Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Influência dos ácidos graxos ômega-3 sobre o sistema glutamatérgico no hipocampo e retina de ratos: parâmetros de desenvolvimento, comportamentais e de neuroproteção.

Júlia Dubois Moreira

Porto Alegre, Janeiro de 2011.

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Porto Alegre, Janeiro de 2011.

"No momento em que uma célula masculina microscópica e serpenteante encaminha-se para a célula – ovo muito maior e liga-se à ela, um ser humano começa a existir, e a Nutrição tem início. Este período de desenvolvimento, quando as coisas podem ser definitivamente 'certas' ou 'erradas', é de vital importância, e a nutrição pode exercer uma profunda influência que se expande por toda a vida." (Roger Willians)

***"Títulos não fazem Mestres, mas sim a maestria com que vivemos e ensinamos."* (Autor desconhecido)**

Dedico esta Tese de Doutorado à minha mãe.

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APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As sessões Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos.

Os itens Introdução, Discussão, Conclusões e Perspectivas encontrados nesta tese apresentam interpretações e comentário gerais sobre os resultados contidos nos artigos científicos do presente trabalho. As referências bibliográficas referem-se somente às citações que aparecem nos itens supracitados.

As informações técnicas mais precisas sobre cada metodologia utilizada poderão ser encontradas nos artigos científicos correspondentes.

SUMÁRIO

PARTE I

RESUMO

O glutamato, principal neurotransmissor excitatório no sistema nervoso central (SNC), está envolvido em várias funções cerebrais e da retina, como aprendizado/memória, desenvolvimento e envelhecimento cerebral e função visual. No entanto, o aumento da concentração do glutamato na fenda sináptica pode levar a neurotoxicidade. A excitotoxicidade glutamatérgica está relacionada a várias desordens cerebrais, tanto agudas quanto crônicas. Manter o nível de glutamato em concentrações fisiológicas relevantes ao adequado funcionamento do SNC se faz necessário. Para tanto, existem transportadores específicos para o glutamato, responsáveis por controlar os níveis de glutamato na fenda sináptica. Os ácidos graxos essenciais ω3 vêm ganhando especial atenção de pesquisadores por seus efeitos sobre o SNC, tanto responsáveis pelo adequado desenvolvimento e funcionamento cerebral e da retina, quanto por sua ação neuroprotetora frente a patologias relacionadas com a excitotoxicidade glutamatérgica. Na presente tese, nós demonstramos que os ácidos graxos ω3 são importantes para a homeostasia do sistema glutamatérgico tanto no hipocampo quanto na retina de ratos. Uma dieta deficiente em ácidos graxos ω3, mantida durante todo o período de desenvolvimento do SNC até a vida adulta, foi capaz de atrasar o desenvolvimento normal das sinapses glutamatérgicas, onde houve uma redução de proteínas sinápticas (NMDA, AMPA e α-CaMKII) no período pós-natal, promovendo alterações comportamentais na vida adulta de ratos, como comportamentos de hiperatividade e ansiedade. Os animais deficientes em ω3 também apresentaram déficits na memória aversiva de longa duração, que foi relacionado com uma baixa interação do receptor NMDA com a proteína cinase Fyn, bem como a redução dos níveis de DHAω3 e BDNF no hipocampo dos ratos. Os ácidos graxos ω3 preveniram as alterações sobre parâmetros do sistema glutamatérgico (queda na captação de glutamato, aumento de transportadores de glutamato GLAST e GLT-1) provocadas por um único evento convulsivo neonatal. Além disso, os animais deficientes em ácidos graxos ω3 apresentaram aumento no conteúdo de GFAP (proteína astrocitária) e redução no conteúdo de NeuN (proteína neuronal) independente do evento convulsivo. Os ácidos graxos ω3 também preveniram o déficit de memória de curta duração na vida adulta dos ratos causado pela convulsão

neonatal. Na retina de ratos, a deficiência dos ácidos graxos ω3 causou alterações na captação de glutamato e no conteúdo do transportador GLT-1. Após um insulto isquêmico na retina, os animais deficientes em ácidos graxos ω3 apresentaram alterações em parâmetros do sistema glutamatérgico (queda na captação de glutamato, aumento de GLT-1 e EAAC1). Já nos animais que receberam ácidos graxos ω3, o conteúdo de GLT-1 foi maior tanto basal quanto isquêmico, e somente o EAAC1 foi modulado. Nossos resultados mostram a importância dos ácidos graxos ω3 em manter a homeostasia glutamatérgica, contribuindo para o adequado desenvolvimento, funcionamento e proteção do SNC e da retina.

ABSTRACT

The main excitatory neurotransmitter in central nervous system (CNS), glutamate, is involved in many functions in brain and retina, such as learning / memory, brain and retina development and aging, and visual process. However, increased glutamate concentrations in synaptic cleft could lead to neurotoxicity. Glutamate excitotoxicity was related to many neurological acute and chronic disorders. Keeping glutamate at relevant physiological concentrations to adequate CNS function is necessary. The high affinity glutamate transporters are responsible to maintain glutamate below neurotoxic levels, preventing excitotoxicity. The essential omega-3 fatty acids (ω3) have received attention from scientific community because of its effects on brain development and neuroprotection against pathologies related to glutamatergic excitotoxicity. In the present thesis, we demonstrated that dietary ω3 fatty acids are important for glutamatergic system homeostasis in the hippocampus and retina of rats. A ω3 deficient diet, maintain since gestation until adulthood, was capable to delay the normal glutamatergic synapse development, with a reduction in synaptic proteins (NMDA, AMPA and αCaMKII) in the postnatal period, promoting behavioral alterations in adulthood in rats (hyperactivity and anxiety-like behavior). The deficient animals also presented aversive long-term memory deficit, which was related to reduction in the interaction of NMDA receptor with Fyn kinase protein, as well as in DHA and BDNF hippocampal content in rats. Dietary ω3 fatty acids prevented alterations on glutamatergic system functionality (decrease in glutamate uptake activity, increase in glutamate transporters GLAST and GLT-1 contents) caused by one single neonatal seizures episode. Dietary ω3 fatty acids also prevented aversive short-term memory deficit in adulthood caused by neonatal seizures. In the retina, ω3 deficiency caused decrease in glutamate uptake and reduction in GLT-1 content in basal conditions. After ischemic insult in the retina, ω3 deficient rats presented alteration on parameters of glutamatergic system evaluated (decrease in glutamate uptake activity, increase in GLT-1 and EAAC1 transporters). In ω3 adequate rats, GLT-1 content was higher in basal and ischemic conditions, and only EAAC1 was increase after ischemia. Taking together, our data show the

importance of dietary ω3 fatty acids to maintain glutamatergic system homeostasis, contributing to adequate development, function and protection of CNS and retina.

LISTA DE ABREVIATURAS

SNC – sistema nervoso central

GluRs – receptores de glutamato

iGluRs – receptores ionotrópicos de glutamato

mGluRs – receptores metabotrópicos de glutamato

NMDA – N-metil-D-aspartato

AMPA - α-amino-3hidroxi-5-metil-4-isoxazol-propionato

EAAT- transportador de aminoácidos excitatórios

DNA – ácido desoxirribonucléico

RNA – ácido ribonucléico

STM – memória de curta duração (do inglês *short-term memory)*

LTM – memória de longa duração (do inglês *long-term memory)*

LTP – potenciação de longa duração (do inglês *long-term potentiation)*

CaMKII – enzima cinase dependente de cálcio e calmodulina tipo II

BDNF – fator neurotrófico derivado do encéfalo

TrkB – receptor tirosina cinase B

SRC – família de proteínas cinase citosólicas

ω3 – ácidos graxos da série ômega-3

ω6 – ácidos graxos da série ômega-6

LA – ácido linoleico

ALA – ácido α-linolênico

LC-PUFA – ácidos graxos poliinsaturados de cadeia longa

ARA – ácido araquidônico

EPA – ácido eicosapentaenóico

DHA – ácido docosahexaenoico

DPA – ácido docosapentaenóico

PLA2 – fosfolipase A2

1. INTRODUÇÃO

1.1. O sistema glutamatérgico

O glutamato é o principal neurotransmissor excitatório no SNC e está envolvido em várias funções cerebrais, como aprendizado/memória, desenvolvimento e envelhecimento cerebral, comunicação celular e interação entre estruturas cerebrais (Ozawa et al, 1998; Danbolt, 2001; Segovia et al, 2001; Izquierdo et al., 2006; Tzingounis e Wadiche, 2007). 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-metil-4-isoxazol-propionato (AMPA), N-metil-D-aspartato (NMDA) e cainato. Os mGluRs são acoplados a proteínas ligadoras de GTP (proteínas G) e modulam a produção de mensageiros intracelulares (Kew e Kemp, 2005).

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 subunidades NR2 (A, B, C e D) ou NR3 (A e B), enquanto que os receptores tipo AMPA são combinações de subunidades GluR1 - GluR4 (Monyer et al., 1994; Kew e 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 (Ozawa et al, 1998; Kew and Kemp, 2005).

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 hiperestimulação 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 e Robinson, 2007).

Manter o nível de glutamato em concentrações fisiológicas relevantes ao adequado funcionamento cerebral se faz necessário. Existem evidências de que a excitotoxicidade glutamatérgica pode ser prevenida pela retirada do glutamato excedente da fenda sináptica (Rothstein et al., 1996; Chen e Swanson, 2003; Belanger e Magistretti, 2009; Tzingounis e Wadiche, 2007). Para isso, 5 distintos transportadores de glutamato com alta afinidade e dependentes de sódio foram identificados em tecidos animais e humanos: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5. Estes diferem em estrutura molecular, propriedades farmacológicas e distribuição em diferentes tecidos (Danbolt, 2001; Dunlop, 2006; Beart and O`Shea, 2007; Bunch et al., 2009). GLAST e GLT-1 são localizados em astrócitos, enquanto que EAAC1 é encontrado em neurônios (Danbolt, 2001; Dunlop, 2006). EAAT4 localiza-se principalmente em células de Purkinje do cerebelo, enquanto que o EAAT5 é localizado em células da retina (Pow and Barnett, 2000). Os transportadores de glutamato podem ser modulados em praticamente todos os níveis, ou seja, durante a transcrição do DNA, processamento do mRNA, síntese de proteínas ou pós-transducional (Danbolt,

2001; Eulenburg and Gomeza, 2010; Tzingounis e Wadiche, 2007). Esta última envolve modulações alostéricas, pelo ácido araquidônico ou Zn^{+}, por exemplo (Vandenberg et al., 2004), ou pela translocação dos transportadores entre a membrana plasmática e compartimentos intracelulares (Gegelashvili et al, 2000; Lortet et al., 1999; O`Shea, 2002; Robinson, 2002). A translocação de transportadores para a membrana envolve ativação de proteínas cinases e fosfatases, modulando diferentemente os transportadores (Gegelashvili et al, 2000; Lortet et al., 1999; O`Shea, 2002; Robinson, 2002).

Estudos têm considerado que alterações na atividade dos transportadores de glutamato pode ser o evento inicial ou parte de uma cascata que implicaria na fisiopatologia de doenças cerebrais agudas e crônicas (Maragakis e Rothstein, 2004; Moussa et al., 2007; Robinson, 2006; Sheldon e Robinson, 2007; Stevens, 2008; Tzingounis e Wadiche, 2007).

1.1.1. O sistema glutamatérgico e memória

O processo de memória é um evento complexo. Estudos mostram que a formação da memória envolve pelo menos duas fases: uma fase independente de síntese de proteínas e mRNA, que dura de 1h a 3h pós-treino (memória de curta duração, STM); e uma fase dependente de síntese protéica e de mRNA, que dura várias horas a dias, semanas ou mais (memória de londa duração, LTM) (Davis & Squire, 1984; Emptage & Carew, 1993; Izquierdo & Medina, 1997; McGaugh, 2000; Schafe & LeDoux, 2000; Scharf et al., 2002; Bekinschtein et al., 2007).

Eventos moleculares no hipocampo que podem influenciar a formação da memória envolvem a ativação de cascatas de sinalização, implicando na ativação

de receptores ionotrópicos de glutamato, essenciais para o evento de *potenciação de longa duração* (LTP), o qual é considerado um modelo das bases celulares e moleculares da memória (Lisman et al., 2002). 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 LTP.

Outro fator importante para a formação da memória e LTP é a síntese do fator neurotrófico derivado do encéfalo (BDNF), uma neurotrofina amplamente distribuída no cérebro de mamíferos, relacionado com processos de plasticidade sináptica e memória, essencial para o desenvolvimento cerebral, modulação da formação das sinapses e arborização dendrítica (Bamji et al., 2006; Tyler & Pozzo-Miller, 2003; Tyler et al., 2002; Bekinschtein et al., 2008). O BDNF, ao ativar seus receptores tirosina cinase B (TrkB), pode ampliar a sinalização glutamatérgica, por aumentar a forforilação dos receptores NMDA (subunidades NR1 e NR2), e desta maneira contribuindo para a formação da memória (Kafitz et al., 1999, Suen et al., 1997; Yamada et al., 2002).

Além do BDNF, a família de proteínas cinases citosólicas SRCs, afetam a ativação de receptores NMDA. Estas proteínas apresentam 5 membros sendo expressos no SNC – SRC, Fyn, Lyn, Lck e Yes- envolvidas em muitas funções celulares, como diferenciação celular, crescimento de neuritos e regulação de canais iônicos (Salter e Kalia, 2004). Dentre estas, Fyn, também está no processo

de envolvida na formação da memória e LTP por, entre outras funções, interagir com o receptor NMDA, fosforilando a subunidade NR2B (Grant et al., 1992; Salter & Kalia, 2004; Kojima et al., 2005; Kojima et al., 1998). A formação da memória espacial e aversiva implica na ativação da Fyn e, consequentemente, na interação desta com o receptor NMDA (Bevilaqua et al., 2003; Mizuno et al., 2003; Isosaka, 2008).

1.1.2. O sistema glutamatérgico e convulsão

A ampliação dos disparos neuronais que levam a episódios de crises convulsivas envolvem a interação de sinais inibitórios GABAérgicos e excitatórios glutamatérgicos (Bradford, 1995; Meldrum, 2000; Naylor, 2010; Treiman, 2001). Desta forma a hiperestimulação glutamatérgica, o bloqueio de transportadores de glutamato ou alterações que ativem os receptores de glutamato podem precipitar crises convulsivas. Estas crises podem ser únicas ou crônicas e recorrentes, as quais chamamos crises epilépticas (Meldrum, 1994; Meldrum, 2000).

Episódios convulsivos são muito comuns na infância, principalmente no primeiro ano de vida (Holmes e Ben-Ari, 2001). Esta alta suscetibilidade do cérebro imaturo a eventos convulsivos se deve ao fato de que o ácido γ-aminobutírico (GABA), neurotransmissor inibitório do SNC, exerce efeitos paradoxais excitatórios no cérebro imaturo (Khazipov et al., 2004; Ben-Ari, 2002). O sistema glutamatérgico, além de um dos responsáveis pelos eventos convulsivos, ainda pode sofrer alterações funcionais, e afetar o funcionamento cerebral tanto após o episódio convulsivo como na vida adulta (Eid et al., 2004;

Ueda et al., 2001; Zhang et al., 2004; Hammer et al., 2008; Cognato et al., 2010; Cornejo et al., 2007; Cornejo et al., 2008).

1.1.3. O sistema glutamatérgico no sistema visual: retina

O glutamato está intimamente envolvido na função visual (Massey, 1990; Bodnarenko et al., 1995). Na retina, o glutamato é o principal neurotransmissor na sinalização vertical dos fotorreceptores para as células ganglionares (Massey & Miller, 1987; 1990). Na camada plexiforme interna, o glutamato é liberado de 2 tipos de células bipolares; as células bipolares-ON liberam o glutamato mediante estimulo luminoso, enquanto as células bipolares-OFF liberam glutamato no escuro (Copenhagen et al, 1983). Na camada plexiforme externa, o glutamato é continuamente liberado dos fotorreceptores para as células ganglionares, as quais levam o impulso nervoso visual para os centros da visão cerebrais (Copenhagen & Jahr, 1989). Os fotorreceptores e as células bipolares não geram potenciais de ação, mas respondem a luz por potenciais que modulam a contínua liberação de glutamato. Então, é de extrema relevância controlar a concentração de glutamato na fenda sináptica, não somente pelo seu efeito excitotóxico quando em latas concentrações, como também porque altas concentrações de glutamato representam o estímulo luminoso (Copenhagen et al., 1983; Romano et al., 1995). Estudos mostram que o acúmulo de glutamato na retina e no humor vítreo é associado com o desenvolvimento do glaucoma, uma doença caracterizada por morte neuronal na retina (Dreyer et al., 1996; Dreyer & Grosskreutz, 1997; Osborne et al., 1999). O glutamato também parece estar envolvido em patologias retinianas como isquemia e retinopatia diabética (Bringmann et al., 2006).

Os transportadores de glutamato são responsáveis por manter a concentração extracelular de glutamato abaixo dos níveis de toxicidade e participam do balanço entre a sinalização fisiológica necessária para o estímulo visual e a hiperestimulação patológica dos receptores glutamatérgicos (Sucher et al., 1997; Holcombe et al., 2007). Na retina, 5 distintos transportadores de glutamato forma identificados por imunocitoquímica: GLAST (EAAT1) é associado a célula glial de Müller (Rauen et al., 1998; Pow & Barnett, 1999); GLT-1 (EAAT2) com os fotorreceptores do tipo cones e células bipolares (Rauen & Kanner, 1994); EAAC1 (EAAT3) às células horizontais, células amacrinas e células ganglionares (Rauen et al., 1996; Schultz and Stell, 1996); EAAT4 a photoreceptores (Pignataro et al., 2005); e o EAAT5 com fotorreceptores e células bipolares (Pow & Barnett, 2000). Em condições fisiológicas, o transporte de glutamato é realizado principalmente pelo GLAST presente nas células de Müller (Rauen et al., 1998; Pow & Barnett, 2000); porém em condições de injúria, como ocorre na isquemia retiniana, a captação de glutamato pelas células de Müller é comprometida (Napper et al., 1999, Barnett et al, 2001), a qual pode afetar a integridade celular na retina, comprometendo seu adequado funcionamento (Osborne et al., 2004).

1.2. Os ácidos graxos essenciais

No fim da década de 20, dois pesquisadores, ao observarem alguns sinais e sintomas em pessoas que tinham restrição de gorduras em suas dietas, constataram que havia compostos que eram essenciais para a saúde do organismo (Burr, 1981). Desde antão, 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; Marszalek & Lodish, 2005).

O ácido graxo essencial linoléico (LA 18:2ω6) (Burr, 1981; Hansen et al., 1962) 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 os ácidos eicosapentaenóico (EPA 20:5ω3) e 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 no cérebro e na 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 (elongases) e dessaturam (dessaturases) 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). Cabe ainda ressaltar que o mesmo sistema enzimático presente no fígado também é encontrado em astrócitos no sistema nervoso central (Williard et al., 2001), o que auxilia a garantir o suprimento de ácidos graxos ω3, principalmente, ao tecido nervoso.

1.2.1. O DHA e sua relação com o sistema nervoso central

1.2.1.1. O DHA e o funcionamento cerebral

O ácido docosaexaenóico (DHA 22:6ω3) é o ácido graxo mais abundante no SNC, tanto no cérebro como na retina (Marszalek & Lodish, 2005). Após ser absorvido no intestino e chegar ao fígado, o DHA chega ao cérebro ligado à

albumina pela corrente sangüínea e passa a barreira hemato-encefálica e retiniana pela ação de proteínas específicas de transporte (Owada et al., 2006; Larqué et al., 2006)

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 e retina 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; 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), além das vesículas sinápticas, 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, pouco se sabe sobre a influência destes ácidos graxos sobre o funcionamento do sistema glutamatérgico.

1.2.1.2 O DHA e o sistema visual: retina

Os ω3PUFAs, principalmente o DHA, são essenciais para a adequada função visual (Rotstein et al., 1996). O DHA encontra-se em uma concentração de até 50 % nos fosfolipídios nos fotorreceptores da retina (Dratz e Deese, 1986). Este ácido graxo atua de forma importante no aumento da fluidez de membrana, proporcionando um ambiente favorável para as mudanças conformacionais da rodopsina (proteína encontrada nos bastonetes e relacionada com visão no escuro) após sua fotoativação; além de modificar a atividade de enzimas na retina (Stubbs & Smith, 1984; Stillwell & Wassall, 2003). Foi demonstrado que o estímulo luminoso provoca a liberação de DHA dos fosfolipídios das membranas na retina (Reinboth et al., 1996). Além disso, a privação de DHA em roedores pode reduzir a eficiência de sinalização da proteína G (Niu et al., 2004). Em mamíferos, a privação dietética de ω3PUFAs resulta em alterações no padrão de ácidos graxos presentes nos fosfolipídios da retina, causando uma redução do conteúdo de DHA

(aproximadamente 40% do nível normal), sendo o suficiente para alterar a função visual (Neuringer et al., 1986; Weisinger eta al., 1998; Anderson et al., 2005).

1.2.1.3. Os ácidos graxos ω3 e sua relação com sistema glutamatérgico

Existem poucos 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 mostrou ser capaz de restaurar a LTP em hipocampo de ratos envelhecidos 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 envelhecidos (Dyall et al., 2006). Recentemente foi demonstrado, em experimentos in vitro, que o DHA pode modular o transporte de glutamato de maneira dose-dependente e em situações de dano cerebral (Grintal et al., 2009; Moreira et al., 2010).

1.2.2. Efeitos benéficos e neuroprotetores do DHA no SNC

1.2.2.1. Efeitos neuroprotetores do DHA no cérebro

Estudos têm demonstrado que o ácido graxo DHA foi capaz de proteger ratos jovens contra eventos de excitotoxicidade, como convulsão e isquemia (Högyes et al., 2003; 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 a um pior 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 (Peet & Stokes, 2005).

Foi recentemente demonstrado que o DHA pode ser liberado das membranas celulares, mediante a ativação da enzima fosfolipase A2 (PLA2) quando em situações de dano neuronal, e dar origem a um composto denominado Neuroprotectina D1, um docosanóide que exerce um papel neuroprotetor no SNC (Lukiw e Bazan, 2008; Bazan, 2006; Bazan et al., 2005).

1.2.2.2. Efeitos neuroprotetores do DHA na retina

O DHA é extremamente importante para o desenvolvimento e diferenciação dos fotorreceptores, bem como atua como um neuroprotetor endógeno contra injúrias nas células da retina (Rotstein et al., 1996; 2003; Moriguchi et al., 2003; Garelli et al., 2006). Foi demonstrado que camundongos *rd*, um modelo de retinite pigmentosa (progressiva degeneração retiniana caracterizada por perda de fotorreceptores por mecanismos apoptóticos), apresentam menor conteúdo de DHA tanto na retina como nos fotorreceptores quando comparados a camundongos normais (Scott et al., 1988). Um menor conteúdo de DHA também foi evidenciado no plasma de pacientes com Sindrome de Usher, caracterizada

por surdez e cegueira precoce como resultado de uma retinite pigmentosa (Bazan, 1989).

Recentemente foi demonstrado que o DHA pode prevenir o dano celular à retina causada por isquemia, bem como suas complicações no padrão de eletroretinografia em animais (Miyauchi et al., 2001; Murayama et al., 2002). Apesar destas evidências, faltam estudos que relacionem o efeito neuroprotetor do DHA a excitotoxicidade glutamatérgica na retina.

2. OBJETIVOS

2.1 Objetivo geral

Investigar a influência dos ácidos graxos ω3 no funcionamento do SNC, por meio de um modelo de privação dietética de ácidos graxos ω3 desde a gestação até a vida adulta, em parâmetros bioquímicos, comportamentais e de neuroproteção relacionados ao sistema glutamatérgico em cérebro e retina de ratos.

2.2. Objetivos específicos

2.2.1 Estudar a influência dos ácidos graxos ω3 no desenvolvimento da sinapse glutamatérgica no hipocampo, bem como em parâmetros comportamentais na vida adulta, em ratos submetidos ao modelo de privação dietética de ácidos graxos ω3;

2.2.2. Estudar a influência dos ácidos graxos ω3 na formação da memória, e o possível mecanismo hipocampal envolvido neste processo, em ratos adultos submetidos ao modelo de privação dietética de ácidos graxos ω3;

2.2.3. Estudar o possível efeito neuroprotetor dos ácidos graxos ω3 em um modelo de convulsão neonatal no hipocampo de ratos submetidos ao modelo de privação dietética de ácidos graxos ω3;

2.2.4. Estudar a influência dos ácidos graxos ω3 em parâmetros do sistema glutamatérgico na retina de ratos adultos submetidos ao modelo de privação dietética de ácidos graxos ω3, em condições basais e de isquemia;

PARTE II

Capítulo 1

Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: Relevance for behavior alterations.

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Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: Relevance for behavior alterations

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ABSTRACT

Essential omega-3 polyunsaturated fatty acids (ω3) are crucial to brain development and function, being relevant for behavioral performance. In the present study we examined the influence of dietary ω3 in the development of the glutamatergic system and on behavior parameters in rats. Female rats received isocaloric diets, either with ω3 (ω3 group) or a ω3 deficient diet (D group). In ontogeny experiments of their litters, hippocampal immunocontent of ionotropic NMDA and AMPA glutamatergic receptors subunits (NR2 A\B and GluR1, respectively) and the alpha isoform of the calcium-calmodulin protein kinase type II (αCaMKII) were evaluated. Additionally, hippocampal [^{3}H]glutamate binding and uptake were assessed. Behavioral performance was evaluated when the litters were adult (60 days old), through the open-field, plus-maze, inhibitory avoidance and flinch-jump tasks. The D group showed decreased immunocontent of all proteins analyzed at 02 days of life (P2) in comparison with the ω3 group, although the difference disappeared at 21 days of life (except for αCaMKII, which content normalized at 60 days old). The same pattern was found for [^{3}H]glutamate binding, whereas [^{3}H]glutamate uptake was not affected. The D group also showed memory deficits in the inhibitory avoidance, increased in the exploratory pattern in open-field, and anxiety-like behavior in plus-maze. Taken together, our results suggest that dietary ω3 content is relevant for glutamatergic system development and for behavioral performance in adulthood. The putative correlation among the neurochemical and behavioral alterations caused by dietary ω3 deficiency is discussed.

1. Introduction

Dietary omega-3 (ω3) polyunsaturated fatty acids (PUFAs) have a great relevance to brain health. It has been established that they are critical for proper infant growth and neurodevelopment (Marszalek and Lodish, 2005). Among the ω3 PUFAs, DHA is the most important ω3 with physiological significance for brain function (Marszalek and Lodish, 2005; Bourre, 2004), especially during prenatal brain development when it is incorporated into nerve growth cones during synaptogenesis (Martin and Bazan, 1992). The human brain growth spurt that takes place from the third trimester of pregnancy until 18 months post birth correlates with DHA accretion into brain phospholipids (Lauritzen et al., 2001). Insufficient dietary supply of ω3 PUFAs during pre- and postnatal development decreases the levels of DHA in neural tissue with a reciprocal increase of docosapentanoic acid (DPA, C22:5 ω6) (Schiefermeier and Yavin, 2002), leading to behavioral deficits in animal models (Lim et al., 2005; Moriguchi et al., 2000). Accordingly, dietary DHA supplementation in breastfeeding has been shown to improve mental development in human children (Hibbeln et al., 2007; Birch et al., 2000; Willatts et al., 1998).

Studies have shown that omega-3 dietary deficiency affect the glutamatergic, dopaminergic and serotoninergic systems (Moreira et al., 2010; Zimmer et al., 2000; Delion et al., 1996). Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), involved in brain plastic processes, such as learning/memory, brain development and ageing (Tzingounis and Wadiche, 2001; Danbolt, 2001; Segovia et al., 2001; Ozawa et al., 1998); however, overstimulation of the glutamatergic system may be highly neurotoxic. The main process responsible for maintaining extracellular glutamate concentration below toxic levels is the

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glutamate uptake by the transporters GLT1, GLAST (astrocytic) and EAAC1 (neuronal) (Sheldon and Robinson, 2007; Maragakis and Rothstein, 2004). Thus, the balance of the physiological/excitotoxic glutamatergic tonus is modulated by the activity of these proteins, especially the astrocytic transporters.

Glutamate exerts its physiological or toxic effects via ionotropic (iGluRs) and/or metabotropic (mGluRs) receptors. iGluRs are cation-specific ion channels, classified as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors, whereas mGluRs are G-proteins-coupled receptors which modulate the production of intracellular messengers (Ozawa et al., 1998; Kew and Kemp, 2005).

Concerning iGluRs, NMDA receptors are arrangements of the NR1 and NR2A-2D subunits, while AMPA receptors are arrangements of GluR1-GluR4 subunits (Kew and Kemp, 2005; Ozawa et al., 1998). α-Calcium/calmodulin-dependent kinase type II (αCaMKII), a synaptic enzyme that interacts with NMDA and AMPA receptors, is involved in memory modulation, and in hippocampal long-term potentiation (LTP), considered as a model for the cellular and molecular basis of memory (Bevilaqua et al., 2005; Lisman et al., 2002).

The glutamatergic system is the focus of several animal behavioral studies. It is involved in numerous behavioral patterns, such as hyperactivity (Dorval et al., 2007; Fadda et al., 2007), anxiety (Kapus et al., 2008; Bergink et al., 2004) and memory deficits (Vicente et al., 2008; Rotta et al., 2008). There are numerous reports related to the effects promoted by ω3 PUFAs on the glutamatergic system. An *in vitro* study showed that DHA differentially modulate glutamate transporters GLT1, GLAST and EAAC1 (Berry et al., 2005). A dietary supplementation with DHA was capable of restoring LTP and glutamate release in hippocampus of aged rats (McGahon et al., 1999). DHA-enriched diet also reversed the age-related decrease in the GluR2 and NR2B, subunits of AMPA and NMDA receptors respectively, in the forebrain of aged rats (Dyall et al., 2006), and it was protective against MK-801-induced neurotoxicity (Ozyurt et al., 2007). In a previous study, our group demonstrated that ω3 PUFAs were capable of strengthening the glutamatergic response in an ischemic injury model (Moreira et al., 2010).

The purpose of the present study was to investigate the influence of dietary ω3 PUFAs on the ontogeny the glutamatergic system in rat hippocampus and on behavioral performance of adult rats.

2. Materials and methods

2.1. Animals and diets

The dietary model used in this study has been previously described by Moreira et al. (2010), and it was designed to control the ω3 PUFAs levels in the maternal milk as well as in the offspring diet after weaning. Two weeks before mating, Wistar female rats were housed in a standard animal house with controlled environment (21–22 °C, 12 h dark–light cycle, food and water *ad libitum*). Rats were divided into two groups: ω3 diet (ω3) and ω3 deficient diet (D); the diets prepared at our own laboratory were isocaloric, containing 8% of total fat and differing only in the fatty acids composition (Tables 1 and 2). Female rats received their respective diets throughout the gestation as well as during suckling. After weaning (21 days old), the litters were maintained with the same diet of their dams until 60 days old. Ontogenetic experiments were done with litters at 02, 21 and 60 days old. Behavioral experiments were performed with male adult rats (60 days old). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

2.2. Neurochemical ontogenetic studies

2.2.1. Western blotting for hippocampal proteins

2.2.1.1. Hippocampal synaptosomal preparations. The synaptosomal preparation was obtained as previously described (Dosemeci et al., 2006). All centrifugation steps were performed in a refrigerated (4 °C) centrifuge. Hippocampi of 02, 21 and 60 days old rats were homogenized (motor-driven small capacity Teflon/glass homogenizer in a final volume of 1 mL/hippocampus) in a 25 mM 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 further transferred to another microfuge tube and centrifuged at 10,000 × *g* × 10 min using the same rotor to obtain a mitochondrial- and synaptosomal-enriched pellet (P2). P2 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. The myelin/light membrane layer at the top of 0.32 M sucrose was removed. Synaptosomal fraction collected at 0.32 M/0.8 M interface was washed twice to remove sucrose excess by centrifugation at 16,000 × *g* × 10 min in 25 mM Hepes (pH 7.4) containing a protease inhibitor cocktail. The final pellet was resuspended in the same solution (200 μL/pellet) for Western blotting analysis.

2.2.1.2. Western blotting analysis. Synaptosomal proteins (30 μg protein/well) were separated in a 7.5% SDS-PAGE mini-gel 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); 1:1000 β-actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1: 5000 (Amersham Pharmacia Biotech) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and band intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the

Table 1
Fatty acids composition of the diet lipids.[a].

	ω3 diet (%)	D diet (%)
Saturated		
C16:0	10.9	11.1
C18:0	2.0	2.4
C20:0	0.5	1.3
C22:0	0.1	2.9
C24:0	0.0	1.5
Monounsaturated		
C16:1	0.2	0.2
C18:1	25.4	46.7
C20:1	0.0	1.6
Polyunsatutared		
C18:2ω6	56.6	32.0
C18:3ω3	1.2	0.0
C20:5ω3[b]	2.2	0.0
C22:6ω3[b]	3.5	0.0

[a] According to O'Brien (2004).
[b] According to manufacturer information (Naturalis, Brazil).

Table 2
Composition of the diets.

	ω3 diet (%)	D diet (%)
Casein[a]	22	22
Corn starch	42	42
D-L-Methionin[b]	0.16	0.16
Sucrose	21	21
Fibers	2	2
Mineral salt mix[c]	4	4
Vitamin mix[d]	1	1
Peanut oil	0	8
Corn oil	7	0
Fish oil	1	0

Salt and vitamin compositions are according to Horwitz (1980).
[a] Casein, purity 87% (from Herzog, Porto Alegre, Brazil).
[b] D-L-Methionin (from Merk, Rio de Janeiro, Brazil).
[c] Mineral salt mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): NaCl, 557; KI, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–H_2O, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; $CoCl$–$6H_2O$, 0.09.
[d] Vitamin mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenic acid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; piridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin (D-(+)-biotin), 0.04; vitamin B12, 0.003.

Internet at http://rsb.info.nih.gov/nih-image/). In order to determine the adequate amount of protein to be assayed, different protein concentrations were carried out in the same gel for each protein tested.

2.2.2. [^{3}H] hippocampal glutamate binding

2.2.2.1. Hippocampal membrane preparations. After the animals being sacrificed, the brains were rapidly removed, hippocampi dissected over ice and homogenized in 0.32 M sucrose. The membrane preparation was performed as previously described (Emanuelli et al., 1998). The final pellet was resuspended in 5 mM Tris–HCl (pH 7.4) for glutamate binding.

2.2.2.2. [^{3}H]glutamate binding assay. Hippocampal plasma membranes were incubated in the absence of sodium, aiming to measure glutamate binding specifically to glutamate receptors (by avoiding binding on sodium dependent glutamate transporters). Incubations were carried out in 50 mM Tris–HCl buffer (pH 7.4), 100 nM [^{3}H]glutamate at 30 °C for 30 min (polycarbonated tubes, in triplicate). The incubation was started by adding 100 μg of membrane protein and terminated by centrifugation at 16,800 × *g* for 30 min at 4 °C. The supernatant was removed, the pellet and tube walls were quickly and carefully washed with ice-cold distilled water, resuspended with 0.1% SDS (w/v), and then left overnight. Radioactivity was measured by liquid scintillation counter (Wallac 1409). Results (specific binding) were considered as the difference between total binding and non-specific binding (in the presence of 50 μM non radioactive glutamate, attaining 10–20% of the total binding).

2.2.3. Hippocampal [^{3}H]glutamate uptake

2.2.3.1. Slices preparation. After the animals being sacrificed, the 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 7.2–7.3. Hippocampi were dissected onto iced Petri dishes with HBSS, and 0.4 mm slices were obtained with a McIlwain tissue chopper. For total and sodium-independent uptake (see below) measurement, slices were transferred to two 24-well culture plates: one was maintained at 35 °C and the other at 4 °C. The slices from the 35 °C plate were washed once with 1 mL of 35 °C HBSS, while the 4 °C with 1 mL of 4 °C sodium-free HBSS.

2.2.3.2. Total and Na^+-independent uptake. Glutamate uptake was performed as previously described (Thomazi et al., 2004). Hippocampal slices were preincubated at 35 °C for 15 min in HBSS, followed by the addition of 100 μM [^{3}H]glutamate. Incubation was stopped after 5 min with two ice-cold washes of 1 mL HBSS, immediately followed by adding 0.5N NaOH, which were then kept overnight. Na^+-independent uptake was measured by using the same protocol described above, except for the temperature (on ice, 4 °C) and medium composition (*N*-methyl-D-glucamine instead of sodium chloride). Na^+-dependent uptake was considered as the difference between the uptake in the presence and in the absence of Na^+. All experiments were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409).

2.2.4. Behavior tasks (60 days old rats)

2.2.4.1. Inhibitory avoidance task. Rats were placed on a 5.0 cm-high, 8.0 cm-wide platform located in the left of a 50 cm × 25 cm × 25 cm task apparatus, with floor composed by a series of parallel bronze bars 1.0 cm apart. The latency to step down from the platform to the grid with all four paws was measured. In the training session, immediately after stepping down on to the grid, the animals received a 0.4 mA, 1.0-s scrambled foot shock; in the test session, performed 1.5 h (short-term memory), 24 h and 7 days after training (long-term memories), the procedures were equal except that the foot shock was omitted. Differences between training and test latencies to step down were taken as index of retention.

2.2.4.2. Flinch-jump task. In order to control unspecific effects on pain sensitivity, which could alter the step down performance, rats were also submitted to the flinch-jump task (Lehner et al., 2006). Rats were individually placed into the inhibitory avoidance apparatus. After 2 min of habituation, foot shocks were sequentially applied every 10 s, in an intensity increasing stepwise manner (0.1–0.7 mA). The flinch threshold was defined as the lowest shock intensity that elicited detectable escape (flinching) response; the jump threshold was defined as the lowest shock intensity that elicited simultaneous removal of at least three paws from the grid. Each animal was evaluated only once and the task terminated as soon as flinch or jump thresholds could be determined.

2.2.4.3. Open-field. In order to control unspecific effects of diets on exploratory and motor activities, which could alter the step down performance, an open-field evaluation was performed as described by Souza et al. (2007). The rats were gently placed in the corner of a 40-cm × 50-cm × 60-cm box, the floor of which was divided into 3 cm × 4 cm squares, and then observed for 5 min. The number of crossings from one square to another and rearings were counted: the first 2 min was considered as a measure of exploratory activity, and the last 3 min as locomotion. All experiments were performed between 9 and 12 h (a.m.).

2.2.4.4. Elevated plus-maze task. The elevated plus-maze consisted of a central platform (10 cm × 10 cm) with two open and two closed arms (45 cm × 10 cm), arranged in such a way that the two arms of each type were opposite to each other. The maze was kept 88 cm above floor and sessions were carried out in a room lighted only with a dim red light. Animals were individually placed on the central platform of the plus-maze facing an open arm and observed for 5 min. Two observers recorded the number of entries and the time spent in the open arms (Walf and Frye, 2007).

2.3. Statistical analysis

For parametric data, results were expressed as means ± standard deviation. For Western blot, glutamate binding and uptake data, one-way ANOVA followed by

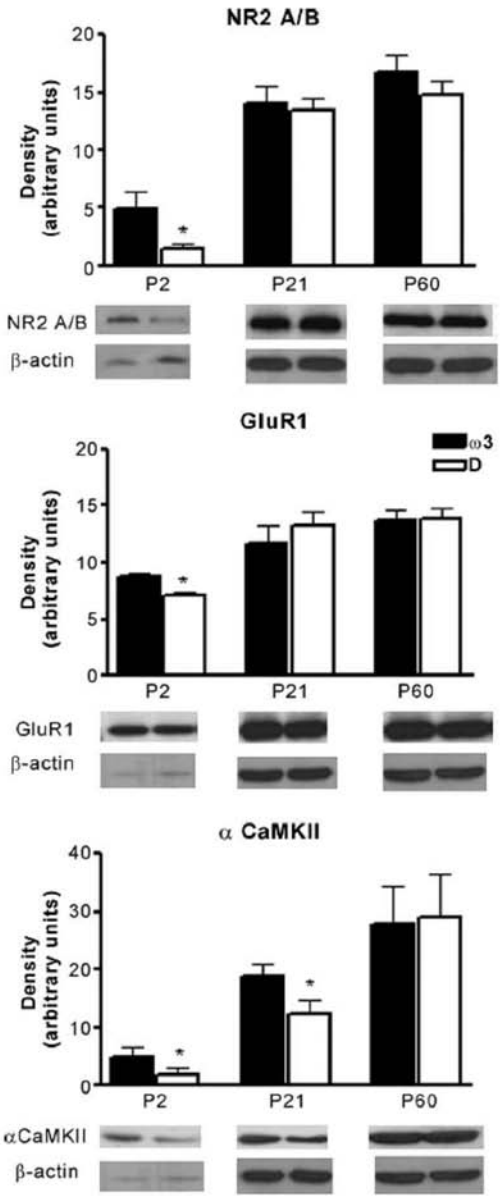

Fig. 1. Effect of dietary ω3 deficiency on the development of the content of ionotropic glutamate receptors subunits NR2 A\B (NMDA) and GluR1 (AMPA), and of the αCaMKII enzyme in the hippocampus of rats. Postnatal age of 02 (P2), 21 (P21) and 60 (P60) days were evaluated. ω3 (ω3 group, black bars; $n = 6$); D (ω3 deficient-group, white bars; $n = 6$). Data are expressed as means ± SD ($^*p < 0.05$ compared to ω3 group at the same postnatal day). One-way ANOVA statistic test was used. Representative Western blot images are shown below respective bars.

Tukey's test as post hoc were used. For open-field, plus-maze, and the flinch-jump tasks, Student *t* test was applied. For inhibitory avoidance, data were expressed as median ± interquartile range, Wilcoxon test was used for analysis within the groups and Mann–Whitney test was used for analysis between the groups. A value of $p < 0.05$ was adopted for statistical significance. The statistics were performed using SPSS 15.0.

3. Results

3.1. Influence of dietary ω3 deficiency on the ontogeny of hippocampal glutamatergic parameters.

As shown in Fig. 1, dietary ω3 deficiency delayed the normal increase in levels of all receptors subunits. At 2 days old, the immunocontent of NR2 A/B (NMDA), GluR1 (AMPA) and αCaMKII subunits in the D group was about 40%, 10% and 50% lower ($p < 0.05$) than in the ω3 group, respectively. At 21 days old, only the αCaMKII immunocontent remained significantly lower in D group (30%; $p < 0.05$). At 60 days old, levels were similar in the ω3 and D groups.

The developmental pattern of glutamate binding was also delayed by ω3 dietary deficiency (Fig. 2a). In the D group, glutamate binding increased with age up to 60 days of life, whereas in the ω3 group the maximum level was attained already at 21 days old. The 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 ω3 group. At 60 days old glutamate binding was similar in both groups.

The ontogenetic profile of glutamate uptake by hippocampal slices was not affected by dietary ω3 deficiency (Fig. 2b). Both groups presented data consistent with previously observed results (Thomazi et al., 2004; Feoli et al., 2006).

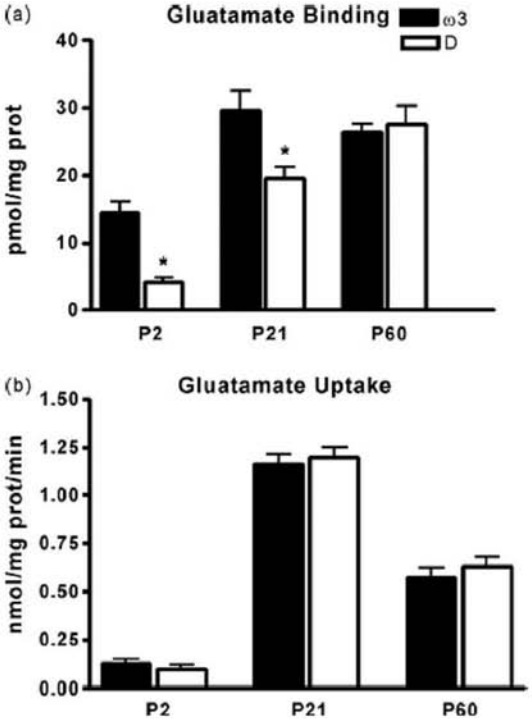

Fig. 2. Effect of dietary ω3 deficiency on the development of [^{3}H]glutamate binding (a) and [^{3}H]glutamate uptake (b) in hippocampal slices of rat. Postnatal age of 02 (P2), 21 (P21) and 60 (P60) days were evaluated. ω3 (ω3 group, black bars; $n = 6$); D (ω3 deficient-group, white bars; $n = 6$). Data are expressed as means ± SD ($^*p < 0.05$ compared to respective ω3). One-way ANOVA statistic test was used.

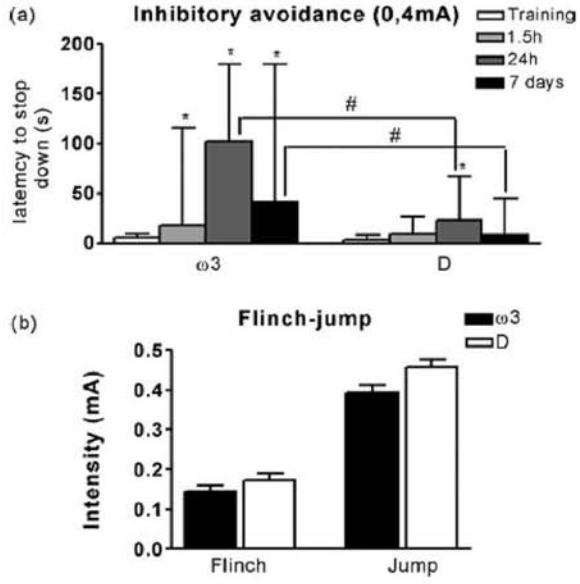

Fig. 3. Effect of dietary ω3 deficiency on inhibitory avoidance (a) and flinch-jump (b) tasks on 60 days old rats. Test sessions were performed 1.5 h (short-term memory), 24 h and 7 days (long-term memories) after training. Sensibility to foot shock was assessed by flinch-jump test. ω3 (ω3 group); D (ω3 deficient-group). For inhibitory avoidance data are expressed as median ± interquartile range, Wilcoxon test was performed within groups and Mann–Whitney test was used between groups; for flinch-jump data are expressed as means ± SD and One-way ANOVA test as statistical analysis was performed ($^*p < 0.05$ compared to respective training; $^\#p < 0.05$ compared to respective ω3). $N = 10$ animals per group in two isolate experiments.

3.2. Dietary ω3 deficiency caused behavioral alterations in adult rats

Dietary ω3 deficiency induced amnesia for short (1.5 h) and long-term [24 h (partially) and 7 days] memories in the inhibitory avoidance task (Fig. 3a). The ω3 deprivation had no effect on the foot-shock sensibility evaluated by flinch-jump task (Fig. 3b).

ω3 Deprivation increased exploratory activity ($p < 0.05$) (Fig. 4a) and had no significant effect in locomotion (Fig. 4b). The number of rearings was higher in the D group (Fig. 4c) but no differences were detected in the locomotion. It is noteworthy that despite effects of ω3 deficiency on exploration, there were no significant differences in training session latencies on inhibitory avoidance between groups.

When anxiety-like behavior was investigated in an elevated plus-maze task (Fig. 5), the D group spent significantly less time in open arms ($p < 0.05$; Fig. 5a) and presented less number of entries in open arm ($p < 0.05$; Fig. 5b) in comparison with the ω3 group.

4. Discussion

Our results indicate that the dietary ω3 deficiency from prenatal period delayed the development of some parameters related to glutamate transmission in hippocampus of rats. These data could support the hypothesis that such changes are associated to behavioral alterations observed in adult age, which include memory impairment in a inhibitory avoidance task, alterations in open-field task and the anxiety-like effect evaluated by elevated plus-maze.

Postsynaptic membranes of brain glutamatergic synapses contain characteristic postsynaptic densities (PSD), specialized domains containing a large number of different proteins, which are essential for signaling transduction and synapse formation (Lardi-Studler and Fritschy, 2007; McAllister, 2007). PSD ontogeny may

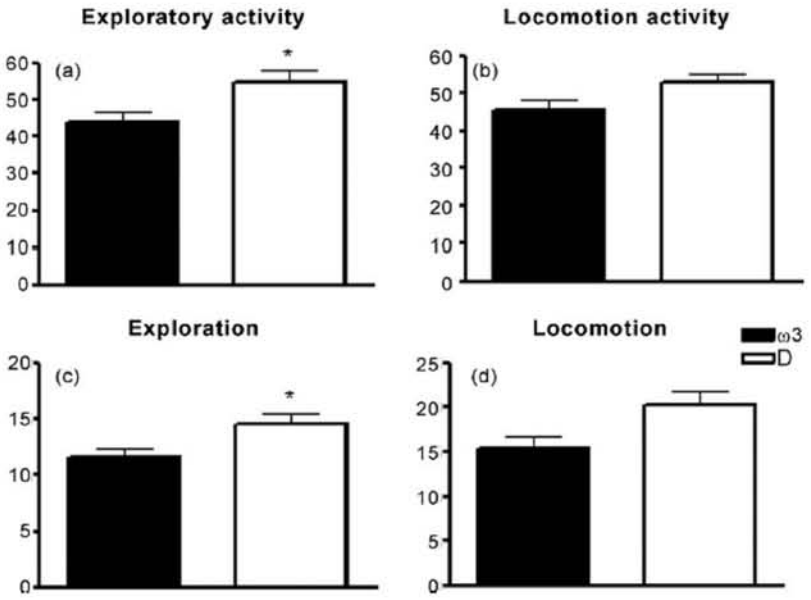

Fig. 4. Effect of dietary ω3 deficiency in the open-field test. The task was performed during 5 min with 60 days old rats ($n = 10$ animals per group). Exploratory activity (a) was evaluated by the number of crossings in the first 2 min, and locomotion (b) in the last 3 min. The number of rearings (c and d) was measured in the same two periods. ω3 (ω3 group); D (ω3 deficient-group). Data are expressed as means ± SD ($^*p < 0.05$). Student t test was used.

be modulated by several factors (Sheng and Hoogenraad, 2007), including nutritional status (Wurtman, 2008), and specifically by ω3 PUFAS in the diet (Cansev et al., 2008; Wurtman et al., 2009).

Glutamate ionotropic receptors interact with a variety of other proteins involved in the spatial and functional organization of PSD, and also with proteins involved in signal transduction pathways, including αCaMKII (Sheng and Hoogenraad, 2007; Meldrum, 2000). In the present report, we show that ω3 deficiency delayed the ontogenetic development of the NMDA, AMPA subunits and αCaKMII content, which were latter normalized in adulthood. Calon et al. (2005) have also shown that ω3 deprivation reduced the immunocontent of NMDA receptor subunits in the cortex and hippocampus and of αCaMKII in the cortex of the transgenic TG2576 mice, a model of Alzheimer's disease. With ageing, DHA content in the hippocampus phospholipids is reduced and coincides with the decrease in normal brain function and neuroplasticity (McGahon et al., 1999; Johnson and Schaefer, 2007). Dyall et al. (2006) showed that rats have age-related decrease in NR2B and GluR2, which was prevented by dietary supplementation with ω3 fatty acids. Here we demonstrate that ω3 nutritional deficiency during development is associated to delayed ontogenetic appearance of these proteins and of the glutamate binding (but not glutamate uptake). ω3 Deprivation could also render rat brain more susceptible to injuries in adult life, as we have previously reported (Moreira et al., 2010).

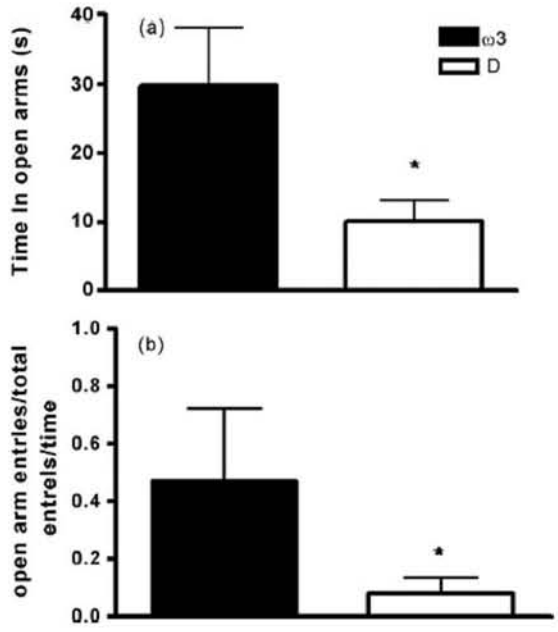

Fig. 5. Effect of dietary ω3 deficiency on the anxiety-like behavior in adult rats. Elevated plus-maze was performed by 5 min using rats at 60 days of age ($n = 10$ animals per group). Time spent in open arms (a) and number of entries in open arms/total entries/min (b) was measured. ω3 (ω3 group); D (ω3 deficient-group). Data are expressed as means ± SD ($^*p < 0.05$ compared to ω3). Student t test was used.

Association between ω3 dietary deficiency and behavioral alterations in rodents have been reported (Frances et al., 1996; Moriguchi et al., 2000), as spatial memory deficits in rodents further reversed by ω3 supplementation (Xiao et al., 2006; Fedorova et al., 2007; Chung et al., 2008). In this study, despite the fact that all the glutamatergic parameters here analyzed were similar in both groups at adult age, rats submitted to ω3 dietary deficiency during development presented behavioral alterations (in adulthood), compared with the ω3 group. Despite in the inhibitory avoidance (aversive) memory task, ω3 deficient-group showed deficits in memory, this was not related to influences on foot shock sensibility nor even on exploratory activity (same training latencies in inhibitory avoidance) or locomotion. Therefore, it is more likely that deficits are related to changes in brain plasticity (Wurtman, 2008).

Frances et al. (1996) verified behavioral changes in mice fed with ω3 deficient diet, and suggested that habituation (a simple form of learning) occurs more slowly in these mice. Here, ω3 deficient mice presented increased exploratory activity (number of crossings and rearings). In fact, locomotor reactivity to novelty has

been shown to be specifically related to lower DHA content in the frontal cortex (Vancassel et al., 2007).

Hyperactivity and anxiety-like behavior have been associated with ω3 deficiency in various animal models (Carrié et al., 2000; Moriguchi et al., 2000; Takeuchi et al., 2003; Vancassel et al., 2007; Lavialle et al., 2008), and corroborated by this study as assessed by the elevated plus-maze test. It is unlikely that the memory deficits are associated with this anxiety trait, given that training latencies in inhibitory avoidance are comparable in ω3 deficient and non-deficient animals. The balance between the inhibitory GABAergic and the excitatory glutamatergic systems in interrelated limbic structures that regulate behavioral performances appears to be important for anxiety pattern (Simon and Gorman, 2006). Accordingly, the glutamate system has recently received more attention as a potential target for anxiolytic drugs (Kapus et al., 2008; Bergink et al., 2004; Engin and Treit, 2008; Nair and Ajit, 2008). Here, we observed that the delayed in the development of the glutamatergic parameters studied in critical periods for synapse formation in early life could be related to anxiety in adulthood. It is noteworthy that attention-deficit/hyperactivity disorder (ADHD) has been suggested to be associated with an imbalance in polyunsaturated fatty acid content (Richardson, 2006), and that inattention, hyperactivity and impulsiveness are core symptoms ADHD.

Neurotransmitter systems play a central developmental role in the CNS (Graaf-Peters and Hadders-Algra, 2006). The trophic effect of glutamate receptors activation is developmental stage-dependent and may play an important role in determining the survival of neural cells, leading to appropriate cell connections. During this sensitive developmental period, interference with glutamate system function may lead to widespread neural loss (Balazs, 2006; Schlett, 2006). It has been shown that reduction of the glutamatergic system (as observed with glutamatergic receptors antagonists) in the initial periods of life in rats causes neurochemical and functional modification in brain, impairing behavioral performances in the adulthood (Latysheva and Rayevsky, 2003), even when the handling of the glutamatergic system finish before the adult age (Gorter and de Bruin, 1992). Although in these studies the underlying mechanisms of these lasting effects were addressed, much of them remain unknown. Accordingly, our results point that omega-3 deficient animals presented an attenuation of the glutamatergic activity, by delaying the ontogeny of the glutamatergic machinery, in the early life of rats. This could be related to the alterations in behavioral performance observed in adult life. The absence of the lipid profile in the hippocampus of the animals in both diets was a limitation of this study; however we based this work on previous studies where this deficiency diet model affects the ω3 fatty acids content in neural membranes (Zimmer et al., 2000; Delion et al., 1996). Accordingly, additional investigations are being performed in our group aiming to elucidate putative neurochemical mechanisms underlying these behavioral effects, including the lipid measurement.

Altogether, our findings support the evidence that dietary ω3 fatty acids are crucially important to an adequate brain development, and point that ω3 PUFAS dietary deficiency could affect the glutamatergic neurotransmission development, specifically by altering proteins involved in signal transduction. These changes in critical timing for neuroplasticity during development could eventually be involved in an anxiety trait, altered response to novelty and memory deficits.

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References

Balazs, R., 2006. Trophic effect of Glu. Curr. Topics Med. Chem. 6 (7), 1–8.
Bergink, V., van Megen, H.J.G.M., Westenberg, H.G.M., 2004. Glutamate and anxiety. Eur. Neuropsychopharmacol. 14, 175–183.
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 Res. 1037, 123–133.
Bevilaqua, L.R., Medina, J.H., Izquierdo, I., Cammarota, M., 2005. Memory consolidation induces N-methyl-D-aspartic acid-receptor- and Ca2+/calmodulin-dependent protein kinase II-dependent modifications in alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor properties. Neuroscience 136 (2), 397–403.
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. Dev. Med. Child Neurol. 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. J. Nutr. 8, 163–174.
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. Eur. J. Neurosci. 22, 617–626.
Cansev, M., Wurtman, R.J., Sakamoto, T., Ulus, I.H., 2008. Oral administration of circulating precursors for membrane phosphatides can promote the synthesis of new brain synapses. Alzheimer's Dementia 4, S153–S168.
Carrié, I., Clément, M., de Javel, D., Francès, H., Bourre, J.M., 2000. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. J. Lipid Res. 41 (3), 473–480.
Chung, W.L., Chen, J.J., Su, H.M., 2008. Fish oil supplementation of control and (n-3) fatty acid-deficient male rats enhances reference and working memory performance and increases brain regional docosahexaenoic acid levels. J. Nutr. 138 (6), 1165–1171.
Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 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 serotonergic neurotransmission in rat frontal cortex. J. Neurochem. 66, 1582–1591.
Dorval, K.M., Wigg, K.G., Crosbie, J., Tannock, R., Kennedy, J.L., Ickowicz, A., Pathare, T., Malone, M., Schachar, R., Barr, C.L., 2007. Association of the glutamate receptor subunit gene GRIN2B with attention-deficit/hyperactivity disorder. Genes Brain Behav. 6 (5), 444–452.
Dosemeci, A., Tao-Cheng, J.H., Vinadé, L., Jaffe, H., 2006. Preparation of postsynaptic density fraction from hippocampal slices and proteomic analysis. Biochem. Biophys. Res. Comments 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. Neurobiol. Aging 28 (3), 424–439.
Emanuelli, T., Antunes, V.F., Souza, D.O., 1998. Characterisation of L-[^{3}H]glutamate binding to fresh and frozen crude plasma membranes isolated from cerebral cortex of adult rats. Biochem. Mol. Biol. Int. 44 (6), 1265–1272.
Engin, E., Treit, D., 2008. The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: a systematic review. Prog. Neuro-Psycopharmacol. Biol. Psychiatry 32, 1399–1419.
Fadda, P., Bedogni, F., Fresu, A., Collu, M., Racagni, G., Riva, M.A., 2007. Reduction of corticostriatal glutamatergic fibers in basic fibroblast growth factor deficient mice is associated with hyperactivity and enhanced dopaminergic transmission. Biol. Psychiatry 62 (3), 235–242.
Fedorova, I., Hussein, N., Di Martino, C., Moriguchi, T., Hoshiba, J., Majchrzak, S., Salem Jr., N., 2007. An n-3 fatty acid deficient diet affects mouse spatial learning in the Barnes circular maze. Prostaglandins Leukot. Essent. Fatty Acids 77 (5–6), 269–277.
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. J. Nutr. 136 (9), 2357–2361.
Frances, H., Monier, C., Clement, M., Lecorsier, A., Debray, M., Bourre, J.M., 1996. Effect of dietary alpha-linolenic acid deficiency on habituation. Life Sci. 58 (21), 1805–1816.
Graaf-Peters, V., Hadders-Algra, M., 2006. Ontogeny of the human central nervous system: what is happening when? Early Hum. Dev. 82, 257–266.
Gorter, J.A., de Bruin, J.P., 1992. Chronic neonatal MK-801 treatment results in an impairment of spatial learning in the adult rat. Brain Res. 580, 12–17.
Hibbeln, J.R., Davis, J.M., Steer, C., Emmett, 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.
Horwitz, W., 1980. Official methods of analysis of the Association of Official Analytical Chemists. AOAC, Washington, DC, USA.
Johnson, E.J., Schaefer, E.J., 2007. Potential role of dietary n-3 fatty acids in the prevention of dementia and macular degeneration. Am. J. Clin. Nutr. 83 (Suppl.), 1494S–1498S.
Kapus, G.L., Gacsályi, I., Vegh, M., Kompagne, H., Hegedus, E., Leveleki, C., Hársing, L.G., Barkóczy, J., Bilkei-Gorzó, A., Lévay, G., 2008. Antagonism of AMPA receptors produces anxiolytic-like behavior in rodents: effects of GYKI 52466 and its novel analogues. Psycopharmacology (Berl.) 198 (2), 231–241.

Kew, J.N., Kemp, J.A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology 179, 4–29.
Lardi-Studler, B., Fritschy, J.M., 2007. Matching of pre- and postsynaptic specializations during synaptogenesis. Neuroscientist 13 (2), 115–126.
Latysheva, N.V., Rayevsky, K.S., 2003. Chronic neonatal N-methyl-D-aspartate receptor blockage induces learning deficits and transient hypoactivity in young rats. Prog. Neuro-Psycopharmacol. Biol. Psychiatry 27, 787–794.
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. Prog. Lipid Res. 40, 1–94.
Lavialle, M., Champeil-Potokar, G., Alessandri, J.M., Balasse, L., Guesnet, P., Papillon, C., Pévet, P., Vancassel, S., Vivien-Roels, B., Denis, I., 2008. An (n-3) polyunsaturated fatty acid-deficient diet disturbs daily locomotor activity, melatonin rhythm, and striatal dopamine in Syrian hamsters. J. Nutr. 138 (9), 1719–1724.
Lehner, M., Taracha, E., Skórzewska, A., Maciejak, P., Wisłowska-Stanek, A., Zienowicz, M., Szyndler, J., Bidziński, A., Płaźnik, A., 2006. Behavioral, immunocytochemical and biochemical studies in rats differing in their sensitivity to pain. Behav. Brain Res. 171 (2), 189–198.
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. J. Neurochem. 5 (3), 848–857.
Lisman, J., Schulman, H., Cline, H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 3 (3), 175–190.
Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.
Marszalek, J.R., Lodish, H.F., 2005. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. Annu. Rev. Cell. Dev. Biol. 21, 633–657.
Martin, R.E., Bazan, N.G., 1992. Changing fatty acid content of growth cone lipids prior to synaptogenesis. J. Neurochem. 59, 318–325.
McAllister, A.K., 2007. Dynamic aspects of CNS synapse formation. Annu. Rev. Neurosci. 30, 425–450.
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, B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr. 130 (4S Suppl.), 1007S–1015S.
Moreira, J.D., Knorr, L., Thomazi, A.P., Simão, F., Battú, C., Oses, J.P., Gottfried, C., Wofchuk, S., Salbego, C., Souza, D.O., Perry, M.L.S., Vinadé, L. Dietary Omega-3 fatty acids attenuate cellular damage after a hippocampal ischemic insult in adult rats. Jouranl of Nutrition Biochemistry, 2010, doi:10.1016/j.jnutbio.2009.01.013.
Moriguchi, T., Greiner, R.S., Salem, N., 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. J. Neurochem. 75 (6), 2563–2573.
Nair, J., Ajit, S.S., 2008. The role of glutamatergic system in posttraumatic stress disorder. CNS Spectr. 13 (7), 585–591.
O'Brien, R., 2004. Fats and Oils: Formulating and Processing for Application. 2nd ed., In: CRC Press (Ed.), USA.
Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54 (5), 581–618.
Ozyurt, B., Sarsilmaz, M., Akpolat, N., Ozyurt, H., Akyol, O., Herken, H., Kus, I., 2007. The protective effects of omega-3 fatty acids against MK-801-induced neurotoxicity in prefrontal cortex of rats. Neurochem. Int. 50, 196–202.
Richardson, A.J., 2006. Omega-3 fatty acids in ADHD and related neurodevelopmental disorders. Int. Rev. Psychiatry 18 (2), 155–172.
Rotta, L.N., Leszczinski, D.N., Brusque, A.M., Pereira, P., Brum, L.F., Nogueira, C.W., Frizzo, M.E., Perry, M.L., Souza, D.O., 2008. Effects of undernutrition on glutamatergic parameters in the cerebral cortex of young rats. Physiol. Behav. 94 (4), 580–585.
Schlett, K., 2006. Glutamate as a modulator of embryonic and adult neurogenesis. Curr. Topics Med. Chem. 6, 1–12.
Schiefermeier, M., Yavin, E., 2002. n-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of developing prenatal rat brain. J. Lipid Res. 43, 124–131.
Segovia, G., Porras, A., Del Arco, A., Mora, F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mech. Ageing Dev. 122 (1), 1–29.
Sheldon, A.L., Robinson, M.B., 2007. The role of glutamate transporters in neurodegenerative and potential opportunities for intervention. Neurochem. Int. 51, 333–355.
Sheng, M., Hoogenraad, C.C., 2007. The postsynaptic architecture of excitatory synapse: a more quantitative view. Annu. Rev. Biochem. 76, 823–847.
Simon, A.B., Gorman, J.M., 2006. Advances in treatment of anxiety: targeting glutamate. NeuroRX 3 (1), 57–68.
Souza, C.G., Moreira, J.D., Siqueira, I.R., Pereira, A.G., Rieger, D.K., Souza, D.O., Souza, T.M., Portela, L.V., Perry, M.L., 2007. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. Life Sci. 81 (3), 198–203.
Takeuchi, T., Iwanaga, M., Harada, E., 2003. Possible regulatory mechanism of DHA-induced anti-stress reaction in rats. Brain Res. 964 (1), 136–143.
Thomazi, A.P., Godinho, G.F., Rodrigues, J.M., Schwalm, F.D., Frizzo, M.E., Moriguchi, E., Souza, D.O., Wofchuk, S.T., 2004. Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. Mech. Ageing Dev. 125 (7), 475–481.
Tzingounis, A.V., Wadiche, J.I., 2001. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. Nat. Rev. Neurosci. 2007 8 (12), 935–947.
Vancassel, S., Blondeau, C., Lallemand, S., Cador, M., Linard, A., Lavialle, M., Dellu-Hagedorn, F., 2007. Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. Behav. Brain Res. 180 (2), 119–126.
Vicente, E., Degerone, D., Bohn, L., Scornavaca, F., Pimentel, A., Leite, M.C., Swarowsky, A., Rodrigues, L., Nardin, P., Vieira de Almeida, L.M., Gottfried, C., Souza, D.O., Netto, C.A., Gonçalves, C.A., 2008. Astroglial and cognitive effects of chronic cerebral hypoperfusion in the rat. Brain Res. 1251, 204–212.
Walf, A.A., Frye, C.H., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 32 (2), 322–328.
Willatts, P., Forsyth, J.S., Di Modugno, M.K., 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.
Wurtman, R.J., 2008. Synapse formation and cognitive brain development: effect of docosahexaenoic acid and other dietary constituents. Metab. Clin. Exp. 57 (2), S6–S10.
Wurtman, R.J., Cansev, M., Sakamoto, T., Ulus, I.H., 2009. Administration of docosahexaenoic acid, uridine and choline increases levels of synaptic membranes and dendritic spines in rodent brain. World Rev. Nutr. Diet 99, 71–96.
Xiao, Y., Wang, L., Xu, R.J., Chen, Z.Y., 2006. DHA depletion in rat brain is associated with impairment on spatial learning and memory. Biomed. Environ. Sci. 19 (6), 474–480.
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. J. Lipid Res. 41, 32–40.

Capítulo 2

Dietary omega-3 deficiency impairs NMDA and Fyn interaction and reduces BDNF content in dorsal hippocampus: implications on persistence of long-term memory in an aversive task in rats.

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Dietary omega-3 deficiency impairs NMDA and Fyn interaction and reduces BDNF content in dorsal hippocampus: implications on persistence of long-term memory in an aversive task in rats.

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Abstract

Wistar rats, receiving omega-3 diet (ω3) or ω3 deficient diet (D) from prenatal period, were submitted to inhibitory avoidance task aiming to elicit the persistence of long-term memory (LTM) and to evaluate some neurochemical parameters involved in memory processes. In D group, the persistence of LTM was abolished; in whole hippocampus there was a decrease in the of DHA (22:6 ω3) levels; in dorsal hippocampus there was a decrease in the interaction of Fyn with NR2B, and in the content of pTyr1336NR2B, pTyr418Fyn and BDNF. Thus, omega-3 fatty acids are important to NMDA-related signaling, BDNF synthesis and persistence of LTM.

Key words: Omega-3; BDNF; NR2B; Fyn; Hippocampus; Memory.

Brain development and function is dependent of ω3 polyunsaturated fatty acids (ω3 PUFAs). Among them, docosahexaenoic acid (DHA 22:6 ω3) is largely enriched in brain, and its deficiency alters the fluidity and the lipid rafts of neural membranes, consequently altering the activity of receptors, ion channels, G proteins and other proteins embedded in the membranes (Stillwell and Wassall 2003; Stillwell et al. 2005; Wurtman 2008). DHA accumulation takes place during brain development, from day 7 prenatal to day 21 postnatal in rats (3rd trimester of gestation to 18 months of life in humans) (Su 2010; Martinez 1992; Green et al. 1999).

It was postulated that an insufficient dietary supply of ω3 PUFAs during pre and postnatal development decreases the DHA levels in neural tissue with a concomitant increase of docosapentanoic acid (DPA, C22:5 ω6) levels (Schiefermeier and Yavin 2002), leading to behavioral alterations and memory deficits in animal models (Moreira et al. 2010; Moriguchi et al. 2000; Lim et al. 2005b). Accordingly, the recovery of brain DHA levels leads to a recovery of memory (Moriguchi and Salem 2003). By using *in vitro* experiments, it was demonstrated that DHA is important for *long-term potentiation* (LTP) induction and hippocampal neuronal development (Cao et al. 2009).

Concerning memory processes, it may be divided at least in two phases: a protein and RNA synthesis-independent phase that last to 1-3h (short-term memory, STM) and a protein and mRNA synthesis-dependent phase that last several hours to days, weeks or even longer (long-term memory, LTM) (Davis and Squire 1984; Izquierdo and Medina 1997; McGaugh 2000). Hippocampal molecular

events that influence memory formation involve activation of signaling cascades, activation of NMDA glutamate receptors, which is very important to the event of LTP, and the BDNF synthesis (Bekinschtein et al. 2007; Bekinschtein et al. 2008).

Glutamate receptors, especially NMDA receptors, play a critical role in synaptic plasticity and, in this way, modulating memory formation. NMDA receptors are composed by NR1 subunit and at least one of NR2 (A, B, C and D) or NR3 (A and B) subunits (Monyer et al. 1994). NMDA receptor has been demonstrated to play a crucial role in the induction of LTP in hippocampus, and NMDA receptor antagonists have been demonstrated to block LTP induction (Frankiewicz et al. 1996). Fyn, a cytosolic kinase protein from SRC family, involved in the activation (phosphorylation) of NMDA receptors (Kalia et al. 2004; Salter and Kalia 2004), is found in post-synaptic densities and is abundantly expressed in hippocampus (Yagi et al. 1993). Studies suggest that Fyn could be involved in myelination processes during brain development (Yagi et al. 1993; Sperber et al. 2001). Spatial memory formation induces phosphorylation of Fyn, which interacts with NR2B, and the administration of PP2, a specific SRC tyrosine kinase inhibitor, decreases memory formation and protein phosphorylation (Bevilaqua et al. 2003; Mizuno et al. 2003). It was demonstrated that Fyn activation, specifically in the dorsal hippocampus, is essential for contextual fear condition, a kind of memory related to an aversive environment (Isosaka et al. 2008).

In this context, the aim of the present study was evaluate the effects of dietary ω3 fatty acids in LTM in an aversive memory task, and the putative

mechanisms involving NMDA receptor activation (pNR2B and Fyn), as well as the levels of BDNF, in dorsal hippocampus of rats.

We used our dietary model as previously described (Moreira et al. 2010). Briefly, 2 weeks before mate, female rats were divided in 2 groups: ω3 adequate diet (ω3) and ω3 deficient-diet (D), housed in an air-conditioned room (21-22ºC) with 12 h dark-light cycle, and food and water were offered *ad libitum*. Both diets were isocaloric, containing 8% total fat and differed only in fatty acids composition (ω3 diet contained α-linolenic acid, EPA and DHA) fatty acids. After weaning at 21 days of postnatal life, male rats were maintained with the same diet of their dams until behavioral experiments in the adult age (60 days old). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

For hippocampal fatty acids profile, total fat was extracted using chloroform and methanol (0.02% butyl hydroxy toluene), as described before (Bligh and Dyer 1959). Fatty acid composition was determined by gas chromatography. Fat was saponified in methanolic KOH solution and then esterified in methanolic H_2SO_4 solution (Hartman and Lago 1973). The fatty acid methyl esters (FAME) were analyzed using an Agilent Technologies gas chromatograph (HP 6890) fitted with a capillary column DB-23 (50% cyanopropyl-methylpolysiloxane, 60 m x 0.25 mm x 0.25 μm) and flame ionization detection. Fatty acids were expressed as percentage of the total fatty acids content.

For the inhibitory avoidance task, rats were placed on a 5.0 cm-high, 8.0 cm-wide platform to the left of a 50 x 25 x 25 cm training apparatus, whose floor

was a series of parallel bronze bars spaced 1.0 cm apart. Latency to step down on to the grid with all four paws was measured. In the training session, immediately after stepping down on to the grid, the animals received a 0.7 mA, 1.0-sec scrambled footshock, aiming to elicit the persistence of long-term memory (LTM), which is dependent of BDNF synthesis (Bekinschtein et al. 2007). In the test session, performed 1.5h (short-term memory - STM), 24h and 7 days after training (LTM), the procedures were similar except that the footshock was omitted. Significant differences in the latency to step down between training and test sessions were taken as a measure of memory.

For biochemical analysis, 12 hours after a 0.7 mA training session in the inhibitory avoidance task, animals from both diets were sacrificed and the dorsal hippocampi were dissected as previously described (Isosaka et al. 2008). Hippocampi were homogenized in a lyses buffer [10mM Tris-HCl (pH 7.4), 1% Trition-X-100, 0.1 SDS, 150 mM NaCl, 1 mM EDTA, 1% protease inhibitor cocktail (Sigma)], centrifuged at 10,000 g x 15 min. Supernatants were used to Western blot, immunoprecipitation analysis and BDNF content. To evaluate the influence of the diets in the interaction of NR2B subunit with Fyn, the immunoprecipitation was performed as described (Muller et al. 2010). Briefly, the dorsal hippocampus homogenates (100 μg) were incubated with the primary antibodies for Fyn (1: 200, Invitrogen) or Yes (1:200, Santa Cruz) overnight, followed by 2h incubation with 20μl of protein A-agarose at 4ºC. After 3 washes with lyse buffer, the A-agarose was pelleted by centrifugation and the samples were submitted to Western blot analysis for NR2B.

Dorsal hippocampus homogenates or immunoprecipitated samples were separated in a 10% SDS-PAGE mini-gel 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: 1:1000 anti-Fyn (Invitrogen), 1:1000 pSRC (Tyr418) (Invitrogen), 1:1000 NR2B (Millipore), 1:1000 pNR2B (Tyr1336), 1:2000 β-actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1: 3000 (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 software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Hippocampal BDNF content was measured 12h after the animals were submitted just to the context (inhibitory avoidance apparatus) or training session. It was used an anti-BDNF sandwich-ELISA, according to the datasheet from DuoSet kit (R&D Systems, Inc, USA) in a Spectra Max M5 molecular Devices (USA).

For hippocampal lipid profile, immunopreciptation and Western blot analysis, the results were expressed as mean ± standard deviation, and statistical analysis was performed by *Student* T test, except for BDNF content, which was used Two-way ANOVA test and Bonferroni as post-hoc. For inhibitory avoidance task, the results were expressed as median ± interquartile range and Wilcoxon test was used for analysis within groups and Mann Whitney test was used for analysis

between the groups. For statistical significance, the value of $P < 0.05$ was adopted. The statistical analysis was performed using SPSS 15.0 software.

The hippocampal fatty acid profile is showed in Table 1. Only fatty acids with contents higher than 0.5% were considered. The D group had a decrease in DHA (22:6ω3) (-24%, $p < 0.05$) content in the hippocampus of adult rats. It was also observed a concomitant increase in hippocampal docosapentaenoic acids (DPA 22:5ω6) content in D group ($p < 0.05$).

An inhibitory avoidance task using 0.7 mA foot shock was performed aiming to elicit the persistence of LTM (Figure 1A). The persistence of LTM (7 days after training session), observed in the ω3 group, was abolished in the D group ($p < 0.001$, compared with ω3 group).

We investigated possible mechanisms involved in memory formation related to NR2B and Fyn by phosphorylation, which is a biochemical process that promotes the activation of both proteins. There was a decrease in the phosphorylation of NR2B and Fyn in dorsal hippocampus of D group 12h after training session (Figure 1B and 1C, respectively - $p < 0.05$); the total content of these proteins was not affected by the diet.

To investigate if these effects were related to the possible reduction in the interaction of Fyn with NR2B, we performed Fyn immunoprecipitation and Western blot for pNR2B Tyr 1336. We also performed immunoprecipitation with other SRC family protein (Yes) as a negative control (Figure 1D). We observed that D group presented a reduction in coimmunoprecipitation of Fyn and pNR2B, suggesting that there was less interaction of these 2 proteins in D group. The hippocampal

BDNF content measured 12h after training session, in animals submitted to the context without foot shock, decreased in D group when compared to ω3 group (Figure 1E, $p < 0.05$). BDNF content was also affected by ω3 fatty acids deficiency, decreasing in D group, independent of foot shock.

In the present study, we observed that ω3 fatty acids deficiency abolished the persistence of LTM (present in ω3 group) possibly by the reduction of the interaction between Fyn and NR2B subunit, observed in dorsal hippocampus, as well as by a decrease in the content of hippocampal DHA and BDNF, pointing to the relevance of dietary ω3 fatty acids for adequate brain plasticity related to aversive memory persistence.

NMDA receptor activation is known to participate in memory formation, and the phosphorylation of its NR2B subunit appears to be an important step for this process (Suen et al. 1997; Black 1999; Xu et al. 2006). Fyn, a cytoplasmic SRC family protein kinase, is involved in phosphorylation of the residue Tyr 13336 in NR2B subunit (Nakazawa et al. 2001). Moreover, Fyn-mediated phosphorylation of NR2B is involved in modulation of aversive memory formation (Kojima et al. 1998; Nakazawa et al. 2001; Isosaka et al. 2008). In our study, we observed that dietary ω3 fatty acids deficiency was able to reduce the phosphorylation of NR2B and Fyn in the dorsal hippocampus, 12h after training session of an inhibitory avoidance task, without affecting Fyn and NR2B total content. Moreover, in the D group there was a decrease in the capacity of interaction between Fyn and NR2B proteins, compared to ω3 group, here observed by the reduction of coimmunoprecititation of

these two proteins. The SRC protein Yes did not coimmunoprecipitated with NR2B, suggesting the specific effect on the interaction of Fyn with NR2B.

Over the past years, it was demonstrated that ω3 fatty acids deficiency alters brain fatty acids profile, especially in the hippocampus, with a reduction in DHAω3 and an increase in DPAω6 brain phospholipids (Miyazawa et al. ; Aid et al. 2003; Chung et al. 2008; Mathieu et al. 2008; Fedorova et al. 2009), resulting in an impairment in memory formation and LPT (Moriguchi et al. 2000; Garcia-Calatayud et al. 2005; Chung et al. 2008; Cao et al. 2009; Fedorova et al. 2009). In our study, we observed that the impairment in the persistence of an aversive LTM was associated with a decrease in the hippocampal DHAω3 levels and with an increase in DPAω6 content.

Step-down inhibitory avoidance task is largely used to evaluate aversive hippocampal-dependent memory in rodents (Izquierdo and Medina 1997; Bevilaqua et al. 2005; Bekinschtein et al. 2007; Rossato et al. 2009). In rats, this inhibitory avoidance task triggers biochemical events in the hippocampus that are required for the retention of this task (Izquierdo and Medina 1997). Passive avoidance task was previous used in ω3-deficient mice, and no effect was observed by the manipulation of the dietary ω3 fatty acids (Carrie et al. 2000). Garía-Calatayud and colleges showed, using the same task, that ω3-deficient rats presented memory deficits (Garcia-Calatayud et al. 2005). Here we showed with a strong foot shock (0.7 mA) that both groups presented similar performance at 1.5 h and 24 h after training session, but the persistence of LTM was abolished in D group. Our previous study using the same dietary protocol used here showed that

using a weaker foot shock (0.4 mA), ω3-deficient rats also presented impairment in inhibitory avoidance task performance compared to ω3 diet (Moreira et al.).

We also observed that hippocampal BDNF content was reduced by dietary ω3 deficiency, independently of the behavioral task, which could have contributed to the abolishment of LTM found in the D group. Bekinschtein and colleges showed that BDNF synthesis in rat hippocampus is required during the time window around 12h after avoidance training for persistence of LTM (Bekinschtein et al. 2007; Bekinschtein et al. 2008). Other studies also showed that BDNF levels are affected by ω3 fatty acids. Decreased levels of BDNF and BDNF mRNA were found in striatum and frontal cortex of animals submitted to a ω3 deficient diet (Miyazawa et al. 2010; Rao et al. 2007). Nevertheless, high levels of BDNF and its receptor (TrKB) mRNA were also implicated in the neuroprotective effect of ω3 fatty acids (Bousquet et al. 2009).

In summary, ω3 fatty acids deficiency during brain development and adulthood decreased the NMDA phosphorylation by Fyn, which could be relevant in the reduction of aversive LTM. Moreover, ω3 fatty acids deficiency reduced hippocampal DHA and BDNF contents, which could be related to the neurochemical and behavioral effects here demonstrated. More investigations need to be performed to elucidate the underling mechanism involved in ω3 fatty acids improvement of memory.

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References

Aid S, Vancassel S, Poumes-Ballihaut C, Chalon S, Guesnet P, Lavialle M. 2003. Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. *J Lipid Res* **44**: 1545-1551.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH. 2007. Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. *Neuron* **53**: 261-277.

Bekinschtein P, Cammarota M, Katche C, Slipczuk L, Rossato JI, Goldin A, Izquierdo I, Medina JH. 2008. BDNF is essential to promote persistence of long-term memory storage. *Proc Natl Acad Sci U S A* **105**: 2711-2716.

Bevilaqua LR, da Silva WN, Medina JH, Izquierdo I, Cammarota M. 2005. Extinction and reacquisition of a fear-motivated memory require activity of the Src family of tyrosine kinases in the CA1 region of the hippocampus. *Pharmacol Biochem Behav* **81**: 139-145.

Bevilaqua LR, Rossato JI, Medina JH, Izquierdo I, Cammarota M. 2003. Src kinase activity is required for avoidance memory formation and recall. *Behav Pharmacol* **14**: 649-652.

Black IB. 1999. Trophic regulation of synaptic plasticity. *J Neurobiol* **41**: 108-118.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911-917.

Bousquet M, Gibrat C, Saint-Pierre M, Julien C, Calon F, Cicchetti F. 2009. Modulation of brain-derived neurotrophic factor as a potential neuroprotective mechanism of action of omega-3 fatty acids in a

parkinsonian animal model. *Prog Neuropsychopharmacol Biol Psychiatry* **33**: 1401-1408.

Cao D, Kevala K, Kim J, Moon HS, Jun SB, Lovinger D, Kim HY. 2009. Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. *J Neurochem* **111**: 510-521.

Carrie I, Clement M, de Javel D, Frances H, Bourre JM. 2000. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J Lipid Res* **41**: 473-480.

Chung WL, Chen JJ, Su HM. 2008. Fish oil supplementation of control and (n-3) fatty acid-deficient male rats enhances reference and working memory performance and increases brain regional docosahexaenoic acid levels. *J Nutr* **138**: 1165-1171.

Davis HP, Squire LR. 1984. Protein synthesis and memory: a review. *Psychol Bull* **96**: 518-559.

Fedorova I, Hussein N, Baumann MH, Di Martino C, Salem N, Jr. 2009. An n-3 fatty acid deficiency impairs rat spatial learning in the Barnes maze. *Behav Neurosci* **123**: 196-205.

Frankiewicz T, Potier B, Bashir ZI, Collingridge GL, Parsons CG. 1996. Effects of memantine and MK-801 on NMDA-induced currents in cultured neurones and on synaptic transmission and LTP in area CA1 of rat hippocampal slices. *Br J Pharmacol* **117**: 689-697.

Garcia-Calatayud S, Redondo C, Martin E, Ruiz JI, Garcia-Fuentes M, Sanjurjo P. 2005. Brain docosahexaenoic acid status and learning in young rats

submitted to dietary long-chain polyunsaturated fatty acid deficiency and supplementation limited to lactation. *Pediatr Res* **57**: 719-723.

Green P, Glozman S, Kamensky B, Yavin E. 1999. Developmental changes in rat brain membrane lipids and fatty acids. The preferential prenatal accumulation of docosahexaenoic acid. *J Lipid Res* **40**: 960-966.

Hartman L, Lago RC. 1973. Rapid preparation of fatty acid methyl esters from lipids. *Lab Pract* **22**: 475-476 passim.

Isosaka T, Hattori K, Kida S, Kohno T, Nakazawa T, Yamamoto T, Yagi T, Yuasa S. 2008. Activation of Fyn tyrosine kinase in the mouse dorsal hippocampus is essential for contextual fear conditioning. *Eur J Neurosci* **28**: 973-981.

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

Kalia LV, Gingrich JR, Salter MW. 2004. Src in synaptic transmission and plasticity. *Oncogene* **23**: 8007-8016.

Kojima N, Ishibashi H, Obata K, Kandel ER. 1998. Higher seizure susceptibility and enhanced tyrosine phosphorylation of N-methyl-D-aspartate receptor subunit 2B in fyn transgenic mice. *Learn Mem* **5**: 429-445.

Lim SY, Hoshiba J, Salem N, Jr. 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. *J Neurochem* **95**: 848-857.

Martinez M. 1992. Treatment with docosahexaenoic acid favorably modifies the fatty acid composition of erythrocytes in peroxisomal patients. *Prog Clin Biol Res* **375**: 389-397.

Mathieu G, Denis S, Lavialle M, Vancassel S. 2008. Synergistic effects of stress and omega-3 fatty acid deprivation on emotional response and brain lipid composition in adult rats. *Prostaglandins Leukot Essent Fatty Acids* **78**: 391-401.

McGaugh JL. 2000. Memory--a century of consolidation. *Science* **287**: 248-251.

Miyazawa D, Yasui Y, Yamada K, Ohara N, Okuyama H. 2010. Regional differences of the mouse brain in response to an alpha-linolenic acid-restricted diet: Neurotrophin content and protein kinase activity. *Life Sci* **87**: 490-494.

Mizuno M, Yamada K, He J, Nakajima A, Nabeshima T. 2003. Involvement of BDNF receptor TrkB in spatial memory formation. *Learn Mem* **10**: 108-115.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**: 529-540.

Moreira JD, Knorr L, Ganzella M, Thomazi AP, de Souza CG, de Souza DG, Pitta CF, Mello e Souza T, Wofchuk S, Elisabetsky E et al. 2010. Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: relevance for behavior alterations. *Neurochem Int* **56**: 753-759.

Moriguchi T, Greiner RS, Salem N, Jr. 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J Neurochem* **75**: 2563-2573.

Moriguchi T, Salem N, Jr. 2003. Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J Neurochem* **87**: 297-309.

Muller AP, Gnoatto J, Moreira JD, Zimmer ER, Haas CB, Lulhier F, Perry ML, Souza DO, Torres-Aleman I, Portela LV. 2010. Exercise increases insulin signaling in the hippocampus: Physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. *Hippocampus*.

Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T. 2001. Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* **276**: 693-699.

Rao JS, Ertley RN, Lee HJ, DeMar JC, Jr., Arnold JT, Rapoport SI, Bazinet RP. 2007. n-3 polyunsaturated fatty acid deprivation in rats decreases frontal cortex BDNF via a p38 MAPK-dependent mechanism. *Mol Psychiatry* **12**: 36-46.

Rossato JI, Bevilaqua LR, Izquierdo I, Medina JH, Cammarota M. 2009. Dopamine controls persistence of long-term memory storage. *Science* **325**: 1017-1020.

Salter MW, Kalia LV. 2004. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* **5**: 317-328.

Schiefermeier M, Yavin E. 2002. n-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of the developing prenatal rat brain. *J Lipid Res* **43**: 124-131.

Sperber BR, Boyle-Walsh EA, Engleka MJ, Gadue P, Peterson AC, Stein PL, Scherer SS, McMorris FA. 2001. A unique role for Fyn in CNS myelination. *J Neurosci* **21**: 2039-2047.

Stillwell W, Shaikh SR, Zerouga M, Siddiqui R, Wassall SR. 2005. Docosahexaenoic acid affects cell signaling by altering lipid rafts. *Reprod Nutr Dev* **45**: 559-579.

Stillwell W, Wassall SR. 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids* **126**: 1-27.

Su HM. 2010. Mechanisms of n-3 fatty acid-mediated development and maintenance of learning memory performance. *J Nutr Biochem* **21**: 364-373.

Suen PC, Wu K, Levine ES, Mount HT, Xu JL, Lin SY, Black IB. 1997. Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic N-methyl-D-aspartate receptor subunit 1. *Proc Natl Acad Sci U S A* **94**: 8191-8195.

Wurtman RJ. 2008. Synapse formation and cognitive brain development: effect of docosahexaenoic acid and other dietary constituents. *Metabolism* **57 Suppl 2**: S6-10.

Xu F, Plummer MR, Len GW, Nakazawa T, Yamamoto T, Black IB, Wu K. 2006. Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. *Brain Res* **1121**: 22-34.

Yagi T, Shigetani Y, Okado N, Tokunaga T, Ikawa Y, Aizawa S. 1993. Regional localization of Fyn in adult brain; studies with mice in which fyn gene was replaced by lacZ. *Oncogene* **8**: 3343-3351.

Figure legend

Figure 1: Effect of ω3 fatty acids deficiency on the persistence of memory (LTM) and on neurochemical parameters. ω3 - ω3 adequate diet; D - ω3 deficient diet. **(A)** Inhibitory avoidance task was used to assess the LTM. Memory was evaluated 1.5 h, 24 h and 7 days after training session. ω3, n = 20; D, n = 20. Results were expressed as median ± interquartile range, Wilcoxon test was used for analysis within groups and Mann Whitney test was used for analysis between the groups (**p* < 0.05 in relation to respective training session; # *p* < 0.05 in relation to respective ω3 group). **(B and C)** Phosphorylation NR2B (pTyr133) and Fyn (pTyr418) 12 h after training session. ω3, n = 6; D, n = 6. Results were expressed as mean ± standard deviation, and *Student* T test was used (**p* < 0.05), representative Western blot images are shown above each respective graphic. **(D)** Interaction of Fyn with NR2B assessed by immunoprecipitation of Fyn and Western blot for pNR2B (Tyr1336); Yes and B+PAg (incubation of lyses buffer with Agarose Protein) were negative controls. ω3, n = 8; D, n = 7; H = total homogenate without immunoprecipitation; results were expressed as mean ± standard deviation, and *Student* T test was used (**p* < 0.05), representative Western blot images are showed above the graphic. **(E)** Hippocampal BDNF content using animals submitted to the context without foot shock (basal) and 12 h after training session (12h shock). ω3, n = 8; D, n = 7. Results were expressed as mean ± standard deviation, and Two-way ANOVA test and Bonferroni as post-hoc were used (**p* < 0.05 in relation to both ω3 groups).

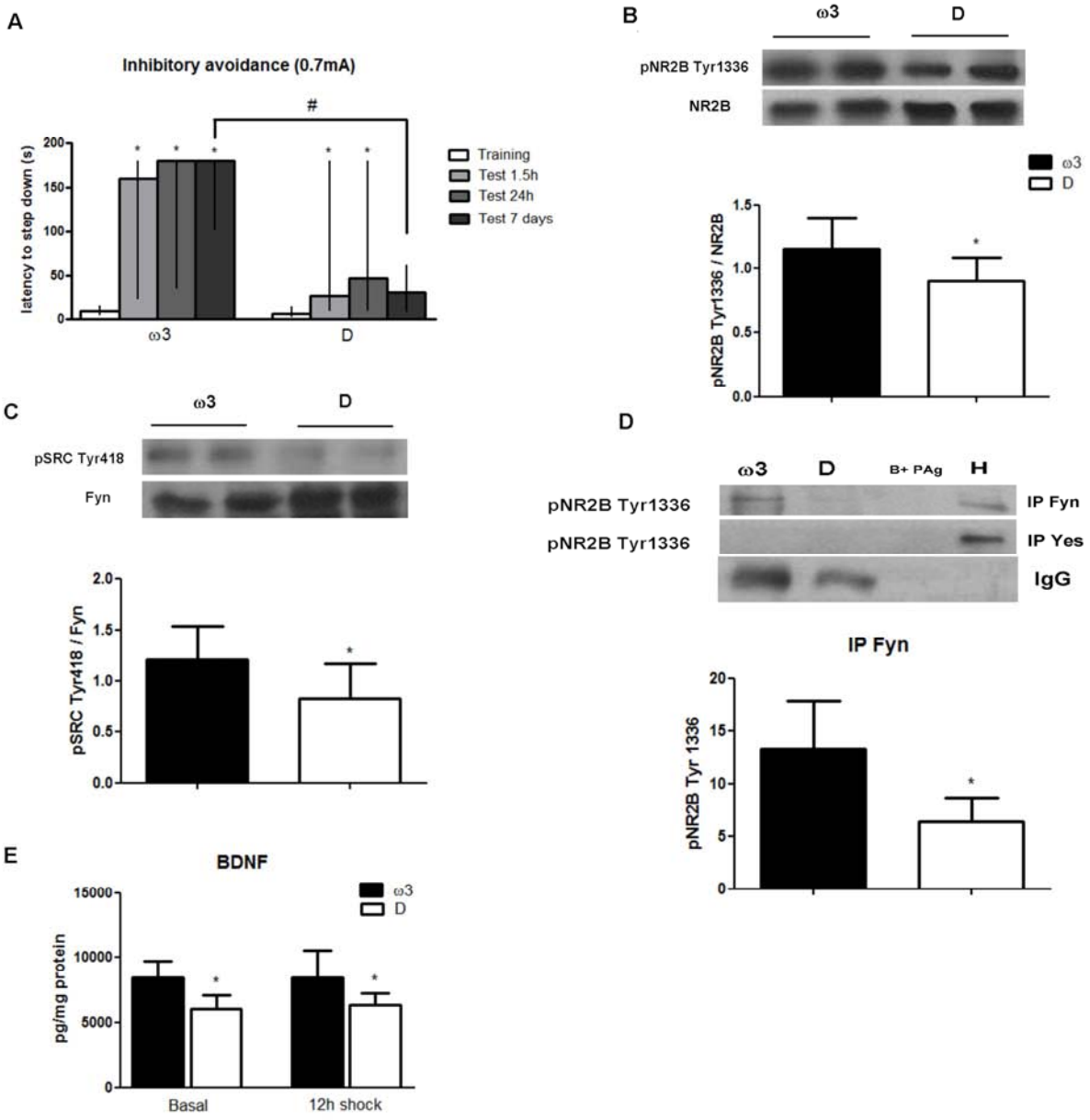
Table 1

Table 1: Hippocampal fatty acids profile (#).

	ω3	D
	%	
Saturated fatty acids		
C 14:0	0.45	0.39
C 16:0	27.29	26.39
C 18:0	19.46	18.02
C 24:0	0.34	0.68
Monounsaturated fatty acids		
C 15:1	2.64	2.52
C 16:1 ω7	0.97	0.95
C 18:1 ω7	2.89	2.80
C 18:1 ω9	18.03	19.45
C 20:1 ω9	0.91	1.25
Polyunsaturated fatty acids		
C 22:6 ω3	11.02	8.41*
C18:2 ω6	2.78	2.38
C 20:4 ω6	9.39	9.15
C 22:4 ω6	2.90	3.70
C 22:5 ω6	0.16	1.70*
ω6:ω3	1.4	2.14

mean value of the fatty acids which content atteined a minimum of 0,5%.
*p<0.001 comparing ω3 group (n=7) and D group (n=5).

Figure 1


A
Inhibitory avoidance (0.7mA)
Latency to step down (s)
Training
Test 1.5h
Test 24h
Test 7 days
ω3
D
B
pNR2B Tyr1336
NR2B
pNR2B Tyr1336 / NR2B
C
pSRC Tyr418
Fyn
pSRC Tyr418 / Fyn
D
B+ PAg
H
IP Fyn
IP Yes
IgG
pNR2B Tyr1336
IP Fyn
pNR2B Tyr 1336
E
BDNF
pg/mg protein
Basal
12h shock

Capítulo 3

Short-term alterations in hippocampal glutamate transport system caused by one-single neonatal seizure episode: implications on behavioral performance in adulthood.

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Short-term alterations in hippocampal glutamate transport system caused by one-single neonatal seizure episode: implications on behavioral performance in adulthood.

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Abstract

Impairment in the activity and expression of glutamate transporters has been found in experimental models of epilepsy in adult animals. However, there are few studies investigating alterations on glutamate transporters caused by epilepsy in newborn animals, especially in the early periods after seizures. In this study, alterations in the hippocampal glutamate transporters activity and immunocontent were investigated in neonatal rats (7 days old) submitted to kainate-induced seizures model. Glutamate uptake, glutamate transporters (GLT-1, GLAST, EAAC1) and glutamine synthetase (GS) were assessed in hippocampal slices obtained 12 h, 24 h, 48 h, 72 h and 60 days after seizures. Immunoreactivity for hippocampal GFAP, NeuN and DAPI were assessed 24 h after seizure. Behavioral analysis (elevated-plus maze and inhibitory avoidance task) was also investigated in the adult animals (60 days old). The decrease on glutamate uptake was observed in hippocampal slices obtained 24 h after seizures. The immunocontent of GLT-1 increased 12 h and decreased 24 h (+62% and -20% respectively), while GLAST increased up to 48 h after seizures. No alterations were observed for EAAC1 and GS. GFAP immunoreactivity increased in all hippocampal subfields (CA1, CA3 and dentate gyrus) with no alterations in NeuN and DAPI staining. In the adulthood, kainate-treated rats showed anxiety-related behavior and lower performance in the inhibitory avoidance task. Our findings indicate that acute modifications on hippocampal glutamate transporters triggered by a single convulsive event in early life may play a role in the behavioral alterations observed in adulthood. **Key words:** kainate; seizures; glutamate transporters; hippocampus; anxiety; memory.

1. Introduction

Epileptic seizures in children are a common and frightening neurological condition. The incidence of seizures is significantly higher in children than in adults, with the highest incidence in the first year of life [1]. This higher susceptibility to seizure of immature brain compared to adult seems to be related to the fact that γ-aminobutiric acid (GABA), an inhibitory neurotransmitter in mammalian brain, exerts paradoxical excitatory effects in early ages [2, 3]. Epidemiological data suggest that prolonged seizures or *status epilepticus* (SE) in childhood may lead to increased risk of epilepsy in adulthood, through mechanisms still unknown (Haut et al., 2004).

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS), involved in essential physiological brain functions, as synaptic plasticity, learning and memory, brain development and ageing [4-7]. Glutamate acts through a*ct*ivation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate ionotropic receptors, and metabotropic receptors (for reviews see [8, 9]. However, overstimulation of the glutamatergic system (by exogenous or endogenous stimuli), which occurs when glutamate levels in the synaptic cleft increase over the physiological range, is involved in various acute and chronic brain diseases (excitotoxicity), including neurodegenerative diseases, traumatic brain injury, cerebral ischemia, and seizures [4, 5, 10-12]. Thus, to keep glutamate at the physiologically relevant concentrations is extremely important.

There are strong evidences pointing that glutamatergic excitotoxicity may be prevented by astrocytic glutamate uptake**,** a process responsible for maintaining the extracellular glutamate levels below toxic levels [9, 13, 14]. To date, five distinct high-affinity, sodium-dependent glutamate transporters have been cloned from animal and human tissue [GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5], differing in molecular structure, pharmacological properties, and tissue distribution [5, 11, 15, 16]. Immunohistochemical studies have revealed that GLAST and GLT-1 are localized primarily in astrocytes, whereas EAAC1 is widely distributed in neurons [5, 16]. The impairment of these glutamate transporters has been also implicated in the pathophysiology of some brain disorders, as epilepsy and seizures [17-21]

Glutamate, after being taken up into astrocytes, may be converted to glutamine by glutamine synthetase (GS), which then is released to extracellular space and taken up by neurons where it is converted again to glutamate and stored in pre synaptic vesicles [5, 22]. Thus, the GS activity is an essential step in the glutamate-glutamine cycle, and its impairment has been implicated in pathogenesis of temporal lobe epilepsy (TLE), since GS expression and activity is reduced in the hippocampus of TLE patients [23]. In adult animals, GS was increased in the latent phase and decreased in the chronic phase of kainate-induced seizures [24].

The consequences of *status epilepticus* (SE) in the developing brain appear to be different from those of mature brain. Comparisons of the findings obtained in the adult and newborn brain reveal a paradox, in that the immature brain has generally been considered ‘resistant’ to the damaging effects of hypoxia and

hypoxia–ischemia, while at the same time exhibiting periods of heightened sensitivity to injury, dependent on the specific developmental stage of the brain [25, 26].

Despite that, the immature brain is not immune to injury in prolonged seizure as SE. Changes in AMPA receptors and EAAC1 transporter expression were reported in SE rats at 10 days post-natal (P10) and these modifications were related to higher susceptibility to another seizure episode [19]. Despite the apparent low susceptibility of immature brain to seizure-induced cell death, seizures in the developing brain can result in irreversible alterations in neuronal connectivity [1]. Neonatal rats, which suffered from SE displayed synaptic alterations and memory impairment in the adulthood [27-29], showing that disturbances in a critical period of brain maturation could persistently compromise its function. Furthermore, neural injuries such as hypoxic or hypoxic–ischemic insult to the developing brain will impact on subsequent maturation, with long-lasting consequences for the adult brain [26].

Although some information is available regarding the involvement of glutamate transporters in events triggered by seizure activity in adult animals [9, 20, 30, 31], little is known about the neonatal brain responses to seizure involving glutamate transporters, especially in the early period post-seizure. The aim of the present study was to investigate whether activity and immunocontent of glial and neuronal glutamate transporters in the hippocampus could be affected by kainate-induced seizure activity in rat pups. Behavioral tasks (anxiety-related behavior and inhibitory avoidance task) were also evaluated in adulthood (60 days after the seizures period).

2. Materials and Methods

2.1 Kainate-induced seizure model

Wistar rats were maintained under controlled environment (21-22° C, 12 h dark-light cycle, food and water *at libitum*). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources of Federal University of Rio Grande do Sul, Brazil. Seizures were induced as previously described [28]. Seven-day-old male Wistar rats were separated from their dams and received a single injection of kainate (KA) (1 mg/kg, s.c.) diluted in saline (NaCl 0.9 g %). Control animals received saline solution. The volume injected in each animal corresponded to 1 % of body weight (ml/g). All animals presented seizures up to 30 min after KA injection. Seizures were characterized by intermittent myoclonic jerks, generalized tonic–clonic jerks, scratching, “swimming”, and “wet-dog shakes”. After spontaneous ending of seizures (around 3 h after KA administration), animals returned to their dams.

2.2 [^{3}H] Glutamate uptake

Hippocampal slices for glutamate uptake were obtained 12, 24, 48, 72 h and 60 days after the end of seizures episode. Animals were euthanized, the hippocampi were dissected out and immediately immersed in ice-cold 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 7.3. Slices from each hippocampus (0.4 mm) were obtained using a McIlwain tissue chopper. They were pre-incubated at 35° C for 15 min and the medium was replaced by HBSS. Glutamate uptake was started by adding 100 μM

[^{3}H] glutamate. Incubation was stopped after 5 minutes by aspiration of the medium and slices were rinsed twice with ice-cold Na^+-free HBSS. Slices were then lysed in 0.5 N NaOH and kept overnight. The uptake was also carried out in Na^+-free HBSS (replaced by *N*-methyl-d-glucamine) at 4º C. Sodium dependent uptake was considered as the difference between the uptake with and without sodium. Incorporated radioactivity was measured using a Wallac liquid scintillation counter.

2.3 Western blotting

Hippocampi were dissected out 12, 24, 48, 72 h and 60 days after the end of seizures episode and immediately homogenized in a 25 mM HEPES solution (pH 7.4) with 0.1 % SDS and protease inhibitor cocktail (Sigma, USA). Samples (20 µg protein/well) were separated in an 8 % SDS-PAGE mini-gel and transferred to a nitrocellulose membrane using a Trans-Blot system (Bio-Rad, São Paulo/SP, Brasil). Membranes were processed as follow: (1) blocking with 5 % bovine serum albumin (Sigma, USA) for 2 h; (2) incubation with primary antibody overnight: 1:1000 rabbit anti-GLAST, rabbit anti-GLT-1 or rabbit anti-EAAC1 glutamate transporters (AlphaDiagnostic International); 1:10,000 rabbit anti-Glutamine synthetase (Sigma); 1:2000 mouse anti-β-Actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1:3000 (Amersham Pharmacia Biotech) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech, São Paulo/SP, Brasil) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and bands intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the web site

(http://rsb.info.nih.gov/nih-image/). In order to determine the adequate amount of protein to be assayed, different protein concentrations were carried out in the same gel for each antibody tested.

2.4 Immunohistochemistry

Perfusion and fixation of the brain from 4 animals/group were performed 24 h after the end of seizures period through transcardiac perfusion with 4 % paraformaldehyde and 0.25 % glutaraldehyde, followed by cryoprotection in 30 % sucrose solution overnight. Brain was sectioned (50 μm coronal sections) using a Leica VT1000S microtome (Leica Microsystems, São Paulo, Brazil). Coronal sections were separated in 4 series throughout the dorsal hippocampus with 300 μm interval between each section and collected in PBS. Free-floating sections of rat brain were processed for immunohistochemistry against the neuronal specific protein neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP), using a primary mouse anti-NeuN (1: 500, Chemicon International, São Paulo/SP, Brasil) as well as rabbit anti-GFAP antibodies (1: 500, Dako, Denmark A/S). Antibodies were diluted in Tris buffer saline (TBS, pH 7.4) containing 0.2 % Triton X-100 and 10 % normal goat serum and incubated for 48 h at 4° C. After incubation, sections were rinsed 4 times for 10 min in TBS and subsequently incubated with secondary fluorescent antibodies overnight: Alexa fluor anti-rabbit 488 and anti-mouse 594 (1: 500, Invitrogen, Porto Alegre/RS), in 0.1 M TBS containing 0.2 % Triton X-100 and 10 % normal goat serum for 24 h at 4° C. After rinsing 4 times for 10 min in TBS, the sections were mounted on slides coated with 2 % gelatin with chromium and potassium sulfate. The slices were mounted in a Vectashield mounting medium

containing the nuclear marker DAPI (4'-6-diamidino-2-phenylindole dilactate) (Vector Laboratories, São Paulo/SP, Brasil). The CA1, CA3 and dentate gyrus (DG) subfields of each hippocampus were examined in the Olympus FluorView 1000 system and the fluorescence was quantified using ImageJ software. The images were captured and a square region of interest (ROI) was created considering the pyramidal layer size. The ROI square of 8019 μm^2 was overlaid on the analyzed subfields with blood vessels and other artifacts being avoided, using a magnification of 20x. Six ROI were analyzed per subfield.

2.5 Behavioral analysis

2.5.1 Elevated plus-maze task

Rats (60-day-old) were exposed to the elevated plus-maze apparatus that consisted of a central platform (10 cm × 10 cm) with 2 open and 2 closed arms (45 cm × 10 cm), arranged in such a way that the 2 arms of each type were opposite to each other. The apparatus was kept 88 cm above the floor and sessions were carried out in a room lighted only with a dim red light. Animals were individually placed in the central platform facing an open arm and observed for 5 min. Two observers blinded to treatments recorded the number of entries and the time spent in the open arms as measurements of anxiety-related behavior (Walf and Frye, 2007).

2.5.2 Inhibitory avoidance task

Rats (60-day old) were placed on a 5.0 cm-high, 8.0 cm-wide platform located in the left side of a 50 cm × 25 cm × 25 cm inhibitory avoidance task apparatus, with floor composed by a series of parallel bronze bars 1.0 cm apart. In the training session, the latency to step down from the platform to the grid with all

four paws was measured; immediately after stepping down onto the grid animals received a 0.4 mA, 1.0-s scrambled foot shock. The test session was performed 1.5 h (short-term memory) and 24 h (long-term memory) after training and procedures were the same, except that the foot shock was omitted. Differences between training and test latencies to step down were taken as an index of memory.

2.6 Statistical Analysis

For glutamate uptake, western blot data and immunohistochemistry, the results were expressed as mean ± standard deviation, and statistical analysis was performed by one-way ANOVA followed by Tukey's test as post-hoc. For elevated plus maze task, the results were expressed as mean ± standard deviation and the Student´s t test was applied. For inhibitory avoidance task, the results were expressed as median ± interquartile range and Wilcoxon test was used for analysis within groups. For statistical significance, the value of $P < 0.05$ was adopted. The statistical analysis was performed using SPSS 15.0. software.

3. Results

3.1 Effect of kainate-induced seizures on hippocampal glutamate uptake activity and on glutamate transporters immunocontent.

Figure 1 shows that the glutamate uptake by hippocampal slices obtained 12 h after kainate-induced seizures showed a trend to be higher ($P = 0.082$), and those obtained 24 h after seizures decreased 20 %, when compared to control

group. Glutamate uptake by hippocampal slices was not affected by seizures after 48 h.

The immunocontent of astrocytic glutamate transporters (GLAST and GLT-1) and of neuronal glutamate transporter (EAAC1) was determined in the whole hippocampus obtained 12, 24, 48, 72 h and 60 days after seizures.

GLT-1 increased (37 %) in hippocampi obtained 12 h after the seizures period, followed by a decrease (20 %) at 24 h (Fig. 2A). GLT-1 showed no alterations after 48 h. The immunocontent of GLAST increased around 2 fold in hippocampi obtained from KA group only up to 48 h after seizures (Fig. 2B).

The immunocontent of the neuronal EAAC1 glutamate transporter was not affected by KA-induced (Fig. 2C).

The time-dependent expression was observed in the immunocontent of all the transporters investigated; in 60 days-old rats the GLT-1 and GLAST immunocontent increased, and the EAAC1 immunocontent decreased, compared with younger animals.

The immunocontent of hippocampal glutamine synthetase (GS) was not affected by KA-induced seizures (Fig. 3).

3.2 Effect of kainate-induced seizures on hippocampal GFAP immunoreactivity.

As the hippocampal glutamate uptake and the immunocontent of astrocytic (GLT1 and GLAST) glutamate transporters were modified in the hippocampus 24 hours after the end of seizures episode, immunohistochemical analysis for GFAP, NeuN and DAPI was performed in this time in all subfields of the hippocampus [CA1, CA3 and dentate gyrus (DG)]. There was an increase in the GFAP immunoreactivity in KA group as compared to control group in all subfields (Fig. 4).

In the regions surrounding pyramidal layer (SPL) and over pyramidal layer (PL) of CA3 there was an increase of 147 % and 100 % for GFAP immunoreactivity compared to control group, respectively (Fig. **3**; first panel). Likewise, surrounding pyramidal layer (SPL) and over pyramidal layer (PL) of CA1 there was an increase of 100 % and 40 % for GFAP immunoreactivity compared to control group, respectively (Fig. 3; second panel). GFAP immunoreactivity increased 100% compared to saline-treated rats in the dentate gyrus (DG) (Fig. 3; third panel). NeuN immunoreactivity and DAPI staining were similar between both groups, indicating absence of neuronal loss 24h after seizure (data not shown).

3.3 Kainate-induced seizures caused anxiety-like behavior and aversive memory impairment in adulthood.

Sixty days after the seizures episode, male rats were submitted to behavioral tasks. In elevated plus-maze task, aiming to assess anxiety-related behavior (Fig. 5), kainate-treated rats presented a decrease on the time spent and the number of entries in open arms compared to saline-treated rats (Fig. 5).

Kainate-treatment abolished the short- (1.5 h after training) and long- (24 h after training) term memory, evaluated in an inhibitory avoidance task (Fig. 6).

4. Discussion

The present study shows that rats presenting KA-induced seizures in early periods of development presented brain acute molecular and biochemical alterations related to the glutamatergic system, and long-term behavioral impairment in adulthood.

The short-term effects investigated were on hippocampal glutamate uptake and on astrocytic glutamate transporters immunocontent. At 12 h after seizures, there was an increase in the glutamate uptake (that did not reach statistical significance) and in both GLT-1 and GLAST immunocontent. At 24 hours after seizures, the GLAST levels remained up regulated, while the glutamate uptake activity and the GLT-1 levels became diminished. The EAAC1 and glutamine synthetase levels did not vary.

Based upon the common pattern of temporal adaptation, GLT-1 seems to be responsible for the transient increase and further decrease on glutamate uptake observed in the hippocampus obtained 12 and 24 h after the end of seizures, respectively. Considering that in the forebrain regions, GLT-1 alone accounts for more than 95 % of the total high-affinity glutamate uptake capacity [21, 32], this correlation may embody a relevant pathophysiological response to seizures (Ueda et al. 2002). It was previously shown that the immunocontent of GLT-1 was transiently increased in newborn rats treated with kainate and the same could be observed for GLT-1 mRNA levels in adult animals submitted to kainate-induced seizures (Simantov et al., 1999). Interestingly, GLAST was the only glutamate transporter in newborn rats treated with kainate that remains up regulated and the same profile for GLAST mRNA levels was also observed in adult animals (Nonaka et al., 1998). Additionally, it is noteworthy that the glutamate uptake apparently follows the ontogeny of GLT-1 during brain development [33]. Our findings ruled out the participation of EAAC1 transporter in the kainate-induced seizures in newborns. Interestingly, the same could not be observed in adult animals

submitted to kainate-induced seizures, since hippocampal EAAC1 mRNA expression remains increased up to 5 days after seizures [34].

As the kainate toxicity depends on the release of endogenous excitatory amino acids [35-37] and *in vitro* studies indicated that glutamate stimulates glutamate transport in primary astrocyte cultures [38], it can be hypothesized that the transient up regulation of both transporters could reflect an attempt to remove the excess of extracellular glutamate that accumulate during seizure periods [39]. As the GLAST immunocontent was more specifically involved in short (Duan et al., 1999) and prolonged [38] stimulatory effect triggered by glutamate on its own uptake by cultured astrocytes, the longer lasting increase in the GLAST immunocontent after KA-induced seizures here observed (up to 48 h) could be interpreted as a neuroprotective response to the increase of hippocampal glutamate extracellular levels.

It is interesting to note that the increase in the immunoreactivity for GFAP-positive astrocytes, which was measured 24 h after the end of seizures, accomplished the increase in the GLAST immunocontent. Epilepsy is characterized by hippocampal sclerosis, which one striking hallmark is astrocytic “reactive gliosis”, accompanied by neuronal cell loss, microvascular proliferation and synaptic reorganization [18, 40]. The occurrence of seizures affects astrocytes functions generating abnormal glutamatergic and GABAergic neurotransmission activities, which precedes neuronal death [41]. Accordingly, it has been shown that kainate treatment caused detectable cell damage 72 h after seizures, in 10 days old rats [42]. The hippocampal damage can also be observed in other seizure models in 15 days old animals [43-45]. In our study, astrogliosis was present in the

hippocampus 24 h after seizures, with no evident signs of neuronal damage; however, it cannot be discarded the occurrence of neuronal damage after this time.

The ontogenetic profile of glutamate transporters levels observed in our findings is in agreement with previous data [33, 46, 47], since GLT-1 and GLAST levels increased, whereas EAAC1 decreased in adult animals. Interestingly, seizures at 7-day old did not modify the immunocontent of glutamate transporters in the adulthood.

It has been reported that patients with medical intractable mesial temporal lobe epilepsy (MTLE) present deficiency in the hippocampal glutamine synthetase (GS) [23]. Likewise, animals treated with methionine sulfoximine, which leads to deficiency in the GS activity, presented recurrent seizures, hippocampal atrophy and neuronal loss [48]. These findings suggest that GS may play a role in the pathogenesis of MTLE that could contribute to glutamate accumulation observed in this condition. In our study, GS hippocampal levels were not affected by kainate-induced seizures.

Even though the short-term alterations in the hippocampal glutamatergic parameters were not persistent over time, in adulthood the rats presented anxiety-related behavior and memory decline in an inhibitory avoidance task, which is in agreement with other studies [27-29, 49]. These long-lasting behavioral alterations might be related to early changes in hippocampal glutamatergic neurotransmission. A previous study of our group also suggested that alterations in early periods after birth could be involved in behavioral deficits in adulthood [50]. The exact mechanism involved in the long-term effects of KA-induced seizures on behavioral performance in adulthood is still unknown, but appears to involve impairment of the

long-term potentiation, enhanced long-term depression and reduction on synaptic proteins levels, such NMDA receptors, SNAP-25 and syntaxin [27, 28]. Apparently, astrogliosis is not persistent up to adulthood in this model [27].

5. Conclusion

The early periods of brain development are of great relevance and determine adequate brain function late in lifespan. Our study indicates that a single convulsive event in early life could induce short-term alterations in relevant parameters involved in the homeostasis of glutamatergic neurotransmission in the hippocampus, which could be involved in the behavioral alterations in adulthood animals. Our findings can contribute to better understand the role of glutamate transporters in seizures during childhood. From clinical point of view, our data suggest that interventions on the glutamatergic system during seizures in children may be relevant for prevention of brain impairment in adulthood.

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References

[1] Holmes GL, Ben-Ari Y. The neurobiology and consequences of epilepsy in the developing brain. Pediatr Res. 2001;49:320-5.

[2] Khazipov R, Khalilov I, Tyzio R, Morozova E, Ben-Ari Y, Holmes GL. Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. Eur J Neurosci. 2004;19:590-600.

[3] Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci. 2002;3:728-39.

[4] Tzingounis AV, Wadiche JI. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. Nat Rev Neurosci. 2007;8:935-47.

[5] Danbolt NC. Glutamate uptake. Prog Neurobiol. 2001;65:1-105.

[6] Segovia G, Porras A, Del Arco A, Mora F. Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev. 2001;122:1-29.

[7] Ozawa S, Kamiya H, Tsuzuki K. Glutamate receptors in the mammalian central nervous system. Prog Neurobiol. 1998;54:581-618.

[8] Kew JN, Kemp JA. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl). 2005;179:4-29.

[9] Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron. 1996;16:675-86.

[10] Maragakis NJ, Rothstein JD. Glutamate transporters: animal models to neurologic disease. Neurobiol Dis. 2004;15:461-73.

[11] Beart PM, O'Shea RD. Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. Br J Pharmacol. 2007;150:5-17.

[12] Sheldon AL, Robinson MB. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int. 2007;51:333-55.

[13] Chen Y, Swanson RA. Astrocytes and brain injury. J Cereb Blood Flow Metab. 2003;23:137-49.

[14] Belanger M, Magistretti PJ. The role of astroglia in neuroprotection. Dialogues Clin Neurosci. 2009;11:281-95.

[15] Bunch L, Erichsen MN, Jensen AA. Excitatory amino acid transporters as potential drug targets. Expert Opin Ther Targets. 2009;13:719-31.

[16] Dunlop J. Glutamate-based therapeutic approaches: targeting the glutamate transport system. Curr Opin Pharmacol. 2006;6:103-7.

[17] Bjornsen LP, Eid T, Holmseth S, Danbolt NC, Spencer DD, de Lanerolle NC. Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures. Neurobiol Dis. 2007;25:319-30.

[18] Binder DK, Steinhauser C. Functional changes in astroglial cells in epilepsy. Glia. 2006;54:358-68.

[19] Zhang G, Raol YS, Hsu FC, Brooks-Kayal AR. Long-term alterations in glutamate receptor and transporter expression following early-life seizures are associated with increased seizure susceptibility. J Neurochem. 2004;88:91-101.

[20] Ueda Y, Doi T, Tokumaru J, Yokoyama H, Nakajima A, Mitsuyama Y, et al. Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. J Neurochem. 2001;76:892-900.

[21] Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science. 1997;276:1699-702.

[22] Suarez I, Bodega G, Fernandez B. Glutamine synthetase in brain: effect of ammonia. Neurochem Int. 2002;41:123-42.

[23] Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, et al. Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. Lancet. 2004;363:28-37.

[24] Hammer J, Alvestad S, Osen KK, Skare O, Sonnewald U, Ottersen OP. Expression of glutamine synthetase and glutamate dehydrogenase in the latent phase and chronic phase in the kainate model of temporal lobe epilepsy. Glia. 2008;56:856-68.

[25] Holmes GL. Effects of seizures on brain development: lessons from the laboratory. Pediatr Neurol. 2005;33:1-11.

[26] Hurn PD, Vannucci SJ, Hagberg H. Adult or perinatal brain injury: does sex matter? Stroke. 2005;36:193-5.

[27] Cognato GP, Agostinho PM, Hockemeyer J, Muller CE, Souza DO, Cunha RA. Caffeine and an adenosine A(2A) receptor antagonist prevent memory impairment and synaptotoxicity in adult rats triggered by a convulsive episode in early life. J Neurochem. 2010; 112:453-62.

[28] Cornejo BJ, Mesches MH, Coultrap S, Browning MD, Benke TA. A single episode of neonatal seizures permanently alters glutamatergic synapses. Ann Neurol. 2007;61:411-26.

[29] Cornejo BJ, Mesches MH, Benke TA. A single early-life seizure impairs short-term memory but does not alter spatial learning, recognition memory, or anxiety. Epilepsy Behav. 2008;13:585-92.

[30] Simantov R, Crispino M, Hoe W, Broutman G, Tocco G, Rothstein JD, et al. Changes in expression of neuronal and glial glutamate transporters in rat hippocampus following kainate-induced seizure activity. Brain Res Mol Brain Res. 1999;65:112-23.

[31] Miller HP, Levey AI, Rothstein JD, Tzingounis AV, Conn PJ. Alterations in glutamate transporter protein levels in kindling-induced epilepsy. J Neurochem. 1997;68:1564-70.

[32] Chao XD, Fei F, Fei Z. The role of excitatory amino acid transporters in cerebral ischemia. Neurochem Res. 2010; 35:1224-30.

[33] Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC. Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur J Neurosci. 1997;9:1646-55.

[34] Nonaka M, Kohmura E, Yamashita T, Shimada S, Tanaka K, Yoshimine T, et al. Increased transcription of glutamate-aspartate transporter (GLAST/GluT-1) mRNA following kainic acid-induced limbic seizure. Brain Res Mol Brain Res. 1998;55:54-60.

[35] Ben-Ari Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience. 1985;14:375-403.

[36] Coyle JT. Neurotoxic action of kainic acid. J Neurochem. 1983;41:1-11.

[37] Sperk G, Lassmann H, Baran H, Kish SJ, Seitelberger F, Hornykiewicz O. Kainic acid induced seizures: neurochemical and histopathological changes. Neuroscience. 1983;10:1301-15.

[38] Gegelashvili G, Civenni G, Racagni G, Danbolt NC, Schousboe I, Schousboe A. Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. Neuroreport. 1996;8:261-5.

[39] Ueda Y, Yokoyama H, Nakajima A, Tokumaru J, Doi T, Mitsuyama Y. Glutamate excess and free radical formation during and following kainic acid-induced status epilepticus. Exp Brain Res. 2002;147:219-26.

[40] Seifert G, Carmignoto G, Steinhauser C. Astrocyte dysfunction in epilepsy. Brain Res Rev. 2010; 63:212-21.

[41] Kang TC, Kim DS, Kwak SE, Kim JE, Won MH, Kim DW, et al. Epileptogenic roles of astroglial death and regeneration in the dentate gyrus of experimental temporal lobe epilepsy. Glia. 2006;54:258-71.

[42] Dunleavy M, Shinoda S, Schindler C, Ewart C, Dolan R, Gobbo OL, et al. Experimental neonatal status epilepticus and the development of temporal lobe epilepsy with unilateral hippocampal sclerosis. Am J Pathol. 2010; 176:330-42.

[43] de Oliveira DL, Fischer A, Jorge RS, da Silva MC, Leite M, Goncalves CA, et al. Effects of early-life LiCl-pilocarpine-induced status epilepticus on memory and anxiety in adult rats are associated with mossy fiber sprouting and elevated CSF S100B protein. Epilepsia. 2008;49:842-52.

[44] Sankar R, Shin DH, Liu H, Mazarati A, Pereira de Vasconcelos A, Wasterlain CG. Patterns of status epilepticus-induced neuronal injury during development and long-term consequences. J Neurosci. 1998;18:8382-93.

[45] Sperber EF, Haas KZ, Romero MT, Stanton PK. Flurothyl status epilepticus in developing rats: behavioral, electrographic histological and electrophysiological studies. Brain Res Dev Brain Res. 1999;116:59-68.

[46] Bar-Peled O, Ben-Hur H, Biegon A, Groner Y, Dewhurst S, Furuta A, et al. Distribution of glutamate transporter subtypes during human brain development. J Neurochem. 1997;69:2571-80.

[47] Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci. 1997;17:8363-75.

[48] Eid T, Ghosh A, Wang Y, Beckstrom H, Zaveri HP, Lee TS, et al. Recurrent seizures and brain pathology after inhibition of glutamine synthetase in the hippocampus in rats. Brain. 2008;131:2061-70.

[49] Sun QJ, Duan RS, Wang AH, Shang W, Zhang T, Zhang XQ, et al. Alterations of NR2B and PSD-95 expression in hippocampus of kainic acid-exposed rats with behavioural deficits. Behav Brain Res. 2009;201:292-9.

[50] Moreira JD, Knorr L, Ganzella M, Thomazi AP, de Souza CG, de Souza DG, et al. Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: relevance for behavior alterations. Neurochem Int. 2010; 56:753-9.

Legends to figures

Fig. 1. Effects of a single episode of neonatal seizures on the hippocampal [^{3}H] glutamate uptake. Hippocampal slices were obtained at 12, 24, 48 and 72 h and 60 days post-seizures. Data are expressed as mean ± SD from 6 animals/group. *P <0.05, significant difference from control group within the time analyzed (One-way ANOVA followed by Tukey`s post-hoc test).

Fig. 2. Effects of a single episode of neonatal seizures on the immunocontent of the glutamate transporters (GLT-1, GLAST and EAAC1), analyzed at 12, 24, 48 and 72 h and 60 days post-seizure. Control (black bars) and kainate (KA, white bars). Data are expressed as mean ± SD from 6 animals/group. At the top of the figure are representative images of the immunocontent of transporters. β-Actin was used as a protein loading control. *P <0.05, significant difference from control group within the specific time. #P <0.05, significant differences within the group from other times analyzed; &P < 0.05, significant difference from K72h (One-way ANOVA followed by Tukey`s post-hoc test).

Fig. 3. Effects of a single episode of neonatal seizures on the immunocontent of glutamine synthetase (GS). GS immunocontent was analyzed at 12, 24, 48 and 72 h and 60 days post-seizure. Control (black bars) and kainate (KA, white bars). Data are expressed as mean ± SD from 6 animals/group. At the top of the figure are representative images of the immunocontent of GS. β-Actin was used as a protein loading control.

Fig. 4. Immunohistochemistry analysis for GFAP, NeuN and DAPI in the rat hippocampus after a single episode of neonatal seizures. GFAP, NeuN and DAPI reactivities were evaluated 24 h after kainate-induced seizures. Fluorescence quantification was achieved at the regions surrounding pyramidal layer (SPL) and over pyramidal layer (PL), except for dentate gyrus (DG), using pictures acquired in 20x of magnification. Control (black bars) and kainate (KA, white bars). Data are expressed as mean ± SD from 4 animals per group. *P<0.005; significant difference from control group (Student's t-test).

Fig. 5. Behavioral performance in adult rats submitted to a single episode of neonatal seizure. Animals were exposed to 5 minutes of exploration in the elevated plus-maze. **A**- time spent in seconds (s) in the open arms; **B** - number of entries in the open arms. Data are expressed as means ± SD from 10 animals per group. *P<0.05; significant difference from control group (Student's t-test).

Fig. 6. Performance in the inhibitory avoidance task in adult rats submitted to a single episode of neonatal seizure. Data are expressed as median ± interquartile range (n=10 per group) of the latencies to step down in seconds (s) in the training and test session performed 1.5 h (short-term memory) and 24 h (long-term memory) later. *P<0.05; significant difference from training and test session within group (Wilcoxon test).

Figures

Figure 1

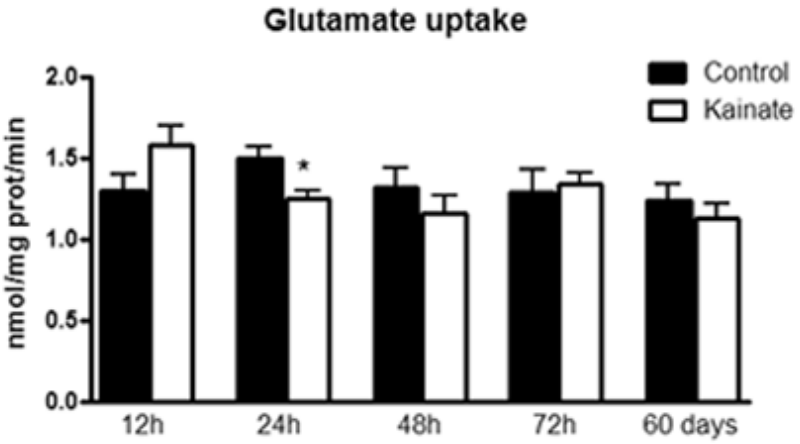

Figure 2

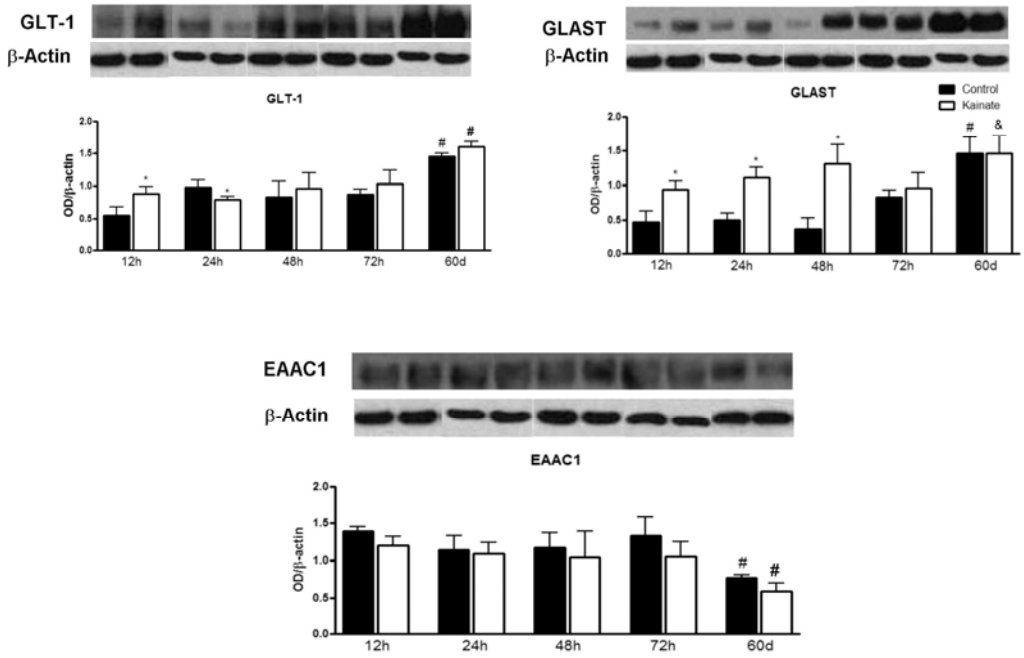
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Figure 3

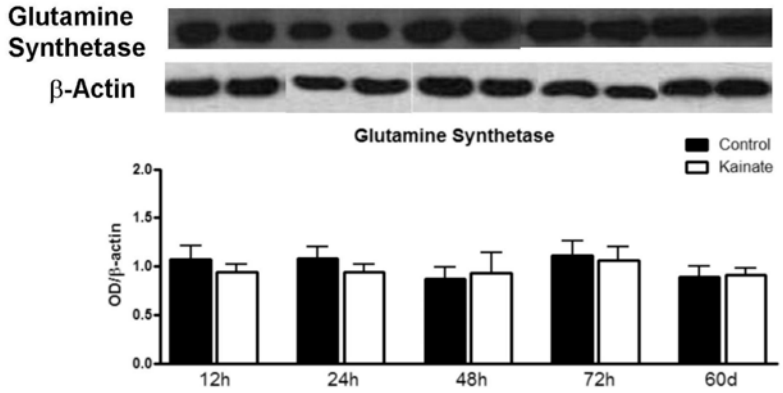
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Figure 4

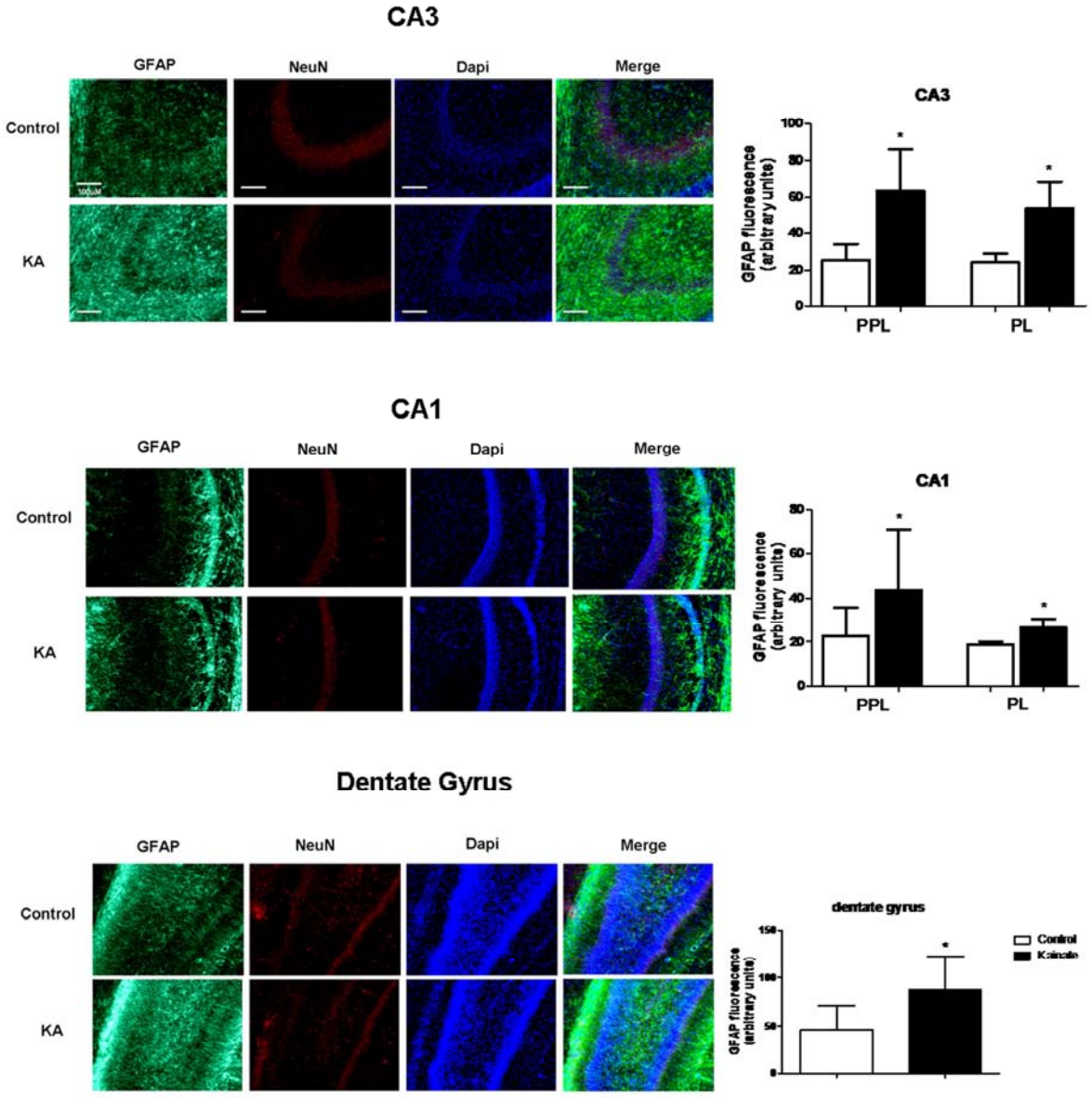
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Figure 5

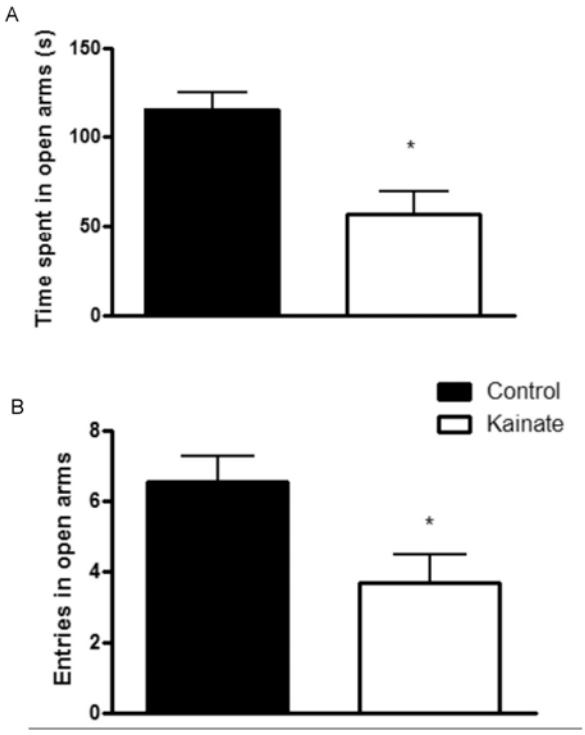
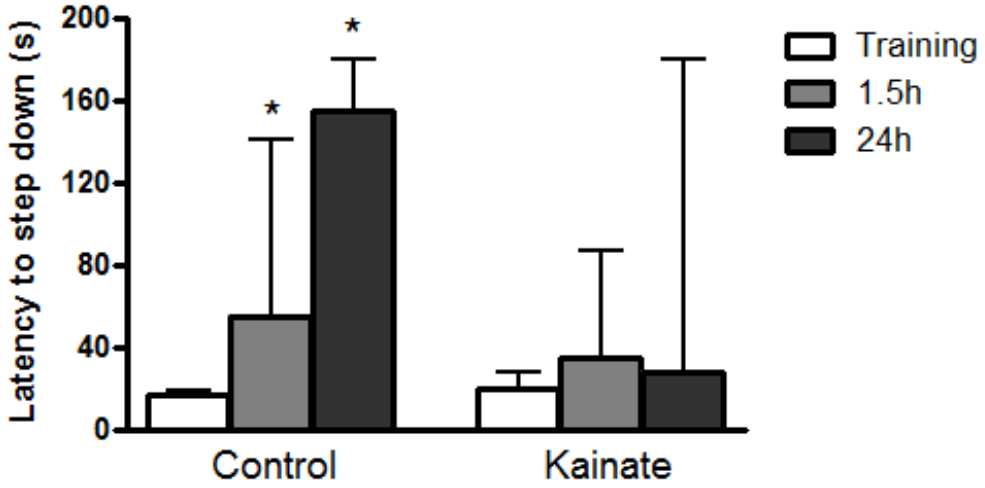


Figure 6



Capítulo 4

Dietary omega-3 fatty acids prevent early alterations in hippocampal glutamatergic system and memory deficits caused by a single neonatal seizure episode.

Júlia D. Moreira, Letícia V. de Siqueira, Vanessa M. Lague, Alexandre P. Müller, Lisiane O. Porciúncula, Lúcia Vinadé, Diogo O. Souza.

Artigo em preparação.

Dietary omega-3 fatty acids prevent early alterations in hippocampal glutamatergic system and memory deficits caused by a single neonatal seizure episode.

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Abstract

The incidence of seizures is higher in children, being glutamatergic excitoxicity effects responsible to brain alteration after seizure period. Here, we investigated the influence of ω3PUFAs on the glutamatergic system modulation after one single neonatal seizures episode and the possible effect on long-lasting behavioral deficits caused by seizures. Wistar male rats, receiving omega-3 diet (ω3) or ω3 deficient diet (D) from prenatal period, were submitted to kainate-induced seizures model at 7 days old. Glutamate transporters activity and content (GLT-1 and GLAST) were assessed in hippocampus 12 h, 24 h, 48 h after seizures. Intensity of fluoresce in glial cells (GFAP), neurons (NeuN) and DAPI (nuclei), were assessed 24 h after seizure in hippocampus. Behavioral analysis (elevated-plus maze and inhibitory avoidance task) was also investigated in the adult animals (60 days old). The D group showed a decrease in glutamate uptake 24h after seizures. D group showed increased in immoncontent of GLT1 at 12h and decrease at 24h after seizures. Moreover in this group GLAST increase up to 48 h after seizure. ω3 Groups showed no difference in glutamate transporters activity or content after seizure. The immunocontent of these transporters were higher in the D group independently of seizures, as well as the GFAP fluorescence intensity, which was increase in ω3 groups only 24h after seizures. NeuN fluorescence intensity decrease in the D group independently of seizures, and presenting no alterations caused by seizures in both groups. DAPI was not affected by diets or seizures. D group presented anxiety-related behavior and memory deficits independently of seizures, but short-term memory (1.5h after

training session) was affected by seizures in D group. Dietary ω3PUFAs are important to glutamatergic homeostasis after neonatal seizures episode, preventing behavioral alterations in adulthood.

Key words: omega-3; kainate; seizures; glutamate transporters; hippocampus; memory.

Introduction

Children are highly susceptible to epileptic seizures, being the incidence higher in the first year of life (Holmes and Ben-Ari 2001). Seizure injury in the immature brain could result in alterations in neural connectivity, causing long-lasting consequences for adult brain (Holmes and Ben-Ari 2001, Hurn, et al. 2005). However, the mechanisms underling brain changes after seizure in immature brain remains unknown.

Glutamate, the main excitatory neurotransmitter in central nervous system (CNS), is involved in many physiological brain functions, such as synaptic plasticity, learning and memory, brain development and ageing (Danbolt 2001, Ozawa, et al. 1998, Segovia, et al. 2001, Tzingounis and Wadiche 2007). However, the overstimulation of the glutamatergic system (excitotoxicity) is involved in various acute and chronic brain diseases, including neurodegenerative diseases, cerebral ischemia, seizures and epilepsy (Beart and O'Shea 2007, Danbolt 2001, Maragakis and Rothstein 2004, Sheldon and Robinson 2007, Tzingounis and Wadiche 2007). In this way, the maintenance of extracellular glutamate concentrations below toxic levels is very important to adequate brain function, preventing excitotoxicity. The extracellular glutamate level are regulated by the activity of glutamate transporters, mainly located in the astrocytic cell membranes, which taken up the glutamate from the synaptic clef protecting neurons from excitotoxicity. Five distinct high-affinity sodium-dependent glutamate transporters were identified: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 (Beart and O'Shea 2007, Bunch, et al. 2009, Danbolt 2001,

Dunlop 2006). GLAST and GLT-1 are primarily localized in astrocytes and appear to be the most relevant to remove glutamate from the synaptic cleft, preventing excitotoxicity (Danbolt 2001, Dunlop 2006)). The impairment of these glutamate transporters activities has been implicated in the pathophysiology of some brain disorders, as epilepsy and seizures (Binder and Steinhauser 2006, Bjornsen, et al. 2007, Ueda, et al. 2001, Zhang, et al. 2004).

Pharmacological strategies have been tried in attempt to control seizure crises and prevent injury to brain, but many patients presented adverse drug effects (Kwan and Brodie 2000). In this order, dietary manipulation had received considered attention as an alternative strategy to the management of seizures (Bourre 2004, Lauritzen, et al. 2001, Marszalek and Lodish 2005). Indeed, several groups have investigated the role of omega-3 polyunsaturated fatty acids (ω3PUFAs), the most abundant fatty acids in CNS, in the management of seizures (Bourre 2004, Lauritzen, Hansen, Jorgensen and Michaelsen 2001, Marszalek and Lodish 2005).

Some clinical and experimental studies using adult animals suggest that ω3PUFAs decrease the duration and frequency of seizures (Schlanger, et al. 2002, Voskuyl, et al. 1998, Yehuda, et al. 1994), prevent the status epilepticus-associated neuropathology changes in hippocampus of adult rats and the decrease of BDNF content caused by seizure (Cysneiros, et al. , Ferrari, et al. 2008). *In vitro* studies demonstrated that ω3PUFAs could inhibit epileptiform activity, synaptic transmission in the hippocampus (Young, et al. 2000) and stabilize neuronal membrane by suppressing voltage-gated Ca^{2+} currents and Na^{+} channels (Xiao, et

al. 1997, Xiao, et al. 1995). Despite these effects, some randomized clinical studies demonstrated only transient or no evidence of beneficial effects in patients (Bromfield, et al. 2008, Yuen, et al. 2005). However, studies involving the neuroprotective potential of ω3PUFAs against seizure in the immature brain are scarce.

Concerning the ω3PUFAs effects on brain, it is well know that they are crucial for adequate brain development and function. The dietary deficiency of ω3PUFAs changes the fluidity of neuronal membranes, altering lipid rafts, and consequently alters the activity of receptors, ion channels, G proteins and other proteins embedded in the membrane (Stillwell, et al. 2005, Stillwell and Wassall 2003, Wurtman 2008). We previously demonstrated that ω3PUFAs deficiency delayed the normal glutamatergic synapse development and could render brain more susceptible to an excitotoxic injury in adulthood (Moreira, et al. 2010, Moreira, et al. 2010).

The aim of the present study was to investigate the neuroprotective potential of ω3PUFAs against one single neonatal seizures episode in rats, investigating the possible early glutamatergic system modulation in hippocampus and the long lasting behavioral deficits promoted by neonatal seizure.

Materials and methods

Animals and diets

The dietary model used in this study was previously described (Moreira et al. 2010), and it was designed to control the ω3 PUFAs levels in the maternal milk

as well as in the offspring diet after weaning. Briefly, 2 weeks before mating, Wistar female rats were housed in a standard animal house with controlled environment (21-22ºC, 12h dark-light cycle, food and water *ad libitum*) and divided into two groups: ω3 diet (ω3) and ω3 deficient-diet (D); the diets prepared in our own laboratory were isocaloric, containing 8% of total fat and differing only in the fatty acids composition. Female rats received their respective diets throughout the gestation as well as during suckling. After weaning (21 days old), the litters were maintained with the same diet of their dams until 60 days old. All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Kainate induced-seizures model

Seizures were induced as previously described (Cornejo, et al. 2007). Seven-day-old male Wistar rats from both diets were separated from their dams and received a single injection of kainate (KA) (1 mg/kg, s.c) diluted in saline (NaCl 0.9 %). Control animals received saline solution. The volume injected in each animal corresponded to 1 % of body weight (ml/g). All animals presented seizures up to 30 min after KA injection. Seizures were characterized by intermittent myoclonic jerks, generalized tonic–clonic jerks, scratching, “swimming”, and “wet-dog shakes”. After spontaneous ending of seizures (around 3 h after KA administration), animals returned to their dams.

[^{3}H] Glutamate uptake

Hippocampal slices for glutamate uptake were obtained from both groups 12 h, 24 h, 48 h and 60 days after the end of seizures episode. Animals were

sacrificed, the hippocampi were dissected out and immediately immersed in ice-cold 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 7.3. Slices from each hippocampus (0.4 mm) were obtained using a McIlwain tissue chopper. They were pre-incubated at 35° C for 15 min and the medium was changed. Glutamate uptake was started by adding 0.66 Ci/mL L-[^{3}H] glutamate, acquiring 100 uM (final concentration) glutamate. Incubation was stopped after 5 minutes by aspiration of the medium and the slices were rinsed twice with ice-cold Na^+-free HBSS. Slices were then lysed in 0.5 N NaOH and kept overnight. The uptake was also carried out in Na^+-free HBSS (replaced by *N*-methyl-d-glucamine) at 4º C. Sodium dependent uptake was considered as the difference between the uptake with and without sodium. Incorporated radioactivity was measured using a HIDEX 300 liquid scintillation counter.

Western blot analysis

Hippocampi from both groups were dissected out 12 h, 24 h, 48 h and 60 days after the end of seizures episode and immediately homogenized in a 25 mM HEPES solution (pH 7.4) with 0.1 % SDS and protease inhibitors cocktail (Sigma, USA). Samples (20 µg protein/well) were separated in an 8 % SDS-PAGE mini-gel and transferred to a nitrocellulose membrane using a Trans-Blot system (Bio-Rad, São Paulo/SP, Brasil). Membranes were processed as follow: (1) blocking with 5 % bovine serum albumin (Sigma, USA) for 2 h; (2) incubation with primary antibody overnight: 1:1000 rabbit anti-GLAST or rabbit anti-GLT-1 glutamate transporters

(AlphaDiagnostic International); 1:2000 mouse anti-β-Actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 or mouse 1:3000 (Amersham Pharmacia Biotech) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech, São Paulo/SP, Brasil) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and bands intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). We determined the adequate amount of protein to be assayed by previously testing different protein concentrations in the same gel for each antibody tested.

Immunohistochemistry

Perfusion and fixation of the brain from 4 animals/group were performed 24 h after the end of seizures period through transcardiac perfusion with 4 % paraformaldehyde and 0.25 % glutaraldehyde, followed by cryoprotection in 30 % sucrose solution overnight. Brain was sectioned (50 µm coronal sections) using a Leica VT1000S microtome (Leica Microsystems, São Paulo, Brazil). Coronal sections were separated in 4 series throughout the dorsal hippocampus with 300 µm interval between each section and collected in PBS. Free-floating sections of rat brain were processed for immunohistochemistry against the neuronal specific protein neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP), using a primary mouse anti-NeuN (1: 500, Chemicon International, São Paulo/SP, Brasil) and rabbit anti-GFAP antibodies (1: 500, Dako, Denmark A/S). Antibodies were diluted in Tris buffer saline (TBS, pH 7.4) containing 0.2 % Triton X-100 and 10 %

normal goat serum and incubated for 48 h at 4° C. After incubation, sections were rinsed 4 times for 10 min in TBS and subsequently incubated with secondary fluorescent antibodies overnight: Alexa fluor anti-rabbit 488 or anti-mouse 594 (1: 500, Invitrogen, Porto Alegre/RS), in 0.1 M TBS containing 0.2 % Triton X-100 and 10 % normal goat serum for 24 h at 4° C. After rinsing 4 times for 10 min in TBS, the sections were mounted on slides coated with 2 % gelatin with chromium and potassium sulfate. The slices were mounted in a Vectashield mounting medium containing the nuclear marker DAPI (4'-6-diamidino-2-phenylindole dilactate) (Vector Laboratories, São Paulo/SP, Brasil). The CA1, CA3 and dentate gyrus (DG) subfields of each hippocampus were examined in the Olympus FluorView 1000 system and the fluorescence was quantified using ImageJ software. The images were captured and a square region of interest (ROI) was created considering the pyramidal layer size. The ROI square of 8019 μm^2 was overlaid on the analyzed subfields with blood vessels and other artifacts being avoided, using a magnification of 20x. Six ROI were analyzed per subfield.

Behavioral tasks

Elevated plus-maze task

Rats (60-day-old) from both diets treated or not with kainate were exposed to the elevated plus-maze apparatus that consisted of a central platform (10 cm × 10 cm) with 2 open and 2 closed arms (45 cm × 10 cm), arranged in such a way that the 2 arms of each type were opposite to each other. The apparatus was kept 88 cm above the floor and sessions were carried out in a room lighted only with a dim red light. Animals were individually placed in the central platform facing an open arm and observed for 5 min. Two observers blinded to treatments

recorded the number of entries and the time spent in the open arms, as measurements of anxiety-related behavior (Walf and Frye 2007).

Inhibitory avoidance task

Rats (60-day old) from both diets treated or not with kainate were placed in a 5.0 cm-high, 8.0 cm-wide platform located in the left side of a 50 cm × 25 cm × 25 cm inhibitory avoidance task apparatus, with floor composed by a series of parallel bronze bars 1.0 cm apart. In the training session, the latency to step down from the platform to the grid with all four paws was measured; immediately after stepping down, the animals received a 0.7 mA, 1.0-s scrambled foot shock. The test session was performed 1.5 h (short-term memory), 24 h and 7 days (long-term memory) after training and the procedures were the same, except that the foot shock was omitted. Differences between training and test latencies to step down were taken as an index of memory.

Statistical Analysis

For glutamate uptake, western blot data, immunohistochemistry analysis and elevated plus maze task, the results were expressed as mean ± standard deviation, and statistical analysis was performed by Two-way ANOVA followed by Bonferroni`s test as post-hoc. For inhibitory avoidance task, the results were expressed as median ± interquartile range and Wilcoxon test was used for analysis within groups. For statistical significance, the value of $P < 0.05$ was adopted. The statistical analysis was performed using SPSS 15.0. software.

Results

Dietary ω3 PUFAs prevented the modulation on glutamatergic system caused by neonatal seizures episode.

Figure 1 shows the glutamate uptake by hippocampal slices obtained 12, 24 48 h, and 60 days after kainate-induced seizures in both groups. We observed that only 24 h after seizures, D group presented a decrease in glutamate uptake compared to the other groups ($p < 0.05$).

The content of astrocytic glutamate transporters (GLAST and GLT-1) were determined in the whole hippocampus obtained 12, 24 and 48 h after seizures (Fig. 2). The results indicate that: the ω3 deprivation increased GLT-1 and GLAST contents compared to ω3 groups in all times analyzed ($p < 0.05$); glutamate transporters were not affected by seizures in ω3 group; in D group seizures increased the content of GLT-1 in hippocampus 12 h after the seizures period, decreased at 24 h, without affecting at 48 h; in D group seizures increased the content of GLAST in all times analyzed ($p < 0.05$).

Dietary ω3 PUFAs deficiency increased GFAP fluorescence intensity and decreased NeuN fluorescence intensity independently of seizures episode.

As the major glutamatergic system alterations occurred 24h after seizures episode (hippocampal glutamate uptake and the astrocytic glutamate transporters contents) in the D group, immunohistochemical analysis for GFAP, NeuN and DAPI were performed at this time in all subfields of the hippocampus [CA1, CA3 and dentate gyrus (DG)]. D group presented higher GFAP fluorescence intensity in all hippocampal subfields and lesser NeuN fluorescence intensity in CA1 and CA3

hippocampal subfields independently of seizures episode, when compared to ω3 groups (Fig. 3, 4 and 5, $p < 0.05$). There was an increase in the GFAP fluorescence intensity in all hippocampal subfields (CA1, CA3 and DG) in ω3 kainate group when compared to the respective saline group (Fig. 3, 4 and 5 $p < 0.05$). NeuN fluorescence intensity was not affected by seizures in both groups. DAPI staining was similar in all groups.

Dietary ω3 PUFAs prevented memory deficits in adulthood caused by the neonatal seizures episode.

Sixty days after the seizures episode, the rats from both groups were submitted to behavioral tasks.

In elevated plus-maze task, aiming to assess anxiety-related behavior, D group presented a decrease on the time spent and the number of entries in open arms compared to ω3 groups independently of seizure episode (Fig. 6), indicating and anxiogenic effect of ω3 deficiency. Kainate-induced seizures did not affect anxiety-related behavior in both groups.

D saline group presented decrease in memory performance 7 days after training session in inhibitory avoidance task (Fig. 7). Additionally, neonatal seizures abolished the short-term memory (1.5 h after training) in D group. No behavioral alterations were observed in ω3 groups.

Discussion

In the present study we observed that dietary ω3PUFAs are very important to maintain the glutamatergic system homeostasis in basal conditions and after

neonatal seizures episode, preventing behavioral alterations in adulthood caused by seizures. Additionally, dietary ω3PUFAs deficiency caused behavioral deficits and morphological alterations in hippocampus, independently of seizures episode, pointing to the importance of ω3PUFAs to adequate brain development.

Dietary ω3PUFAs were proposed as neuroprotective against glutamate excitotoxicity by several groups (Berman, et al. 2010, Cansev, et al. 2008, de Wilde, et al. 2002, Lukiw, et al. 2005, Moreira et al. 2010a).

In the present study, D group decreased the glutamate uptake activity 24h after seizure episode (with no effect in the other times investigated). Few studies related ω3PUFAs with glutamate uptake. We previously showed using *ex vivo* experiments that ω3PUFAs partially prevented the decrease in glutamate uptake caused by ischemia (Moreira et al. 2010b). In this way, dietary ω3PUFAs are very relevant to the homeostasis of glutamatergic system during excitotoxic events in brain.

The modulation of glutamate uptake in D group was accompanied by GLT-1 content increased 12h after seizures and decreased 24h after. Furthermore, GLAST content showed an increase, which persisted up to 48 h after seizure episode. Interesting, both glutamate transporters contents were higher in hippocampus in D groups compared to ω3 groups. As the glutamate transporters could be modulated by the glutamate concentration on synaptic cleft (Gegelashvili, et al. 1996, Gegelashvili, et al. 2000, Kalandadze, et al. 2002, Lortet, et al. 1999), which is increase during seizures period induced by kainate (Ueda et al., 2001), we hypothesized that in D group glutamate uptake activity was reduced, more

glutamate accumulated in synaptic cleft, modulating (increasing) glutamate transporters contents.

We also observed in this study that dietary ω3PUFAs deficiency was capable to cause morphological alterations in hippocampus. D group presented increase GFAP and decrease NeuN fluorescence intensity, independently of seizures episode. Higher GFAP in D group was accomplished by higher glutamate transporters content independently of seizures.

Concerning the lower NeuN fluorescence intensity observed in the D groups, studies demonstrated that ω3PUFAs are very important to adequate neurogenesis and synaptogenesis (Lauritzen, Hansen, Jorgensen and Michaelsen 2001, Marszalek and Lodish 2005, Martin and Bazan 1992). In a previous study, ω3PUFAs deficiency delayed the normal glutamatergic synaptic development, being glutamate receptors decreased in the D group early after birthday (Moreira et al. 2010a). These data could be related to the less NeuN fluorescence intensity observed here, suggesting that ω3PUFAs deficiency caused a delay in brain development.

GFAP fluorescence intensity increased in ω3 kainate group to levels compared to D groups 24 after seizures episode, suggesting that ω3 group presented astrogliosis. NeuN and DAPI fluorescence intensity was not affected by seizures suggesting that there was no neural cell loss caused by kainate-induced seizures. Studies showed that epilepsy is characterized by hippocampal sclerosis, which one striking hallmark is astrocytic “reactive gliosis” (Binder and Steinhauser 2006, Seifert, et al. 2010).

Here, we observed that D groups demonstrated anxiety-like behavior and memory deficits independently of seizure. Additionally, seizure caused short-term memory deficits (1.5 h after training session in inhibitory avoidance task) in D group, but this was not observed in ω3 group. Behavioral alterations in ω3PUFAs deficient animals were observed in many studies (Moreira et al., 2010a; Lim, et al. 2005, Moriguchi, et al. 2000). Dietary ω3PUFAs deficiency induced anxiety-like behavior as well as memory deficits in rats, and these alterations could be related to alterations in normal brain development concerning glutamatergic system (Moreira et al. 2010a). Therefore our present data suggest that ω3PUFAs prevented behavioral alterations in adulthood caused by neonatal seizures episode, probably by preventing glutamatergic system alterations in the early life.

Taken together, our findings pointed to the importance of ω3PUFAs for the homeostasis of glutamatergic system in the immature and mature brain, preventing excitotoxicity caused by neonatal seizure and its consequences. These results also reinforced the relevance of ω3PUFAs for adequate brain development. Nevertheless, more studies have to be performed to elucidate the mechanisms underling the role of ω3PUFAs in brain's heath.

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References

Beart PM, O'Shea RD. (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br J Pharmacol* 150:5-17.

Berman DR, Liu YQ, Barks J, Mozurkewich E. (2010) Docosahexaenoic acid confers neuroprotection in a rat model of perinatal hypoxia-ischemia potentiated by Escherichia coli lipopolysaccharide-induced systemic inflammation. *Am J Obstet Gynecol* 202:469 e461-466.

Binder DK, Steinhauser C. (2006) Functional changes in astroglial cells in epilepsy. *Glia* 54:358-368.

Bjornsen LP, Eid T, Holmseth S, Danbolt NC, Spencer DD, de Lanerolle NC. (2007) Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures. *Neurobiol Dis* 25:319-330.

Bourre JM. (2004) Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. *J Nutr Health Aging* 8:163-174.

Bromfield E, Dworetzky B, Hurwitz S, Eluri Z, Lane L, Replansky S, Mostofsky D. (2008) A randomized trial of polyunsaturated fatty acids for refractory epilepsy. *Epilepsy Behav* 12:187-190.

Bunch L, Erichsen MN, Jensen AA. (2009) Excitatory amino acid transporters as potential drug targets. *Expert Opin Ther Targets* 13:719-731.

Cansev M, Ulus IH, Wang L, Maher TJ, Wurtman RJ. (2008) Restorative effects of uridine plus docosahexaenoic acid in a rat model of Parkinson's disease. *Neurosci Res* 62:206-209.

Cornejo BJ, Mesches MH, Coultrap S, Browning MD, Benke TA. (2007) A single episode of neonatal seizures permanently alters glutamatergic synapses. *Ann Neurol* 61:411-426.

Cysneiros RM, Ferrari D, Arida RM, Terra VC, de Almeida AC, Cavalheiro EA, Scorza FA. (2010) Qualitative analysis of hippocampal plastic changes in rats with epilepsy supplemented with oral omega-3 fatty acids. *Epilepsy Behav* 17:33-38.

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

de Wilde MC, Farkas E, Gerrits M, Kiliaan AJ, Luiten PG. (2002) The effect of n-3 polyunsaturated fatty acid-rich diets on cognitive and cerebrovascular parameters in chronic cerebral hypoperfusion. *Brain Res* 947:166-173.

Dunlop J. (2006) Glutamate-based therapeutic approaches: targeting the glutamate transport system. *Curr Opin Pharmacol* 6:103-107.

Ferrari D, Cysneiros RM, Scorza CA, Arida RM, Cavalheiro EA, de Almeida AC, Scorza FA. (2008) Neuroprotective activity of omega-3 fatty acids against epilepsy-induced hippocampal damage: Quantification with immunohistochemical for calcium-binding proteins. *Epilepsy Behav* 13:36-42.

Gegelashvili G, Civenni G, Racagni G, Danbolt NC, Schousboe I, Schousboe A. (1996) Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. *Neuroreport* 8:261-265.

Gegelashvili G, Dehnes Y, Danbolt NC, Schousboe A. (2000) The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. *Neurochem Int* 37:163-170.

Holmes GL, Ben-Ari Y. (2001) The neurobiology and consequences of epilepsy in the developing brain. *Pediatr Res* 49:320-325.

Hurn PD, Vannucci SJ, Hagberg H. (2005) Adult or perinatal brain injury: does sex matter? *Stroke* 36:193-195.

Kalandadze A, Wu Y, Robinson MB. (2002) Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. *J Biol Chem* 277:45741-45750.

Kwan P, Brodie MJ. (2000) Early identification of refractory epilepsy. *N Engl J Med* 342:314-319.

Lauritzen L, Hansen HS, Jorgensen MH, Michaelsen KF. (2001) The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res* 40:1-94.

Lim SY, Doherty JD, McBride K, Miller-Ihli NJ, Carmona GN, Stark KD, Salem N, Jr. (2005) Lead exposure and (n-3) fatty acid deficiency during rat neonatal development affect subsequent spatial task performance and olfactory discrimination. *J Nutr* 135:1019-1026.

Lortet S, Samuel D, Had-Aissouni L, Masmejean F, Kerkerian-Le Goff L, Pisano P. (1999) Effects of PKA and PKC modulators on high affinity glutamate uptake in primary neuronal cell cultures from rat cerebral cortex. *Neuropharmacology* 38:395-402.

Lukiw WJ, Cui JG, Marcheselli VL, Bodker M, Botkjaer A, Gotlinger K, Serhan CN, Bazan NG. (2005) A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J Clin Invest* 115:2774-2783.

Maragakis NJ, Rothstein JD. (2004) Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis* 15:461-473.

Marszalek JR, Lodish HF. (2005) Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. *Annu Rev Cell Dev Biol* 21:633-657.

Martin RE, Bazan NG. (1992) Changing fatty acid content of growth cone lipids prior to synaptogenesis. *J Neurochem* 59:318-325.

Moreira JD, Knorr L, Ganzella M, Thomazi AP, de Souza CG, de Souza DG, Pitta CF, Mello e Souza T, Wofchuk S, Elisabetsky E, Vinade L, Perry ML, Souza DO. (2010a)Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: relevance for behavior alterations. *Neurochem Int* 56:753-759.

Moreira JD, Knorr L, Thomazi AP, Simao F, Battu C, Oses JP, Gottfried C, Wofchuk S, Salbego C, Souza DO, Perry ML, Vinade L.. (2010b) Dietary omega-3 fatty acids attenuate cellular damage after a hippocampal ischemic insult in adult rats. *J Nutr Biochem* 21:351-356.

Moriguchi T, Greiner RS, Salem N, Jr. (2000) Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J Neurochem* 75:2563-2573.

Ozawa S, Kamiya H, Tsuzuki K. (1998) Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* 54:581-618.

Schlanger S, Shinitzky M, Yam D. (2002) Diet enriched with omega-3 fatty acids alleviates convulsion symptoms in epilepsy patients. *Epilepsia* 43:103-104.

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

Sheldon AL, Robinson MB. (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. *Neurochem Int* 51:333-355.

Stillwell W, Shaikh SR, Zerouga M, Siddiqui R, Wassall SR. (2005) Docosahexaenoic acid affects cell signaling by altering lipid rafts. *Reprod Nutr Dev* 45:559-579.

Stillwell W, Wassall SR. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids* 126:1-27.

Tzingounis AV, Wadiche JI. (2007) Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat Rev Neurosci* 8:935-947.

Ueda Y, Doi T, Tokumaru J, Yokoyama H, Nakajima A, Mitsuyama Y, Ohya-Nishiguchi H, Kamada H, Willmore LJ. (2001) Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. *J Neurochem* 76:892-900.

Voskuyl RA, Vreugdenhil M, Kang JX, Leaf A. (1998) Anticonvulsant effect of polyunsaturated fatty acids in rats, using the cortical stimulation model. *Eur J Pharmacol* 341:145-152.

Walf AA, Frye CA. (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* 2:322-328.

Wurtman RJ. (2008) Synapse formation and cognitive brain development: effect of docosahexaenoic acid and other dietary constituents. *Metabolism* 57 Suppl 2:S6-10.

Xiao YF, Gomez AM, Morgan JP, Lederer WJ, Leaf A. (1997) Suppression of voltage-gated L-type Ca2+ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. *Proc Natl Acad Sci U S A* 94:4182-4187.

Xiao YF, Kang JX, Morgan JP, Leaf A. (1995) Blocking effects of polyunsaturated fatty acids on Na+ channels of neonatal rat ventricular myocytes. *Proc Natl Acad Sci U S A* 92:11000-11004.

Yehuda S, Carasso RL, Mostofsky DI. (1994) Essential fatty acid preparation (SR-3) raises the seizure threshold in rats. *Eur J Pharmacol* 254:193-198.

Young C, Gean PW, Chiou LC, Shen YZ. (2000) Docosahexaenoic acid inhibits synaptic transmission and epileptiform activity in the rat hippocampus. *Synapse* 37:90-94.

Yuen AW, 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 Behav* 7:253-258.

Zhang G, Raol YS, Hsu FC, Brooks-Kayal AR. (2004) Long-term alterations in glutamate receptor and transporter expression following early-life seizures are associated with increased seizure susceptibility. *J Neurochem* 88:91-101.

Legends to figures

Figure 1: Effects of a single episode of neonatal seizures on the hippocampal [^{3}H] glutamate uptake. Hippocampal slices from ω3 adequate (ω3) and deficient (D) groups submitted to kainate-induced seizures were obtained at 12, 24, 48 h and 60 days post-seizures. Data are expressed as mean ± SD from 8 animals/group. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 groups; # P <0.05 in relation to the respective D saline group).

Figure 2: Effects of a single episode of neonatal seizures on the immunocontent of the glutamate transporters (GLT-1 and GLAST), analyzed in hippocampus of ω3 adequate (ω3) and deficient (D) groups submitted to kainate-induced seizures at 12, 24, 48 h pots-seizures. Data are expressed as mean ± SD from 5 animals/group. At the top of the figure are representative images of the immunocontent of transporters. β-Actin was used as a protein loading control. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 groups; # P <0.05 in relation to the respective D saline group).

Figure 3: Immunohistochemistry for GFAP, NeuN and DAPI in the CA1 hippocampal subfield of ω3 adequate (ω3) and deficient (D) 24h after a single episode of neonatal seizures. Fluorescence quantification was achieved using pictures acquired in 20x of magnification. Data are expressed as mean ± SD from

4 animals per group. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 saline group).

Figure 4: Immunohistochemistry analysis for GFAP, NeuN and DAPI in the CA3 hippocampal subfield of ω3 adequate (ω3) and deficient (D) 24h after a single episode of neonatal seizures. Fluorescence quantification was achieved using pictures acquired in 20x of magnification. Data are expressed as mean ± SD from 4 animals per group. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 saline group).

Figure 5: Immunohistochemistry analysis for GFAP, NeuN and DAPI in the dentate gyrus (DG) hippocampal subfield of ω3 adequate (ω3) and deficient (D) 24h after a single episode of neonatal seizures. Fluorescence quantification was achieved using pictures acquired in 20x of magnification. Data are expressed as mean ± SD from 4 animals per group. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 saline group).

Figure 6: Elevated plus-maze behavioral performance in adult rats of ω3 adequate (ω3) and deficient (D) submitted to a single episode of neonatal seizure. (**A)** Time spent in seconds (s) in the open arms; (**B)** Number of entries in the open arms. Data are expressed as means ± SD from 10 animals per group. Two-way ANOVA followed by Bonferroni`s post-hoc test was used (*P <0.05, in relation to ω3 saline group).

Figure 7: Performance in the inhibitory avoidance task in adult rats of ω3 adequate (ω3) and deficient (D) submitted to a single episode of neonatal seizure. Data are expressed as median ± interquartile range (n=10 per group) of the latencies to step down in seconds (s) in the training (dashed line) and test sessions performed 1.5 h (short-term memory), as well as 24 h and 7 days (long-term memory) later. Non-parametric Wilcoxon test was used [*P<0.05; significant difference from training (dashed line) and test session within group].

Figures

Figure 1

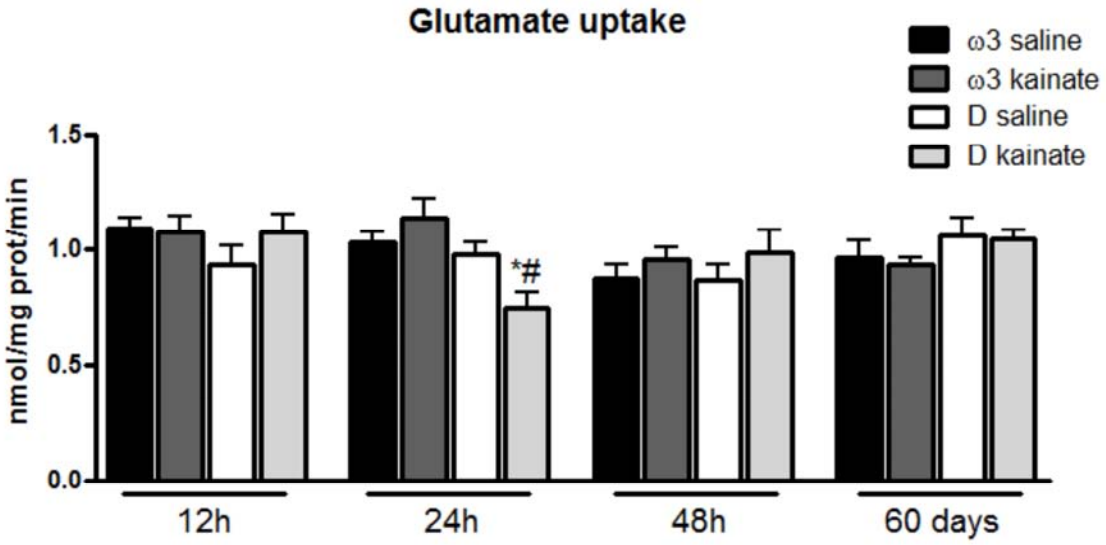
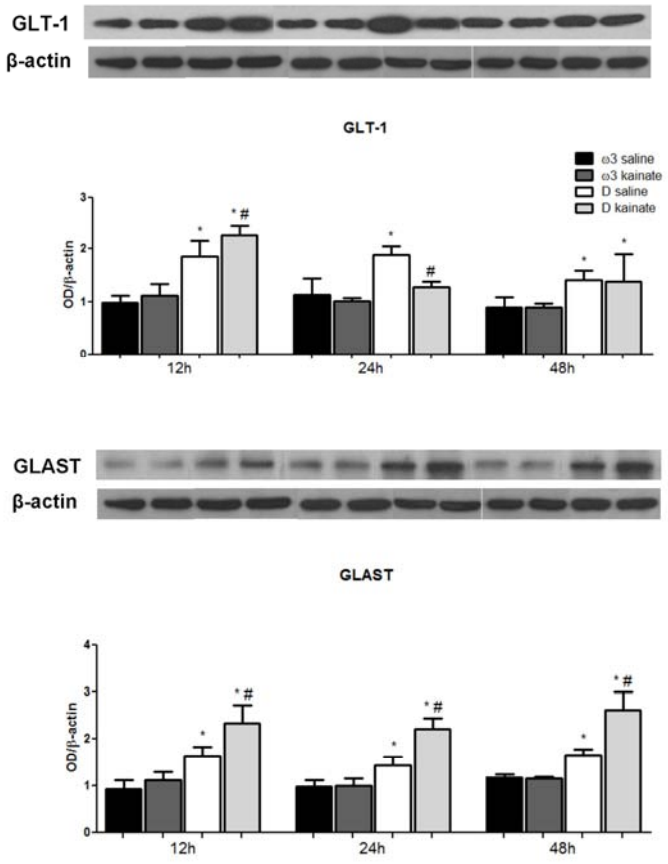


Figure 2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β-actin
GLT-1
ω3 saline
ω3 kainate
D saline
D kainate
OD/β-actin
12h
24h
48h
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GLAST
OD/β-actin
12h
24h
48h

Figure 3

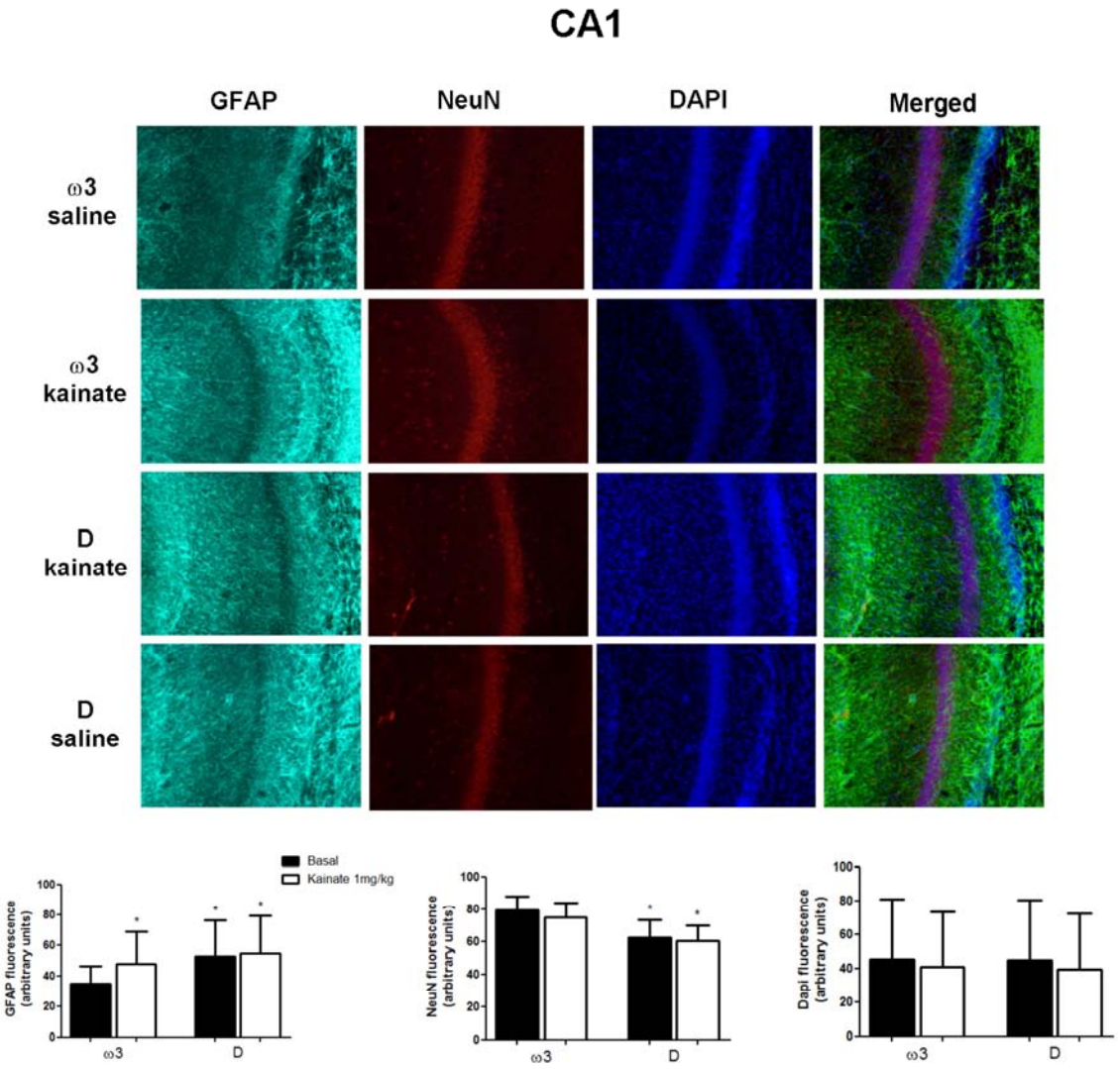

CA1
GFAP
NeuN
DAPI
Merged
ω3 saline
ω3 kainate
D kainate
D saline
Basal
Kainate 1mg/kg
GFAP fluorescence (arbitrary units)
NeuN fluorescence (arbitrary units)
Dapi fluorescence (arbitrary units)
ω3
D

Figure 4

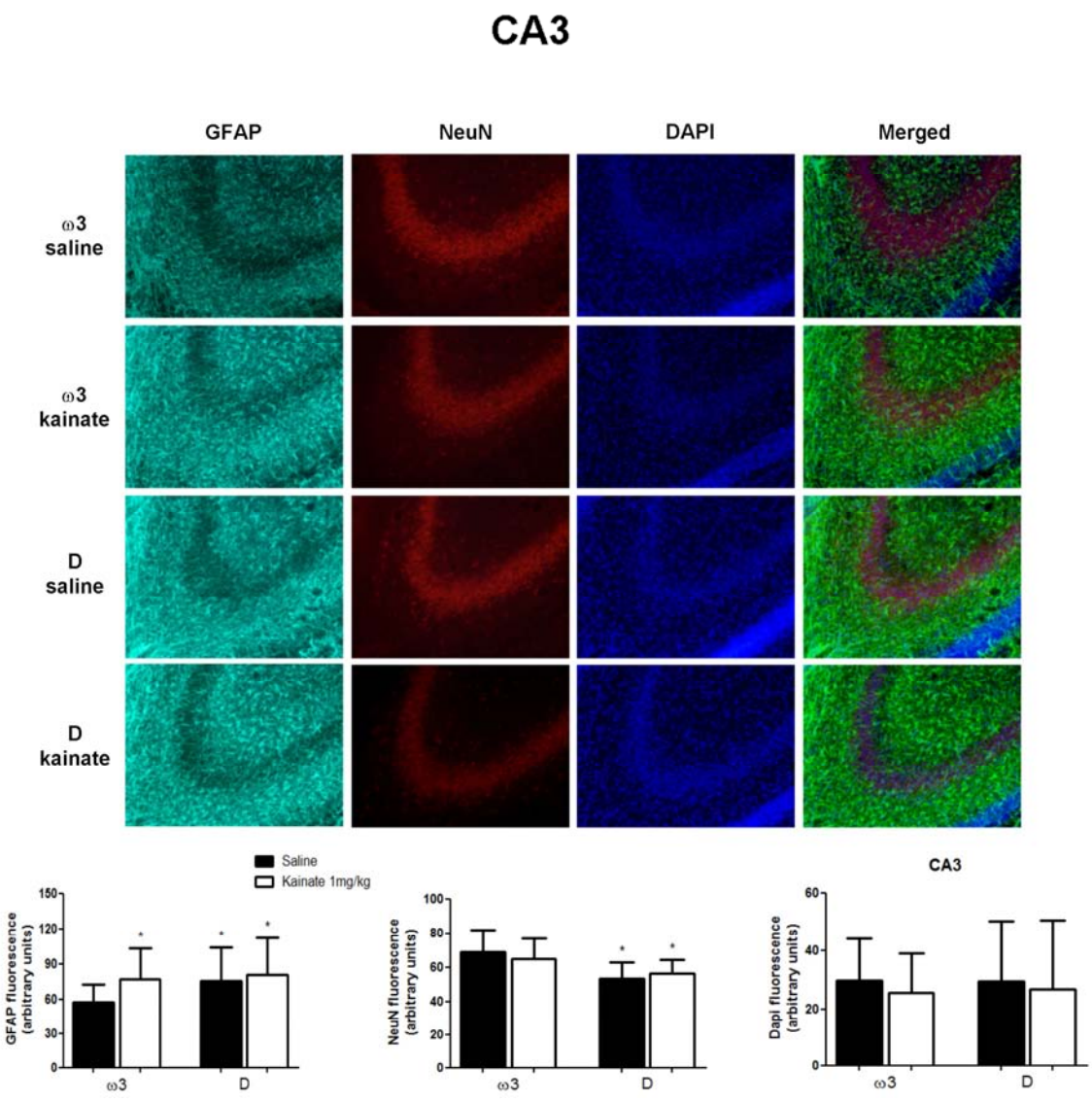

CA3
GFAP
NeuN
DAPI
Merged
ω3 saline
ω3 kainate
D saline
D kainate
Saline
Kainate 1mg/kg
GFAP fluorescence (arbitrary units)
NeuN fluorescence (arbitrary units)
CA3
Dapi fluorescence (arbitrary units)
ω3
D

Figure 5

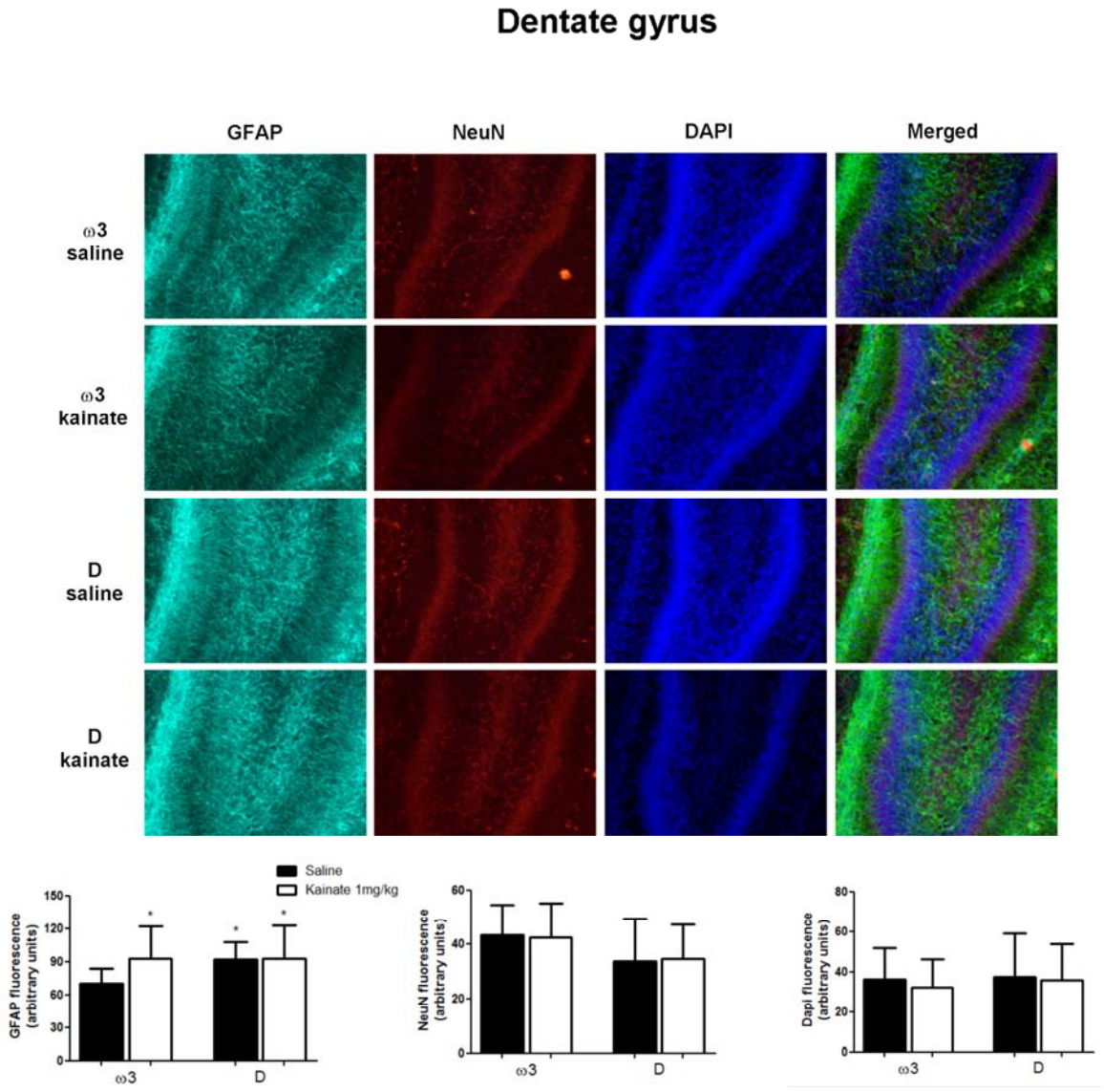

Dentate gyrus
GFAP
NeuN
DAPI
Merged
ω3 saline
ω3 kainate
D saline
D kainate
Saline
Kainate 1mg/kg
GFAP fluorescence (arbitrary units)
NeuN fluorescence (arbitrary units)
Dapi fluorescence (arbitrary units)
ω3
D

Figure 6

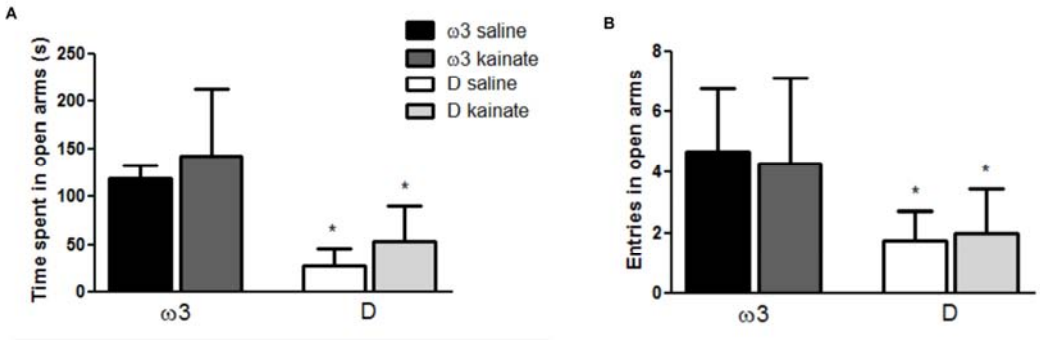
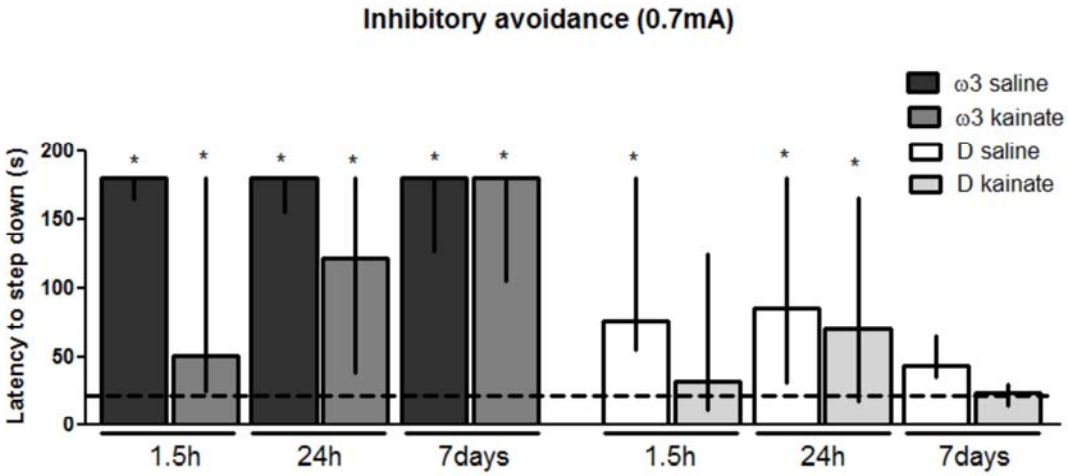


Figure 7



Capítulo 5

The deficiency of dietary omega-3 fatty acids affects glutamatergic transport system in rat retina: relevance for protection against high intraocular pressure.

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Letter submetida ao periódico Experimental Eye Research

The deficiency of dietary omega-3 fatty acids affects glutamatergic transport system in rat retina: relevance for protection against high intraocular pressure

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Abstract

The excitotoxicity of glutamatergic system is an important mechanism by which glaucoma causes retinal cells damage. Thus, the modulation of glutamatergic parameters is a putative therapeutic target to prevent excitotoxic retinal injury. Here we investigated the effect of dietary omega-3 fatty acids (ω3) in retinal glutamate transport system in basal and ischemic conditions. Female Wistar rats were divided in two groups: ω3 diet (ω3 group) and ω3 deficient-diet (D group). Their pups, 60 days old, were used for the experiments. Retinal ischemia, a mechanism involved in physiopathology of glaucoma, was caused by high intraocular pressure (140-180mmHg for 45 min) to impair retinal blood flow. Analyses were performed 7 days after ischemia. D group showed a decreased glutamate uptake in basal conditions and after ischemia, compared to ω3 group. After ischemia, there was a decrease in glutamate uptake in D group, which was not observed in ω3 group ($p < 0.005$). Concerning glutamate transporters, ω3 group presented higher levels of GLT-1 compared to D group in basal and ischemic conditions. After ischemia, EAAC1 was increased in both groups, while GLT-1 increased only in D group, compared to basal levels. GLAST and EAAT5 presented no alterations. The modulation of glutamatergic system by dietary omega-3 fatty acids points to a putative mechanism by which ω3 PUFAs exert their neuroprotection against excitotoxic insult in the retina.

Key words: Omega-3 fatty acids; Retina; Ischemia; Glaucoma; Glutamate transporters.

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), participating in the retinal development and physiology, and consequently in visual processes (Tzingounis and Wadiche 2007; Bringmann et al. 2009). However, elevated levels of extracellular glutamate (excitotoxicity) have been implicated in the pathophysiology of neuronal loss in ophthalmic disorders, such as glaucoma, ischemia, diabetes and inherited photoreceptors degeneration (Ambati et al. 1997; Brooks et al. 1997; Martin et al. 2002; Bringmann et al. 2006; Bringmann et al. 2009). Glutamate transporters are responsible for maintaining the extracellular glutamate concentrations below toxic levels and they play a key role regulating the balance between physiological / pathological signaling through glutamatergic receptors (Holcombe et al. 2008; Bringmann et al. 2009). In the retina, 4 distinct glutamate transporters have been identified: GLAST, associated with the glial Müller cells (Rauen et al. 1998; Pow and Barnett 1999); GLT-1, associated with cone photoreceptors and cone bipolar cells (Rauen and Kanner 1994); EAAC1, present in horizontal cells, some amacrine cells and ganglion cells (Rauen et al. 1996; Wiessner et al. 2002); and EAAT5, associated with photoreceptors and bipolar cells (Pow and Barnett 2000; Barnett and Grozdanic 2004).

Under normal conditions, retinal glutamate transport is mainly exerted by GLAST-mediated uptake into Müller cells (Rauen et al. 1998; Pow and Barnett 2000; Bringmann et al. 2009). However, under pathological conditions, Müller cells uptake of glutamate is reduced (Napper and Kalloniatis 1999; Barnett et al. 2001;

Li and Puro 2002), which can affect cellular integrity (Lam et al. 2003; Osborne et al. 2004). Elevation of intraocular pressure in experimental glaucoma impairs the GLAST activity, resulting in accumulation of glutamate in retinal cells, such as ganglion cells; this reduction in glutamate uptake by Müller cells was related to the excitotoxic damage of the retina (Lam et al. 2003; Barnett and Grozdanic 2004; Holcombe et al. 2008).

Omega-3 polyunsaturated fatty acids (ω3 fatty acids), largely docosahexaenoic acid (DHA 22:6 ω3), are essential polyunsaturated fatty acids (PUFA) in the retina (Neuringer and Connor 1986; Bazan 2006; Bazan 2009). DHA is found composing 43% of the phospholipids in the retinal photoreceptor. This PUFA plays important roles providing a high fluidity of membranes, an adequate environment for conformational rhodopsin changes after photoactivation, and modifying the activity of retinal enzymes (Stubbs and Smith 1984; Stillwell and Wassall 2003). Light exposure can elicit the release of DHA from retinal membrane phospholipids (Reinboth et al. 1996) and DHA deficiency can reduce G proteins-coupled signaling efficiency (Niu et al. 2004). In mammals, dietary deficiency of ω3 fatty acids results in alterations in the retinal PUFA pattern, with a significant decrease in DHA (approximately 40% of normal levels) (Neuringer et al. 1986; Weisinger et al. 1998), which can affect visual function (Anderson et al. 2005; Jensen et al. 2005).

DHA is essential for development and differentiation of photoreceptors and for protection against damage to retinal cells (Moriguchi et al. 2003; Garelli et al. 2006; Bazan 2009). It has been shown that *rd* mice, a model of retinitis pigmentosa

(RP, progressive retinal degeneration characterized by loss of photoreceptor cells via apoptotic mechanisms) present lower levels of DHA in the retina (Scott et al. 1987). Decreased DHA levels also was found in the plasma of patients with Usher`s syndrome, which comprises inherited deafness and early blindness as a result of RP (Bazan 1989a; Bazan 1989b). Recently, it was demonstrated that DHA can prevent retinal cell damage caused by ischemia and its complications (Miyauchi et al. 2001; Murayama et al. 2002).

Despite all investigations, little is known about the influence of ω3 fatty acids status on the retina, concerning the glutamatergic system in basal conditions and in retinal injury models. Thus, in the present study, we investigated whether dietary ω3 fatty acids deficiency affects the glutamate transporter system of retina, in basal and ischemic conditions.

Methods

Animals and diets

Wistar female rats were housed in standard conditions (21-22ºC, 12 h dark-light cycle, food and water *ad libitum*). The dietary model used in this study has been previously described (Moreira et al. 2010). Briefly, 2 weeks before mate, the female rats were divided into two groups: ω3 diet (ω3) and ω3 deficient-diet (D). Both diets were isocaloric, containing 8% total fat and differed only in fatty acids composition. After weaning, the pups were maintained with the same diet of their dams until experiments in the adult age (60 days old). For the experiments only

male rats were used. All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Retinal ischemia model

To produce an ischemic insult by high intraocular pressure (IOP) in retina, animals were anesthetized with ketamine (100mg/kg) and xylazine (12mg/kg). The anterior chamber of one eye was cannulated using a 30-gauge needle connected to a reservoir of sterile balanced salt solution and a perfusion bomb. The pressure was monitored to control the blood flow obstruction (140-180 mmHg) (Dreixler et al.). After 45 min of ischemia, animals returned to standard conditions (12h light/dark cycle, food and water *ad libitum*) for 7 days, to perform the experiments. The other eye was used as control.

Retinal [^{3}H]glutamate uptake

Seven days after ischemia, animals (n=8 per group) were decapitated and their eyes 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. Retinas were dissected and pre-incubated at 37 °C for 15 min, followed by the addition of 100 μM [^{3}H]glutamate (total uptake). Incubation was stopped after 7 min with 2 ice-cold washes with l mL of HBSS, immediately followed by the addition of 1N NaOH, which were then kept overnight. The 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). Na^+-dependent uptake was considered as the difference

between the total uptake and the Na^+-independent uptake. Incorporated radioactivity was measured using a liquid scintillation counter.

Western blot analysis

Retinas were dissected out 7 days after the ischemic episode and immediately homogenized in a 25 mM Hepes solution (pH 7.4) with 0.1 % SDS and protease inhibitor cocktail (Sigma). Samples (20 µg protein/well) were separated in an 8 % SDS-PAGE mini-gel and transferred to nitrocellulose membrane using a Trans-Blot system (Bio-Rad, São Paulo/SP, Brazil). Membranes were processed as follow: (1) blocking with 5% bovine serum albumin (Sigma, São Paulo/SP, Brasil) for 2 h; (2) incubation with primary antibody overnight: 1:1000 rabbit anti-GLAST, anti-GLT-1, anti-EAAC1 or anti-EAAT5 glutamate transporters (AlphaDiagnostic International) or 1:2000 mouse anti-β-Actin (Sigma); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 and mouse 1:3000 (Amersham Pharmacia Biotech) for 2 h; (4) chemiluminescence (ECL, Amersham Pharmacia Biotech, São Paulo/SP, Brasil) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and bands intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In order to determine the adequate amount of protein to be assayed, various protein concentrations were carried out in the same gel for each antibody tested.

Statistical analysis

Tow-way ANOVA followed by Bonferroni post-hoc was used. For statistical significance, the value of $P < 0.05$ was adopted. The statistical analysis was performed using SPSS 15.0 software.

Results

Figure 1 shows the glutamate uptake by whole retina. The ω3 fatty acids deprivation decreased the glutamate uptake measured in basal conditions ($p < 0.05$). Noteworthy, retinal ischemia decreased the glutamate uptake in D group ($p < 0.05$), without affecting the glutamate uptake in the ω3 group.

As ω3 fatty acids deprivation and retinal ischemia decreased the glutamate uptake by whole retina, we also evaluated the glutamate transporters (GLT-1, GLAST, EAAC1, EAAT5) levels (Figure 2). Concerning the ω3 fatty acids deprivation, in basal conditions the glutamate transporters levels were the same in both groups, with exception of GLT-1 levels, which decreased in D group compared to ω3 group. Concerning the retinal ischemia, the GLT-1 levels increased only in D group ($p < 0.05$), persisting reduced compared to ischemic ω3 group ($p < 0.05$); the EEAC1 levels increased in both ischemic groups ($p < 0.05$). GLAST and EAAT5 levels were not affected by ω3 fatty acids deprivation or ischemia.

Discussion

Glutamate excitotoxicity causes damage to retinal cells during neural injuries, as observed in ischemia (Barnett and Pow 2000; Holcombe et al. 2008;

Bringmann et al. 2009), pointing that adequate glutamate metabolism is important for visual function (Pow and Robinson 1994; Barnett and Pow 2000; Bringmann et al. 2009). High intraocular pressure can block retinal blood flow causing ischemia, and this mechanism seems to be involved in the physiopathology of some retinal diseases, such glaucoma (Holcombe et al. 2008; Bringmann et al. 2009).

Here, the experiments were performed 7 days after the ischemic insult. We observed that ω3 fatty acids deprivation and the ischemic insult decreased the glutamate uptake by whole retina. It was accompanied by and increase in the levels of GLT1 (only in D group) and EEAC1 (in both groups).

In the retina, the role of glutamatergic transporters is still not completely understood. It is known that GLAST is the main glutamate transporter responsible to maintain extracellular glutamate below toxic levels, preventing excitotoxic damage to neuronal cells; however GLAST activity is impaired during ischemia (Rauen et al. 1996; Rauen et al. 1998; Barnett et al. 2001; Bringmann et al. 2006; Bringmann et al. 2009). In the present work, the glutamate uptake activity was affected by ω3 deficiency. The D group presented reduced glutamate uptake in basal condition, when compared to ω3 group. After ischemia, it was observed that ω3 fatty acids prevented the decrease in glutamate uptake, which was observed only in D group. However, the reduction of glutamate uptake in D group was not related to a reduction in GLAST levels, once its levels were the same in both groups. Barnett and colleges also observed no changes in GLAST levels after an ischemic insult, despite the reduction in the glutamate uptake capacity of Müller cells (Barnett et al. 2001). As glutamate accumulation is excitotoxic to neurons, the

maintenance of the glutamate removal is essential to prevent cell death in retina. Our results point to the importance of ω3 fatty acids to adequate glutamate uptake activity, putatively involved in the resistance to an ischemic insult.

Little is known about the functional significance of GLT-1 expression in the retina, especially under pathological conditions. It was proposed that GLT-1 could be responsible for maintaining glutamate homeostasis in the retina during ischemia, once GLT-1 levels increased during all ischemic period, while GLAST levels presented no variation (Park et al. 2009). Our results reveal that GLT-1 levels increased in D group 7 days after ischemia, despite these levels remained smaller than in ω3 group. We could postulate that the higher GLT-1 levels in ω3 group could be involved in a prevention of a retinal damage caused by a decrease in glutamate uptake.

In relation to EAAC1, it was demonstrated that it is localized synaptically and extrasynaptically (Wiessner et al. 2002), but its contribution to glutamate uptake is not clear. EAAC1 deficient mice presented spontaneous ganglion cells and optic nerve degeneration (Harada et al. 2007; Namekata et al. 2009), and their ganglion cells were more vulnerable to oxidative stress (Harada et al. 2007). Here, we observed that EAAC1 levels increase in both group after ischemia. We postulate that this also could be a compensatory mechanism against ischemic damage.

EAAT5 is primarily expressed in the retina (Arriza et al. 1997; Pow and Barnett 2000). It has been postulated that EAAT5 presents a high Cl^- conductance and is located close to the glutamate release sites, acting as an inhibitory presynaptic receptor and controlling glutamate release (Wersinger et al. 2006).

Few studies evaluated EAAT5 response to ischemic conditions. It was reported that the localization of this transporter was not affected until 60 days after ischemia (Barnett and Grozdanic 2004). In the present work, we observed that EAAT5 levels were not affected by diets and/or ischemia.

Our data, in accordance with literature data, indicate that dietary ω3 fatty acids are relevant to adequate functionality of the glutamate transport system in basal conditions and in retinal ischemia. They point to a possible relevant therapeutic target in pathologies related to excitotoxicity of glutamatergic system in retina, such as glaucoma.

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References

Ambati, J., Chalam, K.V., Chawla, D.K., D'Angio, C.T., Guillet, E.G., Rose, S.J., Vanderlinde, R.E., Ambati, B.K., 1997. Elevated gamma-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol 115, 1161-1166.

Anderson, G.J., Neuringer, M., Lin, D.S., Connor, W.E., 2005. Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and

brain biochemistry and visual function in rhesus monkeys. Pediatr Res 58, 865-872.

Arriza, J.L., Eliasof, S., Kavanaugh, M.P., Amara, S.G., 1997. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci U S A 94, 4155-4160.

Barnett, N.L., Grozdanic, S.D., 2004. Glutamate transporter localization does not correspond to the temporary functional recovery and late degeneration after acute ocular ischemia in rats. Exp Eye Res 79, 513-524.

Barnett, N.L., Pow, D.V., 2000. Antisense knockdown of GLAST, a glial glutamate transporter, compromises retinal function. Invest Ophthalmol Vis Sci 41, 585-591.

Barnett, N.L., Pow, D.V., Bull, N.D., 2001. Differential perturbation of neuronal and glial glutamate transport systems in retinal ischaemia. Neurochem Int 39, 291-299.

Bazan, N.G., 1989a. The identification of a new biochemical alteration early in the differentiation of visual cells in inherited retinal degeneration. Prog Clin Biol Res 314, 191-215.

Bazan, N.G., 1989b. The metabolism of omega-3 polyunsaturated fatty acids in the eye: the possible role of docosahexaenoic acid and docosanoids in retinal physiology and ocular pathology. Prog Clin Biol Res 312, 95-112.

Bazan, N.G., 2006. Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. Trends Neurosci 29, 263-271.

Bazan, N.G., 2009. Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease. J Lipid Res 50 Suppl, S400-405.

Bringmann, A., Pannicke, T., Biedermann, B., Francke, M., Iandiev, I., Grosche, J., Wiedemann, P., Albrecht, J., Reichenbach, A., 2009. Role of retinal glial cells in neurotransmitter uptake and metabolism. Neurochem Int 54, 143-160.

Bringmann, A., Pannicke, T., Grosche, J., Francke, M., Wiedemann, P., Skatchkov, S.N., Osborne, N.N., Reichenbach, A., 2006. Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25, 397-424.

Brooks, D.E., Garcia, G.A., Dreyer, E.B., Zurakowski, D., Franco-Bourland, R.E., 1997. Vitreous body glutamate concentration in dogs with glaucoma. Am J Vet Res 58, 864-867.

Dreixler, J.C., Shaikh, A.R., Alexander, M., Savoie, B., Roth, S., 2010. Post-ischemic conditioning in the rat retina is dependent upon ischemia duration and is not additive with ischemic pre-conditioning. Exp Eye Res 91, 844-852.

Garelli, A., Rotstein, N.P., Politi, L.E., 2006. Docosahexaenoic acid promotes photoreceptor differentiation without altering Crx expression. Invest Ophthalmol Vis Sci 47, 3017-3027.

Harada, T., Harada, C., Nakamura, K., Quah, H.M., Okumura, A., Namekata, K., Saeki, T., Aihara, M., Yoshida, H., Mitani, A., Tanaka, K., 2007. The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. J Clin Invest 117, 1763-1770.

Holcombe, D.J., Lengefeld, N., Gole, G.A., Barnett, N.L., 2008. The effects of acute intraocular pressure elevation on rat retinal glutamate transport. Acta Ophthalmol 86, 408-414.

Jensen, C.L., Voigt, R.G., Prager, T.C., Zou, Y.L., Fraley, J.K., Rozelle, J.C., Turcich, M.R., Llorente, A.M., Anderson, R.E., Heird, W.C., 2005. Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants. Am J Clin Nutr 82, 125-132.

Lam, T.T., Kwong, J.M., Tso, M.O., 2003. Early glial responses after acute elevated intraocular pressure in rats. Invest Ophthalmol Vis Sci 44, 638-645.

Li, Q., Puro, D.G., 2002. Diabetes-induced dysfunction of the glutamate transporter in retinal Muller cells. Invest Ophthalmol Vis Sci 43, 3109-3116.

Martin, K.R., Levkovitch-Verbin, H., Valenta, D., Baumrind, L., Pease, M.E., Quigley, H.A., 2002. Retinal glutamate transporter changes in experimental glaucoma and after optic nerve transection in the rat. Invest Ophthalmol Vis Sci 43, 2236-2243.

Miyauchi, O., Mizota, A., Adachi-Usami, E., Nishikawa, M., 2001. Protective effect of docosahexaenoic acid against retinal ischemic injury: an electroretinographic study. Ophthalmic Res 33, 191-195.

Moreira, J.D., Knorr, L., Ganzella, M., Thomazi, A.P., de Souza, C.G., de Souza, D.G., Pitta, C.F., Mello e Souza, T., Wofchuk, S., Elisabetsky, E., Vinade, L., Perry, M.L., Souza, D.O., 2010. Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: relevance for behavior alterations. Neurochem Int 56, 753-759.

Moriguchi, K., Yuri, T., Yoshizawa, K., Kiuchi, K., Takada, H., Inoue, Y., Hada, T., Matsumura, M., Tsubura, A., 2003. Dietary docosahexaenoic acid protects against N-methyl-N-nitrosourea-induced retinal degeneration in rats. Exp Eye Res 77, 167-173.

Murayama, K., Yoneya, S., Miyauchi, O., Adachi-Usami, E., Nishikawa, M., 2002. Fish oil (polyunsaturated fatty acid) prevents ischemic-induced injury in the mammalian retina. Exp Eye Res 74, 671-676.

Namekata, K., Harada, C., Guo, X., Kikushima, K., Kimura, A., Fuse, N., Mitamura, Y., Kohyama, K., Matsumoto, Y., Tanaka, K., Harada, T., 2009. Interleukin-1 attenuates normal tension glaucoma-like retinal degeneration in EAAC1-deficient mice. Neurosci Lett 465, 160-164.

Napper, G.A., Kalloniatis, M., 1999. Neurochemical changes following postmortem ischemia in the rat retina. Vis Neurosci 16, 1169-1180.

Neuringer, M., Connor, W.E., 1986. n-3 fatty acids in the brain and retina: evidence for their essentiality. Nutr Rev 44, 285-294.

Neuringer, M., Connor, W.E., Lin, D.S., 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. Proc Natl Acad Sci U S A 83, 4021-4025.

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

Osborne, N.N., Casson, R.J., Wood, J.P., Chidlow, G., Graham, M., Melena, J., 2004. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog Retin Eye Res 23, 91-147.

Park, C.K., Cha, J., Park, S.C., Lee, P.Y., Kim, J.H., Kim, H.S., Kim, S.A., Kim, I.B., Chun, M.H., 2009. Differential expression of two glutamate transporters, GLAST and GLT-1, in an experimental rat model of glaucoma. Exp Brain Res 197, 101-109.

Pow, D.V., Barnett, N.L., 1999. Changing patterns of spatial buffering of glutamate in developing rat retinae are mediated by the Muller cell glutamate transporter GLAST. Cell Tissue Res 297, 57-66.

Pow, D.V., Barnett, N.L., 2000. Developmental expression of excitatory amino acid transporter 5: a photoreceptor and bipolar cell glutamate transporter in rat retina. Neurosci Lett 280, 21-24.

Pow, D.V., Robinson, S.R., 1994. Glutamate in some retinal neurons is derived solely from glia. Neuroscience 60, 355-366.

Rauen, T., Kanner, B.I., 1994. Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. Neurosci Lett 169, 137-140.

Rauen, T., Rothstein, J.D., Wassle, H., 1996. Differential expression of three glutamate transporter subtypes in the rat retina. Cell Tissue Res 286, 325-336.

Rauen, T., Taylor, W.R., Kuhlbrodt, K., Wiessner, M., 1998. High-affinity glutamate transporters in the rat retina: a major role of the glial glutamate transporter GLAST-1 in transmitter clearance. Cell Tissue Res 291, 19-31.

Reinboth, J.J., Clausen, M., Reme, C.E., 1996. Light elicits the release of docosahexaenoic acid from membrane phospholipids in the rat retina in vitro. Exp Eye Res 63, 277-284.

Schuettauf, F., Thaler, S., Bolz, S., Fries, J., Kalbacher, H., Mankowska, A., Zurakowski, D., Zrenner, E., Rejdak, R., 2007. Alterations of amino acids and glutamate transport in the DBA/2J mouse retina; possible clues to degeneration. Graefes Arch Clin Exp Ophthalmol 245, 1157-1168.

Scott, B.L., Reddy, T.S., Bazan, N.G., 1987. Docosahexaenoate metabolism and fatty-acid composition in developing retinas of normal and rd mutant mice. Exp Eye Res 44, 101-113.

Stillwell, W., Wassall, S.R., 2003. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 126, 1-27.

Stubbs, C.D., Smith, A.D., 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 779, 89-137.

Sullivan, R.K., Woldemussie, E., Macnab, L., Ruiz, G., Pow, D.V., 2006. Evoked expression of the glutamate transporter GLT-1c in retinal ganglion cells in human glaucoma and in a rat model. Invest Ophthalmol Vis Sci 47, 3853-3859.

Tzingounis, A.V., Wadiche, J.I., 2007. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. Nat Rev Neurosci 8, 935-947.

Weisinger, H.S., Vingrys, A.J., Abedin, L., Sinclair, A.J., 1998. Effect of diet on the rate of depletion of n-3 fatty acids in the retina of the guinea pig. J Lipid Res 39, 1274-1279.

Wersinger, E., Schwab, Y., Sahel, J.A., Rendon, A., Pow, D.V., Picaud, S., Roux, M.J., 2006. The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. J Physiol 577, 221-234.

Wiessner, M., Fletcher, E.L., Fischer, F., Rauen, T., 2002. Localization and possible function of the glutamate transporter, EAAC1, in the rat retina. Cell Tissue Res 310, 31-40.

Legend to figures

Figure 1: Effect of ω3 deficiency and ischemia on [^{3}H]glutamate uptake by retina. Experiments were performed 7 days after the ischemic insult. ω3 (ω3 group, n = 8), D (ω3 deficient group, n = 8). Data are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-hoc was used (* $p < 0.005$ in relation ω3 groups; & $p < 0.05$ in relation to basal D group).

Figure 2: Effect of ω3 deficiency and ischemia on glutamate transporters levels. Experiments were performed 7 days after the ischemic insult. ω3 (ω3 group, n = 6), D (ω3 deficient group, n = 5). Data are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-hoc was used (* $p < 0.05$ in relation to basal ω3 group; # $p < 0.005$ in relation to ischemic ω3 group; & $p < 0.05$ in relation to basal D group).

Figure 1

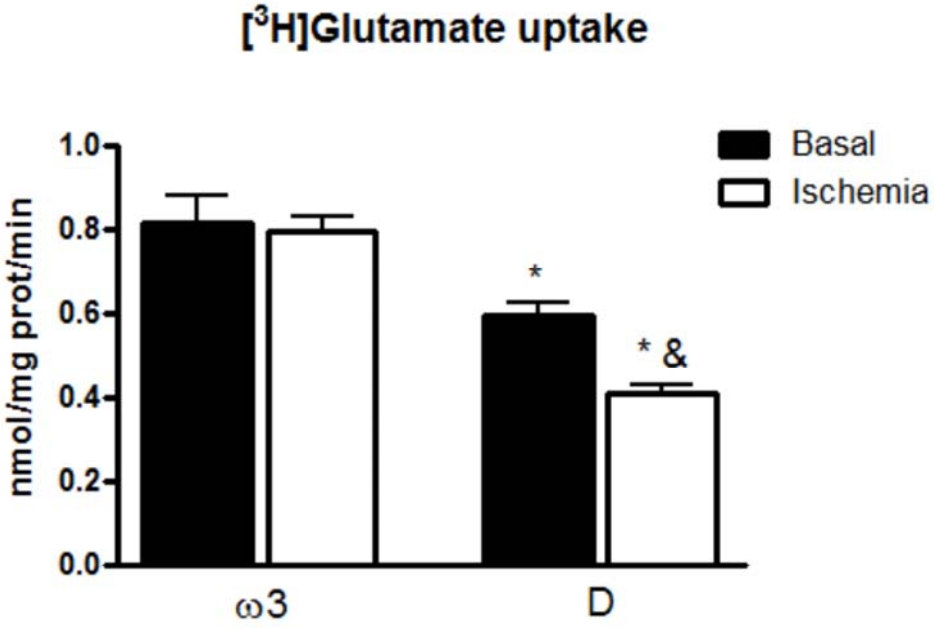
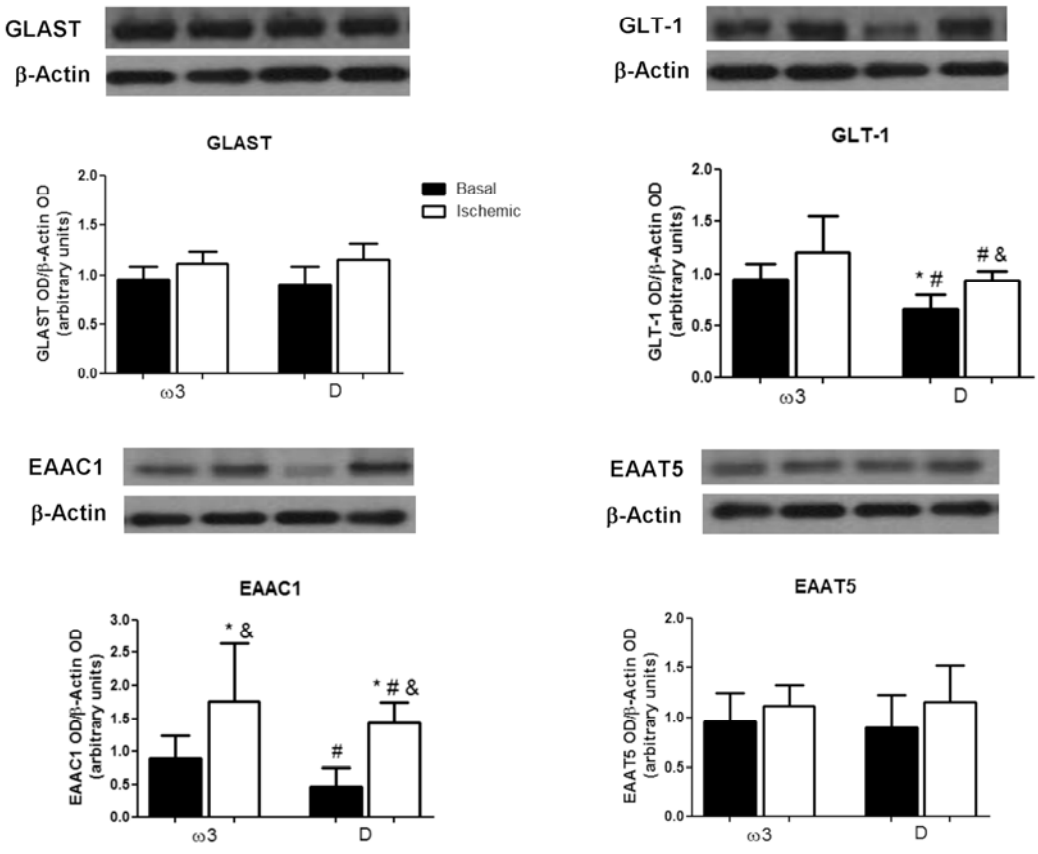

[3H]Glutamate uptake
Basal
Ischemia
nmol/mg prot/min
1.0
0.8
0.6
0.4
0.2
0.0
*
* &
ω3
D

Figure 2


GLAST
β-Actin
GLT-1
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GLAST OD/β-Actin OD (arbitrary units)
Basal
Ischemic
ω3
D
GLT-1
GLT-1 OD/β-Actin OD (arbitrary units)
* #
# &
ω3
D
EAAC1
β-Actin
EAAT5
β-Actin
EAAC1
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PARTE III

3. Discussão

A presente tese teve por objetivo investigar a influência dos ácidos graxos ω3 no funcionamento do SNC, por meio de um modelo de privação dietética de ácidos graxos ω3 desde a gestação até a vida adulta, em parâmetros bioquímicos, comportamentais e de neuroproteção relacionados ao sistema glutamatérgico em cérebro e retina de ratos.

Em linhas gerais, observamos que os ácidos graxos ω3 participam da formação das sinapses glutamatérgicas, influenciando o aparecimento das proteínas sinápticas e a capacidade do glutamato ligar a seus receptores. Estas alterações em períodos iniciais do desenvolvimento podem ter influenciado as alterações comportamentais observadas nos animais deficientes em ácidos graxos ω3 na vida adulta. A deficiência dietética em ácidos graxos ω3 causou déficits de memória, tanto de curta como de longa duração. A persistência da LTM foi abolida pela deficiência de ácidos graxos ω3, por reduzir a sinalização do receptor NMDA glutamatérgico, sua interação com a proteína Fyn, e reduzir o conteúdo de DHA e BDNF no hipocampo; estes dados apontam um papel fundamental dos ácidos graxos ω3 na plasticidade sináptica hipocampal relacionada com a formação da memória. Os ácidos graxos ω3 dietéticos foram capazes de prevenir as alterações no sistema glutamatérgico hipocampal provocadas por um evento convulsivo neonatal, evitando assim as alterações comportamentais na vida adulta. Além disso, ácidos graxos ω3 dietéticos também preveniram as alterações no sistema glutamatérgico na retina de ratos após um insulto ischêmico. Estes dados sugerem que a ingestão de ácidos graxos ω3 pode ter um papel relevante na

prevenção, bem como uma possível estratégia terapêutica, de patologias relacionadas à excitotoxicidade glutamatérgica.

O capítulo 1 teve por objetivo investigar a influência dos ácidos graxos ω3 sobre o desenvolvimento do sistema glutamatérgico e parâmetros comportamentais na vida adulta.

Utilizamos um modelo de deficiência dietética de ácidos graxos ω3, que foi padronizado para os demais trabalhos apresentados na presente tese. Ratas Wistar fêmeas eram separadas em 2 grupos: dieta adequada em ω3 (grupo ω3) e dieta deficiente em ω3 (grupo D). As dietas eram isocalóricas, e só diferiram na composição de ácidos graxos (a dieta ω3 conteve o ácido α-linolênico, EPA e DHA). Estas eram mantidas com as dietas durante todo o período de acasalamento, gestação e amamentação dos filhotes. Aos 21 dias de vida pós-natal das ninhadas, era feito o desmame, sendo que os filhotes mantiveram a mesma dieta materna até a vida adulta (60 dias). Para os experimentos sempre foram utilizados os filhotes machos.

Neste capítulo, mostramos que a deficiência de ω3 causou um atraso do aparecimento de proteínas sinápticas do sistema glutamatérgico (subunidade NR2A/B do receptor NMDA, subunidade GluR1 do receptor AMPA e na isoforma α da enzima CaMKII) no hipocampo de ratos. Os mesmos apresentaram redução da capacidade de união do glutamato aos seus receptores. Apesar de todos estes parâmetros terem sido normalizados na idade adulta, os animais deficientes apresentaram alterações de comportamento, como hiperatividade, ansiedade e déficits na memória aversiva de curta e longa duração.

Neste capítulo mostramos que a deficiência de ácidos graxos ω3 provoca alterações sinápticas no período inicial do desenvolvimento, que mesmo normalizadas, podem estar repercutindo em alterações comportamentais na vida adulta. Estes resultados são de grande relevância, uma vez que níveis reduzidos de ácidos graxos ω3 têm sido relacionados a doenças neurológicas como déficits de atenção e hiperatividade (Raz e Gabis, 2009; Lavialle et al., 2010) e a suplementação de ácidos graxos ω3 tem sido proposta como possível terapêutica para doenças como esquizofrenia e depressão (Peet & Stokes, 2005).

O capítulo 2 teve por objetivo investigar mais profundamente o déficit na persistência da memória de longa duração encontrado nos animais submetidos a deficiência dietética de ácidos graxos ω3.

Para tanto, os animais adultos tratados com ambas as dietas foram submetidos ao protocolo de esquiva inibitória, com um choque mais intenso (0.7mA) para promover a persistência da (LTM). Os animais deficientes em ω3 não apresentaram persistência da LTM. No período de 12h após o treino na esquiva inibitória, os animais deficientes em ω3 apresentaram uma redução da fosforilação da subunidade NR2B do receptor NMDA, da protína cinase Fyn, bem como da interação destas duas proteínas. Além disso, os conteúdos de DHA e BDNF foram reduzidos nos animais deficientes em ω3.

Neste capítulo reproduziu-se o déficit de memória mostrado no capítulo anterior, e mostramos que os ácidos graxos ω3 são importantes para a ativação de proteínas de sinalização sináptica, bem como sua interação (Fyn, NMDA e BDNF), contribuindo para a formação da LTM. A suplementação de ácidos graxos

ω3 em animais deficientes neste ácidos graxos foi capaz de recuperar os déficits de memória presentes neste animais (Moriguchi e Salem, 2003). Juntos, estes dados sugerem que os ácidos graxos ω3 são importantes para eventos de plasticidade sináptica relacionados com a formação da memória.

O capítulo 3 teve por objetivo investigar o efeito de um único episódio convulsivo neonatal sobre parâmetros relacionados à funcionalidade do sistema glutamatérgico no período pós-convulsão e suas conseqüências na vida adulta de ratos. Neste estudo utilizou-se ratos submetidos a uma dieta comercial padrão, para que pudéssemos avaliar, no capítulo 4, os efeitos do mesmo modelo de convulsão neonatal sobre os animais tratados com o modelo dietético aqui mencionadas.

Foi utilizado o modelo de convulsão por injeção subcutânea com cainato, um agonista glutamatérgico, na dose de 1mg/kg, em ratos de 7 dias de vida. Os animais apresentaram sinais de convulsão 30 minutos após a administração de cainato, que persistiram por 3h, quando os filhotes eram devolvidos a suas respectivas mães. As análises bioquímicas foram realizadas 12h, 24h, 48h, 72h e 60 dias após o evento convulsivo no hipocampo dos animais, e as análises comportamentais foram realizadas aos 60 dias de vida dos animais.

Os animais que foram submetidos ao episódio de convulsão neonatal apresentaram redução na capacidade de captar o glutamato, bem como alterações nos níveis dos transportadores GLAST e GLT-1 até 48h após a convulsão. Estes também apresentaram aumento de GFAP 24h após a convulsão, sugerindo uma astrogliose. Não foi detectada perda celular 24h após o episódio

convulsivo. Na vida adulta, quando os parâmetros foram normalizados, os animais apresentaram comportamento ansioso e déficits de memória aversiva.

Este capítulo contribuiu para o entendimento das conseqüências de episódios convulsivos na infância, eventos bem comuns entre as crianças no primeiro ano de vida (Holmes e Bem-Ari, 2001). Este entendimento possibilita que se possam desenvolver terapêuticas para tentar modular estes parâmetros, na tentativa de evitar suas consequências para o funcionamento cerebral na vida adulta.

No capítulo 4, investigamos como o modelo de convulsão neonatal afetaria os animais submetidos à dieta adequada ou deficiente em ácidos graxos ω3.

Os animais deficientes em ácidos graxos ω3 apresentaram inibição na captação de glutamato e alterações no conteúdo dos transpotadores GLAST e GLT-1, bem como alterações comportamentais (ansiedade e déficit de memória aversiva) na vida adulta. Os ácidos graxos ω3 preveniram estas alterações, mas apresentaram astrogliose 24h após a convulsão. Ainda, os animais deficientes apresentaram aumento da reatividade para GFAP (proteína astrocitária) e redução na reatividade para NeuN (proteína neuronal) independente do episódio convulsivo.

Neste capítulo, evidenciamos que ácidos graxos ω3 preveniram algumas alterações bioquímicas causadas pelo episódio convulsivo neonatal, bem como o déficit cognitivo na vida adulta. Mais uma vez evidenciamos a importância dos ácidos graxos ω3 para a cognição e formação da memória, e proteção da função cerebral na vida adulta após um insulto excitotóxico. Ainda, mostramos que os

animais deficientes apresentam menos marcação para NeuN, uma proteína neuronal, podendo indicar menor conteúdo de neurônios no hipocampo destes animais. Estes dados podem estar relacionados aos do capítulo 1, onde as proteínas sinápticas estão reduzidas no período pós-natal nos animais deficientes. Já é sabido da influência dos ácidos graxos ω3 sobre a sinaptogênese e neurogênese (Martin & Bazan, 1992, Lauritzen et al., 2001; Marszalek e Lodish, 2005) Os ácidos graxos ω3 já vêm sendo alvos de estudos para que sejam utilizados na terapêutica da epilepsia (Scorza et al., 2008; Taha et al., 2010) e nossos dados corroboram o benefício, além do efeito preventivo, desta abordagem.

No capítulo 5, investigamos a influência dos ácidos graxos ω3 na homeostasia do sistema glutamatérgico na retina de ratos e seu possível papel protetor frente a alterações sobre este sistema após um insulto isquêmico. Depois do tecido cerebral, a retina é o tecido onde se encontra a maior concentração de ácidos graxos ω3 por quantidade de lipídios totais, até 50% (Dratz and Deese, 1984).

Animais adultos que receberam ambas as dietas foram submetidos a um insulto isquêmico na retina, por aumento da pressão intraocular (IOP), um dos mecanismos relacionados à fisiopatologia do glaucoma. Neste modelo, os animais foram anestesiados, um dos olhos de cada animal foi canulado e infundido com solução salina estéril até que se atingisse uma pressão entre 140 e 180mmHg, sendo mantidos por 45 minutos. Após este período, os animais esperaram 7 dias,

quando então foram feitas as análises bioquímicas. Cada animal era, ao mesmo tempo, basal (controle) e isquêmico.

Observou-se que a retina dos animais deficientes captou menos glutamato em condições basais e, após a isquemia, houve uma inibição da captação de glutamato. Após a isquemia, o conteúdo de GLT-1 e EAAC1 aumentaram nos animais deficientes. Nos animais que receberam dieta adequada em ω3, o conteúdo de GLT-1 foi maior que o dos animais deficientes, tanto basal quanto isquêmico, podendo ser este o motivo da ausência de queda da captação de glutamato observada nestes animais após a isquemia na retina. Além disso, somente o conteúdo de EAAC1 foi afetado pela isquemia neste grupo.

Neste estudo mostramos que os ácidos graxos ω3 são importantes para a homeostasia glutamatérgica, tanto basal quando após um insulto isquêmico. Os ácidos graxos ω3 podem ser relevantes na terapêutica de doenças relacionadas ao sistema visual, prevenindo alterações fisiopatológicas que podem estar relacionadas com distúrbios da função visual em patologias como o glaucoma.

4. Conclusões

Na presente tese, ressaltamos a relevância dos ácidos graxos ω3 presentes na dieta para o adequado desenvolvimento e funcionamento cerebral e da retina.

Estes têm um papel importante na formação das sinapses glutamatérgicas, e possivelmente na quantidade de células neurais no hipocampo de ratos, além de influenciarem eventos de sinalização envolvidos com a memória aversiva e o comportamento na vida adulta.

Em eventos excitotóxicos, como a convulsão e a isquemia, os ácidos graxos ω3 preveniram as alterações causadas por estes, ressaltando o papel neuroprotetor dos ácidos graxos ω3 sobre a excitotoxicidade glutamatérgica tanto no hipocampo quanto na retina.

Na retina, os ácidos graxos ω3 influenciam a homeostasia do sistema glutamatérgico, e também a quantidade de transportadores de glutamato, importantes na prevenção da excitotoxicidade glutamatérgica causada pela isquemia.

5. Perspectivas

A) Avaliar o conteúdo de outras proteínas sinápticas durante o desenvolvimento e buscar possíveis alterações na vida adulta de ratos submetidos ao modelo de deficiência de ácidos graxos ω3;

B) Investigar a sinalização do BDNF e seu receptor TrkB, bem como a ativação de proteínas de sinalização intracelular relacionados a persistência da memória em ratos submetidos ao modelo de deficiência de ácidos graxos ω3;

C) Investigar mais o perfil lipídico (fosfolipídios, colesterol, gangliosídios, esfingolipídios, rafts lipídicos, etc) das membranas hipocampais em ratos submetidos ao modelo de deficiência de ácidos graxos ω3 em diferentes idades;

D) Buscar alterações refefentes ao sistema glutamatérgico em outros tempos após o episódio convulsivo neonatal no hipocampo e demais regiões cerebrais, bem como possíveis efeitos neuroprotetores dos ácidos graxos ω3 frente ao evento convulsivo;

E) Avaliar histologicamente a retina dos ratos submetidos ao modelo de deficiência de ácidos graxos ω3, antes e após o insulto isquêmico, bem como a acuidade visual dos mesmos.

6. Referências bibliográfica

Anderson GJ, Neuringer M, Lin DS, Connor WE. 2005. Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and brain biochemistry and visual function in rhesus monkeys. *Pediatr Res* **58**: 865-872.

André A, Juaneda P, Sebedio JL, Chardigny JM. 2005. Effects of aging and dietary n-3 fatty acids on rat brain phospholipids: focus on plasmalogens. *Lipids* **40**: 799-806.

Bamji SX, Rico B, Kimes N, Reichardt LF. 2006. BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. *J Cell Biol* **174**: 289-299.

Barnett NL, Pow DV, Bull ND. 2001. Differential perturbation of neuronal and glial glutamate transport systems in retinal ischaemia. *Neurochem Int* **39**: 291-299.

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

Bazan NG. 1989. The metabolism of omega-3 polyunsaturated fatty acids in the eye: the possible role of docosahexaenoic acid and docosanoids in retinal physiology and ocular pathology. *Prog Clin Biol Res* **312**: 95-112.

Bazan NG. 2005. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathol* **15**: 159-166.

Bazan NG. 2006. Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends Neurosci* **29**: 263-271.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH. 2007. Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. *Neuron* **53**: 261-277.

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH. 2008. BDNF and memory formation and storage. *Neuroscientist* **14**: 147-156.

Belanger M, Magistretti PJ. 2009. The role of astroglia in neuroprotection. *Dialogues Clin Neurosci* **11**: 281-295.

Belayev L, Marcheselli VL, Khoutorova L, Rodriguez de Turco EB, Busto R, Ginsberg MD, Bazan NG. 2005. Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. *Stroke* **36**: 118-123.

Ben-Ari Y. 2002. Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* **3**: 728-739.

Berry CB, Hayes D, Murphy A, Wiessner M, Rauen T, McBean GJ. 2005. Differential modulation of the glutamate transporters GLT1, GLAST and EAAC1 by docosahexaenoic acid. *Brain Res* **1037**: 123-133.

Bevilaqua LR, Rossato JI, Medina JH, Izquierdo I, Cammarota M. 2003. Src kinase activity is required for avoidance memory formation and recall. *Behav Pharmacol* **14**: 649-652.

Bodnarenko SR, Jeyarasasingam G, Chalupa LM. 1995. Development and regulation of dendritic stratification in retinal ganglion cells by glutamate-mediated afferent activity. *J Neurosci* **15**: 7037-7045.

Bradford HF. 1995. Glutamate, GABA and epilepsy. *Prog Neurobiol* **47**: 477-511.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A. 2006. Muller cells in the healthy and diseased retina. *Prog Retin Eye Res* **25**: 397-424.

Bunch L, Erichsen MN, Jensen AA. 2009. Excitatory amino acid transporters as potential drug targets. *Expert Opin Ther Targets* **13**: 719-731.

Burr GO. 1981. The essential fatty acids fifty years ago. *Prog Lipid Res* **20**: xxvii-xxix.

Chen Y, Swanson RA. 2003. The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *J Neurochem* **84**: 1332-1339.

Cognato GP, Agostinho PM, Hockemeyer J, Muller CE, Souza DO, Cunha RA. 2010. Caffeine and an adenosine A(2A) receptor antagonist prevent memory impairment and synaptotoxicity in adult rats triggered by a convulsive episode in early life. *J Neurochem* **112**: 453-462.

Collingridge GL, Isaac JT. 2003. Functional roles of protein interactions with AMPA and kainate receptors. *Neurosci Res* **47**: 3-15.

Copenhagen DR, Ashmore JF, Schnapf JK. 1983. Kinetics of synaptic transmission from photoreceptors to horizontal and bipolar cells in turtle retina. *Vision Res* **23**: 363-369.

Copenhagen DR, Jahr CE. 1989. Release of endogenous excitatory amino acids from turtle photoreceptors. *Nature* **341**: 536-539.

Cornejo BJ, Mesches MH, Benke TA. 2008. A single early-life seizure impairs short-term memory but does not alter spatial learning, recognition memory, or anxiety. *Epilepsy Behav* **13**: 585-592.

Cornejo BJ, Mesches MH, Coultrap S, Browning MD, Benke TA. 2007. A single episode of neonatal seizures permanently alters glutamatergic synapses. *Ann Neurol* **61**: 411-426.

Danbolt NC. 2001. Glutamate uptake. *Prog Neurobiol* **65**: 1-105.

Davis HP, Squire LR. 1984. Protein synthesis and memory: a review. *Psychol Bull* **96**: 518-559.

Delion S, Chalon S, Guilloteau D, Besnard JC, Durand G. 1996. alpha-Linolenic acid dietary deficiency alters age-related changes of dopaminergic and serotoninergic neurotransmission in the rat frontal cortex. *J Neurochem* **66**: 1582-1591.

Dreyer EB, Grosskreutz CL. 1997. Excitatory mechanisms in retinal ganglion cell death in primary open angle glaucoma (POAG). *Clin Neurosci* **4**: 270-273.

Dreyer EB, Zurakowski D, Schumer RA, Podos SM, Lipton SA. 1996. Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. *Arch Ophthalmol* **114**: 299-305.

Dunlop J. 2006. Glutamate-based therapeutic approaches: targeting the glutamate transport system. *Curr Opin Pharmacol* **6**: 103-107.

Dunlop J, Butera JA. 2006. Ligands targeting the excitatory amino acid transporters (EAATs). *Curr Top Med Chem* **6**: 1897-1906.

Dyall SC, Michael GJ, Whelpton R, Scott AG, Michael-Titus AT. 2007. Dietary enrichment with omega-3 polyunsaturated fatty acids reverses age-related

decreases in the GluR2 and NR2B glutamate receptor subunits in rat forebrain. *Neurobiol Aging* **28**: 424-439.

Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC, Ottersen OP, de Lanerolle NC. 2004. Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. *Lancet* **363**: 28-37.

Emptage NJ, Carew TJ. 1993. Long-term synaptic facilitation in the absence of short-term facilitation in Aplysia neurons. *Science* **262**: 253-256.

Eulenburg V, Gomeza J. 2010. Neurotransmitter transporters expressed in glial cells as regulators of synapse function. *Brain Res Rev* **63**: 103-112.

Farooqui AA, Horrocks LA. 2001. Plasmalogens, phospholipase A2, and docosahexaenoic acid turnover in brain tissue. *J Mol Neurosci* **16**: 263-272; discussion 279-284.

Garcia MC, Ward G, Ma YC, Salem N, Jr., Kim HY. 1998. Effect of docosahexaenoic acid on the synthesis of phosphatidylserine in rat brain in microsomes and C6 glioma cells. *J Neurochem* **70**: 24-30.

Garelli A, Rotstein NP, Politi LE. 2006. Docosahexaenoic acid promotes photoreceptor differentiation without altering Crx expression. *Invest Ophthalmol Vis Sci* **47**: 3017-3027.

Gegelashvili G, Dehnes Y, Danbolt NC, Schousboe A. 2000. The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. *Neurochem Int* **37**: 163-170.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER. 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* **258**: 1903-1910.

Grintal B, Champeil-Potokar G, Lavialle M, Vancassel S, Breton S, Denis I. 2009. Inhibition of astroglial glutamate transport by polyunsaturated fatty acids: evidence for a signalling role of docosahexaenoic acid. *Neurochem Int* **54**: 535-543.

Haag M. 2003. Essential fatty acids and the brain. *Can J Psychiatry* **48**: 195-203.

Hammer J, Alvestad S, Osen KK, Skare O, Sonnewald U, Ottersen OP. 2008. Expression of glutamine synthetase and glutamate dehydrogenase in the latent phase and chronic phase in the kainate model of temporal lobe epilepsy. *Glia* **56**: 856-868.

Hansen AE, Stewart RA, Hughes G, Soderhjelm L. 1962. The relation of linoleic acid to infant feeding. *Acta Paediatr Suppl* **137**: 1-41.

Heird WC, Lapillonne A. 2005. The role of essential fatty acids in development. *Annu Rev Nutr* **25**: 549-571.

Hibbeln JR, Davis JM, Steer C, Emmett 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**: 578-585.

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

Holcombe DJ, Lengefeld N, Gole GA, Barnett NL. 2008. The effects of acute intraocular pressure elevation on rat retinal glutamate transport. *Acta Ophthalmol* **86**: 408-414.

Holman RT, Johnson SB. 1982. Linolenic acid deficiency in man. *Nutr Rev* **40**: 144-147.

Holmes GL, Ben-Ari Y. 2001. The neurobiology and consequences of epilepsy in the developing brain. *Pediatr Res* **49**: 320-325.

Isosaka T, Hattori K, Kida S, Kohno T, Nakazawa T, Yamamoto T, Yagi T, Yuasa S. 2008. Activation of Fyn tyrosine kinase in the mouse dorsal hippocampus is essential for contextual fear conditioning. *Eur J Neurosci* **28**: 973-981.

Izquierdo I, Bevilaqua LR, Rossato JI, Bonini JS, Medina JH, Cammarota M. 2006. Different molecular cascades in different sites of the brain control memory consolidation. *Trends Neurosci* **29**: 496-505.

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

Kafitz KW, Rose CR, Thoenen H, Konnerth A. 1999. Neurotrophin-evoked rapid excitation through TrkB receptors. *Nature* **401**: 918-921.

Kew JN, Kemp JA. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl). 2005;179:4-29.

Khazipov R, Khalilov I, Tyzio R, Morozova E, Ben-Ari Y, Holmes GL. 2004. Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. *Eur J Neurosci* **19**: 590-600.

Kojima N, Ishibashi H, Obata K, Kandel ER. 1998. Higher seizure susceptibility and enhanced tyrosine phosphorylation of N-methyl-D-aspartate receptor subunit 2B in fyn transgenic mice. *Learn Mem* **5**: 429-445.

Kojima N, Sakamoto T, Endo S, Niki H. 2005. Impairment of conditioned freezing to tone, but not to context, in Fyn-transgenic mice: relationship to NMDA receptor subunit 2B function. *Eur J Neurosci* **21**: 1359-1369.

Larqué E, Demmelmair H, Klingler M, De Jonge S, Bondy B, Koletzko B. 2006. Expression pattern of fatty acid transport protein-1 (FATP-1), FATP-4 and heart-fatty acid binding protein (H-FABP) genes in human term placenta. *Early Hum Dev* **82**: 697-701.

Lauritzen L, Hansen HS, Jorgensen MH, Michaelsen KF. 2001. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res* **40**: 1-94.

Lavialle M, Denis I, Guesnet P, Vancassel S. 2010. Involvement of omega-3 fatty acids in emotional responses and hyperactive symptoms. *J Nutr Biochem* **21**: 899-905.

Lim SY, Doherty JD, Salem N, Jr. 2005a. Lead exposure and (n-3) fatty acid deficiency during rat neonatal development alter liver, plasma, and brain polyunsaturated fatty acid composition. *J Nutr* **135**: 1027-1033.

Lim SY, Hoshiba J, Moriguchi T, Salem N, Jr. 2005b. N-3 fatty acid deficiency induced by a modified artificial rearing method leads to poorer performance in spatial learning tasks. *Pediatr Res* **58**: 741-748.

Lipton SA, Rosenberg PA. 1994. Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* **330**: 613-622.

Lisman J, Schulman H, Cline H. 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* **3**: 175-190.

Lortet S, Samuel D, Had-Aissouni L, Masmejean F, Kerkerian-Le Goff L, Pisano P. 1999. Effects of PKA and PKC modulators on high affinity glutamate uptake in primary neuronal cell cultures from rat cerebral cortex. *Neuropharmacology* **38**: 395-402.

Lukiw WJ, Bazan NG. 2008. Docosahexaenoic acid and the aging brain. *J Nutr* **138**: 2510-2514.

Maragakis NJ, Rothstein JD. 2004. Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis* **15**: 461-473.

Marszalek JR, Lodish HF. 2005. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. *Annu Rev Cell Dev Biol* **21**: 633-657.

Martin RE, Bazan NG. 1992. Changing fatty acid content of growth cone lipids prior to synaptogenesis. *J Neurochem* **59**: 318-325.

Massey SC, Miller RF. 1990. N-methyl-D-aspartate receptors of ganglion cells in rabbit retina. *J Neurophysiol* **63**: 16-30.

Massey SC, Redburn DA. 1987. Transmitter circuits in the vertebrate retina. *Prog Neurobiol* **28**: 55-96.

McGahon BM, Martin DS, Horrobin DF, Lynch MA. 1999. Age-related changes in synaptic function: analysis of the effect of dietary supplementation with omega-3 fatty acids. *Neuroscience* **94**: 305-314.

McGaugh JL. 2000. Memory--a century of consolidation. *Science* **287**: 248-251.

Meldrum BS. 1994. The role of glutamate in epilepsy and other CNS disorders. *Neurology* **44**: S14-23.

Meldrum BS. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr* **130**: 1007S-1015S.

Miyauchi O, Mizota A, Adachi-Usami E, Nishikawa M. 2001. Protective effect of docosahexaenoic acid against retinal ischemic injury: an electroretinographic study. *Ophthalmic Res* **33**: 191-195.

Mizuno M, Yamada K, He J, Nakajima A, Nabeshima T. 2003. Involvement of BDNF receptor TrkB in spatial memory formation. *Learn Mem* **10**: 108-115.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**: 529-540.

Moreira JD, Knorr L, Thomazi AP, Simao F, Battu C, Oses JP, Gottfried C, Wofchuk S, Salbego C, Souza DO et al. 2010. Dietary omega-3 fatty acids attenuate cellular damage after a hippocampal ischemic insult in adult rats. *J Nutr Biochem* **21**: 351-356.

Moriguchi K, Yuri T, Yoshizawa K, Kiuchi K, Takada H, Inoue Y, Hada T, Matsumura M, Tsubura A. 2003. Dietary docosahexaenoic acid protects against N-methyl-N-nitrosourea-induced retinal degeneration in rats. *Exp Eye Res* **77**: 167-173.

Moriguchi T, Greiner RS, Salem N, Jr. 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J Neurochem* **75**: 2563-2573.

Moriguchi T, Salem N, Jr. 2003. Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J Neurochem* **87**: 297-309.

Moussa CE, Rae C, Bubb WA, Griffin JL, Deters NA, Balcar VJ. 2007. Inhibitors of glutamate transport modulate distinct patterns in brain metabolism. *J Neurosci Res* **85**: 342-350.

Murayama K, Yoneya S, Miyauchi O, Adachi-Usami E, Nishikawa M. 2002. Fish oil (polyunsaturated fatty acid) prevents ischemic-induced injury in the mammalian retina. *Exp Eye Res* **74**: 671-676.

Napper GA, Pianta MJ, Kalloniatis M. 1999. Reduced glutamate uptake by retinal glial cells under ischemic/hypoxic conditions. *Vis Neurosci* **16**: 149-158.

Naylor DE. 2010. Glutamate and GABA in the balance: convergent pathways sustain seizures during status epilepticus. *Epilepsia* **51 Suppl 3**: 106-109.

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. *Proc Natl Acad Sci U S A* **83**: 4021-4025.

Niu SL, Mitchell DC, Lim SY, Wen ZM, Kim HY, Salem N, Jr., Litman BJ. 2004. Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J Biol Chem* **279**: 31098-31104.

O'Shea RD. 2002. Roles and regulation of glutamate transporters in the central nervous system. *Clin Exp Pharmacol Physiol* **29**: 1018-1023.

Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J. 2004. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res* **23**: 91-147.

Osborne NN, Safa R, Nash MS. 1999. Photoreceptors are preferentially affected in the rat retina following permanent occlusion of the carotid arteries. *Vision Res* **39**: 3995-4002.

Owada Y, Abdelwahab SA, Kitanaka N, Sakagami H, Takano H, Sugitani Y, Sugawara M, Kawashima H, Kiso Y, Mobarakeh JI et al. 2006. Altered emotional behavioral responses in mice lacking brain-type fatty acid-binding protein gene. *Eur J Neurosci* **24**: 175-187.

Ozawa S, Kamiya H, Tsuzuki K. 1998. Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* **54**: 581-618.

Pang PT, Lu B. 2004. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. *Ageing Res Rev* **3**: 407-430.

Peet M, Stokes C. 2005. Omega-3 fatty acids in the treatment of psychiatric disorders. *Drugs* **65**: 1051-1059.

Pignataro L, Sitaramayya A, Finnemann SC, Sarthy VP. 2005. Nonsynaptic localization of the excitatory amino acid transporter 4 in photoreceptors. *Mol Cell Neurosci* **28**: 440-451.

Popescu G, Auerbach A. 2004. The NMDA receptor gating machine: lessons from single channels. *Neuroscientist* **10**: 192-198.

Pow DV, Barnett NL. 2000. Developmental expression of excitatory amino acid transporter 5: a photoreceptor and bipolar cell glutamate transporter in rat retina. *Neurosci Lett* **280**: 21-24.

Rauen T, Kanner BI. 1994. Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. *Neurosci Lett* **169**: 137-140.

Rauen T, Rothstein JD, Wassle H. 1996. Differential expression of three glutamate transporter subtypes in the rat retina. *Cell Tissue Res* **286**: 325-336.

Rauen T, Taylor WR, Kuhlbrodt K, Wiessner M. 1998. High-affinity glutamate transporters in the rat retina: a major role of the glial glutamate transporter GLAST-1 in transmitter clearance. *Cell Tissue Res* **291**: 19-31.

Raz R, Gabis L. 2009. Essential fatty acids and attention-deficit-hyperactivity disorder: a systematic review. *Dev Med Child Neurol* **51**: 580-592.

Robinson MB. 2002. Regulated trafficking of neurotransmitter transporters: common notes but different melodies. *J Neurochem* **80**: 1-11.

Robinson MB. 2006. Acute regulation of sodium-dependent glutamate transporters: a focus on constitutive and regulated trafficking. *Handb Exp Pharmacol*: 251-275.

Romano C, Price MT, Olney JW. 1995. Delayed excitotoxic neurodegeneration induced by excitatory amino acid agonists in isolated retina. *J Neurochem* **65**: 59-67.

Rothstein JD. 1996. Excitotoxicity hypothesis. *Neurology* **47**: S19-25; discussion S26.

Rotstein NP, Politi LE, German OL, Girotti R. 2003. Protective effect of docosahexaenoic acid on oxidative stress-induced apoptosis of retina photoreceptors. *Invest Ophthalmol Vis Sci* **44**: 2252-2259.

Salter MW, Kalia LV. 2004. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* **5**: 317-328.

Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins SJ, 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. *Arch Neurol* **63**: 1545-1550.

Schafe GE, LeDoux JE. 2000. Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J Neurosci* **20**: RC96.

Scharf MT, Woo NH, Lattal KM, Young JZ, Nguyen PV, Abel T. 2002. Protein synthesis is required for the enhancement of long-term potentiation and long-term memory by spaced training. *J Neurophysiol* **87**: 2770-2777.

Schiefermeier M, Yavin E. 2002. n-3 Deficient and docosahexaenoic acid-enriched diets during critical periods of the developing prenatal rat brain. *J Lipid Res* **43**: 124-131.

Schultz K, Stell WK. 1996. Immunocytochemical localization of the high-affinity glutamate transporter, EAAC1, in the retina of representative vertebrate species. *Neurosci Lett* **211**: 191-194.

Scorza FA, Cysneiros RM, Arida RM, Terra-Bustamante VC, de Albuquerque M, Cavalheiro EA. 2008. The other side of the coin: Beneficiary effect of

omega-3 fatty acids in sudden unexpected death in epilepsy. *Epilepsy Behav* **13**: 279-283.

Scott BL, Racz E, Lolley RN, Bazan NG. 1988. Developing rod photoreceptors from normal and mutant Rd mouse retinas: altered fatty acid composition early in development of the mutant. *J Neurosci Res* **20**: 202-211.

Segovia G, Porras A, Del Arco A, Mora F. 2001. Glutamatergic neurotransmission in aging: a critical perspective. *Mech Ageing Dev* **122**: 1-29.

Sheldon AL, Robinson MB. 2007. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. *Neurochem Int* **51**: 333-355.

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

Stevens B. 2008. Neuron-astrocyte signaling in the development and plasticity of neural circuits. *Neurosignals* **16**: 278-288.

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**: 547-553.

Stubbs CD, Smith AD. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim Biophys Acta* **779**: 89-137.

Sucher NJ, Lipton SA, Dreyer EB. 1997. Molecular basis of glutamate toxicity in retinal ganglion cells. *Vision Res* **37**: 3483-3493.

Suen PC, Wu K, Levine ES, Mount HT, Xu JL, Lin SY, Black IB. 1997. Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic N-methyl-D-aspartate receptor subunit 1. *Proc Natl Acad Sci U S A* **94**: 8191-8195.

Treiman DM. 2001. GABAergic mechanisms in epilepsy. *Epilepsia* **42 Suppl 3**: 8-12.

Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD. 2002. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learn Mem* **9**: 224-237.

Tyler WJ, Pozzo-Miller L. 2003. Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *J Physiol* **553**: 497-509.

Tzingounis AV, Wadiche JI. 2007. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat Rev Neurosci* **8**: 935-947.

Ueda Y, Doi T, Tokumaru J, Yokoyama H, Nakajima A, Mitsuyama Y, Ohya-Nishiguchi H, Kamada H, Willmore LJ. 2001. Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. *J Neurochem* **76**: 892-900.

Vandenberg RJ, Ju P, Aubrey KR, Ryan RM, Mitrovic AD. 2004. Allosteric modulation of neurotransmitter transporters at excitatory synapses. *Eur J Pharm Sci* **23**: 1-11.

Weisinger HS, Vingrys AJ, Abedin L, Sinclair AJ. 1998. Effect of diet on the rate of depletion of n-3 fatty acids in the retina of the guinea pig. *J Lipid Res* **39**: 1274-1279.

Willatts P, Forsyth JS, DiModugno MK, Varma S, Colvin M. 1998. Influence of long-chain polyunsaturated fatty acids on infant cognitive function. *Lipids* **33**: 973-980.

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

Zhang G, Raol YS, Hsu FC, Brooks-Kayal AR. 2004. Long-term alterations in glutamate receptor and transporter expression following early-life seizures are associated with increased seizure susceptibility. *J Neurochem* **88**: 91-101.

Zimmer L, Delpal S, Guilloteau D, Aioun J, Durand G, Chalon S. 2000. Chronic n-3 polyunsaturated fatty acid deficiency alters dopamine vesicle density in the rat frontal cortex. *Neurosci Lett* **284**: 25-28.