Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

PPG Ciências Biológicas – Bioquímica

DISSERTAÇÃO DE MESTRADO

Influência da privação dietética de ácidos graxos ω3 no sistema glutamatérgico no cérebro de ratos: parâmetros ontogenéticos e neuroproteção.

Aluna

Nut. Júlia Dubois Moreira

Orientadora

Profa. Dra. Lúcia Helena do Canto Vinadé

Co-orientadores

Prof. Dr. Marcos Luiz Santos Perry

Porto Alegre, Março de 2008.

Dedico esta dissertação de Mestrado à minha querida e amadíssima mãe,

Maria Teresa Dubois Moreira.

“Títulos não fazem Mestres, mas sim a maestria

com que vivemos e ensinamos.”

“Deixa teu medicamento ser teu alimento,

e teu alimento o teu medicamento.”

Hipócrates

Agradecimentos

Primeiramente, agradeço a minha família. À minha mãe por achar que "estudo não é dinheiro posto fora, mas um investimento", além de exemplo de mulher guerreira e batalhadora. Á minha irmã pelo exemplo de força e personalidade. À minha afilhada pela alegria e esperança. Aos demais familiares pelos momentos inesquecíveis e incentivos.

Agradeço à nutricionista Carmem Franscisco (HCPA) pelo primeiro artigo sobre os ácidos graxos omega-3 e pelos ensinamentos, tanto profissionais como pessoais.

Agradeço ao Prof. Dr. Severo Dutra por me receber no departamento de bioquímica tão prontamente e por me indicar o laboratório 26, que viria a se tornar minha segunda casa.

Agradeço ao querido e sábio Prof. Dr. Diogo O. Souza, pelo exemplo de profissional e de pessoa, sempre pronto para "dar um cabo de vassoura para um maluco" como eu.

Agradeço ao querido e sábio Prof. Dr. Marcos L. S. Perry, pelo acolhimento em seu laboratório e pelo incentivo em nos tornarmos pessoas mais sábias e capazes.

Agradeço a minha querida orientadora Lúcia Vinadé, que de uma colega passou a ser uma mestra, um exemplo e uma amiga.

Agradeço à Profa. Dra. Suzana Wofchuk, pela alegria, entusiasmo, exemplo e suporte.

Agradeço à Profa. Dra. Christianne Salbego e colegas do laboratório 37, pelos préstimos, paciência e disposição.

Agradeço à Profa. Dra. Carmem Gottfried, pela disposição e suporte.

Agradeço aos queridos Prof. Dr. Luiz Valmor Portela e Prof. Dr. Diogo Lara, pelos préstimos, suportes, risadas e afins.

Agradeço aos queridos amigos dos laboratórios 26 e 28, pela amizade, companheirismo, incentivo, convivência, sonhos, lágrimas e tragos na ASCHCLIN.

Agradeço aos queridos amigos do laboratório 27, pela paciência, convivência e amizade.

Agradeço aos colegas do laboratório 35, pelo companheirismo e suporte.

Agradeço imensamente aos funcionários do Biotério, da secretaria e demais funcionários deste departamento que tornam todo o nosso trabalho suado possível.

Por fim, agradeço a todas as pessoas que, de alguma forma, enriquecem e fazem parte da minha vida. Amigos, companheiros de estrada e todos que me ensinam a ser uma pessoa melhor, com suas lições de vida e seus ricos exemplos.

Índice

Parte 1

Resumo

O presente estudo tem por objetivo investigar a influência da privação dietética de ácidos graxos ω3 no sistema glutamatérgico no hipocampo de ratos Wistar em parâmetros ontogenéticos e relacionados à neuroproteção. Os ratos foram alimentados, desde a gestação até a vida adulta, com duas dietas diferentes: uma dieta contendo ácidos graxos ω3 (grupo controle, C) e outra deficiente nestes ácidos graxos (grupo deficiente, D). Nos experimentos ontogenéticos, foram avaliados os imunoconteúdos das subunidades de receptores glutamatérgicos ionotrópicos do tipo NMDA (NR2 A/B) e AMPA (GluR1), bem como da isoforma alfa da cinase dependente de cálcio e calmodulina do tipo II (αCaMKII). Também foram avaliados a capacidade do glutamatode ligar-se a seus receptores e a captação de [^{3}H]glutamato em fatias de hipocampo. Para investigar o efeito da privação de ácidos graxos ω3 na resposta cerebral frente à injúria isquêmica, fatias de hipocampo foram submetidas à privação de glicose e oxigênio (OGD), um modelo de isquemia *in vitro*. O grupo D apresentou imunoconteúdo reduzido de todas as proteínas avaliadas aos 02 dias de vida, o que foi sendo normalizado aos 21 dias (exceto pela αCaMKII) e aos 60 dias. O mesmo padrão foi observado na capacidade de ligação do glutamato aos seus receptores. Porém a captação de [^{3}H]glutamato não foi afetada pelas dietas. Nos experimentos de OGD, o grupo D apresentou maior dano celular e uma queda na captação de [^{3}H]glutamato mais acentuada que o grupo controle. Ainda, a privação de ácidos graxos ω3 influenciou a resposta celular anti-apoptótica após OGD, afetando a fosforilação das proteínas GSK3β e ERK1/2, mas não a fosforilação da Akt. Estes resultados sugerem que os ácidos graxos ω3 são importantes para o desenvolvimento do sistema glutamatérgico e para a proteção celular após dano isquêmico, além de ser importante para ativação de rotas de sinalização anti-apoptóticas.

Abstract

The effect of dietary deprivation of essential omega-3 fatty acids on parameters of the glutamatergic system related to brain ontogeny and neuroprotection was investigated in this study. Rats were fed with two different diets: omega-3 diet (control group) and omega-3 deprived-diet (D group). In ontogeny experiments, it was evaluated the hippocampal immunocontent subunits of the of ionotropic glutamatergic receptor NMDA and AMPA (NR2 A\B and GluR1, respectively) and the alfa isoform of the calcium-calmodulin protein kinase tipe II (αCaMKII), as well as the binding and uptake of [^{3}H]glutamate. To assess the influence of omega-3 fatty acids deprivation on brain responses to ischemic insult, hippocampal slices were submitted to oxygen and glucose deprivation (OGD), a model of *in vitro* ischemia. The D group showed lower immunocontent of all proteins analyzed at 02 days of life (P2) than the control group, and it was normalized at P21 (except for αCaMKII) and P60. The same pattern was found for [^{3}H]glutamate binding, while [^{3}H]glutamate uptake was not affected. In the OGD studies, the D group showed higher cell damage and stronger decrease in the [^{3}H]glutamate uptake. Moreover, omega-3 deprivation influenced anti-apoptotic cell response after OGD, affecting GSK-3beta and ERK1/2, but not Akt phosphorylation. Taken together, these results suggest that omega-3 fatty acids are important for the glutamatergic development and cell protection after ischemia, and also seem to play an important role on activation of anti-apoptotic signaling pathways.

Lista de Abreviaturas

ALA – ácido α-linolênico

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazolepropionato

ARA – ácido araquidônico

αCaMKII – isoforma alfa da cinase dependente de cálcio e calmodulina tipo II

DHA – ácido docosaexaenóico

DPA – ácido docosapentaenóico

EPA – ácido eicosapentaenóico

ERK 1/2 – cinase reguladora do sinal extracelular tipo 1 e 2

GluRs – receptores glutamatérgicos

iGluRs – receptores glutamatérgicos ionotrópicos

mGluRs – receptores glutamatérgicos metabotrópicos

GSK3β - isoforma beta da glicogênio sintase cinase tipo 3

GTP – guanosina trifosfato

LA – ácido linoléico

LDH – lactato desidrogenase

LTP – potenciação de longa duração

MAPK – proteína cinase ativada por mitógenos

MTT - brometo tetrazólíco 3(4,5-dimetiltiazol-2-yl)-2,5-difenil

NMDA - N-methil-D-aspartato

NSE – proteína enolase específica de neurônio

LC-PUFA – ácidos graxos poiinsaturados de cadeia longa

SNC – sistema nervoso central

ω6 – acido graxo da série omega-6

ω3 – ácido graxo da série omega-3

Introdução

Ácidos graxos essenciais

No fim da década de 20, dois pesquisadores, ao observar alterações em pessoas que não consumiam gorduras na sua dieta, constataram que haviam compostos que eram essenciais para a saúde do organismo (Burr & Burr, 1929). Desde então, os ácidos graxos essenciais vem sendo estudados, porém, somente na década de 70 é que houve uma maior evolução nos estudos envolvendo os ácidos graxos essenciais. Os ácidos graxos com ligações duplas nos carbonos omega-6 (ω6) e omega-3 (ω3) são essenciais ao bom funcionamento do organismo de mamíferos, incluindo os seres humanos, porém não podem ser sintetizados endogenamente. Estes devem estar presentes na alimentação para que possam ser utilizados pelos tecidos corporais. São eles os ácidos linoléico (LA 18:2ω6) e α-linolênico (ALA 18:3ω3). Pela ação de enzimas específicas no fígado, estes dão origem a ácidos graxos poliinsaturados de cadeia longa (LC-PUFAs), compostos que tem um importante papel no processo inflamatório e de defesa do organismo (Haag, 2003).

O ácido graxo linoléico (LA 18:2ω6) é reconhecido como nutriente essencial a bastante tempo (Burr & Burr, 1929; Hansen et al., 1962). Este ácido graxo é amplamente encontrado em óleos vegetais e pode ser convertido ao ácido araquidônico (ARA 20:4ω6). O ARA é muito abundante nos fosfolipídios das membranas celulares e desempenha um importante papel imunológico, dando origem a mediadores inflamatórios como os eicosanóides (prostaglandinas, tromboxanos e leucotrienos). Os sintomas de deficiência

deste ácido graxo são retardo de crescimento, lesões de pele, insuficiência reprodutora, esteatose hepática e polidipsia, entre outros (Marszalek & Lodish, 2005).

O ácido graxo α-linolênico (ALA 18:3ω3) somente foi reconhecido como nutriente essencial há poucas décadas atrás (Heird & Lapillonne, 2005). Ele está presente em óleos vegetais como linhaça, canola e soja. Deste ácido graxo derivam o ácido eicosapentaenóico (EPA 20:5ω3) e ácido docosaexaenóico (DHA 22:6ω3). Estes PUFAs também estão presentes nos óleos de peixes, como salmão, sardinha, atum e cavalinha. Eles se apresentam compondo fosfolipídios de membrana e desempenham papéis diferentes no organismo. O EPA, assim como o ARA, também pode dar origem a eicosanóides, porém com uma ação mais anti-inflamatória no organismo (Marszalek & Lodish, 2005). O DHA é o mais abundante ácido graxo nas membranas celulares do cérebro e da retina, tendo um importante papel funcional nestes sistemas. Entre os sintomas de deficiência destes ácidos graxos estão, além de crescimento e reprodução prejudicados, problemas de visão e redução de aprendizado (Holman et al., 1982).

Os ácidos graxos ω6 e ω3 competem pelas mesmas enzimas que os alongam e dessaturam no fígado para dar origem aos seus respectivos PUFAs. Por essa razão, estes devem estar em equilíbrio na alimentação. Estudos mostram que uma relação ω6:ω3 de 5:1 é a mais adequada para que ambos tenham seu melhor aproveitamento pelo organismo (Marszalek & Lodish, 2005, Heird & Lapillonne, 2005).

DHA e sua relação com o sistema nervoso central

O ácido docosaexaenóico (DHA 22:6ω3) é o ácido graxo mais abundante no sistema nervoso central, tanto no cérebro como na retina (Marszalek & Lodish, 2005). Ligado a albumina, o DHA chega ao cérebro pela corrente sangüínea e passa a barreira hemato-encefálica pela ação de proteínas específicas de transporte. Além disso, os astrócitos também possuem as enzimas necessárias para sintetizar DHA (Williard et al., 2001).

O DHA é especialmente importante durante o desenvolvimento cerebral pré-natal, onde participa ativamente da sinaptogênese (Martin & Bazan, 1992). Este ácido graxo passa da mãe para o feto pela barreira placentária e, após o nascimento, pelo leite materno. O crescimento cerebral humano, que ocorre do terceiro trimestre de gestação até o 18º mês de vida, é correlacionado com o acréscimo de DHA nos fosfolipídios de membrana do cérebro (Lauritzen et al., 2001). O DHA está presente nos fosfolipídios de membrana, principalmente fosfatidiletanolamina e fosfatidilserina, e nos plasmalogenios, compostos que estão relacionados à proteção celular contra o estresse oxidativo (André et al., 2005; Farooqui & Horrocks, 2001). O conteúdo de DHA nos fosfolipídios chega a 50% do total de ácido graxos insaturados no cérebro de ratos adultos (Garcia et al., 1998). Um fornecimento insuficiente de ácidos graxos ω3 durante o desenvolvimento pré e pós-natal diminui o conteúdo de DHA nos tecidos neurais com um aumento recíproco de ácido docosapentaenóico (DPA 22:5ω6; Schiefermeier & Yavin, 2002), levando a uma variedade de déficits visuais, olfatórios, cognitivos e comportamentais em modelos animais (Lim et al., 2005; Lim et al., 2005[1]; Niu et al., 2004; Moriguchi et al., 2000). Porém, o suprimento

de DHA através do aleitamento materno tem mostrado melhorar o desenvolvimento mental em crianças (Hibbeln et al., 2007; Birch et al., 2000; Willatts et al., 1998).

A influência do DHA nas propriedades de membrana, como fluidez, permeabilidade e capacidade de fusão (Stillwell & Wassall, 2003), afeta a atividade de várias proteínas de membrana (canais iônicos, receptores, transportadores, enzimas), assim modulando vários sistemas de neurotransmissores. Muitos estudos mostram que os sistemas dopaminérgico e serotoninérgico são afetados pela privação de ácidos graxos ω3 (Zimmer et al., 2000; Delion et al., 1996). Porém, muito pouco se sabe sobre a influência destes ácidos graxos sobre o funcionamento do sistema glutamatérgico.

O DHA e sua possível relação com sistema glutamatérgico

O glutamato é o principal neurotransmissor excitatório do sistema nervoso central (SNC) e está envolvido em várias funções cerebrais, como aprendizado/memória e desenvolvimento e envelhecimento cerebral (Ozawa et al, 1998; Danbolt, 2001; Segovia et al, 2001). O glutamato exerce seus efeitos através de receptores específicos (GluRs) que são divididos em ionotrópicos (iGluRs) e metabotrópicos (mGluRs). Os iGluRs são canais iônicos cátion-específicos, subdivididos em α-amino-3-hidroxi-5-methil-4-isoxazolepropionato (AMPA), cainato e N-methil-D-aspartato (NMDA). Os mGluRs são acoplados a proteínas ligadoras de GTP (proteínas G) e modulam a produção de mensageiros intracelulares.

Em relação aos iGluRs, eles são essenciais para as funções cerebrais (Popescu & Auerbach, 2004; Collingridge & Isaac, 2003; Kew and Kemp, 2005), e sua

estrutura molecular influencia em sua atividade. Os receptores tipo NMDA são combinações de subunidades NR1 e NR2A-2D, enquanto que os receptores tipo AMPA são combinações de subunidades GluR1-GluR4 (Ozawa et al, 1998; Kew and Kemp, 2005). A composição específica e as interações destas subunidades são responsáveis pela modulação da atividade destes receptores, nas diferentes regiões e estágios de desenvolvimento do SNC. Os receptores tipo AMPA e NMDA podem interagir com a enzima α-cinase dependente de cálcio e calmodulina tipo II (α-CaMKII), muito abundante em membranas sinápticas. Esta enzima está envolvida, junto aos receptores do tipo AMPA e NMDA, na modulação da memória e no evento de potenciação de longa duração (LTP) no hipocampo, considerado um modelo das bases celulares e moleculares da memória (Lisman et al., 2002).

No entanto, apesar do papel essencial do glutamato para as funções cerebrais normais, o aumento da concentração deste na fenda sináptica pode levar a neurotoxicidade. O evento excitotóxico do glutamato pela superestimulação de seus receptores está relacionado a várias desordens cerebrais, tanto agudas, como hipóxia, isquemia, convulsão e trauma, quanto crônicas, como doença de Parkinson, Alzheimer, Huntington e epilepsia (Lipton & Rosenberg, 1994; Ozawa et al, 1998; Danbolt, 2001; Maragakis & Rothstein, 2004; Sheldon and Robinson, 2007).

Existem alguns estudos que relacionam o sistema glutamatérgico com os ácidos graxos ω3, principalmente o DHA. Um estudo *in vitro* mostrou que o DHA é capaz de modular diferentemente os transportadores de glutamato GLT-1, GLAST e EAAC1 (Berry et al., 2005). A suplementação com DHA demostrou ser capaz de restaurar a LTP em hipocampo de ratos idosos com a concomitante normalização da liberação e glutamato, que

estava reduzida nestes animais (McGahon et al., 1999). Uma dieta enriquecida em DHA também foi capaz de reverter o decréscimo de subunidades de receptores NMDA e AMPA que ocorre em animais idosos (Dyall et al., 2006).

Efeitos benéficos e neuroprotetores do DHA

O ácido graxo DHA foi capaz de proteger ratos jovens contra excitotoxicidade e convulsão (Högyes et al., 2003; Xiao & Li, 1999). DHA também inibiu a atividade epileptiforme no hipocampo de ratos (Young et al., 2000) e reduziu a injúria neuronal em modelo experimental de isquemia (Belayev et al., 2005; Strokin et al., 2006; Bas et al., 2007). Em humanos, DHA também parece exercer um efeito neuroprotetor, uma vez que baixos níveis deste ácido graxo foram associados com doenças neurodegenerativas, como a doença de Alzheimer (Schaefer et al., 2006; Soderberg et al., 1991). A deficiência dietética e baixos níveis endógenos de ácidos graxos ω3 têm sido associados com a emergência e o prognóstico de doenças psiquiátricas, e alguns estudos clínicos têm mostrado que a suplementação destes ácidos graxos foi benéfica em pacientes com depressão, doença bipolar e esquizofrenia (Yuen et al., 2005; Peet & Stokes, 2005).

Apesar das evidências dos efeitos benéficos do DHA para a saúde cerebral, o mecanismo desta proteção ainda não está completamente entendido. Dois possíveis mecanismos envolvidos nesta proteção é o envolvimento das proteínas ERK1/2, uma enzima da rota das MAPK, e da rota da Akt, que mostrou ser ativada pelo DHA em células injuriadas (German et al., 2006; Florent et al., 2006; Akbar et al., 2005). Estas enzimas estão relacionadas com rotas de sobrevivência celular às mais variadas injúrias.

Objetivos

Objetivo geral

O presente estudo tem por objetivo a investigação da influência da privação dietética de ácidos graxos ω3 no sistema glutamatérgico no cérebro de ratos relacionada a parâmetros ontogenéticos e neuroproteção.

Objetivos específicos

1. Investigar os parâmetros do sistema glutamatérgico no hipocampo de ratos;

2. Investigou-se o imunoconteúdo de subunidades de receptores ionotrópicos glutamatérgicos NMDA e AMPA e da enzima αCaMKII;

3. Avaliar parâmetros de neuroproteção em fatias de hipocampo submetidas à privação de oxigênio e glicose (OGD);

Parte 2

Influence of dietary ω3 fatty acid deprivation on the glutamatergic system of rat brain: ontogenetic profile and neuroprotection.

Júlia D. Moreira[a*], Luisa Knorr[a], Ana Paula Thomazi[a], Fabrício Simão[a], Cíntia Battú[a], Jean Pierre Oses[a], Marcelo Ganzella[a], Carolina G. de Souza[a], Carolina F. Pitta[a], Suzana Wofchuk[a], Christianne Salbego[a], Diogo O. Souza[a], Marcos L. S. Perry[a], Lúcia Vinadé[a,b].

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

[b]Departamento Didático, CCRSG, Universidade Federal do Pampa, Rua Antonio Mercado 1357, CEP 97300-000, São Gabriel, RS, Brazil.

[*]Corresponding author: Tel: +55 51 33085559; fax: +55 51 33085540.

Email address: juliamoreira@gmail.com.br (J. D. Moreira)

Submitted to Neurochemistry International

1. Introduction

Omega-3 (ω3) is a group of essential fatty acids that can not be synthesized by mammalian organism, and therefore have to be present in the diet. α-Linolenic acid (18:3 ω3) is the precursor of eicosapentaenoic acid (EPA 20:5 ω3) and docosahexaenoic acid (DHA 22:6ω3), polyunsaturated fatty acids (PUFAs) of great relevance to the organism's health. Research over the past 30 years has established that PUFAs are critical for proper infant growth and neurodevelopment. Among the ω3 fatty acids, DHA is the fatty acid of the most physiological significance for brain function (Bourre 2004; Marszalek & Lodish, 2005).

DHA is especially important during prenatal brain development, when it is incorporated into nerve growth cones in events leading to synaptogenesis (Martin & Bazan, 1992). The human brain growth spurt, that takes place from the third trimester of pregnancy until 18 months after birth, correlates with DHA accretion into brain phospholipids (Lauritzen et al., 2001). DHA is present in membrane phospholipids, like phosphatidylethanolamine and phosphatidylserine, and in plasmalogens, compounds that seem to protect cells against oxidative damage (André et al., 2005; Farooqui & Horrocks, 2001). The content of DHA in the sn-2 position of phospholipids reaches up to 50% of the total amount of unsaturated fatty acids in the brain of adult rats (Garcia et al., 1998). An insufficient supply of ω3 fatty acids during prenatal and postnatal development decreases the levels of DHA in neural tissue with a reciprocal increase of docosapentanoic acid (DPA, C22:5ω6) (Schiefermeier & Yavin, 2002), leading to a variety of visual, olfactory, cognitive and behavioral deficits in animal models (Lim et al., 2005; Lim et al., 2005[1]; Niu et al., 2004; Moriguchi et al., 2000). Conversely, dietary supply of DHA through

breastfeeding has been shown to improve mental development in human children (Hibbeln et al., 2007; Birch et al., 2000; Willatts et al., 1998).

DHA's actions on membrane properties, such as fluidity, permeability and capacity of fusion (Stillwell & Wassall, 2003), affect the activity of various brain membrane proteins (ion channels, receptors, transporters, enzymes), thus modulating the activity of neurotransmitter systems. Previous studies have shown that the dopaminergic and serotoninergic systems are affected by DHA deprivation (Zimmer et al., 2000; Delion et al., 1996), whereas less is known about its effects on the glutamatergic system.

Glutamate is the major excitatory neurotransmitter in the CNS involved in various brain functions, such as learning/memory and brain development and ageing (Ozawa et al, 1998; Danbolt, 2001; Segovia et al, 2001). The glutamatergic receptors (GluRs) are categorized into ionotropic (iGluRs) and metabotropic (mGluRs) receptors. iGluRs are cation-specifc ion channels, subdivided into a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. mGluRs are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular messengers.

Regarding iGluRs, they are essential to brain functions (Popescu & Auerbach, 2004; Collingridge & Isaac, 2003; Kew and Kemp, 2005), and their molecular structure assembly modulates their activities. NMDA receptors are combinations of the NR1 and NR2A-2D subunits, while AMPA receptors are combinations of GluR1-GluR4 subunits (Ozawa et al, 1998; Kew and Kemp, 2005). The specific composition and interaction of these subunits modulate the activity of iGluRs. α-Calcium/calmodulin-dependent kinase type II (αCaMKII) is an enzyme abundant in synaptic membranes that interacts with NMDA and

AMPA receptors. It is involved in memory modulation of some tasks and in long-term potentiation (LTP) in the hippocampus, which has been considered a model for the cellular and molecular basis of memory (Lisman et al., 2002).

However, besides the essential role of glutamate for normal brain functions, it has been well established that increased amounts of glutamate in the synaptic cleft leads to neurotoxicity (excitotoxicity). The excitotoxic events by over-stimulation of glutamate receptors are involved in various acute (hypoxia, ischemia, seizure and trauma) and chronic (Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy) brain disorders (Lipton & Rosenberg, 1994; Ozawa et al, 1998; Danbolt, 2001; Maragakis & Rothstein, 2004; Sheldon and Robinson, 2007).

There are some reports about the effects of DHA on the glutamatergic system. An *in vitro* study showed that DHA differentially modulate the glutamate transporters GLT1, GLAST and EAAC1 (Berry et al., 2005). Dietary supplementation with DHA was capable of restoring LTP in hippocampus of old rats with concomitant normalization of glutamate release, which was reduced in these animals (Mcgahon et al., 1999). A diet enriched with DHA also reversed the age-related decrease in the GluR2 and NR2B, subunits of AMPA and NMDA receptors respectively, in the forebrain of old rats (Dyall et al., 2006).

Concerning the neuroprotective roles of DHA against the glutamatergic excitotoxicity in animal models, DHA protected infant rats against excitotoxicity and convulsion (Högyes et al., 2003; Xiao & Li, 1999), inhibited epileptiform activity in rat hippocampus (Young et al., 2000) and reduced neuronal injury in experimental brain ischemia (Belayev et al., 2005; Strokin et al., 2006; Bas et al., 2007).

In humans, DHA appears to be neuroprotective as decreased levels of this fatty acid were associated with neurodegenerative diseases, such as Alzheimer's disease (Schaefer et

al., 2006; Soderberg et al., 1991). Deficient dietary intake and low endogenous levels of ω3 fatty acids have been associated with the emergence and prognosis of psychiatric disorders, and many small clinical trials have shown that their supplementation was beneficial in patients with depression, bipolar disorder and schizophrenia (Yuen et al., 2005; Peet & Stokes, 2005). Although the evidence indicates the beneficial effect of DHA to brain health, underlying mechanisms are not well understood. Putative mechanism is the involvement of extracellular signal-regulated kinase-1 and –2 (ERK 1/2), an enzyme of mitogen-activated protein kinase (MAPK) pathway, and Akt pathways, which has been shown to be activated by DHA in injured cells (German et al., 2006; Florent et al., 2006; Akbar et al., 2005).

Based upon these data, here we investigated the effects of dietary deprivation of essential ω3 fatty acids on: i) the ontogeny of the glutamatergic system in the rat hippocampus and ii) the hippocampal response against an *in vitro* injury model in terms of cell damage and viability, glutamate uptake and apoptotic / anti-apoptotic pathway profile.

2. Materials and methods

2.1 Animals and diets

Wistar female rats were housed in an air-conditioned room (21-22ºC) with 12h dark-light cycle and food and water were offered *ad libitum*. Both diets were isocaloric, containing 8% fat and differed only in oil composition. The nutrients (proteins, carbohydrates, vitamins and minerals) composition of the diets was according to Ximenes da Silva et al. (2002), with some modifications. The lipid composition, as recommended for dietary intake of ω6 and ω3 fatty acids, was according to Simopoulos and Salem Jr, 2000.

In order to manage the ω3 fatty acids maternal milk supply, two weeks before mating female rats were divided into two groups: control diet and the ω3 deprived-diet (D diet). The control diet contained a mixture of fish oil (Naturalis, Brazil) plus corn oil (attaining ~500mg DHA/100g of chow), which completed the optimum ω6:ω3 PUFA fatty acid ratio of 5:1. The D diet contained peanut oil, which only supplies ω6 fatty acid. Pups were maintained with the same diet of their dams until adult age (60 days old). For the ontogenetic experiments, pups of 2, 21 and 60 days old were used (n = 10 per group). For the *in vitro* brain injury model, only 60 days old male rats were used (n = 10 per group). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

2.2 Synaptosomal preparations

Synaptosomal fraction was prepared according to Dosemeci et al. (2006). All centrifugation steps were carried out in a refrigerated (4ºC) microfuge. Hippocampi of 2, 21 and 60 days old rats were homogenized (motor-driven small capacity Teflon/glass homogenizer in a final volume of 1mL/hippocampus) in a 25mM Hepes buffer (pH 7.4) with 0.32 M sucrose, 1 mM $MgCl_2$ and a protease inhibitor cocktail (Sigma). The homogenate was transferred to microfuge tubes (1.5 mL per tube) and centrifuged at 470*g* × 2 min using a fixed angle rotor. The resultant supernatant was transferred to another microfuge tube and centrifuged at 10,000*g* × 10 min using the same rotor to obtain a mitochondria- and synaptosome-enriched pellet (P2). The (P2) pellet was resuspended into 0.32 M sucrose (500 µl per tube) and the suspension was layered onto 750 µl of 0.8 M sucrose in a microfuge tube. The samples were centrifuged at 9100*g* ×15 min using a swinging bucket rotor. Following centrifugation, the myelin/light membrane layer at the top

of 0.32M sucrose was removed. Synaptosomal fraction collected at 0.32M/0.8M interface was washed twice to remove sucrose excess by centrifugation at 16000*g* ×10min in 25 mM Hepes (pH 7.4) containing a protease inhibitor cocktail. The final pellet was resuspended in the same solution (200 μ/pellet) for the Western blotting analysis.

2.3 Western Blotting Analysis

In ontogenetic experiments, synaptosomal proteins (30 μg protein/well) were separated in a 7.5% SDS-PAGE mini-gel (Vinadé et al., 2003) and transferred to nitrocellulose membrane using a Trans-Blot system (Bio-Rad, Hercules CA). Membranes were processed as follow: (1) blocking with 5 % bovine serum albumin (Sigma) for 2 h; (2) incubation with primary antibody overnight: 1:200 anti-αCaMKII (Chemicon International); 1:1000 anti-GluR1(Upstate cell signaling solutions); 1:5000 anti-NR2A/B (Chemicon International); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1: 5000 (Amersham Pharmacia Biotech) for 2h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and band intensity was analyzed using Image J (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). DHA group was considered as the control and results were expressed in percentage compared with this group.

For Western blotting analysis of *in vitro* brain injury (OGD experiments - see below), hippocampal slices were homogenized in 25 mM Hepes (pH 7.4) containing the protease inhibitor cocktail. Samples were normalized to 2 μg protein/μL with a sample buffer (4% sodium dodecylsulfate (SDS), 2.1 mM EDTA, 50 mM Tris and 5 % β-mercaptoethanol). Samples (30μg protein/well) were submitted to electrophoresis and

transferred to a nitrocellulose membrane. The same Western blot procedures were performed with the following primary antibodies at 1:1000 concentration: p-AKT; p-GSK3β; p-ERK1/2 (CellSignal). The loaded protein was always verified by Coomassie blue gel stain (data not shown).

2.4 [^{3}H] Glutamate Binding

Animals were killed by decapitation, the brains were rapidly removed and hippocampi were dissected and homogenized. Experiments were carried out according to Emanuelli et al., 1998. Hippocampal plasma membranes were incubated in the absence of sodium, aiming to obtain glutamate binding specifically to glutamatergic receptors (and not to glutamate carriers, which depend on sodium). Binding incubations were carried out in triplicate in polycarbonated tubes contained 50 mM Tris-HCl buffer, pH 7.4, 100nM [^{3}H]glutamate at 30ºC for 30 min. The incubation was started by adding 100μg of protein membrane and was stopped by centrifugation at 16,800xg for 15 min at 4ºC. The pellet and the wall of the tube were carefully washed with ice-cold distilled water and resuspended with 0.1% sodium dodecyl sulfate (w/v) overnight. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Results (specific binding) were considered as the difference between total binding and non-specific binding (in the presence of 50μM non radioactive glutamate, attaining 10-20% of the total binding).

2.5 [3H]Glutamate Uptake

2.5.1 Slices preparation

Animals were decapitated and their brains were immediately removed and humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63

Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. Hippocampi were dissected onto Petri dishes with HBSS and slices (0.4 mm) were obtained using a McIlwain tissue chopper. Slices were transferred to two 24-well culture plates: one plate was maintained at 35°C and the other at 4ºC. The slices from the first plate were washed once with l mL of 35 °C HBSS and the second with l mL of 4 °C sodium-free HBSS for measurement of sodium-independent uptake (see below).

2.5.2 *Total and Na^+- independent uptake*

Glutamate uptake was performed according to Thomazi et al. (2004). Hippocampal slices were pre-incubated at 35 °C for 15 min, followed by the addition of 100μM [^{3}H]glutamate. Incubation was stopped after 5 min with two ice-cold washes of l mL HBSS, immediately followed by the addition of 0.5N NaOH, which were then kept overnight. Na^+-independent uptake was measured by using the same protocol described above, with differences in the temperature (on ice - 4°C) and medium composition (*N*-methyl-D-glucamine instead of sodium chloride). Results (Na^+-dependent uptake) were considered as the difference between the total uptake and the Na^+-independent uptake. Both uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). This protocol was used in both ontogeny and OGD experiments.

2.6 Oxygen and Glucose Deprivation Experiments (OGD)

After decapitation, hippocampi were immediately isolated and transverse sections (400 μm) were prepared using a McIlwain tissue chopper. Hippocampal slices were divided into two equal sets: control and OGD (*in vitro* brain injury), placed into separate 24-well

culture plates, and preincubated for 30 min in a tissue culture incubator at 37°C with 95% air/5% CO_2 in a modified Krebs–Henseleit solution (pre-incubation solution, pH 7.4): in mM - 120 NaCl, 2 KCl, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose. After pre-incubation, the medium in the control plate was replaced with another modified Krebs–Henseleit solution (KHS incubation solution, pH 7.4): in mM - 120 NaCl, 2 KCl, 2 CaCl2, 2.6 NaHCO3, 1.19 MgSO4, 1.18 KH2PO4, 11 glucose and the slices were incubated 60 min in the culture incubator. In the ischemic plate, OGD slices were washed twice with Krebs–Henseleit medium without glucose and incubated for 60 min (OGD period) at 37 °C in an anaerobic chamber saturated with N_2, as detailed in Strasser and Fischer (1995) and Cimarosti et al. (2001). After incubation, the medium of both plates was removed, received Krebs–Henseleit solution with glucose and the slices were incubated for 3 h (recovery period) in the culture incubator. Control and OGD sets were used concomitantly with 4 slices from the same animal in each plate. After reoxigenation, slices were used for determination of glutamate uptake, cellular damage and viability and for Western blot analysis.

2.7 Cellular damage and viability

2.7.1 *LDH assay*

Membrane damage was determined by measuring lactate dehydrogenase (LDH) released into the medium (Koh and Choi, 1987). After the reoxigenation period, LDH activity was determined using a kit (Labtest, Minas Gerais, Brazil). Total LDH activity (100%) was evaluated by disrupting the slices by freezing/thawing and homogenization. LDH activity released into the medium was quantified as a percent of total activity. Results are expressed as a percentage of control.

2.7.2 MTT colorimetric assay

Slice viability assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, Sigma) method. After the recovery time, slices were incubated with 0.5 mg/mL of MTT, followed by incubation at 37°C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 560 and 630 nm. Only viable slices are able to reduce MTT. Results are expressed as a percentage of control.

2.7.3 Trypan blue incorporation

Membrane permeability was evaluated by Trypan blue assay. Briefly, at the end of the recovery time, slices were incubated for 5 min in a solution containing 400 μL of Trypsin/EDTA (Gibco) and fetal calf serum at 37^{o}C, gently dissociated by a sequential passage through a Pasteur pipette and allowed to settle during 10 min to remove residual intact tissue. An aliquot of the cell suspension was blended with 1.2% trypan blue solution. After 2 min, cells were counted in a hemocytometer by phase-contrast in an inverted light microscope at X 100 magnification. Each value indicates the percentage trypan blue labeled cells, counted in four squares of the chamber in 4 - 6 separated experiments.

2.7.4 NSE release assay

Neuron-specific enolase (NSE) released in the medium was used as marker of neuronal damage. NSE was measured using an eletrochemiluminescent assay kit. It consists of a double sandwich assay that use an anti-NSE antibody bound with ruthenium, which is the luminescent molecule. The reaction and quantification were performed by Elecsys-2010 (Roche Diagnostics Corporation®). The assay was carried out in duplicate and the variation coefficient was less than 5%. NSE levels are expressed as ng/ml (Oses et al., 2004).

2.8 Protein determination

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

2.9 Statistical analysis

Data are expressed as mean ± standard deviation. One-way ANOVA was used followed by Tukey test as *post-hoc*, when significant effects ($p < 0.05$) were found. Analyses were performed with the SPSS 8.0 software.

3. Results

3.1 Influence of dietary ω3 deprivation on the ontogeny of glutamatergic parameters in the hippocampus

We initially evaluated the immunocontent of glutamatergic ionotropic (AMPA and NMDA) receptor subunits and of the protein αCaMKII, an enzyme involved in phosphorylation of these receptors and in hippocampal LTP, which are events strongly related to learning and memory. As shown in Figure 1, at 02 days post-natal, the immunocontent of αCaMKII, NMDA subunit NR2 A/B and AMPA subunit GluR1 in the D group was about 40%, 50% and 10% smaller than in the control group, respectively ($p < 0.05$). At 21 days, only the αCaMKII immunocontent was still reduced (30% smaller than DHA group, $p < 0.05$). At 60 days, their levels were similar between both groups.

The ontogenetic pattern of glutamate binding was different in control and D group (Fig.2a). In the D group glutamate binding increased with age up to 60 days of life, whereas in the control group the maximum level was attained yet at 21 days of life. Glutamate binding was significantly reduced in the D group at 02 days (-73%, $p < 0.05$) and at 21 days

(-34% $p<0.05$) of life, compared with the control group. At 60 days post-natal glutamate binding was similar between groups.

In contrast to the results on glutamate receptors, the ontogenetic profile of glutamate uptake in hippocampal slices was not affected by ω3 deprivation (Fig.2b), being consistent with the pattern previously observed by us (Thomazi et al., 2004; Feoli et al., 2006).

3.2 ω3 Deprivation affected cellular injury in hippocampal slices submitted to OGD

To compare the hippocampal response of the control and D groups to brain injury, hippocampal slices from both groups were submitted to oxygen and glucose deprivation (OGD), a model of *in vitro* ischemia. Figure 3 shows the results of cellular viability, measured by various approaches, after OGD. Fig. 3a shows that after 1h and 3h of recovery, OGD increased LDH release, compared with the basal conditions, and that the D group released more LDH after 3h of recovery ($p < 0.01$). As no difference was found between the groups after 1h of recovery, we choose 3h of recovery to assess the other parameters of cellular viability.

Similarly, both groups differed from its basal after recovery in Trypan blue assay, with D group showing higher membrane injury ($p < 0.05$) (Fig 3b).

MTT measurement, which assesses mitochondrial viability, also showed the same pattern, confirming higher cell death in the D group after 3h of recovery. (Fig. 3c).

To assess neuronal damage by OGD, we measured NSE (a neuronal specific enolase) liberation. Figure 3d shows that, after 3 h of recovery, the D group released more NSE ($p < 0.01$) after OGD.

3.3 ω3 Deprivation influenced [^{3}H]glutamate uptake after OGD

To assess the involvement of astrocytes in hippocampal responses to OGD injury, we measured the glutamate uptake by hippocampal slices. This parameter reflects the physiological capacity of astrocytes to keep glutamate concentration in the synaptic cleft below toxic levels, protecting neurons from excitotoxic damage (Fig 4). OGD decreased glutamate uptake measured after 3h recovery in both groups ($p < 0.05$). However, again, in the D group this decrease was more pronounced than in the D group. There was no difference between both groups in basal conditions (not submitted to OGD) in the parameters evaluated in Figures 3 and 4.

3.4 Influence of ω3 deprivation on signaling pathways involved in apoptosis after OGD,

In this set of experiments, by using Westerns blot in hippocampal slices, we investigated the phosphorylation state of proteins involved in apoptotic (glycogen synthase kinase-3β, GSK3β, inactive when phosphorylated) or anti-apoptotic (ERK 1/2 and Akt, both active when phosphorylated) signaling pathways (Figure 5), known to be involved in the protective effect of DHA (German et al., 2006; Florent et al., 2006; Akbar et al., 2005). There was a decrease in the phosphorylation state of GSK3β after OGD, which was much more accentuated in D than in control group (to 15% and 25% respectively, $p < 0,01$) (Figure 5a). Similarly, there was a decrease in the phosphorylation state of ERK1\2 after OGD in both groups, but it also was more accentuated in the D than in control group (to 25% and 40% respectively, $p < 0.01$) (Figure 5b). Akt phosphorylation was not significantly affected by OGD or by diet (Figure 5c).

4. Discussion

The present results show that dietary deficiency of ω3 fatty acids delayed the development of glutamatergic parameters, which could be related to the fact that it also yields the mature brain more susceptible to ischemic injury, with alteration in glutamate uptake and in phosphorylation states of proteins involved in apoptotic and anti-apoptotic pathways.

Glutamate ionotropic receptors interact with a variety of proteins involved in the spatial and functional organization of postsynaptic densities, and also with proteins involved in signal transduction, like MAPK family and αCaMKII (Meldrum, 2000). Here, we have showed that ω3 deprivation led to a delay in the ontogenetic development of NMDA and AMPA subunits and αCaKMII immunocontent, which was normalized in adulthood. Calon and colleagues (2005) have also shown that ω3 deprivation reduced the immunocontent of NMDA receptor subunits in the cortex and hippocampus and αCaMKII in the cortex of TG2576 mice, a model of Alzheimer's disease. In other studies, similar results have been found in old rats. With aging, DHA content in the hippocampus phospholipids is reduced and coincides with the decrease in normal brain function and neuroplasticity (Mcgahon et al., 1999; Johnson & Schaefer, 2007). Dyall and colleagues (2006) showed that rats have age-related decrease of NR2B and GluR2, which was prevented by supplementation with ω3 fatty acids. The reduction in the immunocontent of these proteins in early life may have impaired the adequate brain development, which is possibly related to the increased susceptibility to brain injury in adults animals, as observed

here, and cognitive deficits found in ω3 deprived-animals in other studies (Lim et al., 2005; Lim et al., 2005[1]; Niu et al., 2004; Moriguchi et al., 2000).

Glutamate is the main excitatory neurotransmitter in mammalian brain and glutamatergic function plays an essential role in CNS development, contributing to neuronal differentiation, migration and survival in the developing brain (Meldrum, 2000; Segovia et al, 2001; Manent & Represa, 2007). However, excessive amounts of glutamate are highly toxic to neurons, a process called excitotoxicity (Choi, 1992). The glutamate toxicity may contribute to cell damage observed in various acute and chronic brain diseases (Danbolt, 2001; Maragakis & Rothstein, 2004; Gardoni & Luca, 2006; Sheldon & Robinson, 2007). Thus, excessive glutamate has to be removed from the synaptic cleft, and the equilibrium between the physiological/toxic tonus of the glutamatergic system is modulated by the uptake of extracellular glutamate, which is performed mainly by glutamate transporters located is astrocytic cell membranes surrounding the synaptic cleft (Maragakis & Rothstein, 2004; Danbolt, 2001; Chen & Swanson, 2003; Schousboe & Waagepetersen, 2005).

To assess the neuroprotective role of ω3 fatty acids, we submitted hippocampal slices of adult rats to 1 hour of oxygen and glucose deprivation (OGD) and 3 hours of recovery, and measured the cell viability through various parameters. Nervous tissue is particularly sensitive to reactive oxygen species (ROS; Halliwell, 1997), which are generated in excessive amounts during post-ischemic recovery. Here, we have showed that after recovery, the D group showed an increased susceptibility to the cellular injury, including neuronal injury specifically (marked with NSE), than the control group. Strokin and colleagues (2006) showed similar results with the same model of damage. They

showed a release of DHA during ischemia; both free DHA and the preservation of DHA-containing phospholipids were able to reduce cellular damage. DHA could be acting by itself and by its products, such as Neuroprotectin D1 (NPD1), a docosanoid derived from DHA (Bazan 2005). This docosanoid appears to be synthesized against brain injury (Bazan, 2006; Lukiw et al., 2005), can activate signaling pathways that culminate in cellular survival and may contribute to DHA-induced prevention of apoptosis in injured neurons and retina photoreceptors (Bazan, 2005).

By searching putative neurochemical mechanisms for the neuroprotective effects of ω3 fatty acids, we investigated the effect of OGD on glutamate uptake and on apoptotic and anti-apoptotic signaling pathways.

It has been shown that after oxygen and glucose deprivation (OGD), the extracellular glutamate concentration increases (Danbolt, 2001). Here, we showed that after OGD there was a decrease in the glutamate uptake, which is in agreement with previous studies (Fontella et al., 2005). But, in the D group, this decrease was more pronounced. Since baseline glutamate uptake was similar between diet groups, this difference could be related to the less pronounced cell damage found in the control group after ischemia and/or to a lower capacity of astrocytes to protect neurons by keeping extracellular glutamate concentration below toxic levels. Moreover, ω3 fatty acids can be modulating glutamate transporters density after ischemia. More studies have to be done to elucidate this parameter.

Akbar and colleagues (2005) showed that DHA promotes neuronal survival via activation of Akt signaling. Akt has direct effects on the apoptotic pathway, by inhibiting pro-apoptotic proteins, such Bad, caspase 9 and GSK3β (Song et al., 2005). Moreover, a

previous study showed that blocking increased Akt phosphorylation increases subsequent DNA fragmentation (Noshita et al., 2001). Similarly to Akt signaling, MAPK signaling pathway is required for the anti-apoptotic effect and neuronal survival in the brain following ischemic insults and one study suggests that DHA can modulates MAPK pathway (German et al, 2006). We showed that ω3 PUFAs consumption was able to partially prevent the increase in apoptotic and the decrease in anti-apoptotic signaling pathways, involving GSK3β and ERK1\2 phosphorylation. Despite that, in this study Akt phosphorylation was not affected by both diets. Although it has been previously showed that p-Akt could phosphorylate GSK3β (Pap & Cooper, 1998), in this study we did not observed it, pointing to the involvement of other mechanism(s) responsible for the increase in the phosphorylation state (inactivation) of GSK3β. DHA can also modulate other proteins involved in apoptosis, such as Bcl-2 and Bcl-xl (Lukiw et al., 2005) and caspases (Calon et al., 2005).

In summary, our data suggest that ω3 fatty acids are important for the ontogeny of the glutamatergic system, as well as for neural cells survival against OGD injury, partially preventing the decrease in the glutamate uptake, and partially decreasing the apoptotic response and increasing the cellular capacity to activate anti-apoptotic pathways.

Acknowledgements

This work was support by CAPES, FAPERGS, National Research Council of Brazil (CNPq) and IBN.Net FINEP/FADESP Proc. n^{o} 01.06.0842-00. Special thanks to Prof. Dr. Luiz Valmor Portela, Prof. Dr. Carmem Gottfried and Prof. Dr. Diogo Lara, for the support.

References

Akbar M., Calderon F., Wen Z., Kim HY., 2005. Docosahexaenoic acid: A positive modulator of AKT signaling in neuronal survival. Proceedings of the National Academy of Science USA, 102 (31), 10858-10863.

André A., Juanéda P., Sébédio JL., Chardigny JM., 2005. Plasmalogen metabolism-related enzymes in rat brain during aging: influence of n-3 fatty acid intake. Biochimie, 88, 103-111.

Bas O., Songur A., Sahin O., Mollaoglu H., Ozen O. A., Yaman M., Eser O., Fidan H., Yagmurca M., 2007. The protective effect of fish oil n-3 fatty acids on cerebral ischemia in rat hippocampus. Neurochemistry International, 50, 548-554.

Bazan N. G., 2005. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. Brain Pathology, 15 (2), 159-166.

Bazan N. G., 2006. The Onset of Brain Injury and Neurodegeneration Triggers the Synthesis of Docosanoid Neuroprotective Signaling. Cellular and Molecular Neurobiology, 26 (4-6), 901-913.

Belayev L., Marchezelli VL., Khoutorova L., Rodriguez de Turco E. B., Busto R., Ginsberg M. D., Bazan N. G., 2005. Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. Stroke, 36 (1), 118-123.

Berry C. B., Hayes D., Murphy A., Weibner M., Rauen T., McBean G. J., 2005. Differential modulation of glutamate transporters GLT1, GLAST and EAAC1 by docosahexaenoic acid. Brain Research, 1037, 123-133.

Birch E. E., Garfield S., Hoffman D. R., Uauy R., Birch D. G., 2000. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Develop Medicine and Child Neurology, 42, 174-181.

Bourre J. M., 2004. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. Journal of Nutrition, Health and Aging, 8:163–74.

Calon F., Lim G. P., Morihara T., Yang F., Ubeda O., Salem N., Frautschy S. A., Cole G. M., 2005. Dietary n-3 polyunsaturated depletion activates caspase and decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease. European Journal of Neuroscience, 22, 617-626.

Caplan L. R., 1993. Stroke. A clinical approach. 2nd ed. Boston: Butterworth-Heinemann; 23-66.

Chen Y, Swanson RA. 2003. Astrocytes and brain injury. Journal of Cerebral Blood Flow and Metabolism, 23(2):137-149

Choi D. W., 1992. Excitotoxic cell death. Journal of Neurobiology, 23, 1261-1276.

Choi D. W., 1994. Glutamate receptors and the induction of excitotoxic neuronal death. Progress in Brain Research, 100, 47-51.

Cimarosti H., Rodnight R., Tavares A., Paiva R., Valentim L., Rocha E., Salbego C., 2001. An investigation of the neuroprotective effect of lithium in organotypic slice cultures of rat hippocampus exposed to oxygen and glucose deprivation. Neuroscience Letters, 315 (1-2), 33-36.

Collingridge G. L. & Isaac J. T. R., 2003. Functional role of protein interactions with AMPA and kainite receptors. Neuroscience Research, 47, 3-15.

Danbolt N. C., 2001. Glutamate uptake. Progress in Neurobiology, 65, 1-105.

Delion S., Chalon S., Guilloteau D., Besnard J-C., Durand G., 1996. α-Linolenic acid dietary deficiency alters age-related changes of dopaminergic and serotoninergic neurotransmission in rat frontal cortex. Journal of Neurochemistry, 66, 1582-1591.

Dosemeci A., Tao-Cheng J. H., Vinadé L., Jaffe H., 2006. Preparation of postsynaptic density fraction from hippocampal slices and proteomic analysis. Biochemical and Biophysical Research Communications, 339 (2), 687-694.

Dyall S. C., Michael G. J., Whelpton R., Scott A. G., Michael-Tittus A. T., 2006. Dietary enrichment with omega-3 polyunsaturated fatty acids reverses age-related decrease in the GluR2 and NR2B glutamate receptors subunits in rat forebrain. Neurobiology of Aging, 28 (3), 424-439.

Emanuelli T., Antunes V. F., Souza D. O., 1998. Characterisation of L-[3H]glutamate binding to fresh and frozen crude plasma membranes isolated from cerebral cortex of adult rats. Biochemistry and Molecular Biology Internation, 44 (6), 1265-1272.

Farooqui A. A. & Horrocks L. A., 2001. Plasmalogens, phospholipase A2, and docosahexaenoic acid turnover in brain tissue. Journal of Molecular Neuroscience, 16, 263-272.

Feoli A. M., Siqueira I., Almeida L. M., Tramontina A. C., Battu C., Wofchuk S. T., Gottfried C., Perry M. L., Gonçalves C. A., 2006. Brain glutathione content and

glutamate uptake are reduced in rats exposed to pre- and postnatal protein malnutrition. Journal of Nutrition, 136 (9), 2357- 61.

Florent S., Malaplate-Armand C., Youssef I., Kriem B., Koziel V., Escanyé M. C., Fifre A., Sponne I., Leininger-Muller B., Olivier J. L., Pillot T., Oster T., 2006. Docosahexaenoic acid prevents neuronal apoptosis induced by soluble amyloid-beta oligomers. Journal of Neurochemistry, 96 (2), 385-395.

Fontella F. U., Cimarosti H., Crema L. M., Thomazi A. P., Leite M. C., Gonçalves C. A., Wofchuk S., Dalmaz C., Netto C. A., 2005. Acute and repeated restraint stress influences cellular damage in rat hippocampal slices exposed to oxygen and glucose deprivation. Brain Research Bulletin, 65 (5), 443-450.

Garcia M. C., Ward G., Ma Y. C., Salem N., Kim H. Y., 1998. Effect of docosahexaenoic acid on the synthesis of phosphatidylethanolserine in rat brain in microssomes and C6 glioma cells. Journal of Neurochemistry, 70, 24-30.

Gardoni F., Di Luca, M., 2006. New targets for pharmacological intervention in the glutamatergic synapse. European Journal of Pharmacology, 545, 2-10.

Garland M., Sacks F., Colditz G., Rimm E., Sampson L., 1998. The relation between dietary intake and adipose tissue composition of selected fatty acids in U.S. women. American Journal of Clinical Nutrition, 67, 25-30.

German O. L., Insua M. F., Gentili C., Rotstein N. P., Politi L. E., 2006. Docosahexaenoic acid prevents apoptosis of retina photoreceptors by activating the ERK/MAPK pathway. Journal of Neurochemistry, 98 (5), 1507-1520.

Halliwell B., 1997. Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochemical Society Transactions, 24 (4), 1023-1027.

Hertz L., Zielke H. R., 2004. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neuroscience, 27 (12), 735-743.

Hibbeln J. R., Davis J. M., Steer C., Emmet P., Rogers I., Williams C., Golding J., 2007. Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. Lancet, 369 (9561), 578-585.

Högyes E., Nyakas C., Kiliaan A., Faskas T., Penke B., Luiten P. G. M., 2003. Neuroprotective effect of developmental docosahexaenoic acid supplement against excitotoxic brain damage in infant rats. Neuroscience, 119, 999-1012.

Holman R. T., Johnson S. B., Hatch T. F., 1982. A case of human linolenic acid deficiency involving neurological abnormalities. American Journal of Clinical Nutrition, 35 (3), 617-623.

Johnson E. J. & Schaefer E. J., 2007. Potential role of dietary n-3 fatty acids in the prevention of dementia and macular degeneration. American Jouranl of Clinical Nutrition, 83 (suppl), 1494S-1498S.

Kew J. N. & Kemp J. A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology, 179, 4-29.

Koh J. Y, Choi D. W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. Journal of Neuroscience Methods, 20 (1), 83-90.

Lauritzen L., Hansen H. S., Jorgensen M. H., Michaelsen K. F., 2001. The essentiality of long chain omega-3 fatty acids in relation to development and function of the brain and retina. Progress in Lipid Research, 40, 1-94.

Lim S. Y., Hoshiba J., Salem N., 2005. An extraordinary degree of structural specificity is required in neural phospholipids for optimal brain function: n-6 docosapentaenoic acid substitution for docosahexaenoic acid leads to a loss in spatial task performance. Journal of Neurochemistry, 95 (3), 848-857.

Lim S. Y., Doherty J. D., McBride K., Miller-Ihli N. J., Carmona G. N., Stark K. D., Salem N., 2005[1]. Lead exposure and (n-3) fatty acid deficiency during rat neonatal development affect subsequent spatial task performance and olfactory discrimination[1.] Journal of Nutrition, 135 (5), 1019-1026.

Lipton P., 1999. Ischemic cell death in brain neurons. Physiological Reviews, 79, 1431-1568.

Lipton S. A. & Rosenberg P. A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. New England Journal of Medicine, 330, 613-622.

Lisman J., Schulman H., Cline H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nature Review Neuroscience, 3 (3), 175-190.

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193 (1), 265-275.

Lukiw W. J., Cui J. G., Marcheselli V. L., Bodker M., Botkjear A., Gotlinger K., Serhan C. N., Bazan N. G., 2005. A role for docosahexaenoic acid-derived neuroprotectin

D1 in neural cell survival and Alzheimer disease. Journal of Clinical Investigation, 115 (10), 2774-2783.

Manent J. B., Represa A., 2007. Neurotransmitters and brain maturation: early paracrine actions of GABA and glutamate modulate neuronal migration. The Neuroscientist, 13 (3), 268-279.

Maragakis N. J., Rothstein J. D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiology of Disease, 15, 461-473.

Martin R. E., Bazan N. G., 1992. Changing fatty acid content of growth cone lipids prior to synaptogenesis. Journal of Neurochemistry, 59, 318-325.

Marszalek J. R., Lodish H. F. 2005. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. Annual Review of Cellular Development Biology, 21, 633-657.

Mehta S. L., Manhas N., Raghubir R., 2007. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Research Reviews, 54 (1), 34-66.

McGahon B. M., Martin D. S. D., Horrobin D. F., Lynch M. A., 1999. Age-related changes in synaptic function: analysis of the effect of dietary supplementation with ω-3 fatty acids. Neuroscience, 94 (1), 305-314.

Meldrum BS., 2000. Glutamate as a neurotransmitter in the brain: Review of physiology and pathology. The Journal of Nutrition, 130 (4S suppl), 1007S-1015S.

Moriguchi T, Greiner RS, Salem N., 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. Journal of Neurochemistry, 75 (6), 2563-2573.

Neuringer M, Connor WE, Lin DS, Barstad L, Luck S., 1986. Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. Proceedings of the National Academy of Sciences USA, 83, 4021-4025.

Niu S. L., Mitchell D. C., Lim S. Y., Wen Z. M., Kim H. Y., Salem Jr. N., Litman B. J., 2004. Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. Journal of Biological Chemistry, 279 (30), 31098-31104.

Noshita N, Lewen A, Sugawara T, Chan PH., 2001. Evidence of phosphorilation of Akt and neuronal survival after transient focal cerebral ischemia in mice. Journal of Cerebral Blood Flow Metabolism, 21, 1442-1450.

Oses JP, Leke R, Portela LV, Lara DR, Schmidt AP, Casali EA, Wofchuk S, Souza DO, Sarkis JJ., 2004. Biochemical brain markers and purinergic parameters in rat CSF after seizure induced by pentylenetetrazol. Brain Research Bulletin, 64 (3) 237-242.

Ozawa S, Kamiya H, Tsuzuki K., 1998. Glutamate receptors in the mammalian central nervous system. Progress in Neurobiology 54 (5) 581-618.

Pap M. & Cooper G. M., 1998. Role of glycogen synthase kinase-3 in the phosphathidylinositol 3-kinase/Akt cell survival pathway. Journal of Biological Chemistry, 273 (32), 19929-19932.

Peet M & Stokes C., 2005. Omega-3 fatty acids in the treatment of psychiatric disorders. Drugs, 65 (8), 1051-1059.

Phinney SD, Tang AB, Johnson SB, Holman RT., 1990. Reduced adipose 18:3 omega 3 with weight loss by very low calorie dieting. Lipids, 25, 798-806.

Popescu G & Auerbach A., 2004. The NMDA receptor gating machine: lessons from single channels. The Nueroscientist, 10 (3), 192-198.

Rossi D. J., Oshima T., Attwell D., 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature, 403 (6767), 316-321.

Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins JS, Au R, Tucker KL, Kyle DJ, Wilson PW, Wolf PA., 2006. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. Archives of Neurology, 63 (11), 1545-1550.

Schousboe A, Waagepetersen HS. 2005 Role of astrocytes in glutamate homeostasis: implications for excitotoxicity. Neurotoxic Research 8(3-4): 221-225.

Schiefermeier M & Yavin E., 2002. n-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of developing prenatal rat brain. Journal of Lipid Research, 43, 124-131.

Segovia G, Porras A, Del Arco A, Mora F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mechanisms of Ageing and Development, 122(1), 1-29.

Sheldon A. L. & Robinson M. B., 2007. The role of glutamate transporters in neurodegenerative and potential opportunities for intervention. Neurochemistry International, 51, 333-355.

Simopoulos A. P., Leaf A., Salem N. Jr., 2000. Workshop statement on the essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. Prostaglandins, Leukotrienes and Essential Fatty Acids, 63 (3), 119-121.

Söderberg M, Edlind C, Kristensson K, Dallner G., 1991. Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. Lipids, 26, 421-425.

Song G, Ouyang G, Bao S., 2005. The activation of Akt/PKB signaling pathway and cell survival. Journal of Cellular and Molecular Medicine, 9 (1), 59-71.

Stillwell W, Wassall SR., 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chemistry and Physics of Lipids, 126, 1-27, 2003.

Strasser U & Fischer G., 1995. Protection from neuronal damage induced by combined oxygen and glucose deprivation in organotypic hippocampal cultures by glutamate receptor antagonists. Brain Research, 687 (1-2), 167-174.

Strokin M, Chechneva O, Reymann KG, Reiser G., 2006. Neuroprotection of rat hippocampal slices exposed to oxygen-glucose deprivation by enrichment with docosahexaenoic acid and by inhibition of hydrolysis of docosahexaenoic acid-containing phospholipids by calcium independent phospholipase A2. Neuroscience, 140 (2), 547-553.

Thomazi AP, Godinho GF, Rodrigues JM, Schwalm FD, Frizzo ME, Moriguchi E, Souza DO, Wofchuk ST., 2004. Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. Mechanisms of Ageing and Development, 125 (7), 475-481.

Vinadé L., Chang M., Schlief M. L., Petersen J. D., Reese T. S., Tao-Cheng J. H., Dosemeci A., 2003. Affinity purification of PSD-95-containing postsynaptic complexes. Journal of Neurochemistry, 87 (5), 1255-1261.

Voloboueva L. A., Suh S. W., Swanson R. A., Giffard R. G., 2007. Inhibition of mitochondrial function in astrocytes: implications for neuroprotection. Journal of Neurochemistry, 102 (4), 1383-1394.

Willatts P, Forsyth JS, Di Modugno MK, Varma S, Colvin M., 1998. Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age. Lancet, 352 (9129), 688-691.

Xiao YF, Li X, 1999. Polyunsaturated fatty acids modify mouse hippocampal neuronal excitability during excitotoxic or convulsant stimulation. Brain Research, 846, 112-121.

Ximenes da Silva A, Lavialle F, Gendrot C, Guesnet P, Alessandri JM, Lavialle M., 2002. Glucose transport and utilization are altered in brain of rats deficient in n-3 polyunsaturated fatty acid. Journal of Neurochemistry, 81, 1328-1337.

Young C, Gean PW, Chiou LC, Shen YZ., 2000. Docosahexaenois acid inhibit synaptic transmission ans epileptiform activity in the rat hippocampus. Synapse, 37, 90-94.

Yuen AWC, sander JW, Fluegel D, Patsalos PN, Bell GS, Johnson T, Koepp MJ., 2005. Omega-3 fatty acid supplementation in patients with chronic epilepsy: A randomized trial. Epilepsy Behavior, 7 (2), 253-258.

Zimmer L, Delion-Vancassel S, Durand G, Guilloteau D, Bodard S, Besnard J-C, Chalon S., 2000. Modifications of dopamine neurotransmission in the nucleous accumbens of rats deficient in n-3 polyunsaturated fatty acids. Journal of Lipid Research, 41, 32-40.

Figures

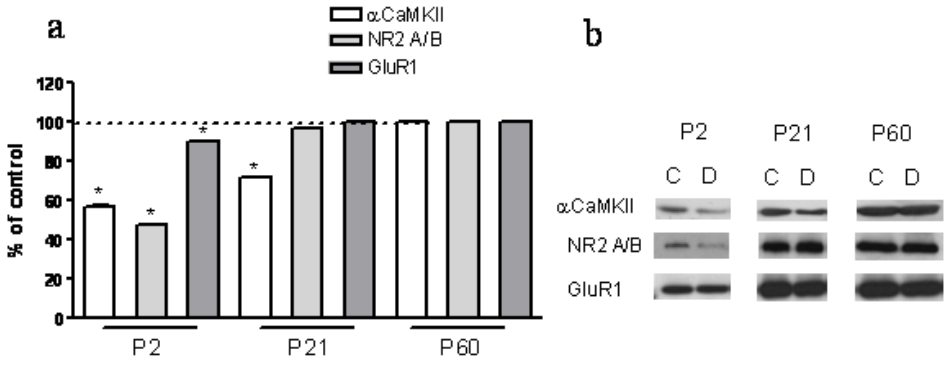

Figure 1: ω3 Deprivation affects the ontogeny of the immunocontent of αCaMKII enzyme and ionotropic glutamate receptors subunits NR2 A\B (NMDA) and GluR1 (AMPA) in the hippocampus of rats at postnatal age 02 (P2), 21 (P21) and 60 (P60) days. In Figure 1a, results are quantified as percentage of control group (100%, dashed line). Panel b shows representative Western blot images. C (control group); D (ω3 deprived-group). Data are expressed as means ± SD (*p<0.05, compared to 100%).

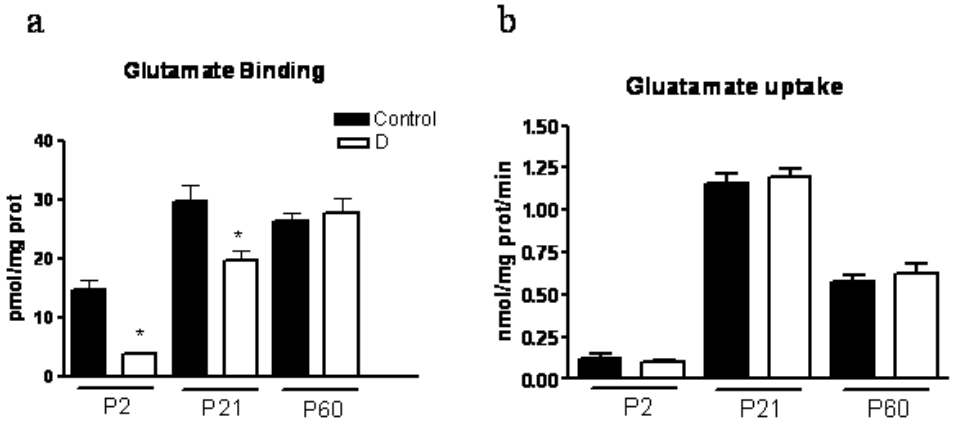

Figure 2: Effect of ω3 deprivation on the ontogeny of [^{3}H]glutamate binding (a) and [^{3}H]glutamate uptake (b) in hippocampal slices of rats at postnatal age of 02 (P2), 21 (P21) and 60 (P60) days. Control (control group); D (ω3 deprived-group). Data are expressed as means ± SD (*p<0.05 in relation to the respective control).

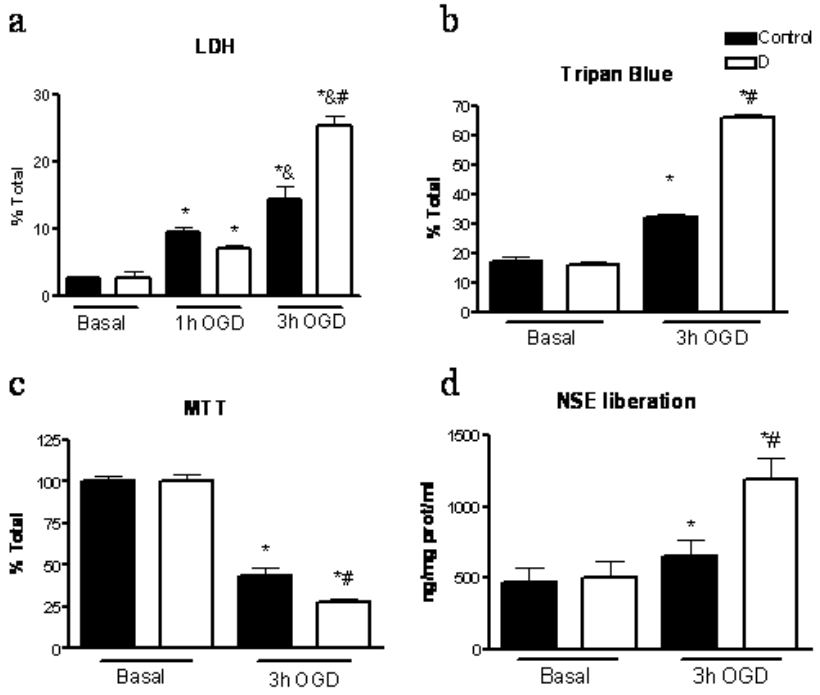

Figure 3: Effect of ω3 deprivation on cellular viability parameters of hippocampal slices of rats at 60 days of age, submitted to oxygen and glucose deprivation (OGD) for 60 min. LDH content released in the medium (a) was measured in basal conditions (basal) and after 1h (1h OGD) and 3h (3h OGD) of recovery. Trypan blue (b), MTT (c) and NSE released in the medium (d) were measured in basal conditions and after 3h of reperfusion. Control (control group); D (ω3 deprived-group); (* p<0.05 in relation to its basal; & p<0.01 in relation to 1h OGD; # p< 0.01 in relation to control 3h OGD).

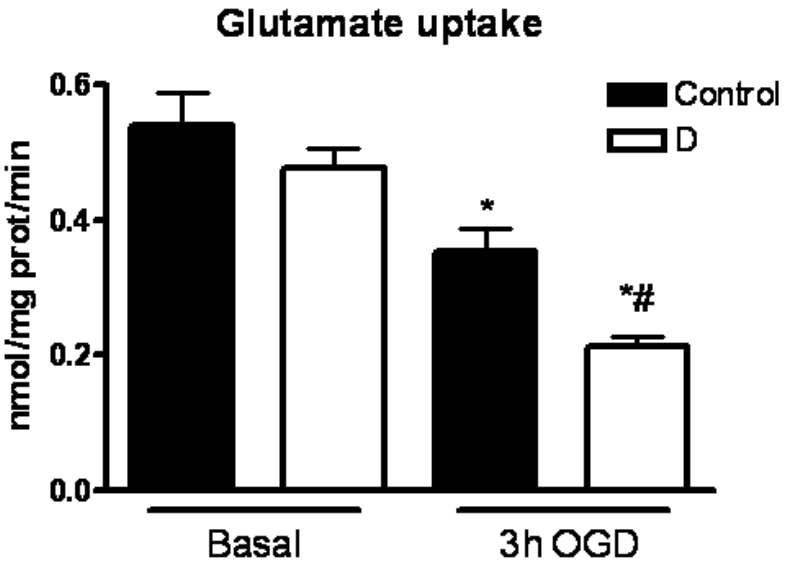

Figure 4: ω3 Deprivation affects [^{3}H]glutamate uptake measured after 3h of recovery in hippocampal slices of rats at 60 days of age submitted to oxygen and glucose deprivation (OGD) for 60 min. Control (control group); D (ω3 deprived-group); (* p<0.05 in relation to its basal; # p< 0.01 in relation to control 3h OGD).

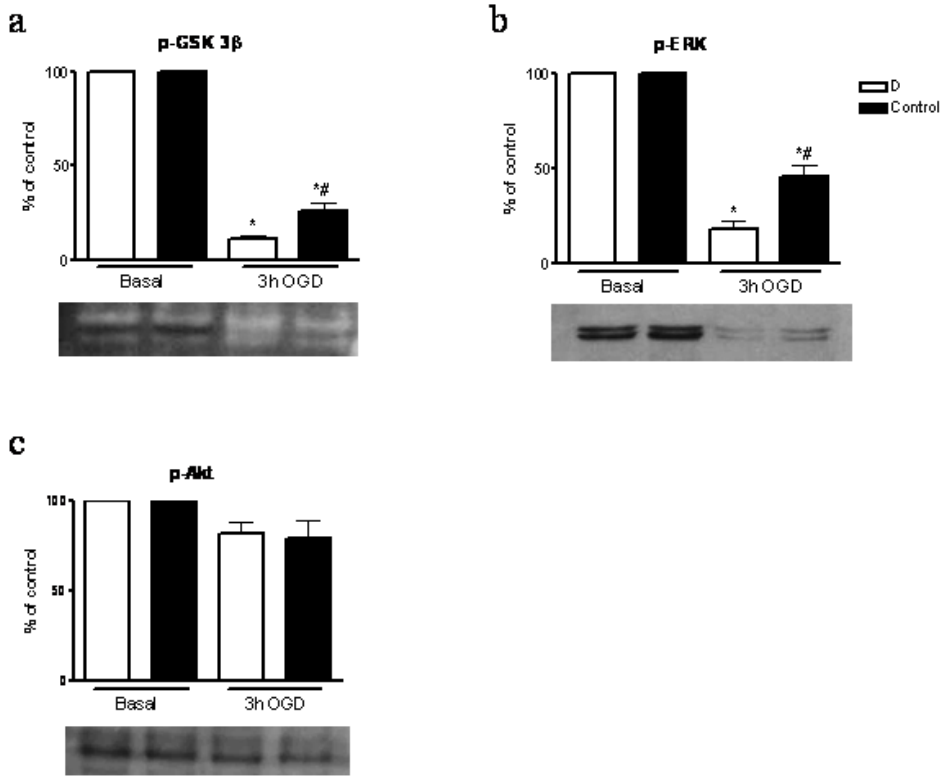

Figure 5: Effect of ω3 deprivation on the phosphorylation state of proteins p-GSK3β (a), p-ERK 1/2 (b) and p-Akt (c), involved in signaling pathways related to apoptosis/anti-apoptosis. Phosphorylation was measured in basal conditions (basal) and after 3h of recovery in hippocampal slices of rats at 60 days of age submitted to oxygen and glucose deprivation (OGD) for 60 min. Control (control group); D (ω3 deprived-group); (* $p<0.05$ in relation to its basal; # $p< 0.01$ in relation to D 3h OGD). Representative Western blot images are shown below the respective bars.

Parte 3

Discussão

O presente estudo mostrou que a deficiência de ácidos graxos ω3 na dieta de ratos foi capaz de causar atraso no desenvolvimento de parâmetros glutamatérgicos no hipocampo de ratos, o que pode estar relacionado com uma maior susceptibilidade do cérebro maduro à injúria isquêmica, com alterações na captação de glutamato e na fosforilação de proteínas envolvidas em rotas de sinalização apoptóticas e anti-apoptóticas.

Os receptores ionotrópicos glutamatérgicos interagem com uma variedade de proteínas envolvidas na organização espacial e funcional da densidade pós-sináptica, e também com proteínas envolvidas na transdução de sinal, como a família das MAPK e CaMKII (Meldrum 2000). Neste estudo foi mostrado que a deficiência de ácidos graxos ω3 atrasou o desenvolvimento da sinapse glutamatérgica, visto pelo imunoconteúdo das subunidades dos receptores NMDA e AMPA e da enzima αCaKMII, que foi normalizado na vida adulta. Calon e colaboradores (2005) também mostraram que a deficiência de ácidos graxos ω3 reduziu o imunoconteúdo das subunidades do receptor NMDA no córtex e hipocampo e da αCaKMII no córtex de camundongos TG2576, um modelo utilizado para estudar doença de Alzheimer. Em outro estudo, foram encontrados resultados similares em ratos idosos. Com a idade, o conteúdo de DHA nos fosfolipídios do hipocampo é reduzido, o que coincide com um decréscimo da função cerebral normal e da neuroplasticidade (Mcgahon et al., 1999; Johnson & Schaefer, 2007). Dyall e colaboradores (2006) mostraram que ratos apresentam um decréscimo com a idade das subunidades NR2B e GluR2 (receptores NMDA e AMPA, respectivamente), o que é prevenido com a

suplementação de ácidos graxos ω3. A redução do imunoconteúdo destas proteínas no início da vida pode prejudicar o adequado desenvolvimento cerebral, que pode estar relacionado com um aumento da suscetibilidade a injúrias cerebrais em animais adultos, como observado neste estudo, e déficits cognitivos encontrados em animais que consumiram dietas deficientes em ácidos graxos ω3 em outros estudos (Lim et al., 2005; Lim et al., 2005[1]; Niu et al., 2004; Moriguchi et al., 2000).

O glutamato é o principal neurotransmissor excitatório no cérebro de mamíferos e a função glutamatérgica é essencial para o desenvolvimento do SNC, contribuindo para a diferenciação neuronal, migração e sobrevivência no cérebro em desenvolvimento (Meldrum, 2000; Segovia et al, 2001; Manent & Represa, 2007). No entanto, o excesso de glutamato é altamente tóxico para os neurônios, um processo chamado excitotoxicidade (Choi, 1992). A toxicidade do glutamato pode contribuir para o dano celular observado em várias doenças cerebrais agudas e crônicas (Danbolt, 2001; Maragakis & Rothstein, 2004; Gardoni & Luca, 2006; Sheldon & Robinson, 2007). Então, o excesso de glutamato deve ser removido da fenda sináptica, e o equilíbrio entre o tônus fisiológico/tóxico do sistema glutamatérgico é modulado pela captação do glutamato extracelular, que é feita principalmente pelos transportadores de glutamato localizados nos astrócitos que circundam a fenda sináptica (Maragakis & Rothstein, 2004; Danbolt, 2001; Chen & Swanson, 2003; Schousboe & Waagepetersen, 2005).

Para verificar o efeito neuroprotetor dos ácidos graxos ω3, submetemos fatias de hipocampo dos ratos adultos por 1h de OGD e 3h de recuperação, e mensuramos a viabilidade celular por vários parâmetros. O tecido nervoso é particularmente sensível às espécies reativas de oxigênio (ROS, Halliwell, 1997), que são produzidos em quantidades

elevadas durante a recuperação pós-isquemia. Neste trabalho foi mostrado que, após a recuperação, o grupo deficiente em ácidos graxos ω3 apresentou uma suscetibilidade aumentada à injúria celular, incluindo dano neuronal especificamente (marcado pela liberação de NSE) que o grupo controle. Strokin e colaboradores (2006) mostraram resultados similares com o mesmo modelo de injúria. Eles mostraram que ocorre liberação de DHA durante a isquemia e que tanto o DHA livre como a preservação dos fosfolipídios que contém DHA são capazes de reduzir o dano celular. O DHA pode agir *per se* e por seus derivados, como a neuroprotectina D1, um docosanóide derivado do DHA (Bazan 2005). Este docosanóide parece ser sintetizado mediante uma injúria neuronal (Bazan, 2006; Lukiw et al., 2005), pode ativar rotas de sinalização que culminam em sobrevivência celular e pode contribuir para o efeito protetor do DHA no cérebro e retina (Bazan, 2005).

Buscando um mecanismo neuroquímico que possa explicar o efeito deletério da deficiência de ácidos graxos ω3, foi investigado o feito da OGD na captação de glutamato e em rotas de sinalização apoptóticas e anti-apoptóticas.

Tem sido mostrado que, após a OGD, a concentração extracelular de glutamato aumenta (Danbolt, 2001). No presente trabalho foi mostrado que após a OGD, há um decréscimo na captação de glutamato, o que está em acordo com outros estudos (Fontella et al., 2005). Porém, no grupo deficiente, este decréscimo foi mais pronunciado. Como a captação basal de glutamato foi similar entre os grupos, esta diferença pode estar relacionada com um dano celular maior neste grupo e/ou com uma menor capacidade dos astrócitos de proteger neurônios mantendo a concentração extracelular de glutamato abaixo dos níveis tóxicos. Além disso, os ácidos graxos ω3 podem estar modulando a densidade de

transportadores de glutamato após a isquemia. Para tanto, mais estudos necessitam ser feitos para elucidar este parâmetro.

Akbar e colaboradores (2005) mostraram que o DHA promove sobrevivência neuronal pela sinalização da Akt. Esta enzima tem efeito direto em rotas apoptóticas, inibindo a ativação de proteínas pró-apoptóticas, como Bad, caspase-9 e GSK3β (Song et al., 2005). Estudos prévios mostraram que bloqueando o aumento da fosforilação da Akt ocorre um aumento da fragmentação do DNA subseqüente (Noshita et al., 2001). Similarmente a sinalização da Akt, a rota de sinalização das MAPK é requerida para um efeito anti-apoptótico e sobrevivência neuronal no cérebro após um insulto isquêmico, e um estudo sugere que o DHA pode modular esta via (German et al, 2006). No presente trabalho foi mostrado que o consumo de ácidos graxos ω3 foi capaz de, parcialmente, prevenir o aumento na sinalização apoptótica e o decréscimo da anti-apoptótica, envolvendo a fosforilação de GSK3β e ERK1/2. Porém, neste estudo a fosforilação da Akt não foi afetada pelas dietas. Apesar de estudos prévios mostrarem que a Akt fosforilada fosforila a GSK3β (Pap & Cooper, 1998), neste estudo não foi observado tal efeito, apontando para o envolvimento de outros mecanismos responsáveis pela inativação da GSK3β. O DHA também pode modular outras proteínas envolvidas na rota apoptótica, como Bcl-2 e Bcl-xl (Lukiw et al., 2005) e caspases (Calon et al., 2005).

Este estudo sugere que o consumo de ácidos graxos ω3 é importante para a ontogenia do sistema glutamatérgico, bem como para a sobrevivência neuronal frente a injúria isquêmica, parcialmente prevenindo o decréscimo da captação de glutamato, diminuindo a resposta apoptótica e aumentando a capacidade celular de ativar rotas anti-apoptóticas.

References

Akbar M., Calderon F., Wen Z., Kim HY., 2005. Docosahexaenoic acid: A positive modulator of AKT signaling in neuronal survival. Proceedings of the National Academy of Science USA, 102 (31), 10858-10863.

André A., Juanéda P., Sébédio JL., Chardigny JM., 2005. Plasmalogen metabolism-related enzymes in rat brain during aging: influence of n-3 fatty acid intake. Biochimie, 88, 103-111.

Bas O., Songur A., Sahin O., Mollaoglu H., Ozen O. A., Yaman M., Eser O., Fidan H., Yagmurca M., 2007. The protective effect of fish oil n-3 fatty acids on cerebral ischemia in rat hippocampus. Neurochemistry International, 50, 548-554.

Bazan N. G., 2005. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. Brain Pathology, 15 (2), 159-166.

Bazan N. G., 2006. The Onset of Brain Injury and Neurodegeneration Triggers the Synthesis of Docosanoid Neuroprotective Signaling. Cellular and Molecular Neurobiology, 26 (4-6), 901-913.

Belayev L., Marchezelli VL., Khoutorova L., Rodriguez de Turco E. B., Busto R., Ginsberg M. D., Bazan N. G., 2005. Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. Stroke, 36 (1), 118-123.

Berry C. B., Hayes D., Murphy A., Weibner M., Rauen T., McBean G. J., 2005. Differential modulation of glutamate transporters GLT1, GLAST and EAAC1 by docosahexaenoic acid. Brain Research, 1037, 123-133.

Birch E. E., Garfield S., Hoffman D. R., Uauy R., Birch D. G., 2000. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Develop Medicine and Child Neurology, 42, 174-181.

Bourre J. M., 2004. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. Journal of Nutrition, Health and Aging, 8:163–74.

Burr G. O. & BurrM. M. 1929. A new deficiency disease produced by the rigid exclusion of fat from the diet. Journal os Biological Chemistry, 82, 345-367.

Calon F., Lim G. P., Morihara T., Yang F., Ubeda O., Salem N., Frautschy S. A., Cole G. M., 2005. Dietary n-3 polyunsaturated depletion activates caspase and decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease. European Journal of Neuroscience, 22, 617-626.

Caplan L. R., 1993. Stroke. A clinical approach. 2nd ed. Boston: Butterworth-Heinemann; 23-66.

Chen Y, Swanson RA. 2003. Astrocytes and brain injury. Journal of Cerebral Blood Flow and Metabolism, 23(2):137-149

Choi D. W., 1992. Excitotoxic cell death. Journal of Neurobiology, 23, 1261-1276.

Choi D. W., 1994. Glutamate receptors and the induction of excitotoxic neuronal death. Progress in Brain Research, 100, 47-51.

Cimarosti H., Rodnight R., Tavares A., Paiva R., Valentim L., Rocha E., Salbego C., 2001. An investigation of the neuroprotective effect of lithium in organotypic slice cultures

of rat hippocampus exposed to oxygen and glucose deprivation. Neuroscience Letters, 315 (1-2), 33-36.

Collingridge G. L. & Isaac J. T. R., 2003. Functional role of protein interactions with AMPA and kainite receptors. Neuroscience Research, 47, 3-15.

Danbolt N. C., 2001. Glutamate uptake. Progress in Neurobiology, 65, 1-105.

Delion S., Chalon S., Guilloteau D., Besnard J-C., Durand G., 1996. α-Linolenic acid dietary deficiency alters age-related changes of dopaminergic and serotoninergic neurotransmission in rat frontal cortex. Journal of Neurochemistry, 66, 1582-1591.

Dosemeci A., Tao-Cheng J. H., Vinadé L., Jaffe H., 2006. Preparation of postsynaptic density fraction from hippocampal slices and proteomic analysis. Biochemical and Biophysical Research Communications, 339 (2), 687-694.

Dyall S. C., Michael G. J., Whelpton R., Scott A. G., Michael-Tittus A. T., 2006. Dietary enrichment with omega-3 polyunsaturated fatty acids reverses age-related decrease in the GluR2 and NR2B glutamate receptors subunits in rat forebrain. Neurobiology of Aging, 28 (3), 424-439.

Emanuelli T., Antunes V. F., Souza D. O., 1998. Characterisation of L-[3H]glutamate binding to fresh and frozen crude plasma membranes isolated from cerebral cortex of adult rats. Biochemistry and Molecular Biology Internation, 44 (6), 1265-1272.

Farooqui A. A. & Horrocks L. A., 2001. Plasmalogens, phospholipase A2, and docosahexaenoic acid turnover in brain tissue. Journal of Molecular Neuroscience, 16, 263-272.

Feoli A. M., Siqueira I., Almeida L. M., Tramontina A. C., Battu C., Wofchuk S. T., Gottfried C., Perry M. L., Gonçalves C. A., 2006. Brain glutathione content and glutamate uptake are reduced in rats exposed to pre- and postnatal protein malnutrition. Journal of Nutrition, 136 (9), 2357- 61.

Florent S., Malaplate-Armand C., Youssef I., Kriem B., Koziel V., Escanyé M. C., Fifre A., Sponne I., Leininger-Muller B., Olivier J. L., Pillot T., Oster T., 2006. Docosahexaenoic acid prevents neuronal apoptosis induced by soluble amyloid-beta oligomers. Journal of Neurochemistry, 96 (2), 385-395.

Fontella F. U., Cimarosti H., Crema L. M., Thomazi A. P., Leite M. C., Gonçalves C. A., Wofchuk S., Dalmaz C., Netto C. A., 2005. Acute and repeated restraint stress influences cellular damage in rat hippocampal slices exposed to oxygen and glucose deprivation. Brain Research Bulletin, 65 (5), 443-450.

Garcia M. C., Ward G., Ma Y. C., Salem N., Kim H. Y., 1998. Effect of docosahexaenoic acid on the synthesis of phosphatidylethanolserine in rat brain in microssomes and C6 glioma cells. Journal of Neurochemistry, 70, 24-30.

Gardoni F., Di Luca, M., 2006. New targets for pharmacological intervention in the glutamatergic synapse. European Journal of Pharmacology, 545, 2-10.

Garland M., Sacks F., Colditz G., Rimm E., Sampson L., 1998. The relation between dietary intake and adipose tissue composition of selected fatty acids in U.S. women. American Journal of Clinical Nutrition, 67, 25-30.

German O. L., Insua M. F., Gentili C., Rotstein N. P., Politi L. E., 2006. Docosahexaenoic acid prevents apoptosis of retina photoreceptors by activating the ERK/MAPK pathway. Journal of Neurochemistry, 98 (5), 1507-1520.

Haag M. 2003. Essential fatty acids and the brain. Canadian Journal of Psychiatry, 48 (3), 195-203.

Halliwell B., 1997. Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochemical Society Transactions, 24 (4), 1023-1027.

Hansen A. E., Steward R. A., Hughes G., Soderhjelm L. 1962. The relation of linoleic acid to infant feeding. Acta of Paediatric, 51 (137) 1-41.

Heird WC & Lapillonne A. 2005. The role of essential fatty acids in development. Annual Reviews of Nutrition, 25, 549-571.

Hertz L., Zielke H. R., 2004. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neuroscience, 27 (12), 735-743.

Hibbeln J. R., Davis J. M., Steer C., Emmet P., Rogers I., Williams C., Golding J., 2007. Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. Lancet, 369 (9561), 578-585.

Högyes E., Nyakas C., Kiliaan A., Faskas T., Penke B., Luiten P. G. M., 2003. Neuroprotective effect of developmental docosahexaenoic acid supplement against excitotoxic brain damage in infant rats. Neuroscience, 119, 999-1012.

Holman R. T., Johnson S. B., Hatch T. F., 1982. A case of human linolenic acid deficiency involving neurological abnormalities. American Journal of Clinical Nutrition, 35 (3), 617-623.

Johnson E. J. & Schaefer E. J., 2007. Potential role of dietary n-3 fatty acids in the prevention of dementia and macular degeneration. American Jouranl of Clinical Nutrition, 83 (suppl), 1494S-1498S.

Kew J. N. & Kemp J. A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology, 179, 4-29.

Koh J. Y, Choi D. W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. Journal of Neuroscience Methods, 20 (1), 83-90.

Lauritzen L., Hansen H. S., Jorgensen M. H., Michaelsen K. F., 2001. The essentiality of long chain omega-3 fatty acids in relation to development and function of the brain and retina. Progress in Lipid Research, 40, 1-94.

Lim S. Y., Hoshiba J., Salem N., 2005. An extraordinary degree of structural specificity is required in neural phospholipids for optimal brain function: n-6 docosapentaenoic acid substitution for docosahexaenoic acid leads to a loss in spatial task performance. Journal of Neurochemistry, 95 (3), 848-857.

Lim S. Y., Doherty J. D., McBride K., Miller-Ihli N. J., Carmona G. N., Stark K. D., Salem N., 2005[1]. Lead exposure and (n-3) fatty acid deficiency during rat neonatal development affect subsequent spatial task performance and olfactory discrimination[1.] Journal of Nutrition, 135 (5), 1019-1026.

Lipton P., 1999. Ischemic cell death in brain neurons. Physiological Reviews, 79, 1431-1568.

Lipton S. A. & Rosenberg P. A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. New England Journal of Medicine, 330, 613-622.

Lisman J., Schulman H., Cline H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nature Review Neuroscience, 3 (3), 175-190.

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193 (1), 265-275.

Lukiw W. J., Cui J. G., Marcheselli V. L., Bodker M., Botkjear A., Gotlinger K., Serhan C. N., Bazan N. G., 2005. A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. Journal of Clinical Investigation, 115 (10), 2774-2783.

Manent J. B., Represa A., 2007. Neurotransmitters and brain maturation: early paracrine actions of GABA and glutamate modulate neuronal migration. The Neuroscientist, 13 (3), 268-279.

Maragakis N. J., Rothstein J. D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiology of Disease, 15, 461-473.

Martin R. E., Bazan N. G., 1992. Changing fatty acid content of growth cone lipids prior to synaptogenesis. Journal of Neurochemistry, 59, 318-325.

Marszalek J. R., Lodish H. F. 2005. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. Annual Review of Cellular Development Biology, 21, 633-657.

Mehta S. L., Manhas N., Raghubir R., 2007. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Research Reviews, 54 (1), 34-66.

McGahon B. M., Martin D. S. D., Horrobin D. F., Lynch M. A., 1999. Age-related changes in synaptic function: analysis of the effect of dietary supplementation with ω-3 fatty acids. Neuroscience, 94 (1), 305-314.

Meldrum BS., 2000. Glutamate as a neurotransmitter in the brain: Review of physiology and pathology. The Journal of Nutrition, 130 (4S suppl), 1007S-1015S.

Moriguchi T, Greiner RS, Salem N., 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. Journal of Neurochemistry, 75 (6), 2563-2573.

Neuringer M, Connor WE, Lin DS, Barstad L, Luck S., 1986. Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. Proceedings of the National Academy of Sciences USA, 83, 4021-4025.

Niu S. L., Mitchell D. C., Lim S. Y., Wen Z. M., Kim H. Y., Salem Jr. N., Litman B. J., 2004. Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. Journal of Biological Chemistry, 279 (30), 31098-31104.

Noshita N, Lewen A, Sugawara T, Chan PH., 2001. Evidence of phosphorilation of Akt and neuronal survival after transient focal cerebral ischemia in mice. Journal of Cerebral Blood Flow Metabolism, 21, 1442-1450.

Oses JP, Leke R, Portela LV, Lara DR, Schmidt AP, Casali EA, Wofchuk S, Souza DO, Sarkis JJ., 2004. Biochemical brain markers and purinergic parameters in rat CSF after seizure induced by pentylenetetrazol. Brain Research Bulletin, 64 (3) 237-242.

Ozawa S, Kamiya H, Tsuzuki K., 1998. Glutamate receptors in the mammalian central nervous system. Progress in Neurobiology 54 (5) 581-618.

Pap M. & Cooper G. M., 1998. Role of glycogen synthase kinase-3 in the phosphathidylinositol 3-kinase/Akt cell survival pathway. Journal of Biological Chemistry, 273 (32), 19929-19932.

Peet M & Stokes C., 2005. Omega-3 fatty acids in the treatment of psychiatric disorders. Drugs, 65 (8), 1051-1059.

Phinney SD, Tang AB, Johnson SB, Holman RT., 1990. Reduced adipose 18:3 omega 3 with weight loss by very low calorie dieting. Lipids, 25, 798-806.

Popescu G & Auerbach A., 2004. The NMDA receptor gating machine: lessons from single channels. The Nueroscientist, 10 (3), 192-198.

Rossi D. J., Oshima T., Attwell D., 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature, 403 (6767), 316-321.

Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins JS, Au R, Tucker KL, Kyle DJ, Wilson PW, Wolf PA., 2006. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. Archives of Neurology, 63 (11), 1545-1550.

Schousboe A, Waagepetersen HS. 2005 Role of astrocytes in glutamate homeostasis: implications for excitotoxicity. Neurotoxic Research 8(3-4): 221-225.

Sciefermeier M & Yavin E., 2002. n-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of developing prenatal rat brain. Journal of Lipid Research, 43, 124-131.

Segovia G, Porras A, Del Arco A, Mora F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mechanisms of Ageing and Development, 122(1), 1-29.

Sheldon A. L. & Robinson M. B., 2007. The role of glutamate transporters in neurodegenerative and potential opportunities for intervention. Neurochemistry International, 51, 333-355.

Simopoulos A. P., Leaf A., Salem N. Jr., 2000. Workshop statement on the essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. Prostaglandins, Leukotrienes and Essential Fatty Acids, 63 (3), 119-121.

Söderberg M, Edlind C, Kristensson K, Dallner G., 1991. Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. Lipids, 26, 421-425.

Song G, Ouyang G, Bao S., 2005. The activation of Akt/PKB signaling pathway and cell survival. Journal of Cellular and Molecular Medicine, 9 (1), 59-71.

Stillwell W, Wassall SR., 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chemistry and Physics of Lipids, 126, 1-27, 2003.

Strasser U & Fischer G., 1995. Protection from neuronal damage induced by combined oxygen and glucose deprivation in organotypic hippocampal cultures by glutamate receptor antagonists. Brain Research, 687 (1-2), 167-174.

Strokin M, Chechneva O, Reymann KG, Reiser G., 2006. Neuroprotection of rat hippocampal slices exposed to oxygen-glucose deprivation by enrichment with docosahexaenoic acid and by inhibition of hydrolysis of docosahexaenoic acid-containing phospholipids by calcium independent phospholipase A2. Neuroscience, 140 (2), 547-553.

Thomazi AP, Godinho GF, Rodrigues JM, Schwalm FD, Frizzo ME, Moriguchi E, Souza DO, Wofchuk ST., 2004. Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. Mechanisms of Ageing and Development, 125 (7), 475-481.

Vinadé L., Chang M., Schlief M. L., Petersen J. D., Reese T. S., Tao-Cheng J. H., Dosemeci A., 2003. Affinity purification of PSD-95-containing postsynaptic complexes. Journal of Neurochemistry, 87 (5), 1255-1261.

Volobоueva L. A., Suh S. W., Swanson R. A., Giffard R. G., 2007. Inhibition of mitochondrial function in astrocytes: implications for neuroprotection. Journal of Neurochemistry, 102 (4), 1383-1394.

Williard D. E., Harmon S. D., Kaduce T. L., Preuss M., Moore S. A., Robins M. E., Spector A. A. 2001. Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes. Journal of Lipid Research, 42 (9), 1368-1376.

Willatts P, Forsyth JS, Di Modugno MK, Varma S, Colvin M., 1998. Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age. Lancet, 352 (9129), 688-691.

Xiao YF, Li Xiangyang., 1999. Polyunsaturated fatty acids modify mouse hippocampal neuronal excitability during excitotoxic or convulsant stimulation. Brain Research, 846, 112-121.

Ximenes da Silva A, Lavialle F, Gendrot C, Guesnet P, Alessandri JM, Lavialle M., 2002. Glucose transport and utilization are altered in brain of rats deficient in n-3 polyunsaturated fatty acid. Journal of Neurochemistry, 81, 1328-1337.

Young C, Gean PW, Chiou LC, Shen YZ., 2000. Docosahexaenois acid inhibit synaptic transmission ans epileptiform activity in the rat hippocampus. Synapse, 37, 90-94.

Yuen AWC, sander JW, Fluegel D, Patsalos PN, Bell GS, Johnson T, Koepp MJ., 2005. Omega-3 fatty acid supplementation in patients with chronic epilepsy: A randomized trial. Epilepsy Behavior, 7 (2), 253-258.

Zimmer L, Delion-Vancassel S, Durand G, Guilloteau D, Bodard S, Besnard J-C, Chalon S., 2000. Modifications of dopamine neurotransmission in the nucleous accumbens of rats deficient in n-3 polyunsaturated fatty acids. Journal of Lipid Research, 41, 32-40.