12 horas após a última injeção e o córtex cerebral, o hipocampo, o hipotálamo e o cerebelo foram imediatamente isolados e mantidos a -70°C até o momento das análises subseqüentes.

3.4. Extração lipídica das estruturas cerebrais

As estruturas cerebrais foram degeladas a 4°C por alguns minutos, pesadas sobre folha de alumínio e homogeneizadas numa mistura de clorofórmio e metanol 2:1 (C:M, 2:1, v/v) numa diluição de 20 vezes a massa do tecido, mantidas em contato com os solventes por 10 minutos e centrifugadas a 4.000×g por 5 minutos. O sedimento foi, novamente, homogeneizado em C:M, 1:2 numa diluição de 10 vezes a massa original (FOLCH et al., 1957). Os extratos foram combinados, tiveram o volume determinado e foram mantidos a -20°C em tubos de vidro com capacidade para 12 ml para as análises que se seguiram.

3.5. Avaliação de gangliosídios das estruturas cerebrais

Uma alíquota correspondente à décima parte do extrato lipídico total de cada amostra foi evaporada e utilizada na determinação do ácido N-acetil neuramínico (NANA), de acordo com o método do resorcinol (SVENNERHOLM, 1957, modificado por MIETTINEN e TAKKI-LUUKKAINEN, 1959). O perfil das espécies de gangliosídios foi analisado por cromatografia em camada delgada (TLC) utilizando placas de sílica gel 60 da Merck, de 10 x 10 cm com aplicação de alíquotas dos extratos lipídicos totais contendo 4 nmoles de NANA, como determinado anteriormente, evaporados e suspensos em C:M 1:1. A análise foi realizada pela utilização de duas migrações sucessivas: a primeira utilizando como fase móvel uma mistura de solventes clorofórmio e metanol na proporção de 4:1 v/v, e a segunda, utilizando como fase móvel uma mistura de solventes clorofórmio, metanol e uma solução de $CaCl_2$ 0,25% em água, numa proporção de 60:35:8 v/v/v (IRWIN e IRWIN, 1979). Esta última migração foi realizada numa

cuba de migração descrita por NORES e colaboradores, 1994. O cromatograma foi revelado pelo método do resorcinol (SVENNERHOLM, 1957, modificado por LAKE e GOODWIN, 1976) e quantificado por densitometria por escaneamento a 580 nm num densitômetro CS 9301 PC Shimadzu (Tóquio, Japão). A quantidade absoluta total de gangliosídio-NANA e as espécies individuais de gangliosídios foram expressas como nmol/mg de massa da estrutura, calculando as espécies de gangliosídios pela relação da porcentagem individual com a quantidade absoluta total de NANA-gangliosídio. A nomenclatura utilizada é a recomendada por SVENNERHOLM, 1963.

3.6. Quantificação de colesterol

Alíquotas de 100 μL do extrato lipídico total foram evaporadas, suspensas em isopropanol e quantificadas de acordo com *kit* colesterol liquiform (Labtest) que é baseado no método enzimático de Trinder (BERGMEYER, 1974) A quantidade absoluta total de colesterol foi expressa como μg/mg de massa do tecido.

3.7. Avaliação de fosfolipídios

Alíquotas correspondendo à quarta parte do volume do extrato lipídico total foram evaporadas e analisadas de acordo com o método de BARTLETT, 1959. A quantidade absoluta total de Pi-fosfolipídos foi expressa como nmol Pi/mg massa do tecido.

3.8. Preparação da membrana sináptica de córtex

Os animais foram mortos por decapitação sem anestesia, o cérebro foi rapidamente removido e o córtex cerebral foi imediatamente dissecado sobre placa de Petri em gelo. A estrutura foi homogeneizada em 10 volumes (1:10, massa por volume) de solução de sacarose 0,32 M contendo HEPES 5,0 mM e EDTA 0,1 mM, pH 7,4. Após a homogeneização, a membrana plasmática sináptica foi preparada de acordo com o método de JONES e MATUS, 1974, com algumas modificações (WYSE et al., 1995). Estas membranas foram obtidas utilizando um gradiente de densidade descontínuo de sacarose consistindo de camadas sucessivas de 0,3, 0,8 e 1,0 M. Após centrifugação a 69.000×g por 2 horas, a fração na interface de sacarose 0,8 e 1,0 M foi tomada como a preparação de membrana sináptica. Esta fração foi, então, lavada com tampão Tris-HCl 5mM, pH 7,4 e armazenada a -20°C até a utilização nas análises subseqüentes.

3.9. Isolamento das frações de membrana sináptica solúvel e resistente a detergente e análise dos gangliosídios constituintes.

3.9.1 Tratamento com Triton X-100 a 4°C

As frações de membrana solúvel e resistente a detergente foram isoladas como previamente descrito (CRESPO et al., 2004). O *pellet* de membrana sináptica foi suspenso em tampão TNE (Tris-HCl 25mM pH=7,5; NaCl 150mM, EDTA 5mM, Triton X-100 1%) e deixado em contacto, sob agitação a 4°C, por 1 hora. A suspensão foi então centrifugada por 1 hora a 100.000×g, isolando uma fração resistente (sedimento) que foi seca à temperatura ambiente, e uma fração

solúvel (sobrenadante), seca por liofilização usando uma centrífuga Speed-Vac por duas horas.

3.9.2. Extração lipídica das frações solúvel e resistente a detergente

Os resíduos das frações foram tratados com uma mistura de clorofórmio e metanol 2:1 (C:M, 2:1, v/v) e centrifugados a 10.000×g, por 10 minutos, a 4°C. Os extratos foram ajustados para C:M 1:1(v/v) e submetidos a uma cromatografia em coluna de troca iônica (DEAE-Sephadex A-50) para a eliminação do Triton X-100. Nestas condições, os glicolipídios neutros (glicosil-ceramida e lactosil-ceramida) co-eluem com o Triton X-100 na fração não retida pela coluna. Os glicolipídios ácidos (gangliosídios) foram eluídos com acetato de sódio 0,1M em metanol. Os eluatos salinos foram elevados à fase superior teórica (C:M: Água, 3:48:47) e o sal removido através de uma cromatografia de fase reversa (Sep-Pack C18) (WILLIAMS e MCCLUER, 1980). Os eluatos purificados (extratos lipídicos) das frações solúvel e insolúvel (resistente) a detergente foram analisados como segue abaixo.

3.9.3. Avaliação de gangliosídios nas frações solúvel e resistente a detergente

Uma alíquota correspondente à terça parte do extrato lipídico de cada fração foi evaporada e utilizada na determinação do ácido N-acetil neuramínico (NANA), de acordo com o método do ácido tiobarbitúrico (SKOZA e MOHOS, 1976). O perfil das espécies de gangliosídios foi analisado por cromatografia em camada delgada de alta performance (HPTLC) conforme descrito no item 3.5.

3.9.4. Quantificação de proteínas

Os sedimentos protéicos obtidos após a extração lipídica das frações solúvel e resistente a Triton X-100 foram solubilizados da seguinte forma: à fração solúvel, foi adicionado volume suficiente de tampão Tris-HCl 500 mM, pH 6,8; à fração insolúvel, foi adicionado volume suficiente de tampão Tris-HCl 500 mM com EDTA 2 mM e SDS 4%, pH 6,8. A determinação do conteúdo de proteína foi feita de acordo com o método de PETERSON e colaboradores (1977), utilizando como padrão solução de albumina sérica bovina contendo 0,5 mg proteína/ml.

3.10. Análise estatística

Os dados obtidos foram tratados, estatìsticamente, através da Análise de Variância (Anova) de duas vias, seguida pelo teste de Ducan para a comparação entre as médias, quando o valor de *F* foi significativo. Quando indicado, o teste *t* de Student também foi utilizado para comparação entre as médias. Os resultados foram expressos como média ± erro padrão da média. Todas as análises foram feitas utilizando o software *Statistical Package for the Social Sciences* (SPSS) em um computador compatível. As diferenças foram consideradas estatisticamente significantes, quando $p<0,05$.

4. RESULTADOS

Os resultados desta dissertação serão mostrados na forma de um artigo científico a ser submetido à revista *International Journal of Developmental Neuroscience,* cujas instruções encontram-se no anexo (item 9).

Effects of chronic proline administration

on lipid contents of rat brain

Luciene P. Vianna, Daniela Delwing, Fábria Chiarani, Ana Carolina Breier,

Fernando Kreutz, Angela T. S. Wyse and Vera M. Treis Trindade.

Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul,
Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

Correspondence to: Dr. Vera M. Treis Trindade
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 3316 5550 Fax: 55 51 3316 5555
E-mail: vmtt@ufrgs.br

Running title: membrane lipids in hyperprolinemic rat brain.

Abstract

In the present work we investigated the effects of chronic proline administration on ganglioside, cholesterol and phospholipid total contents, as well as on ganglioside profile in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats. We also evaluated the ganglioside content and profile in detergent- soluble and resistant fractions isolated from synaptic membranes obtained from cerebral cortex. Wistar rats were divided into two groups: 1) saline (control) and 2) proline injected (hyperprolinemic). Proline solution or saline were administered from 6th to 28th postnatal day, according to body weight. Twelve hours after the last injection, the animals were sacrificed by decapitation without anesthesia. Brain structures were homogenized with chlorophorm:methanol 1:1 for lipid extraction. Synaptic membrane was extracted by differential centrifugation and detergent- soluble and resistant fractions were isolated by cold Triton X-100 treatment. Results showed that rats subjected to chronic proline treatment presented a significant increase of ganglioside content on cortex and hippocampus, while phospholipid and cholesterol contents only increased in hippocampus. However, the content of these components were not altered in hypothalamus and cerebellum of hyperprolinemic rats. On the other hand, ganglioside content decreased in detergent- soluble and resistant fractions isolated from synaptic membrane obtained from hyperprolinemic cortex. Although ganglioside profiles were apparently not modified, the individual absolute quantities were altered in cortex total lipid extract and membrane microdomains obtained from cerebral cortex. Our findings suggest that chronic proline treatment affects, in a distinct manner, different cerebral regions concerning the lipid composition of the cell membranes, reflecting on its distribution in the cortex membrane microdomains. Among these phenomena consequences, different modulations in synaptic transmission may be suggested which may contribute to the impairment in cognition and/or other neurological disfunctions found in hyperprolinemia type II patients.

Keywords: gangliosides, phospholipids, cholesterol, hyperprolinemia, brain, synaptic membranes, membrane rafts.

1. Introduction

Plasma membrane lipids of the central nervous system (CNS) include gangliosides, phospholipids and cholesterol (Agranoff and Hajra, 1993). Gangliosides are glicosphingolipids containing a sialic acid in the molecule, related to cell to cell signalling and interaction, cellular growth and differentiation and may be involved in neural development (synaptogenesis, myelogenesis), as neural membranes are enriched in these lipids (Ando, 1983; Zeller and Marchase, 1992; Nagai, 1995). Phospholipids perform structural functions in the membrane, as well as cellular signaling functions (Ohvo-Rekilä et al., 2002). Cholesterol is an essential lipid component of plasma membranes, modulating its physicochemical properties and contributing to raft formation through changes in behavior and function of membrane proteins (Harder and Simons, 1997; Ohvo-Rekilä et al., 2002; Suzuki, 2002). Membrane rafts are small (100-200 nm), heterogeneous, highly dynamic domains, enriched in sterols and glycosphingolipids that compartimentalize cellular processes (Gil et al., 2006). They are also named as glycosphingolipid-enriched membrane microdomain's (GEM) or detergent-resistant membrane microdomain's (DRM) (Simons and Toomre, 2000).

Some studies have evaluated changes on lipid membranes caused by experimental CNS damages. In general, models of malnutrition (Morgan and Naismith, 1982; Trindade et al., 1992), neonatal hypoxia-ischemia (Qi and Xue, 1991; Ramirez et al., 2003); malonic and propionic acidemia's (Wajner et al., 1988; Brusque et al., 1998; Trindade et al., 2002) and neurodegeneration as Creutzfield-Jakob disease (Ohtani et al., 1996) determined lipid loss in the membrane of neural structures. Recently, we demonstrated a decrease in ganglioside,

phospholipid and cholesterol contents in cerebral cortex of rats caused by chronic hypermethioninemia model (Stefanello et al., 2007).

Hyperprolinemia type II (HP II) is an inborn error of amino acid metabolism, caused by deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity, leading to tissue accumulation of proline (Pro). Patients are usually asymptomatic, but some display neurological manifestations, such as seizures and mental retardation (Phang et al., 2001). Studies have shown a correlation between clinical manifestations and hyperprolinemia (Flynn et al., 1989; Phang et al., 2001). Proline in high concentrations (>100 mM) activates NMDA and AMPA receptors, and is therefore thought to modulate glutamate neurotransmission (Nadler, 1987; Nadler et al., 1992; Fremeau et al., 1992; Fremeau et al., 1995). This is supported by findings of high proline levels in cerebrospinal fluid of hyperprolinemic patients and in some of them high glutamate levels could be also found (Van Harreveld and Fifkova, 1973; Rhoads et al., 1983; Phang et al., 2001).

As the mechanism of these neurological symptoms is yet unknown, studies have been made to elucidate it. Cognitive deficit (Cherkin et al., 1976; Bavaresco et al., 2005; Delwing et al., 2006a), decrease in Na^+,K^+-ATPase activity in synaptic plasma membrane (Pontes et al., 1999; 2001) and increase in lipid peroxidation while decrease in tissue antioxidant potential (Delwing et al., 2003a; 2003b; 2005a; 2006a; 2007a) in brain of rats have been shown due to hyperprolinemic conditions. Proline administration also alters nucleotide hydrolisis in synaptosomes obtained from rats' cerebral cortex (Delwing et al., 2007b) and serum (Delwing et al., 2006b) as well as acetilcholinesterase and butirylcholinesterase activities in serum (Delwing et al., 2003c; 2005b).

Considering that proline administration causes lipid peroxidation, reduces Na^+,K^+-ATPase activity, activates NMDA and AMPA receptors and that probably modulates the relation between membrane lipids and these proteins, specially those present in microdomains, in the present study we evaluated the content and profile of gangliosides, as well as the phospholipids and cholesterol concentrations in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats submitted to a chronic hyperprolinemia II model. Furthermore, we determined ganglioside content and profile in soluble and resistant (insoluble cold) detergent membrane fractions obtained from cortex synaptic membranes.

2. Experimental procedures

2.1. Material

Proline; GM1, GD1a, GD1b and GT1b gangliosides; EDTA; N-acetyl-neuramic acid; 1-amino, 2-naphtol, 4-sulphonic acid; TritonX-100 and DEAE-Sephadex A-50 were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Silica-gel 60 thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) sheets were supplied by Merck (Darmstadt, Germany). Sep-Pack C18 cartridges were obtained from Waters (Milford, MA, USA). Cholesterol liquiform kit was purchased from Labtest Diagnóstica (Lagoa Santa, MG, Brazil). All other chemicals and solvents used were of analytical grade.

2.2. Animals

Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were culled to groups of eight with their dams on the day they were born. They were maintained on a 12:12h light/dark cycle (lights on from 7 am to 7 pm) in an air-conditioned constant temperature (22 ° C) colony room. The rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

2.3. Proline treatment

Proline was dissolved in 0.9% NaCl and the pH adjusted to 7.2–7.4 with 0.1 N NaOH. Proline solution was administered subcutaneously twice a day at 10-h intervals from the 6th to the 28th days of post-natal age. Doses of proline were calculated as described by Moreira et al. (1989). During the first 8 days of treatment, the rats received 12.8 μmol of proline/g body weight; from day 14 to 17, they received 14.6 μmol of proline/g body weight; from 18 to 21 days, the animals were injected with 16.4 μmol of proline/g body weight; and from 22 to 28 days, the rats received 18.2 μmol of proline/g body weight. Plasma proline levels achieved by rats subjected to this treatment were between 1.0 and 2.0 mM, similar to those found in plasma of hyperprolinemic type II patients (Phang et al., 2001). Brain proline concentrations achieved by this model were 0.25–0.55 μmol /g tissue (~ 0.25–0.55 mM) (Delwing et al., 2003a). Control animals received saline injections in the same volumes as those applied to Proline-treated rats. The animals were killed 12h after the last injection and the cerebral cortex, hippocampus, hypothalamus and cerebellum were immediately isolated and kept at –70°C until used.

2.4. Cerebral structures lipid extraction

The structures were left to thaw at 4°C for a few minutes, weighed and homogenized in a 2:1 mixture of chloroform and methanol (C:M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 600×g for 5 minutes. The pellet was re-homogenized in C:M, 1:2 to a 10-fold dilution of original mass (Folch et al.,

1957). The extracts were combined, had their volume measured and were kept in 12 ml glass tubes at –20°C for the determinations that followed.

2.5. Cerebral structures ganglioside evaluation

An aliquot corresponding to the tenth part of total lipid extract of each sample was evaporated and used for N-acetyl neuraminic acid (NANA) determination, according to the resorcinol method (Svennerholm, 1957, modified by Miettinen and Takki-Luukkainen, 1959). The ganglioside species profile was analyzed by thin-layer chromatography (TLC) performed on 10 x 10 cm Merck plates of silica gel 60, applied with aliquots of total extract (containing 4 nmol of N-acetyl neuraminic acid), evaporated and suspended in chloroform and methanol 1:1. Analysis was made by utilization of two successive migrations: the first utilizing as mobile phase a solvent mixture of chloroform and methanol in a 4:1 proportion, v/v, and the second utilizing a mixture of chloroform, methanol and a solution of $CaCl_2$ 0.25% in water, in a 60:35:8 proportion, v/v/v (Irwin and Irwin, 1979). This last migration was done in a developing tank designed by Nores et al. (1994). The chromatogram was revealed by the resorcinol method (Svennerholm, 1957, modified by Lake and Goodwin, 1976) and quantified by scanning densitometry at 580 nm in a CS 9301 PC Shimadzu Densitometer. The absolute total quantity of ganglioside-N-acetyl neuraminic acid and the individual species were expressed as nmol/mg tissue weight, calculated by relating their respective percentage to the absolute total quantity of ganglioside-N-acetyl neuraminic acid. The terminology used is that recommended by Svennerholm (1963).

2.6. Cholesterol quantification

Aliquots of 100 μL of total lipid extract were evaporated, suspended in isopropanol and quantified according to the Trinder enzymatic method (Bergmeyer, 1974). The absolute total quantity of cholesterol was expressed as μg / mg tissue weight.

2.7. Phospholipid evaluation

Aliquots corresponding to the fourth part of the total lipid extract original volume were evaporated and quantified according to the Bartlett method (1959). The absolute total quantity of phospholipids-inorganic phosphorus was expressed as nmol/mg weight.

2.8. Synaptic membrane preparation

Cerebral cortex was homogenized in 10 volumes of sucrose 0.32 M with 5 mM HEPES (pH=7.4) and 0.1 mM EDTA. Following this, synaptic plasmatic membrane was isolated according to the method described by Jones and Matus (1974), with modifications (Wyse et al., 1995). This isolation was achieved with a sucrose discontinuous density gradient consisting of layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000×g for 2 h, the fraction on 0.8 and 1.0 M interface was taken as synaptic membrane preparation. This fraction was washed with 5 mM Tris-HCl (pH 7.4) buffer and stored frozen at -20°C (no more than two months) until the next experimental procedures.

2.9. Isolation of detergent- soluble and resistant fractions from cortex synaptic membrane and their ganglioside analysis

2.9.1 Triton X-100 treatment under cold conditions

The synaptic membrane pellet was treated with 0.5 mL of TNE buffer (25mM Tris-HCl (pH=7.5), 150mM NaCl, 5mM EDTA, 1% Triton X-100) under shaking, at 4°C for 1h. It was then centrifuged at 4°C for 1h at 100,000×g (Crespo et al., 2004). The detergent soluble fraction (supernatant) was dried by a Speed-Vac centrifugation for two hours and the detergent resistant fraction (insoluble fraction, pellet) was dried at environment temperature.

2.9.2. Lipid extraction of detergent- soluble and resistant fractions

Glycolipids from the supernatant (detergent soluble fraction) and from the pellet (detergent resistant fraction) were subjected to chloroform:methanol extraction (Folch et al., 1957). The resulting lipid extracts were freed from Triton X-100 (non ionic molecule) by passing through DEAE–Sephadex A-50 column. Under this condition, neutral lipids, such as glucosylceramide and lactosylceramide were co-eluted with Triton X-100 in the non-retained fraction. The acidic lipid fraction retained in the column (gangliosides) was eluted with 0.1M sodium acetate and the salt removed by passing through Sep-Pak C18 cartridge column (Williams and McCluer, 1980).

2.9.3. Ganglioside evaluation in detergent- soluble and resistant fractions

An aliquot corresponding to the third part of detergent- soluble and resistant fractions were evaporated and dried to residue and it was used for N-acetyl neuraminic acid (NANA)-ganglioside determination, according to the thiobarbituric acid method (Skoza e Mohos, 1976). Ganglioside species profile was analysed on

high performance thin layer chromatography (HPTLC) plates as described above (item 2.5.).

2.9.4. Protein quantification

Proteic pellets obtained after lipid extraction from membrane fractions were solubilized as follows: to the detergent soluble fraction was added sufficient volume of Tris-HCl buffer 500 mM, pH=6.8; to the detergent resistant fraction was added sufficient volume of 500 mM Tris-HCl buffer with 2 mM EDTA and 4%SDS, pH=6.8 (Crespo et al., 2004). Protein content determination was done according to the Lowry-modified method (Peterson et al., 1977), using bovine serum albumine solution as standard.

2.10. Statistical analysis

Data were statistically treated by two-way analysis of variance (Anova) followed by Duncan's test when *F* value was significant. When it was indicated, Student's t test was also used. The results were expressed as mean ± standard error of mean. All analysis was performed using the Statistical Package for the Social Sciences (SPSS) software in a compatible computer. Differences were considered statistically significant if $p<0.05$.

3. Results

3.1. Total ganglioside, phospholipid and cholesterol contents in brain structures

Figure 1 shows data relative to the total ganglioside (A), phospholipid (B) and cholesterol (C) contents in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats submitted to chronic model of hyperprolinemia. Statistical analysis by two-way Anova showed, on total ganglioside content, a proline treatment effect [$F(1,72)=127.15$, $p<0.001$], a cerebral structure effect [$F(5,72)=78.91$, $p<0.001$] and a significant interaction of both factors [$F(5,72)=16.14$, $p<0.001$] suggesting that proline treatment affects total ganglioside content in the studied structures in different ways. Duncan's test showed that this lipid content variation was alike in right cortex and hippocampus, as well as in left cortex and hippocampus. This may indicate that the treatment effect may be hemisphere dependent. By comparing total ganglioside contents from control and treated rats in each structure, it was noted that this lipid content was statistically higher in cortex and hippocampus of hyperprolinemic animals than in control rats (right cortex [$t(7)=-11.428$; $p<0.05$]; left cortex [$t(7)=-6.125$; $p<0.05$] and right hippocampus [$t(7)=-4.945$; $p<0.05$]; left hippocampus $t(7)=-4.594$; $p<0.05$]).

On total phospholipid content, two-way Anova analysis showed a proline treatment effect [$F(1,71)=15.22$, $p<0.001$] and a cerebral structure effect [$F(5,71)=7.04$, $p<0.001$], without a significant interaction of these factors. Duncan's test did not show influence of proline treatment within cerebral hemisphere. By comparing total phospholipid contents from control and treated rats in each structure, it was noted that this lipid content was statistically higher in hippocampus

of hyperprolinemic animals than in control rats (right hippocampus [t(7)=-5.302; p<0.05] and left hippocampus [t(7)=-2.394; p<0.05].

On cholesterol content, statistic analysis showed only a proline treatment effect [F(1,72)=8.96, p<0.05]. There was no region influence by treatment effect on this lipid content. By comparing total cholesterol contents from control and treated rats in each structure, it was observed that this lipid content was only statistically higher in right hippocampus of hyperprolinemic animals than in control rats [t(7)=-4.945; p<0.05].

There were no significant differences for these biochemical evaluations in hypothalamus and cerebellum of hyperprolinemic rats in comparison to control rats.

3.2. Ganglioside profile in brain structures

The four main gangliosides present in the CNS (GM1, GD1a, GD1b and GT1b) were detected in the cerebral structures studied. The profile of the different ganglioside species from control and hyperprolinemic rat structures was apparently unaffected (TLC not shown). Table I shows the distribution of ganglioside specie contents. These contents treated by two-way Anova showed an effect of proline treatment [F(1,24)=13,74, p<0,05] and an effect of cerebral structure [F(5,24)=10,39, p<0,001] on GM1; an effect of proline treatment, [F(1,24)=27,69, p<0,001], an effect of cerebral structure [F(5,24)=22,66, p<0,001] and a significant interaction of both factors [F(5,24)=5,94, p<0,05] on GD1a; an effect of proline treatment [F(1,24)=18,76, p<0,001], an effect of cerebral structure,

[F(5,24)=17,05, p<0,001] and a significant interaction of both factors [F(5,24)=4,16, p<0,05] on GD1b; an effect of proline treatment [F(1,24)=27,46, p<0,001] and an effect of cerebral structure [F(5,24)=11,40, p<0,001] on GT1b. By comparing the ganglioside species of the studied structures, it was observed that their contents were statistically higher in cortex and hippocampus of hyperprolinemic rats than in control animals (Table I).

3.3. Ganglioside analysis in detergent- soluble and resistant fractions isolated from cortex synaptic membrane

Fig. 2 shows data relative to the total ganglioside contents in detergent-soluble and resistant fractions isolated from synaptic membranes of cerebral cortex of rats submitted to the chronic model of hyperprolinemia. These data confirmed a greater proportion of glycosphingolipids in the detergent-resistant microdomains than in the detergent–soluble microdomains, and it could be observed in the fractions isolated from control, as well as in those obtained from hyperprolinemic rats. Statistical analysis by two-way Anova showed effect of proline treatment [F(1,8)=28.44, p<0.001] membrane solubility effect [F(1,8)=52.78, p<0.001] and significant interaction of both factors [F(1,8)=9.70, p<0.05], indicating that proline treatment affects diferently total ganglioside content in the membrane fractions. By comparing the total ganglioside contents in detergent- soluble and resistant microdomains it was noted that both fractions were statistically lower in hyperprolinemic rats than in control animals.

The four main gangliosides present in the CNS (GM1, GD1a, GD1b and GT1b) were detected in the isolated microdomains (Figure 3). The profile of the

different ganglioside species from control and hyperprolinemic rat structures was apparently unaffected. Table II shows the distribution of ganglioside specie contents. These contents treated by two-way Anova showed a effect of proline treatment [$F(1,8)=0.43$, $p<0.05$] and an effect of membrane solubility [$F(1,8)=8.22$, $p<0.05$] on GM1; an effect of proline treatment [$F(1,8)=10.53$, $p<0.05$] and an effect of membrane solubility [$F(1,8)=5.67$, $p<0.05$] on GD1a; an effect of proline treatment [$F(1,8)=7.58$, $p<0.05$ on GD1b]; and an effect of proline treatment [$F(1,8)=9.04$, $p<0.05$ on GT1b]. By comparing the ganglioside species of the detergent- soluble and resistant fractions, it was noted that their contents were statistically lower in both microdomains of hyperprolinemic rats than in control animals (Table II).

4. Discussion

In the present study we evaluated the total content and profile of gangliosides, as well as the content of phospholipids and cholesterol in rat cerebral cortex, hippocampus, hypothalamus and cerebellum, and the total content and profile of gangliosides in detergent-soluble and resistant microdomains isolated from synaptic membranes obtained from cerebral cortex of rats subjected to a chronic hyperprolinemia model. Previous studies concerning neural membranes composition in animal models of CNS damages have shown a decrease in membrane components, represented by a reduction in membrane lipid contents (Wajner et al., 1988; Trindade et al., 1992; Brusque et al., 1998; Trindade et al., 2002; Ramirez et al. 2003; Stefanello et al., 2007). In this respect, the present results showed an unusual increase in the membrane lipid contents, with different effects of proline treatment according to the cerebral structure studied on ganglioside content, effect of treatment independent of structure on phospholipid content and effect of proline treatment on cholesterol content. There was a regional effect on total ganglioside content, where the right side cerebral structures had similar variations, as well as the left side structures. The ganglioside profile was apparently unaffected, but individual species increased their content with proline treatment and cerebral structure, according to the total content.

As has been reported in the literature, the detergent-resistant microdomains showed an enrichment on ganglioside content in comparison to the soluble detergent microdomains (Harder and Simons, 1997; Gil et al., 2006) and this was detect in cortex synaptic membrane microdomains isolated from control, as well as from hyperprolinemic rats. A decrease on total ganglioside contents was observed

in both, detergent- soluble and resistant microdomains, in the hyperprolinemic group. There was no alteration in the studied gangliosides profile, although their individual specie contents decrease with treatment and membrane solubility. As far as we know, this is the first report relating synaptic membrane microdomains and an amino acid metabolism disorder.

At the present time, we do not have a definitive explanation for the enhanced content of plasma membrane lipids when proline is chronically administered. We postulate that a reactive gliosis mechanism may have contributed to the increase of the studied lipids, once glial cells have similar plasma membrane characteristics as neurons and appear as a consequence of tissue damage (Pekny et al., 2007) for example the increase of oxidative stress by chronic proline administration (Baydas et al., 2004; Delwing et al., 2003a; Delwing et al., 2003b). This mechanism may be region specific, as there was difference in right and left side structures.

However, the ganglioside reduction in soluble- and detergent-resistant microdomains isolated from synaptic membranes obtained from cerebral cortex, however, may indicate modifications in the synaptic structure with the proline chronic administration and may be due to membrane loss by lipid peroxidation mechanism, since it has been reported that acute proline administration increases oxidative stress parameters in cerebral cortex (Delwing et al., 2003a; Delwing et al., 2003b). The loss of ganglioside from the detergent-soluble and resistant microdomains synaptic membrane may also indicate loss of stability and function of normal lipids of this membrane and may represent a loss of stability and damage to neuronal cell function (Busch et al., 1998).

Although the proline concentrations used in our study (which significantly altered plasma membrane lipids content) are similar to those observed in hyperprolinemic patients, it is difficult to extrapolate our findings to the human condition. However, if that is the case, the results of the present study demonstrating the damage to normal plasma membrane by proline administration in the brain may represent, at least in part, one of the mechanisms underlying the neurological damage in HPII.

In summary, we reported that cerebral cortex and hippocampus of rats submitted to chronic proline treatment present distinct increase in their plasma membrane lipids contents (ganglioside, phospholipid and cholesterol contents), whereas in detergent- soluble and resistant microdomains isolated from synaptic membranes obtained from cerebral cortex showed a decrease in ganglioside contents. This last effect may express the loss of stability and function of membrane, possibly leading to cell death. According to our results, it seems reasonable to postulate that the decrease of synaptic membrane components contents may contribute to the neurological dysfunction characteristic of hyperprolinemia. However, further studies investigating hyperprolinemic patients should be conducted in order to confirm the hypothesis that the alteration in membrane lipids is involved in the pathology of this disorder.

Acknowledgments

This work was supported by grants from CNPq, PRONEX, FAPERGS and PROPESQ/UFRGS. The authors wish to thank to Dr. Carla Dalmaz (Dep. Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil) for helpful statistical discussions and to Tatiana Dourado Hoffmann for technical assistance in some experiments.

References

Agranoff, B.W. And Hajra, A.K. (1993) Lipids. In Siegel, G.J., Agranoff, B.W., Albers, R.W. And Molinoff, P.B., Basic Neurochemistry, New York: Raven Press, p. 97-116.

Ando, S. (1983). Gangliosides in the nervous system. Neurochem. Int., vol. 5, n. 5, pp. 507-537.

Bartlett, G.R. (1959). Phosphorus assay in column chromatography. J. Biol. Chem., vol. 234, pp. 466 – 468.

Bavaresco, C.S., Streck, E.L., Netto, C.A. and Wyse, A.T. (2005). Chronic hyperprolinemia provokes a memory deficit in the Morris water maze task. Metab. Brain Dis., vol. 20, n. 1, pp. 73-80.

Baydas, G., Tuzcu, M., Yasar, A., Baydas, B. (2004) Early changes in glial reactivity and lipid peroxidation in diabetic rat retina: effects of melatonin. Acta Diabetol., vol. 41, n. 3, pp. 123-128.

Bergmeyer, H.U. (1974). Cholesterol and Esterified Cholesterol. In Methods of Enzymatic Analysis, Verlag Chemie, vol. 4, second ed., pp. 1890 – 1893.

Brusque, A.M., Terracciano, S.T., Fontella, F.U., Vargas, C.. Da Silva, C.G.; Wyse, A.T., Trindade, V.M.; Wannmacher, C.M. and Wajner, M. (1998). Chronic administration of propionic acid reduces ganglioside N-acetylneuraminic acid concentration in cerebellum of young rats. J. Neurol. Sci., vol. 158, n. 2, pp. 121-124.

Busch, N.A., Yarmush, M.L. and Toner, M. (1998) A theoretical formalism for aggregation of peroxidized lipids and plasma membrane stability during photolysis. Biophys. J., vol. 75, n. 6, pp. 2956-2970.

Cherkin, A., Eckardt, M.J. and Gerbrandt, L.K. (1976). Memory: proline induces retrograde amnesia in chicks. Science, vol. 193, pp. 242–244.

Crespo, P.M., Zurita, A.R., Giraudo, C.G., Maccioni, H.J. and Daniotti, J.L. (2004). Ganglioside glycosyltransferases and newly synthesized gangliosides are excluded from detergent-insoluble complexes of Golgi membranes. Biochem. J., vol. 377, pp.561–568.

Delwing, D., Bavaresco, C.S., Chiarani, F., Wannmacher, C.M.D., Wajner, M., Dutra-Filho, C.S. and Wyse, A.T.S. (2003a). In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. Brain Res., vol. 991, pp. 180–186.

Delwing, D., Bavaresco, C.S., Monteiro, S.C., Matté, C., Netto, C.A. and Wyse, A.T. (2006a). alpha-Tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav. Brain Res., vol. 168, n. 2, pp. 185-189.

Delwing, D., Bavaresco, C.S., Wannmacher, C.M.D., Wajner, M., Dutra-Filho, C.S. and Wyse, A.T.S. (2003b). Proline induces oxidative stress in cerebral cortex of rats. Int. J. Dev. Neurosci., vol. 21, pp. 105–110.

Delwing, D., Chiarani, F., Bavaresco, C.S., Wannmacher, C.M., Wajner, M., Dutra-Filho, C.S. and Wyse, A.T. (2005a). Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci. Res., vol. 52, n. 1, pp. 69-74.

Delwing, D., Chiarani, F., Delwing, D, Bavaresco, C.S., Wannmacher, C.M.D., Wajner, M. and Wyse, A.T.S. (2003c). Proline reduces acetylcholinesterase activity in cerebral cortex of rats. Metab. Brain Dis., vol. 18, pp. 79–86.

Delwing, D., Chiarani, F., Wannmacher, C.M., Wajner, M. and Wyse, A.T. (2005b). Effect of hyperprolinemia on acetylcholinesterase and butyrylcholinesterase activities in rat. Amino Acids, vol. 28, n. 3, pp. 305-308.

Delwing, D.; Delwing, D.; Chiarani, F.; Kurek, A.G.; Wyse, A.T. (2007a) Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int. J. Dev. Neurosci. vol.25, n.1, pp.17-22.

Delwing, D., Delwing, D., Sarkis, J.J. and Wyse, A.T. (2006b). Proline induces alterations in nucleotide hydrolysis in rat blood serum. Mol. Cell Biochem., vol. 292, n. 1-2, pp. 139-144.

Delwing, D., Delwing, D., Sarkis, J.J. and Wyse, A.T. (2007b). Proline induces alterations on nucleotide hydrolysis in synaptosomes from cerebral cortex of rats. Brain Res., vol. 1146, pp. 210-215.

Flynn, M.P., Martin, M.C., Moore, P.T., Stafford, J.A., Fleming, G.A. and Phang, J.M. (1989). Type II hyperprolinemia in a pedigree of Irish travelers (nomads). Arch. Dis. Child, vol. 64, pp. 1699–1707.

Folch, J., Leel, M. and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., vol. 226, pp. 497 – 509.

Fremeau, R.T., Caron, M.G. and Blakely, R.D. (1992). Molecular cloning and expression of a high affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. Neuron, vol. 8, pp. 915–926.

Fremeau, R.T., Chan, J., Pohorille, A., Nadler, J.V., Milner, T.A. and Pickel, V.M. (1995). Mammalian brain-specific L-proline transporter: preferential localization of transporter protein to glutamatergic nerve terminals in rat forebrain. Soc. Neurosci. Abstr., vol. 21, pp. 2062.

Gil, C., Cubí, R., Blasi, J. and Aguilera, J. (2006) Synaptic proteins associate with a sub-set of lipid rafts when isolated from nerve endings at physiological temperature. Biochem Biophys. Res. Commun., vol. 348, pp. 1334–1342.

Harder, T. and Simons, K. (1997). Caveolae, DIGs, and the dynamics of sphingolipid cholesterol microdomains. Curr. Opin. Cell Biol., vol. 9, pp. 534- 542.

Irwin, C. and Irwin, L.N. (1979). A simple method for ganglioside isolation from small amounts of tissue. Anal. Biochem, vol. 94, pp. 335 – 339.

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

Lake, B.D. and Goodwin, H.J. (1976). Lipids. In Chromatographic and Eletrophoretic Techniques. Paper and Thin Layer Chromatography, Smith, I., Seakins, J.W.T. (eds.), William Neinemann Medical Books Ltd., London, vol.1, pp. 345 – 366.

Miettinen, T. and Takki-Luukkainen, I.T. (1959). Use of butyl acetate in determination of sialic acid. Acta Chem. Scand., vol. 13, pp. 856 – 858.

Moreira, J.C.F., Wannmacher, C.M.D., Costa, S.M. and Wajner, M. (1989). Effect of proline administration on rat behavior in aversive and nonaversive tasks. Pharmacol. Biochem. Behav., vol. 32, pp. 885–890.

Morgan, B.L. and Naismith, D.J. (1982). The effect of early postnatal undernutrition on the growth and development of the rat brain. Br. J. Nutr., vol. 48, n. 1, pp. 15-23.

Nadler, J.V. (1987). Sodium-dependent proline uptake in the rat hippocampal formation: association with ipsilateral – commissural projections of CA3 pyramidal cells. J. Neurochem., vol. 49, pp. 1155– 1160.

Nadler, J.V., Bray, S.D. and Evenson, D.A. (1992). Autoradiographic localization of proline uptake in excitatory hippocampal pathways. Hippocampus, vol. 2, pp. 269–278.

Nagai, Y., (1995). Functional roles of gangliosides in bio-signaling. Behav. Brain Res., vol. 66, pp. 99-104.

Nores, G.A., Mitzumari, R.K. and Kremer, D.M. (1994). Chromatographic tank designed to obtain highly reproducible high-performance thin-layer chromatograms of gangliosides and neural glycosphingolipds. J. Chromatogr. A., vol. 686, pp. 155 – 157.

Ohvo-Rekilä , H., Ramstedt, B., Leppimäki, P. and Slotte, J.P. (2002). Cholesterol interactions with phospholipids in membranes. Prog. Lipid Res., vol. 41, pp. 66-97.

Ohtani, Y., Tamai, Y., Ohnuki, Y. and Miura, S. (1996). Ganglioside alterations in the central and peripheral nervous system of patients with Creutzfeldt-Jakob disease. Neurodegeneration , vol. 5, pp. 331-338.

Pekny, M., Wilhelmsson, U., Bogestål, Y.R. and Pekna, M. (2007). The role of astrocytes and complement system in neural plasticity. Int. Rev. Neurobiol., vol. 82, pp. 95-111.

Peterson, G.L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem., vol. 83, n. 2, pp.p346-56.

Phang, J.M., Hu, C.A. and Valle, D. (2001). Disorders of proline and hydroxyproline metabolism. In The Metabolic and Molecular Bases of Inherited Disease, Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (eds.), McGraw-Hill, New York, 8th ed., pp. 1821 – 1838.

Pontes, Z.L., Oliveira, L.S., Bavaresco, C.S., Dutra-Filho, C.S., Wajner, M., Wannmacher, C.M.D. and Wyse, A.T.S. (1999). Proline administration decreases Na+,K+-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. Metab. Brain Dis., vol. 26, pp. 1321–1326.

Pontes, Z.L., Oliveira, L.S., Franzon, R., Wajner, M., Wannmacher, C.M.D. and Wyse, A.T.S. (2001). Inhibition of Na+,K+-ATPase activity from rat hippocampus by proline. Neurochem. Res., vol. 26, pp. 1321– 1326.

Qi, Y. and Xue, Q.-M. (1991). Ganglioside levels in hipoxic brains from neonatal and premature infants. Mol. Chem. Neuropathol., vol. 14, pp. 87-97.

Ramirez, M.R., Muraro, F., Zylbersztejn, D.S., Abel, C.R., Arteni, N.S., Lavinsky, D., Netto, C.A. and Trindade, V.M. (2003). Neonatal hypoxia-ischemia reduces ganglioside, phospholipid and cholesterol contents in the rat hippocampus. Neurosci. Res., vol. 46, n. 3, pp. 339-347.

Rhoads, D.E., Peterson, N.A. and Raghupathy, E. (1983). Selective inhibition of synaptosomal proline uptake by leucine and methionine enkephalins. J. Biol. Chem., vol. 258, pp. 12233– 12237.

Simons, K. and Toomre D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. vol.1, pp.31–39.

Skoza, L. and Mohos, S. (1976). Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Application to sialic acid assay in analytical de-O-acetylation. Biochem J., vol. 159, n. 3, pp. 457-462.

Stefanello, F.M., Kreutz, F., Scherer, E.B., Breier, A.C., Vianna, L.P., Trindade, V.M. and Wyse, A.T. (2007) Reduction of gangliosides, phospholipids and cholesterol content in cerebral cortex of rats caused by chronic hypermethioninemia. Int. J. Dev. Neurosci., vol. 25. n. 7, pp. 473-477.

Suzuki, T. (2002). Lipid rafts at postsynaptic sites: distribution, function and linkage to postsynaptic density. Neurosci. Res., vol. 44, pp. 1- 9.

Svennerholm L. (1963). Chromatographic separation of human brain gangliosides. J. Neurochem., vol. 10, pp. 613 – 623.

Svennerholm, L. (1957). Quantitative estimation of sialic acids: a colorimetric resorcinol – hydrochloric acid method. Biochim. Biophys. Acta, vol. 24, pp. 604 – 611.

Trindade, V.M., Perry, M.L. and Bernard, E.A. (1992). Gangliosides and sialoglycoproteins in hypothalamus of normal, postnatal, and pre- and postnatal protein undernourished rats. J. Neurol. Sci., vol. 107, n. 1, pp. 93-97.

Trindade, V.M., Brusque, A.M., Raasch, J.R., Pettenuzzo, L.E., Rocha, H.P., Wannmacher, C.M. and Wajner, M. (2002). Ganglioside alterations in the central nervous system of rats chronically injected with methylmalonic and propionic acids. Metab. Brain. Dis., vol. 17, n. 2, pp. 93-102.

Van Harreveld, A. and Fifkova´, E. (1973). Effects of amino acids on the isolated chicken retina, and on its response to glutamate stimulation. J. Neurochem., vol. 20, pp. 947– 962.

Wajner, M., Brites, E.C., Dutra, J.C., Buchalter, M.S., Pons, A.H., Pires, R.F., Wannmacher, L.E., Rosa Júnior, A., Trindade, V.M. and Wannmacher, C.M. (1988). Diminished concentrations of ganglioside N-acetylneuraminic acid (G-NeuAc) in cerebellum of young rats receiving chronic administration of methylmalonic acid. J. Neurol. Sci., vol. 85, n. 2, pp. 233-238.

Williams, M.A. and McCluer, R.H. (1980). The use of Sep-Pak C18 cartridges during the isolation of gangliosides. J. Neurochem. vol. 35, pp 266-269.

Wyse, A.T.S., Bolognesi, G., Brusque, A.M., Wajner, M. and Wannmacher, C.M.D. (1995). Na+,K+-ATPase activity in the synaptic plasma membrane from the cerebral cortex of rats subjected to chemically induced phenylketonuria. Med. Sci. Res., vol. 23, pp. 261–263.

Zeller, C.B. and Marchase, R.B. (1992). Gangliosides as modulators of cell function. Am. J. Physiol., vol. 262, n. 6, pt. 1, pp. C1341- C1355.

Table I. Ganglioside distribution in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats after chronic administration of proline (HP).

Gangliosides	Right cortex		Left cortex		Right hippocampus		Left hippocampus		Hypothalamus		Cerebellum	
	Control	HP	Control	HP	Control	HP	Control	HP	Control	HP	Control	HP
GM1	0.28±0.01	0.52±0.05*	0.56±0.01	0.76±0.10	0.39±0.07	0.50±0.04*	0.39±0.10	0.95±0.27*	0.19±0.02	0.26±0.02	0.09±0.02	0.16±0.09
GD1a	0.69±0.03	1.62±0.07*	1.39±0.04	1.70±0.15*	1.03±0.10	1.41±0.09*	1.15±0.10	1.50±0.19*	0.72±0.10	0.70±0.08	0.69±0.10	0.63±0.07
GD1b	0.35±0.04	0.80±0.11*	0.60±0.05	0.90±0.10*	0.44±0.07	0.89±0.08*	0.87±0.13	0.97±0.07*	0.43±0.09	0.46±0.09	0.29±0.03	0.18±0.06
GT1b	0.41±0.07	1.08±0.11*	0.77±0.07	1.15±0.22*	0.65±0.10	1.17±0.15*	0.87±0.05	1.32±0.15*	0.56±0.05	0.50±0.10	0.44±0.06	0.42±0.03

Individual ganglioside values (nmol NANA/mg structure weight) were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NANA. The percentages were obtained by scanning densitometry of the TLC. Values are expressed as mean ± standard error for three independent TLC migrations. (*) proline value different from control ($p < 0.05$) using Student's *t*-test.

Table II. Ganglioside species distribution in detergent-soluble membrane microdomains (DSM) and detergent-resistant membrane microdomains (DRM) of synaptic membranes of cerebral cortex of rats after chronic administration of proline (HP).

Gangliosides	DSM		DRM	
	Control	HP	Control	HP
GM1	1.92±1.03	0.46±0.04*	7.47±2.17	1.87±0.36*
GD1a	5.38±2.90	0.93±0.07*	17.42±5.07	2.85±0.54*
GD1b	2.27±1.22	0.15±0.01*	4.63±1.35	1.67±0.32*
GT1b	4.02±2.26	0.86±0.07*	10.51±3.06	8.72±1.65*

Individual ganglioside values (pmol NANA/μg protein) were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NANA. The percentages were obtained by scanning densitometry of the TLC. Values are expressed as mean ± standard error for two independent TLC migrations. (*) proline value different from control ($p < 0.01$) using Student's *t*-test.

Legends

Fig. 1. Effects of chronic administration of proline on ganglioside (A) phospholipid (B) and cholesterol (C) contents in cerebral cortex, hippocampus, hypothalamus and cerebellum of rats. Bars represent mean ± S.E. (standard error of mean) of 14 determinations (duplicates from n = 7). Ganglioside contents are expressed in nmol NANA/ mg structure weight, phospholipid contents are expressed in nmol Pi/ mg structure weight and cholesterol contents are expressed in μg cholesterol/ mg structure weight. C, control; P, hyperprolinemic; RCX, right cortex; LCX, left cortex; RHC, right hippocampus; LHC, left hippocampus; HT, hypothalamus; CB, cerebellum.

(*) proline value different from control ($p < 0.05$) using Student's *t*-test.

Fig. 2. Effects of chronic administration of proline on ganglioside contents in detergent-soluble (DSM) and detergent-insoluble (resistant) membrane microdomain's (DRM) of synaptic membrane from rat cerebral cortex. Bars represent mean ± S.E. (standard error of mean) of 6 determinations (duplicates from n = 3). Ganglioside contents are expressed in nmol NANA/μg protein. C, control; P, hyperprolinemic; DSM, detergent-soluble membrane microdomains; DRM, detergent-resistant membrane microdomains. (a) DRM value different from DSM ($p < 0.01$) using Student's *t*-test. (*) proline value different from control ($p < 0.01$) using Student's *t*-test.

Fig.3. Ganglioside profiles from detergent-soluble membrane (DSM) and detergent-resistant membrane microdomains (DRM) of synaptic membranes from rat cortex cerebral after chronic administration of proline. HPTLC was developed and the ganglioside bands were revealed as described in experimental procedures. The positions of co-chromatographed ganglioside standards are indicated. Gangliosides were named as recommend by Svennerholm (1963). C, control; P, treated with proline. This is a representative experiment from two independent HPTLC migrations.

Figure 1

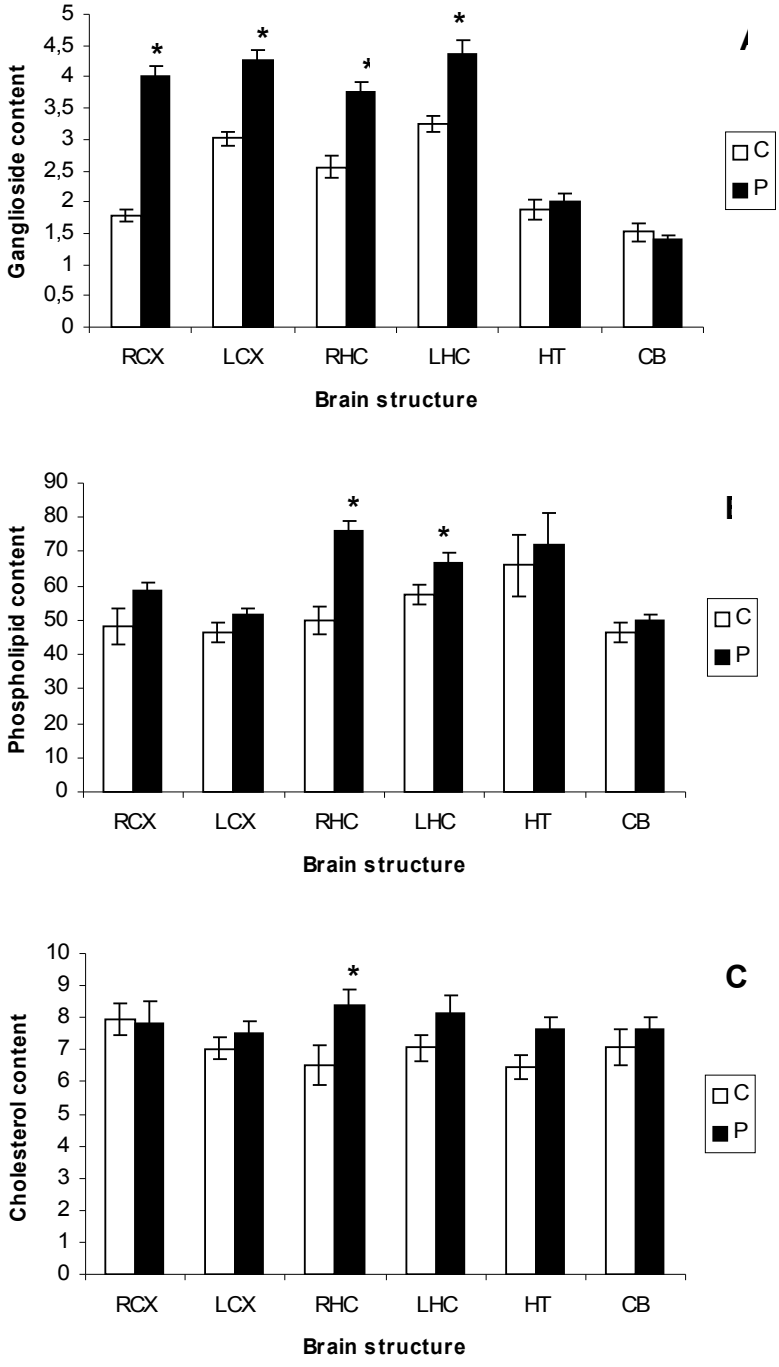

A
Ganglioside content
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
RCX
LCX
RHC
LHC
HT
CB
Brain structure
C
P
B
Phospholipid content
90
80
70
60
50
40
30
20
10
0
RCX
LCX
RHC
LHC
HT
CB
Brain structure
C
P
C
Cholesterol content
10
9
8
7
6
5
4
3
2
1
0
RCX
LCX
RHC
LHC
HT
CB
Brain structure
C
P

Figure 2

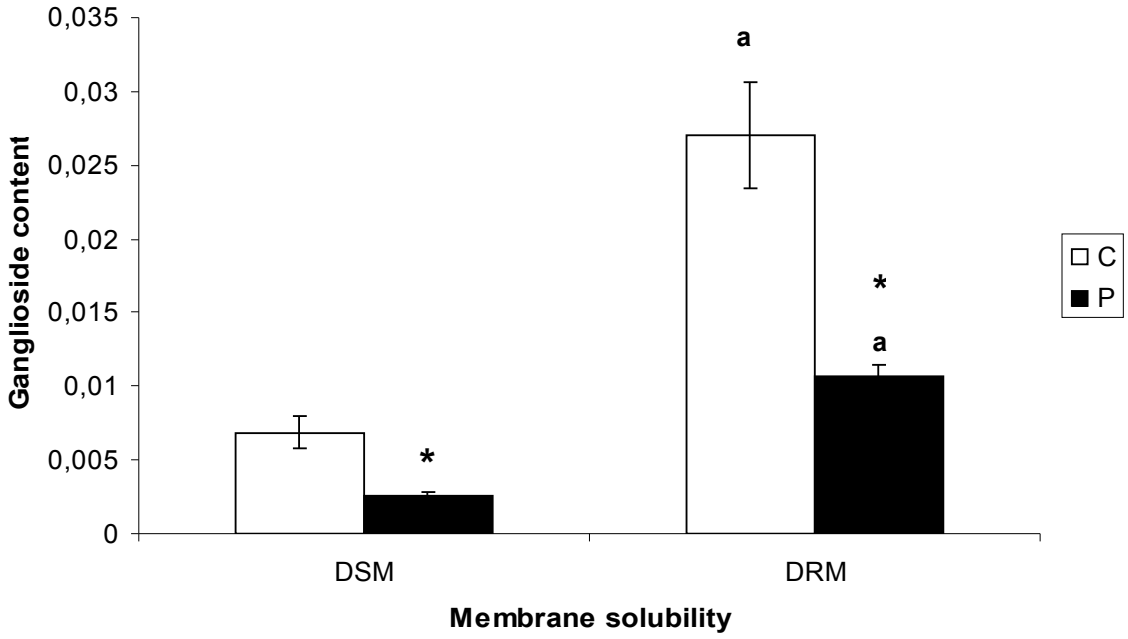
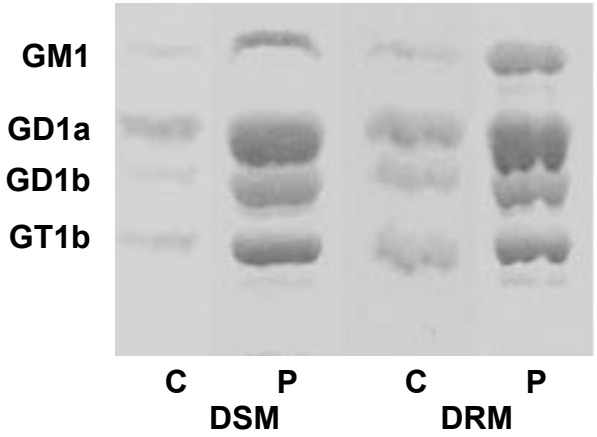


Figure 3



5. DISCUSSÃO

Neste estudo foram avaliados o conteúdo total e o perfil de gangliosídios, assim como a concentração de fosfolipídios e de colesterol em córtex cerebral, hipocampo, hipotálamo e cerebelo de ratos submetidos a um modelo crônico de hiperprolinemia. Além disso, foram determinados o conteúdo total e o perfil de gangliosídios nas frações de membranas solúveis e resistentes a detergente, isoladas de membrana sináptica de córtex cerebral. Estudos anteriores tratando da composição de membranas neurais em diversos modelos de dano ao sistema nervoso central mostraram perda de membrana, representada pela redução no conteúdo de lipídios (MORGAN and NAISMITH, 1982; WAJNER et al., 1988; QI and XUE, 1991; TRINDADE et al., 1992; OHTANI et al., 1996; BRUSQUE et al., 1998; TRINDADE et al., 2002; STEFANELLO et al., 2007).

A esse respeito, nossos resultados mostraram um aumento nos lipídios de membrana (gangliosídios, fosfolipídios e colesterol). Pela análise de variância de duas vias foi demonstrado um efeito do tratamento com prolina, um efeito da estrutura cerebral estudada e um efeito diferencial do tratamento, de acordo com a estrutura e o hemisfério a que ela pertence, sobre o conteúdo total de gangliosídios. Em relação ao conteúdo de fosfolipídios, houve um efeito do tratamento com prolina independente da estrutura cerebral e sobre o conteúdo de colesterol houve apenas efeito do tratamento.

Também, foi demonstrada uma variação no conteúdo total de gangliosídio no grupo controle de acordo com o hemisfério cerebral . Estudos anteriores em córtex sensorimotor (NALIVAEVA et al., 1995) mostraram não haver variação desses

lipídios, de acordo com o hemisfério. Entretanto, neste trabalho foi utilizado córtex total de rato, que talvez no somatório das diferentes sub-áreas corticais revele variações fisiológicas, quanto aos hemisférios cerebrais, que as mesmas isoladamente não apresentem.

O perfil de gangliosídios não foi alterado nas estruturas estudadas, embora as espécies gangliosídicas tenham variado individualmente com o tratamento e de acordo com a região cerebral.

Como já relatado na literatura, os microdomínios resistentes a detergente mostraram-se enriquecidos em gangliosídios, quando comparados aos microdomínios solúveis a detergente (HARDER and SIMONS, 1997; GIL et al., 2006). Da mesma forma, isto foi detectado nos microdomínios de membrana sináptica obtidos de córtex cerebral tanto de ratos controles como de hiperprolinêmicos. Foi observada uma diminuição no conteúdo de gangliosídios em ambas as frações (solúvel e resistente a detergente) com a administração de prolina, afetando de maneira diferente as frações de membrana. Não houve alteração no perfil dos gangliosídios estudados, embora as espécies tenham variado de acordo com o conteúdo total. Dentro do que sabemos, este é o primeiro estudo relacionando microdomínios de membrana e uma desordem metabólica de aminoácidos.

Até o momento, não temos uma explicação definitiva para a elevação no conteúdo de lipídios de membrana em animais submetidos à administração crônica de prolina. Postulamos que um mecanismo de gliose reativa possa contribuir no aumento verificado, uma vez que as células gliais têm características similares às membranas plasmáticas dos neurônios, e que surgem como

conseqüência de um dano tecidual (PEKNY et al., 2007), como por exemplo, o estresse oxidativo desencadeado pela administração de Prolina (BAYDAS et al., 2004; DELWING et al., 2003a; DELWING et al., 2003b). Este mecanismo pode ser específico para cada região cerebral, e para cada hemisfério, já que a análise estatística mostra que as estruturas do lado direito tiveram variação semelhante entre si, assim como as estruturas do lado esquerdo.

A redução no conteúdo dos gangliosídios em microdomínios solúveis e resistentes a detergente, isolados de membrana sináptica de córtex cerebral, pode representar que a membrana na região da sinapse sofra modificações específicas secundárias à administração crônica de prolina e indicar perda de componentes de membrana por peroxidação lipídica, uma vez que foi relatado que a administração aguda de prolina aumenta o estresse oxidativo em córtex cerebral (DELWING et al., 2003a; DELWING et al., 2003b). A diminuição dos gangliosídios presentes nos microdomínios da membrana sináptica sugere uma mudança na dinâmica normal dos lipídios desta membrana, representando uma perda de estabilidade e um dano à função normal dos neurônios (BUSCH, YARMUSH and TONER, 1998).

Embora as concentrações de prolina utilizadas em nosso estudo sejam similares às observadas em pacientes hiperprolinêmicos e tenham alterado de maneira significativa os lipídios de membrana, é difícil extrapolar nossa hipótese à patologia em humanos. Entretanto, se for o caso, o resultado do presente estudo demonstra dano à dinâmica normal da membrana neuronal pela administração crônica de prolina pode representar, pelo menos, um dos mecanismos envolvidos no dano neurológico observado na hiperprolinemia tipo II.

Em resumo, mostramos que o córtex cerebral e o hipocampo de ratos submetidos a um modelo crônico de hiperprolinemia apresentaram um distinto aumento nos conteúdos de lipídios de membrana (gangliosídios, fosfolipídios e colesterol), enquanto os microdomínios solúveis e resistentes a detergente, isolados de membrana sináptica de córtex cerebral, apresentaram uma diminuição no conteúdo de gangliosídios. Este último efeito pode expressar a perda de estabilidade e função da membrana, possivelmente, levando à morte celular. De acordo com nossos resultados, parece razoável postular que a perda da função da membrana na região da sinapse pode contribuir para a disfunção neurológica característica da hiperprolinemia. Entretanto, mais estudos investigando pacientes hiperprolinêmicos devem ser realizados, com a finalidade de avaliar a hipótese de que a alteração nos lipídios de membrana esteja envolvida na patologia deste distúrbio metabólico, assim como já relatado para os pacientes com doença de Alzheimer (MOLANDER-MELIN et al., 2005).

6. CONCLUSÕES

- O córtex cerebral e o hipocampo de ratos submetidos a um modelo crônico de hiperprolinemia apresentaram um aumento nos conteúdos de lipídios de membrana (gangliosídios, fosfolipídios e colesterol). Esse aumento pode ser conseqüência de uma reação de gliose ao dano tecidual causado pelas condições do modelo.

- Os microdomínios solúveis e resistentes a detergente, isolados de membrana sináptica de córtex cerebral, apresentaram uma diminuição no conteúdo de gangliosídios. Esta redução pode representar que a membrana na região da sinapse sofra modificações específicas com a administração crônica de prolina e indicar perda de componentes de membrana por peroxidação lipídica, uma vez que foi relatado que a administração aguda de prolina aumenta o estresse oxidativo em córtex cerebral.

- Os principais gangliosídios encontrados no SNC foram detectados nas estruturas cerebrais estudas, assim como, nas frações solúvel e resistente a detergente obtida de membrana sináptica de córtex (GM1, GD1a, Gd1b e GT1b). Embora os perfis de gangliosídios não tenham sido aparentemente modificados, as quantidades absolutas das espécies foram alteradas tanto nos extratos totais de córtex e hipocampo, assim como nos microdomínios de membrana obtidos do córtex.

- De acordo com nossos resultados, parece razoável postular que a perda de lipídios na membrana sináptica pode contribuir para a disfunção neurológica característica da hiperprolinemia. Entretanto, mais estudos investigando pacientes hiperprolinêmicos devem ser realizados, com a finalidade de avaliar a hipótese de que a alteração nos lipídios de membrana esteja envolvida na patologia deste distúrbio metabólico.

- Até onde sabemos, este é o primeiro estudo relacionando microdomínios de membrana e uma desordem metabólica de aminoácidos.

7. PERSPECTIVAS

Dentre as possíveis perspectivas deste trabalho destacam-se:

- O tratamento dos animais com substâncias de ação antioxidante, como a vitamina E (tocoferol), concomitante ao modelo de injeção crônica de prolina e análise dos efeitos sobre os componentes lipídicos dos microdomínios de membrana obtidos de membrana sináptica de córtex cerebral.
- A verificação da co-localização de proteínas associadas aos microdomínios, como a Na^+, K^+ ATPase e dos gangliosídios analisados no modelo estudado.
- A verificação do processo de gliose reativa, através de imunohistoquímica para a proteína glial fibrilar ácida (GFAP).

8. REFERÊNCIAS BIBLIOGRÁFICAS

AGRANOFF, B.W.; HAJRA, A.K. Lipids. In SIEGEL, G.J.; AGRANOFF, B.W.; ALBERS, R.W.; MOLINOFF, P.B., **Basic Neurochemistry**, New York: Raven Press, 1993. p. 97-116.

ALCHORN, A. L. Lipid rafts observed in cell membranes. **Science & Technology Review**, mai. 2007. Disponível em http://www.llnl.gov/str/May07/Ratto.html, acessado dia 10 de outubro de 2007, às 10:00.

ALLEN, J.A.; HALVERSON-TAMBOLI, R.A.; RASENICK, M.M. Lipid raft microdomains and neurotransmitter signalling. **Nat. Rev. Neurosci.** v.8, n.2, p.128-140, fev. 2007.

ANDO, S. Gangliosides in the nervous system. **Neurochem. Int.** v.5, n.5, p.507-537, nov. 1983.

BARTLETT, G.R. Phosphorus assay in column chromatography. **J. Biol. Chem.** v.234, n.3, p. 466 – 468, mar. 1959.

BAVARESCO, C.S.; STRECK, E.L.; NETTO, C.A.; WYSE, A.T. Chronic hyperprolinemia provokes a memory deficit in the Morris water maze task. **Metab. Brain Dis.** v.20, n.1, p.73-80, mar. 2005.

BAYDAS, G.; TUZCU, M.; YASAR, A.; BAYDAS, B. Early changes in glial reactivity and lipid peroxidation in diabetic rat retina: effects of melatonin. **Acta Diabetol.** v.41, n.3, p.123-128, set. 2004.

BERG, J.M.; TYMOCZKO, J.L.; STRYER, L. **Biochemistry.** 5th ed. New York: W.H. Freeman and company, 2003. 974p.

BERGMEYER, H.U. Cholesterol and Esterified Cholesterol. In **Methods of Enzymatic Analysis.** Verlag Chemie, v.4, 2nd ed., 1974. p. 1890 – 1893.

BRUSQUE, A.M.; TERRACCIANO, S.T.; FONTELLA, F.U.; VARGAS, C.; DA SILVA, C.G.; WYSE, A.T.; TRINDADE, V.M.; WANNMACHER, C.M.; WAJNER, M. Chronic administration of propionic acid reduces ganglioside N-acetylneuraminic acid concentration in cerebellum of young rats. **J. Neurol. Sci.** v.158, n.2, p.121-124, jun. 1998.

BUSCH, N.A.; YARMUSH, M.L.; TONER, M. A theoretical formalism for aggregation of peroxidized lipids and plasma membrane stability during photolysis. **Biophys. J.** v.75, n.6, p.2956-2970, dez. 1998.

CHEN, X.; MORRIS, R.; LAWRENCE, M.J.; QUINN, P.J. The isolation and structure of membrane lipid rafts from rat brain. **Biochimie** v.89, n.2, p.192-196, fev. 2006.

CHERKIN, A.; ECKARDT, M.J.; GERBRANDT, L.K. Memory: proline induces retrograde amnesia in chicks. **Science** v.193, n.4249, p.242–244, jul. 1976.

CHRONISTER, R.B.; HARDY, S.G.P. The limbic system. In HAINES, D.E. (Ed.) **Fundamental neuroscience.** New York: Churchil Livingstone Inc., 1997. p.443 – 454.

CRESPO, P.M.; ZURITA, A.R.; GIRAUDO, C.G.; MACCIONI, H.J.; DANIOTTI, J.L. Ganglioside glycosyltransferases and newly synthesized gangliosides are excluded from detergent-insoluble complexes of Golgi membranes. **Biochem. J.** v.377, p.561–568, fev. 2004.

DALSKOV, S.M.; IMMERDAL, L.; NIELS-CHRISTIANSEN, L.L.; HANSEN, G.H.; SCHOUSBOE, A.; DANIELSEN, E.M. Lipid raft localization of GABA A receptor and Na+, K+-ATPase in discrete microdomain clusters in rat cerebellar granule cells. **Neurochem Int.** v.46, n.6, p.489-499, mai. 2005.

DELWING, D.; BAVARESCO, C.S.; CHIARANI, F.; WANNMACHER, C.M.D.; WAJNER, M.; DUTRA-FILHO, C.S.; WYSE, A.T.S. In vivo and in vitro effects of proline on some parameters of oxidative stress in rat brain. **Brain Res.** v.991, n.1-2, p.180–186, nov. 2003a.

DELWING, D.; BAVARESCO, C.S.; MONTEIRO, S.C.; MATTÉ, C.; NETTO, C.A.; WYSE, A.T. alpha-Tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. **Behav. Brain Res.** v.168, n.2, p.185-189, abr. 2006a.

DELWING, D., BAVARESCO, C.S., WANNMACHER, C.M.D., WAJNER, M., DUTRA-FILHO, C.S. AND WYSE, A.T.S. Proline induces oxidative stress in cerebral cortex of rats. **Int. J. Dev. Neurosci.** v.21, n.2, p.105–110, abr. 2003b.

DELWING, D.; CHIARANI, F.; BAVARESCO, C.S.; WANNMACHER, C.M.; WAJNER, M.; DUTRA-FILHO, C.S.; WYSE, A.T. Protective effect of antioxidants on brain oxidative damage caused by proline administration. **Neurosci. Res.** v.52, n.1, p.69-74, mai. 2005a.

DELWING, D.; CHIARANI, F.; DELWING, D.; BAVARESCO, C.S.; WANNMACHER, C.M.D.; WAJNER, M.; WYSE, A.T.S. Proline reduces acetylcholinesterase activity in cerebral cortex of rats. **Metab. Brain Dis.** v.18, n.1, p.79–86, mar. 2003c.

DELWING, D.; CHIARANI, F.; WANNMACHER, C.M.; WAJNER, M.; WYSE, A.T. Effect of hyperprolinemia on acetylcholinesterase and butyrylcholinesterase activities in rat. **Amino Acids**, v.28, n.3, p.305-308, mai. 2005b.

DELWING, D.; DELWING, D.; CHIARANI, F.; KUREK, A.G.; WYSE, A.T. Proline reduces brain cytochrome c oxidase: prevention by antioxidants. **Int. J. Dev. Neurosci.** v.25, n.1, p.17-22, fev. 2007a.

DELWING, D.; DELWING, D.; SARKIS, J.J.; WYSE, A.T. Proline induces alterations in nucleotide hydrolysis in rat blood serum. **Mol. Cell Biochem.** v.292, n.1-2, p.139-144, nov. 2006b.

DELWING, D.; DELWING, D.; SARKIS, J.J.; WYSE, A.T. Proline induces alterations on nucleotide hydrolysis in synaptosomes from cerebral cortex of rats. **Brain Res**. v.1146, p.210-215, mai. 2007b.

FLYNN, M.P.; MARTIN, M.C.; MOORE, P.T.; STAFFORD, J.A.; FLEMING, G.A.; PHANG, J.M. Type II hyperprolinemia in a pedigree of Irish travelers (nomads). **Arch. Dis. Child** v.64, n.12, p.1699–1707, dez. 1989.

FOLCH, J.; LEEL, M.; SLOANE-STANLEY, G.H. A simple method for the isolation and purification of total lipids from animal tissues. **J. Biol. Chem.** v.226, p.497 – 509, mai. 1957.

FREMEAU, R.T.; CARON, M.G.; BLAKELY, R.D. Molecular cloning and expression of a high affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. **Neuron** v.8, n.5, p.915–926, mai. 1992.

FREMEAU, R.T.; CHAN, J.; POHORILLE, A.; NADLER, J.V.; MILNER, T.A.; PICKEL, V.M. Mammalian brain-specific L-proline transporter: preferential localization of transporter protein to glutamatergic nerve terminals in rat forebrain. **Soc. Neurosci. Abstr.** v.21, p.2062, 1995.

GIL, C.; CUBÍ, R.; BLASI, J.; AGUILERA, J. Synaptic proteins associate with a sub-set of lipid rafts when isolated from nerve endings at physiological temperature. **Biochem Biophys. Res. Commun.** n.348. p1334–1342, ago. 2006.

HAINES, D.E. Orientation to the central nervous system. In HAINES, D.E. (Ed.) **Fundamental neuroscience.** New York: Churchil Livingstone Inc., 1997. p. 2 – 8.

HAINES, D.E.; MIHAILOFF, G.A.; BLOEDEL, J. R. The cerebellum. In HAINES, D.E. (Ed.) **Fundamental neuroscience.** New York: Churchil Livingstone Inc., 1997. p. 379 – 398.

HANZAL-BAYER M.F; HANCOCK, J.F. Lipid rafts and membrane traffic. **FEBS Lett.** v.581, n.11, p.2098-2104, mai. 2007.

HARDER, T.; SIMONS, K. Caveolae, DIGs, and the dynamics of sphingolipid cholesterol microdomains. **Curr. Opin. Cell Biol.** v.9, n.4, p.534- 542, ago. 1997.

HERING, H.; LIN, C.C.; SHENG, M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. **J Neurosci.** v.23, n.8, p.3262-3271, abr. 2003.

IRWIN, C.C.; IRWIN, L.N. A simple rapid method for ganglioside isolation from small amounts of tissue. **Anal. Biochem.** , v.94, pp. 335-339, 1979.

JACOBSON, K.; MOURITSEN, O.G.; ANDERSON, R.G.W. Lipid rafts: at a crossroad between cell biology and physics. **Nat. Cell. Biol.** V.9, n.1, p.7-14, jan. 2007.

JONES, D.H.; MATUS, A.I. Isolation of plasma synaptic membrane from brain by combination flotation-sedimentation density gradient centrifugation. **Biochim. Biophys. Acta** v.356, p.276–287, 1974.

LAKE, B.D.; GOODWIN, H.J. Lipids. In SMITH, I.; SEAKINS, J.W.T. (Eds.) **Chromatographic and Eletrophoretic Techniques. Paper and Thin Layer Chromatography**, London:William Neinemann Medical Books Ltd., 1976, v.1, p.345 – 366.

LYNCH, J.C. The cerebral cortex. In HAINES, D.E. (Ed.) **Fundamental neuroscience.** New York: Churchil Livingstone Inc., 1997. p. 455 – 470.

MIETTINEN, T.; TAKKI-LUUKKAINEN, I.T. Use of butyl acetate in determination of sialic acid. **Acta Chem. Scand.** v. 13, p. 856 – 858, 1959.

MIHAILOFF, G.A.; HAINES, D.E. The Diencephalon. In HAINES, D.E. (Ed.) **Fundamental neuroscience.** New York: Churchil Livingstone Inc., 1997. p. 189 – 202.

MOLANDER-MELIN, M.; BLENNOW, K.; BOGDANOVIC, N.; DELLHEDEN, B.; MÅNSSON, J.E.; FREDMAN, P. Structural membrane alterations in Alzheimer brains found to be associated with regional disease development; increased density of gangliosides GM1 and GM2 and loss of cholesterol in detergent-resistant membrane domains. **J. Neurochem.** v.92, n.1, p.171-182, jan. 2005.

MOREIRA, J.C.F.; WANNMACHER, C.M.D.; COSTA, S.M.; WAJNER, M. Effect of proline administration on rat behavior in aversive and nonaversive tasks. **Pharmacol. Biochem. Behav.** v.32, p.885–890, abr.1989.

MORGAN, B.L.; NAISMITH, D.J. The effect of early postnatal undernutrition on the growth and development of the rat brain. **Br J Nutr.** v.48, n.1, p.15-23, jul. 1982.

NADLER, J.V. Sodium-dependent proline uptake in the rat hippocampal formation: association with ipsilateral – commissural projections of CA3 pyramidal cells. **J. Neurochem.** v.49, n.4, p.1155– 1160, out. 1987.

NADLER, J.V.; BRAY, S.D.; EVENSON, D.A. Autoradiographic localization of proline uptake in excitatory hippocampal pathways. **Hippocampus** v.2, n.3, p.269–278, jul. 1992.

NAGAI, Y. Functional roles of gangliosides in bio-signaling. **Behav. Brain Res.** v.66, n.1-2, p.99-104, jan. 1995.

NALIVAEVA, N.; PLESNEVA, A.; CHEKULAEVA, U.; ZHURAVIN, I.; DUBROVSKAYA, N.; KLEMENTJEV, B. Hypoxic hypoxia induces different biochemical changes in the cortex of the right and left hemispheres of rat brain. **Mol. Chem. Neuropathol.** v.25, n.2-3, p.255-263, jun.-ago. 1995.

NELSON, D.L.; COX, M.M. **Lehninger Principles of Biochemistry.** 4. ed. New York: W. E. Freeman, 2005. 1172 p.

NORES, G.A.; MITZUMARI, R.K.; KREMER, D.M. Chromatographic tank designed to obtain highly reproducible high-performance thin-layer chromatograms of gangliosides and neural glycosphingolipds. **J. Chromatogr. A**. v.686, p.155 – 157, 1994.

OHVO-REKILÄ, H.; RAMSTEDT, B.; LEPPIMÄKI, P.; SLOTTE, J.P. Cholesterol interactions with phospholipids in membranes. **Prog. Lipid Res.** v.41, n.1, p.66-97, jan. 2002.

OHTANI, Y.; TAMAI, Y.; OHNUKI, Y.; MIURA, S. Ganglioside alterations in the central and peripheral nervous system of patients with Creutzfeldt-Jakob disease. **Neurodegeneration** v.5, p.331-338, 1996.

PEKNY, M.; WILHELMSSON, U.; BOGESTÅL, Y.R.; PEKNA, M. The role of astrocytes and complement system in neural plasticity. **Int. Rev. Neurobiol.** v.82, p.95-111, ago. 2007.

PETERSON, G.L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. **Anal Biochem.** v.83, n.2, p.346-56, dez. 1977.

PHANG, J.M.; HU, C.A.; VALLE, D. Disorders of proline and hydroxyproline metabolism. In SCRIVER, C.R.; BEAUDET, A.L;, SLY, W.S.; VALLE, D. (eds.), **The Metabolic and Molecular Bases of Inherited Disease**, 8. ed., New York : McGraw-Hill, 2001. p.1821 – 1838.

PIKE, L.J. Rafts defined: a report on the Keystone symposium on lipid rafts and cell function. **J. Lipid Res.** v.47, n.7, p.1597–1598, jul. 2006.

PONTES, Z.L.; OLIVEIRA, L.S.; BAVARESCO, C.S. ; DUTRA-FILHO, C.S.; WAJNER, M.; WANNMACHER, C.M.D.; WYSE, A.T.S. Proline administration decreases Na+,K+-ATPase activity in the synaptic plasma membrane from cerebral cortex of rats. **Metab. Brain Dis.** v.26, n.4, p.1321–1326, dez. 1999.

PONTES, Z.L.; OLIVEIRA, L.S.; FRANZON, R.; WAJNER, M.; WANNMACHER, C.M.D.; WYSE, A.T.S. Inhibition of Na+,K+-ATPase activity from rat hippocampus by proline. **Neurochem. Res.** v.26, n.12, p.1321– 1326, dez. 2001.

QI, Y.; XUE, Q.M. Ganglioside levels in hipoxic brains from neonatal and premature infants. **Mol. Chem. Neuropathol.** v.14, p.87-97, 1991.

RAMIREZ, M.R.; MURARO, F.; ZYLBERSZTEJN, D.S.; ABEL, C.R.; ARTENI, N.S.; LAVINSKY, D.; NETTO, C.A.; TRINDADE, V.M. Neonatal hypoxia-ischemia reduces ganglioside, phospholipid and cholesterol contents in the rat hippocampus. **Neurosci Res.** v.46, n.3, p.339-347, jul. 2003.

RHOADS, D.E.; PETERSON, N.A.; RAGHUPATHY, E. Selective inhibition of synaptosomal proline uptake by leucine and methionine enkephalins. **J. Biol. Chem.** v.258, n.20, p.12233– 12237, out. 1983.

SCHRATTENHOLZ, A.; SOSKIC, V. NMDA receptors are not alone: dynamic regulation of NMDA receptor structure and function by neuregulins and transient cholesterol-rich membrane domains leads to disease-specific nuances of glutamate-signalling. **Curr Top Med Chem.** v.6, n.7, p.663-686, abr. 2006.

SCRIVER, C.R.; BEAUDET, A.L;, SLY, W.S.; VALLE, D. (eds.), **The Metabolic and Molecular Bases of Inherited Disease**, 8. ed., New York : McGraw-Hill, 2001. p.3-45.

SIMONS, K.; TOOMRE D. Lipid rafts and signal transduction. **Nat. Rev. Mol. Cell Biol.** v1, p.31–39, oct. 2000.

SKOZA, L.; MOHOS, S. Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Application to sialic acid assay in analytical de-O-acetylation. **Biochem. J.** v.159, n.3, p.457-462, dez.1976.

STEFANELLO, F.M.; KREUTZ, F.; SCHERER, E.B.; BREIER, A.C.; VIANNA, L.P.; TRINDADE, V.M.; WYSE, A.T. Reduction of gangliosides, phospholipids and cholesterol content in cerebral cortex of rats caused by chronic hypermethioninemia. **Int. J. Dev. Neurosci.** v.25. n.7, p.473-477, ago. 2007.

SUZUKI, T. Lipid rafts at postsynaptic sites: distribution, function and linkage to postsynaptic density. **Neurosci. Res.** v.44, n.1, p.1- 9, set. 2002.

SVENNERHOLM L. Chromatographic separation of human brain gangliosides. **J. Neurochem.** v. 10, p. 613 – 623, set. 1963.

SVENNERHOLM, L. Quantitative estimation of sialic acids: a colorimetric resorcinol – hydrochloric acid method. **Biochim. Biophys. Acta** v. 24, n.3, p. 604 – 611, jun. 1957.

TRINDADE, V.M.; BRUSQUE, A.M.; RAASCH, J.R.; PETTENUZZO, L.E.; ROCHA, H.P.; WANNMACHER, C.M.; WAJNER, M. Ganglioside alterations in the central nervous system of rats chronically injected with methylmalonic and propionic acids. **Metab Brain Dis.** v.17, n.2, p.93-102, jun. 2002.

TRINDADE, V.M.; PERRY, M.L.; BERNARD, E.A. Gangliosides and sialoglycoproteins in hypothalamus of normal, postnatal, and pre- and postnatal protein undernourished rats. **J. Neurol. Sci.** v.107, n.1, p.93-97, jan. 1992.

VAN HARREVELD, A.; FIFKOVÁ, E. Effects of amino acids on the isolated chicken retina, and on its response to glutamate stimulation. **J. Neurochem.** v.20, n.4, p.947– 962, abr. 1973.

WAJNER, M.; BRITES, E.C.; DUTRA, J.C.; BUCHALTER, M.S.; PONS, A.H.; PIRES, R.F.; WANNMACHER, L.E.; ROSA JÚNIOR, A.; TRINDADE, V.M.; WANNMACHER, C.M. Diminished concentrations of ganglioside N-acetylneuraminic acid (G-NeuAc) in cerebellum of young rats receiving chronic administration of methylmalonic acid. **J. Neurol. Sci.** v. 85, n.2, p. 233-238, jun. 1988.

WILLIAMS, M.A.; McCLUER, R.H. The use of Sep-Pak C18 cartridges during the isolation of gangliosides. **J. Neurochem.** v.35, p.266-269, jul. 1980.

WOODS, A.S.; JACKSON, S.N. Brain Tissue Lipidomics: Direct Probing Using Matrix-assisted Laser Desorption/Ionization Mass Spectrometry. **AAPS J.** v.8, n.2, p.E391-E395, jun. 2006.

WYSE, A.T.S.; BOLOGNESI, G.; BRUSQUE, A.M.; WAJNER, M.; WANNMACHER, C.M.D. Na+,K+-ATPase activity in the synaptic plasma membrane from the cerebral cortex of rats subjected to chemically induced phenylketonuria. **Med. Sci. Res.** v. 23, p.261–263, 1995.

ZELLER, C.B.; MARCHASE, R.B. Gangliosides as modulators of cell function. **Am. J. Physiol.** v.262, n.6, Pt.1, p.C1341- C1355, jun. 1992.

9. ANEXO

INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE
The Official Journal of the International Society for Developmental Neuroscience

Guide for Authors

GENERAL

The International Journal of Developmental Neuroscience is devoted to the prompt publication of the results of original research on any aspect of the scientific study of the nervous system. **The type of paper most suitable for publication is that in which new observations are clearly and succinctly reported and in which an attempt is made to make the purpose and outcome of the study meaningful to scientists of other disciplines.**
Detailed and/or very specialized papers will only be considered for publication if the authors have followed the latter suggestion. Since one of the chief aims is to promote communication between neuroscientists, the journal will also include occasional commentaries on specific areas of neuroscience.

The International Editorial Board, which is appointed by the Chief Editor in consultation with the Publications Committee of the **International Society for Developmental Neuroscience**, consists of specialists in all major branches of neuroscience. Each paper is read by at least two Editors or referees. Further opinions are sought if necessary.
Papers reporting original research are considered for publication provided that they describe **significant, new and carefully confirmed findings and that adequate experimental details are given.** Preliminary communications are not accepted. No distinction is made between short and long papers. Accelerated publication of Brief Communications (under 2000 words in length) will be reserved for very novel findings. All papers must be prepared according to the instructions given in Section II.
Commentaries or review articles on a specific topic in neuroscience will normally be published after *an invitation* to the author from the Chief Editor or an Associate Editor. Other prospective authors should obtain the agreement of the Chief Editor before submitting a manuscript. Commentaries are not exhaustive reviews but short articles (3000-10,000 words in length) intended either to draw attention to developments in a specific field for workers in other scientific disciplines, or to bring together observations over a wide area that seem to point in a new direction. Authors should always endeavour to make their commentary understandable to neuroscientists of other disciplines.
Submission of a paper for International Journal of Developmental Neuroscience will be held to imply that it represents original research not previously published (except in the form of an abstract), and that it is not being considered for publication elsewhere in similar form.

SUBMISSION

All manuscripts should be submitted through *International Journal of Developmental Neuroscience's* online submission and peer review website (http://ees.elsevier.com/dn). Authors are requested to submit the text, tables and artwork in electronic form. In a Cover letter, authors should state that the manuscript, or parts of it, have not been and will not be submitted elsewhere for publication.

MANUSCRIPT REQUIREMENTS

Form

Manuscripts should be typewritten with **double or triple spacing** throughout and wide margins at least 2.5 cm wide. If possible, A4 size (206 x 294 mm) paper should be used. *Each manuscript must have a title page* which includes only the title, the authors' names, the laboratory or origin, the name and address of the person to whom proofs should be mailed and any necessary footnotes. Original manuscripts and diagrams are discarded 1 month after publication unless the Publisher is requested to return original material to the author.

Corrections to the proof should be restricted to printer's errors. Substantial alterations may be charged to the author.

The title should be as short as is consistent with clarity. Papers should not be numbered in series, but subtitles are accepted. A list of abbreviations used *in the text* should be put at the bottom of the title page (see 2a and 6).

A running title, not to exceed 56 letter spaces, should be included on a separate sheet.

Pages should be numbered in succession, the title page being page 1.

Tables and figures should be on separate pages placed at the end of the manuscript. Their desired approximate locations should be indicated in the margin of the text.

Footnotes to the text should be used sparingly; where they must be used their locations should be indicated by the symbols *, †, ‡, §, [], ¶, in that order.

Greek characters should be clearly indicated.

Isotopic specifications should conform to the IUPAC system [*Biochem. J.* (1975) **145,** 1-20].

Drug names should be the official or approved names; trade names or common names may be given in parentheses where the drug is first mentioned. The manufacturer's name must be given. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg. Concentrations should be given in terms of molarity, e.g. μM or M, or as unit weight/unit volume solution, stating whether the weight refers to the salt or the active component of the drug. The molecular weight, inclusive of water crystallizations, should be stated if doses are given as unit weight.

Style

Manuscripts should be concisely written in English in a readily understandable style. Authors who need assistantce **before** they submit their article for peer review or **before** it is accepted for publication may find the language editing companies listed here ⇨http://authors.elsevier.com/LanguageEditing.html useful.

Technical jargon, 'laboratory slang' or words not defined in dictionaries should not be used. **Abbreviations should be avoided unless they conform to the instructions** under sub-section 6. They must not be used as a short form for experimental procedures or for concepts. Redundant words, phrases, and sentences should not be used. For example, the captions of Tables and Figures, with or without paraphrasing, should not be repeated in the text.

The Editor reserves the right to revise the wording of manuscripts accepted for publication in *International Journal of Developmental Neuroscience.*

Organization

Each paper must begin with a brief Abstract. It should not exceed 5% of the length of the paper. The first paragraph of the abstract should summarize the results obtained, *the final paragraph should summarize the major conclusions in such a way that a reader not familiar with the techniques used can see any implications for his area of neuroscience*. **Abbreviations must not be used in the Abstract.**

The remaining text of all papers, however short, should be organized in the following four main sections:

(1) An introductory statement should first set 'the scene' for a non-specialist and then continue with the specific reasons for undertaking the investigation. Exhaustive reviews of the literature should be avoided and no attempt should be made to indicate the results obtained. The heading 'Introduction' should be avoided.

(2) *Experimental procedures*. Procedures used should be given in sufficient detail to permit the repetition of the work by others. However, published procedures should only be briefly summarized and only described in detail if they have been substantially modified. Special chemicals, drugs, etc. with their sources of supply should be grouped under a separate sub-headings Materials.

(3) *Results*. In this section findings should be described without discussion of their significance. *Sub-sections should be used to clarify the expression of the results.*

(4) *Discussion*. In this section the authors' interpretations of their findings should be accompanied by an assessment of their significance in relation to previous work. Repetition of material given under 'Results' should be avoided. *Sub-sections should be used wherever possible* and sub-sections dealing with technical or highly

specialized matter should be clearly separated from the result of the text so that they can be printed in small type.

Presentation of data in Tables or Figures

In general, Tables and Figures should be so constructed what they, together with their captions and legends, will be intelligible with minimal reference to the text.

Each figure must be accompanied by a caption and explanatory legend typed on a separate sheet.

Care should be taken to present data in a precise manner. For example, histographs should not be used when the data can equally well be given in a Table.

Figures

For full guidelines on the preparation and submission of your artwork please refer to http://www.authors.elsevier.com/artwork. The use of colour plates is encouraged when appropriate. A charge for one full page of ca. $1000 is made for reproducing colour photographs. The exact cost will be given on request.

References

In the text, cite references by name and date (Harvard system). For more than two authors, use the first surname and et al. In the final list, they should be in alphabetical order, including the complete title of the article cited, and names of all authors. In the following examples notice the punctuation; do not use all capitals, do not underline.

The reference list should be typed on a separate sheet(s) of paper at the end of the manuscript, in alphabetical order and arragned as follows: author's name and initials, year, title of the article, abbreviated title of the journal, volume, first and last page numbers. Journal titles should be:

Journal Articles
Vogt M. and Wilson G., (1972) Concentration of 5-hydroxytryptamine and its acid metabolite in ventricle-near regions of the rat brain. Journal of Neurochemistry 19, 1599-1600.

References to books should include the authors' name and initials, year, title of book, volume, publisher and place of publication. Where relevant, the title of a paper within a book, and the editor's name, should be given. For example:

Book Chapters
Baker, P.F. (1972). The sodium pump in animal tissues and its role in the control of cellular metabolism and function. In Metabolic Pathways, Academic Press, New York, Vol. 6, pp. 243-268.

Unpublished experiments may be mentioned only in the text; they must not be included in the list of References; initials as well as surnames must be given for authors whose unpublished experiments are quoted.

A paper which has been accepted for publication but which has not appeared may be cited in References with the abbreviated name of the Journal followed by the words 'In press'. The date of acceptance of each such paper should be indicated when the manuscript is submitted to IJDN.

Personal communications may be used only when written authorization from the communicator is submitted with the original manuscript: they may be mentioned only in the text.

Abbreviations

Symbols for physical units should be restricted to the Systeme Internationale (S.I.) Units. Examples
of commonly used symbols can be found in Biochem. J. (1975) 145, 1-20 and more detailed descriptions, in Quantities, Units and Symbols (1971) The Royal Society, London.

The excessive use of abbreviations in the text is strongly discouraged. In particular, awkward and unfamiliar abbreviations and those intended to express concepts or experimental techniques will not be permitted. In order to aid communication between scientists of different disciplines, authors should only use abbreviations sparingly and should always define the abbreviation when first used by placing it in parentheses after the full term, e.g. Acetylcholinesterase (AChE). A list of abbreviations used in the text should be put on the bottom of the title page.

Chemical and biochemical nomenclature

As far as possible authors should follow the conventions used in the The Biochemical Journal, See Biochem. J. (1975) 145, 1-20. **Author enquiries**
For enquiries relating to the submission of articles (including electronic submission where available) please visit this journal's homepage at
http://www.elsevier.com/locate/ijdevneu. You can track accepted articles at
http://www.elsevier.com/trackarticle, and set up e-mail alerts to inform you of when an article's status has changed, as well as copyright information, frequently asked questions and more.

Contact details for questions arising after acceptance of an article, especially those relating to proofs, are provided after registration of an article for publication.