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

Mecanismos Celulares e Moleculares Envolvidos no Efeito Neuroprotetor da Curcumina em Modelos Experimentais da Doença de Alzheimer

JULIANA BENDER HOPPE

Porto Alegre

2013

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

Mecanismos Celulares e Moleculares Envolvidos no Efeito Neuroprotetor da Curcumina em Modelos Experimentais da Doença de Alzheimer

JULIANA BENDER HOPPE

Orientadora: Dra. Christianne Gazzana Salbego

Tese apresentada ao curso de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

Porto Alegre
2013

CIP - Catalogação na Publicação

Bender Hoppe, Juliana
Mecanismos Celulares e Moleculares Envolvidos no Efeito Neuroprotetor da Curcumina em Modelos Experimentais da Doença de Alzheimer / Juliana Bender Hoppe. -- 2013.
216 f.

Orientador: Christianne Gazzana Salbego.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2013.

1. Doença de Alzheimer. 2. Peptídeo Beta-Amiloide. 3. Curcumina. 4. Neuroproteção. 5. Sinalização Celular. I. Gazzana Salbego, Christianne , orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

„Ich habe mich sozusagen verloren“

Auguste Deter (1850-1906)

I

AGRADECIMENTOS

À Christianne por toda a confiança depositada em mim. E, principalmente, pela amizade, apoio e liberdade concedidos durante todos estes anos.

A todos os colegas que ainda estão ou que já passaram pelo Grupo de Neuroproteção e Sinalização Celular pelo companheirismo e parceria de sempre.

Ao Rudimar e a Andressa pela amizade, por toda a ajuda, conversas, ensinamentos, boas ideias e o apoio que sempre me deram e que, mesmo distantes, se fizeram presentes sempre solicitos.

À Dra. Helena Cimarosti e aos Doutores Benjamin Whalley e Marcus Rattray por tão bem me acolherem em seus laboratórios durante o meu doutorado sanduíche na Faculdade de Farmácia da Universidade de Reading, na Inglaterra.

Aos colegas de laboratório na Inglaterra, em especial ao Vasco, Marcus, Henry e Alison por toda ajuda em meu trabalho.

À Karine e ao Prof. Ruy Beck da Faculdade de Farmácia-UFRGS, pela excelente colaboração que realizamos neste trabalho.

À Cristianne Matté e seu grupo, pela ótima parceria formada no Laboratório 23.

Aos Coordenadores, professores e funcionários do PPG Bioquímica, por fazerem deste um programa de pós-graduação de reconhecida qualidade.

A todos os amigos do Departamento de Bioquímica agradeço pelo espírito de colaboração e amizade.

À UFRGS e ao CNPq pela oportunidade e pela bolsa concedida durante o doutorado e o doutorado sanduíche.

A minha família pela dedicação e carinho que sempre demonstraram por mim e por me encorajarem a seguir minhas escolhas com confiança e otimismo.

SUMÁRIO

APRESENTAÇÃO

Esta Tese está organizada em seções dispostas da seguinte maneira: *Introdução, Objetivos, Capítulos* (I, II, III e IV – referentes a artigos científicos), *Discussão, Conclusões, Perspectivas e Bibliografia.*

A seção *Introdução* apresenta o embasamento teórico que levou a formular as propostas da Tese, as quais estão descritas na seção *Objetivos*.

A seção *Capítulos* contém os artigos científicos publicados, submetidos ou em fase de preparação para serem submetidos, os quais estão apresentados de acordo com os objetivos específicos. Esta seção também apresenta os materiais, os métodos e as referências bibliográficas específicas de cada artigo e está dividida em *Capítulos I, II, III e IV*. Os *Capítulos I e IV* foram realizados no Laboratório de Neuroproteção e Sinalização Celular – Departamento de Bioquímica (UFRGS), coordenado pela Profa. Dra. Christianne Gazzana Salbego; no *Capítulo IV* houve a colaboração com o Laboratório de Sistemas Nanoestruturados para a Administração de Fármacos – Faculdade de Farmácia (UFRGS), através do Prof. Dr. Ruy Carlos Ruver Beck. Os *Capítulos II e III* foram realizados nos laboratórios de Farmacologia e Eletrofisiologia da Faculdade de Farmácia da Universidade de Reading, Inglaterra, durante a realização do doutorado sanduíche sob a supervisão do Prof. Dr. Benjamin Whalley e da Profa. Dra. Helena Cimarosti.

A seção *Discussão* contém uma interpretação geral dos resultados obtidos nos diferentes artigos científicos. A seção *Conclusões* aborda as conclusões gerais obtidas na Tese. A seção *Perspectivas* aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos dando continuidade a esta linha de pesquisa.

A seção *Bibliografia* lista as referências citadas na *Introdução* e *Discussão* da Tese.

LISTA DE FIGURAS

Introdução

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

LISTA DE TABELAS

Capítulo IV

LISTA DE ABREVIATURAS

Aβ – peptídeo beta-amiloide (*Amyloid-beta peptide*)

Aβ1-42 – peptídeo beta-amiloide contendo 42 aminoácidos

AD – Doença de Alzheimer (*Alzheimer Disease*)

ALT – alanina aminotransferase (A*lanine aminotransferase*)

AST – aspartato aminotransferase (*Aspartate aminotransferase*)

AMPA – α-amino-3hidroxi-5-metil-4-isoxazol (*α-amino-3hydroxy-5-methyl-4-isoxazole*)

APP – proteína precursora amilóide (*Amyloid Precursor Protein*)

BACE-1 – β-secretase (*Beta-Site Amyloid Precursor Protein-Cleaving Enzyme-1*)

BDNF – fator neurotrófico derivado do encéfalo (*Brain-derived Neurotrophic Factor*)

BBB – barreira hemato-encefálica *(Blood-Brain Barrier)*

BHE – Barreira Hemato-Encefálica

B-LNC – nanocápsulas sem a droga (*Blank lipid-core nanocapsules*)

CaMKII – quinase dependente de cálcio/calmodulina II (C*alcium/calmodulin-dependent Protein Kinase II)*

CMC- carboximetilcelulose

CNS – sistema nervoso central *(Central Nervous System)*

COX-2 – ciclooxigenase-2 (C*yclooxygenase-2*)

Cur – curcumina livre

Cur-LNC – nanocápsulas de curcumina (*Curcumin-loaded lipid-core nanocapsules*)

DA – Doença de Alzheimer

DCF – diclorofluoresceína (D*ichlorofluorescein*)

DCFH2-DA – 2',7'-diclorofluoresceína diacetato (*2',7'-dichlorofluorescin diacetate*)

DMSO- dimetilsulfóxido

ELISA – ensaio de imunoabsorção enzimática (E*nzyme-linked Immunosorbent Assay)*

GFAP - proteína glial fibrilar ácida (*Glial Fibrillary Acidic Protein*)

HBSS - solução salina balenceada de Hank's *(Hank's Balanced Salt Solution)*

GSK-3β - glicogênio sintase quinase-3beta (*Glycogen Synthase Kinase-3beta)*

γ-GT – Gama-glutamil transferase *(Gamma-glutamyltransferase*)

IB4 – isolectina B4 *(Isolectin B4)*

ICV - intracerebroventricular

IL-1β – interleucina-1beta *(Interleukin-1beta)*

IL-6 – interleucina-6 *(Interleukin-6)*

IL-10 – interleucina-10 *(Interleukin-10)*

iNOS – óxido nitrico sintase induzível *(inducible Nitric Oxide Synthase)*

JNK – proteína quinase c-Jun N-terminal *(c-Jun N-terminal Kinase)*

LDH – lactato desidrogenase

LFP – potencial de campo local (*Local Field Potential*)

L-JNKi1 – inibidor da proteína JNK (*L-JNK inhibitor 1*)

LPS – lipopolissacarídeo (*Lipopolysaccharide)*

LTM – memória de longa duração (*Long-Term Memory*)

LTP – potencial de longa duração (*Long-Term Potentiation*)

MAPKs – proteínas quinase mitógeno-ativadas (*Mitogen-Activated Protein Kinase)*

MEA – matriz multieletrodo (*Multi-Electrode Array*)

MEM – meio essencial mínimo *(Minimum Essential Medium)*

NF-κB – fator nuclear kappa B (N*uclear Factor κ-light-chain-enhancer of activated B cells*)

NGF – fator de crescimento neuronal (*Nerve Growth Factor*)

NMDARs – receptores N-metil D-aspartato (*N-methyl- D-aspartate Receptors*)

NO – óxido nítrico *(Nitric Oxide)*

P38 - proteína ativada por mitógeno p38-cinase (*p38 Mitogen Activated Protein Kinase*)

PDPK - proteínas quinases dependentes de prolina (*Proline-Directed* Protein *Kinase*)

PI – iodeto de propídeo *(Propidium Iodide)*

PI3K – fosfatidilinositol 3-quinase *(Phosphoinositide 3-kinase)*

PKA – proteína quinase A (*Protein Kinase A)*

PKC – proteína quinase C (*Protein Kinase C)*

PP-2A – proteína fosfatase-2A (*Protein phosphatase 2A*)

ROS – espécies reativas de oxigênio (*Reactive Oxygen Species*)

SENP – protease desconjugadora de SUMO (*SUMO-specific protease*)

SNC – Sistema Nervoso Central

STM – memória de curta duração (*Short-Term Memory*)

SUMO – pequeno modificador do tipo ubiquitina (*Small Ubiquitin-like Modifier*)

TGF-β – fator de crescimento tumoral-beta (*Tumoral Growth Factor-beta*)

TNF-α - fator de necrose tumoral- alfa *(Tumor Necrosis Factor alpha)*

Ubc9 – proteína conjugadora de SUMO (SUMO-*conjugating enzyme*)

RESUMO

O aumento da longevidade da população mundial tem como consequência uma maior prevalência de doenças neurodegenerativas. A Doença de Alzheimer (DA) é a desordem neurodegenerativa mais prevalente e a principal causa de demência após os 60 anos. A DA caracteriza-se por um crescente declínio na função mental e memória do paciente. Esses sintomas são acompanhados pela presença de alterações estruturais no tecido cerebral: os emaranhados neurofibrilares constituídos pela proteína tau hiperfosforilada e as placas senis constituídas pelo peptídeo beta-amiloide (Aβ). O peptídeo Aβ tem sido considerado o principal responsável pelos processos de disfunção sináptica, morte neuronal e consequente declínio cognitivo dos pacientes com DA. O cenário atual ao qual se enquadram nossos conhecimentos sobre a etiologia, fisiopatologia e terapêutica da DA ainda não permitem um entendimento completo e satisfatório sobre essa doença e seu tratamento. A curcumina, um polifenol de origem vegetal, tem atraído considerável interesse devido aos seus potenciais benefícios à saúde humana. Alguns estudos têm demonstrado que a curcumina possui propriedades antioxidantes, antiinflamatórias e anti-amiloidogênicas em modelos animais de DA. Entretanto, as bases moleculares para a sua neuroproteção ainda não estão totalmente esclarecidas. Neste trabalho, avaliamos diversos mecanismos que são modulados pela curcumina em modelos *in vivo* e *in vitro* de toxicidade induzida pelo peptídeo Aβ1-42. Inicialmente, demonstramos que o co-tratamento com curcumina reduziu a morte celular induzida pela exposição ao peptídeo Aβ por 48 h em culturas organotípicas de hipocampo de ratos. Em paralelo, a curcumina preveniu a redução da proteína sinaptofisina, a geração de espécies reativas, a ativação astrocitária e microglial, bem como, a alteração na secreção de importantes mediadores inflamatórios. Além disso, o mecanismo envolvido com sua neuroproteção pode estar associado com a regulação da fosforilação da proteína β-catenina e da modulação da via de sinalização PI3K, através da regulação dos substratos Akt e GSK-3β, visto que o uso do inibidor LY294002 bloqueou o efeito neuroprotetor da curcumina. Diante da intensa ativação astrocitária observada no primeiro capítulo, investigamos o efeito da curcumina na reatividade astrocitária em cultura primária de astrócitos expostos ao peptídeo Aβ. Neste trabalho observamos que os mecanismos de proteção da curcumina envolvem a prevenção da ativação da proteína JNK e a regulação do perfil de sumoilação de astrócitos, visto que a superexpressão de SUMO-1 diminui a reatividade astrocitária induzida pelo peptídeo Aβ. Além do efeito neuroprotetor contra a morte celular, a curcumina demonstrou ter efeitos benéficos na transmissão sináptica, evitando a diminuição da excitabilidade neuronal em fatias organotípicas de hipocampo de ratos expostas por 24 h ao peptídeo Aβ e este efeito parece estar associado com a modulação das proteínas CaMKII e sinapsina I. Para a avaliação do efeito neuroprotetor da curcumina no modelo *in vivo* de toxicidade ao peptídeo Aβ1-42, devido a sua baixa biodisponibilidade, comparamos a eficiência de nanocápsulas de curcumina e curcumina livre na prevenção dos danos cognitivos induzidos pela injeção icv do peptídeo Aβ. De modo interessante, observamos um significativo aumento do desempenho *in vivo* da curcumina nanoencapsulada, a qual apresentou efeitos similares ou melhores com uma dose 20 vezes menor do que a dose efetiva de curcumina livre. Os mecanismos de neuroproteção da curcumina neste modelo parecem envolver o fator neurotrófico BDNF e a via de sinalização PI3K/Akt. Juntos, nossos resultados não somente confirmam o potencial da curcumina no tratamento dos processos neurodegenerativos como também oferecem uma via efetiva para melhorar o efeito neuroprotetor da curcumina através da nanobiotecnologia.

ABSTRACT

The increased longevity of the population has resulted in a higher prevalence of neurodegenerative diseases. Alzheimer's disease (AD) is the most common neurodegenerative disorder and the leading cause of dementia in persons over 60 years. The AD is characterized clinically by progressive impairments in cognition and memory. These clinical features are accompanied by the presence of structural changes in brain tissue: neurofibrillary tangles formed by hyperphosphorylated tau protein and amyloid plaques formed by amyloid beta-peptide (Aβ). Aβ has been considered the main processes responsible for synaptic dysfunction, neuronal death and subsequent cognitive decline in patients with AD. The current scenario of our knowledge of the etiology, pathophysiology and therapy of AD does not allow a full and satisfactory understanding of this disease and its treatment. Curcumin, a naturally occurring polyphenol, has attracted considerable interest for its beneficial potentials for human health. Over the past decade, studies have been shown that curcumin is associated with antioxidant, anti-inflammatory and anti-amyloidogenic properties in models of AD; however, the molecular basis for its neuroprotection is not yet fully understood. In this study, we evaluated several mechanisms that are modulated by curcumin in *in vitro* and *in vivo* models of Aβ1-42-induced toxicity. Initially, we demonstrated that co-treatment with curcumin reduced the cell death induced by exposure to peptide Aβ for 48 h in organotypic hippocampal slice cultures. In parallel, curcumin prevented the reduction of synaptophysin protein, generation of reactive species, astrocytic and microglial activation and the changes in the release of important inflammatory mediators. Furthermore, the mechanism involved in this neuroprotection may be associated with regulation of phosphorylation of β-catenin protein and modulation of PI3K signaling pathway, by regulating the substrates Akt and GSK-3β, since the use of inhibitor LY294002 blocked the neuroprotective effect of curcumin. Given the intense astrocytic activation observed in the first study, we investigated the effect of curcumin on astrocytic reactivity in primary culture of astrocytes exposed to Aβ1-42. In this study we observed that the protective mechanisms of curcumin involve the prevention of JNK activation and regulation of SUMOylation profile of astrocytes, whereas overexpression of SUMO-1 reduces astrocyte reactivity induced by Aβ. Besides the neuroprotective effect against cell death, curcumin shown to have beneficial effects in synaptic transmission, avoiding the reduction of neuronal excitability in organotypic hippocampal slices exposed for 24 h to Aβ1-42 and this effect could be associated with CaMKII and synapsin I modulation by curcumin. For evaluating the neuroprotective effect of curcumin in an *in vivo* model of Aβ-induced toxicity, due to its poor bioavailability, we compared the efficiency of curcumin-loaded lipid-core nanocapsules and free curcumin in preventing cognitive impairments induced by icv injection of Aβ1-42. Interestingly, we observed a significant increase in the *in vivo* performance of nanoencapsulated curcumin, which presented similar or better neuroprotective results in a dose 20-fold lower compared to the effective dose of free curcumin. The neuroprotective mechanisms of curcumin in this model appear to involve the neurotrophic factor BDNF and PI3K/Akt signaling pathway. Taken together, our results not only confirm the potential of curcumin in treating neurodegenerative processes but also offer an effective way to improve the neuroprotective efficiency of curcumin through nanobiotechnology.

1. INTRODUÇÃO

1.1. Doença de Alzheimer

A população mundial vem passando por uma significante transição demográfica, as sociedades estão deixando de ser formadas predominantemente por jovens e adultos para se transformarem em sociedades compostas por pessoas cada vez mais idosas. Embora esse dado demonstre um avanço na qualidade de vida de parte da população, ele também acena para a possibilidade do crescimento alarmante do número de pessoas acometidas pelas doenças relacionadas ao envelhecimento, dentre as quais se destaca a Doença de Alzheimer (DA).

Descrita em 1906 pelo médico alemão Alois Alzheimer durante sua conferência intitulada *"Über eine eigenartige Erkrankung der Hirnrinde"* (Sobre uma doença peculiar do córtex cerebral) (Alzheimer, 1907). Alzheimer definiu seu achado como uma patologia neurológica não reconhecida, que cursa com demência, e destacou os sintomas de déficit de memória, alterações de comportamento e incapacidade para as atividades rotineiras. Esses sintomas foram observados em uma paciente do sexo feminino de 51 anos, admitida por Alzheimer no Hospital de Frankfurt em 1901, apresentando perda de memória progressiva, delírios e alucinações. Em 1906, após a morte da paciente seu cérebro foi analisado por Alzheimer que identificou as duas principais características histopatológicas da doença: as placas senis e os emaranhados neurofibrilares (Alzheimer, 1911). Estes dois achados patológicos, em uma doente com severas perturbações neurocognitivas, e na ausência de evidência de compromisso ou lesão intravascular, permitiram a Alzheimer caracterizar este quadro clínico como distinto de outras patologias orgânicas do cérebro. Em 1910, o também médico alemão Emil Kraepelin, após estudar casos semelhantes aos relatos de Alzheimer, propôs o nome da doença em homenagem ao seu descobridor (Moller and Graeber, 1998; Goedert et al., 2006; Goedert and Spillantini, 2006).

A DA é a desordem neurodegenerativa mais comum e a principal causa de demência após os 60 anos de idade (Forman et al., 2004; Querfurth and LaFerla, 2010). A DA acomete 8 a 15% da população com mais de 65 anos correspondendo aproximadamente a 35 milhões de pessoas com a DA no mundo, o que representa 70% do conjunto de doenças que afetam a população idosa (Philipson et al., 2010; Querfurth and LaFerla, 2010). Segundo relatório produzido pela Alzheimer's Disease International (ADI), um novo caso de demência aparece a cada sete segundos fazendo com que o número de pessoas com demência duplique a cada 20 anos, com 4,6 milhões de novos casos anualmente (Ferri et al., 2005).

A DA constitui um quadro complexo envolvendo a combinação de fatores genéticos, moleculares e ambientais. Desta forma, compreender os mecanismos moleculares pelos quais essas alterações patológicas comprometem a função e integridade neuronal e levam aos sintomas clínicos observados na DA tem sido uma busca incessante na investigação desta patologia. A idade de início das manifestações clínicas características desta demência é mais precoce e sua progressão mais acelerada em pacientes com história familiar desse transtorno (Casserly and Topol, 2004; Blennow et al., 2006). As manifestações da DA iniciam com leves déficits de memória, primeiramente afetando a memória de curto prazo. Os primeiros sintomas geralmente surgem como déficits sutis e intermitentes em lembrar pequenos eventos do cotidiano. Nos estágios mais avançados, há o desenvolvimento progressivo de uma profunda demência afetando as esferas cognitivas e comportamentais. Embora a incapacitação seja progressiva, o paciente com a DA pode conviver com esta desordem durante muitos anos, em média oito, mas podendo chegar até vinte anos (Alzheimer's Association, 2011). O cérebro de um paciente com DA apresenta atrofia cortical mais acentuada no lobo temporal, principalmente no córtex e na formação hipocampal. O volume cerebral

reduzido é decorrente de uma profunda degeneração de sinapses e morte neuronal (Mattson, 2004; Heneka and O'Banion, 2007).

O diagnóstico definitivo da DA somente é obtido por meio da análise *post mortem* com a demonstração das alterações histopatológicas encontradas por Alzheimer (Ballard et al., 2011). As placas senis extracelulares são formadas pelo acúmulo e agregação do peptídeo beta-amiloide (Aβ), formando filamentos cercados por neuritos distróficos, microglia ativada e astrócitos reativos. Enquanto que os emaranhados neurofibrilares são formados pela deposição da proteína tau, cuja principal função é estabilizar os microtúbulos responsáveis por modular a organização funcional do neurônio. Porém na DA, a tau encontra-se hiperfosforilada desprendendo-se dos microtúbulos, acumulando-se intracelularmente na forma de filamentos emaranhados helicoidais pareados (Selkoe and Schenk, 2003; LaFerla et al., 2007; Ittner and Gotz, 2011).

1.2. Peptídeo beta-amiloide

O peptídeo Aβ é produzido pela proteólise da proteína precursora amiloide (Amyloid Precursor Protein – APP), uma glicoproteína transmembrana amplamente expressa na superfície celular de neurônios e glia. A clivagem e o processamento da APP podem ser divididos em duas vias distintas: a via amiloidogênica e a via não-amiloidogênica (Figura 1). O processamento proteolítico anormal, ou amiloidogênico, da APP ocorre através da ação sequencial de duas proteases denominadas β-secretase (BACE 1) e γ-secretase, resultando na secreção do Aβ em fragmentos amiloides de 38 a 43 aminoácidos (Haass et al. 1992; Herz and Beffert, 2000). Produzido em sua forma solúvel o peptídeo Aβ tende a se aglomerar. Inicialmente são formados pequenos dímeros, trímeros e, posteriormente, oligômeros (Klein et al., 2002). Esses oligômeros

se agregam dando origem as fibrilas que por sua vez darão origem as placas senis (Stine et al., 2003).

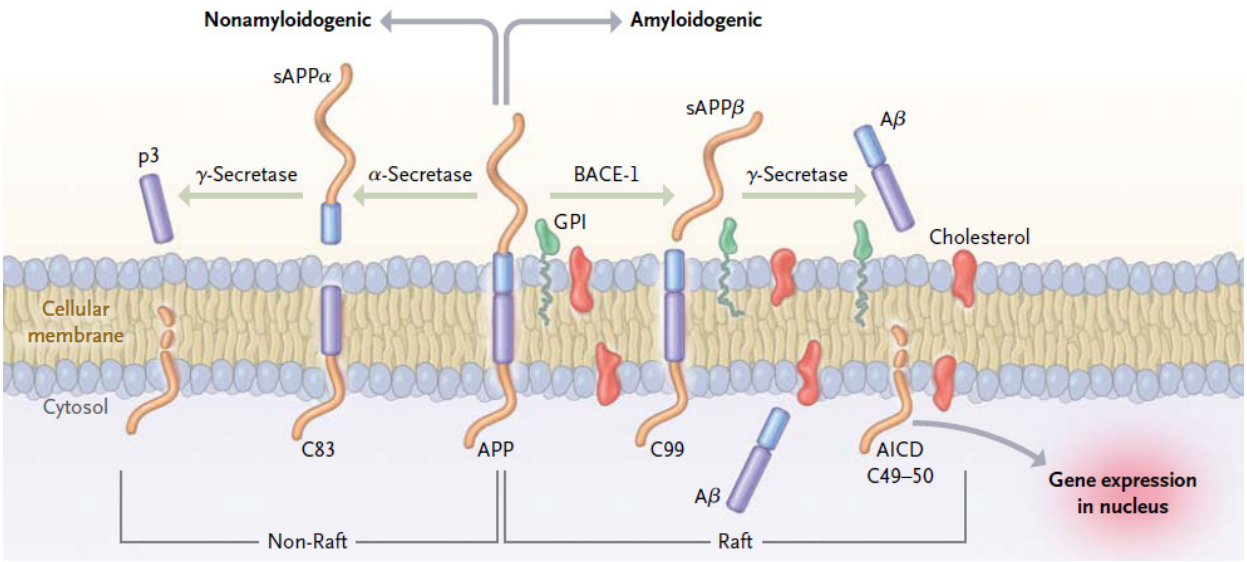


Figura 1. Vias de processamento da APP. Processamento amiloidogênico, através das enzimas β-secretase (BACE-1) e γ-secretase, gerando o peptídeo Aβ. Processamento não amiloidogênico, mediado pelas enzimas α-secretase e γ-secretase, não havendo geração do peptídeo Aβ (Adaptado de Querfurth and LaFerla, 2010).

No estado monomérico, o peptídeo Aβ parece não exercer efeitos tóxicos, enquanto que a espécie solúvel oligomérica demonstra alto potencial neurotóxico com capacidade de interferir na plasticidade sináptica, bloqueando os potenciais de longa duração e afetando de forma significativa diferentes vias de sinalização celular. Na forma de fibrilas, insolúveis, o peptídeo Aβ possui a capacidade de se auto-agregar formando as placas senis (Eckert et al., 2008; Plimplikar, 2009). Apesar das fibrilas insolúveis que formam as placas senis serem relativamente inativas, isso não significa que as placas não tenham um papel na degeneração neuronal progressiva. De fato, os oligômeros difusíveis do peptídeo Aβ encontrados cercando as placas, foram associados à perda local de espinhos dendríticos e à distrofia neurítica (Knowles et al., 1999; Koffie et al., 2009). Além disso, a presença de dímeros e oligômeros maiores "presos" junto às placas

senis insolúveis sugere fortemente que estas placas servem como uma reserva local de pequenos oligômeros, e estes podem difundir para locais mais distantes das placas causando dano sináptico/neurítico (Shankar et al., 2008; Selkoe, 2011).

O peptídeo Aβ tem sido considerado o principal responsável pelos processos de disfunção sináptica e morte neuronal e consequente declínio cognitivo dos pacientes com DA (Crews and Masliah, 2010). Desta forma, acredita-se que um aumento acentuado dos níveis cerebrais do peptídeo Aβ levaria a uma maior formação de oligômeros desse peptídeo gerando importantes alterações na função sináptica e na sinalização celular. Em paralelo, ocorreria a formação das fibrilas, com consequente, acúmulo e deposição em forma de placas. A progressão da doença ocorre com dano sináptico e neuronal, acompanhada da ativação de diferentes vias de sinalização, perda da homeostase iônica, dano oxidativo e resposta inflamatória. O aumento dos níveis do peptídeo Aβ também é acompanhado por alterações na atividade de quinases e fosfatases, levando a hiperfosforilação da tau e formação dos emaranhados neurofibrilares com consequente dano no transporte axonal (Hardy and Selkoe, 2002; Mattson and Chan, 2003; Haass and Selkoe, 2007).

1.3. Proteína tau

Os emaranhados neurofibrilares são constituídos principalmente pela deposição de filamentos helicoidais pareados da proteína tau hiperfosforilada. A principal função da proteína tau é estabilizar os microtúbulos, atuando na regulação da polimerização/despolimerização destes durante o processo de extensão axonal. Ao ligar-se às unidades de tubulina a proteína tau possibilita a organização em hélice das mesmas e o alongamento dos microtúbulos. Porém, na forma hiperfosforilada a proteína tau se desprende das unidades de tubulina, causando a desestabilização dos microtúbulos bem

como a deposição da proteína tau no corpo celular e dendritos dos neurônios na forma de emaranhados neurofibrilares (Figura 2) (Golde, 2007; Hooper et al., 2008; Ittner and Gotz, 2011).

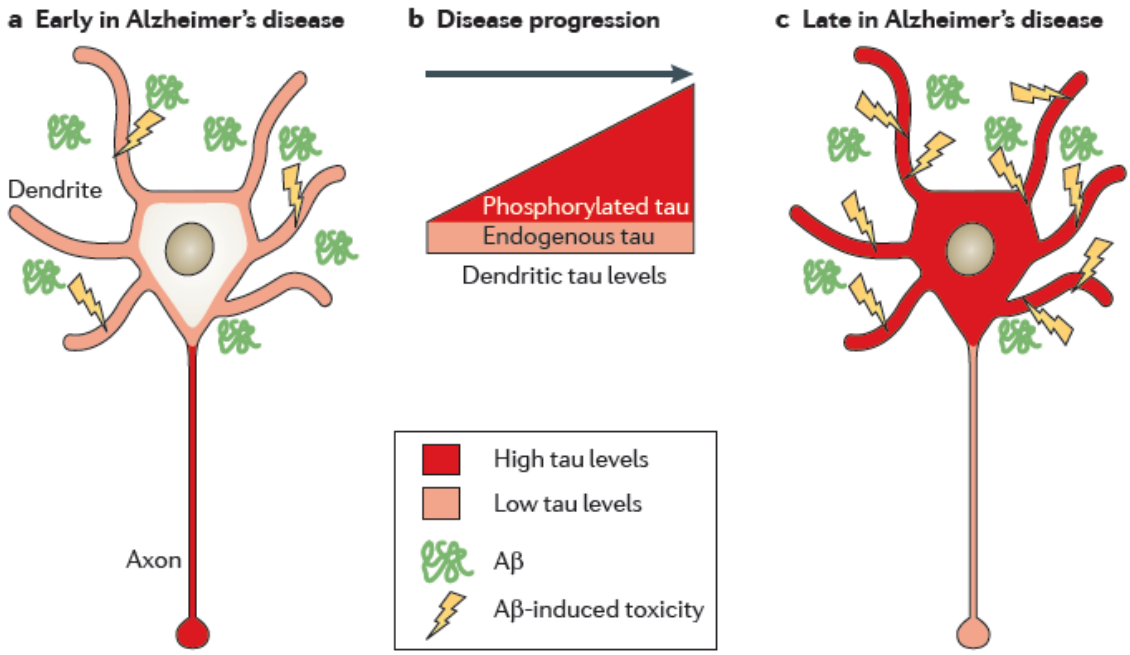


Figura 2. Hiperfosforilação da proteína tau na DA. Em neurônios a proteína tau é normalmente encontrada em axônios, porém na DA ela se redistribui para o corpo celular e dendritos, em função de seu desprendimento dos microtúbulos (Adaptado de Ittner and Gotz, 2011).

A atividade da proteína tau é regulada, a nível pós-traducional, por mecanismos de fosforilação e desfosforilação. Na DA, a hiperfosforilação da proteína tau parece resultar de uma maior atividade das tau quinases, ou da subsensibilização de suas fosfatases, ou ainda por ambos os mecanismos citados. Esses processos parecem ser desencadeados pelo acúmulo do peptídeo Aβ, acelerando a agregação da proteína tau e a formação dos emaranhados na região somatodendrítica de neurônios (Mazanetz and Fischer, 2007; Citron, 2010). A proteína tau possui mais de 30 sítios de fosforilação, dos quais muitos são em motivos formados por um resíduo serina ou treonina seguido de uma prolina (Ser/Thr-Pro) e, dessa maneira, fosforilados por proteínas quinases

dependentes de prolina (PDPK). Já foi demonstrado que a proteína tau pode ser fosforilada por muitas proteínas quinases, dentre essas a glicogênio sintase quinase 3-β (GSK-3β), que é considerada a principal quinase com a função de manter a estabilidade da proteína tau. Dentre as proteínas fosfatases, a proteína fosfatase 2A (PP-2A) tem sido descrita como a principal fosfatase com atividade sobre a proteína tau (Gong et al., 2006; Crespo-Biel et al., 2007). A hiperfosforilação da proteína tau, com o consequente desmonte dos microtúbulos e desorganização do transporte axonal, levam a importantes alterações neuronais, culminando em um processo de perda de função e morte neuronal (Hernandez et al. 2010; Avila et al., 2012). Na DA parece ocorrer um círculo vicioso entre as atividades do peptídeo Aβ e da proteína tau, enquanto os níveis elevados da proteína tau hiperfosforilada na região somatodendrítica estão associados com a maior vulnerabilidade neuronal para os efeitos tóxicos do Aβ, esse aumento da toxicidade do peptídeo Aβ, por sua vez, exacerba a fosforilação da proteína tau e do seu acúmulo sensibilizando ainda mais os neurônios para os efeitos tóxicos do peptídeo Aβ (Ittner and Gotz, 2011). Embora o mecanismo pelo qual ocorreria essa interconexão ainda não foi completamente compreendido, sugere-se possíveis envolvimentos da ativação de quinases pelo peptídeo Aβ tais como: a GSK-3β, que é principal enzima envolvida na fosforilação da proteína tau (Avila et al., 2012); a inibição da atividade do proteassoma pelo peptídeo Aβ, envolvido na degradação de proteínas não usuais, favorecendo o depósito dos emaranhados neurofibrilares (Blurton-Jones and LaFerla, 2006); a liberação de citocinas pró-inflamatórias, decorrentes do processo de inflamação desencadeado pelo peptídeo Aβ, as quais podem modular o processo de fosforilação da proteína tau (Quintanilla et al., 2004; Garwood et al., 2011).

1.4. Neuroinflamação e DA

O processo inflamatório crônico, desencadeado pelo peptídeo Aβ, parece ter importante contribuição no processo neurodegenerativo da DA. Os principais mediadores da inflamação observada na DA são os astrócitos reativos e a microglia ativada circundantes as placas senis (Wyss-Coray, 2006; Zaheer et al., 2008; McGeer and McGeer, 2010). A ativação prolongada e generalizada destas células gliais no cérebro se correlaciona com a extensão da atrofia cerebral e o declínio cognitivo (Cagnin et al., 2001; Parachikowa et al., 2007; Garwood et al., 2011). O componente neuroinflamatório presente na DA é caracterizado, além da ativação glial, por uma resposta local de fase aguda mediada por citocinas, ativação do sistema complemento, liberação de glutamato, indução de enzimas inflamatórias como a óxido nítrico sintase induzível (iNOS) e ciclooxigenase-2 (COX-2), com consequente aumento da geração de óxido nítrico (NO) e espécies reativas de oxigênio (ROS) (Rock et al., 2004; Heneka and O'Banion, 2007).

As citocinas representam um grupo de substâncias multifuncionais que estão envolvidas nos principais pontos da resposta inflamatória. O sistema imune influencia o sistema nervoso central (SNC) primariamente através de citocinas. Até o momento, mais de 100 membros das famílias das citocinas e seus respectivos receptores foram identificados (Barnes, 2001; Haddad, 2002). De maneira geral, as citocinas podem ser classificadas como pró ou anti-inflamatórias, dependendo do momento que irão interferir no processo inflamatório (Hopkins, 2003). Citocinas pró-inflamatórias, como IL-1β, IL-6, IL-18 e TNF-α, estão envolvidas na iniciação e amplificação do processo inflamatório, enquanto citocinas anti-inflamatórias, como IL-10 e TGF-1β, modulam negativamente esses eventos (Dinarello, 2000; Opal and DePalo, 2010). O hipocampo é a estrutura do SNC que apresenta a maior densidade de receptores para citocinas e

parece ser particularmente vulnerável ao processo inflamatório (Wilson et al., 2002; Mattson and Magnus, 2006). As citocinas influenciam mecanismos complexos que envolvem uma variedade de circuitos neurais como termo-regulação, apetite, padrões de sono e comportamento. Evidências também sugerem que redes de citocinas estão envolvidas em mecanismos centrais de memória e aprendizado (Wilson et al., 2002; Medeiros et al., 2007; Belarbi et al., 2012)

Dentre as citocinas alteradas na DA uma das mais estudadas é o TNF-α. O tratamento com o peptídeo Aβ no hipocampo acarreta um aumento na produção desta citocina pró-inflamatória principalmente pelas células microgliais (Ryu and McLarnon, 2008). Esse aumento de TNF-α parece ser um dos substratos biológicos associados aos déficits cognitivos ocasionados por processos neuroinflamatórios, uma vez que a ativação desta citocina, por modelo farmacológico ou genético, é capaz de atenuar esses déficits (Medeiros et al., 2007; Belarbi et al., 2012). Outra importante citocina pró-inflamatória alterada na DA é a IL-1β. Estudos com astrócitos já demonstraram que estas células gliais desempenham um papel importante na elevação de IL-1β na presença do peptídeo Aβ (Hou et al., 2011). Em modelos animais já foi observado que o aumento de IL-1β ocasiona déficits de aprendizado e que seu bloqueio pode reverter déficits cognitivos ocasionados por estímulos inflamatórios (Gibertini et al., 1995). Assim como o TNF-α e a IL-1β, a IL-6 parece ter uma importante função no processo inflamatório observado na DA. A exposição de neurônios hipocampais a IL-6 ocasiona o aumento da fosforilação da proteína tau através do aumento da atividade de quinases específicas (Quintanilla et al., 2004). Também já foram demonstradas correlações entre polimorfismos que alteram a produção da citocina antiinflamatória IL-10 e a ocorrência de DA (Ribizzi et al., 2010). Pacientes com DA apresentam menos capacidade de produção de IL-10 do que pacientes sadios (Saresella et al., 2011). Porém, ainda é

necessário mais informações sobre a relação entre IL-10 e a DA, especialmente sobre o papel desempenhado pelo peptídeo Aβ sobre esta citocina.

Durante a progressão do processo inflamatório, a presença quantitativa das neurotrofinas diminui concomitantemente ao aumento dos mediadores pró-inflamatórios (Figura 3) (Murer et al., 2001; Tong et al., 2008).

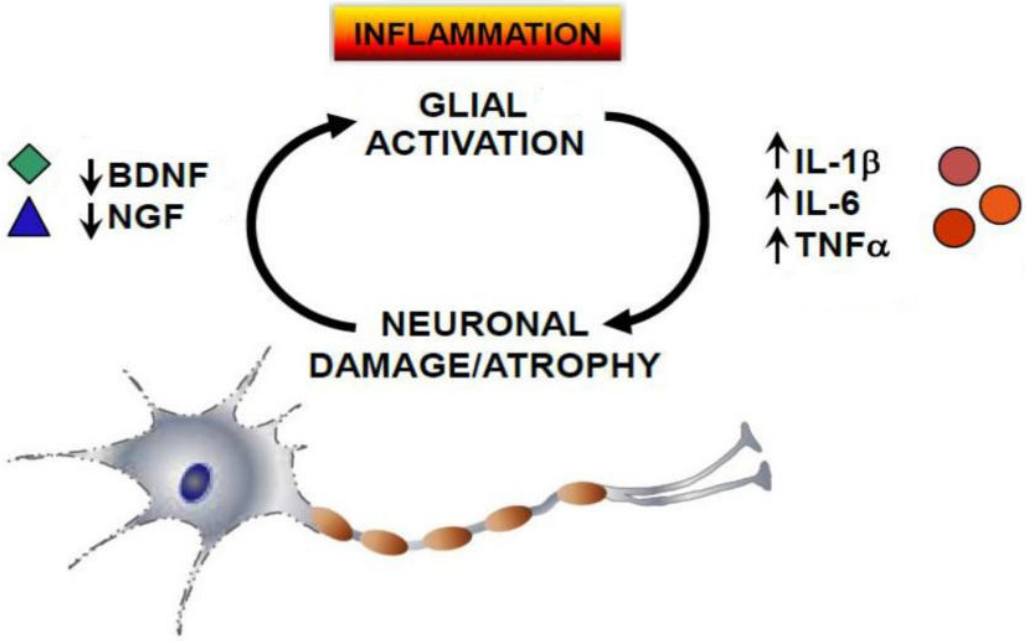


Figura 3. Envolvimento de citocinas e neurotrofinas na comunicação glia-neurônio no processo neuroinflamatório (Adaptado de Jurgens and Johnson, 2012).

1.5. BDNF

As neurotrofinas são uma família de proteínas que regulam diversos aspectos do desenvolvimento e funções neuronais, incluindo a formação de sinapses e plasticidade sináptica, além de serem importantes mediadores da comunicação intercelular neurônio-glia (Chao, 2003). Entre os mamíferos, os principais componentes da família são o fator de crescimento neuronal (NGF), fator neurotrófico derivado do encéfalo (BDNF), NT-3 e NT-4/5. O papel das neurotrofinas tem sido de extremo interesse, particularmente no que tange pesquisas envolvendo as doenças neurodegenerativas (Chao et al., 2006). O

estudo da expressão das neurotrofinas na DA tem dado importância especial ao BDNF, molécula altamente expressa no hipocampo que possui a capacidade de modulação da plasticidade sináptica e mediação do processo de sobrevivência e morte neuronal (McAllister et al., 1999; Tyler et al., 2002, Qian et al., 2007). O BDNF é considerado fator essencial no mecanismo de formação de aprendizagem e memória (Bliss and Collingridge, 1993; Bekinschtein et al., 2008; Lou et al., 2008). Análises *post mortem* de pacientes com DA mostraram níveis reduzidos de BDNF em tecidos cerebrais, embora a natureza desta observação permaneça parcialmente compreendida até o momento (Mattson, 2008; Querfurth and LaFerla, 2010; Wang et al., 2011). A ação do BDNF ocorre através de sua ligação aos receptores TrkB, promovendo uma cascata de sinalizadores intracelulares e de transcrição através de distintas vias neuroquímicas (MAPK, PI3K, PKA) (Brunet et al., 2001; Reichardt, 2006; Zeng et al., 2010). Deste modo, o BDNF é capaz de modular moléculas importantes para a transmissão sináptica, tais como sinapsina I, cálcio/calmodulina proteína quinase ativada II (CaMKII) e o fator de transcrição proteína de ligação ao elemento de resposta do AMPc (CREB) (Gómez-Pinilla et al., 2007). Estudos em diferentes modelos animais têm demonstrado que a ativação dos receptores TrkB por BDNF promove a sobrevivência neuronal em grande parte através da ativação da via de sobrevivência celular PI3K/Akt (Yoshii and Constantine-Paton, 2007; Jantas et al., 2009).

1.6. Sinalização Celular e DA

Sinalização celular é o mecanismo pelo qual sinais que estão presentes no meio extracelular são transmitidos via receptores a fim de serem interpretados intracelularmente. O controle inadequado das vias de sinalização contribui para a perda da homeostase neural, responsável pelo desencadeamento e progressão de muitas das

doenças neurodegenerativas incluindo a DA (Fuentealba et al., 2004; Park and Bowers, 2010). Os mecanismos envolvidos no dano sináptico e na morte celular da DA, como formação de espécies reativas (ERs), neuroinflamação, distúrbios na homeostase do cálcio intracelular, hiperfosforilação da tau, toxicidade do peptídeo Aβ e apoptose estão diretamente relacionados com alterações em vias de sinalização (Chiang et al., 2010). Dessa forma, a pesquisa das vias de sinalização envolvidas na patogênese da DA é extensa e bastante complexa. A integração destas vias foi realizada recentemente através da elaboração de um mapa chamado "AlzPathway", no qual estão catalogadas muitas das vias de sinalização envolvidas com a DA. Para dimensionar a complexidade do tema, este mapa é atualmente composto por 1347 moléculas (proteínas, genes, RNAs, íons, produtos de degradação, fenótipos), 1070 reações (em neurônios, região pré-sináptica e pós-sináptica, astrócitos, microglia, barreira hematoencefálica) e suas localizações celulares (Figura 4) (Mizuno et al., 2012).

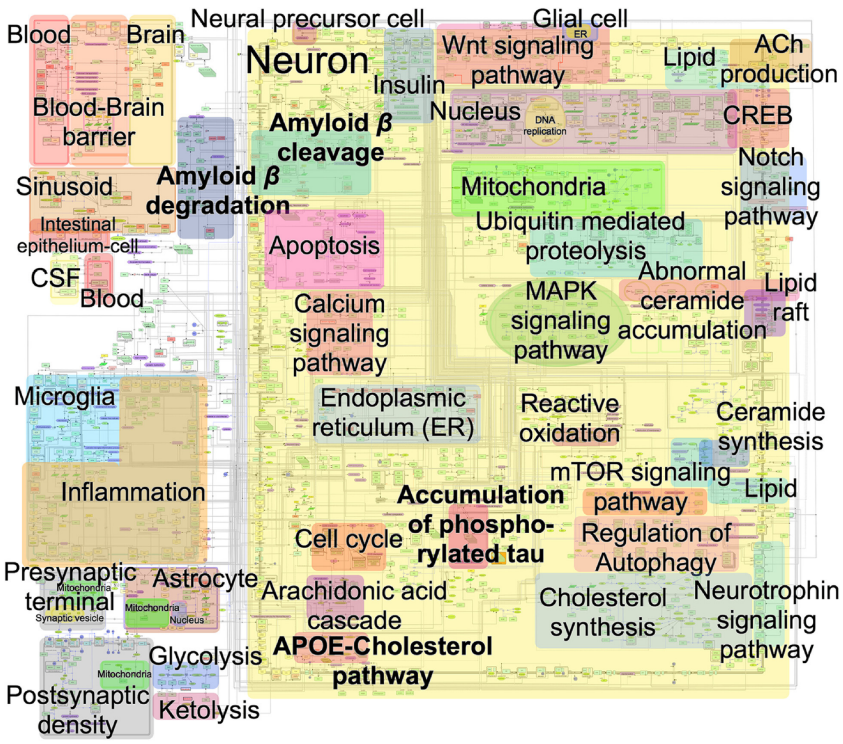


Figura 4. Complexidade da sinalização celular envolvida com a patogênese da DA. "AlzPathway" é um mapa global contento as vias de sinalização intra, inter e

extracelulares correlacionadas a DA. O banco de dados completo encontra-se disponível em http://alzpathway.org/. (Adaptado de Mizuno et al., 2012).

No entanto, o conhecimento adquirido sobre as vias de sinalização envolvidas na patogênese da DA e a inter-relação destas com as placas senis e os emaranhados neurofibrilares permanece longe de estar elucidado (Cakala et al., 2007; Chiang et al., 2010; Mizuno et al., 2012).

1.6.1 A via de sinalização celular PI3K/Akt

A via de sinalização fosfatidilinositol-3-quinase (PI3K)/Akt possui um importante papel na sobrevivência celular em vários tipos neuronais (Lawlor and Alessi, 2001; Kim and Chung, 2002). Mais recentemente estudos também têm demonstrado o papel da via PI3K/Akt na plasticidade sináptica (Horwood et al., 2006; Chen et al., 2011). Além disso, crescentes evidências sugerem que a via PI3K/Akt é diretamente influenciada pela exposição ao peptídeo Aβ e encontra-se alterada no cérebro de pacientes com AD (Jimenez et al., 2011). PI3K é ativada através da ligação de vários estímulos aos receptores tirosina quinase (RTKs), como fatores de crescimento, BDNF, insulina, fator de crescimento tipo insulina-I (IGFI) e citocinas (Lawlor and Alessi, 2001). Uma vez ativada gera fosfatidilinositol-3,4,5-trifosfato (PIP3), o qual é fundamental para a ativação da proteína quinase B (PKB/Akt) (Brunet et al., 2001; Sarbassov et al., 2005).

1.6.1.1. Akt

Akt está altamente expressa no SNC, constituindo um promotor de sobrevivência e proteção neuronal (Brunet et al., 2001). Quando ativa, a Akt inibe a apoptose por fosforilar e inativar uma variedade de substratos pertencentes à maquinaria celular apoptótica, tais como a BAD - proteína pró-apoptótica pertencente à família das Bcl-2

(oncogene da célula B) (Yuan and Yankner, 2000), GSK-3β (Cross et al., 2001), caspase-9, e a família de fatores da transcrição em "forquilha" (*Forkhead*, FKHR) (Brunet et al., 1999). Akt também parece fosforilar diretamente a proteína tau (Vivanco and Sawyers, 2002; Ksiezak-Reading et al., 2003) (Figura 5).

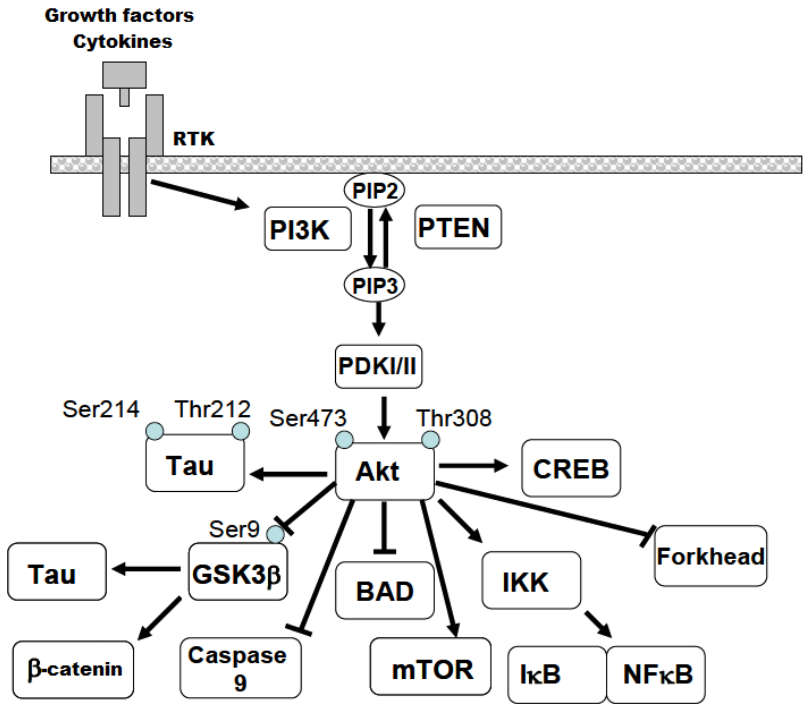


Figura 5. Via de sinalização celular PI3K/Akt simplificada. Detalhe da ativação da Akt e a regulação de seus substratos (Adaptado de Vivanco and Sawyers, 2002).

1.6.1.2. GSK-3β

Um dos principais alvos da proteína Akt é a proteína glicogênio sintase quinase 3-β. A GSK-3β é uma proteína constitutivamente ativa, no entanto ao ser fosforilada em Serina 9, pela proteína Akt, torna-se inativa (Cross et al., 2001). Diversas evidências têm demonstrado que a proteína GSK3-β desempenha papel fundamental na cascata de eventos envolvidos com a DA, visto que esta quinase está envolvida na indução da hiperfosforilação da proteína tau, modula negativamento mecanismos envolvidos com aprendizagem e memória, e estimula a liberação de citocinas envolvidas com a resposta

inflamatória (Mandelkov et al., 1992; Hooper et al., 2008; Dewachter et al., 2009; Green et al., 2012). Além de ser uma das principais quinases envolvidas na hiperfosforilação da proteína tau, a GSK-3β é considerada uma enzima pró-apoptótica por inibir a ativação de uma variedade de proteínas e fatores de transcrição importantes para sobrevivência, proliferação e crescimento celular (Li et al., 2002).

Exemplo disso é seu envolvimento na regulação dos níveis intracelulares da proteína β-catenina, componente fundamental da via de sinalização Wnt (Lee et al., 2000; Salinas and Zou, 2008). Quando a GSK-3β está ativa, atua fosforilando a β-catenina, facilitando sua ubiquitinação e degradação proteolítica, inibindo assim sua atividade. Quando desfosforilada a β-catenina é estável e capaz de se translocar para o núcleo ativando a família de fatores de transcrição do fator promotor de célula T/linfócitos (Tcf/Lef) importantes para a expressão de genes que codificam para a sobrevivência e homeostase neuronal (Inestrosa et al., 2007; Chong et al., 2007; Zhang et al., 2011).

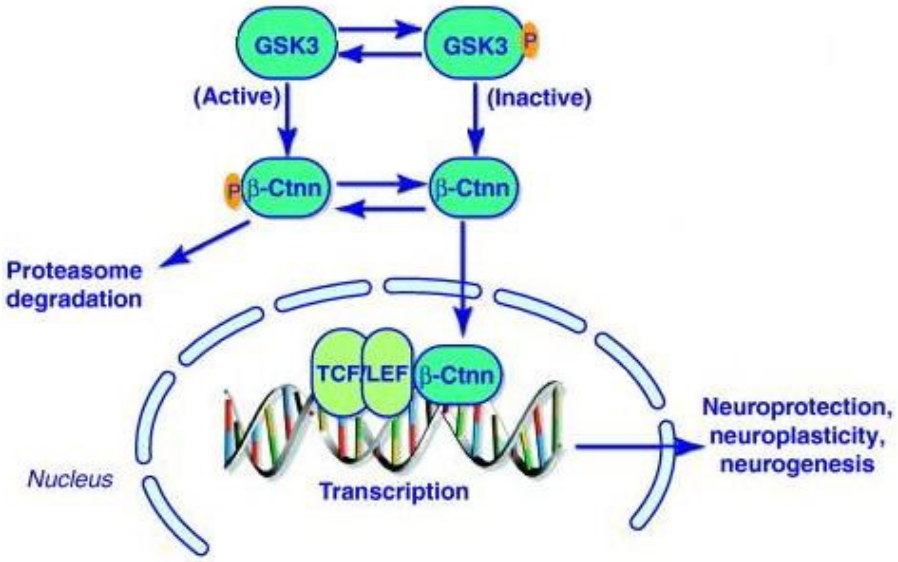


Figura 6. Sinalização celular envolvendo GSK-3β e Wnt/β-catenina. A atividade aumentada da GSK-3β reduz a estabilidade da β-catenina encaminhando-a para degradação, enquanto que a inibição da GSK-3β facilita a translocação da β-catenina para o núcleo promovendo a expressão gênica (Adaptado de Duman and Voleti, 2012).

1.6.2. Sumoilação e DA

Como já mencionado acima, a modificação pós-traducional de proteínas é crítica para a regulação espacial e temporal das cascatas de sinalização, e isto é especialmente importante no sistema nervoso central, onde os processos que afetam diferenciação, crescimento, segmentação e comunicação entre os neurônios são altamente complexos e muito bem regulados. Na última década, observou-se que a modificação de proteínas por membros da família SUMO (Small Ubiquitin-like Modifier) de proteínas está relacionada com a função neural. Dessa forma, a sumoilação de uma variedade de proteínas intra e extra nucleares têm sido relacionadas a uma gama diversificada de processos que possuem implicações na função e na fisiopatologia neuronal (Geiss-Friedlander et al., 2007; Wilkinson et al., 2010; Wilkinson and Henley, 2010).

A sumoilação envolve uma ligação covalente de um membro da família de proteínas SUMO a um resíduo de lisina em proteínas alvo específico através de uma cascata enzimática análoga, mas distinta, da via de ubiquitinação. Existem três parálogos SUMO presentes em mamíferos, designadas de SUMO-1 a SUMO-3, e um número crescente de proteínas estão sendo identificadas como alvo da SUMO, porém pouco se sabe como esta sumoilação é regulada (Wilkinson et al., 2010; Lee et al., 2011).

Durante o ciclo de conjugação da SUMO, a molécula responsável por conjugar a SUMO ao seu alvo é a sua enzima de conjugação (Ubc9). A sumoilação, apesar de ser uma ligação covalente, é prontamente revertida com a ação de proteases específicas (SENPs). O equilíbrio entre a conjugação mediada por Ubc9 e a desconjugação mediada por SENP determina o estado de sumoilação de uma proteína específica (Figura 7) (Gareau and Lima, 2010; Wilkinson et al., 2010; Wilkinson and Henley, 2010).

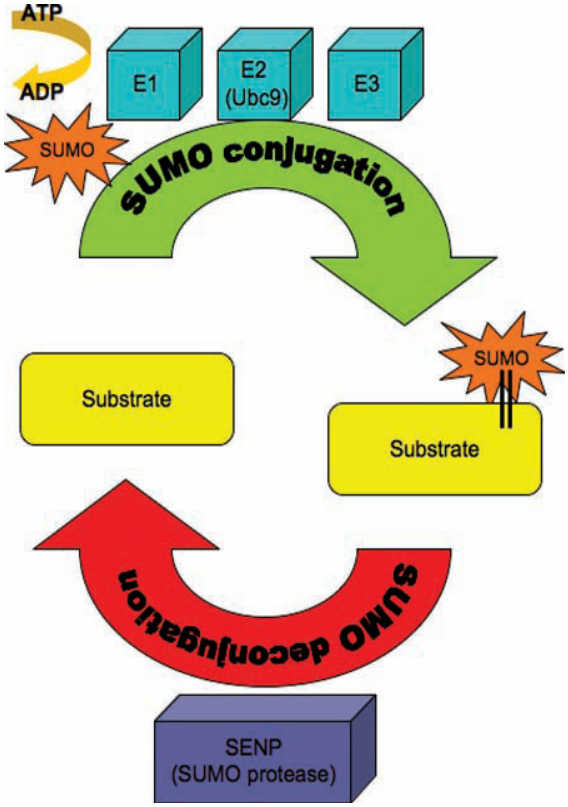


Figura 7. Ciclo de Sumoilação. A proteína SUMO é transcrita como um precursor inativo que é clivada por membros da SENP para a forma madura. Esta forma madura é ativada através do uso da energia do ATP, e será conjugada com o auxílio da proteína conjugadora Ubc9, a qual catalisa a transferência da SUMO para a proteína alvo. A desumoilação é mediada por membros da família SENP, liberando a SUMO para realizar mais ciclos de conjugação a proteínas alvo (Adaptado de Dorval and Fraser, 2007).

A principal função da sumoilação é modificar, inibir ou ativar interações proteína-proteína (Geiss-Friedlander et al., 2007). A sumoilação se mostrou essencial em células eucarióticas e a supressão ou deleção da Ubc9, um componente crítico da sumoilação, é fatal para células de mamíferos (Hayashi et al., 2002; Nacerddine et al., 2005). Visto que a sumoilação desempenha papel importante na sinalização, desenvolvimento e excitabilidade neuronal, não surpreende que a desregulação desta via tem sido relacionada com a patofisiologia de doenças neurodegenerativas (Dorval and Fraser, 2007; Anderson et al., 2009). A dinâmica de fosforilação/desfosforilação de proteínas

desempenha papel crítico em várias vias de sinalização celular e um número de quinases, fosfatases e proteínas de estruturação envolvidas na ativação destas vias têm sido relatadas como sendo alvos de conjugação por SUMO (Gareau and Lima, 2010; Wilkinson et al, 2010).

Os estudos sobre o papel da sumoilação na DA ainda são recentes e bastante controversos. Já foi observado que a proteína APP, que dá origem ao peptídeo Aβ, pode ser sumoilada e que esta sumoilação regula negativamente a geração do peptídeo Aβ, reduzindo dessa maneira os níveis do peptídeo agregado (Zhang and Sarge, 2008). Porém, também existem resultados na literatura apontando que a sumoilação pode estar relacionada com o aumento da produção deste peptídeo (Dorval et al, 2007). Os resultados obtidos até o momento ainda não são claros quanto à implicação da proteína SUMO na DA, mas mostram-se promissores para um importante envolvimento na patogênese da doença. Níveis elevados de oligômeros do Aβ podem contribuir para a morte neuronal através da via da c-Jun-N- terminal quinase (JNK), uma proteína quinase associada a estímulos de estresse celular (Thakur et al., 2007; Mehan et al., 2011). Estudos demonstram uma ligação entre a via da JNK e a via da sumoilação protéica na resposta ao estresse celular, porém estes estudos também permanecem controversos. Alguns estudos já demonstraram que muitos dos fatores de transcrição ativados por JNK como c-Jun, p53, Elk-1, Smad4 são também regulados por SUMO-1 (Mueller et al., 2000; Lee et al., 2005; Feligione et al., 2011).

1.7. Memória e Aprendizado: déficit cognitivo na DA

O comprometimento da memória é o sintoma clínico de maior magnitude na DA. Nos estágios iniciais, geralmente encontramos perda de memória episódica e dificuldades na aquisição de novas habilidades, evoluindo gradualmente com prejuízos

em outras funções cognitivas, tais como julgamento, raciocínio e habilidades visuo-espaciais (Budson and Price, 2005; Sperling et al., 2010).

Embora geralmente confundidos, memória e aprendizado possuem conceitos distintos. Aprendizado é a aquisição de novas informações resultado da interação do sujeito com o meio. A retenção das informações aprendidas para a evocação mais adiante é chamada de memória (Izquierdo and Medina, 1997). As memórias mantidas durante o tempo necessário para sua análise (frações de segundos ou poucos segundos) são chamadas de memórias imediatas. As memórias que são armazenadas por um curto período de tempo (segundos, minutos, poucas horas) são chamadas de memória de curto prazo (short-term memory, STM), e as memórias mantidas por um longo período (muitas horas, dias, semanas, anos, permanentemente) são conhecidas como memória de longo prazo (long-term memory, LTM) (McGaugh, 2000). A consolidação é o processo pelo qual as informações aprendidas são armazenadas, e ocorre através de uma série de eventos bioquímicos envolvidos com a sinalização intracelular de formação da memória. Estes eventos parecem ser similares aos descritos para a potenciação de longa duração (LTP) no hipocampo, mecanismo celular relacionado ao aprendizado e memória. A LTP é caracterizada pelo o aumento persistente da transmissão sináptica glutamatérgica entre o neurônio pré- e o pós-sináptico (Bliss and Collingridge, 1993; Izquierdo and Medina, 1997; Granger et al., 2013). Dentre os eventos de sinalização intracelular envolvidos na transmissão sináptica, a proteína quinase II dependente de cálcio/calmodulina (CaMKII) apresenta função chave na modulação da plasticidade sináptica, indução de LTP e formação da memória (Figura 8) (Yamauchi, 2005; Miyamoto, 2006; Lisman et al., 2012). Recentemente, a desregulação da fosforilação da proteína CaMKII nas sinapses tem sido implicada na toxicidade induzida pelo peptídeo

Aβ e no déficit de memória observado na DA (Zeng et al., 2010; Ly and Song, 2011; Wang et al., 2013).

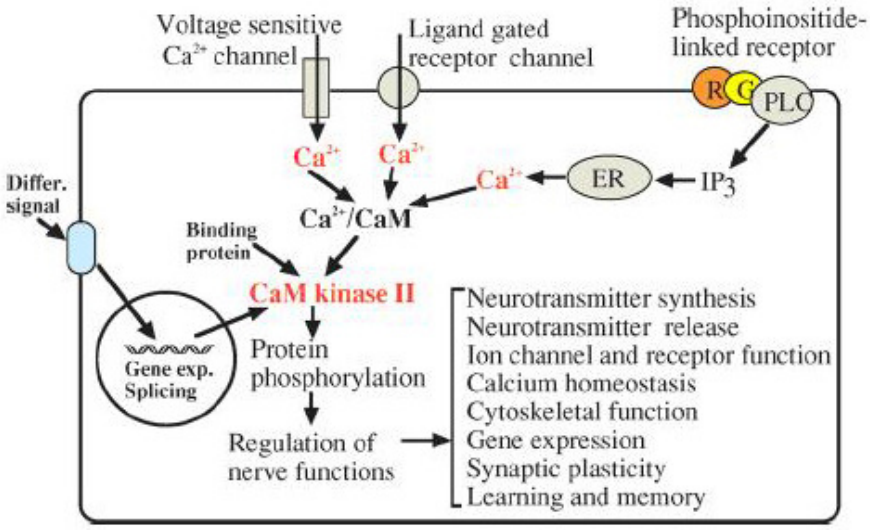


Figura 8. As múltiplas funções da proteína CaMKII. O aumento do influxo de Ca^{2+} intracelular leva a formação do complexo Ca^{2+}/Calmodulina (CaM), que por sua vez ativa a autofosforilação da proteína CaMKII. CaMKII ativada possui a capacidade de fosforilar e ativar múltiplas proteínas envolvidas na plasticidade sináptica tanto em neurônios pré-sinápticos (Sinapsina I) quanto nos pós-sinápticos (receptores glutamatérgicos do tipo AMPA) (Adaptado de Yamauchi, 2005).

1.7.1. Neuroeletrofisiologia através de matrizes multieletrodos

A utilização de técnicas *in vitro* para registro de correlatos eletrofisiológicos da atividade neural é uma importante ferramenta empregada para o estudo das patologias do sistema nervoso. As sinapses são locais chaves para a modificação de propriedades funcionais das redes neuronais, possivelmente fornecendo a base para o fenômeno de aprendizagem e memória. A atividade elétrica produzida pelo cérebro pode ser detectada por eletrodos em diferentes níveis de localização e reflete o comportamento médio de populações de neurônios, onde cada célula neuronal contribui através de seus potenciais pós-sinápticos inibitórios (PIPS), excitatórios (PEPS) e disparos de

potenciais de ação (Srinivasan and Nunez, 2006). O registro de um potencial de ação pode ser realizado a partir de duas configurações básicas, intracelular ou extracelular. Na realização de medidas extracelulares, o sinal captado representa não a atividade individual de um neurônio, mas sim o comportamento geral da população neural, chamado de potencial de campo local (LFP).

A maioria dos estudos eletrofisiológicos funcionais *in vitro* dos efeitos do peptídeo Aβ sobre os neurônios até o momento foram realizados utilizando o método "patch clamp" (Lambert et al., 1998; Jhamandas et al., 2001; Gureviciene et al., 2004). Um avanço tecnológico recente para o monitoramento crônico e não-invasivo da atividade elétrica de neurônios é a utilização de matrizes multieletrodos (MEAs) para medida dos LFPs (Chong et al., 2011; Liu et al., 2012; Spira and Hai, 2013). Em contraste com as técnicas eletrofisiológicas intracelulares mais comumente utilizadas, que geralmente permitem o monitoramento da atividade das células individualmente e apenas em curto prazo, as MEAs são ideais para investigar os efeitos de longa duração sem limitar o número de células que podem ser analisadas em um dado momento (Jung et al., 1998; Hofmann e Balding, 2006). Além disso, a MEA constitui um sistema *in vitro* ideal para monitorar os efeitos de drogas e toxinas, promovendo conclusões importantes sobre a atuação neuroquímica específica para o estudo de fármacos (Potter and De-Marse, 2001; Varghese et al., 2010).

Dessa forma, as MEAs representam um importante refinamento do conceito de interface bioeletrônica. Uma interface bioeletrônica pode ser descrita como um sistema onde células ou fatias de tecido nervoso, mantidas viáveis em cultura celular, são conectadas a um circuito elétrico, a fim de estabelecer um intercâmbio bidirecional de informação (Figura 9) (Besl and Fromherz, 2002; Fromherz, 2002).

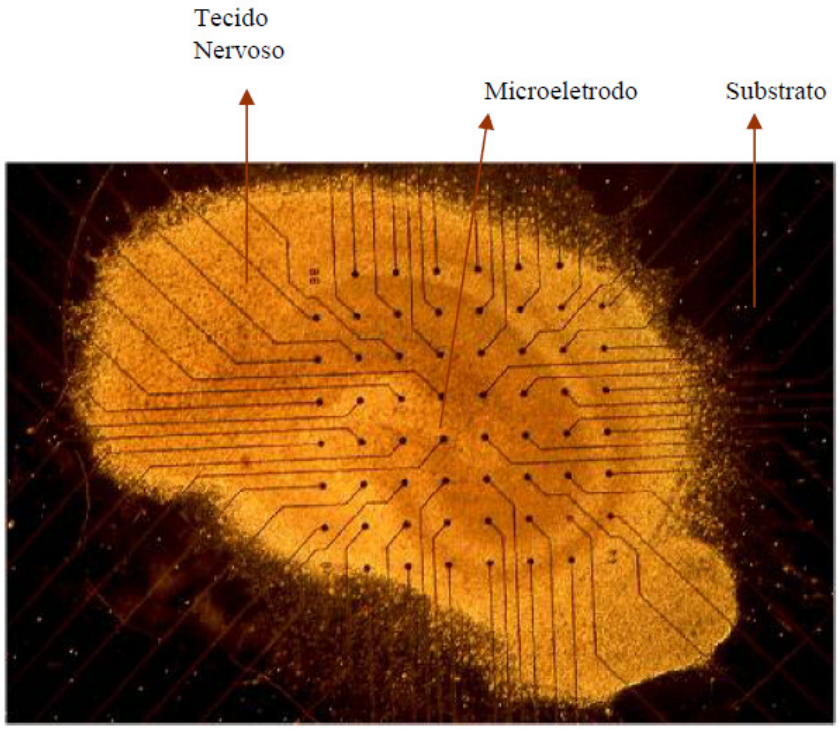


Figura 9. Conjunto de eletrodos em uma fatia organotípica hipocampal. Matrizes multieletrodos são dispositivos utilizados para estimular e registrar *in vitro* a atividade elétrica de culturas celulares ou fatias cerebrais, e consistem em microchips que apresentam configurações de microeletrodos (Adapatada de Rennaker et al., 2005).

1.8. Curcumina

O desenvolvimento de terapias efetivas para a DA representa um grande desafio atual e futuro, visto o grande número de pessoas afetadas atualmente pela DA e diante das evidências do aumento significativo de pessoas com essa patologia nas próximas décadas. As terapias atuais para a DA apenas fornecem benefícios discretos para os sintomas clínicos da doença, particularmente nos estágios iniciais. Infelizmente, ainda não existem terapias capazes de afetar o desenvolvimento do processo neurodegenerativo dessa desordem. Diante dessa realidade, muitas estratégias terapêuticas neuroprotetoras com o objetivo de retardar ou impedir a neurodegeneração têm sido amplamente pesquisadas. Derivados de compostos naturais representam uma

grande fonte para o desenvolvimento de medicamentos, cerca de metade das drogas aprovadas como novos medicamentos durante os anos de 1995 a 2005 foram desenvolvidos a partir de produtos naturais (Refolo and Fillit, 2004; Harvey, 2008). Dentre os muitos compostos investigados, a curcumina tem emergido como um agente com múltiplas propriedades biológicas e seu efeito neuroprotetor na DA tem sido objeto de intensa pesquisa na última década. A curcumina (1,7-bis-(4-hidroxi-3-metoxifenil)-1,6-heptadieno-3,5-diona), caracterizada quimicamente em 1910 (Figura 10), é um composto polifenólico naturalmente encontrado no rizoma de *Curcuma Longa,* amplamente usado como corante amarelo e tempero em alimentos, como o curry (Park et al., 2008). O interesse nos efeitos neuroprotetores da curcumina na DA iniciou a partir de estudos epidemiológicos na Índia, onde a *Curcuma Longa* é amplamente usada na alimentação, mostrarem que a incidência de pacientes com Doença de Alzheimer neste país foi significativamente menor que os observados nos Estados Unidos (Ganguli et al., 2000; Ng et al., 2006; Mishra and Palanivelu, 2008).

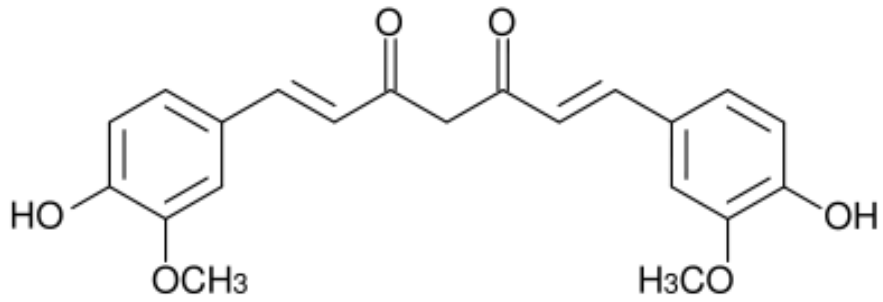


Figura 10. Estrutura química da curcumina. A curcumina é um polifenólico lipossolúvel, estruturalmente composto de dois anéis metoxifenol unidos por uma β-dicetona.

A curcumina demonstrou ter propriedades antioxidantes, anti-inflamatórias e anti-amiloidogênicas (Zhao et al., 1989), e desta forma reduzir a quantidade de placas amilóides em modelos animais da DA (Lim *et al*., 2001; Begum et al., 2008). A

curcumina antagoniza muitas etapas do processo inflamatório, incluindo a ativação de NFκ-B, iNOS e JNK (Weber et al., 2006). Além disso, o tratamento com curcumina aumenta a sobrevivência celular em diferentes modelos *in vitro* de toxicidade pelo peptídeo Aβ (Kim et al., 2001; Park et al., 2008; Qin et al., 2010; Huang et al., 2012). O hipocampo e as regiões corticais são os principais locais de transmissão colinérgica para o processamento do aprendizado e memória, e parecem ser as mais propensas aos danos oxidativos observados na DA. Uma vez que o dano nas sinapses destas regiões contribui para o déficit cognitivo, a curcumina, por sua vez, também apresenta um papel benéfico nas funções de memória observado em modelos animais de DA (Pan et al., 2008; Agrawal et al., 2010; Ahmed et al., 2010). Apesar dos avanços na compreensão dos efeitos neuroprotetores da curcumina na DA, seus mecanismos moleculares permanecem distantes de estarem elucidados.

A maioria dos estudos demonstrando efeitos benéficos do tratamento com curcumina na DA até o momento foram realizados em culturas celulares ou modelos animais da doença. Nos modelos *in vivo* da doença as doses de curcumina que se mostraram efetivas foram geralmente altas devido a baixa solubilidade em água e o curto tempo de meia-vida da curcumina, gerando uma biodisponibilidade reduzida deste composto no plasma e tecidos. Estudos em animais demonstraram que a biodisponibilidade oral de curcumina em ratos é de apenas 1% (Yan et al., 2011). Além disso, estudos clínicos com o uso de curcumina em pacientes com DA não apresentaram evidências clínicas ou bioquímicas de sua eficácia, e sugeriram que a baixa disponibilidade deste composto foi o fator limitante (Baum et al., 2008; Ringman et al., 2012).

Nesse sentido, alguns grupos de pesquisa têm utilizado a nanobiotecnologia e os efeitos de nanopartículas contendo curcumina já vêm sendo promissor (Thangapazham

et al., 2008; Ray et al., 2011; Jaques et al., 2013; Zanotto-Filho et al., 2013). Extensivas estratégias têm sido realizadas a fim de aumentar a vetorização da curcumina ao SNC (Ma et al., 2008; Chen et al., 2009; Shaik et al., 2009). As nanopartículas poliméricas são sistemas carreadores de fármacos que apresentam diâmetros inferiores a 1µm. Dentre as nanopartículas poliméricas amplamente utilizadas para a entrega de fármacos destacam-se as nanocápsulas. Estas são formadas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica, dependendo das propriedades físico-químicas e da composição das nanocápsulas (Figura 11). Dessa forma, nanocápsulas podem ser consideradas um sistema "reservatório" (Couvreur et al., 2002). As nanocápsulas apresentam alto potencial de encapsulamento de substâncias, especialmente as lipofílicas, são capazes de controlar a liberação de fármacos em sítios de ação específicos e otimizar a velocidade de cedência, gerando respostas adequadas por prolongados períodos de tempo. Além disso, aumentam a estabilidade de armazenamento e no organismo, melhoram regimes de dosagens de substâncias e o índice terapêutico por aumentarem a eficácia e/ou reduzirem a toxicidade de fármacos, diminuindo a absorção sistêmica (Meier, 2000; Barrat, 2003; Schaffazick et al., 2003; Guterres et al., 2007). Dentre os polímeros utilizados, a poli(ε-caprolactona) se destaca devido sua biocompatibilidade e biodegradabilidade (Guterres et al., 2007). O revestimento das nanocápsulas poliméricas com polissorbato 80 já foi relatado pela literatura como fundamental para a vetorização cerebral (Ambruosi et al., 2006; Zhang et al., 2006).

Desta maneira, o encapsulamento de fármacos é uma alternativa importante para mascarar suas propriedades físico-químicas, aumentar sua interação com as membranas, bem como o transporte através delas, além de permitir sua liberação sustentada e

aumentar a estabilidade, a solubilidade e a disponibilidade em fluidos biológicos e tecidos (Roney et al., 2005; Faraji et al., 2009).

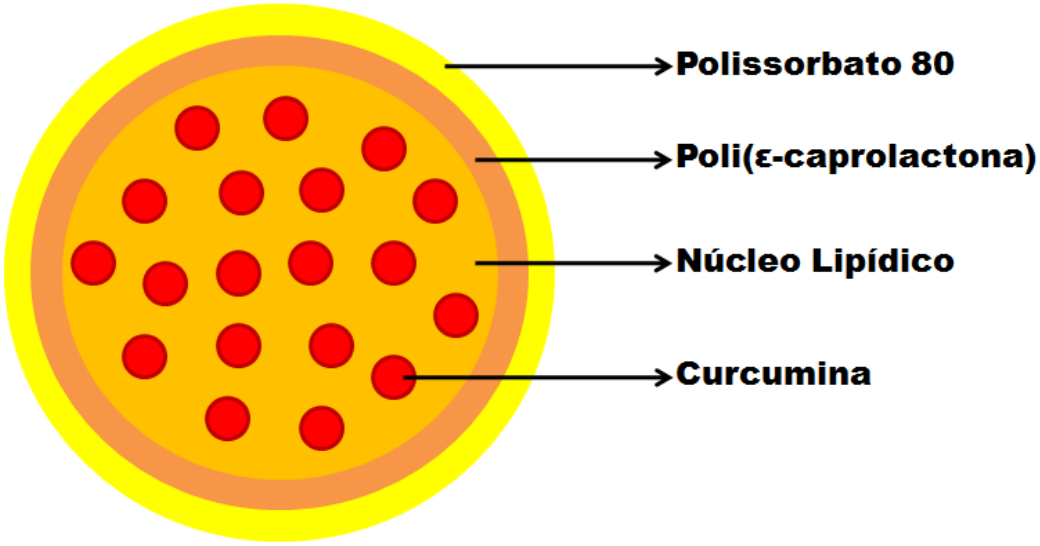


Figura 11. Composição e organização estrutural da nanocápsula polimérica de curcumina utilizada neste trabalho.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar o potencial efeito neuroprotetor da curcumina em modelos *in vitro* e *in vivo* de toxicidade induzida pelo peptídeo beta-amiloide (Aβ).

2.2. Objetivos Específicos

1) Investigar o efeito da curcumina sobre a morte celular, o estresse oxidativo e a neuroinflamação induzidos pelo peptídeo $A\beta_{1-42}$ em modelo de cultura organotípica de hipocampo de ratos e verificar o possível envolvimento da via de sinalização PI3K/Akt na neuroproteção mediada pela curcumina;

2) Avaliar o efeito da curcumina sobre o perfil de sumoilação em modelo de cultura primária de astrócitos expostos ao peptídeo $A\beta_{1-42}$ e verificar se a alteração deste perfil pode conferir aumento ou diminuição da reatividade astrocitária;

3) Verificar o efeito da curcumina na alteração da excitabilidade neuronal induzida pelo peptídeo $A\beta_{1-42}$ em culturas organotípicas de hipocampo de ratos e verificar o possível envolvimento das proteínas CaMKII e sinapsina I;

4) Comparar o efeito do tratamento com curcumina livre e curcumina nanoencapsulada em um modelo *in vivo* de toxicidade induzida pelo peptídeo $A\beta_{1-42}$ e verificar o possível envolvimento da via de sinalização PI3K/Akt na neuroproteção mediada pela curcumina.

3. CAPÍTULO I

Artigo: *The curry spice curcumin attenuates β-amyloid-induced toxicity through β-catenin and PI3K signaling in rat organotypic hippocampal slice culture –* ***Status:*** *Publicado no periódico Neurological Research*

The curry spice curcumin attenuates β-amyloid-induced toxicity through β-catenin and PI3K signaling in rat organotypic hippocampal slice culture

Juliana Bender Hoppe[a*], Rudimar Luiz Frozza[b], Elisa Nicoloso Simões Pires[a], André Bevilacqua Meneghetti[a] and Christianne Salbego[a].

[a] Laboratory of Neuroprotection and Cell Signaling, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b] Laboratory of Neurodegenerative Diseases, Institute of Medical Biochemestry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[*]Corresponding author. Address: Laboratory of Neuroprotection and Cell Signaling, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil, Telephone: +55 (51) 3308.5548, Fax: +55 (51) 3308.5535.

E-mail address: juhoppe@hotmail.com (JB Hoppe)

Abstract

Objective: Accumulating evidence indicates that curcumin potently protects against Aβ due to its oxygen free radicals scavenging and anti-inflammatory properties. However, cellular mechanisms that may underlie the neuroprotective effect of curcumin in Aβ-induced toxicity are not fully understood yet. The present study was undertaken to investigate the mechanisms involved in these curcumin neuroprotective effects, particularly involving Wnt/β-catenin and PI3K pathways.

Methods: Organotypic hippocampal slice cultures were treated with curcumin and exposed to $A\beta_{1-42}$ by 48 h. Synaptic dysfunction, cell death, ROS formation, neuroinflammation and β-catenin, Akt and GSK-3β phosphorylation were measured to determine the effects of curcumin against Aβ toxicity.

Results: Curcumin significantly attenuated Aβ-induced cell death, loss of synaptophysin and ROS generation. Furthermore, curcumin was able to decrease IL-6 release and increase IL-10 release, and prevented glial activation. The phosphorylation of β-catenin was avoided and the levels of free β-catenin were increased by curcumin to promote cell survival upon treatment with Aβ. Curcumin, in the presence of Aβ, activated Akt which in turn phosphorylates GSK-3β, and resulted in the inhibition of GSK-3β. The presence of LY294002, an inhibitor of PI3K pathway, blocked the pro survival effect of curcumin.

Discussion: These results reinforce the neuroprotective effects of curcumin on Aβ toxicity and add some evidence that its mechanism may involve β-catenin and PI3K signaling pathway in organotypic hippocampal slice culture.

Keywords: β-amyloid, β-catenin, curcumin, organotypic hippocampal slice culture, PI3K signaling pathway

Introduction

Alzheimer's disease (AD) is the most common dementia disorder and is characterized by widespread neurodegeneration, accumulation of beta-amyloid (Aβ) aggregates, and formation of neurofibrillary tangles (NFTs) in the brain[1,2]. Aβ has been emphasized as a key molecule in AD pathogenesis. The amyloid cascade hypothesis suggests that increased Aβ results in inflammatory response, oxidative stress, altered central nervous system homeostasis, synaptic dysfunction, cellular death, and dementia[3,4,5].

The mechanisms underlying Aβ-induced neurotoxicity are not fully characterized, and treatments, including dietary interventions, which may reduce neuroinflammation and oxidative stress, are of great therapeutic interest. Curcumin, the curry flavoring and coloring agent, is the principal curcuminoid constituent of the spice turmeric[6]. Extensive studies have been carried out to investigate the pharmacological properties of curcumin. Curcumin modulates the expression of various molecular targets, such as transcription factors, enzymes, cytokines, cell cycle proteins, receptors and adhesion molecules[7,8]. In fact, curcumin has shown wide range of pharmacological activities including powerfull anti-inflammatory[9] and anti-oxidant[10] effects. The pleiotropic activities of curcumin derive from its complex chemistry as well as its ability to influence multiple signaling pathways, including survival pathways[11,12]. Over the past decade, curcumin has been reported to have potent neuroprotective effect in different *in vitro* and *in vivo* AD models[13,14,15]. So far, studies examining the molecular mechanisms mediating the therapeutic effect of curcumin particularly in AD are still incomplete.

More and more studies have proposed that neuroprotective substances can prevent Aβ-induced neuronal death by altering several intracellular signaling pathways

and these changes are tightly correlated with the genesis and development of AD. The phosphatidylinositol 3-kinase (PI3K) pathway is known to be important in neuronal cell survival and death and to be affected by inflammatory response and oxidative stress[16,17]. Akt, the direct downstream effector of PI3K, in turn regulates a wide range of target proteins such as glycogen synthase kinase 3β (GSK-3β) which regulates cell survival, proliferation and growth[18]. GSK-3β can phosphorylate β-catenin, facilitating its proteolysis to promote the neuronal degeneration in AD. β-catenin, as an integral component in the canonical Wnt signaling pathway, is involved in controlling several development processes including cell fate specification, axis patterning, neural development, and synapse development[19,20].

In the present work, we investigated whether curcumin could protect organotypic hippocampal slice cultures against Aβ-induced toxicity and the mechanisms by which it did so, with a special focus on the β-catenin and PI3K/Akt/GSK3-β signaling pathway.

Materials and Methods

Organotypic hippocampal culture

All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and were approved by the Ethics Committee on the Use of Animals (CEP-UFRGS, protocol number 20005). Hippocampal slice cultures were prepared from 6- to 8-day-old male Wistar rats[21]. Briefly, the animals were sacrificed, the brains were removed, the hippocampi were isolated, and transverse hippocampal slices (350 μm thickness) were prepared by using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, UK). The slices were placed on membrane inserts (0.4 mM Millicell®-CM culture plate inserts) in

six-well plates. Each well contained 1 ml of culture medium consisting of 50% minimum essential medium (MEM), 25% Hank' balanced salt solution (HBSS), 25% horse serum, supplemented with (mM, final concentration): glucose 36, HEPES 25, and $NaHCO_3$; Fungizone® 1%, and gentamicin 0.100 $mg.mL^{-1}$, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with a 5%CO_2/95%O_2 atmosphere at 37 ºC. Culture medium was changed three times weekly for the subsequent 4 weeks until the experiments commenced.

Preparation of drugs and treatments

Curcumin (#C1386, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and stored at -20ºC. Single aliquots were further diluted in culture media and added to the cells to provide the required final concentration, alongside vehicle control (DMSO 0.1%). $A\beta_{1-42}$ (Bachem, Torrance, CA, USA) was prepared by incubating the peptide in Milli-Q water with 0.1% ammonium hydroxide at 37 ºC for 72 h[22]. On the 28 DIV, cultures were co-treated with $A\beta_{1-42}$ (2 µM) and curcumin (0.5-10 µM) for 48 h. The control group was set up with DMSO instead of curcumin.

Quantification of cellular death

Cell damage was assessed by fluorescent image analysis of propidium iodide (PI, Calbiochem, San Diego, CA, USA) uptake. PI is a polar compound that is impermeable to an intact cell membrane, but it penetrates damaged cell membranes of dying cells and binds to nuclear DNA to generate a bright red fluorescence. Forty-six hours after the Aβ peptides exposure, organotypic cultures were stained with PI (5 µM) for 2h. PI fluorescence was observed by an inverted fluorescence microscope (Nikon

Eclipse TE 300). Images were captured using a CCD camera (Visitron Systems, Puchheim, Germany), stored and subsequently analyzed by using Scion Image software. The amount of PI fluorescence was determined densitometrically after transforming the red values into grey values. For quantification of neural damage, the percentage of area expressing PI fluorescence above background level was calculated in relation to the total area of each slice. PI intensity, meaning cell death, was expressed as a percentage of cell damage: cell death (%) = F_d/F_0 X 100, where F_d is the PI uptake fluorescence of dead area of hippocampal slices and F_0 is the total area of each hippocampal slice[23].

Evaluation of intracellular ROS formation

Formation of intracellular peroxides was detected using an oxidant sensing fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes Inc., Eugene, OR, USA), which is de-esterified within cells by endogenous esterases to the ionized free acid, 2',7'-dichlorofluorescin; 2',7'- dichlorofluorescin ($DCFH_2$) is then oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) by peroxides. The cultures were incubated with 10 μM DCFH-DA in PBS at 37ºC for 30 min[24]. The fluorescent DCF images were observed under a fluorescence microscope (Nikon Eclipse TE 300), captured by a digital camera, stored and subsequently analyzed by using the Scion Image software. For quantification of ROS formation, the percentage of area expressing DCF fluorescence above background level was calculated in relation to the total area of each slice. DCF intensity, meaning ROS formation, was expressed as a percentage of fluorescence: fluorescence (%) = F_d/F_0 X 100, where F_d is the DCF fluorescence of ROS formation area of hippocampal slices and F_0 is the total area of each hippocampal slice.

Determination of IL-6 and IL-10 levels in the culture medium of organotypic cultures

Pro- and anti-inflammatory cytokines release were analyzed. The culture medium was collected, rapidly frozen, and stored at -20 ºC for later measurement of IL-6, and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's recommendations. The values of cytokines were expressed as $pg.ml^{-1}$ medium.

Western blot analysis

Organotypic slices were homogenized in lyses buffer 4% sodium dodecylsulfate -SDS-, 2 mM EDTA, 50 mM Tris, 1% mammalian protease inhibitor (Roche, San Francisco, CA, USA) at 4 ºC and stored at -70 ºC until use. Equal amounts of protein were resolved and immunodetected as described previously[25], using anti-GFAP (1:3000; Sigma-Aldrich), anti-synaptophysin (1:2000; Calbiochem, Temecula, CA, USA), anti-Phospho-β-catenin Ser45 (1:1000; Sigma-Aldrich), anti-β-catenin (1:2000; Sigma-Aldrich), anti-Phospho-Akt Ser473 (1:1000; Cell Signaling, Beverly, MA, USA), anti-Akt (1:1000; Cell Signaling), anti-Phospho-GSK-3β Ser9 (1:1000; Cell Signaling), anti-GSK-3β (1:1000; Cell Signaling) and anti-β-actin (1:1000; Cell Signaling). The films were scanned and the digitalized images analysed using Optiquant (Bio-Rad Laboratories, Hertfordshire, UK). The average optical density for the vehicle control was designated as 100%.

Immunofluorescence staining

Cultured slices were fixed for 2 h with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M PBS. The slices were then incubated at 4ºC for 1 h in blocking solution containing 0.2% Triton X-100 in 5% bovine serum albumin (BSA) in PBS[26]. Slices were incubated for 18 h at 4ºC in the primary antibodies anti-

synaptophysin (1:1000; Chemicon), anti-CD11b (1:1000; Millipore) and anti-GFAP (1:3000; Sigma-Aldrich). After three washes with PBS, secondary antibodies Alexa Fluor® 488 or Alexa Fluor® 568 (1:500; Invitrogen, Carlsbad, CA, USA) were applied to the slices. Dapi (1:1000, Sigma) was used to stain the nuclei (blue). Slices were mounted with Pro-Long® Gold Antifade Reagent (Molecular Probes), the images were acquired using an inverted fluorescence microscopy (Olympus IX-70, Germany) and a digital camera connected to a microscope (Camera model U-LH100HGAPO) (Magnification x4 and x20). For quantitative analysis, the images were captured using the 4x objective lens and digitalized. Three to 6 sections from each rat were randomly selected and images were quantified using ImageJ software (NIH, USA).

Statistical analysis

Values are means ± SEM of at least three separate experiments. Data were analyzed by ANOVA to test the hypothesis for the comparison of independent groups using Prism Graph Pad (v.5.0, Graph Pad Software Inc., San Diego, CA, USA). Student Newman-Keuls post hoc test was applied to the means to determine differences between groups. *p-Values* < 0.05 were considered to be statistically significant.

Results

Curcumin enhances cell survival against Aβ-induced toxicity in organotypic hippocampal culture

The exposure of organotypic cultures to $A\beta_{1-42}$ for 48 h caused a significant increase in PI fluorescence in hippocampal slices, which means an increase in the cellular damage. This damage was reduced by the treatment with curcumin (Figure 1A). Quantification of PI fluorescence showed that Aβ caused damage to around 28 ± 3%

(n=8) of the cells in the hippocampus, a significant increase when compared with control cultures 4 ± 1% (n=12). The treatments with low-doses curcumin (0.5 and 1 μM) did not protect from Aβ-induced cell death; however, when cultures were treated with 5 (15 ± 5%, n=10) and 10 μM (9 ± 4%, n=10) the toxicity was significantly decreased (Figure 1B). The exposure of cultures to Aβ 12 and 24 h did not cause a significant rise in fluorescence in hippocampal slices (data not shown). Cultures exposed to the same concentration of scramble sequence of $A\beta_{42-1}$ showed no differences in cell survival compared to untreated control cultures (data not shown). Curcumin 10 μM is a concentration commonly used for *in vitro* studies and was adopted for subsequent experiments.

Curcumin prevents synaptic dysfunction triggered by Aβ

To investigate the synaptic integrity in organotypic hippocampal slice cultures, we performed Western blotting and immunofluorescence analyses for the synaptic protein synaptophysin, a specific synaptic marker[26,27]. A reduction in the synaptophysin immunofluorescence intensity was found in Aβ-treated organotypic hippocampal slice cultures, which were prevented with curcumin treatment (Figure 2A, B, C, D). Western blot analysis of synaptophysin confirmed the imaging data: Aβ significantly decreased synaptophysin levels to 54 ± 12% of controls (n=5), whereas, concomitant treatment with curcumin blocked this decrease (101 ± 15%, n=5) (Figure 2E).

Oxidant effect of Aβ and antioxidant effect of curcumin in organotypic hippocampal culture

To determine the oxidant effect of Aβ, the amount of free radicals in hippocampal slices treated with Aβ and/or curcumin for 48 h was measured using

DCFH-DA. As can be seen in Figure 3C, Aβ increased the fluorescence staining in organotypic slices. Free radical levels were significantly higher in cells treated with Aβ (37 ± 6%, n=6) (Figure 3C, E) when compared to control slices (Figure 3A, B, E). Treatment with 10 μM curcumin was able to prevent the production of free radicals induced by Aβ (14 ± 2%, n=6) (Figure 3D, E).

Curcumin prevents Aβ-induced glial reactivity and cytokine levels changes

Considering the involvement of neuroinflammation in the physiopathology of AD, the anti-inflammatory properties of curcumin were assayed in organotypic hippocampal cultures exposed to Aβ. We first analyzed the effects of treatment with 2 μM Aβ and 10 μM curcumin on astrocytes by analyzing the content of glial fibrillary acidic protein (GFAP) by immunofluorescence. Aβ-treated slices exhibited an increase in GFAP immunofluorescence intensity (0.85 ± 0.1), which were prevented by curcumin treatment (0.32 ± 0.04) (Figure 4A, B). In Western blotting analysis GFAP immunocontent was increased on slices exposed to Aβ (152 ± 14%, n=5), whereas curcumin significantly prevented the increase in GFAP (116 ± 12%, n=5) (Figure 4C). We also analyzed the effects of treatment with Aβ and curcumin on microglial reactivity by immunofluorescence. Aβ-treated slices exhibited an increase in CD11b immunofluorescence intensity (0.68 ± 0.18), which were prevented by curcumin treatment (0.26 ± 0.05) (Figure 5A, B). We investigated some cytokines thought to play a central role in self-propagation of neuroinflammation, including the pro-inflammatory IL-6 and the anti-inflammatory IL-10. The anti-inflammatory cytokine IL-10 levels significantly decreased in Aβ-treated slices medium culture (160 ± 91%, n=8) (Figure 5C) and the pro-inflammatory cytokine IL-6 levels significantly increased in the medium culture after exposure to Aβ (2236 ± 321, n=10) (Figure 5D). Curcumin was

able to prevent these alterations levels induced by Aβ of both cytokines analyzed, IL-10 (1270 ± 190%, n=10) and IL-6 (1008 ± 153%, n=10). Moreover, curcumin treatment *per se* increased the IL-10 levels (1332 ± 254%, n=8) when compared to control (886 ± 123%, n=12) (Figure 5C).

Regulation of β-catenin in curcumin-mediated neuroprotection

We next ascertained the effect of curcumin on intracellular signaling proteins, which are critical for cellular survival, in Aβ-treated organotypic slices. Wnt pathway disruption by Aβ represents a pivotal event in the neuronal death occurring in AD, and it is well established that regulation of β-catenin stability is a crucial control mechanism in Wnt signaling[28]. To detect the role of β-catenin phosphorylation status in the neuroprotective effect of curcumin after Aβ-induced toxicity in hippocampus, we analyzed β-catenin levels and phophorylated β-catenin levels by Western blotting. Aβ treated slices displayed increased phosphorylated β-catenin (168 ± 19%, n=5) (Figure 6A) and reduced β-catenin levels in cytoplasm (53 ± 8%, n=6) (Figure 6B). However, these β-catenin stability alterations induced by Aβ were recovered with co-treatment with 10 μM curcumin (116 ± 16%, n=5 and 94 ± 12%, n=6, respectively).

Curcumin is protective against Aβ through the PI3K pathway

The PI3K pathway has been emphasized to play an important role in cell survival by both activating the expression of antiapoptotic proteins and inhibiting the expression of pro-apoptotic proteins[16]. Activated PI3K phosphorylates its downstream target Akt. Phosphorylated Akt directly affects GSK-3β by phosphorylating it at Ser 9 and thus inhibiting its activity[18]. In order to determine whether the PI3K signaling pathway by Akt activation and GSK-3β inactivation was involved in the neuroprotective

effect of curcumin, we carried out this experiment using LY294002, a specific inhibitor of PI3K. LY294002 does not induce significant cell death in intact cell at the low concentrations used as inhibitor[29,30]. LY294002 5 μM abolished the neuroprotection induced by 10 μM curcumin, increasing the PI incorporation from 13 ± 3% (Aβ+Cur) to 24 ± 4% (n=8) (Aβ+Cur+LY) (Figure 7A). Considering that LY294002 abolished the neuroprotective effect of curcumin, we analyzed the status of Akt phosphorylation. Curcumin remained significantly increased the percentage of pAkt/Akt ratio 152 ± 17% (n=6) in cultures treated for 48 h with Aβ (Figure 7B), and this increase was abolished by LY294002 (95 ± 7%, n=6). Treatment with LY294002 reduced the ratio of pAkt/Akt in control and Aβ treated cultures, confirming the efficacy of the inhibitor. We subsequently found that LY 2940002 also blocked (108 ± 12%, n=7) the increase of GSK-3β phosphorylation mediated by curcumin in Aβ treated slices for 48 h (157 ± 18%, n=7) (Figure 7C), indicating that GSK-3β inhibition is modulated by PI3K pathway in curcumin neuroprotective mechanism against Aβ-induced neurotoxicity in organotypic hippocampal slices.

Discussion

The development of therapies for neurodegenerative diseases represents a major challenge to academic, biotechnology, and pharmaceutical scientists. To contribute in this field, we have used an organotypic hippocampal slice cultures model of Aβ-induced neurotoxicity, which are a valuable alternative to animal experiments. Organotypic cell culture models represent an *in vitro* model that maintains the *in vivo* three-dimensional structure, neuron-neuron and neuronal-glial interactions and neurons and glial cells survive long-term and physiologically mature in culture, allowing relevant studies to understand the mechanisms of neurodegeneration and neuroprotection at a cellular level

in AD[31,32]. To the best of our knowledge, this is the first report of neuroprotective effects of curcumin against β-Amyloid-induced toxicity in organotypic hippocampal slice culture.

Aβ protein, Aβ fibrils, and Aβ oligomers, which are abnormally accumulated in human brains afflicted with AD, have long been proposed as the most likely culprit in the pathogenesis of the disease[3]. Synaptic loss and death of specific neurons population in AD are provoked by a cascade of multiple deleterious molecular and cellular events. There is extensive evidence that Aβ contributes significantly to the synaptic loss and neuronal death in AD[33]. Our results showed a significant decrease in a synaptic marker and a significant increase in cell death when cultures were exposed to Aβ, whereas treatment with curcumin was able to prevent the Aβ-induced toxicity. This hypothesis is reinforced by effects of curcumin against synaptotoxicity and cell death triggered by Aβ, since curcumin prevented changes in the major synaptic vesicle protein, synaptophysin, and PI uptake in organotypic hippocampal slice cultures.

Neuroinflammation is common place in various central nervous system (CNS) conditions including neurodegenerative disorders such as AD[34]. Glia-neuron crosstalk through the release of several neurotrophic factors is a primary event in the maintenance of CNS homeostasis. Microglial and astrocytic gliosis occurs with neuroinflammation and there is production of proinflammatory chemokines and cytokines[15, 35]. The pro-inflammatory cytokine IL-6 has been associated with severe cognitive impairments[36]. IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6 by activated glia cells[37]. Neuroinflammatory and oxidative stress responses are interdependent and bidirectional events. The concept of free radical toxicity actually has its roots in inflammation biology, where the secretion of reactive oxygen and nitrogen species by inflammatory

cells is a major mechanism for attacking opsonized targets[33]. Physiologically, the cellular expression of cytokines in the CNS is strictly controlled; however, under certain pathological conditions such high reactive species formation, the expression of various cytokine genes may become spatially and temporally modified. These parallel or sequential relations between neuroinflammation and oxidative stress might imply the same regulatory or auto regulatory mechanisms through commonly implicated signaling pathways[38]. Receptor binding of cytokines stimulates a variety of intracellular signaling pathways that have been implicated in AD, including the activation of protein kinase C (PKC), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38/MAPK), PI3K/Akt, extracellular signaling-related kinase (ERK), as well as activation of caspase-1 and -3[39,40].

In this study, we also demonstrate that curcumin exerts a neuroprotective effect by blunting inflammatory response and oxidative stress characteristics that were pivotal for the development of Aβ-induced toxicity. Inhibition of glial activation, release alteration of cytokines IL-6 and IL-10, and diminish formation of reactive species with curcumin treatment, suggest that this natural compound may be effective in combating these processes closely associated with AD. These results are in accordance with findings obtained in others *in vivo* and *in vitro* models of AD[10,13,15], however we may further investigate its neuroprotective mechanism. So this study confirms previous findings showing that curcumin is protective against Aβ-induced toxicity, and goes beyond adding new information on the mechanism of neuroprotection.

In the attempt to better establish the neuroprotective effects of curcumin in AD, we next proposed that curcumin could mediate its effects observed here by acting of two important signaling pathways implicated in supporting neuronal survival: Wnt/β-catenin and PI3K. Wnt pathway disruption by Aβ represents a pivotal event in the neuronal

death occurring in AD, and drugs that rescue the Wnt pathway have been proposed as attractive novel AD therapeutics[41]. In the Wnt pathway, the phosphorylation of β-catenin targets it for ubiquitination and degradation by the proteasome, while the unphosphorylated β-catenin is stable and translocates into nuclei to promote cell survival by activating its T cell factor/lymphocyte enhancer factor(TCF/LEF)-dependent transcriptional activity[27]. We observed that Aβ was able to increase phosphorylated β-catenin and decrease unphosphorylated β-catenin in cytoplasm of organotypic slices, thereby leading to the degradation of β-catenin and the inactivation of Wnt signaling. These data also show that curcumin could promote its neuroprotective effect preventing this degradation of β-catenin by Aβ. However, further studies are needed to clarify the involvement of the Wnt/β-catenin pathway in the neuroprotective effect demonstrated by curcumin against Aβ toxicity.

The PI3K pathway is known to be important in neuronal cell survival and death and to be highly affected by inflammatory response and oxidative stress. It has been shown that the protective effects of PI3K are mediated primarily by one of its downstream targets, Akt[42]. Akt has direct effects on the apoptosis pathway, by inhibiting the pro-apoptotic proteins as, among others, GSK-3β[43]. GSK-3β is a cross link between PI3K and Wnt/β-catenin pathways, it can also phosphorylate β-catenin, facilitating its proteolysis to promote the neuronal degeneration in AD. Therefore, we investigated whether curcumin could act in the PI3K pathway using the PI3K inhibitor, LY294002. Firstly, we observed that PI3K inhibitor prevented the curcumin protection against Aβ-induced cell death. The possible reason for this fact is that LY294002 was able to abolish the increase levels of phospho-Akt and phospho-GSK-3β observed with curcumin treatment in presence of Aβ. Curcumin appears to act as a potent modulator of PI3K/Akt pathway in organotypic hippocampal slices exposed to Aβ, while LY294002

blocks this survival pathway and thereby reduces the neuroprotective effects of curcumin in our model. It is known Aβ can modify multiple intracellular signaling pathways, which contribute to its toxicity. A reason for we did not observe differences in phosphorylation of Akt and GSK-3β in organotypic hippocampal slices treated with Aβ for 48 h can be due to the fact that these changes may happen earlier that 48 h. Our previous studies have shown that 12 h of Aβ exposure in organotypic hippocampal slices, the phosphorylation of Akt and GSK-3β were significantly decreased and at 48 h exposure returned to control levels[25,44]. Although Akt inactivation by Aβ appears be transient *in vitro*, it may be sufficient to transient signals for apoptosis to downstream targets. Our goal in this study was to demonstrate that curcumin, even after long time of exposure, acts to hold this important pro-survival pathway activates during the cell injury stimulus induced by Aβ in organotypic hippocampal slice cultures.

In conclusion, the results presented here indicate a close association between neuroinflammation and oxidative stress to synaptic loss and cell death promoted by Aβ, and suggests that suppression of these responses by curcumin may represent a valid therapeutic strategy for the treatment of AD. We also considered that PI3K/Akt-mediated inactivation of GSK-3β and the stabilization of β-catenin could contribute to the neuroprotective activity of curcumin and open a new light for future investigations. Moreover, the inhibition of PI3K signaling pathway plays critical roles in curcumin mediated neuroprotection, suggesting that the drug may induce activation of this survival pathway against Aβ-induced toxicity.

Acknowledgments

This work was supported by the Brazilian funding agencies CNPq, CAPES, FAPERGS, PRONEX and PROPESQ/UFRGS.

References

1 Trojanowski JQ, Lee VM. "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. Ann N Y Acad Sci 2000; 924:62-7.

2 Shim YS, Morris JC. Biomarkers predicting Alzheimer's disease in cognitively normal aging. J Clin Neurol 2011; 7:60-8.

3 Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell 2005; 120:545-55.

4 Verdile G, Fuller S, Atwood CS, et al. The role of beta amyloid in Alzheimer's disease: still a cause of everything or the only one who got caught? Pharmacol Res 2004; 50:397-409.

5 Walsh DM, Selkoe DJ. Deciphering the molecular basis of memory failure in Alzheimer's disease. Neuron 2004; 44:181-93.

6 Maheshwari RK, Singh AK, Gaddipati J, et al. Multiple biological activities of curcumin: a short review. Life Sci 2006; 78:2081-7.

7 Shishodia S, Sethi G, Aggarwal BB. Curcumin: getting back to the roots. Ann N Y Acad Sci 2005; 1056:206-17.

8 Belkacemi A, Doggui S, Dao L, et al. Challenges associated with curcumin therapy in Alzheimer disease. Expert Rev Mol Med 2011; 13:e34.

9 Moon DO, Kim MO, Choi YH, et al. Curcumin attenuates inflammatory response in IL-1beta-induced human synovial fibroblasts and collagen-induced arthritis in mouse model. Int Immunopharmacol 2010; 10:605-10.

10 Lim GP, Chu T, Yang F, et al. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci 2001; 21:8370-7.

11 Hatcher H, Planalp R, Cho J, et al. Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 2008;65:1631-52.

12 Zhou H, Beevers CS, Huang S. The targets of curcumin. Curr Drug Targets 2011;12:332- 47.

13 Begum AN, Jones MR, Lim GP, et al. Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. J Pharmacol Exp Ther 2008; 326:196-208.

14 Hamaguchi T, Ono K, Murase A, et al. Phenolic compounds prevents Alzheimer's pathology through different effects on the amyloid-beta aggregation pathway. Am J Pathol 2009; 175:2557-65.

15 Wang HM, Zhao YX, Zhang S, et al. PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes. J Alzheimers Dis 2010; 20:1189-99.

16 Lee KY, Koh SH, Noh MY, et al. Phosphatidylinositol-3-kinase activation blocks amyloid beta-induced neurotoxicity. Toxicology 2008; 243:43-50.

17 de Oliveira AC, Candelario-Jalil E, Langbein J, et al. Pharmacological inhibition of Akt and downstream pathways modulates the expression of COX-2 and mPGES-1 in activated microglia. J Neuroinflammation 2012; 9:2.

18 Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem 1998; 273:19929-32.
19 Lee SM, Tole S, Grove E, McMahon AP. A local Wnt-3a signal is required for development of the mammalian hippocampus. Development 2000; 127:457-67.
20 Salinas PC, Zou Y. Wnt signaling in neural circuit assembly. Annu Rev Neurosci 2008; 31:339-58.
21 Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 1991; 37:173-82.
22 Bernardi A, Frozza RL, Meneghetti A, et al. Indomethacin-loaded lipid-core nanocapsules reduce the damage triggered by Aβ1-42 in Alzheimer's disease models. Int J Nanomedicine 2012;7:4927-42.
23 Frozza RL, Horn AP, Hoppe JB, et al. A comparative study of beta-amyloid peptides Abeta1-42 and Abeta25-35 toxicity in organotypic hippocampal slice cultures. Neurochem Res. 2009;34:295-303.
24 Liu R, Liu W, Doctrow SR, et al. Iron toxicity in organotypic cultures of hippocampal slices: role of reactive oxygen species. J Neurochem. 2003;85:492-502.
25 Hoppe JB, Frozza RL, Horn AP, et al. Amyloid-beta neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3beta, tau and neuroinflammation. J Pineal Res 2010; 48:230-8.
26 Suh EC, Jung YJ, Kim YA, et al. Abeta25-35 induces presynaptic changes in organotypic hippocampal slice cultures. Neurotoxicology 2008; 29:691-9.
27 Inestrosa NC, Varela-Nallar L, Grabowski CP, et al. Synaptotoxicity in Alzheimer's disease: the Wnt signaling pathway as a molecular target. IUBMB Life 2007; 59:316-21.
28 Zhang X, Yin WK, Shi XD, et al. Curcumin activates Wnt/β-catenin signaling pathway through inhibiting the activity of GSK-3β in APPswe transfected SY5Y cells. Eur J Pharm Sci 2011; 42:540-6.
29 Crowder RJ, Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci. 1998;18:2933-43.
30 Cimarosti H, Zamin LL, Frozza R, et al. Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates GSK-3beta. Neurochem Res. 2005;30(2):191-9.
31 Holopainen IE. Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. Neurochem Res 2005; 30:1521-8.
32 Noraberg J, Poulsen FR, Blaabjerg M, et al. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. Curr Drug Targets CNS Neurol Disord 2005; 4:435-52.
33 Akiyama H, Barger S, Barnum S, et al. Inflammation and Alzheimer's disease. Neurobiol Aging 2000; 21:383-421.
34 McGeer EG, McGeer PL. Neuroinflammation in Alzheimer's disease and mild cognitive impairment: a field in its infancy. J Alzheimers Dis 2010; 19:355-61.
35 Garwood CJ, Pooler AM, Atherton J, et al. Astrocytes are important mediators of Aβ-induced neurotoxicity and tau phosphorylation in primary culture. Cell Death Dis 2011; 2:e167.
36 Beurel E, Jope RS. Lipopolysaccharide-induced interleukin-6 production is

controlled by glycogen synthase kinase-3 and STAT3 in the brain. J Neuroinflamm 2009; 6:9.
37 Dagvadorj J, Naiki Y, Tumurkhuu G, et al. Interleukin (IL)-10 attenuates lipopolysaccharide-induced IL-6 production via inhibition of IkappaB-zeta activity by Bcl-3. Innate Immun 2009;15:217-224
38 Rosales-Corral S, Tan DX, Reiter RJ, et al. Kinetics of the neuroinflammation-oxidative stress correlation in rat brain following the injection of fibrillar amyloid-beta onto the hippocampus in vivo. J Neuroimmunol 2004; 150:20-8.
39 Van Eldik LJ, Thompson WL, Ralay Ranaivo H, et al. Glia proinflammatory cytokine upregulation as a therapeutic target for neurodegenerative diseases: function-based and target-based discovery approaches. Int Rev Neurobiol 2007; 82:277-96.
40 Anisman H. Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. J Psychiatry Neurosci 2009; 34:4-20.
41 Garrido JL, Godoy JA, Alvarez A, et al. Protein kinase C inhibits amyloid beta peptide neurotoxicity by acting on members of the Wnt pathway. FASEB J 2002; 16:1982-4.
42 Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. Cell 1997; 88:435-7.
43 Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 2005; 9:59-71.
44 Nassif M, Hoppe J, Santin K, et al. β-Amyloid peptide toxicity in organotypic hippocampal slice culture involves Akt/PKB, GSK-3b, and PTEN. Neurochem Int 2007;50:229-35.

Figure legends

Figure 1 Effects of curcumin on cell damage triggered by Aβ in organotypic hippocampal cultures. **A** Representative images of propidium iodide (PI) uptake in hippocampal slices treated with Aβ and curcumin. **B** Quantitative analysis of hippocampal damage 48 h after Aβ and curcumin (0.5, 1, 5 and 10 μM) exposure. Bars represent the mean ± SEM, n= 8-10 animals per group. $^{\#}P < 0.01$ significantly different from controls group; $^{**}P < 0.01$ significantly different from the Aβ group; $^{***}P < 0.001$ significantly different from the Aβ group.

Figure 2 Curcumin attenuates sinaptotoxicity after exposure of organotypic hippocampal slices cultures to Aβ. Representative images of synaptophysin (Syn) immunoreactivity in CA1 cell body layer of organotypic hippocampal slices at x20 magnification (Scale bar, 100μm), **A** Control, **B** Curcumin 10 μM, **C** $A\beta_{1-42}$ 2 μM and **D**

$A\beta_{1-42}$ + Curcumin are shown. Experiments were performed at least three times. **e** Representative Western blotting analysis for synaptophysin and β-actin protein (loading control) performed in organotypic hippocampal cultures. Graphic shows quantification of synaptophysin immunocontent normalized by β-actin protein (loading control). Bars represent the mean ± SEM, n=5 animals per group. $^{**}P < 0.01$ significantly different from the other groups.

Figure 3 Curcumin prevents ROS formation triggered by Aβ in rat hippocampus. Representative images of hippocampal slices using the fluorescent probe DCFH-DA, **A** Control, **B** Curcumin 10 μM, **C** $A\beta_{1-42}$ 2 μM and **D** $A\beta_{1-42}$ + Curcumin. **e** Quantitative analysis of hippocampal DCF fluorescence after Aβ exposure and curcumin treatment. Bars represent the mean ± SEM, n= 6 animals per group. $^{\#}P < 0.01$ significantly different from the controls group; $^{*}P < 0.05$ significantly different from the Aβ group.

Figure 4 Effect of curcumin on Aβ-induced astrocyte reactivity. **A** Immunofluorescence images of GFAP immunoreactivity in organotypic hippocampal slices at 4x and 20x magnification (Scale bars, 20 μm and 100 μm, respectively). **B** Analysis of GFAP immunofluorescence intensity (6 slices from each rat, 4 animals per group) showed Aβ-treated hippocampal slices had significantly more immunofluorescence intensity (arbitrary units/mm^2) than other groups. Bars represent the mean ± SEM ($^{**}P < 0.01$). **C** Western blotting analysis of GFAP and β-actin protein (loading control) performed in organotypic hippocampal cultures. Bars represent the mean ± SEM, n=5. $^{*}P < 0.05$ significantly different from the other groups.

Figure 5 Effect of curcumin on Aβ-induced microglial reactivity and altered levels of cytokines. **A** Immunofluorescence images of CD11b immunoreactivity in organotypic hippocampal slices at 4x (Scale bars: 10 μm). **B** Analysis of CD11b immunofluorescence intensity (6 slices from each rat, 3 animals per group) showed Aβ-treated hippocampal slices had significantly more immunofluorescence intensity (arbitrary units/mm^2) than other groups. Bars represent the mean ± SEM ($^*P < 0.05$). **C** Levels of anti-inflammatory cytokine IL-10 in the culture medium by organotypic slices. $^{\#}P < 0.01$ significantly different from the other groups; $^*P < 0.05$ significantly different from the control group (n=8-10). **D** Release of pro-inflammatory cytokine IL-6 in the culture medium by organotypic slices. $^{\#}P < 0.001$ significantly different from the control groups; $^*P < 0.05$ significantly different from the Aβ group (n=8-12).

Figure 6 Effect of Aβ phosphorylated β-catenin and unphosphorylated β-catenin in organotypic slices in the absence or in the presence of curcumin. **A** Representative Western blot and graphic showing quantification of β-catenin phosphorylated immunocontent normalized by β-actin protein (loading control). Bars represent the mean ± SEM, n= 5. $^{\#}P < 0.01$ significantly different from the control groups; $^*P < 0.05$ significantly different from the Aβ group. **B** Representative Western blot and graphic showing quantification of β-catenin immunocontent normalized by β-actin protein (loading control). Bars represent the mean ± SEM, n= 6. $^{\#}P < 0.05$ significantly different from the other groups (n=6).

Figure 7 Effect of PI3K inhibitor on neuroprotective effect of curcumin against Aβ. **A** Representative images and graphic showing quantification of PI uptake in organotypic slices in presence of LY294002. Bars represent the mean ± SEM, n= 8. $^{\#}P < 0.05$

significantly different from the Aβ groups; *$P < 0.05$ significantly different from the control groups. **B** Representative Western blot and graphic showing quantification of p-Akt and Akt levels in organotypic slices. Bars represent the mean ± SEM, n= 6. **$P < 0.01$ significantly different from the other groups; *$P < 0.05$ significantly different from the other groups. **C** Representative Western blot and graphic showing quantification of p-GSK-3β (Ser 9) and GSK-3β levels in organotypic slices. Bars represent the mean ± SEM, n= 7. *$P < 0.05$ significantly different from the other groups.

Figure 1

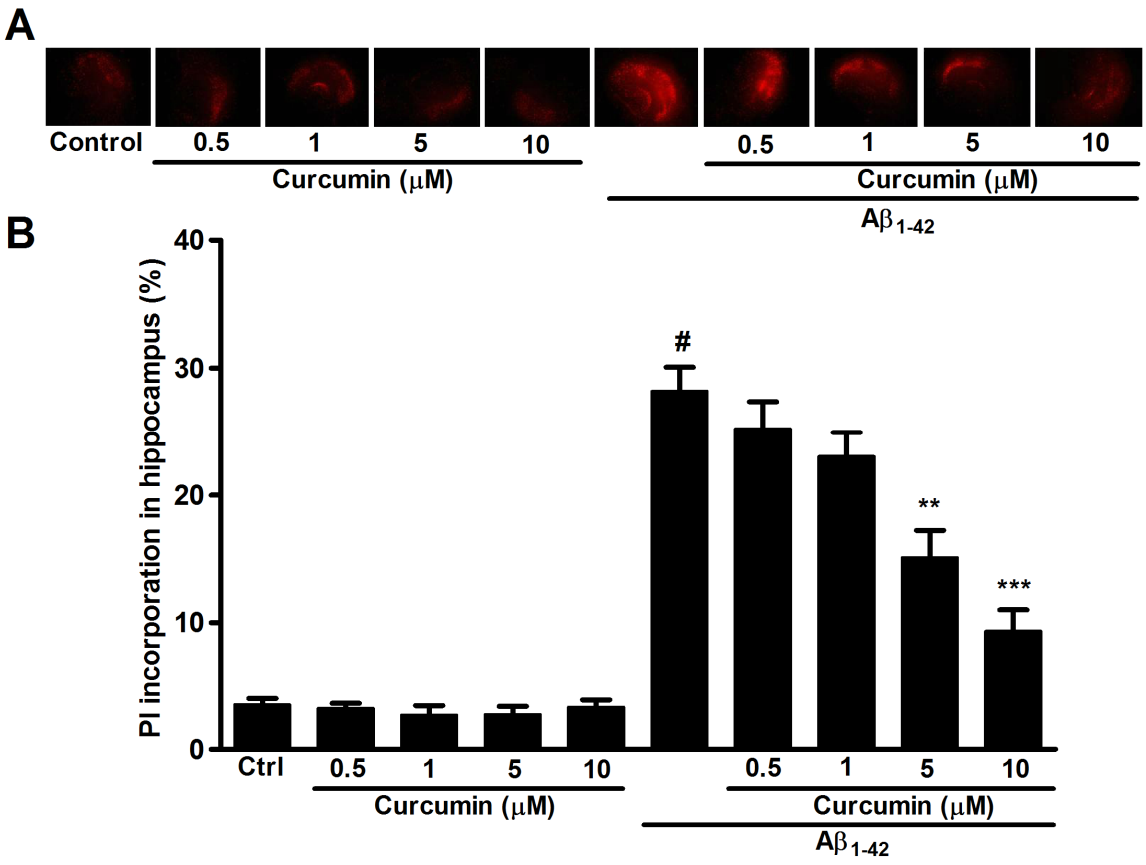

A
Control
0.5
1
5
10
0.5
1
5
10
Curcumin (μM)
Curcumin (μM)
Aβ1-42
B
PI incorporation in hippocampus (%)
40
30
20
10
0
#
**
***
Ctrl
0.5
1
5
10
0.5
1
5
10
Curcumin (μM)
Curcumin (μM)
Aβ1-42

Figure 2

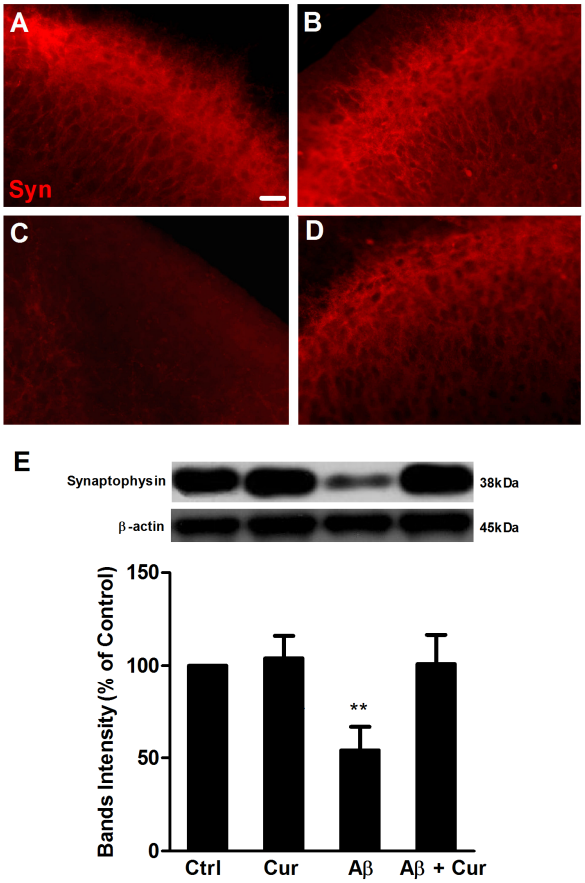

A
B
Syn
C
D
E
Synaptophysin
38kDa
β-actin
45kDa
Bands Intensity (% of Control)
150
100
50
0
**
Ctrl
Cur
Aβ
Aβ + Cur

Figure 3

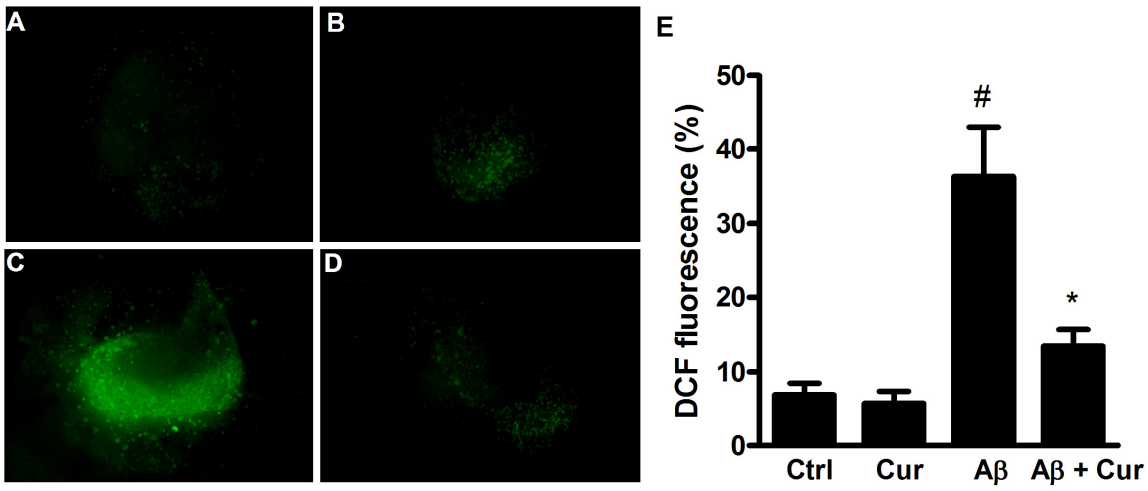


Figure 4

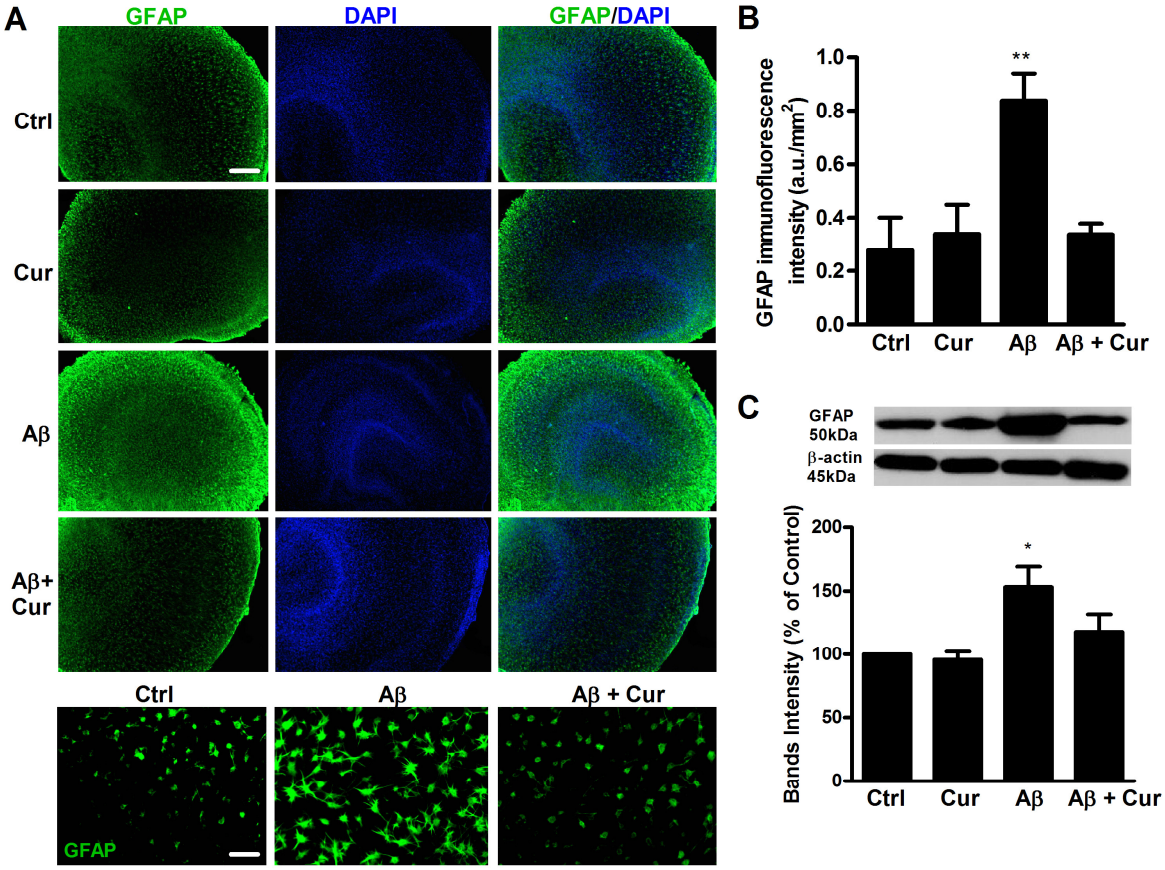

A
GFAP
DAPI
GFAP/DAPI
Ctrl
Cur
Aβ
Aβ+
Cur
Ctrl
Aβ
Aβ + Cur
GFAP
B
GFAP immunofluorescence intensity (a.u./mm2)
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
Cur
Aβ
Aβ + Cur
C
GFAP
50kDa
β-actin
45kDa
Bands Intensity (% of Control)
200
150
100
50
0
*
Ctrl
Cur
Aβ
Aβ + Cur

Figure 5

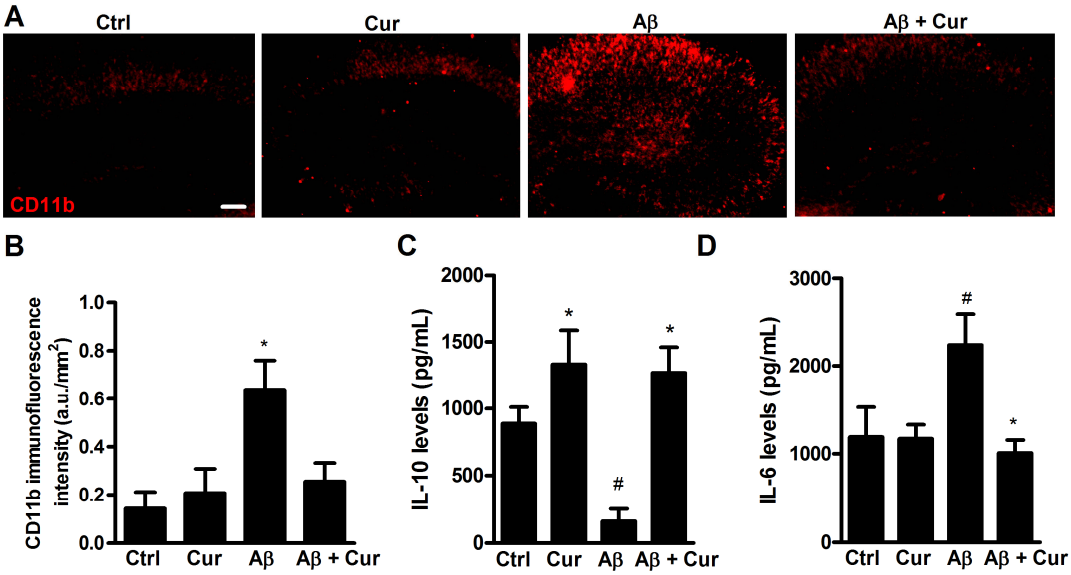

A
Ctrl
Cur
Aβ
Aβ + Cur
CD11b
B
CD11b immunofluorescence intensity (a.u./mm²)
1.0
0.8
0.6
0.4
0.2
0.0
*
Ctrl
Cur
Aβ
Aβ + Cur
C
IL-10 levels (pg/mL)
2000
1500
1000
500
0
*
#
*
Ctrl
Cur
Aβ
Aβ + Cur
D
IL-6 levels (pg/mL)
3000
2000
1000
0
#
*
Ctrl
Cur
Aβ
Aβ + Cur

Figure 6

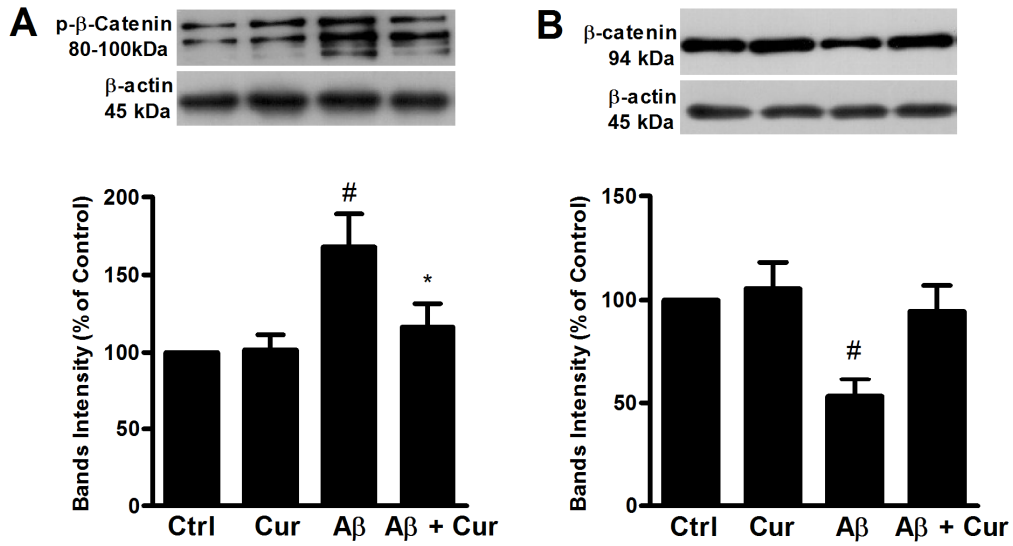
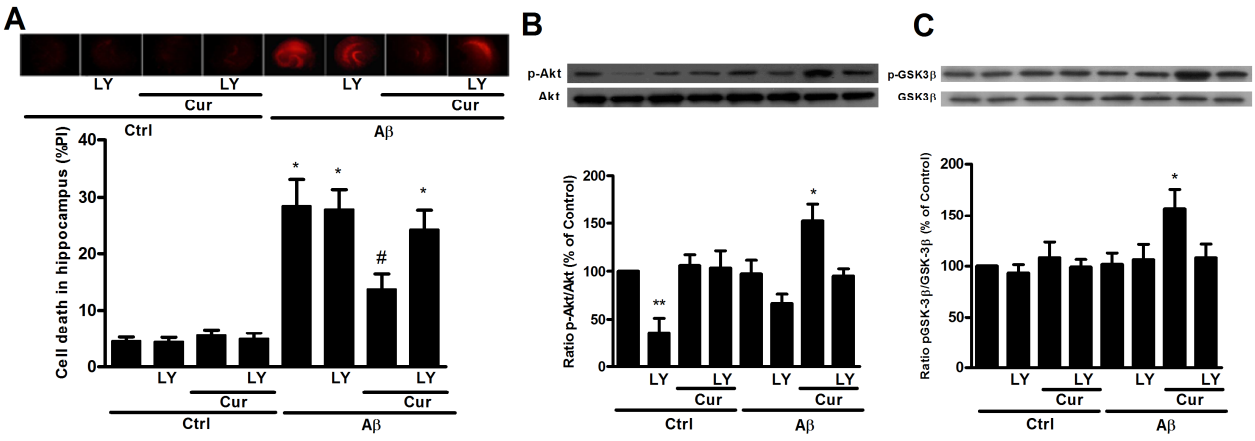

A
p-β-Catenin
80-100kDa
β-actin
45 kDa
Bands Intensity (% of Control)
200
150
100
50
0
#
*
Ctrl
Cur
Aβ
Aβ + Cur
B
β-catenin
94 kDa
β-actin
45 kDa
Bands Intensity (% of Control)
150
100
50
0
#
Ctrl
Cur
Aβ
Aβ + Cur

Figure 7


A
LY
LY
LY
LY
Cur
Cur
Ctrl
Aβ
Cell death in hippocampus (%PI)
40
30
20
10
0
*
*
#
*
LY
LY
LY
LY
Cur
Cur
Ctrl
Aβ
B
p-Akt
Akt
Ratio p-Akt/Akt (% of Control)
200
150
100
50
0
**
*
LY
LY
LY
LY
Cur
Cur
Ctrl
Aβ
C
p-GSK3β
GSK3β
Ratio pGSK-3β/GSK-3β (% of Control)
200
150
100
50
0
*
LY
LY
LY
LY
Cur
Cur
Ctrl
Aβ

4. CAPÍTULO II

Artigo: *SUMO-1 conjugation blocks beta-amyloid-induced astrocytes reactivity –*

Status: *Publicado no periódico Neuroscience Letters*

SUMO-1 conjugation blocks beta-amyloid-induced astrocyte reactivity

Juliana B. Hoppe[1, 2], Henry Tu[1], Marcus Rattray[1], Christianne G. Salbego[2] and Helena Cimarosti[1,*]

[1]Reading School of Pharmacy, University of Reading, Reading, RG6 6UB, UK

[2]Laboratory of Neuroprotection and Cell Signaling, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil

[*]Please address correspondence to:

Helena Cimarosti, Reading School of Pharmacy, University of Reading, Reading, RG6 6UB, UK

E-mail: h.i.cimarosti@reading.ac.uk

Abstract

Astrocyte reactivity is implicated in the neuronal loss underlying Alzheimer's disease. Curcumin has been shown to reduce astrocyte reactivity, though the exact pathways are incomplete. Here we investigated the role of the small ubiquitin-like modifier (SUMO) conjugation in mediating this effect of curcumin. In beta-amyloid (Aβ)-treated astrocytes, morphological changes and increased glial fibrillary acidic protein (GFAP) confirmed reactivity, which was accompanied by c-jun N-terminal kinase (JNK) activation. Moreover, the levels of SUMO-1 conjugated proteins, as well as the conjugating enzyme, Ubc9, were decreased, with concomitant treatment with curcumin preventing these effects. Increasing SUMOylation in astrocytes, by over-expression of constitutively active SUMO-1, but not its inactive mutant, abrogated Aβ-induced increase in GFAP, suggesting astrocytes require SUMO-1 conjugation to remain non-reactive.

Keywords: Alzheimer's disease; beta-amyloid peptide; c-jun N-terminal kinase; curcumin; reactivity; SUMOylation

1. Introduction

Astrocytes are receiving increasing attention as key contributors to neurodegenerative disease, including Alzheimer's disease (AD) [21]. Loss of function in astrocytes, associated with astrocyte reactivity, is considered to have an important role in neurodegeneration [28]. In human patients, and mouse models of AD, there is increased astrocyte reactivity, assessed by up-regulation of the intermediate filament protein, glial fibrillary acidic protein (GFAP), and morphological changes in astrocytes [19, 25, 26]. AD-related beta-amyloid (Aβ) peptides cause astrocyte reactivity [6, 13]. Specifically, Aβ-treated reactive astrocytes have been shown to be involved in inflammatory responses and to exacerbate neurotoxicity [1, 2, 4, 11, 20].

The mechanisms underlying astrocyte reactivity are not fully characterized, and treatments, including dietary interventions, which may reduce astrocyte reactivity, are of great therapeutic interest. One well-known modulator of astrocyte properties is curcumin, a major constituent of turmeric (*Curcuma longa*). Curcumin has been shown to reduce astrocyte reactivity and up-regulate a number of cytoprotective pathways in astrocytes, including those involved in antioxidant defense [14, 23, 24, 29]. The exact pathways underlying the effects of curcumin are incomplete and may include regulation of the posttranslational modification of proteins by the small ubiquitin-like modifier (SUMO). SUMOylation [10] is a pathway recently implicated in AD [17, 31] and is usually regarded as an endogenous cytoprotective mechanism [5, 30]. Little is currently known about the role of SUMOylation in AD, in particular in astrocytes, therefore the aims of this study were to determine whether Aβ caused changes in SUMOylation in astrocytes associated with reactivity, and if these could be blocked by curcumin.

Furthermore, we determined whether increasing SUMOylation could prevent Aβ-induced astrocyte reactivity.

2. Materials and Methods

2.1. Primary cultures of mouse astrocytes

Animals were maintained and used according to the UK Animals (Scientific Procedures) Act, 1986 and institutional ethical approval. Animals were killed using cervical dislocation (Schedule 1 procedure) according to Home Office guidelines. Cerebral cortex astrocytes were prepared from 15-day-old Swiss mouse embryos (NIH, Harlan, UK) [27]. Cultures were treated at 12-14 days *in vitro* (DIV). For expression of recombinant protein, astrocytes at 10-12 DIV were transfected with 1.75 μg of DNA encoding pEYFP-SUMO-1GG and pEYFP-SUMO-1ΔGG (gifts of Jeremy Henley, University of Bristol), active and inactive forms of SUMO-1, respectively, using Lipofectamine 2000 (Invitrogen, Paisley, UK). To evaluate cytotoxicity, lactate dehydrogenase (LDH) release was measured using Tox7 (Sigma-Aldrich), according to the manufacturer's instructions.

2.2. Preparation of drugs and treatments

Curcumin (#C1386, Sigma-Aldrich, Poole, UK) was dissolved in dimethyl sulfoxide (DMSO, Merck, Beeston, UK) and stored at -20ºC. Aliquots were further diluted in culture media and added to the cells to provide the final concentration (0.1-10 μM), alongside vehicle-control (0.1% DMSO). $A\beta_{1-42}$ (Bachem, Torrance, CA, USA) was incubated in Milli-Q water at 37ºC for 72 h prior to use [12]. Cells were treated with $A\beta_{1-42}$ (2 μM) and curcumin for 48 h.

2.3. Protein analysis

Astrocytes grown on 13 mm glass coverslips were fixed at 12 DIV using 4% paraformaldehyde for 30 min. Coverslips were processed as described previously [27] using rabbit polyclonal anti-GFAP (1:2000; Dako, Ely, UK) and Hoechst 33342 (1:1000; Calbiochem, La Jolla, CA, USA). Images were acquired using fluorescence microscopy (AxioScop2, Zeiss). Five arbitrarily selected microscopic fields of view at a 40-fold magnification were photographed for each experimental condition of three separate experiments. Morphometric analysis of GFAP-immunolabelled astrocytes was quantified with NIH ImageJ software (National Institutes of Health, USA) [7, 22]. For Immunoblotting astrocytes were lysed in ice-cold buffer containing: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% mammalian protease inhibitor (Roche, Hertforshire, UK) and 20 mM NEM (Sigma-Aldrich). Protein samples were resolved, immunodetected and analysed as described previously [5]. The antibodies used were rabbit polyclonal anti-GFAP (1:4000; Dako), anti-phospho-SAPK/JNK1/2 (1:1000; Cell Signaling, Danvers, MA, USA), anti-SUMO-1 (1:1000; Cell Signaling), anti-SUMO-2/3 (1:1000; Invitrogen) and anti-SENP1 (1:1000; Imgenex, San Diego, CA, USA), mouse polyclonal anti-JNK3 (1:1000; Cell Signaling) and anti-Ubc9 (1:250; Santa Cruz Biotechnology, Wembley, UK), and mouse monoclonal anti-β-actin (1:1000; Cell Signaling).

2.4. Statistical analysis

Values are means ± SEM of at least three independent experiments. One-way analysis of the variance (ANOVA) followed by Tukey's post hoc test was applied to the means to determine differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin prevents Aβ-induced astrocyte reactivity and JNK activation

Astrocytes exhibit altered morphology in the course of AD, and are referred to as "reactive" astrocytes. Reactive astrocytes feature an up-regulation of GFAP expression and an apparent hypertrophy of cell bodies and processes [25, 26]. We first analyzed the effects of treatment with 2 μM $A\beta_{1-42}$ (Aβ) and 10 μM curcumin on astrocyte morphology by GFAP immunofluorescence. Aβ-treated astrocytes exhibited morphological signs of reactivity (Fig. 1A). The morphometric assessments of GFAP expression in astrocyte cultures show the marked changes in astroglial morphology observed in the different experimental groups. Aβ-treated cultures presented a greater increase in astrocytic cell area (hypertrophy) (179 ± 16 μm^2) compared with control group (89 ± 13 μm^2), which were prevented by curcumin (114 ± 15 μm^2) (n=3). In addition, we found that the number of primary processes leaving the soma was significantly increased in Aβ-treated astrocytes (6.7 ± 0.46) compared with control (2.4 ± 0.25) or Aβ + curcumin treated astrocytes (3.2 ± 0.55) (Fig. 1B). Additionally, Aβ significantly enhanced GFAP levels to 155 ± 18% of controls (n=6), whereas, concomitant treatment with curcumin blocked this increase in Western blot analysis (Fig. 1C). The Aβ-induced reactivity was not a consequence of glial cell damage or loss of viability, as evidenced by no differences in the release of LDH from control vs. Aβ-treated astrocytes (Fig. 1D).

In 3xTg-AD mice, curcumin has been shown to reduce activation of JNK, a key kinase in cellular stress, though the cell types in which this occurs has not been reported [16]. We therefore measured the effect of Aβ and curcumin on JNK in astrocytes. Aβ caused

a marked increase (160 ± 20%, n=4) in the phosphorylation status of pJNK, which was significantly reduced by concomitant treatment with curcumin (116 ± 11%, n=4) (Fig. 1E).

3.2. Aβ decreases SUMO-1 conjugated proteins in astrocytes

Next, we investigated whether SUMO conjugation to proteins was involved in the curcumin-mediated inhibition of Aβ-induced reactivity. Probing untreated astrocytes with anti-SUMO-1 antibody revealed four major bands of molecular weights approximately 15, 75, 150 and 250 kDa, with long film exposures required to reveal all of the bands (Fig. 2A, left lane). Treatment with Aβ resulted in a highly significant decrease (59 ± 9%, n=4) in the SUMO-1-conjugated proteins in astrocytes (Fig. 2A). This effect was blocked by concomitant treatment with curcumin (94 ± 8%, n=4). The JNK inhibitor L-JNKI1 (3μM) (Enzo Life Sciences, Exeter, UK) prevented the Aβ-induced decrease in protein conjugation by SUMO-1 (106 ± 16%, n=4) (Fig. 3A) in a similar way to curcumin, consistent with the concept that curcumin acts as a JNK inhibitor in astrocytes. This decrease in SUMOylation was specific to SUMO-1, since SUMO-2/3 conjugated proteins remained unaltered (Fig. 2B). In line with the decreased SUMO-1 conjugation, levels of the sole conjugating enzyme, Ubc9, were reduced (57± 12%, n=4) and treatment with curcumin was able to prevent this decrease (95 ± 7%, n=4) (Fig. 2C). No changes in the deconjugating enzyme, SENP1 were observed (Fig. 2D).

3.3. Over-expression of SUMO-1 in astrocytes blocks Aβ-induced increase in GFAP levels

To determine whether the decreased SUMO-1 conjugation was involved in the Aβ-induced astrocyte activation, we altered SUMOylation in astrocytes by transiently over-expressing the inactive SUMO-1 construct (SUMO-1ΔGG) or the constitutively active SUMO-1 construct (SUMO-1GG). The inactive SUMO-1contains a di-glycine deletion, which makes it unable to conjugate to proteins, as confirmed by detection of only one of the main SUMO-1 immunoreactive bands in astrocytes over-expressing SUMO-1ΔGG compared to SUMO-1GG (Fig. 3B). Astrocyte viability was unaffected by transfection (data not shown). Next, we exposed transfected astrocytes to Aβ. Over-expression of active SUMO-1 completely blocked the Aβ-induced increase in GFAP protein levels (94 ± 8%, n=4), whereas the inactive mutant exerted no effect in Western blot analysis (143 ± 14%, n=4) (Fig. 3C). In GFAP immunofluorescence analysis, Aβ-treated astrocytes over-expressing SUMO-1ΔGG exhibited morphological signs of reactivity (Fig. 3D). The increased cell area (hypertrophy) of GFAP immunostained astrocytes is a sensitive parameter used to evaluate the astroglial reactivity [22]. The morphometric analysis of the individual astrocytic cell area showed that astrocytes over-expressing SUMO-1 GG treated with Aβ presented a significantly decrease in cell area (95 ± 15 μm^2) compared with astrocytes over-expressing SUMO-1 ΔGG treated with Aβ (160 ± 22 μm^2, n=3) (Fig. 3E).

4. Discussion

We have used an *in vitro* model of Aβ-induced astrocyte activation, which is relevant to understanding the role of astrocyte reactivity in neurodegenerative disorders. Because of our prior interest in SUMOylation in AD [17], we hypothesized that SUMOylation was linked to Aβ-induced astrocyte reactivity. This paper is the first report of SUMOylation

patterns in astrocytes, and their regulation after Aβ exposure. The data show that astrocytes have a number of proteins which are SUMOylated under resting conditions, with relatively few targets of SUMO-1 compared to SUMO-2/3. This finding contrasts to the pattern observed when analyzing samples from neuronal cultures [5] or whole brain [17], where there are as many SUMO-1 bands as there are SUMO-2/3 bands. This restricted pattern of SUMO-1 conjugation suggests a key role in cellular regulation. Following exposure to Aβ, there is a marked decrease in conjugation by SUMO-1, but not by SUMO-2/3, an entirely unexpected and novel observation. Furthermore, increasing the ability of astrocytes to conjugate proteins with SUMO-1, by over-expressing SUMO-1GG, abolishes the ability of Aβ to elevate GFAP levels in astrocytes, suggesting a critical role for SUMO-1 in this pathway associated with astrocyte reactivity.

Curcumin is an effective suppressor of astrocyte activation, reducing Aβ-induced increase in GFAP levels and morphological changes, consistent with its reported effects *in vitro* and in animal models of AD [15, 29]. To our knowledge, the effects of curcumin on SUMOylation have not been reported previously. Our data show that curcumin prevents Aβ-induced reduction in SUMO-1 conjugation in astrocytes and that curcumin is acting mainly as a JNK inhibitor, since it is able to prevent the Aβ-induced activation of JNK. An effect of curcumin on JNK is consistent with the literature: treatment with curcumin has been shown to reduce activated JNK in an AD transgenic mouse model [16]. JNK and SUMOylation pathways have previously been associated [8, 18] and JNK itself is likely to be a target for SUMOylation. Indeed, in a human neuroblastoma cell line, increasing SUMO-1 conjugation increases JNK activation under conditions of moderate oxidative stress [8].

Altogether our data suggest astrocytes require SUMO-1 conjugation to remain in a non-reactive state and predict that increased SUMO-1 conjugation in astrocytes will be neuroprotective. SUMOylation is usually associated with a cytoprotective response to cellular stressors, e.g ischaemia [5, 30]. While there is little data on SUMO in astrocytes, one report has shown that the mRNA levels for SUMO-1, Ubc9 and SENP1 are down-regulated following lipopolysaccharide treatment of astrocytes, suggesting that loss of the SUMO pathway in astrocytes is associated with pathophysiological processes [3]. However, we note that SUMOylation of certain proteins in astrocytes, namely the C-terminal proteolytic fragment of the glutamate transporter, EAAT2, has been associated with gliotoxicity [8]. In our working model, SUMO is downstream of JNK, with JNK inhibition preventing reduction of SUMO-1 conjugation. Further research is required to elucidate the exact nature of astrocyte SUMO-1 conjugation and its regulation by JNK in neurodegeneration and neuroprotection.

Acknowledgments

The authors thank Jeremy Henley for SUMO-1 constructs and Vasco Silveirinha for assistance. This study was supported by a Royal Society Research Grant to HC. JBH was recipient of a Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) PhD Studentship. The authors declare no conflicts of interest.

References

[1] R. Abeti, A.Y. Abramov, M.R. Duchen, Beta-amyloid activates PARP causing astrocytic metabolic failure and neuronal death, Brain : a journal of neurology 134 (2011) 1658-1672.

[2] A.Y. Abramov, L. Canevari, M.R. Duchen, Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of

neurons through activation of NADPH oxidase, The Journal of neuroscience : the official journal of the Society for Neuroscience 24 (2004) 565-575.
[3] C.A. Akar, D.L. Feinstein, Modulation of inducible nitric oxide synthase expression by sumoylation, Journal of neuroinflammation 6 (2009) 12.
[4] I. Allaman, M. Gavillet, M. Belanger, T. Laroche, D. Viertl, H.A. Lashuel, P.J. Magistretti, Amyloid-beta aggregates cause alterations of astrocytic metabolic phenotype: impact on neuronal viability, The Journal of neuroscience : the official journal of the Society for Neuroscience 30 (2010) 3326-3338.
[5] H. Cimarosti, E. Ashikaga, N. Jaafari, L. Dearden, P. Rubin, K.A. Wilkinson, J.M. Henley, Enhanced SUMOylation and SENP-1 protein levels following oxygen and glucose deprivation in neurones, Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 32 (2012) 17-22.
[6] B. DaRocha-Souto, T.C. Scotton, M. Coma, A. Serrano-Pozo, T. Hashimoto, L. Sereno, M. Rodriguez, B. Sanchez, B.T. Hyman, T. Gomez-Isla, Brain oligomeric beta-amyloid but not total amyloid plaque burden correlates with neuronal loss and astrocyte inflammatory response in amyloid precursor protein/tau transgenic mice, Journal of neuropathology and experimental neurology 70 (2011) 360-376.
[7] F.S. Estrada, V.S. Hernandez, E. Lopez-Hernandez, A.A. Corona-Morales, H.Solis, A. Escobar, L. Zhang, Glial activation in a pilocarpine rat model for epileptogenesis: A morphometric and quantitative analysis, Neurosci Lett 514 (2012) 51-56.
[8] M. Feligioni, E. Brambilla, A. Camassa, A. Sclip, A. Arnaboldi, F. Morelli, X. Antoniou, T. Borsello, Crosstalk between JNK and SUMO signaling pathways: deSUMOylation is protective against H2O2-induced cell injury, PloS one 6 (2011) e28185.
[9] E. Foran, A. Bogush, M. Goffredo, P. Roncaglia, S. Gustincich, P. Pasinelli, D. Trotti, Motor neuron impairment mediated by a sumoylated fragment of the glial glutamate transporter EAAT2, Glia 59 (2011) 1719-1731.
[10] J.R. Gareau, C.D. Lima, The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition, Nature reviews. Molecular cell biology 11 (2010) 861-871.
[11] C.J. Garwood, A.M. Pooler, J. Atherton, D.P. Hanger, W. Noble, Astrocytes are important mediators of Abeta-induced neurotoxicity and tau phosphorylation in primary culture, Cell death & disease 2 (2011) e167.
[12] J.B. Hoppe, R.L. Frozza, A.P. Horn, R.A. Comiran, A. Bernardi, M.M. Campos, A.M. Battastini, C. Salbego, Amyloid-beta neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3beta, tau and neuroinflammation, Journal of pineal research 48 (2010) 230-238.
[13] S. Itagaki, P.L. McGeer, H. Akiyama, S. Zhu, D. Selkoe, Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease, Journal of neuroimmunology 24 (1989) 173-182.
[14] S. Lavoie, Y. Chen, T.P. Dalton, R. Gysin, M. Cuenod, P. Steullet, K.Q. Do, Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit, Journal of neurochemistry 108 (2009) 1410-1422.
[15] G.P. Lim, T. Chu, F. Yang, W. Beech, S.A. Frautschy, G.M. Cole, The curry spice curcumin reduces oxidative damage and amyloid pathology in an

Alzheimer transgenic mouse, The Journal of neuroscience : the official journal of the Society for Neuroscience 21 (2001) 8370-8377.
[16] Q.L. Ma, F. Yang, E.R. Rosario, O.J. Ubeda, W. Beech, D.J. Gant, P.P. Chen, B. Hudspeth, C. Chen, Y. Zhao, H.V. Vinters, S.A. Frautschy, G.M. Cole, Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin, The Journal of neuroscience : the official journal of the Society for Neuroscience 29 (2009) 9078-9089.
[17] L.E. McMillan, J.T. Brown, J.M. Henley, H. Cimarosti, Profiles of SUMO and ubiquitin conjugation in an Alzheimer's disease model, Neuroscience letters 502 (2011) 201-208.
[18] S. Muller, M. Berger, F. Lehembre, J.S. Seeler, Y. Haupt, A. Dejean, c-Jun and p53 activity is modulated by SUMO-1 modification, The Journal of biological chemistry 275 (2000) 13321-13329.
[19] M. Olabarria, H.N. Noristani, A. Verkhratsky, J.J. Rodriguez, Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease, Glia 58 (2010) 831-838.
[20] J.A. Orellana, K.F. Shoji, V. Abudara, P. Ezan, E. Amigou, P.J. Saez, J.X. Jiang, C.C. Naus, J.C. Saez, C. Giaume, Amyloid beta-induced death in neurons involves glial and neuronal hemichannels, The Journal of neuroscience : the official journal of the Society for Neuroscience 31 (2011) 4962-4977.
[21] V. Parpura, M.T. Heneka, V. Montana, S.H. Oliet, A. Schousboe, P.G. Haydon, R.F. Stout, Jr., D.C. Spray, A. Reichenbach, T. Pannicke, M. Pekny, M. Pekna, R. Zorec, A. Verkhratsky, Glial cells in (patho)physiology, Journal of neurochemistry 121 (2012) 4-27.
[22] A.J. Ramos, M.D. Rubio, C. Defagot, L. Hischberg, M.J. Villar, A. Brusco, The 5HT1A receptor agonist, 8-OH-DPAT, protects neurons and reduces astroglial reaction after ischemic damage caused by cortical devascularization, Brain Res 1030 (2004) 201-220.
[23] G. Scapagnini, C. Colombrita, M. Amadio, V. D'Agata, E. Arcelli, M. Sapienza, A. Quattrone, V. Calabrese, Curcumin activates defensive genes and protects neurons against oxidative stress, Antioxidants & redox signaling 8 (2006) 395-403.
[24] G. Scapagnini, R. Foresti, V. Calabrese, A.M. Giuffrida Stella, C.J. Green, R. Motterlini, Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers, Molecular pharmacology 61 (2002) 554-561.
[25] J.E. Simpson, P.G. Ince, G. Lace, G. Forster, P.J. Shaw, F. Matthews, G. Savva, C. Brayne, S.B. Wharton, M.R.C.C. Function, G. Ageing Neuropathology Study, Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain, Neurobiology of aging 31 (2010) 578-590.
[26] J.E. Simpson, P.G. Ince, P.J. Shaw, P.R. Heath, R. Raman, C.J. Garwood, C. Gelsthorpe, L. Baxter, G. Forster, F.E. Matthews, C. Brayne, S.B. Wharton, M.R.C.C. Function, G. Ageing Neuropathology Study, Microarray analysis of the astrocyte transcriptome in the aging brain: relationship to Alzheimer's pathology and APOE genotype, Neurobiology of aging 32 (2011) 1795-1807.
[27] A. Spilsbury, D. Vauzour, J.P. Spencer, M. Rattray, Regulation of NF-kappaB activity in astrocytes: effects of flavonoids at dietary-relevant concentrations, Biochemical and biophysical research communications 418 (2012) 578-583.

[28] A. Verkhratsky, M.V. Sofroniew, A. Messing, N.C. deLanerolle, D. Rempe, J.J. Rodriguez, M. Nedergaard, Neurological diseases as primary gliopathies: a reassessment of neurocentrism, ASN neuro 4 (2012).
[29] H.M. Wang, Y.X. Zhao, S. Zhang, G.D. Liu, W.Y. Kang, H.D. Tang, J.Q. Ding, S.D. Chen, PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes, Journal of Alzheimer's disease : JAD 20 (2010) 1189-1199.
[30] W. Yang, H. Sheng, H.M. Homi, D.S. Warner, W. Paschen, Cerebral ischemia/stroke and small ubiquitin-like modifier (SUMO) conjugation - a new target for therapeutic intervention?, Journal of neurochemistry 106 (2008) 989-999.
[31] Y.Q. Zhang, K.D. Sarge, Sumoylation of amyloid precursor protein negatively regulates Abeta aggregate levels, Biochemical and biophysical research communications 374 (2008) 673-678.

Legends to figures

Figure 1. Effects of curcumin on Aβ-induced astrocytic reactivity and JNK activation. Astrocytes were untreated or treated with 2 μM $A\beta_{1-42}$ in the presence or absence of 0.1, 1 and 10 μM curcumin for 48 h. A) Representative images of control and Aβ-treated astrocytes, with or without 10 μM curcumin, immunostained using anti-GFAP antibody (green) and Hoechst 333342 (blue). B) Morphometric analysis of cell area and number of primary process leaving the soma based on GFAP. Values are shown as mean ± SEM (n=3). Asterisks (* and **) indicate significant difference $p < 0.05$ and $p < 0.01$, respectively. C) Representative GFAP immunoreactivity and β-actin loading controls. The cumulative results are presented as percentage of control ± SEM (n=6). Asterisks (* and **) indicate significant difference $p < 0.05$ and $p < 0.01$, respectively. D) Cellular viability as measured by LDH assay. Values represent the amount of LDH released into the culture medium in relation to total LDH and are presented as percentage of control ± SEM (n=8). E) Representative phospho-JNK (Thr183/Tyr185) and JNK3 immunoreactivities and β-actin loading controls. The cumulative results represent the

ratio p-JNK/JNK and are presented as percentage of control ± SEM (n=4). Asterisks (* and **) indicate significant difference $p < 0.05$ and $p < 0.01$, respectively.

Figure 2. Effects of curcumin on protein SUMOylation in Aβ-treated astrocytes. A) Representative pattern of SUMO-1 immunoreactivity, β-actin loading controls and cumulative SUMO-1 results. B) Representative pattern of SUMO-2/3 immunoreactivity, β-actin loading controls and cumulative SUMO-2/3 results. C) Representative SENP1 immunoreactivity, β-actin loading controls and cumulative SENP1 results. D) Representative Ubc9 immunoreactivity, β-actin loading controls and cumulative Ubc9 results. The quantified data in A-D are presented as percentage of control ± SEM (n=4). Asterisk (*) indicates significant difference $p < 0.05$.

Figure 3. Effects of L-JNKI1 on protein SUMOylation and SUMO-1 overexpression on GFAP levels in Aβ-treated astrocytes. A) Representative pattern of SUMO-1 immunoreactivity, β-actin loading controls and cumulative SUMO-1 results using L-JNKI1. The quantified data are presented as percentage of control ± SEM (n=5). Asterisk (*) indicates significant difference $p < 0.05$. B) Representative pattern of SUMO-1 immunoreactivity and β-actin loading control from astrocytes over-expressing SUMO-1ΔGG (inactive) and SUMO-1GG (active). C) Representative GFAP immunoreactivity and β-actin loading controls and cumulative GFAP results from astrocytes over-expressing SUMO-1ΔGG (inactive) and SUMO-1GG (active). The quantified data are presented as percentage of control ± SEM (n=4). Asterisk (*) indicates significant difference $p < 0.05$. D) Representative images of control and Aβ-treated astrocytes over-expressing SUMO-1ΔGG (green) and SUMO-1GG (green)

immunostained using anti-GFAP antibody (red). E) Morphometric analysis of cell area based on GFAP. Values are shown as mean ± SEM (n=3). Asterisks (* and **) indicate significant difference $p < 0.05$ and $p < 0.01$, respectively.

Figure 4. Schematic diagram representing the key mediators of β-amyloid (Aβ)-induced astrocyte reactivity. Treatment with Aβ increases pJNK, which leads to a decrease in global SUMO-1 conjugation and increase in GFAP levels, causing astrocytes to become reactive. Treatment with curcumin is able to prevent Aβ effects. By decreasing pJNK, global SUMO-1 conjugation and GFAP levels are kept at basal levels, restoring the non-reactive state of astrocytes.

Figure 1

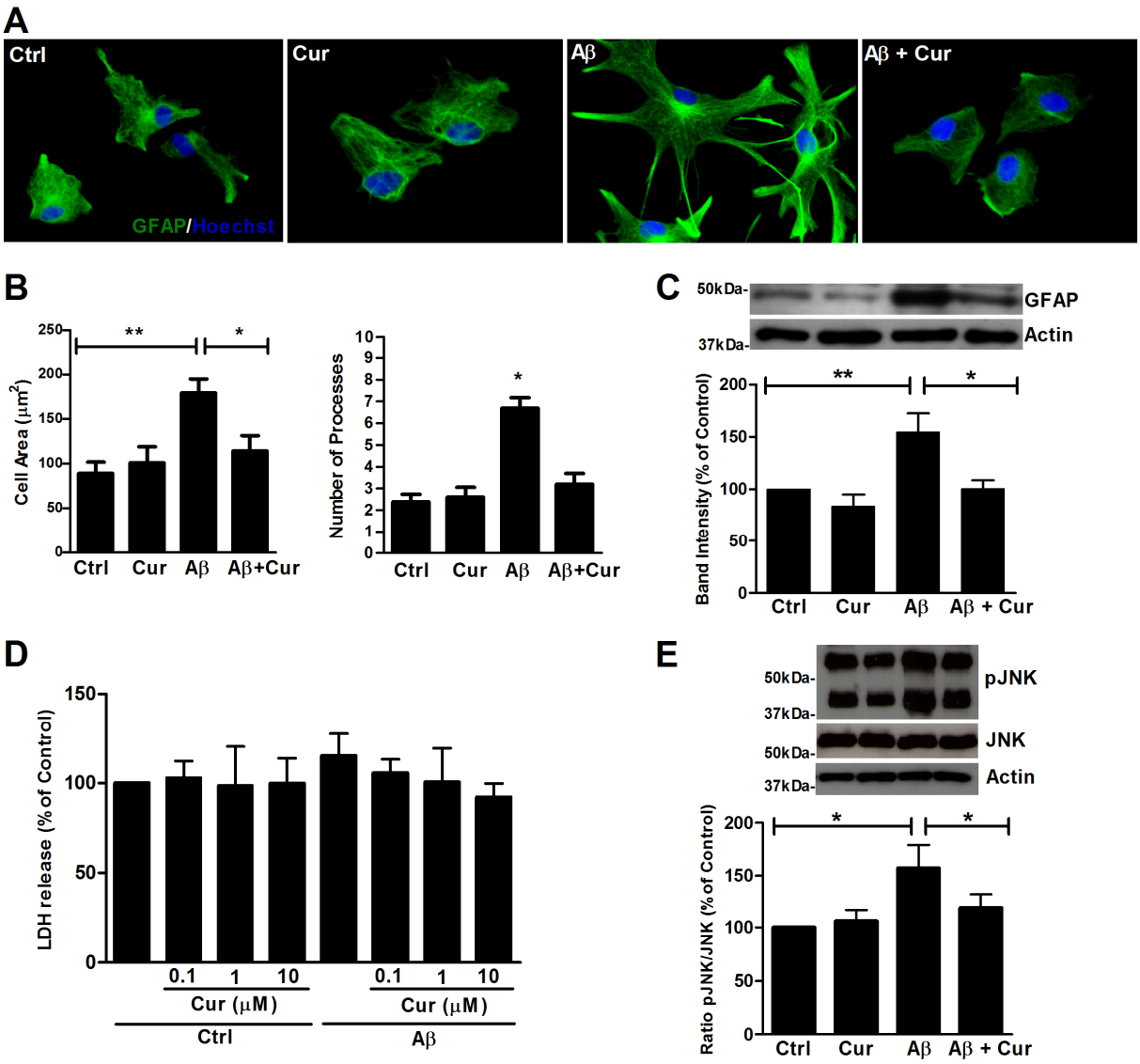

A
Ctrl
Cur
Aβ
Aβ + Cur
GFAP/Hoechst
B
Cell Area (μm²)
Number of Processes
Ctrl
Cur
Aβ
Aβ+Cur
C
50kDa-
37kDa-
GFAP
Actin
Band Intensity (% of Control)
Aβ + Cur
D
LDH release (% of Control)
0.1
1
10
Cur (μM)
E
pJNK
JNK
Actin
Ratio pJNK/JNK (% of Control)

Figure 2

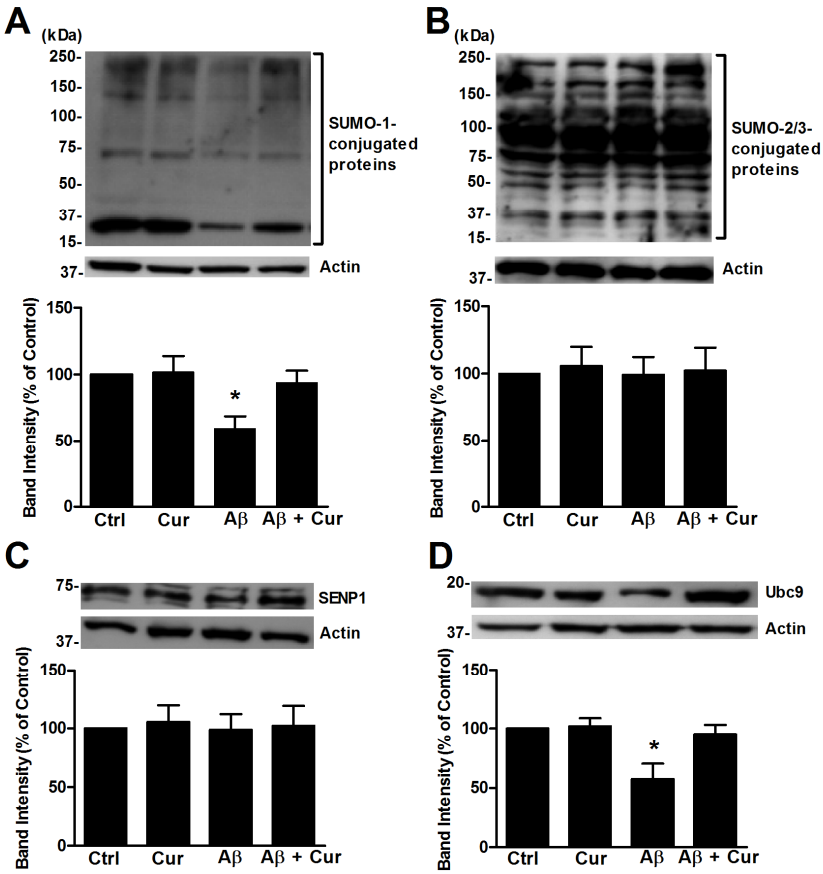

A
(kDa)
250-
150-
100-
75-
50-
37-
15-
SUMO-1-conjugated proteins
37-
Actin
Band Intensity (% of Control)
150
100
50
0
*
Ctrl
Cur
Aβ
Aβ + Cur
B
(kDa)
250-
150-
100-
75-
50-
37-
15-
SUMO-2/3-conjugated proteins
37-
Actin
Band Intensity (% of Control)
150
100
50
0
Ctrl
Cur
Aβ
Aβ + Cur
C
75-
SENP1
37-
Actin
Band Intensity (% of Control)
150
100
50
0
Ctrl
Cur
Aβ
Aβ + Cur
D
20-
Ubc9
37-
Actin
Band Intensity (% of Control)
150
100
50
0
*
Ctrl
Cur
Aβ
Aβ + Cur

Figure 3

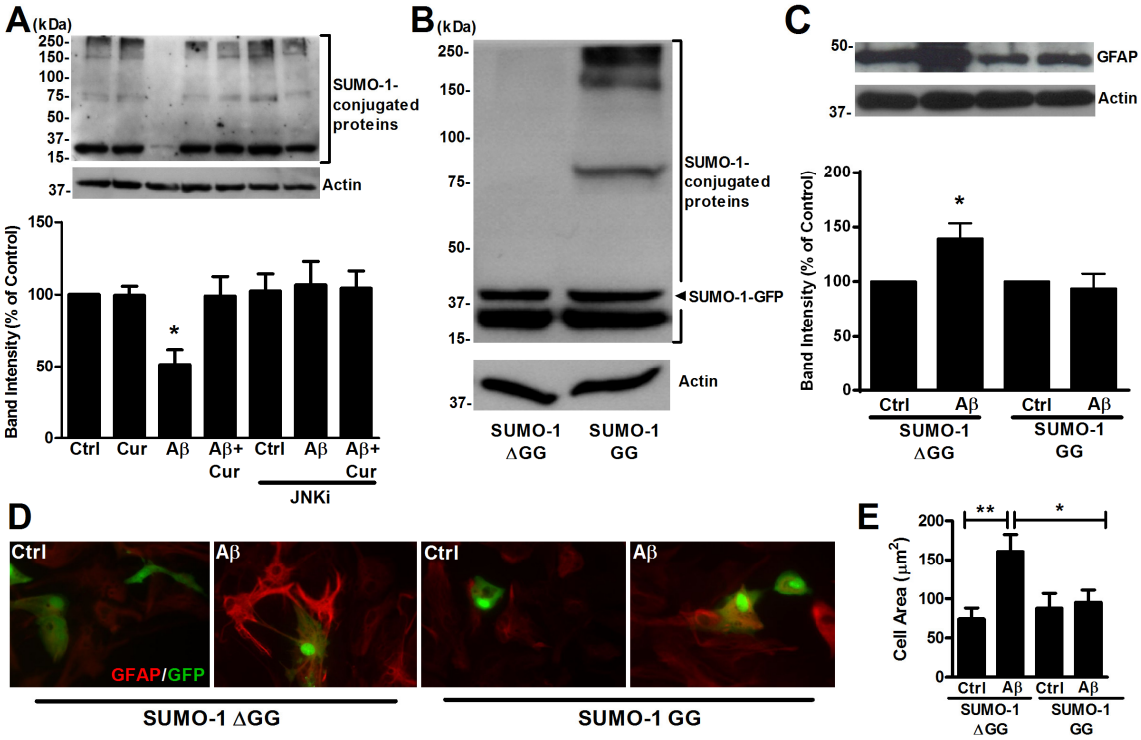
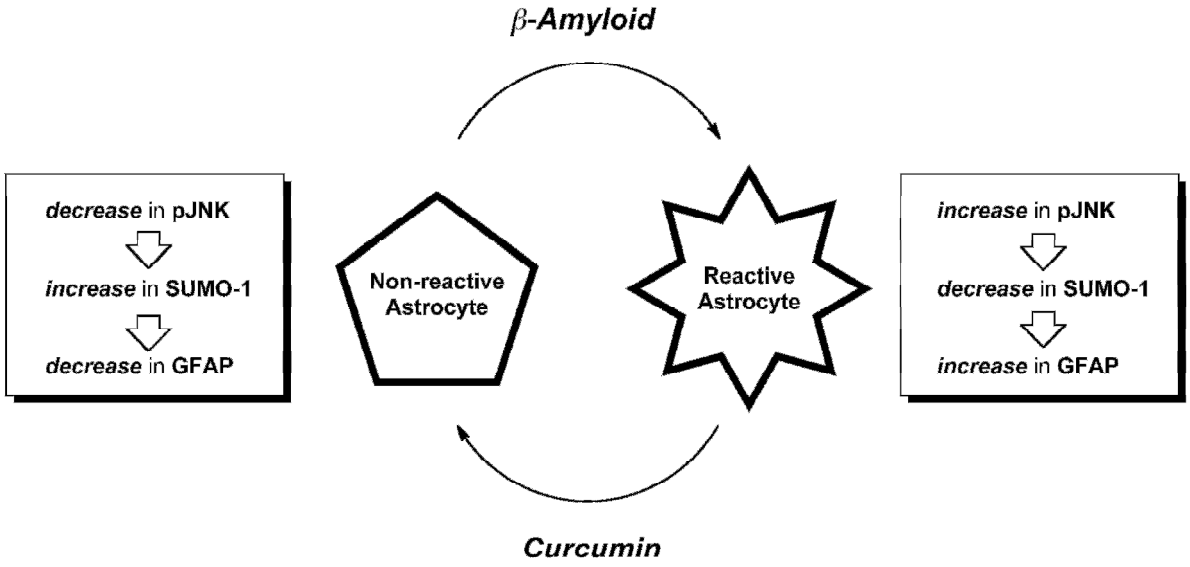

A
(kDa)
250-
150-
100-
75-
50-
37-
15-
SUMO-1-conjugated proteins
37-
Actin
Band Intensity (% of Control)
150
100
50
0
*
Ctrl
Cur
Aβ
Aβ+ Cur
Ctrl
Aβ
Aβ+ Cur
JNKi
B
(kDa)
250-
150-
100-
75-
50-
37-
15-
SUMO-1-conjugated proteins
SUMO-1-GFP
37-
Actin
SUMO-1 ΔGG
SUMO-1 GG
C
50-
GFAP
37-
Actin
Band Intensity (% of Control)
200
150
100
50
0
*
Ctrl
Aβ
Ctrl
Aβ
SUMO-1 ΔGG
SUMO-1 GG
D
Ctrl
Aβ
Ctrl
Aβ
GFAP/GFP
SUMO-1 ΔGG
SUMO-1 GG
E
Cell Area (μm²)
200
150
100
50
0
**
*
Ctrl
Aβ
Ctrl
Aβ
SUMO-1 ΔGG
SUMO-1 GG

Figure 4


β-Amyloid
decrease in pJNK
increase in SUMO-1
decrease in GFAP
Non-reactive Astrocyte
Reactive Astrocyte
increase in pJNK
decrease in SUMO-1
increase in GFAP
Curcumin

5. CAPÍTULO III

Artigo: *Curcumin protects organotypic hippocampal slice cultures from* $A\beta_{1-42}$*-induced synaptic toxicity* ***– Status:*** *a ser submetido ao periódico Neuropharmacology*

Curcumin protects organotypic hippocampal slice cultures from $A\beta_{1-42}$-induced synaptic toxicity

Juliana B. Hoppe[1,2], Marcus Haag[1], Benjamin J. Whalley[1], Christianne G. Salbego[2] and Helena Cimarosti[1,*]

[1]Reading School of Pharmacy, University of Reading, Whiteknights, Reading, RG6 6UB, UK

[2]Laboratory of Neuroprotection and Cell Signaling, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, 90035-003, Brazil

[*]Please address correspondence to:

Dr Helena Cimarosti, Reading School of Pharmacy, University of Reading, Reading, RG6 6UB, UK

Phone: +44 (0)118 378 8015

Fax: +44 (0)118 378 6562

E-mail: h.i.cimarosti@reading.ac.uk

Abstract

Synaptic plasticity is crucial for the process of memory formation and synaptic degeneration (synaptotoxicity) is involved in the early stages of Alzheimer's disease (AD). Increasing evidence demonstrates that beta-amyloid (Aβ) is toxic to synapses, resulting in the progressive dismantling of neuronal circuits. Therapeutic strategies that could counteract the synaptotoxic effects of Aβ could be particularly relevant for providing effective treatments for this devastating neurodegenerative disease. Curcumin was recently reported to reverse Aβ detrimental synaptic effects and improve memory in animal models of AD, though the exact pathways are incomplete. Little is currently known about the specific mechanisms by which Aβ affects neuronal excitability and whether curcumin can ameliorate synaptic transmission in the hippocampus. Here, organotypic hippocampal slice cultures exposed to $A\beta_{1-42}$ were used to study the proposed neuroprotective effects of curcumin. Curcumin counteracted both deleterious effects of Aβ; the initial synaptic dysfunction and the later neuronal death. A spectral analysis of multi-electrode array (MEA) recordings of spontaneous neuronal activity showed an attenuation of signal propagation induced by Aβ before cell death and curcumin-induced alterations to LFP phase coherence. Curcumin-mediated attenuation of Aβ-induced synaptic dysfunction involved regulation of synaptic proteins, namely phospho-CaMKII and phospho-synapsin I (Ser603). Taken together, our results expand the neuroprotective role of curcumin to a synaptic level. The identification of these mechanisms underlying the effects of curcumin may lead to new targets for future therapies for AD.

Keywords: Beta-amyloid peptide; CaMKII; curcumin; multi-electrode array; organotypic hippocampal slice culture; synapsin I

1. Introduction

Increasing evidence suggests that impaired memory and cognitive decline in Alzheimer's disease (AD) begin with synaptic dysfunction, are followed by synaptic loss and progress to include widespread neuronal cell death and network failure (Coleman *et al.* 2004, Koffie *et al.* 2009, Masliah *et al.* 2001, Zeng *et al.* 2010). Although well accepted as a key player in AD pathogenesis, the specific mechanisms underlying detrimental effects of beta-amyloid (Aβ) peptide on synaptic function have not been fully elucidated (Koffie *et al.* 2009, Tanzi & Bertram 2005). Several studies have demonstrated Aβ-induced synaptic toxicity that results in the progressive dismantling of neuronal circuits (Hartley *et al.* 1999, Jo *et al.* 2011, Selkoe 2008, Tsai *et al.* 2004), and multiple molecular and cellular signaling pathways contributing to this process have been proposed (Kamenetz *et al.* 2003, Ma & Klann 2012, Palop & Mucke 2010, Shankar & Walsh 2009). Apart from a mechanistic understanding of Aβ's ability to disrupt normal synaptic function, therapeutic strategies, including dietary interventions, which could counteract synaptotoxic effects of Aβ, warrant serious consideration as effective treatments for this devastating neurodegenerative disease.

Curcumin, a major polyphenol from curry spice (*Curcuma longa*), was recently reported to reverse Aβ detrimental synaptic effects and improve memory in animal models of AD (Ishrat *et al.* 2009, Ma *et al.* 2013, Varghese *et al.* 2010). Preventing loss of synaptic proteins, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and synapsin I, has been suggested to mediate the synaptoprotective effects of curcumin (Sun *et al.* 2013, Wu *et al.* 2011, Xu *et al.* 2009). CaMKII and synapsin I are key proteins implicated in presynaptic neurotransmitter release *(Benfenati et al. 1996, Chi et al.* 2003) and are essential for synaptic plasticity (Hilfiker *et al.* 1999, Ninan & Arancio

2004). During the induction of long-term potentiation (LTP), CaMKII undergoes autophosphorylation and activation following activity-triggered Ca^{2+} influx through N-methyl-*D*-aspartate (NMDA) receptors (Sanhueza & Lisman 2013). Aβ has been shown to block CaMKII autophosphorylation/activation in rat hippocampal slices and CaMKII activity is decreased in the hippocampus of AD patients (Reese *et al.* 2011, Zeng *et al.* 2010, Zhao *et al.* 2004). CaMKII modulates various synaptic proteins, including synapsin I which, under resting conditions, anchors synaptic vesicles to cytoskeletal elements, whereas once phosphorylated it dissociates from synaptic vesicles, increasing their availability at the presynaptic terminal for neurotransmitter release (Chi *et al.* 2003). Aβ-induced synapse loss in primary hippocampal or cortical neurons has been shown to be accompanied by a reduction in synapsin I, an effect also observed in the hippocampus of AD patients (Evans *et al.* 2008, Qin *et al.* 2004). Multiple Ca^{2+}-regulated signaling pathways are involved in mediating Aβ action at hippocampal synapses and Aβ-induced accumulation of cytosolic Ca^{2+}, which contributes to abnormal levels of phosphorylation of several synaptic proteins, including CAMKII, is an early and pivotal event in Aβ neurotoxicity (Xie *et al* 2004; Zeng *et al* 2010).

Little is currently known about the specific mechanisms by which Aβ can affect neuronal excitability and whether curcumin can ameliorate Aβ effects upon synaptic transmission in the hippocampus, a brain area critical for learning and memory, especially vulnerable to damage at early stages of AD. Thus, the present study assesses early effects of Aβ upon synaptic transmission and whether they are beneficially modulated by curcumin. The effects of Aβ treatment, in the presence or absence of curcumin, were assessed via multi-electrode array (MEA) electrophysiological recordings (Hill *et al.* 2010, Varghese et al. 2010) of spontaneous local field potential (LFP) activity from organotypic hippocampal slice cultures. Aβ exerted detrimental

effects upon synaptic transmission in hippocampal networks that were ablated by curcumin. Furthermore, we demonstrate that curcumin prevented Aβ-induced inactivation of CaMKII and synapsin I, key synaptic proteins, which could effectively rescue Aβ-induced LFP deficits at hippocampal synapses. Taken together, our results demonstrate that curcumin can act via a CAMKII-dependent pathway to prevent early stage Aβ-induced synaptic dysfunction in functional hippocampal neuronal networks.

2. Materials and Methods

2.1. Organotypic hippocampal slice cultures

Animals were maintained and used according to the UK Animals (Scientific Procedures) Act, 1986 and killed by cervical dislocation. All efforts were made to minimise animal suffering and the number of animals used. Organotypic hippocampal slice cultures were prepared from 6-8-day-old Wistar rats (NIH, Harlan, UK) as described previously (Cimarosti *et al.* 2009). Briefly, transverse hippocampal slices (350 μm) were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, UK) and transferred to ice-cold Hank's balanced salt solution (HBSS, Invitrogen). Slices were placed on membrane inserts (Millicell®-CM 0.4 μm, Millipore) in 24-well plates. Each well contained 250 μL of culture medium (pH 7.3) comprising minimum essential medium (MEM, Invitrogen) 50%, HBSS 25% and horse serum 25%, supplemented with glucose 36 mM, HEPES 25 mM, $NaHCO_3$ 4 mM, fungizone 1%, and gentamicin 0.100 $mg.mL^{-1}$. Cultures were incubated at 37ºC in an atmosphere of 5% CO_2 for 14 days *in vitro* (DIV) prior to use. Medium was changed every 3 days.

2.2. Preparation of drugs and treatments

Curcumin (#C1386, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) and stored at -20ºC until use. Aliquots were further diluted in culture media and added to the slices to provide the final concentration, alongside vehicle-control (0.1% DMSO). $A\beta_{1-42}$ (Bachem) was incubated in Milli-Q water at 37ºC for 72 h prior to use. On DIV 14 cultures were treated with $A\beta_{1-42}$ (2 μM) and curcumin (10 μM) for 24 h and 48 h (Hoppe *et al.* 2010).

2.3. Assessment of cell death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Cimarosti *et al.* 2009, Hoppe *et al.* 2010) and lactate dehydrogenase (LDH) release into the medium. Twenty-four and forty-eight hours after $A\beta_{1-42}$ and curcumin treatments, PI 7.5 mM was added to cultures and incubated for 1 h. Slices were observed with an inverted Nikon Eclipse TE 300 microscope (Nikon, Japan; magnification ×5) fitted with a standard rhodamine filter set. Images were captured using a CCD camera (Visitron Systems, Puchheim, Germany) and subsequently analyzed using Scion Image software (Scion Corporation, www.scioncorp.com). For quantification of cellular damage, the pixel intensity and area in which PI fluorescence was detectable above background level was determined using the 'density slice' function of the software. This tool converts portions of the greyscale images into red, based on the density value of the pixel, providing a value correspondent to the amount of PI fluorescence in each slice. The results were expressed as a percentage in relation to the total area of each slice. For measurement of LDH release into the culture medium, Tox7 kit (Sigma-Aldrich) was used according to the manufacturer's instructions. Samples of medium from the wells containing the inserts with slices were combined

with the assay mixture consisting of cofactor, substrate and dye solutions. The conversion of the tetrazolium dye into a soluble, coloured compound by nicotinamide adenine dinucleotide (NADH), which is proportional to LDH activity, was quantified using a microplate spectrophotometer.

2.4. Electrophysiological recordings

Substrate-integrated MEAs (Multi Channel Systems, Reutlingen, Germany) were used to record spontaneous LFP activity from each organotypic hippocampal slice culture. MEAs comprise 60 planar electrodes (including reference ground) of 30 µm diameter, arranged in an 8x8 array with 200 µm spacing between electrodes (100 µm recording radius). Electrical activity was amplified (×1100, MEA60 System, Multi Channel Systems GmbH, Reutlingen, Germany), sampled at 10 kHz and lowpass filtered (1-200 Hz; 2^{nd} order Butterworth filter) for analyses of LFPs. Recordings ($n \geq 6$ per condition) were made at DIV 15 using a heated MEA headstage (25ºC). Data acquisition was to a PC using MC Rack software (Multi Channel Systems GmbH, Reutlingen, Germany) and offline analysis performed using MC_Rack and MATLAB 7.0.4 (Mathworks Inc., Natick, MA). LFP power was calculated via multitaper spectrums for a recording period of 120 s using a 1-40 Hz frequency range using the Chronux toolbox (Mitra & Bokil, 2007; http://chronux.org/; time-bandwidth product: 3; taper number: 5). Spectrograms were averaged to produce a single power value for each MEA channel which was scaled to the channel showing the highest power value on a given MEA (P_{max}). Power values for each channel were categorized by distance from the focus (P_{dist}), normalised to P_{max} and averaged for each distance per MEA and condition ($\Sigma\ P_{max}/P_{dist}$). Resulting normalised power values were averaged across all MEAs per condition. Phase coherences were calculated using multitaper spectrums

estimated for representative LFP cutouts of 5 s duration and a 1-40 Hz frequency range for each MEA. The phase coherence $C_{max,dist}$ between the underlying spectra of the channel with the highest power and channels at various distances from it (see above) was calculated based on the multitaper spectrums resulting in a value between 0 (no coherence) and 1 (strongest coherence). Coherence values were then averaged across distances before averaging across all MEAs per condition ($\Sigma C_{max,dist}$)), resulting in a single coherence value for each distance per condition.

2.5. Western blots and densitometry

Slices were lysed in ice-cold buffer containing: Tris-HCl (pH 7.4) 50 mM, NaCl 150 mM, EDTA 1 mM, SDS 0.1%, Triton X-100 1%, mammalian protease inhibitor 1% and NEM 20 mM. Protein samples were resolved, immunodetected and analysed as described previously (Cimarosti et al. 2009). The antibodies used were rabbit polyclonal anti-phospho-CaMKII (Thr286) (1:1000; Cell Signaling), anti-CaMKII (1:1000; Cell Signaling), anti-phospho-synapsin I (Ser09) (1:1000; Cell Signaling), anti-phospho-synapsin I (Ser603) (1:250; Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (1:1000; Cell Signaling).

2.6. Statistical analysis

Data are presented as mean ± SEM of the indicated number of independent experiments. Frequency values for all data points on MEAs were averaged over five MEAs. One-way analysis of the variance (ANOVA) followed by Tukey's post hoc test was applied to the means to determine significant differences between experimental

groups. Electrophysiological data are presented as median ± MEM[1] (for LFP power) and interquartile and absolute range from the median (for phase coherence) for normalised channels per distance. Normalised frequency and phase coherence values for all distances and MEAs were analysed for differences using a Kruskal-Wallis test followed by Dunn's post-hoc tests when appropriate. For all statistical tests used, $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin prevents Aβ-induced cellular death

We first analysed the effects of treatment with $A\beta_{1-42}$ (2 μM) and $A\beta_{1-42}$ plus curcumin (2 μM and 10 μM, respectively) for 24 h and 48 h on cell viability. Organotypic hippocampal slice cultures treated with Aβ for 24 h showed a slightly increased cell death (12.5 ± 2.7%, n = 8) compared to control slices (4.3 ± 0.7%, n = 10), as assessed by the incorporation of PI uptake, whereas treatment for 48 h caused a significant increase in PI uptake (25.4 ± 3.5%, n = 8, $P \leq 0.001$) (Figure 1A, B). This Aβ-induced increase in PI uptake was accompanied by a 42 ± 9% increase (n = 6, $P \leq 0.05$) in LDH released into the culture medium compared to control slices (Figure 1C). Concomitant curcumin treatment prevented the Aβ-induced increase in PI uptake (10.8 ± 2.2%, n = 8) and LDH release (104 ± 15%, n = 6).

3.2. *Assessment of spontaneous neuronal activity using MEA recordings*

Spontaneous LFPs (Figure 2A; inset) were recorded from organotypic hippocampal slice cultures at DIV 15. In control conditions, median total LFP power progressively decreased with increasing distance from the site at which the highest

[1] Standard error of the median absolute deviation

power LFP was recorded (Figure 2A). Whilst 24 h $A\beta_{1-42}$ (2 μM) treatment appeared to increase the extent to which total LFP decreased, this was not statistically significant ($P \geq 0.05$). However, total LFP power under $A\beta_{1-42}$ plus curcumin (2 μM and 10 μM, respectively) conditions declined at a significantly ($P \leq 0.05$) lower rate than in cultures treated with $A\beta_{1-42}$ (2 μM) alone. These results demonstrate that concomitant curcumin treatment can reverse the tendency for $A\beta_{1-42}$ to attenuate synaptically propagated neuronal activity. In order to probe the manner in which curcumin effects upon LFP propagation arose, we next examined the effects of drug treatments upon phase coherence assessed with reference to the channel where the highest power LFP was observed ('focus'). In comparison to control conditions, although $A\beta_{1-42}$ (2 μM) treatment had no effect upon phase coherence at any distance from the focus, phase coherence was significantly reduced 200 μm from the focus in $A\beta_{1-42}$ plus curcumin (2 μM and 10 μM, respectively) conditions (Figure 2B).

3.3. Curcumin prevents Aβ-induced decrease in the phosphorylation/activation of synapsin I and CaMKII

In order to investigate possible mechanisms involved in the curcumin-mediated attenuation of Aβ-induced synaptic dysfunction observed in our model, we evaluated phosphorylation/activation of synapsin I and CaMKII. Synapsin I is phosphorylated at multiple sites by various protein kinases, including CaMKs, which phosphorylate synapsin I at Ser9 (CaMKI) and Ser603 (CaMKII) (Yamagata 2003). We measured the effects of Aβ and curcumin on these two important phosphorylation sites of synapsin I. Neither Aβ nor curcumin affected the phosphorylation state of synapsin I at Ser9 (Figure 3A, B). However, Aβ caused a marked decrease in the phosphorylation of synapsin I at Ser603 (50 ± 12%, n = 5, $P \leq 0.05$), which was completely blocked by

concomitant curcumin treatment (96 ± 8%, n = 5, $P \leq 0.01$, Figure 3A, C). Because the phosphorylation of synapsin I at Ser603 is generally considered to be CaMKII-dependent (Yamagata 2003), we further investigated the phosphorylation state of CaMKII. CaMKII autophosphorylation at Thr286 results in a persistently active form of the kinase, which is required for CAMKII to phosphorylate/activate synapsin I (Hilfiker *et al.* 1999). Treatment with Aβ resulted in a significant decrease in the ratio phospho-CaMKII (Thr286)/CaMKII (54 ± 13%, n = 5, $P \leq 0.05$), while curcumin abrogated this effect (85 ± 9%, n = 5, Figure 3D).

4. Discussion

Here, we have used an organotypic hippocampal slice culture model of demonstrate functional neuroprotective effects of curcumin againist Aβ-induced neuronal death and synaptic dysfunction.

Firstly, we demonstrated that extended exposure to Aβ (48 h) was necessary to induce significant neuronal death in organotypic hippocampal slices, which is in agreement with previous reports (Lu *et al.* 2004; Ishige *et al.* 2007; Patel & Good 2007; Suh *et al.* 2008). Interestingly, co-treatment with curcumin for 48 h prevented cellular damage. Whilst treatment with Aβ for 24 h did not cause significant neuronal loss, it exerted effects upon functional synaptic transmission in organotypic slices, which were also prevented by curcumin. Curcumin-induced blockade of Aβ effects upon LFP power is consistent with prevention of synapse loss and/or synaptic dysfunction. Of particular interest is the effect of curcumin upon phase coherence, a measure of the extent to which spatially separated LFP events retain elements of synchronisation, where reduced phase coherence was observed. Aβ has been shown to reduce glutamate reuptake (Palop

& Mucke 2010) leading to remodeling of synaptic synaptic plasticity that culminates in synaptic depression. Furthermore, other studies have shown that Aβ attenuates NMDA receptor currents by direct effects upon GluN2B containing NMDARs (Koffie *et al.* 2011) again leading to reduced Ca^{2+} influx into spines the promotion of long-term depression (LTD) and ultimately, impairments in neuronal network function (Decker *et al.* 2010; Corona *et al.* 2011). Given the pivotal role of sustained reduction of synaptically evoked Ca^{2+} influx and activation of cellular pathways that lead to spine loss and synapse weakening in Aβ-induced synaptotoxicity (Shankar *et al.* 2007), this desynchronizing effect of curcumin may underlie an element of its neuroprotective properties against Aβ-induced loss of electrophysiologically active synapses.

These results are reinforced by our findings on the profile of CaMKII phosphorylation. It is known that Aβ-induced reduction of Ca^{2+} influx through NMDA receptors limits CAMKII function (Koffie *et al.* 2011). We observed that curcumin was able to prevent Aβ-induced decrease in CaMKII function in organotypic hippocampal slices. This contributed to the induction of more robust and synaptically efficient neurons, reflected in inhibition of synaptic dysfunction and neuronal death. Moreover, CaMKII activation facilitates the induction of LTP, which promotes dendritic spine enlargement and synaptic strength (Pi *et al.* 2010; Koffie *et al.* 2011).

Our results are in agreement with previous studies reporting the roles of synapsin I and CaMKII in the neuroprotective effects of curcumin. For example, dietary supplementation of curcumin improved the outcome of traumatic brain injury not only by dramatically reducing oxidative damage, but also by restoring the levels of phospho-synapsin I that had been reduced after the insult (Wu *et al.* 2006). Additionally, treatment with a curcumin derivative, which exhibits better brain absorption and biological activity, counteracted the post-traumatic deficits in cognition and locomotion,

as well as prevented the reductions in synapsin I and CaMKII in the hippocampus and spinal cord (Wu *et al.* 2011). Moreover, phospho-CaMKII down-regulation was observed in the hippocampus of senescence-accelerated mouse prone 8 (SAMP8 mice), a model displaying age-related deficits of cognition. Treatment of SAMP8 mice with curcumin attenuated the cognitive impairment by improving the expression of phospho-CaMKII (Sun *et al.* 2013).

Taken together, our results support the emerging view that Aβ-mediated synaptic dysfunction appears to be an important driving factor in AD pathogenesis and understanding the molecular underpinnings may provide effective therapeutic targets for combating the disease. Our results show that the protective role of curcumin goes beyond its capacity to modulate neuronal death since it is able to counteract early and later deleterious effects of Aβ; synaptic dysfunction and later neuronal loss respectively. Our results suggest that curcumin modulates Aβ-induced decrease in synaptic function via a CaMKII-dependent pathway.

Acknowledgments

This study was supported by a Royal Society Research Grant to HC. JBH was recipient of a Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) PhD Studentship. The authors declare no conflicts of interest.

References

Benfenati, F., Onofri, F., Czernik, A. J. and Valtorta, F. (1996) Biochemical and functional characterization of the synaptic vesicle-associated form of CA2+/calmodulin-dependent protein kinase II. *Brain Res Mol Brain Res,* **40,** 297-309.

Chi, P., Greengard, P. and Ryan, T. A. (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. *Neuron,* **38,** 69-78.

Cimarosti, H., Kantamneni, S. and Henley, J. M. (2009) Ischaemia differentially regulates GABA(B) receptor subunits in organotypic hippocampal slice cultures. *Neuropharmacology,* **56,** 1088-1096.

Coleman, P., Federoff, H. and Kurlan, R. (2004) A focus on the synapse for neuroprotection in Alzheimer disease and other dementias. *Neurology,* **63,** 1155-1162.

Corona, C., Pensalfini, A., Frazzini, V., Sensi, S.L. (2011) New theratpeutic targets in Alzheimer's disease: brain deregulation of calcium and zinc. *Cell Death Dis*, **2**, e176.

Decker, H., Jürgensen, S., Adrover, M.F., Brito-Moreira, J., Bomfim, T.R., Klein, W.L., Epstein, A.L., De Felice, F.G., Jerusalinsky, D., Ferreira, S.T. (2010) N-methyl-D-aspartate receptors are required for synaptic targeting of Alzheimer's toxic amyloid-β peptide oligomers. *J Neurochem*, **115**, 1520-9.

Evans, N. A., Facci, L., Owen, D. E., Soden, P. E., Burbidge, S. A., Prinjha, R. K., Richardson, J. C. and Skaper, S. D. (2008) Abeta(1-42) reduces synapse number and inhibits neurite outgrowth in primary cortical and hippocampal neurons: a quantitative analysis. *J Neurosci Methods,* **175,** 96-103.

Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vasquez, S., Vassilev, P. M., Teplow, D. B. and Selkoe, D. J. (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci,* **19,** 8876-8884.

Hilfiker, S., Pieribone, V. A., Czernik, A. J., Kao, H. T., Augustine, G. J. and Greengard, P. (1999) Synapsins as regulators of neurotransmitter release. *Philos Trans R Soc Lond B Biol Sci,* **354,** 269-279.

Hill, A. J., Jones, N. A., Williams, C. M., Stephens, G. J. and Whalley, B. J. (2010) Development of multi-electrode array screening for anticonvulsants in acute rat brain slices. *J Neurosci Methods,* **185,** 246-256.

Ho, R., Ortiz, D. and Shea, T. B. (2001) Amyloid-beta promotes calcium influx and neurodegeneration via stimulation of L voltage-sensitive calcium channels rather than NMDA channels in cultured neurons. *J Alzheimers Dis,* **3,** 479-483.

Hoppe, J. B., Frozza, R. L., Horn, A. P., Comiran, R. A., Bernardi, A., Campos, M. M., Battastini, A. M. and Salbego, C. (2010) Amyloid-beta neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3beta, tau and neuroinflammation. *J Pineal Res,* **48,** 230-238.

Ishrat, T., Hoda, M. N., Khan, M. B., Yousuf, S., Ahmad, M., Khan, M. M., Ahmad, A. and Islam, F. (2009) Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). *Eur Neuropsychopharmacol,* **19,** 636-647.

Jo, J., Whitcomb, D. J., Olsen, K. M. et al. (2011) Abeta(1-42) inhibition of LTP is mediated by a signaling pathway involving caspase-3, Akt1 and GSK-3beta. *Nat Neurosci,* **14,** 545-547.

Joseph, M. S., Ying, Z., Zhuang, Y. et al. (2012) Effects of diet and/or exercise in enhancing spinal cord sensorimotor learning. *PLoS One,* **7,** e41288.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S. and Malinow, R. (2003) APP processing and synaptic function. *Neuron,* **37,** 925-937.

Koffie, R. M., Meyer-Luehmann, M., Hashimoto, T. et al. (2009) Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. *Proc Natl Acad Sci U S A,* **106,** 4012-4017.

Koffie, R.M., Hyman, B.T., Spires-Jones, T.L. (2011) Alzheimer's disease: synapses gone cold. *Mol Neurodegener*, **6**, 63.

Lambert, M. P., Barlow, A. K., Chromy, B. A. et al. (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A,* **95,** 6448-6453.

Ma, Q. L., Zuo, X., Yang, F. et al. (2013) Curcumin suppresses soluble tau dimers and corrects molecular chaperone, synaptic, and behavioral deficits in aged human tau transgenic mice. *J Biol Chem,* **288,** 4056-4065.

Ma, T. and Klann, E. (2012) Amyloid beta: linking synaptic plasticity failure to memory disruption in Alzheimer's disease. *J Neurochem,* **120 Suppl 1,** 140-148.

Masliah, E., Mallory, M., Alford, M., DeTeresa, R., Hansen, L. A., McKeel, D. W., Jr. and Morris, J. C. (2001) Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology,* **56,** 127-129.

Mitra, P., Bokil, H. (2007) Observed Brain Dynamics. Oxford University Press, USA, 408.

Ninan, I. and Arancio, O. (2004) Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. *Neuron,* **42,** 129-141.

Palop, J. J. and Mucke, L. (2010) Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat Neurosci,* **13,** 812-818.

Pi, H.J., Otmakhovm N., El Gaamouch, F., Lemelin, D., De Koninck, P., Lisman, J. (2010) CaMKII control of spine size and synaptic strength: role of

phosphorylation states and nonenzymatic action. *Proc Natl Acad Sci USA*, **107**, 14437-42.

Qin, S., Hu, X. Y., Xu, H. and Zhou, J. N. (2004) Regional alteration of synapsin I in the hippocampal formation of Alzheimer's disease patients. *Acta Neuropathol,* **107,** 209-215.

Reese, L. C., Laezza, F., Woltjer, R. and Taglialatela, G. (2011) Dysregulated phosphorylation of Ca(2+) /calmodulin-dependent protein kinase II-alpha in the hippocampus of subjects with mild cognitive impairment and Alzheimer's disease. *J Neurochem,* **119,** 791-804.

Sanhueza, M. and Lisman, J. (2013) The CaMKII/NMDAR complex as a molecular memory. *Mol Brain,* **6,** 10.

Selkoe, D. J. (2008) Soluble oligomers of the amyloid beta-protein impair synaptic plasticity and behavior. *Behav Brain Res,* **192,** 106-113.

Shankar, G. M. and Walsh, D. M. (2009) Alzheimer's disease: synaptic dysfunction and Abeta. *Mol Neurodegener,* **4,** 48.

Shankar, G.M., Bloodgood, B.L., Townsend, M., Walsh, D.M., Selkoe, D.J., Sabatini, B.L. (2007) Natural oligomers of the Alzheimer amyloid beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci*, **27**, 2866-75.

Sun, C. Y., Qi, S. S., Zhou, P., Cui, H. R., Chen, S. X., Dai, K. Y. and Tang, M. L. (2013) Neurobiological and pharmacological validity of curcumin in ameliorating memory performance of senescence-accelerated mice. *Pharmacol Biochem Behav,* **105C,** 76-82.

Tanzi, R. E. and Bertram, L. (2005) Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell,* **120,** 545-555.

Tsai, J., Grutzendler, J., Duff, K. and Gan, W. B. (2004) Fibrillar amyloid deposition leads to local synaptic abnormalities and breakage of neuronal branches. *Nat Neurosci,* **7,** 1181-1183.

Varghese, K., Molnar, P., Das, M., Bhargava, N., Lambert, S., Kindy, M. S. and Hickman, J. J. (2010) A new target for amyloid beta toxicity validated by standard and high-throughput electrophysiology. *PLoS One,* **5,** e8643.

Wu, A., Ying, Z. and Gomez-Pinilla, F. (2006) Dietary curcumin counteracts the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition. *Exp Neurol,* **197,** 309-317.

Wu, A., Ying, Z., Schubert, D. and Gomez-Pinilla, F. (2011) Brain and spinal cord interaction: a dietary curcumin derivative counteracts locomotor and cognitive deficits after brain trauma. *Neurorehabil Neural Repair,* **25,** 332-342.

Xie, C.W. (2004) Calcium-regulated signaling pathways: role in amyloid beta-induced synaptic dysfunction. *Neuromolecular Med,* **6**, 53-64.

Xu, Y., Lin, D., Li, S., Li, G., Shyamala, S. G., Barish, P. A., Vernon, M. M., Pan, J. and Ogle, W. O. (2009) Curcumin reverses impaired cognition and neuronal plasticity induced by chronic stress. *Neuropharmacology,* **57,** 463-471.

Yamagata, Y. (2003) New aspects of neurotransmitter release and exocytosis: dynamic and differential regulation of synapsin I phosphorylation by acute neuronal excitation in vivo. *J Pharmacol Sci,* **93,** 22-29.

Zeng, Y., Zhao, D. and Xie, C. W. (2010) Neurotrophins enhance CaMKII activity and rescue amyloid-beta-induced deficits in hippocampal synaptic plasticity. *J Alzheimers Dis,* **21,** 823-831.

Zhao, D., Watson, J. B. and Xie, C. W. (2004) Amyloid beta prevents activation of calcium/calmodulin-dependent protein kinase II and AMPA receptor

phosphorylation during hippocampal long-term potentiation. *J Neurophysiol,* **92,** 2853-2858.

Legends to figures

Figure 1. Effects of curcumin on Aβ-induced cell death. Organotypic hippocampal slice cultures were untreated or treated with 2 μM $A\beta_{1-42}$ in the presence or absence of 10 μM curcumin for 24 h and/or 48 h. A) Representative images of control and Aβ-treated slices, with or without curcumin, stained with propidium iodide (PI). B) Quantitative analysis of incorporation of PI in all regions of control and Aβ-treated slices, with or without curcumin, in relation to the whole slice area. The results are presented as percentage of total slice area ± SEM (n = 8). C) Cellular viability as measured by lactate dehydrogenase (LDH) assay. Values represent the amount of LDH released into the culture medium in relation to total LDH and are presented as percentage of control ± SEM (n = 8-10). One-way analysis of the variance (ANOVA) followed by Tukey's post hoc test, *: $P \leq 0.05$, ***: $P \leq 0.001$.

Figure 2. Aβ-induced impairment of signal propagation in organotypic hippocampal slice cultures on MEAs. Organotypic slices exhibit spontaneous local field potential (LFP) events that arise from a single arbitrary focus to propagate across the tissue. A) Variation of median spontaneous LFP power (1-40 Hz) with distance from site of highest power LFP ('focus'). Power values are normalised with reference to total power at the focus. Error bars show standard error of the median absolute deviation. Inset shows representative LFP traces for a control recording (low-pass filtered at 1 kHz). $A\beta_{1-42}$ (2 μM) treatment significantly reduces total power compared to $A\beta_{1-42}$ (2 μM)

plus curcumin (10 μM) conditions. B) Effect of $A\beta_{1-42}$ and curcumin treatment upon median LFP phase coherence (black line). Phase coherence values at specified distances from, and with respect to, LFP focus were calculated from spectrograms of representative 5 s periods during which LFPs occurred. Error bars represent maxima and minima, blocks represent 25th and 75th percentiles. A significant effect of curcumin treatment to reduce phase coherence at a distance 200 μm was observed. Kruskal Wallis followed by Dunn's post hoc tests, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$.

Figure 3. Effects of curcumin on phosphorylation of synapsin I and CaMKII in Aβ-treated organotypic hippocampal slice cultures. A) Representative immunoreactivities for phospho-synapsin I (Ser09 and Ser603), phospho-CAMKII (Thr286), CaMKII and β-actin loading controls. B) Cumulative results represent phospho-synapsin I Ser09 immunocontent normalized by β-actin. C) Cumulative results represent phospho-synapsin I Ser603 immunocontent normalized by β-actin. D) Cumulative results represent the ratio phospho-CaMKII/CaMKII. The quantified data in B-D are presented as percentage of control ± SEM (n = 4-5). One-way analysis of the variance (ANOVA) followed by Tukey's post hoc test, *: $P \leq 0.05$, **: $P \leq 0.01$.

Figure 1

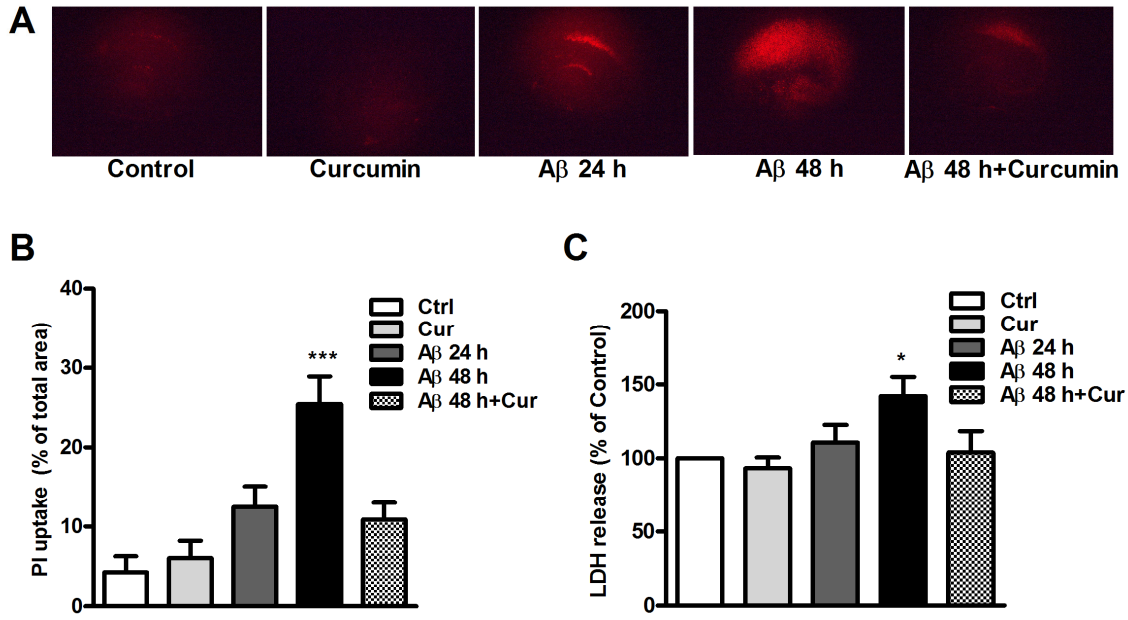

A
Control
Curcumin
Aβ 24 h
Aβ 48 h
Aβ 48 h+Curcumin
B
PI uptake (% of total area)
40
30
20
10
0
***
Ctrl
Cur
Aβ 24 h
Aβ 48 h
Aβ 48 h+Cur
C
LDH release (% of Control)
200
150
100
50
0
*
Ctrl
Cur
Aβ 24 h
Aβ 48 h
Aβ 48 h+Cur

Figure 2

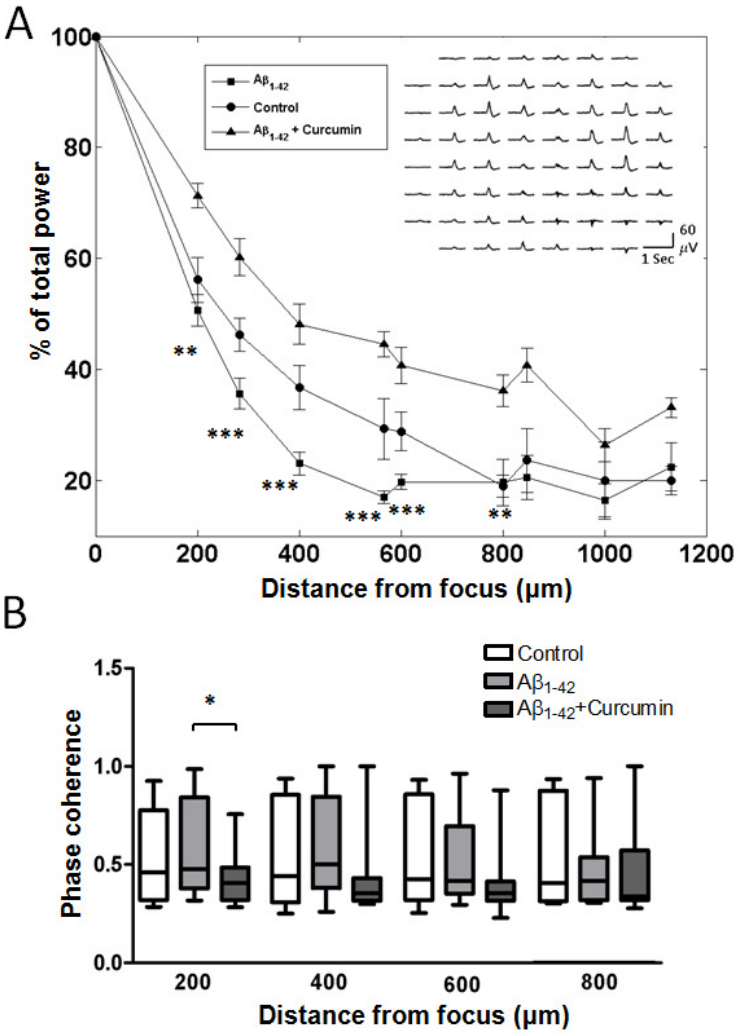
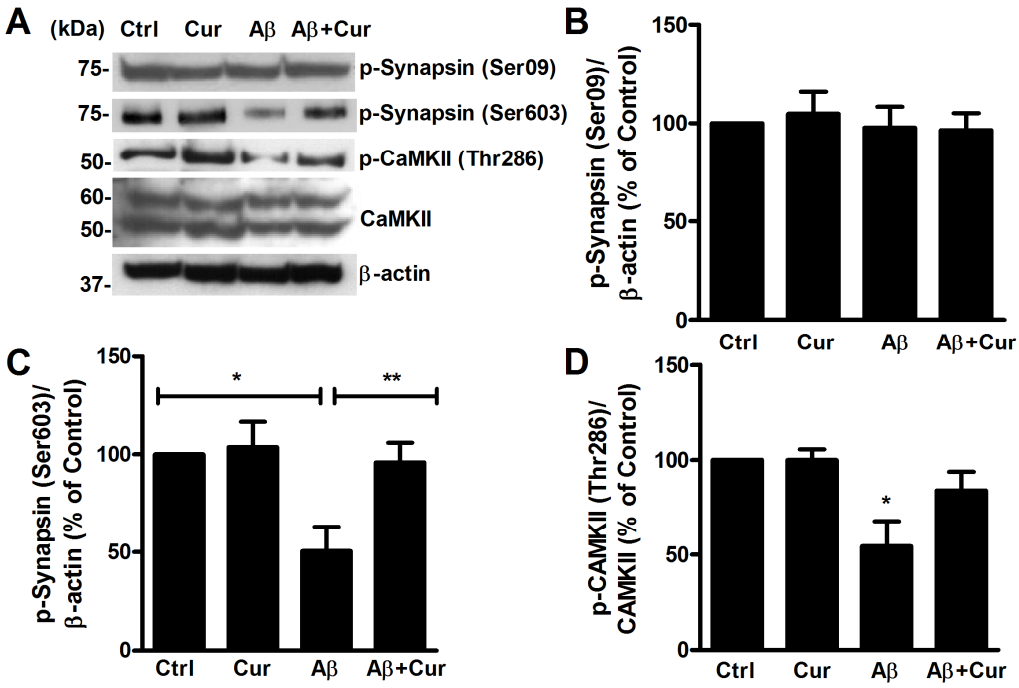

A
100
80
60
40
20
% of total power
Aβ1-42
Control
Aβ1-42 + Curcumin
60 μV
1 Sec
**
***
***
***
***
**
0
200
400
600
800
1000
1200
Distance from focus (μm)
B
Control
Aβ1-42
Aβ1-42+Curcumin
*
1.5
1.0
0.5
0.0
Phase coherence
200
400
600
800
Distance from focus (μm)

Figure 3


A
(kDa) Ctrl Cur Aβ Aβ+Cur
75-
p-Synapsin (Ser09)
75-
p-Synapsin (Ser603)
50-
p-CaMKII (Thr286)
60-
50-
CaMKII
37-
β-actin
B
p-Synapsin (Ser09)/ β-actin (% of Control)
150
100
50
0
Ctrl Cur Aβ Aβ+Cur
C
p-Synapsin (Ser603)/ β-actin (% of Control)
*
**
150
100
50
0
Ctrl Cur Aβ Aβ+Cur
D
p-CAMKII (Thr286)/ CAMKII (% of Control)
*
150
100
50
0
Ctrl Cur Aβ Aβ+Cur

6. CAPÍTULO IV

Artigo: *Nanoencapsulated curcumin improves the neuroprotective effect against β-amyloid-induced cognitive impairments in rats by the involvement of BDNF and Akt/GSK-3β signaling pathway –* ***Status:*** *Submetido ao periódico Neurobiology of Learning and Memory*

Nanoencapsulated curcumin improves the neuroprotective effect against β-amyloid-induced cognitive impairments in rats by the involvement of BDNF and Akt/GSK-3β signaling pathway

Juliana B. Hoppe, Karine Coradini, Rudimar L. Frozza, Claudia M. Oliveira, André B. Meneghetti, Andressa Bernardi, Elisa Simões Pires, Aline Matté, Ruy C. R. Beck and Christianne G. Salbego

J. B. Hoppe (✉) · A. B. Meneghetti · E.S. Pires · C. G. Salbego

Programa de Pós-Graduação em Bioquímica, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul - UFRGS, Rua Ramiro Barcelos, 2600, Porto Alegre, Rio Grande do Sul 90035-003, Brasil
Tel.: +55 (51) 3308.5548, Fax: +55 (51) 3308.5535
Email: juhoppe@hotmail.com

K. Coradini · C.M. Oliveira · R.C.R Beck

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, Rio Grande do Sul 90610-000, Brasil

R. L. Frozza
Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro - UFRJ, Rio de Janeiro, Rio de Janeiro 21944-590, Brasil

A. Bernardi
Instituto Oswaldo Cruz - Fundação Oswaldo Cruz – Rio de Janeiro, Rio de Janeiro 21040-900, Brasil

Abstract

Alzheimer's disease (AD), a neurodegenerative disorder exhibiting progressive loss of memory and cognitive functions, is characterized by the presence of neuritic plaques composed of neurofibrillary tangles and β-amyloid (Aβ) peptide. Drug delivery to the brain still remains highly challenging for the treatment of AD. Several studies have been shown that curcumin is associated with anti-amyloidogenic properties, but therapeutic application of its beneficial effects is limited. Here we investigated possible mechanisms involved in curcumin protection against Aβ(1-42)-induced cognitive impairment and, due to its poor bioavailability, we developed curcumin-loaded lipid-core nanocapsules in an attempt to improve the neuroprotective effect of this polyphenol. Animals received a single intracerebroventricular injection of Aβ(1-42) and they were administered either free curcumin or curcumin-loaded lipid-core nanocapsules (Cur-LNC) intraperitoneally for 10 days. Aβ(1-42)-infused animals showed a significant impairment on learning-memory ability, which was paralleled by a significant decrease in hippocampal synaptophysin levels. Furthermore, animals exhibited activated astrocytes and microglial cells, as well as disturbance in BDNF expression and Akt/GSK-3β signaling pathway, beyond tau hyperphosphorylation. Our findings demonstrate that administration of curcumin was effective in preventing behavioral impairments, neuroinflammation, tau hyperphosphorylation as well as cell signaling disturbances triggered by Aβ *in vivo*. Of high interest, Cur-LNC in a dose 20-fold lower presented similar or better neuroprotective results compared to the effective dose of free curcumin. Considered overall, the data suggest that curcumin is a potential therapeutic agent for neurocognition and nanoencapsulation of curcumin in LNC is an important strategy to enhance its pharmacological efficacy in the treatment of neurodegenerative diseases.

Keywords Alzheimer's disease, Lipid-core nanocapsules, Curcumin, β-Amyloid, BDNF, Akt/GSK-3β pathway, Hippocampus

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common form of dementia in the elderly population. It has a complex pathophysiology, which, although not completely understood, is characterized by β-amyloid (Aβ) peptide aggregation originated from cleavage of the amyloid precursor protein (APP) and neurofibrillary tangles (NFTs) formed from hyperphosphorylated microtubule protein tau (Tiraboschi et al., 2004). Synaptic loss is another characteristic feature of the condition and probably the best correlate of the cognitive decline that develops progressively in AD patients (Terry et al., 1991). The deposition of Aβ in brain areas involved in cognitive functions is assumed to initiate an array of molecular and cellular cascades that lead to synaptic dysfunction (Walsh & Selkoe, 2004). However, mechanistic molecular processes that link Aβ and memory impairment remain to be firmly established.

Current therapies for neurodegenerative diseases provide effective symptomatic relief, particularly in early stages of the disease. However, there are too few, if any therapies that affect the underlying disease process. Furthermore, the presence of a blood-brain barrier (BBB) presents a huge challenge for effective entry of the majority of drugs, and thus severely restricts the therapy of many diseases affecting the central nervous systems (CNS), including AD. Therapeutic strategies for the treatment of neurodegenerative diseases have been widely researched. One of the most significant challenges facing CNS drug development is the availability of effective brain drug targeting technology (Pardridge, 2005). Therefore, the application of technological advances in neurological research is expected to have a major impact leading to the development of newer therapeutic modalities (Modi et al., 2009). In this way,

nanoparticles have become an important area of research in the field of drug delivery because they have the ability to deliver a wide range of drugs to varying areas of the body for a sustained period of time (Hans & Lowman, 2002; Song et al., 2011).

Curcumin (diferuloylmethane), a naturally occurring polyphenol derived from the root of the rhizome *Curcuma longa*, possesses multiple biological activities (Ono et al., 2004; Maheshwari et al., 2006). In particular, experimental evidences have demonstrated that curcumin exhibits neuroprotective effects in a variety of AD models (Lim et al., 2001; Begum et al., 2008; Wang et al., 2010). These curcumin neuroprotective effects have been associated with its antioxidant, anti-inflammation and anti-amyloidogenic properties. Curcumin has shown to modulate functions of many regulatory proteins involved in signal transduction, and to affect a variety of cellular activities, including cell growth, survival and death (Ma et al., 2009; Qin, Cheng & Yu, 2010; Zhang et al., 2011). However, the clinical advancement of this promising natural compound is hampered by its poor water solubility and short biological half-life, resulting in low bioavailability in both plasma and tissues (Yang et al., 2007; Anand et al., 2007). The oral bioavailability of curcumin is very low (only 1% in rats) (Yan et al., 2011). Facing this problem, many research groups have employed nanotechnology to overcome these limitations and the effects of nanoparticles formulations of curcumin have been promising (Thangapazham et al., 2008; Ray et al., 2011; Jaques et al., 2013; Zanotto-Filho et al., 2013). In this context, we developed a curcumin-loaded lipid-core nanocapsules formulation in an attempt to stabilize the drug and improve its biological activities.

Although many studies have reported the protective effects of curcumin against Aβ, the mechanisms underlying these neuroprotective effects still remain to be

determinate. Thereby, the present study compared the ability of curcumin-loaded lipid-core nanocapsule and non-encapsulated curcumin to protect against Aβ1-42-induced synaptotoxicity, neuroinflammation and memory impairment and investigated some underlying mechanisms.

2. Materials and Methods

2.1. Preparation and characterization of lipid-core nanocapsules

Curcumin-loaded lipid-core nanocapsules (Cur-LNC) were prepared in triplicate (3 batches) by the interfacial deposition of preformed polymer (Jäger et al., 2009). An organic phase containing poly(ε-caprolactone) as a biodegradable polymer (0.25 g), grape seed oil (413 μL), sorbitan monostearate (0.0957 g) and curcumin (0.0125 g) dissolved in acetone (67 mL) at 40 °C was poured into the aqueous phase containing polysorbate 80 (0.1915 g), under moderate magnetic stirring. After 10 minutes, a rotary evaporator (Büchi R-114, Flawil, Switzerland) was used to remove acetone and adjust the final volume (25 mL) under reduced pressure at 40 ºC. Unloaded-nanocapsules, called blank lipid-core nanocapsules (B-LNC), were also prepared, but omitting the curcumin in the organic phase. Lipid-core nanocapsules were characterized by means of particle size, polydispersity index, zeta potential, pH measurements and drug content analysis. To ensure the presence of only nanometric particles, formulations were evaluated by laser diffraction using a Mastersizer® 2000 instrument (Malvern Instruments, Malvern, UK). Particle mean diameters and polydispersity index of formulations were evaluated at 25 °C by photon correlation spectroscopy using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) after dilution in previously filtered (0.45 μm) ultrapure water. Using the same instrument, zeta potential was measured after dilution of the suspension in 10 mM NaCl aqueous solution by

electrophoretic mobility. The pH values were determined directly in the formulations. The curcumin content was assayed by high performance liquid chromatography (HPLC) after dissolution of the sample in acetonitrile (Perkin-Elmer, Series 200, Waltham, MA, USA). A C-18 column (Sigma-Aldrich, St. Louis, MO, USA) was used and the mobile phase was composed of acetonitrile and 0.1% trifluoracetic acid (50/50 v/v), (adjusted with pH 3.0 with triethylamine) at the flow rate of 0.6 mL/min (Zanotto-Filho et al., 2013).

2.2. Peptide preparation

The Aβ(1-42) peptide (Bachem Americas Inc. Torrance, CA, USA) or the inactive control Aβ(42-1) peptide (American Peptide Co., Sunnyvale, CA, USA) were dissolved in sterilized bi-distilled water with 0.1% ammonium hydroxide (Merck, NJ, USA) at a concentration of 1 mg/mL and stored to -20 °C. Aliquots of Aβ peptides were allowed aggregating by incubation at 37 ºC for 72 h before in vivo infusion (Frozza et al., 2013).

2.3. *Animals*

Male adult *Wistar* rats (300–350 g) were obtained from in-house breeding colonies at the Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS- Porto Alegre, Brazil). Animals were housed in cages under optimum light conditions (12:12 h light–dark cycle), temperature (22 ± 1 °C), and humidity (50 to 60%), with food and water provided *ad libitum*. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and were approved by the local Ethics Committee on the Use of Animals (CEP-UFRGS, protocol number 20005). All efforts were made to minimize the number of animals and their suffering.

2.4. Surgical procedure

Animals were anesthetized with Equithesin (3.5 mL/kg intraperitoneally – i.p.) and placed in a stereotaxic frame. After sterilized using standard procedures a middle saggital incision was made in the scalp. Bilateral holes were drilled in the skull using a dental drill over the lateral ventricles. Injection coordinates were chosen according to the atlas of Paxinos and Watson (Paxinos & Watson, 2005): 0.8 mm posterior to bregma; 1.5 mm lateral to the saggital suture; 3.5 mm beneath the surface of brain. Rats received a single infusion of 5 μL into each lateral ventricle of Aβ(1-42) or Aβ(42-1) (total of 2 nmol in 10 μL). Controls animals received bilateral intracerebroventricular (i.c.v.) injections of equal volume of bi-distilled water with 0.1% ammonium hydroxide. Microinjections were performed using a 10-μL Hamilton syringe fitted with a 26-gauge needle. All infusions were made at a rate of 1 μL/min over a period of 5 min. At the end of infusion, the needle was left in place for an additional 3-5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the injection, the scalp was sutured and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5 °C. The animals were submitted to behavioral tasks 2 weeks after Aβ injection.

2.5. Drug administration

The animals were randomly divided into eight groups. Control animals infused i.c.v. with water plus 0.1% ammonium hydroxide (Aβ-vehicle) were split into the following three groups: (1) untreated (Control group), (2) treated with free curcumin (Cur group), and (3) treated with curcumin-loaded lipid-core nanocapsules (Cur-LNC group). Animals infused i.c.v. with Aβ(1-42) were divided into the following groups: (4) untreated (Aβ group), (5) treated with free curcumin vehicle (Aβ Veh group), (6) treated

with blank lipid-core nanocapsules (Aβ B-LNC group), (7) treated with free curcumin (Aβ Cur group), and (8) treated with curcumin-loaded lipid-core nanocapsules (Aβ Cur-LNC group). Animals injected with Aβ(42-1) received no treatment and completed behavioral tasks 2 weeks after Aβ injection. Suspension of free curcumin (Cur) (Sigma-Aldrich) was made in 0.5% w/v aqueous solution of carboxymethylcellulose (CMC). Curcumin is nonpolar and must be suspended in CMC (Ghosh et al., 2009). This solution was freshly made up immediately before each administration, and lipid-core nanocapsules containing curcumin (Cur-LNC) were prepared as described above. Daily, doses reaching 50 mg/kg/day of Cur and 2.5 mg/kg/day Cur-LNC were administered i.p. to the animals for 10 consecutive days beginning 4 days after the surgical procedure. Similarly, vehicle treated groups with identical volumes to those treated with Cur or Cur-LNC were run in parallel in rats infused with Aβ. The behavior tests were started on day 13 after Aβ infusion and were carried out sequentially.

2.6. Behavioral analysis

Spontaneous alternation. Hippocampal-dependent memory performance was assessed by measuring spontaneous alternation performance during 8 min in the Y-maze test, which evaluates cognitive searching behavior, although it does not allow isolating memory performance (reviewed in Huges, 2004). Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory. The experimental apparatus used in the present study consisted of three arms (40 cm long, 25 cm high and 10 cm wide, labeled A, B, and C) constructed of plywood and painted black with an equilateral triangular central area. This apparatus was used in a testing room with constant illumination. Each rat was placed at the end of one arm and allowed to move freely through the apparatus for 8 min. Behavior was recorded by a

video camera mounted vertically above the test arena for later analysis using a video-tracking software (ANY-maze®, Stoelting Co., USA). The number of arm choices and pattern of choices were recorded for each animal. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices in overlapping triplet sets (i.e. ABC, BCA, CBA). The percentage of alternation was calculated as [total alternations/(total arm entries-2) × 100].

Novel object recognition task. The object recognition task was performed following recently reviewed guidelines (Bevins & Besheer, 2006). This task is based on the spontaneous tendency of rodents to explore novel objects (Ennaceur & Delacour, 1988). The task was performed in an apparatus made of wood covered with impermeable Formica (dimensions: 40 × 50 × 50 cm) that had black floor and walls. The apparatus was used in a testing room with a constant illumination. The objects used in this test had similar textures, colors and sizes, but had different shapes. Objects were placed near the two corners at either end of one side of the apparatus. The objects chosen were two cuboids glass blocks, a cylindrical bottle filled with water, and a dodecahedron-shaped block. These objects were heavy enough to prevent the rats from moving them. A day before the tests, rats were submitted to a habituation session in which they were allowed to explore the apparatus for 5 min without objects. On the following day, rats were acclimated in the testing room during 1 h before the beginning of the sessions. First, rats completed a training session (24h after habituation) that consisted of leaving the animals in the apparatus containing two identical objects (A and A1). After training, rats were placed in their home-cages for 3 h. The testing session to evaluate short-term recognition memory was performed 3 h after the training session. Rats were once again allowed to explore the apparatus, but during this session, the apparatus contained two

dissimilar objects: the familiar object from the training session, and a novel one (A and B, respectively). Long-term recognition memory was evaluated 24 h after the training session and a different pair of dissimilar objects (a familiar and a novel one; A and C, respectively) were presented. In all sessions, each rat was always placed in the apparatus facing the wall and allowed to explore the objects for 5 min, after which the rat was returned to its home cage. Behavior was recorded by a video camera mounted vertically above the test arena and analyzed using appropriated video-tracking software (ANY-maze®, Stoelting Co., USA). Each animal underwent three trials, including the training and two test sessions. The animals started to explore the objects 1 min after they had been placed in the apparatus. The percentage of time spent exploring the novel object was calculated as a function of the total amount of time spent exploring both objects during testing [time spent with novel object/(time spent with novel object + time spent with familiar object)]. A higher percentage of time spent exploring the novel object was considered to be an index of enhanced cognitive performance (Recognition Index). Between trials, the objects were cleaned with 10% ethanol solution. Active exploration was defined by directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaws. Sitting on the object was not considered exploratory behavior.

2.7. Western blotting assay

In order to evaluate any cell signaling disturbances triggered by i.c.v. injection of Aβ1-42 as well as curcumin treatments, animals were killed by a lethal dosage of anesthesia following completion of the behavioral tasks and blood samples were collected by cardiac puncture. The brain was rapidly removed from the skull, and the hippocampus was dissected on dry ice. The hippocampi were then homogenized in ice-

cold lysis buffer (4% sodium dodecylsulfate [SDS], 2 mM EDTA, 50 mM Tris) containing a protease/phosphatase inhibitor cocktail. The homogenates were denatured for 5 min at 100 °C, and then centrifuged at 10,000 × *g* for 30 min. The supernatant containing the cytosolic fraction was collected, the protein concentration was determined (Peterson, 1972), and β-mercaptoethanol was added to a final concentration of 5%. Equal amounts of proteins were resolved (35 µg per lane) on 8-14% SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany) using a semi-dry transfer apparatus (Bio-Rad, Trans-Blot SD, Hercules, CA, USA). Membranes were incubated for 60 min at 4 ºC in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4ºC. The primary antibodies against the following proteins were used: anti-phospho GSK-3β [pSer^{9}] (1:1000, Cell Signaling Technology Beverly, MA, USA), anti-GSK-3β (1:1000, Cell Signaling Technology), anti-phospho Akt[pSer^{473}] (pAkt, 1:1000, Cell Signaling Technology), anti-Akt (1:1000, Cell Signaling Technology), anti-phospho Tau [pSer$^{396/404}$] (pTau, 1:500, Invitrogen, Grand Island, NY, USA), anti-Tau (1:1000, Invitrogen), anti-glial fibrillary acidic protein (GFAP, 1:3000, Sigma-Aldrich), anti-BDNF (1:250, Sigma-Aldrich), anti-synaptophysin (1:3000, Millipore® Bradford, MA, USA), and anti-β-actin (1:2000, Sigma-Aldrich). After washing, the membranes were incubated with adjusted secondary antibodies coupled to horseradish peroxidase (1:1000, Amersham Pharmacia Biotech Piscataway, NJ, USA) for 2 h. The immunocomplexes were visualized by using Western blot detection SuperSignal West Pico chemiluminescent reagent (Pierce, Rockford, IL, USA). Band density

measurements were performed by using Optiquant software (Packard Instrument). The average optical density for the control group was designated as 100%.

2.8. Isolectin B4 (IB4) reactivity

In an attempt to evaluate the activation of microglial cells after Aβ i.c.v. injection, we analyzed the IB4 (Sigma Chemical Co.) reactivity. Proteins (25 μg per line) were resolved on 8% SDS-PAGE, and electro-transferred to nitrocellulose membranes as described for the Western blotting assay. Membranes were incubated overnight at 4 °C in albumin solution (5% albumin and 2% Tween 20 in PBS, pH 7.4). After washing, IB4 peroxidase conjugate was incubated in PBS containing 0.05% Tween 20 overnight in a final concentration of 0.250 μg/mL. Chemioluminescence was detected by Western blot detection SuperSignal West Pico chemiluminescent reagent (Pierce) using X-ray films (Kodak X-Omat, Rochester, NY, USA).

2.9. Measurement of proinflammatory cytokines

Levels of the proinflammatory cytokines Tumor Necrosis Factor-α (TNF-α) and Interleukin 1-β (IL-1β) were measured in hippocampal extract supernatants by specific enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's recommendations. For cytokines measurement, hippocampus was homogenized in phosphate buffer saline (PBS, pH 7.4), containing 1mM ethylene glycol tetraacetic acid (EGTA) and 1mM PMSF. The homogenate was centrifuged at 1000 x g for 5 min at 4 ºC and the supernatant was used. For each assay, samples were analyzed in duplicate and compared with known concentrations of protein standard. The values of cytokines are shown as mean picograms of each cytokine per milliliter (±SEM) of supernatant.

2.10. Measurement of hepatic enzymes in serum

In an attempt to evaluate whether treatments caused hepatic alterations, the serum levels of hepatic enzymes were evaluated at the end of the treatments. The blood samples collected by cardiac puncture were analyzed by activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase and aspartate aminotransferase, which were used as markers of metabolic and tissue toxicity. These experiments were performed in a LabMax 240 analyzer (Labtest Diagnostica, Lagoa Santa, Brazil).

2.11. Data analysis

All experiments were carried out at least in triplicate except for behavioral tests. The results are presented as the mean ± SEM of five to fifteen animals per group. The statistical comparisons of the data were performed by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls *post hoc* test using GraphPad Prism software version 5.01 (GraphPad Software Inc. La Jolla, CA, USA). A p value lower than 0.05 ($p<0.05$) was considered significant.

3. Results

3.1. Physicochemical characterization of lipid-core nanocapsules

Laser diffraction analysis showed a unimodal distribution without micrometric particle population (Fig. S1). Nanocapsules suspensions presented homogeneous aspect with nanometric size around 200 nm and polydispersity index below 0.2. Formulations presented negative zeta potential of -9.6 (Cur-LNC), -7.5 (B-LNC), and pH values ranged from 6.2 and 6.4. Regarding the mean curcumin content, the Cur-LNC showed values of 0.49 mg/mL, corresponding to a drug recovery of 98% in relation to the amount of drug initially added to the formulations (0.50 mg/mL) (Table 1). The physicochemical characteristics presented here are in accordance with our previous

work who demonstrated the nanotechnological features of this formulation (Zanotto-Filho et al., 2013).

3.2. Effects of curcumin on Aβ(1-42)-induced cognitive impairments

Since AD is characterized clinically by a progressive decline in learning and memory processes, we evaluated the potential neuroprotective effects of curcumin against neurotoxicity using behavioral tests in Aβ(1-42)–treated rats. Spontaneous alternation and novel object recognition tasks were used to investigate behavioral impairments involved in Aβ-induced cognitive decline 2 weeks after Aβ(1-42)-infused rats. Furthermore, we compared the effects of treatment with free curcumin (Cur) to those of curcumin-loaded lipid-core nanocapsules (Cur-LNC) against Aβ. We observed that 2 weeks after a single i.c.v. infusion of 2 nmol of Aβ(1-42) the rats displayed a decrease of spontaneous alternation in the Y-maze ($p<0.01$) (Fig. 1). In order to evaluate the effects of curcumin, animals were treated daily with 50 mg/kg/day of Cur (Cur 50) or 2.5 mg/kg/day of Cur-LNC (Cur-LNC 2.5) for 10 days. As shown in Fig. 1, Cur 50 and Cur-LNC 2.5 treatments increase the spontaneous alternation behavior in Aβ-infused rats ($p<0.05$). However, it is important to note that the treatment with Cur-LNC 2.5, in a dose 20-fold lower, significantly attenuated this impairment triggered by Aβ(1-42). The number of arm entries did not differed among all groups (data not shown), indicating that changes in alternation behavior were not due to generalized exploratory, locomotor or motivational effects. Next, we evaluated the effects of Aβ1-42 infusion, as well as Cur 50 and Cur-LNC 2.5 treatments on recognition memory by submitting the animals to a novel object recognition task. As is seen in Fig. 2B, when the animals were placed in the arena 3h after first exploration period (training session, Fig. 2A), Aβ-infused rats were not able to discriminate between the familiar and novel

objects, as indicated by similar exploration times for both objects (Fig. 2B). Treatment with Cur 50 and Cur-LNC 2.5 were able to improve short-term recognition memory ($p<0.05$ and $p<0.01$, respectively; Fig. 2B). Similar results were found when long-term recognition memory was evaluated, indicating that rats treated with Cur 50 and Cur-LNC 2.5 were able to distinguish between familiar and new one objects following Aβ infusion ($p<0.05$ and $p<0.01$, respectively; Fig. 2C). Lower dose of free curcumin showed no effect on behavioral tasks (10 mg/kg/day) (data not shown). Animals i.c.v. infused with water plus 0.1% ammonium hydroxide (Aβ vehicle) were treated with Cur 50 or Cur-LNC 2.5 at the same schedule in order to test for possible intrinsic toxicity. These animals showed no impairment in either the spontaneous alternation task or the recognition memory task when compared to control animals. Treatment with vehicles (CMC or B-LNC, respectively) had no effects on the spontaneous alternation and recognition memory impairments triggered by Aβ (Figs. 1, 2). Animals i.c.v. infused with Aβ(42-1) had no differences in the spontaneous alternation and memory recognition tasks (data not shown).

3.3. Curcumin decreases synaptotoxicity triggered by Aβ

To investigate the synaptic integrity in our treatment groups, we performed Western blotting analysis for the synaptic protein synaptophysin, a specific presynaptic marker. A significant reduction in synaptophysin levels was found in Aβ(1-42)-infused rats 15 days after i.c.v. injection ($p<0.05$), suggesting that some form of synaptic dysfunction had been induced (Fig. 3). Consistent with the behavioral results, treatments with Cur 50 and Cur-LNC 2.5 were able to block the decrease in synaptophysin levels following Aβ(1-42) infusion ($p<0.05$; Fig. 3).

3.4. Effect of curcumin in abnormal hyperphosphorylation of tau in Aβ-infused rats

Abnormal hyperphosphorylation of tau is a critical event in the cascade leading to AD pathology. Aberrant tau phosphorylation in response to elevated Aβ contributes to tau aggregation and fibrillisation in NFTs (Hernández et al., 2010). To investigate whether our treatment groups influence the phosphorylation of microtubule protein tau, Western blotting was performed with antibodies against the phosphorylated form of tau as well as against its total immunocontent. In the rat hippocampus, tau protein phosphorylation was found to increase after Aβ intracerebroventricularly infused rats without modifying the total levels of tau ($p<0.05$; Fig. 4). Again, treatment with both Cur 50 and Cur-LNC 2.5 was able to decrease the tau phosphorylation ($p<0.05$ and $p<0.01$, respectively; Fig. 4).

3.5. Effect of curcumin on glial activation and proinflammatory cytokines induced by Aβ

Considering that neuroinflammation is a prominent feature of AD brain, with inflammatory responses playing a significant role in modulating disease progression, we tested the possible requirement of astrocytes and microglial activation after Aβ-infused rats. We also investigated TNF-α and IL-1β proinflammatory cytokines thought to play a central role in the self-propagation of neuroinflammation (Inoue, 2002). The results showed that Aβ infusion caused a highly significant increase in activated astrocytes, as seen by increased glial fibrillary acidic protein (GFAP) immunocontent (Fig. 5A). Cur 50 and Cur-LNC 2.5 were effective at blocking astrocytes activation, the treatments significantly decreased the levels of GFAP immunocontent ($p<0.05$; Fig. 5A). Similarly, the reactivity for IB4 was increased in the hippocampi of Aβ(1-42)-infused rats, meaning microglial activation and treatments with Cur 50 and Cur-LNC 2.5 allowed the reduced activated microglia (n= 4; Fig. 5B). Performing ELISA assays, we

observed that TNF-α and IL-1β levels were greatly increased in the hippocampi of Aβ-infused rats (p<0.001 and p<0.01, respectively; Fig. 5B and D). Only the treatment with Cur-LNC 2.5 was able to significantly attenuate the TNF-α and IL-1β increase levels following Aβ(1-42) infusion (p<0.05; Figs. 5C, D).

3.6. Curcumin attenuates BDNF reduction in the Aβ-infused rats

Some important neurotrophic factors are critical for neuronal survival and plasticity and could be implicated in AD development (Yasutake et al., 2006). Because brain-derived neurotrophic factor (BDNF) supports neuronal survival, maintains several neuronal activities, modulates neurotransmitter release, participates in neuronal plasticity, and mediates long-term potentiation and memory fixation (Jantas et al., 2009), we further measured BDNF concentrations in rat hippocampus. As shown in Fig. 6, an evident decrease in BDNF protein expression was observed following Aβ(1-42) injection (p<0.05). Western blot analysis revealed that treatments with both Cur 50 and Cur-LNC 2.5 were able to block this Aβ-induced decrease in BDNF levels (p<0.05).

3.7. Akt and GSK-3β phosphorylation is involved in curcumin-mediated neuroprotection

Increasing evidence suggests that the PI3K/Akt/GSK-3β signaling pathway is directly impacted by Aβ exposure and is altered in AD brains (Jimenez et al., 2011). Neurotrophins, activating the PI3K/Akt signaling pathway, control neuronal survival and plasticity. Akt can be activated by BDNF (Troca-Marín, Alves-Sampaio & Montesinos, 2011). We therefore hypothesize that the PI3K/Akt pathway may have an important role in curcumin-mediated neuroprotection against Aβ-induced cognitive impairments in rats. To detect the role of Akt and GSK-3β activity in hippocampus after Aβ infusion in rats, we performed Western blots. As shown in Fig. 7A, a significant decrease in Akt phosphorylation was found in the hippocampi of Aβ1-42–infused rats

15 days after ICV injection ($p<0.01$; Fig. 7A). Cur 50 and Cur-LNC 2.5 treatments were able to significantly increase the levels of Akt phosphorylation without modifying the total levels of Akt ($p<0.05$ and $p<0.01$, respectively; Fig. 7A). GSK3-β is constitutively active in most cells and is negatively regulated by phosphorylation at its Ser-9 by a variety of upstream signalling cascades, including PI3K/Akt. The GSK-3β activity could play a central role controlling the development of AD. GSK-3β accounts for several features of this pathology such as memory impairment, Tau hyperphosphorylation, microglia-mediated inflammation, proinflammatory cytokines production, synaptic failure and neuronal death (Hooper, Killick & Lovestone, 2008). As seen in Fig. 7B, while Aβ(1-42) induced activation of GSK-3β ($p<0.001$), an increase in phosphorylation/inactivation of GSK-3β was noticed after both Cur 50 and Cur-LNC 2.5 treatments ($p<0.01$ and $p<0.001$, respectively).

3.8. Investigation of possible side effects of curcumin treatments

Treatment with Cur (50 mg/kg/day, i.p.) or Cur-LNC (2.5 mg/kg/day, i.p.), as well as the vehicles did not induce mortality or alter body weight within 10 days of treatment (data not shown). The activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase, and aspartate aminotransferase were assessed in rat blood serum. None of the treated animals presented with significant alterations in the investigated enzymes, suggesting no hepatic or metabolic alterations in the animals under the tested conditions (data not shown).

4. Discussion

In this study we demonstrated that a single intracerebroventricular injection of Aβ induces cognitive and behavioural deficits which may be related to synaptic dysfunction, neuroinflammation and tau hyperphosphorylation, cardinal features of AD.

This Aβ disease model has been a useful complement to transgenic approaches to AD neuropathology in development and evaluation of therapeutic approaches (Woodruff-Pak, 2008). Drug delivery to the brain still remains highly challenging for the treatment of AD. The development of new practical treatment modalities for the treatment of AD is currently a highly active area of research (Fazil et al., 2012). In this scenario, we developed an aqueous dispersion constituted by curcumin-loaded lipid core-nanocapsules. This nanomaterial was characterized and showed unimodal particle size distribution at the nanoscale. These nanocapsules are coated by polysorbate 80, whose strategy has been used to enhance the brain delivery of nanoencapsulated drugs (Su et al., 2010; Benvegnú et al., 2012). Regarding the in vivo experiments, our findings demonstrate that administration of both curcumin free and nanoencapsulated was effective in preventing behavioral impairments, neuroinflammation, tau hyperphosphorylation as well as cell signaling disturbances triggered by Aβ *in vivo*. Non-encapsulated curcumin only exerted significant effects against Aβ at a high dose of 50 mg/kg/day. Of high interest, Cur-LNC at a dose 20-fold lower (2.5 mg/kg/day) presented similar or better neuroprotective results compared to the effective dose of non-encapsulated curcumin in our experimental model of AD in rats.

Previous studies by our group showed that lipid-core nanocapsules (LNCs) are able to increase indomethacin and resveratrol accumulation in rat brain after daily i.p. injection when compared to the free drug (Frozza et al., 2010; Bernardi et al., 2012). Recently, Su et al. (2010) showed a 2.5-fold increase in the brain concentration of curcumin and a 14-fold increase in mean residence time (MRT) of curcumin after intravenous injections when this polyphenol was associated with poly(butylcyanoacrylate) nanoparticles coated with polysorbate 80. Pharmacokinetics

analysis in the different brain regions revealed that the half-time ($t_{1/2}$) and the MRT of curcumin-loaded PLGA nanoparticles were increased in the hippocampus and cerebral cortex (Tsai et al., 2011), both regions of vital importance in the cognition and memory processes (Medina & Izquierdo, 1995). Thereby, data obtained herein show an improvement in the *in vivo* performance of the curcumin, strongly suggesting that LNCs has the potential of increasing the brain bioavailability of curcumin. Moreover, the absence of systemic side effects, such as alterations in serum markers and tissue histology in treatments with Cur-LNC, suggest that the LNCs were nontoxic in this model and similar treatments (Zanotto-Filho et al., 2013). Recently, toxicological studies indicated the safety of LNC after single and repeated-dose treatments during 28 days, by i.p. administration (Bulcão et al., 2013).

Neuroprotective effects of curcumin against cognitive and memory impairments induced by Aβ remain to be established. Here, we have observed that treatments with 50 mg/kg/day of free curcumin and 2.5 mg/kg/day of Cur-LNC improve the cognitive damage induced by i.c.v. Aβ injection. Because curcumin treatments were initiated after Aβ infusion, these data suggest that curcumin not only prevented memory loss, but also restored the cognitive deficit. Thereby, we suppose that improvement in spontaneous alternation and recognition index performances in treated animals reflects neuroprotective effect of curcumin on hippocampal regions damaged by Aβ. After the evaluation of memory parameters, we aimed to elucidate some mechanisms involved in the memory impairment caused by Aβ and by what routes curcumin exerts its protective effect. In the next experiments, we focused our interest on the hippocampus, an important region involved in the cognition and memory processes related to AD (Broadbent et al., 2010). Synapse loss in hippocampal region of AD patients

significantly correlates with the severity of their cognitive symptoms (Scheff et al., 2006). In our experimental model, Aβ caused a significant decrease in hippocampal levels of synaptophysin, a specific presynaptic marker. The decreased synaptophysin levels and the impaired spontaneous alternation and object recognition tasks in Aβ(1-42)-exposed rats greatly suggest a link between both events. The synaptotoxic effects of Aβ might be crucial in causing the observed memory deficits. Our results are in accordance with previous studies related to synaptic loss due to toxicity induced by i.c.v. injection of Aβ (Medeiros et al., 2007; Canas et al., 2009; Passos et al., 2009). This hypothesis is reinforced by effects of curcumin against synaptotoxicity triggered by Aβ, since curcumin rescued synaptic changes as can be seen by increasing synaptophysin levels, the major synaptic vesicle protein.

Synaptic loss and death of specific neurons population in AD are provoked by a cascade of multiple deleterious molecular and cellular events rather than a single pathogenic factor. One feature present in AD brain and analyzed in the present study is the presence of reactive astrocytes and activated microglial cells triggered by Aβ, which plays a prominent role in the neurodegenerative process (Ralay et al., 2006; Lue et al., 2010). Some of those roles include phagocytizing cell debris after synaptic rewiring and memory formation and the release of proinflammatory cytokines, such as TNF-α and IL-1β. These proinflammatory cytokines could cause a vicious cycle of inflammation that has been characterized in a number of neurodegenerative diseases including AD (Drew et al., 2006; Garwood et al., 2011). Our results clearly show that both treatments, free curcumin (50 mg/kg/day) and nanoencapsulated curcumin (2.5 mg/kg/day), were able to decrease both astrocyte and microglial activation induced by i.c.v. injection of Aβ(1-42). Despite treatment with free curcumin shows a tendency to decrease the levels

of TNF-α and IL-1β in Aβ-infused rats, only treatment with Cur-LNC significantly exhibited decreased levels of these proinflammatory cytokines in hippocampus. A possible mechanism underlying this superior efficacy of treatment with Cur-LNC may include an enhanced brain biodistribution of curcumin afforded by lipid-core nanocapsules. Glia cells also release factors vital for neuronal survival that are important intercellular communication between neurons and glia. One factor that is released is brain derived neurotrophic factor involved in neuronal growth, maintenance, and in different aspects of activity-dependent synaptic physiology by acting in neuronal plasticity and mediating long-term potentiation and memory fixation (McAllister et al., 1999; Tyler et al., 2002). Contextually, it may be noted that during neurodegenerative diseases, the quantitative presence of BDNF and other neurotrophins decreases with a parallel increase in proinflammatory products (Murer et al., 2001; Ge & Lahiri, 2002). For this reason, the content of BDNF within hippocampal region was measured following Aβ i.c.v. injection and curcumin administrations as a means to assess the possible involvement of neurotrophic factors in the action of curcumin. Our results demonstrate that Aβ-infused rats showed significantly decreased BDNF protein levels in the hippocampus, while curcumin rescued BDNF content. These results indicate that curcumin could up-regulate the BDNF level in rat hippocampus, an important finding that may be related with the improvements of memory performance impaired by Aβ.

Trophic factors such as NGF, insulin-like growth factor I, or BDNF activate a variety of signaling cascades, including the PI3K/Akt, the Ras–mitogen-activated protein kinase (MAPK), and the cAMP/protein kinase A (PKA) (Brunet, Datta & Greenberg, 2001; Johnson-Farley, Travkina & Cowen, 2006). It was shown that activation of TrkB by BDNF promotes neuronal survival largely through the Akt

(Yoshii et al., 2007; Jantas et al., 2009). Increasing evidence suggests that the PI3K/Akt survival pathway is directly impacted by Aβ exposure and is altered in AD brains (Jimenez et al., 2011). A variety of substrates of Akt have been implicated in the regulation of cell survival, including Bcl-2 family member Bad, caspase 9, the transcription factors Forkhead, nuclear factor-κB and protein kinase GSK-3β (Datta, Brunnet & Greenberg, 1999; Song, Feng & Bao, 2005). We focused on GSK-3β because of its known role in tau phosphorylation, production of proinflammatory cytokines and a modulator in learning and memory (Brunet, Datta & Greenberg 2001; Hooper, Killick & Lovestone, 2008; Green et al., 2012). Based on this information, we hypothesized that the PI3K/Akt/GSK-3β signaling pathway could be involved in curcumin neuroprotective effect in our experimental model. We observed that Akt phosphorylation was strongly decreased and GSK-3β was activated in hippocampus of Aβ-infused rats, while curcumin treatments were able to maintain Akt phosphorylation and inhibit GSK-3β activity by serine 9 phosphorylation. This effect leads us to believe that curcumin may be exerting its neuroprotective effect through up-regulating BDNF which in turns activates Akt and inhibits GSK-3β.

GSK-3β is abundant in the central nervous system and can modify several neuronal proteins like tau (Hernández et al., 2010; Avila et al., 2012). Overexpression of GSK-3β in a conditional transgenic model produced tau hyperphosphorylation and neuronal death (Lucas et al., 2001; Engel et al., 2006). Furthermore, pharmacological inhibition of the GSK-3β activity by lithium decreased Aβ production and plaque accumulation (Sofola et al., 2010), improved performance in memory tests, preserved the dendritic structure, and reduced the Tau-dependent pathology in AD transgenic models (Noble et al., 2005; Rockenstein et al., 2008). Aβ directly affects neurons,

leading to neurodegeneration and tau protein phosphorylation (Kar et al., 2004). Once hyperphosphorylated, tau protein affinity for microtubules is reduced and it can detach, leading to changes in microtubule dynamics which perturb axon transport of synaptic vesicles, resulting in the degeneration of synapses (Sorrentino & Bonavita, 2007). A growing body of evidence suggests that the effects of Aβ on tau phosphorylation could be the direct consequence of increased kinase or decreased phosphatase activities (Mazanetz & Fischer, 2007). Putative tau kinases that phosphorylate tau in response to Aβ include cyclin dependent kinase-5, GSK-3β, p38/MAPK, JNK-1/2 and ERK, several of which are activated by increased production or release of cytokines (Ma et al., 2009). Indeed, of the proline-directed kinases, GSK-3β is the major contributor to the phosphorylation of tau (Ryder, Su & Ni, 2004). Our current results showed that Aβ-infused rats can significantly increase GSK-3β activity and it may explain the result observed in tau phosphorylation, where there was a strong increase in tau phosphorylation in hippocampus of Aβ-infused rats. Curcumin was able to promote phosphorylation/inactivation of GSK-3β, thereby suppressing GSK-3β-dependent phosphorylation of tau.

In conclusion, the present findings reinforce and extend the notion of the hazardous effects of Aβ1-42 toward hippocampal synaptic homeostasis and cognitive performance. To the best of our knowledge, this is the first report of an *in vivo* effect of curcumin-loaded lipid-core nanocapsules in an animal model of AD. Cur-LNC presented efficacy at a low dose in contrast to the high doses of nonencapsulated curcumin, suggesting an improvement in the *in vivo* performance of the curcumin by its nanoencapsulation. Furthermore, we showed that neuroprotective effect of curcumin on Aβ induced memory impairments could be connected with the activation of Akt/GSK-

3β signaling pathway and the increased expression of BDNF. These results, taken together, provide a new insight into the neuroprotective action of curcumin, its beneficial effects in chronic neurodegenerative disorders and its nanoencapsulation as a promising approach for the development of an effective nanomedicine.

Acknowledgments

This study was supported by the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

References

Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B.B. (2007). Bioavailability of curcumin: problems and promises. *Mol Pharm*, 4, 807-18.

Avila, J., León-Espinosa, G., García, E., García-Escudero, V., Hernández, F., Defelipe, J. (2012). Tau phosphorylation by GSK3 in different conditions. *Int J Alzheimers Dis*, 2012, 578373.

Begum, A.N., Jones, M.R., Lim, G.P., Morihara, T., Kim, P., Heath, D.D., et al. (2008). Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J Pharmacol Exp Ther*, 326, 196-208.

Benvegnú, D.M., Barcelos, R.C., Boufleur, N., Pase, C.S., Reckziegel, P., Flores, F.C., et al. (2012). Haloperidol-loaded polysorbate coated polymeric nanocapsules decrease its adverse motor side effects andoxidative stress markers in rats. *Neurochem Int,* 61, 623-31.

Bernardi, A., Frozza, R.L., Meneghetti, A., Hoppe, J.B., Battastini, A.M., Pohlmann, A.R., et al. (2012). Indomethacin-loaded lipid-core nanocapsules reduce the damage triggered by Aβ1-42 in Alzheimer's disease models. *Int J Nanomedicine*, 7, 4927-42.

Bevins, R.A. & Besheer, J. (2006). Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat Protocols*, 1, 1306-11.

Broadbent, N.J., Gaskin, S., Squire, L.R., Clark, R.E. (2010). Object recognition memory and the rodent hippocampus. *Learn Mem*, 17, 5-11.

Brunet, A., Datta, S.R., Greenberg, M.E. (2001). Transcription-dependent and independent control of neuronal survival by the PI3K/Akt signaling pathway. *Curr Opin Neurobiol*, 11, 297-305.

Bulcão, R.P., Freitas, F.A., Venturini, C.G., Dallegrave, E., Durgante, J., Göethel, G., et al. (2013). Acute and subchronic toxicity evaluation of poly(epsilon-caprolactone) lipid-core nanocapsules in rats. *Toxicol Sci*, 132, 162-176.

Canas, P.M., Porciúncula, L.O., Cunha, G.M.A., Silva, C.G., Machado, N.J., et al. (2009). Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogenactivated protein kinase pathway. *J Neurosci*, 29, 14741-51.

Datta, S.R., Brunet, A., Greenberg, M.E. (1999). Cellular survival: a play in three Akt. *Genes Dev*, 13, 2905-27.

Drew, P., Xu, J., Storer, P., Chavis, J., Racke, M. (2006). Peroxisome proliferator-activated receptor agonist regulation of glial activation: Relevance to CNS inflammatory disorders. *Neurochem Int*, 49:183-89.

Engel, T., Hernández, F., Avila, J., Lucas, J.J. (2006). Full reversal of Alzheimer's disease-like phenotype in a mouse model with conditional overexpression of glycogen synthase kinase-3. *J Neurosci*, 25, 5083-90.

Ennaceur, A. & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1. Behavioral data. *Behav Brain Res*, 31, 47-59

Fazil, M., Shadab, Baboota, S., Sahnim J.K., Ali, J. (2012). Nanotherapeutics for Alzheimer's disease (AD): Past, present and future. *J Drug Target*, 20:97-113.

Frozza, R.L., Bernardi, A., Paese, K., Hoppe, J.B., da Silva, T., Battastini, A.M., et al. (2010). Characterization of trans-resveratrol-loaded lipid-core nanocapsules and tissue distribution studies in rats. *J Biomed Nanotechnol*, 6, 694-703.

Frozza, R.L., Bernardi, A., Hoppe, J.B., Meneghetti, A.B., Matté, A., Battastini, A.M., et al. (2013). Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-Core Nanocapsules. *Mol Neurobiol* [Epub ahead of print]

Garwood, C.J., Pooler, A.M., Atherton, J., Hanger, D.P., Noble, W. (2011). Astrocytes are important mediators of Aβ-induced neurotoxicity and tau phosphorylation in primary culture. *Cell Death Dis*, 2, 1–9.

Ge, Y.W. & Lahiri, D.K. (2003). Regulation of promoter activity of the APP gene by cytokines and growth factors: implications in Alzheimer's disease. *Ann N Y Acad Sci*, 973, 463-7.

Ghosh, S.S., Massey, H.D., Krieg, R., Fazelbhoy, Z.A., Ghosh, S., Sica, D.A., et al. (2009). Curcumin ameliorates renal failure in 5/6 nephrectomized rats: role of inflammation. *Am J Physiol Renal Physiol,* 296, 1146-57.

Green, H.F. & Nolan Y.M. (2012). GSK-3 mediates the release of IL-1b, TNF-a and IL-10 from cortical glia. *Neurochemistry International*, 61, 666-71.

Hans, M.L. & Lowman, A,M. (2002). Biodegradable nanoparticles for drug delivery and targeting. *Curr Opin Solid State Mater Sci*, 6, 319-27.

Hernández, F., Gómez de Barreda, E., Fuster-Matanzo, A., Lucas, J.J., Avila, J. (2010). GSK3: a possible link between beta amyloid peptide and tau protein. *Exp Neurol*, 223, 322-5.

Hooper, C., Killick, R., Lovestone, S. (2008). The GSK3 hypothesis of Alzheimer's disease. *Journal of Neurochemistry*, 104, 1433-39.

Huges, R.N. (2004). The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neurosci Biobehav,* 28, 497–505

Inoue, K. (2003). Microglial activation by purines and pyrimidines. *Glia*, 40, 156-63.

Jäger, E., Venturini, C.G., Poletto, F.S., Colomé, L.M., Pohlmann, J.P., Bernardi, A., et al. (2009). Sustained release from lipid-core nanocapsules by varying the core viscosity and the particle surface area. *J Biomed Nanotechnol,* 5, 130-40.

Jantas, D., Szymanska, M., Budziszewska, B., Lason, W. (2009). An involvement of BDNF and PI3-K/Akt in the anti-apoptotic effect of memantine on staurosporine-evoked celldeath in primary cortical neurons. *Apoptosis*, 14, 900-12.

Jaques, J.A., Doleski, P.H., Castilhos, L.G., da Rosa, M.M., Souza, V.D., Carvalho, F.B., et al. (2013). Free and nanoencapsulated curcumin prevents cigarrete smoke-induced cognitive impairment and redox imbalance. *Neurobiol Learn Mem*, 100, 98-107.

Jimenez, S., Torres, M., Vizuete, M., Sanchez-Varo, R., Sanchez-Mejias, E., Trujillo-Estrada, L., et al. (2011). Age-dependent accumulation of solubre beta (Abeta) oligomers reverses neuroprotectvive effect of soleble amyloid precursor protein-alpha (sAPP(alpha)) by modulating phosphatidylinositol 3-kinase (PI3K)/Akt-GSK-3beta pathway in Alzheimer mouse model. *J Biol Chem*, 286, 18414-25.

Johnson-Farley, N.N., Travkina, T., Cowen, D.S. (2006). Cumulative activation of Akt and consequent inhibition of glycogen synthase kinase 3 by brain-derived neurotrophic factor and insulin-like groth factor-1 in cultured hippocampal neurons. *Pharmacol Exp Ther*, 316, 1062-9.

Kar, S., Slowikowski, S.P., Westaway, D., Mount, H.T. (2004). Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheime's disease. *J Psychiatry Neurosci*, 29, 427-41.

Lim, G.P., Chu, T., Yang, F., Beech, W., Frautschy, S.A., Cole, G.M. (2001). The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J Neurosci*, 21, 8370-7.

Lucas, J.J., Hernández, F., Gómez-Ramos, P., Morán, M.A., Hen, R., Avila, J. (2001). Decreased nuclear beta-
catenin, tau hyperphosphorylation and neurodegeneration in GSK-
3beta conditionaltransgenic mice. *EMBO J,* 20, 27-39.

Lue, L.F., Kuo, Y.M., Beach, T., Walker, D.G. (2010). Microglia activation and anti-inflammatory regulation in Alzheimer's disease. *Mol Neurobiol,* 41, 115-28.

Ma, Q.L., Yang, F., Rosario, E.R., Ubeda, O.J., Beech, W., Gant, D.J., et al. (2009). Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. *J Neurosci*, 29, 9078-89.

Maheshwari, R.K., Singh, A.K., Gaddipati, J., Srimal, R.C. (2006). Multiple biological activities of curcumin: a short review. *Life Sci,* 78, 2081-7.

Mazanetz, M.P. & Fischer, P.M. (2007). Untangling tau hyperphosphorylation in drug design for neurodegenerative disease. *Nat Rev*, 6, 464-79.

McAllister, A.K., Katz, L.C., Lo, D.C. (1999). Neurotrophins and synaptic plasticity. *Annu Rev Neurosci*, 22, 295-318.

Medeiros, R., Prediger, R.D.S., Passos, G.F., Pandolfo, P., Duarte, F.S., et al. (2007). Connecting TNF-α signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: relevance for the behavioral and synaptic deficits induced by amyloid β protein. *J Neurosci*, 27, 5394–5404.

Medina, J.H. & Izquierdo, I. (1995). Retrograde messengers, long-term potentiation and memory. *Brain Res Rev,* 21, 185-94.

Modi, G., Pillay, V., Choonara, Y.E., Ndesendo, V.M.K., du Toit, L.C., Naidoo, D. (2009). Nanotechnological applications for the treatment of neurodegenerative disorders. *Progress Neurobiol*, 88, 272-85.

Murer, M., Yanb, Q., Raisman-Vozari, R. (2001). Brain derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Progress in Neurobiology*, 63, 71-124.

Noble, W., Planel, E., Zehr, C., Olm, V., Meyerson, J., Suleman, F., et al. (2005). Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proc Natl Acad Sci U S A,*102, 6990-5.

Ono, K., Hasegawa, K., Naiki, H., Yamada, M. (2004). Curcumin has potent anti-amyloidogenic effects for Alzheimer's beta-amyloid fibrils in vitro. *J Neurosci Res*, 75, 742-50.

Pardridge, W.M. (2005). The blood–brain barrier: bottleneck in brain drug development. *NeuroRx*, 2, 3-14.

Passos, G.F., Figueiredo, C.P., Prediger, R.D.S., Pandolfo, P., Duarte, F.S., Medeiros, R., Calixto, J.B. (2009). Role of the macrophage inflammatory protein-1α/CC

chemokine receptor 5 signaling pathway in the neuroinflammatory response and cognitive deficits induced by β- amyloid peptide. *Am J Pathol*, 175, 1586-97.

Paxinos, G. & Watson, C.(2005). The rat brain in stereotaxic coordinates. (5th ed). San Diego: Elsevier Academic.

Peterson, G.L. (1979). Review of the Folin-phenol protein quantification method of Lowry, Rosebrough, Farr and Randall. *Anal Biochem*, 100, 201-20.

Qin, X.Y., Cheng, Y., Yu, L.C. (2010). Potential protection of curcumin against intracellular amyloid beta-induced toxicity in cultured rat prefrontal cortical neurons. *Neurosci Lett*, 480, 21-24.

Ralay Ranaivo, H., Craft, J.M., Hu, W., Guo, L., Wing, L.K., Van Eldik, L.J., Watterson, D.M. (2006). Glia as a therapeutic target: selective suppression of human amyloid-beta-induced upregulation of brain proinflammatory cytokine production attenuates neurodegeneration. *J Neurosci,* 26, 662-70.

Ray, B., Bisht, S., Maitra, A., Lahiri, D.K. (2011). Neuroprotective and neurorescue effects of a novel polymeric nanoparticle formulation of curcumin (NanoCurc) in the neuronal cell culture and animal model: Implications for Alzheimer's disease. *JAD*, 23, 61–77.

Rockenstein, E., Torrance, M., Adame, A., Mante, M., Bar-on, P., Rose, J.B., et al. (2007). Neuroprotective effects of regulators of the glycogen synthase kinase-3beta signaling pathway in a transgenicmodel of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. *J Neurosci*, 27, 1981-91.

Ryder, J., Su, Y., Ni, B. (2004). Akt/GSK3b serine/threonine kinases: evidence for a signaling pathway mediated by familial Alzheimer's disease mutations. *Cell Signal*, 16, 187–200.

Scheff, S.W., Price, D.A., Schmitt, F.A., Mufson, E.J. (2006). Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. *Neurobiol Aging*, 27, 1372–1384.

Sofola, O., Kerr, F., Rogers, I., Killick, R., Augustin, H., Gandy, C., Allen, M.J., et al. (2010). Inhibition of GSK-3 ameliorates Abeta pathology in an adult-onset Drodophila model of Alzheimer's disease. *PLoS Genet,* 6, e1001087.

Song, G., Ouyang, G., Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*, 9, 59-71.

Song, Z., Feng, R., Sun, M., Guo, C., Gao, Y., Li, L., et al. (2011). Curcumin-loaded PLGA-PEG-PLGA triblock copolymeric micelles: Preparation, pharmacokinetics and distribution in vivo. Journal of Colloid and Interface *Science*, 354, 116-23.

Sorrentino, G. & Bonavita, K. (2007). Neurodegeneration and Alzheimer's disease: the lesson from tauopathies. *Neurol Sci*, 28, 63-71.

Su, C.C., Wang, M.J., Chiu, T.L. (2010). The anti-cancer efficacy of curcumin scrutinized through core signaling pathways in glioblastoma. *Int J Mol Med*, 26, 217-24.

Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill. R., et al. (1991). Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol,* 30, 572-80.

Thangapazham, R.L., Puri, A., Tele, S., Blumenthal, R., Maheshwari, R.K. (2008). Evaluation of a nanotechnology-based carrier for delivery of curcumin in prostate cancer cells. *International Journal of Oncology,* 32, 1119-23.

Tiraboschi, P., Hansen, L.A., Thal, L.J., Corey-Bloom, J. (2004). The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology*, 62, 1984-9.

Troca-Marín, J.A., Alves-Sampaio, A., Montesinos, M.L. (2011). An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse modelo f Down's syndrome. *J Neurosci*, 31, 9445-55.

Tsai, Y.M., Chien, C.F., Lin, L.C., Tsai, T.H. (2011). Curcumin and its nano-formulation: the kinetics of tissue distribution and blood-brain barrier penetration. *Int J Pharm*, 416, 331-38.

Tyler, W.J., Alonso, M., Bramham, C.R., Pozzo-Miller, L.D. (2002). From acquisition to consolidation: on the role of brain-derived neurotrophic factor signalingin hippocampal-dependent learning. *Learn Mem*, 9, 224-37.

Walsh, D.M. & Selkoe, D.J. (2004). Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron*, 44, 181-93.

Wang, H.M., Zhao, Y.X., Zhang, S., Liu, G.D., Kang, W.Y., Tang, H.D., et al. (2010). PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes. *J Alzheimers Dis*, 20, 1189-99.

Woodruff-Pak, D.S. (2008). Animal models of Alzheimer's disease: therapeutic implications. *J Alzheimers Dis*, 15, 507-21.

Yan, Y.D., Kim, J.A., Kwak, M.K., Yoo, B.K., Yong, C.S., Choi, H.G. (2011). Enhanced oral bioavailability of curcumin via a solid lipid-based self-emulsifying drug delivery system using a spray-drying technique. *Biol Pharm Bull,* 34, 1179-86.

Yang, K.Y., Lin, L.C., Tseng, T.Y., Wang, S.C., Tsai, T.H. (2007). Oral bioavailabil-ity of curcumin in rat and the herbal analysis from Curcuma longa by LC-MS/MS. *J Chromatogr B: Analyt Technol Biomed Life Sci*, 853, 183-89.

Yasutake, C., Kuroda, K., Yanagawa, T., Okamura, T., Yoneda, H. (2006). Serum BDNF, TNF-a and IL-1b levels in dementia patients. *Eur Arch Psychiatry Clin Neurosci,* 256, 402–6.

Yoshii, A. & Constantine-Paton, M. (2007). BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nature Neuroscience*, 10, 702-11.

Zanotto-Filho, A., Coradini, K., Braganhol, E., Schröder, R., de Oliveira, C.M., et al. (2013). Curcumin-loaded lipid-core nanocapsules as strategy to improve pharmacological efficacy of curcumin in glioma treatment. *Eur J Pharm Biopharm*, 83, 156-67.

Zhang ,X., Yin, W.K., Shi, X.D., Li, Y. (2011). Curcumin activates Wnt/β-catenin signaling pathway through inhibiting the activity of GSK-3β in APPswe transfected SY5Y cells. *Eur J Pharm Sci*, 42, 540-6.

Figure legends

Table 1. Physicochemical characteristics of curcumin-loaded lipid-core nanocapsules (Cur-LNC) and unloaded-lipid-core nanocapsules (B-LNC) after preparation. Data are mean ± SEM, n = 3. PDI, Polydispersity index.

Fig. 1 Effect of free curcumin (Cur) and curcumin-loaded lipid-core nanocapsules (Cur-LNC) on spontaneous alternation behavior. Rats were injected (2 nmol, i.c.v.) with Aβ1-42 or Aβ-vehicle and daily administered with Cur (50 mg/kg, i.p.) or Cur-LNC (2.5 mg/kg, i.p.), for 10 days. Spontaneous alternation behavior during 8 min session in the Y-maze task was measured after 14 days of treatment. Columns indicate mean ± SEM, n= 12-15 animals in each experimental group. *Significant differences between the indicated columns ($p<0.05$) (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 2 Effect of free curcumin (Cur) and curcumin-loaded lipid-core nanocapsules (Cur-LNC) on the novel object recognition memory. Rats were injected (2 nmol, i.c.v.) with Aβ1-42 or Aβ-vehicle and daily administered with Cur (50 mg/kg, i.p.) or Cur-LNC (2.5 mg/kg, i.p.), for 10 days. Graphics show object recognition index during 5 min in the training session **(A)**, short-term memory test session performed 3 h after training **(B)**, and long-term memory test session performed 24 h after training session **(C)**.

Columns indicate mean ± SEM, n= 10-16 animals in each experimental group. *Significant differences between familiar and new object for each group (p<0.05) and **Significant differences between familiar and new object for each group (p<0.01). (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 3 Aβ1-42 injection causes synaptotoxicity, which is reduced by curcumin treatments. Western blot analysis for synaptophysin and β-actin protein (loading control) was performed in the hippocampus of animals after being injected with Aβ1-42 (2 nmol, i.c.v.) and treated by 10 days with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day). Graphic shows representative quantification of synaptophysin immunocontent normalized to β-actin protein (loading control). The values represent synaptophysin level expressed as the average percentage increase (mean ± SEM) over basal levels, n= 6 animals in each experimental group. *Significantly different from all control and curcumin groups (p<0.05) (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 4 Effect of curcumin on tau phosphorylation triggered by Aβ1-42. Western blot analysis were performed in hippocampi of animals after being injected with Aβ1-42 (2 nmol, i.c.v.) and treated by 10 days with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day). **(A)** Representative Western blot of ratio ptau/tau immunocontent normalized to β-actin protein (loading control). **(B)** The values represent ptau/tau level expressed as the average percentage increase (mean ± SEM) over basal levels, n= 7 animals in each experimental group. *Significant differences between the indicated columns (p<0.05) and ** Significant differences between the indicated columns (p<0.01) (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 5 Curcumin-loaded lipid-core nanocapsules treatment enhances the prevention of neuroinflammation in the hippocampus after i.c.v. injection of Aβ1-42. Representative image showing Isolectin B4 (IB4) reactivity **(A)**, GFAP expression **(B),** TNF-α levels **(C),** and IL-1β levels **(D)** in the hippocampus 15 days after i.c.v. injection of Aβ1-42 (2 nmol) and treatment with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day) for 10 days. Graphic shows representative quantification of GFAP immunocontent normalized to β-actin protein (loading control). The values represent GFAP level expressed as the average percentage increase (mean ± SEM) over basal levels, n= 5 animals in each experimental group. *Significantly different from all control and curcumin groups (p<0.05) **(B)**. Graphic shows quantification of the levels of TNF-α and IL-1β (mean ± SEM), n=6-8 in each experimental group. *Significantly differences between the indicated column (p<0.05), **Significantly different from all control groups (p<0.01) and ***Significantly different from all control groups (p<0.001) **(C)** and **(D)** (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 6 The up-regulation of BDNF levels by curcumin in Aβ-infused rats. **(A)** Representative Western blot of BDNF expression normalized to β-actin protein (loading control) in the hippocampus 15 days after i.c.v. injection of Aβ1-42 (2 nmol) and treated by 10 days with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day). **(B)** Graphic shows representative quantification of BDNF immunocontent normalized to β-actin protein (loading control). The values represent BDNF level expressed as the average percentage increase (mean ± SEM) over basal levels, n= 5 animals in each experimental group. *Significantly different from all control and curcumin groups (p<0.05) (one-way ANOVA followed by Newman–Keuls post hoc test).

Fig. 7 Akt and GSK-3β dephosphorylation induced by Aβ1-42 peptide can be reduced by curcumin. **(A)** Representative Western blot and graphic showing quantification of ratio pAkt/Akt immunocontent in the hippocampus 15 days after i.c.v. injection of Aβ1-42 (2 nmol) and treatment with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day) for 10 days. The values are expressed as the average percentage increase (mean ± SEM) over basal levels, n= 6 animals in each experimental group. *Significant differences between the indicated columns ($p<0.05$) and **Significant differences between the indicated columns ($p<0.01$). **(B)** Representative Western blot and graphic showing quantification of ratio pGSK-3β/GSK-3β immunocontent in the hippocampus 15 days after i.c.v. injection of Aβ1-42 (2 nmol) and treatment with Cur (50 mg/kg/day) or Cur-LNC (2.5 mg/kg/day) for 10 days. The values represent pGSK-3β/GSK-3β level expressed as the average percentage increase (mean ± SEM) over basal levels, n= 5 animals in each experimental group. **Significant differences between the indicated columns ($p<0.01$) and ***Significant differences between the indicated columns ($p<0.001$) (one-way ANOVA followed by Newman–Keuls post hoc test).

Supplementary Fig. 1 Granulometric profiles obtained by laser diffraction for Cur-LNC and B-LNC considering the particles volume.

Table 1

Formulations	Cur-LNC	B-LNC
Particle size (nm)	192 ± 6	200 ± 3
PDI	0.09 ± 0.01	0.10 ± 0.01
Zeta potential (mV)	-9.6 ± 3.5	-7.5 ± 0.3
pH	6.4 ± 0.5	6.2 ± 0.4
Drug loaded (mg/mL)	0.49 ± 0.01	-

Figure 1

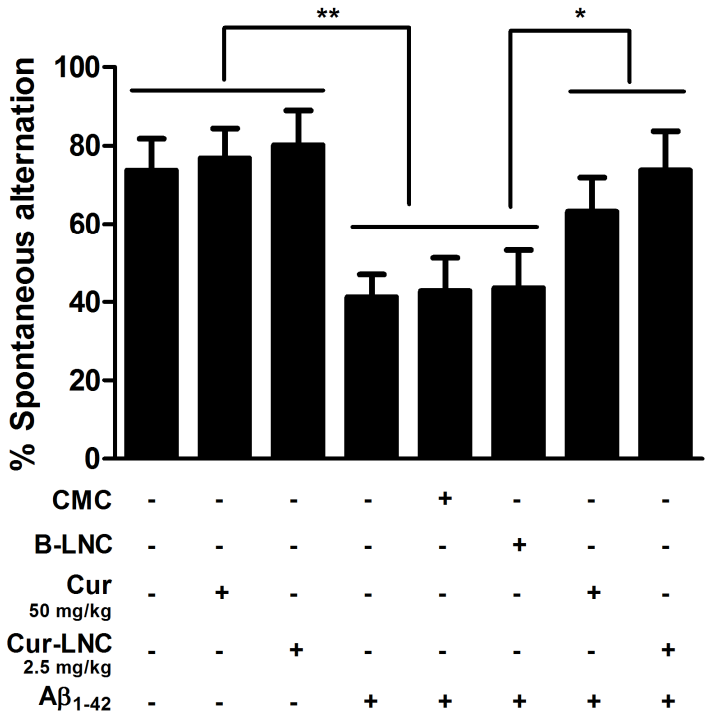

**
*
% Spontaneous alternation
100
80
60
40
20
0
CMC - - - - + - - -
B-LNC - - - - - + - -
Cur 50 mg/kg - + - - - - + -
Cur-LNC 2.5 mg/kg - - + - - - - +
Aβ1-42 - - - + + + + +

Figure 2

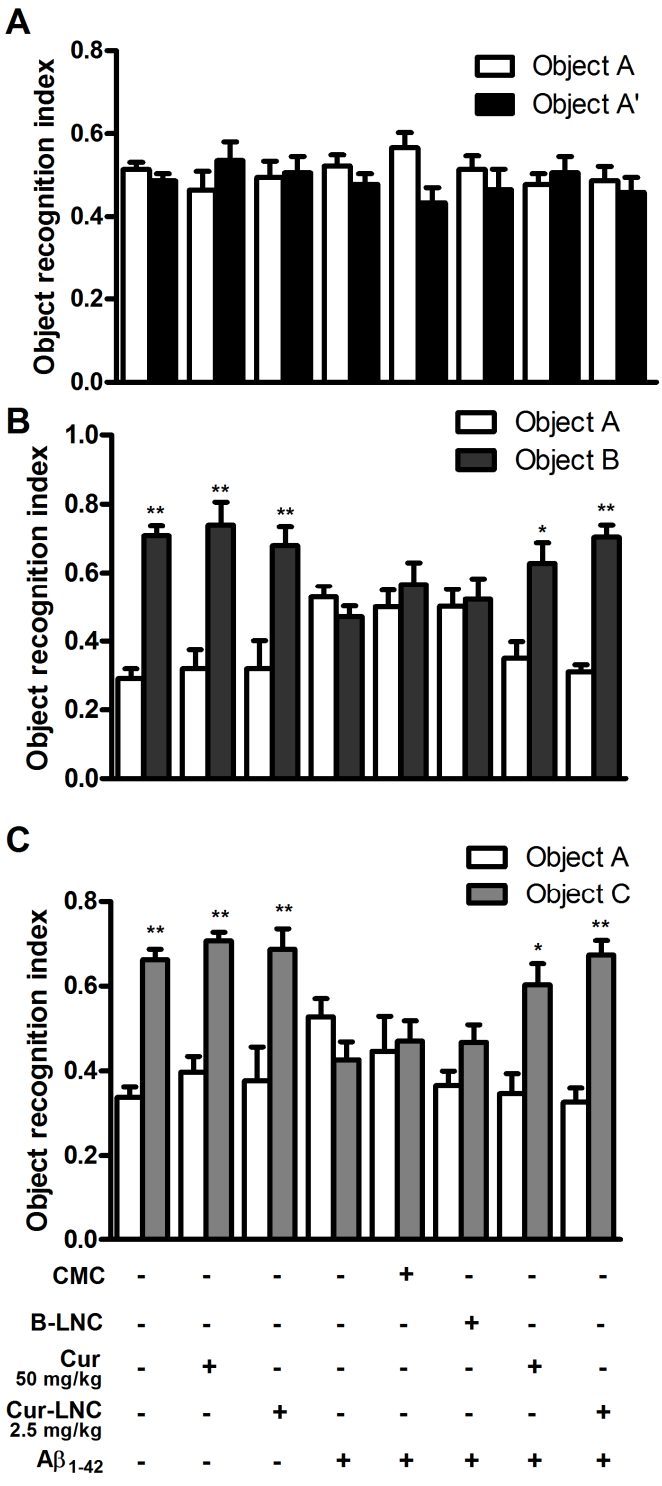

A
Object recognition index
0.0
0.2
0.4
0.6
0.8
Object A
Object A'
B
Object recognition index
0.0
0.2
0.4
0.6
0.8
1.0
Object A
Object B
**
**
**
*
**
C
Object recognition index
0.0
0.2
0.4
0.6
0.8
Object A
Object C
**
**
**
*
**
CMC - - - - + - - -
B-LNC - - - - - + - -
Cur 50 mg/kg - + - - - - + -
Cur-LNC 2.5 mg/kg - - + - - - - +
Aβ1-42 - - - + + + + +

Figure 3

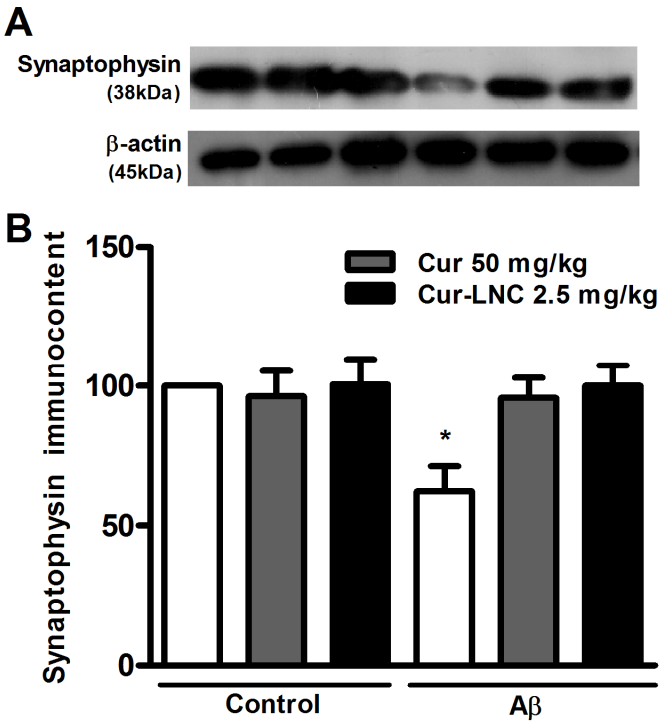

A
Synaptophysin
(38kDa)
β-actin
(45kDa)
B
Synaptophysin immunocontent
150
100
50
0
Cur 50 mg/kg
Cur-LNC 2.5 mg/kg
*
Control
Aβ

Figure 4

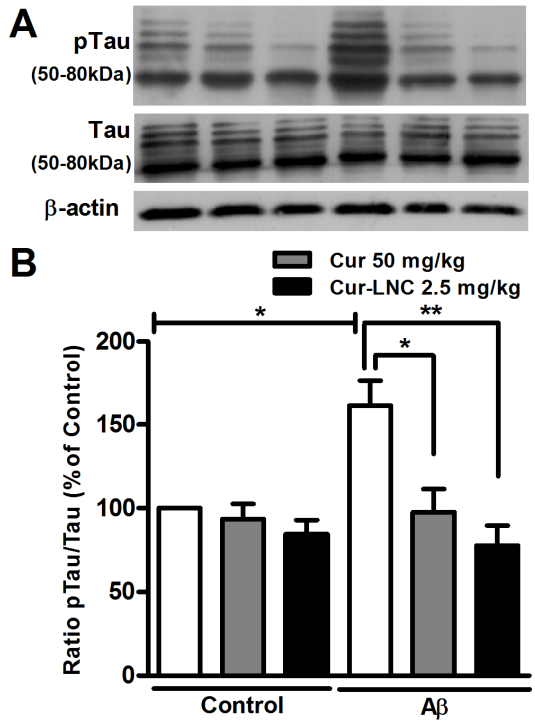

A
pTau
(50-80kDa)
Tau
(50-80kDa)
β-actin
B
Cur 50 mg/kg
Cur-LNC 2.5 mg/kg
*
**
*
Ratio pTau/Tau (% of Control)
200
150
100
50
0
Control
Aβ

Figure 5

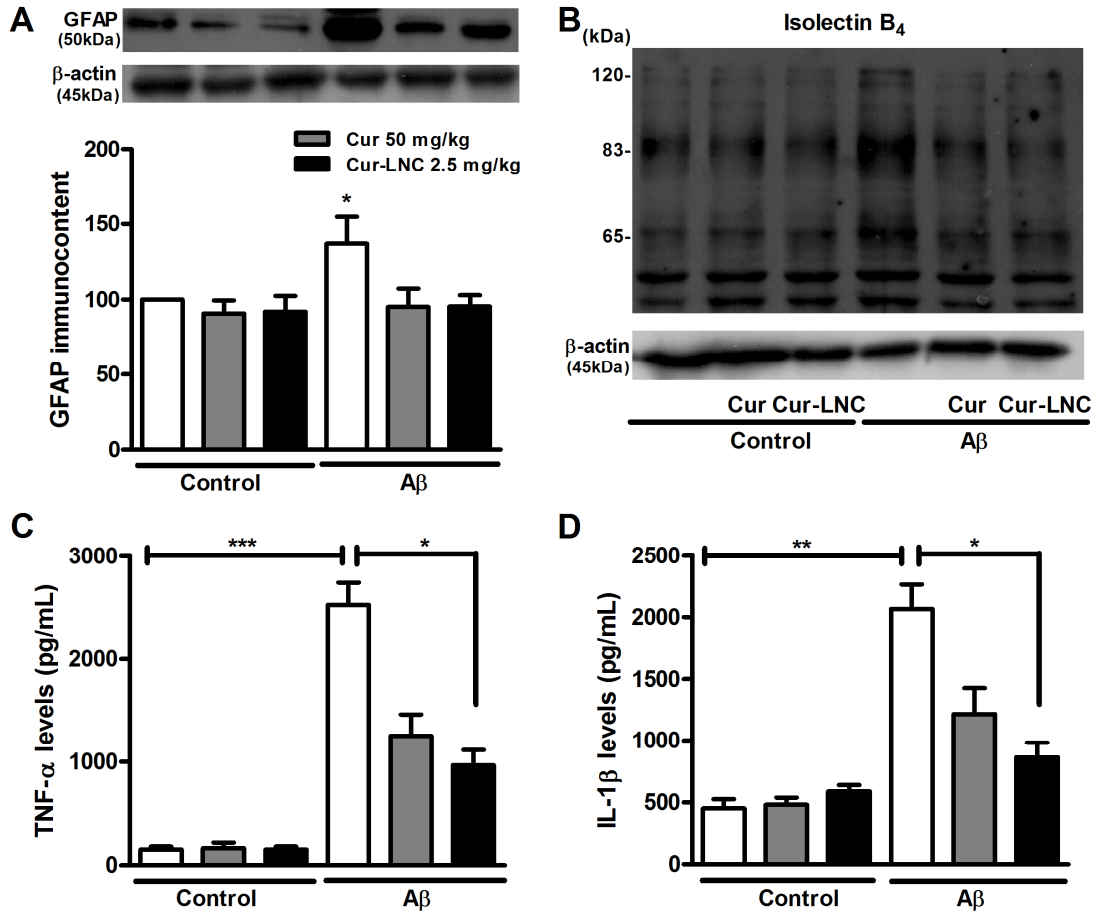

A
GFAP
(50kDa)
β-actin
(45kDa)
Cur 50 mg/kg
Cur-LNC 2.5 mg/kg
GFAP immunocontent
200
150
100
50
0
*
Control
Aβ
B
(kDa)
Isolectin B4
120-
83-
65-
β-actin
(45kDa)
Cur Cur-LNC
Cur Cur-LNC
Control
Aβ
C
TNF-α levels (pg/mL)
3000
2000
1000
0
***
*
Control
Aβ
D
IL-1β levels (pg/mL)
2500
2000
1500
1000
500
0
**
*
Control
Aβ

Figure 6

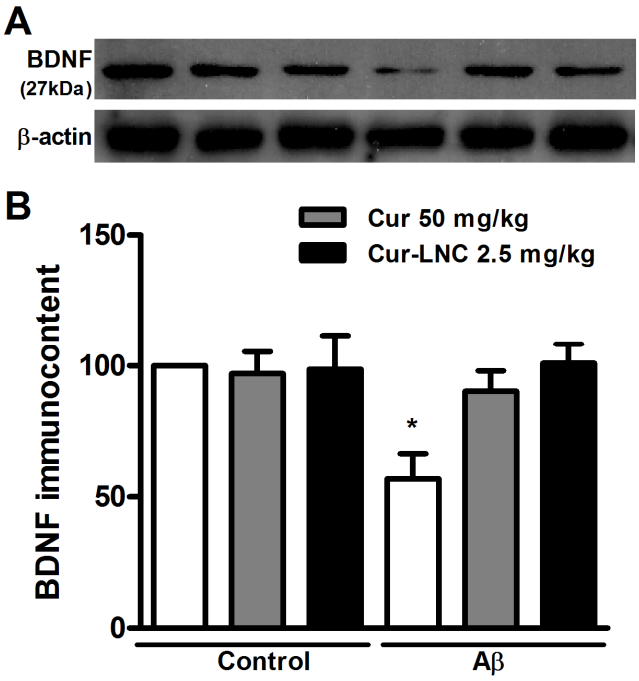
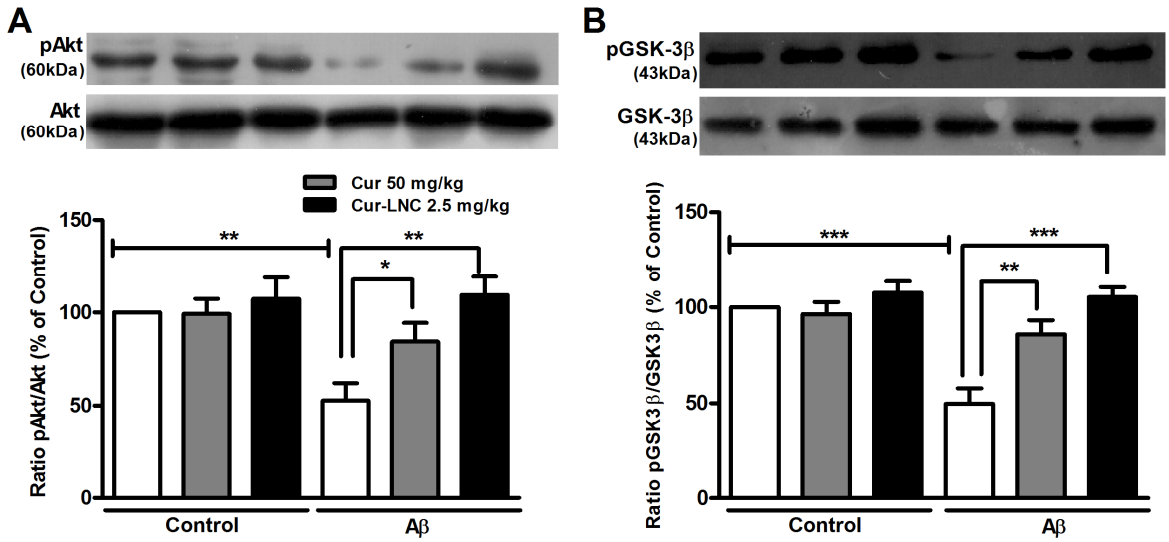
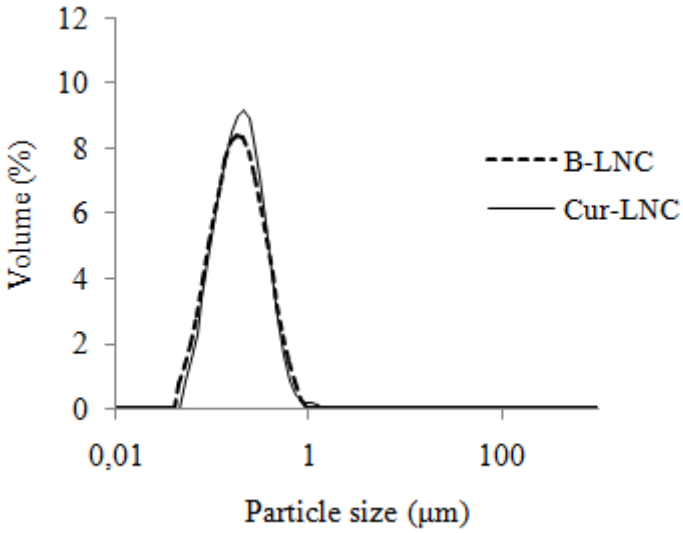

A
BDNF
(27kDa)
β-actin
B
Cur 50 mg/kg
Cur-LNC 2.5 mg/kg
BDNF immunocontent
150
100
50
0
*
Control
Aβ

Figure 7


A
pAkt
(60kDa)
Akt
(60kDa)
Cur 50 mg/kg
Cur-LNC 2.5 mg/kg
Ratio pAkt/Akt (% of Control)
150
100
50
0
**
**
*
Control
Aβ
B
pGSK-3β
(43kDa)
GSK-3β
(43kDa)
Ratio pGSK3β/GSK3β (% of Control)
150
100
50
0
***
***
**
Control
Aβ

Supplementary Figure 1



7. DISCUSSÃO

Atualmente, é elevado o número de pessoas afetadas pela DA e esse aumento alarmante é também esperado para as próximas décadas em decorrência do aumento da expectativa de vida da população. A farmacoterapia atual se mostra limitada a benefícios sintomáticos e apenas por um curto período de tempo, e, portanto, a descoberta e o desenvolvimento de terapias eficazes contra a DA é ainda um grande desafio da área médica, farmacêutica e biotecnológica (Citron, 2002; Refolo and Fillit, 2004). Durante as últimas décadas, diversos estudos epidemiológicos têm consistentemente demonstrado uma associação inversa entre o consumo de vegetais e frutas e o desenvolvimento de uma grande variedade de condições patofisiológicas, como câncer, doenças neurodegenerativas, diabetes, doenças inflamatórias e envelhecimento, possivelmente em decorrência das propriedades farmacológicas dos seus metabólitos secundários (Zhuang et al., 2003; Craggs and Kalaria, 2011; Ferrís-Tortajada et al., 2012). Os compostos polifenólicos constituem um dos mais numerosos e amplamente distribuídos grupos de metabólitos secundários de plantas. Estes compostos ocorrem naturalmente e são encontrados em quantidades significativas em uma variedade de plantas. Os polifenóis encontrados nestas plantas são conhecidos como agentes multifuncionais por apresentarem uma ampla variedade de funções biológicas, incluindo antibacteriana, antiviral, anticarcinogênica, antimutagênica e antienvelhecimento, sendo muitas das suas atividades atribuídas ao potencial antioxidante e antiinflamatório destes compostos (Soobrattee et al., 2005). Os efeitos protetores de muitos destes compostos também têm sido atribuídos, além da ação sobre o estresse oxidativo e a inflamação, a mecanismos alternativos como a modulação das cascatas de transdução dos sinais de apoptose e sobrevivência celular ou efeitos na expressão gênica (Kesley et al., 2010).

Dentre estes compostos a curcumina é um polifenol de baixo peso molecular encontrado de 2 a 8% nos rizomas de cúrcuma (Maheshwari et al., 2006; Park et al., 2008). Seus efeitos biológicos têm sido extensivamente estudados, demonstrando principalmente ação antioxidante, antiinflamatória e antitumoral (Motterlini et al., 2000; Moon et al., 2010; Zanotto-Filho et al., 2012). Na última década, diversos estudos tanto *in vitro* como *in vivo*, têm sugerido que a curcumina desempenha um importante papel neuroprotetor, impulsionando o interesse em determinar a sua eficácia na prevenção ou recuperação de doenças neurodegenerativas (Lim et al., 2001; Wang et al., 2009; Sun et al., 2011). Estes dados nos guiaram para formular nossa hipótese de trabalho que foi verificar o efeito neuroprotetor da curcumina sobre a toxicidade induzida pelo peptídeo Aβ, utilizando para comprovar esta hipótese modelos experimentais *in vitro* e *in vivo*. Os resultados obtidos nesta tese não só confirmam a hipótese de trabalho como também contribuem para aprofundar os conhecimentos sobre os efeitos da curcumina, visando o melhor entendimento do seu mecanismo de neuroproteção contra a toxicidade induzida pelo peptídeo Aβ.

No primeiro capítulo deste trabalho, na tentativa de melhor compreender os mecanismos envolvidos no efeito neuroprotetor da curcumina contra o peptídeo Aβ, utilizamos o modelo de toxicidade do peptídeo $A\beta_{1-42}$ em culturas organotípicas de hipocampo de ratos. Culturas organotípicas representam um modelo *in vitro* que mantém a estrutura tridimensional e a multiplicidade celular original do tecido, além de suas conexões intraneurais intactas (Stoppini et al., 1991; Holopainen, 2005; Noraberg et al., 2005). Neste sistema, neurônios e células gliais sobrevivem por longo período de tempo "amadurecendo" fisiologicamente em cultivo, permitindo o estudo de fatores fisiológicos e de compostos farmacológicos que possam contribuir com a sobrevivência celular e com a plasticidade sináptica, bem como a biologia de proteínas específicas e

cascatas de sinalização celular (Frotscher et al., 1995; Holopainen, 2005). Além disso, o modelo de cultura organotípica de fatias hipocampais é um modelo já bem estabelecido em nosso grupo de trabalho, e amplamente empregado para o estudo de dano celular, envolvimento de vias de sinalização e investigação de potenciais agentes neuroprotetores nos modelos de privação de oxigênio e glicose (Valentim et al., 2003; Horn et al., 2005; Zamin et al. 2006; Simão et al., 2009) e no modelo de toxidade induzida pelo peptídeo Aβ (Nassif et al., 2007; Frozza et al., 2009; Hoppe et al., 2010; Bernardi et al., 2012). Dessa maneira, a cultura organotípica constitui-se um excelente modelo para estudar a resposta dos neurônios, dos astrócitos e da microglia frente à toxicidade induzida pelo peptídeo Aβ e favorece a investigação de mecanismos moleculares envolvidos na neuroproteção da curcumina contra o dano provocado no tecido hipocampal, uma área importante para o aprendizado e memória e uma das principais regiões afetadas na DA (Jarrard, 1993; Broadbent et al; 2004).

No modelo de lesão do presente trabalho, o tecido foi mantido *in vitro* durante 28 dias, a partir de cultura realizada com ratos de 6-8 dias, visto que dados da literatura indicam que culturas organotípicas de hipocampo amadurecidas são mais sensíveis ao peptídeo Aβ (Bruce et al., 1996; Ishige et al., 2007; Frozza et al., 2009). Embora evidências sugiram que agregados oligoméricos sejam os principais responsáveis pela toxicidade do Aβ, em nosso estudo utilizamos o peptídeo Aβ1-42 fibrilado conforme processo de fibrilação amplamente descrito na literatura. Entretanto, nós não excluímos a possibilidade da presença de oligômeros em nossa preparação. Após o período de 28 dias de cultivo as culturas foram expostas à concentração de 2 μM do peptídeo Aβ1-42, previamente fibrilado, e 0.5-10 μM de curcumina concomitantemente por 48h, totalizando 30 dias de cultivo. O tempo de exposição de 48 h do peptídeo Aβ foi determinado a partir de uma curva de tempo de 6, 12, 24, 48 e 72 h realizada em

trabalhos prévios do nosso grupo, nos quais observamos morte celular significativa das fatias expostas ao peptídeo Aβ sem comprometimento da viabilidade de fatias controle no tempo de 48 h (Nassif et al 2007; Hoppe et al.; 2010). Os resultados mostraram uma morte celular significativa, analisada pela incorporação do corante Iodeto de Propídeo (IP), após 48 h de exposição ao peptídeo Aβ1-42. O longo tempo de exposição ao peptídeo Aβ necessário para observar essa toxicidade está de acordo com outros estudos que também relatam a necessidade de um prolongado tempo de exposição (Lu et al. 2004; Ishige et al., 2007; Patel and Good, 2007; Suh et al., 2008). Considerando as limitações dos estudos *in vitro,* o pós-tratamento com curcumina não apresentou neuroproteção em nosso modelo de toxicidade do peptídeo Aβ em fatias organotípicas de hipocampo de ratos. Porém, o resultado apresentado na Figura 1 (Capítulo I) mostra que o co-tratamento com curcumina nas concentrações de 5 μM e 10 μM preveniu significativamente a morte celular induzida pela exposição de culturas organotípicas de hipocampo ao peptídeo Aβ1-42. O método de tratamento e as concentrações de curcumina que se mostraram neuroprotetoras em nosso trabalho estão de acordo com outros estudos em modelos *in vitro* de toxidade do peptídeo Aβ (Qin et al., 2010; Wang et al., 2010; Zhang et al., 2011; Huang et al., 2012). Um estudo importante realizou uma curva de concentração de curcumina (1-100 μM) em cultura primária de neurônios expostas ao peptídeo Aβ e demonstrou que o perfil de proteção da curcumina segue uma curva em forma de U, na qual as concentrações efetivas para a manutenção da viabilidade celular dos neurônios expostos ao peptídeo Aβ foram entre 5-30 μM, sem diferença significativa entre elas, enquanto que em doses maiores a curcumina não demonstrou mais eficácia e em doses elevadas (60-100 μM) foi tóxica para os neurônios (Ye and Zhang, 2012). Visto que curcumina 10 μM é comumente usada em estudos *in*

vitro de toxicidade do peptídeo Aβ e diante do resultado observado em nosso modelo, essa concentração foi adotada para os experimentos posteriores.

Visto que a perda sináptica pode levar ao desenvolvimento de importantes alterações no aprendizado e na memória muito antes da morte neuronal generalizada ser observada na DA (Selkoe et al., 2002), a integridade sináptica nas fatias organotípicas de hipocampo de ratos foi avaliada através da proteína sinaptofisina. A disfunção de sinapses na DA é decorrente de uma cascata de múltiplos eventos celulares e moleculares deletérios desencadeados pelo peptídeo Aβ (Shankar and Walsh, 2009; Palop and Mucke, 2010). O resultado apresentado na Figura 2 (Capítulo I) mostra que o co-tratamento com curcumina 10 μM preveniu significativamente a redução do imunoconteúdo de sinaptofisina induzida pela exposição de fatias organotípicas de hipocampo ao peptídeo Aβ1-42. A sinaptofisina é a principal proteína de membrana da vesícula sináptica, estando presente em todos os neurônios do cérebro e medula espinhal que participam da transmissão sináptica e, dessa maneira, tem sido usada para a quantificação de sinapses (Calhoun et al., 1996; Suh et al., 2008).

Na sequência da nossa avaliação, procuramos investigar os mecanismos envolvidos na neuroproteção da curcumina contra a toxicidade sináptica e morte celular induzidas pelo peptídeo Aβ nas fatias hipocampais. A neuroinflamação tem sido reconhecida por exercer um papel central na patogenia da DA, uma vez que a produção anormal de citocinas e quimiocinas, bem como de espécies reativas, parece desempenhar um papel importante na toxicidade induzida pelo peptídeo Aβ, levando a distúrbios na homeostasia celular, perda sináptica e morte celular (Agostinho et al., 2010). Nossos resultados reforçam dados prévios, nos quais a curcumina tem sido proposta como um potente composto antioxidante e antiinflamatório em resposta ao peptídeo Aβ (Figura 3, 4 e 5 - Capítulo I) (Lim et al., 2001; Begum et al., 2008; Wang et al., 2010). Também

ampliamos estes achados ao demonstrarmos mudanças nos níveis de importantes citocinas, como a IL-6 e a IL-10, para o meio de cultivo das fatias organotípicas hipocampais expostas ao peptídeo Aβ (Figura 5 - Capítulo I). A redução da formação de espécies reativas, a inibição da ativação astrocitária e microglial e a prevenção da alteração na liberação das citocinas IL-6 e IL-10 na presença de curcumina, sugerem que este composto polifenólico pode ser eficaz no combate a estes processos intimamente associadas com a DA. Enquanto a citocina pró-inflamatória IL-6 tem sido associada com danos cognitivos graves, a citocina antiinflamatória IL-10 atua reprimindo a expressão de citocinas pró-inflamatórias, como a IL-6, TNF-α e IL-1β por células gliais ativadas (Beurel et al., 2009; Dagvadorj et al., 2009; Garwood et al., 2011). As respostas ao estresse oxidativo e a inflamação são eventos interdependentes e bidirecionais, visto que o conceito da toxicidade por espécies reativas tem como raiz a biologia da inflamação, onde a formação de espécies reativas por astrócitos e microglia é um mecanismo importante para atacar alvos opsonizados (Akiyama et al., 2000; Block et al., 2007). Essas relações paralelas ou sequenciais entre inflamação e dano oxidativo podem implicar em mecanismos de regulação comuns através das mesmas vias de sinalização (Rosales-Corral et al., 2004). Ainda que inicialmente astrócitos e microglia atuem promovendo a remoção do peptídeo Aβ, os elevados níveis de fatores pró-inflamatórios e pró-oxidativos gerados levam ao agravamento da neurodegeneração. Em um ambiente inflamatório e altamente oxidativo, os astrócitos e a microglia podem sofrer alterações em seus fenótipos metabólicos reduzindo sua capacidade de controlar as alterações homeostáticas e diminuindo sua habilidade em proteger os neurônios (Gavillet et al., 2008; Luo and Chen, 2012). A geração deste microambiente inflamatório pode desencadear a ativação de diversas vias de sinalização celular, comprometento a sobrevivência neuronal e contribuindo para a geração de mais

mediadores tóxicos (Van Eldik et al., 2007). A ligação de citocinas aos seus receptores estimulam uma variedade de vias de sinalização intracelulares que têm sido implicadas na DA, incluindo a ativação da proteína quinase C (PKC), c-Jun N-terminal quinase (JNK), a proteína ativada por mitógeno p38-quinase (p38), PI3K/Akt, quinase de sinalização relacionada extracelular (ERK), bem como a ativação de caspases -1 e -3 (Van Eldik et al., 2007; Anisman et al., 2009).

Além do importante efeito antioxidante e anti-inflamatório que a curcumina apresenta, também cresce o número de evidências mostrando que este composto pode modular vias de sinalização intracelulares reguladas por modificações pós-traducionais de proteínas (Hatcher et al., 2008; Zhou et al., 2011). A fosforilação de proteínas é uma das mais importantes modificações pós-traducionais com efeito direto na atividade celular. Na tentativa de melhor estabelecer os efeitos neuroprotetores da curcumina em nosso modelo, nos baseamos na hipótese de que a curcumina poderia mediar seus efeitos até agora observados, agindo em duas importantes vias de sinalização envolvidas na sobrevivência neuronal: Wnt/β-catenina e PI3K/Akt. A desregulação da via de sinalização Wnt/ β-catenina pelo peptídeo Aβ representa um evento central na morte neuronal que ocorre na DA (Garrido et al., 2002; De Ferrari et al., 2003; Li et al., 2011). Os níveis citoplasmáticos da β-catenina são regulados por fosforilação de tal maneira que livre a β-catenina pode migrar para o núcleo e induzir a expressão de diversos genes envolvidos na sobrevivência e na homeostase neuronal ou ser direcionada às sinapses para modular o fortalecimento sináptico em resposta à despolarização (Caricasole et al., 2003). Entretanto, uma vez fosforilada a β-catenina é degradada via sistema ubiquitina-proteossoma (Aberle et al., 1997). Na figura 6 (Capítulo I) observamos que o peptídeo Aβ foi capaz de aumentar a proteína β-catenina fosforilada e diminuir a β-catenina não fosforilada no citoplasma das fatias organotípicas, possivelmente conduzindo à

degradação da β-catenina. Nossos dados também demonstram que a curcumina pode promover o seu efeito neuroprotetor ao prevenir essa degradação da proteína β-catenina induzida pelo peptídeo Aβ.

A via de sinalização PI3K é conhecida por ser importante na sobrevivência e na morte neuronal, e por ser altamente afetada por ambientes de estresse oxidativo e inflamação (Lee et al., 2008; De Oliveira et al., 2012). Tem sido demonstrado que os efeitos protetores da via de sinalização PI3K são mediados principalmente por um dos seus alvos a jusante, a proteína Akt. A Akt tem efeitos diretos sobre a via de apoptose, por inibir diversas proteínas pró-apoptóticas, como a GSK-3β (Pap et al., 1998). A proteína GSK-3β é uma importante inter-relação entre as vias PI3K e Wnt/β-catenina, pois pode atuar diretamente na fosforilação da β-catenina (Doble and Woodgett, 2003). Para investigar o envolvimento da via de sinalização PI3K na neuroproteção mediada pela curcumina contra a toxicidade induzida pelo peptídeo Aβ em fatias organotípicas de hipocampo, utilizamos LY294002, um inibidor específico da PI3K, e este suprimiu quase que totalmente o efeito neuroprotetor da curcumina. Sendo assim, foi investigado o efeito da curcumina sobre a proteína Akt, principal efetora desta via, e os resultados mostraram que a curcumina foi capaz de aumentar a fosforilação, e consequente ativação, apenas nas fatias expostas ao peptídeo Aβ, e o uso do LY parcialmente aboliu esta ativação da Akt. Seguindo a via da PI3K/Akt, investigou-se o efeito da curcumina sobre a proteína pró-apoptótica GSK-3β. Seguindo o mesmo perfil observado na Akt, a curcumina aumentou a fosforilação da GSK-3β, e consequente inativação, apenas nas fatias expostas ao peptídeo Aβ, enquanto o LY foi capaz de bloquear esta inativação da GSK-3β. Este conjunto de resultados, onde a curcumina aumentou a fosforilação da Akt e da GSK-3β em fatias expostas ao peptídeo Aβ, e o efeito da inibição da via de sinalização PI3K pelo LY294002 abolindo a neuroproteção mediada pela curcumina,

sugerem que este pode ser um importante mecanismo pelo qual a curcumina protege as culturas organotípicas de hipocampo de rato do dano sináptico e da morte celular induzidos pelo peptídeo Aβ (Figura 7 - Capítulo I).

Uma explicação para não observarmos diferença na fosforilação de Akt e GSK-3β em fatias organotípicas de hipocampo tratados com o peptídeo Aβ após 48 h de exposição, possivelmente seja devido ao fato de que estas alterações estejam ocorrendo antes deste período e sejam transientes. Nossos estudos anteriores já demonstraram este perfil, onde com a exposição de 12 h ao peptídeo Aβ em fatias organotípicas de hipocampo a fosforilação das proteínas Akt e GSK-3β encontram-se significativamente diminuídas e no tempo de 48 h de exposição já retornaram aos níveis basais (Nassif et al., 2007; Hoppe et al., 2010). Embora a fosforilação da Akt e GSK-3β pelo peptídeo Aβ parece acontecer de forma transiente *in vitro*, neste estudo observamos que a curcumina, mesmo após longo período de exposição, atua para manter esta importante via pró-sobrevivência ativa em culturas organotípicas de hipocampo expostas ao Aβ.

A ativação prolongada e difundida de astrócitos e microglia caracteriza a neuroinflamação na DA, onde a severidade da ativação glial é correlacionada à extensão da atrofia cerebral e do declínio cognitivo (Parachikova et al., 2007; Garwood et al., 2011). Considerando os resultados da curcumina contra a neuroinflamação induzida pelo peptídeo Aβ em fatias organotípicas de hipocampo, no segundo capítulo desta tese procuramos aprofundar a investigação dos efeitos da curcumina contra a toxicidade induzida pelo peptídeo Aβ em células gliais astrocitárias. Esta hipótese foi baseada no fato de que os astrócitos têm recebido grande foco como contribuintes fundamentais no desenvolvimento do processo neurodegenerativo. Em estudos recentes, a reatividade astrocitária tem sido fortemente implicada na perda neuronal induzida pelo peptídeo Aβ na DA (Garwood et al., 2011). Os mecanismos envolvidos na reatividade dos astrócitos

não estão totalmente caracterizados e tratamentos que podem reduzir a reatividade dos astrócitos são de grande interesse terapêutico. A curcumina já demonstrou capacidade em reduzir a reatividade astrocitária e regular um número significativo de vias citoprotetoras nos astrócitos (Lavoie et al., 2009; Wang et al., 2010). Como discutido anteriormente, a regulação destas vias de sinalização envolve a regulação de modificações pós-traducionais de proteínas. Dentre estas modificações, recentemente tem sido atribuído o papel da sumoilação na regulação da atividade de proteínas através dos pequenos modificadores do tipo ubiquitina (SUMO) na DA, geralmente considerado um mecanismo endógeno citoprotetor (Yang et al., 2008; Zhang et al., 2008, McMillan et al., 2011).

Neste segundo capítulo utilizamos um modelo *in vitro* de toxicidade induzida pelo peptídeo Aβ em cultura primária de astrócitos. Em nosso estudo, observamos que a exposição por 48 h ao peptídeo Aβ causou a reatividade astrocitária das culturas, evidenciado pelo aumento do imunoconteúdo da proteína glial fibrilar ácida (GFAP) e pelas alterações morfológicas astrogliais como hipertrofia e aumento de processos celulares, sem provocar morte celular das culturas. O co-tratamento com curcumina 10 μM foi um supressor eficaz da ativação astrocitária, reduzindo os níveis da proteína GFAP e as alterações morfológicas induzidas pelo peptídeo Aβ em nosso modelo, resultados estes que estão em conformidade com estudos prévios *in vitro* e em modelo animal da DA (Figura 1 - Capítulo II) (Lim et al., 2001; Wang et al., 2010).

A partir destes resultados, prosseguiu-se a investigação dos possíveis mecanismos de indução da ativação dos astrócitos pelo peptídeo Aβ e de citoproteção mediada pela curcumina. A ativação da JNK, membro da família das proteínas quinases ativadas por mitógenos (MAPK), tem sido descrita em modelos da DA e sua inibição pode contribuir significativamente para a redução da toxicidade desencadeada pelo Aβ (Herrera et al.,

2012). Além disso, a JNK pode estar envolvida nos processos de ativação glial e na indução da expressão de genes responsáveis pela síntese de diversas citocinas pró-inflamatórias, bem como com a ativação de COX-2 e iNOS (Waetzig and Herdegen, 2004; Mehan et al., 2011). A análise por western blotting da JNK demonstrou que a curcumina foi capaz de inibir a fosforilação/ativação desta proteína induzida pelo peptídeo Aβ nas culturas de astrócitos (Figura 1 – Capítulo II). Este efeito da curcumina sobre a proteína JNK é consistente com a literatura, na qual o tratamento com curcumina mostrou reduzir a ativação de JNK em um modelo transgênico da DA (Ma et al., 2009).

Com a finalidade de ampliar a investigação sobre os mecanismos envolvidos no efeito citoprotetor mediado pela curcumina avaliou-se o envolvimento da sumoilação na indução da ativação astrocitária pelo peptídeo Aβ. Os resultados mostraram que os astrócitos têm certo número de proteínas que são sumoiladas sob condições de repouso, com um número relativamente pequeno de alvos para a SUMO-1 em comparação com os para SUMO-2/3. Este achado contrasta ao padrão observado na análise de amostras de culturas neuronais (Cimarosti et al., 2012) ou de homogeneizado de tecido cerebral (McMillan et al., 2011), em que existe um número equivalente de bandas protéicas marcadas com SUMO-1 e SUMO-2/3. Este padrão restrito de conjugação de SUMO-1 nos sugere um papel importante deste na regulação celular dos astrócitos. Após a exposição das culturas ao peptídeo Aβ, há uma diminuição acentuada da conjugação por SUMO-1, mas não por SUMO-2/3, uma observação até agora não encontrada na literatura (Figura 2 – Capítulo II). Assim como, os resultados sobre a ação da curcumina, bem como do peptídeo inibidor da proteína JNK (L-JNKi), prevenindo a diminuição da conjugação por SUMO-1 em astrócitos expostos ao peptídeo Aβ. Sugerindo que a curcumina possa estar atuando como um inibidor da proteína JNK,

visto que ela foi capaz de prevenir a ativação de JNK neste modelo. Sumoilação e a via da JNK já foram associadas em estudos anteriores, porém os resultados destes estudos ainda permanecem controversos. Estudos já demonstraram que enquanto a JNK fosforila e ativa os fatores de transcrição c-Jun e p53, o aumento da conjugação por SUMO-1 parece regular negativamente estes fatores de transcrição (Mueller et al., 2000). Além disso, o aumento da sumoilação parece inibir a proteína sinal de apoptose quinase 1 (ASK1), um ativador de JNK (Lee et al., 2005). Em outro estudo, com linhagem de neuroblastoma humano, observou-se que aumentando a conjugação por SUMO-1 ocorre um aumento da ativação de JNK em condições de estresse oxidativo moderado (Feligione et al., 2011).

Diante da diminuição da conjugação por SUMO-1 nos astrócitos expostos ao peptídeo Aβ e na prevenção desta alteração pela curcumina, decidimos investigar um possível papel da SUMO-1 na reatividade astrocitária induzida pelo peptídeo Aβ. O aumento da capacidade dos astrócitos para conjugar proteínas com SUMO-1, através da superexpressão com SUMO-1GG, aboliu a capacidade do peptídeo Aβ em elevar os níveis de GFAP e induzir alterações morfológicas em astrócitos, sugerindo um papel crítico da conjugação por SUMO-1 no mecanismo envolvido com a reatividade dos astrócitos expostos ao peptídeo Aβ (Figura 3 - Capítulo II). Os resultados obtidos neste capítulo sugerem que os astrócitos exigem a conjugação por SUMO-1 para permanecer em um estado não-reativo. Desse modo, a prevenção da alteração da conjugação de SUMO-1 pela curcumina pode ser um importante mecanismo citoprotetor contra a toxicidade induzida pelo peptídeo Aβ em astrócitos (Figura 4 - Capítulo II). Até o momento existem poucos dados sobre a sumoilação em astrócitos, um importante estudo mostrou que os níveis de SUMO-1 são regulados negativamente em astrócitos tratados com lipopolissacarídeo (LPS), reforçando a ideia que a perda da conjugação

por SUMO-1 em astrócitos esteja associada com processos patofisiológicos (Akar and Feinstein, 2009).

Tendo em mente os resultados obtidos no Capítulo I, onde a morte celular significativa em fatias organotípicas hipocampais somente foi observada após a exposição por 48 h ao peptídeo Aβ, no Capítulo III desta tese decidimos utilizar o mesmo modelo de culturas organotípicas expostas ao peptídeo Aβ, porém por um menor período de tempo, para investigar a atividade sináptica através de medidas eletrofisiológicas nestas fatias hipocampais e o efeito do tratamento com curcumina neste modelo. Segundo a nova versão da hipótese da cascata amilóide a disfunção sináptica constitui a melhor correlação com os distúrbios cognitivos associados com a DA precedendo a morte neuronal generalizada (Koffie et al., 2009; Zeng et al., 2010). Apesar de bem aceito como componente principal na patogênese da DA, os mecanismos envolvidos nos efeitos prejudiciais do peptídeo Aβ na função sináptica não estão totalmente caracterizados (Tanzi and Bertram, 2005; Koffie et al., 2009). Vários estudos têm mostrado que o peptídeo Aβ é tóxico para as sinapses, resultando na eliminação progressiva dos circuitos neuronais (Hartley et al., 1999; Tsai et al., 2004; Selkoe, 2008; Jo et al., 2011) e alguns importantes mecanismos de sinalização celular têm sido envolvidos neste processo de disfunção sináptica (Shankar and Walsh, 2009; Palop and Mucke, 2010; Ma and Klann, 2012). Apesar de diversos estudos demonstrarem um maior potencial tóxico dos oligômeros do Aβ para as sinapses, já foi demonstrado que a perda de sinapses é mais proeminente nas imediações das placas senis sugerindo que estas placas podem ser um reservatório de oligômeros sinaptotóxicos (Tsai et al., 2004; Koffie et al., 2009; Shankar and Walsh, 2009).

Neste estudo, confirmamos a morte celular das fatias organotípicas hipocampais após 48 h de exposição ao peptídeo Aβ1-42 (2μM), bem como a sua prevenção através

do co-tratamento destas fatias com curcumina (10 μM). Este resultado foi confirmado através de duas técnicas complementares de análise de viabilidade celular nestas fatias, a incorporação de IP nas fatias e a liberação da enzima lactato desidrogenase (LDH) no meio de cultivo, sendo que o perfil de dano celular foi similar com as duas técnicas. Além disso, observamos que após 24 h de exposição ao peptídeo Aβ as fatias ainda não apresentavam morte celular significativa e, portanto, este foi o tempo escolhido para os experimentos seguintes realizados no presente trabalho (Figura 1 - Capítulo III).

Desta forma, utilizamos fatias organotípicas co-tratadas com peptídeo Aβ e curcumina por 24 h e avaliamos possíveis efeitos deletérios e protetores, respectivamente, destes compostos sobre a funcionalidade celular através do registro dos sinais eletrofisiológicos das fatias organotípicas hipocampais com o auxílio de matrizes multieletrodo (MEAs). As fatias organotípicas hipocampais controles e tratadas após 15 dias *in vitro* foram acopladas nas MEAs para a gravação da atividade neuronal espontânea de cada fatia. Cada MEA era composta de 60 eletrodos de 30 μm de diâmetro dispostos numa matriz 8 x 8 com espaçamento de 200 μm entre cada eletrodo (Multi Channel Systems, Reutlingen, Germany). A fatia foi posicionada corretamente sobre a matriz e os sinais elétricos obtidos, a partir dos eletrodos extracelulares, foram ampliados, gravados e processados através do uso de software específico (MC Rack Software), para posterior análise dos potenciais de campo local (LFPs). Os LFPs são caracterizados como os potenciais elétricos registrados por eletrodos extracelulares e refletem a atividade sináptica de uma grande população de neurônios (Lindén et al., 2011; Lewis, 2012). Estudos já demonstraram que as oscilações de LFPs no hipocampo estão associadas com aprendizagem e memória em ratos (Siegel et al., 2009; Pangalos et al., 2013). A análise dos espectros gravados com os potenciais de campo local espontâneos de 60 eletrodos por fatia de hipocampo

demonstrou uma atenuação da propagação do sinal induzida pelo peptídeo Aβ quando comparado com o controle, o qual foi significativamente prevenido pela curcumina (Figura 2 A – Capítulo III). A maioria dos estudos *in vitro* envolvendo a atividade eletrofisiológica de neurônios tratados com o peptídeo Aβ utilizaram a técnica de "patch clamp" (Jhamandas et al., 2001; Gureviciene et al., 2004; Tan et al., 2012). Nossos dados reforçam os achados de um único estudo existente na literatura até o momento utilizando a técnica eletrofisiológica de MEAs para avaliar os efeitos do peptídeo Aβ na atividade sináptica, neste os autores compararam os resultados obtidos com a técnica de MEAs com a técnica eletrofisiológica de "patch clamp" em cultura primária de neurônios hipocampais e observaram a inibição da atividade sináptica espontânea induzida por oligômeros do Aβ e a atenuação desta condição por curcumina (Varghese et al., 2010). A fim de investigar um possível mecanismo eletrofisiológico pelo qual a curcumina atenuou a diminuição da propagação do sinal induzido pelo peptídeo Aβ, avaliamos o efeito do co-tratamento com curcumina nas fatias hipocampais expostas ao peptídeo Aβ1-42 sobre a coerência de fase, uma medida da extensão em que os eventos de LFPs mantêm elementos de sincronização. Observamos que a coerência de fase foi significativamente reduzida 200 µm a partir do foco nas fatias co-tratadas com peptídeo Aβ1-42 e curcumina (Figura 2 B - Capítulo III). Fortes evidências têm demonstrado que o peptídeo Aβ é capaz de reduzir a recaptação de glutamato (Palop and Mucke, 2010) levando a remodelação da plasticidade sináptica e culminando na depressão sináptica. Além disso, outros estudos têm demonstrado que o peptídeo Aβ atenua a atividade dos receptores N-metil-D-aspartato (NMDARs) por efeitos diretos sobre GluN2B NMDAR (Koffie et al., 2011) levando a diminuição do influxo de Ca^{2+} nos espinhos dendríticos, promovendo a depressão a longo prazo (LTD) e, finalmente, gerando o dano na funcionalidade da rede neuronal (Decker et al., 2010; Corona et al., 2011). Visto o

importante papel da diminuição sustentada do influxo Ca^{2+} e a consequente ativação de vias celulares que levam ao enfraquecimento sináptico na sinaptotoxicidade provocada pelo peptídeo Aβ (Shankar et al., 2007), este efeito de dessincronização da transmissão sináptica pode estar envolvido com o efeito neuroprotetor da curcumina na prevenção da disfunção sináptica induzida pelo peptídeo Aβ em fatias organotípicas de hipocampo. Além disso, estes resultados são reforçados pelo estudo do perfil de fosforilação das proteínas sinápticas CaMKII e sinapsina I em nosso modelo.

Considerando que pouco se sabe sobre os mecanismos específicos pelos quais o peptídeo Aβ afeta a excitabilidade neuronal e a curcumina pode melhorar a transmissão sináptica no hipocampo, avaliamos a fosforilação/ativação de uma proteína importante para a liberação de neurotransmissores, a sinapsina I, e uma proteína essencial para a plasticidade sináptica, a CaMKII (Hilfiker et al., 1999; Ninan and Arancio, 2004). A perda de sinapses induzida pelo peptídeo Aβ em neurônios hipocampais e corticais tem sido mostrado estar acompanhado por uma redução de sinapsina I, um efeito também observado em hipocampo de pacientes com DA (Qin et al., 2004; Evans et al., 2008). O influxo reduzido de Ca^{2+} através dos NMDARs induzido pelo peptídeo Aβ tem demonstrado impedir a funcionalidade da proteína CaMKII (Zhao et al., 2004; Koffie et al., 2011). Por sua vez, a ativação da proteína CaMKII facilita a indução da potenciação de longo prazo (LTP), promovendo o alargamento de espinhos dendríticos e o fortalecimento sináptico (Pi et al 2010; Koffie et al., 2011). Isso contribuiu para a indução de neurônios mais robustos e sinapticamente eficientes, refletido na inibição da disfunção sináptica e morte neuronal. Além disso, a CaMKII quando ativada modula várias proteínas envolvidas com a transmissão sináptica, incluindo a sinapsina I, a qual sob condições de repouso, ancora as vesículas sinápticas a elementos do citoesqueleto, enquanto que fosforilada se dissocia das vesículas sinápticas, aumentando a

disponibilidade destas para o terminal pré-sináptico e, consequentemente, a liberação de neurotransmissores (Chi et al. 2003). Na figura 3 (Capítulo III) avaliamos o estado de fosforilação da sinapsina I em dois sítios importantes Ser 09 e Ser 603, e observamos que apenas na Ser 603 houve mudança do perfil de fosforilação pelo peptídeo Aβ em nosso modelo, e que esta foi bloqueada com curcumina. Diante deste resultado, e visto que o sítio de fosforilação 603 é CaMKII específico, analisamos os níveis de fosforilação desta importante proteína sináptica e encontramos uma significativa redução dos níveis de p-CaMKII (Thr 286) na fatias expostas ao peptídeo Aβ por 24 h, enquanto nas fatias co-tratadas com curcumina esta redução foi prevenida. Estes resultados nos levam a acreditar que a curcumina possa regular a atividade da proteína CaMKII e, por consequência, a fosforilação da sinapsina I Ser 603 e, desta forma, a regulação desta via de sinalização esteja contribuindo para o seu efeito protetor no comprometimento da transmissão sináptica de fatias hipocampais expostas ao peptídeo Aβ.

Este achado está de acordo com alguns estudos prévios encontrados na literatura, envolvendo outros modelos de danos ao SNC, que também relacionam a regulação de CaMKII e sinapsina I aos efeitos neuroprotetores da curcumina. A suplementação dietética com curcumina melhorou o resultado de uma lesão cerebral traumática, não apenas através da redução substancial do dano oxidativo, mas também através da restauração dos níveis de fosfo-sinapsina I que haviam sido reduzidos após o insulto (Wu et al. 2006). Em outro estudo, o tratamento com curcumina bloqueou os déficits cognitivos pós-trauma, bem como preveniu a redução dos níveis de sinapsia I e CaMKII no hipocampo e na medula espinhal (Wu et al., 2011).

Tomados em conjunto, os resultados obtidos neste Capítulo suportam a emergente visão de que a deficiência funcional dos neurônios pode ser mais importante para o

desenvolvimento dos sintomas da DA do que a morte celular que ocorre em fases mais avançadas da doença (Varghese et al. 2010; Palop and Mucke, 2010). Nossos resultados revelaram que o tratamento com a curcumina vai além de sua capacidade de modular a morte neuronal, uma vez que a mesma foi capaz de neutralizar tanto os efeitos deletérios do peptídeo Aβ na morte celular mais tardia quanto na disfunção sináptica mais precoce em culturas organotípicas de hipocampo de ratos. Além disso, nossos resultados sugerem que a curcumina modula a diminuição da função sináptica induzida pelo peptídeo Aβ através de uma via dependente da proteína CaMKII.

Diante dos resultados obtidos nos três primeiros Capítulos utilizando modelos experimentais *in vitro*, na última abordagem experimental desta tese foi avaliado o efeito da curcumina em um modelo *in vivo* de toxicidade induzida pelo peptídeo Aβ. Para tanto, utilizamos o modelo de injeção intracerebroventricular (icv) do peptídeo Aβ em ratos Wistar. Apesar da maioria dos estudos *in vivo* abordando as alterações características da DA utilizarem roedores transgênicos, a utilização do modelo de administração icv do peptídeo Aβ tem sido especialmente útil no desenvolvimento e na avaliação de novas modalidades terapêuticas. Embora variável em termos de tamanho e estado de agregação do peptídeo Aβ utilizado, do procedimento e do local de injeção e dos testes comportamentais empregados, diversos estudos tem consistentemente demonstrado a ocorrência de déficits comportamentais relacionados à perda de memória após a injeção intracerebral do peptídeo Aβ em roedores (Medeiros et al., 2007; Prediger et al., 2007; Canas et al., 2009; Ahmed et al., 2010; Frozza et al., 2013). Da mesma forma como discutido no Capítulo I, o peptídeo $A\beta_{1-42}$ utilizado em nosso estudo foi submetido aos processos de formação de fibrilas amplamente empregados pela literatura. Porém conforme acima citado, não é excludente a presença de oligômeros em nossa preparação. Os resultados obtidos no Capítulo IV desta tese mostram que a

injeção icv do Aβ1-42 (2 nmol) causou um significativo déficit comportamental, como pode ser evidenciado pela redução significativa na alternação espontânea e pela diminuição significativa no índice de reconhecimento de objetos (Figura 1 e 2 – Capítulo IV). Estes resultados corroboram com estudos prévios mostrando que a injecção icv de 2 nmol de oligômeros do Aβ1-42 também levou a diminuição de desempenho nos testes de alternação espontânea e de reconhecimento de objetos após um período de 15 dias (Cunha et al., 2008; Canas et al., 2009). O teste de alternação espontânea tem sido utilizado para a avaliação da memória espacial de curta duração onde se podem analisar alterações nos processos colinérgicos hipocampais (Hughes, 2004). O teste de reconhecimento de objetos constitui um teste de memória não-espacial utilizado para analisar memória de curta e longa duração a qual traduz alterações do lobo temporal medial, principalmente no córtex perirrinal e no hipocampo (Winters and Bussey, 2005; Furini et al., 2010). O processo de reconhecimento é composto por pelo menos dois componentes, um é o julgamento de familiaridade de itens e o outro é a lembrança da informação contextual onde os itens foram encontrados. Alguns estudos têm sugerido que as diferentes regiões do lobo temporal possuem funções distintas no processo de consolidação da memória de reconhecimento, enquanto o córtex perirrinal está envolvido com a discriminação de familiaridade o hipocampo parece estar envolvido com o contexto em que elas ocorrem (Winters et al., 2004; Balderas et al., 2008). Esta tarefa é particularmente útil para estudar as memórias declarativas em roedores, pois se baseia em sua preferência inata por objetos novos sobre objetos familiares (Clarke et al., 2010). Visto que as alterações no desempenho destes dois testes comportamentais são dependentes de regiões cerebrais que constituem os primeiros alvos do peptídeo Aβ, dessa maneira podem fornecer uma medida sensível

das alterações cognitivas iniciais da DA (Dodart et al., 2002; Sipos et al., 2007; Francis et al., 2012).

Considerando que os efeitos da curcumina sobre o dano cognitivo induzido pelo peptídeo Aβ em animais foram pouco explorados até o momento, decidimos avaliar o efeito da administração de curcumina sobre o dano cognitivo induzido pela injeção icv do peptídeo Aβ1-42 em ratos *Wistar*. Em nossos estudos iniciais utilizamos a dose de 10 mg/kg/dia de curcumina em suspensão (i.p.) por 10 dias consecutivos, e nestas condições, não observamos nenhum efeito benéfico da nossa droga sobre os parâmetros comportamentais testados nos ratos infundidos com o peptídeo Aβ1-42. Embora um significativo número de evidências demonstre o elevado potencial terapêutico da curcumina na DA, sua aplicação em estudos *in vivo* e na clínica são limitadas pelas baixas concentrações plasmáticas atingidas após sua administração. A baixa biodisponibiladade da curcumina é atribuída a uma baixa absorção no trato gastrointestinal, rápida biotransformação e uma rápida eliminação sistêmica. Além disso, sua natureza lipofílica conferindo pequena solubilidade em soluções aquosas também é considerada um fator importante para a sua baixa biodisponibilidade (Sharma et al., 2004; Yan et al., 2011). Com o objetivo de aumentar a biodisponibilidade da curcumina estão sendo realizados estudos envolvendo a síntese de análogos, a utilização conjunta com inibidores do metabolismo da curcumina, como a piperina, ou o desenvolvimento de novas formulações como as nanopartículas, micelas, dispersões sólidas (Anand et al., 2007; Aggarwal and Harikumar, 2009; Ray et al., 2011). Nesse estudo optamos por uma alternativa nanotecnológica para minimizar as limitações relativas a biodisponibilidade da droga, e para tanto utilizamos nanocápsulas poliméricas de curcumina (Cur-LNC) revestidas com polissorbato. Esta estratégia de revestimento tem sido usada para aumentar a disponibilidade das nanocápsulas ao SNC

(Su et al., 2010; Benvegnú et al., 2012). De modo interessante, seguindo o modelo de tratamento de 10 dias consecutivos de injeção i.p. iniciados após 4 dias da infusão do peptídeo Aβ, observamos que a dose de 2,5 mg/kg/dia de Cur-LNC foi capaz de reduzir os danos cognitivos induzidos pelo peptídeo Aβ enquanto que uma dose 20 vezes maior de curcumina livre (50 mg/kg/dia) foi necessária para os mesmos efeitos serem observados em nosso modelo (Figura 1 e 2 – Capítulo IV).

Na investigação dos possíveis mecanismos envolvidos nos efeitos benéficos observados com os tratamentos com curcumina, confirmamos em nosso modelo *in vivo* o envolvimento de importantes alterações já observadas em nosso modelo *in vitro* e anteriormente explanadas no Capítulo I desta tese, como o envolvimento da alteração da proteína sinaptofisina (Figura 3 - Capítulo IV), da ativação glial e produção de citocinas pró-inflamatórias (Figura 5 - Capítulo IV), bem como, da modulação das proteínas Akt e GSK-3β (Figura 7 - Capítulo IV), nos quais o tratamento com uma baixa dose de Cur-LNC apresentou resultados similares de proteção das alterações observadas no hipocampo do que a alta dose de curcumina livre efetiva em nosso modelo. Além disso, neste estudo observamos o envolvimento de um importante fator trófico, o fator neurotrófico derivado do encéfalo (BDNF), na neuroproteção mediada pela curcumina contra o dano cognitivo induzido pelo peptídeo Aβ (Figura 6 - Capítulo IV). Este é um resultado interessante, visto que estudos prévios já demonstraram que o BDNF é um importante fator para a formação da memória, além de atuar na manutenção da plasticidade e da sobrevivência neuronal através da ativação de importantes vias de sinalização, entre elas a via PI3K/Akt (Yoshii et al., 2007; Jantas et al., 2009). Este efeito da curcumina sobre a regulação do BDNF demonstrado pela primeira vez em um modelo de toxicidade induzida pelo peptídeo Aβ neste estudo, está de acordo com o efeito da curcumina observado em alguns modelos de depressão em roedores, nos quais

a curcumina parece regular positivamente os níveis deste fator neurotrófico no hipocampo (Huang et al., 2011; Zhang et al., 2012; Hurley et al., 2013).

Outro dado interessante encontrado neste estudo foi a restauração dos níveis de fosforilação da proteína tau através de ambos os tratamentos com curcumina utilizados neste estudo (Figura 4 - Capítulo IV). Este efeito da curcumina pode ser explicado pela sua capacidade de regular a proteína GSK-3β, inibindo a sua ativação induzida pelo peptídeo Aβ e, desta maneira, pode atuar prevenindo o aumento da fosforilação da tau, visto que a GSK-3β é a principal proteína quinase responsável pela fosforilação da proteína tau (Ryder et al., 2004; Gong et al., 2006).

Os dados apresentados neste último Capítulo reforçam alguns dos mecanismos envolvidos no efeito neuroprotetor da curcumina contra a toxicidade induzida pelo peptídeo Aβ *in vitro* encontrados no primeiro Capítulo desta tese, além de ampliarem o conhecimento dos mecanismos envolvidos nesta neuroproteção. Tomados em conjunto, os resultados obtidos neste modelo *in vivo* da Doença de Alzheimer não somente confirmam o potencial da curcumina como um importante agente contra os danos cognitivos induzidos pelo peptídeo Aβ como também sugerem o nanoencapsulamento desta droga como uma estratégia eficiente para aumentar seu efeito farmacológico no tratamento de doenças do SNC.

8. CONCLUSÕES

Esta tese apresentou os resultados obtidos no estudo dos efeitos da curcumina sobre parâmetros moleculares, eletrofisiológicos e comportamentais em modelos experimentais da Doença de Alzheimer. Como importantes contribuições para o tema de pesquisa desenvolvido nesta tese destacam-se:

- O envolvimento da via de sinalização celular PI3K/Akt e o bloqueio do seu substrato GSK-3β na neuroproteção mediada pela curcumina, observados tanto no modelo *in vitro* quanto no modelo *in vivo* de toxicidade desencadeada pelo peptídeo Aβ (Capítulo I e IV);
- A capacidade da curcumina em reduzir a ativação astrocitária e microglial induzida pelo peptídeo Aβ, e desta forma, modular a produção de citocinas, importantes moléculas envolvidas na sinalização celular (Capítulo I e IV);
- Este foi o primeiro trabalho a verificar o envolvimento da SUMO-1 na reatividade astrocitária induzida pelo peptídeo Aβ, bem como a regulação da sumoilação pela curcumina em astrócitos (Capítulo II);
- A capacidade da curcumina em prevenir a disfunção sináptica induzida pelo peptídeo Aβ através da regulação das proteínas sinápticas CaMKII e sinapsina I (Capítulo III);
- O tratamento com curcumina nanoencapsulada apresentou eficácia com uma dose 20 vezes menor quando comparada com o tratamento com curcumina livre sobre a diminuição de desempenho nos testes comportamentais induzida pelo Aβ, demonstrando ser uma eficiente ferramenta para superar as limitações intrínsicas da curcumina (Capítulo IV);
- A curcumina parece mediar este efeito através da regulação dos níveis de BDNF, neurotrofina envolvida no processo de formação da memória e na ativação da via PI3K/Akt (Capítulo IV).

A sequência de trabalhos apresentados nesta tese permitiu aprofundar o conhecimento sobre o efeito neuroprotetor da curcumina contra a toxicidade induzida pelo peptídeo beta-amiloide, considerando os benefícios e as limitações do uso de modelos translacionais na pesquisa científica envolvendo a Doença de Alzheimer e o desenvolvimento de fármacos para a intervenção desta doença.

9. PERSPECTIVAS

Como continuação deste trabalho, pretende-se trabalhar com os seguintes objetivos:

- Avaliar o envolvimento do fator de transcrição CREB na neuroproteção mediada pela curcumina nos modelos de cultura organotípica de hipocampo de ratos expostas ao peptídeo Aβ e no modelo i*n vivo* de injeção icv do peptídeo Aβ;

- Testar a hipótese que a injeção de LY294002 bloqueia os efeitos benéficos da curcumina contra os danos cognitivos induzidos pela injeção do peptídeo Aβ;

- Determinar através de microscopia confocal a disponibilidade de nanocápsulas de curcumina e curcumina livre no cérebro dos ratos.

10. BIBLIOGRAFIA

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997). β-Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J*, 16: 3797-3804.

Aggarwal BB, Harikumar KB (2009). Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int J Biochem Cell Biol*, 4: 40-59.

Agostinho P, Cunha RA, Oliveira C (2010). Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. *Curr Pharm Des*, 16: 2766-78.

Agrawal R, Mishra B, Tyagi E, Nath C, Shukla R (2010). Effect of curcumin on brain insulin receptors and memory functions in STZ (ICV) induced dementia model of rat. *Pharmacol Res,* 61: 247-52.

Ahmed T, Enam SA, Gilani AH (2010). Curcuminoids enhance memory in an amyloid-infused rat model of Alzheimer's disease. *Neuroscience*, 169, 1296-306.

Akar CA, Feinstein DL (2009). Modulation of inducible nitric oxide synthase expression by sumoylation. *J Neuroinflamm*, 6: 12.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, et al. (2000). Inflammation and Alzheimer's disease. *Neurobiol Aging*, 21:383-421.

Alzheimer A (1907). Uber eigenartige Erkrankung der Hirnrinde. *Allg Z Pzychiat Psych Gerichtl Med*, 64: 146-148.

Alzheimer A (1911). Uber eigenartige Krankheitsfälle des späteren Alters. *Zbl Ges Neurol Psych*, 4: 356-385.

Alzheimer's Association, 2011 (2011). Alzheimer's Disease Facts and Figures, *Alzheimer's & Dementia* 7: 1-63.

Ambruosi A, Khalansky AS, Yamamoto H, Gelperina SE, Begley DJ, Kreuter J (2006). Biodistribution of polysorbate 80-coated doxorubicin-loaded [14C]-poly(butyl cyanoacrylate) nanoparticles after intravenous administration to glioblastoma-bearing rats. *J Drug Target*, 14: 97-105.

Anderson DB, Wilkinson KA, Henley JM (2009). Protein SUMOylation in neuropathological conditions. *Drug News Perspect*, 22: 255–265.

Anisman H (2009). Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. *J Psychiatry Neurosci,* 34: 4-20.

Avila J, de Barreda EG, Pallas-Bazarra N, Hernandez F (2013). Tau and neuron aging. *Aging Dis*, 4:23-8.

Balderas I, Rodriguez-Ortiz CJ, Salgado-Tonda P, Chavez-Hurtado J, McGaugh JL, Bermudez-Rattoni F (2008). The consolidation of object and context recognition memory involve different regions of the temporal lobe. *Learn Mem*, 15: 618-24.

Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E (2011). Alzheimer's disease. *Lancet*, 377: 1019-31.

Barnes PJ (2001). Cytokine modulators as novel therapies for airway disease. *Eur Respir J Suppl*, 34: 67-77.

Barrat G (2003). Colloidal drug carriers: achievements and perspectives. *Cel Mol Life Sci*, 60: 21-37.

Baum L, Lam CW, Cheung SK, Kwok T, Lui V, Tsoh J, Lam L, Leung V, Hui E, Ng C, Woo J, Chiu HF, Goggins WB, Zee BC, Cheng KF, Fong CY, Wong A, Mok H, Chow MS, Ho PC, Ip SP, Ho CS, Yu XW, Lai CY, Chan MH, Szeto S, Chan IH, Mok V (2008). Six-month randomized, placebo-controlled, double-blind, pilot clinical trial of curcumin in patients with Alzheimer disease. *J Clin Psychopharmacol*, 28: 110-3.

Begum AN, Jones MR, Lim GP, Morihara T, Kim P, Heath DD, Rock CL, Pruitt MA, Yang F, Hudspeth B, Hu S, Faull KF, Teter B, Cole GM, Frautschy SA (2008). Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J Pharmacol Exp Ther*, 326: 196-208.

Belarbi K, Jopson T, Tweedie D, Arellano C, Luo W, Greig NH, Rosi S (2012). TNF-α protein synthesis inhibitor restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. *J Neuroinflammation*, 9: 23.

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

Benvegnú DM, Barcelos RC, Boufleur N, Pase CS, Reckziegel P, Flores FC, Ourique AF, Nora MD, da Silva Cde B, Beck RC, Bürger ME (2012). Haloperidol-loaded polysorbate coated polymeric nanocapsules decrease its adverse motor side effects andoxidative stress markers in rats. *Neurochem Int*, 61: 623-31.

Bernardi A, Frozza RL, Meneghetti A, Hoppe JB, Battastini AM, Pohlmann AR, Guterres SS, Salbego CG (2012). Indomethacin-loaded lipid-core nanocapsules reduce the damage triggered by Aβ1-42 in Alzheimer's disease models. *Int J Nanomedicine*, 7: 4927-42.

Besl B, Fromherz P (2002). Transistor array with an organotypic brain slice: field potential records and synaptic currents. *Eur J Neurosci*, 15: 999-1005.

Beurel E, Jope RS (2009). Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J Neuroinflamm*, 6: 9.

Blennow K, De Leon MJ, Zetterberg H (2006). Alzheimer's disease. *Lancet,* 368: 387-403.

Bliss TV, Collingridge GL (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361: 31-9.

Block ML, Zecca L, Hong JS (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci*, 8: 57-69.

Blurton-Jones M, Laferla FM (2006). Pathways by which Abeta facilitates tau pathology. *Curr Alzheimer Res*, 3: 437-48.

Broadbent NJ, Squire LR, Clark RE (2004). Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci U S A*, 101: 14515-20.

Bruce AJ, Malfroy B, Baudry M (1996). β-amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic catalytic free radical scavenger. *Proc Nati Acad Sci USA*, 93: 2312-2316.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, 96: 857-68.

Brunet A, Datta SR, Greenberg ME (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol*, 11: 297-305.

Budson AE, Price BH (2005). Memory dysfunction. *N Engl J Med*, 352: 692-9.

Cagnin A, Brooks DJ, Kennedy AM, Gunn RN, Myers R, Turkheimer FE, Jones T, Banati RB (2001). In-vivo measurement of activated microglia in dementia. *Lancet*, 358: 461-7.

Cakała M, Malik AR, Strosznajder JB (2007). Inhibitor of cyclooxygenase-2 protects against amyloid beta peptide-evoked memory impairment in mice. *Pharmacol Rep*, 59: 164-72.

Canas PM, Porciúncula LO, Cunha GMA, Silva CG, Machado, NJ, Oliveira JMA, Oliveira CR, Cunha RA (2009). Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogenactivated protein kinase pathway. *J Neurosci*, 29: 14741-51.

Caricasole A, Copani A, Caruso A, Caraci F, Iacovelli L, Sortino MA, Terstappen GC, Nicoletti F (2003). The Wnt pathway, cell-cycle activation and β-amyloid: novel therapeutic strategies in Alzheimer's disease? *TRENDS Pharmacol Sc*i, 24: 233-238.

Casserly I, Topol E (2004). Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded protein. *Lancet***,** 363: 1139-46.

Chao MV (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci*, 4: 299-309.

Chao MV, Rajagopal R, Lee FS (2006). Neurotrophin signalling in health and disease. Clin Sci (Lond), 110: 167-73.

Chen C, Johnston TD, Jeon H, Gedaly R, McHugh PP, Burke TG, Ranjan D (2009). An in vitro study of liposomal curcumin: Stability, toxicity and biological activity in human lymphocytes and Epstein-Barr virus-transformed human B-cells. *Int J Pharm*, 366: 133-139.

Chen Q, Prior M, Dargusch R, Roberts A, Riek R, Eichmann C, Chiruta C, Akaishi T, Abe K, Maher P, Schubert D (2011). A novel neurotrophic drug for cognitive enhancement and Alzheimer's disease. *PLoS One*, 6: e27865.

Chiang HC, Wang L, Xie Z, Yau A, Zhong Y (2010). PI3 kinase signaling is involved in Abeta-induced memory loss in Drosophila. *Proc Natl Acad Sci U S A*, 107: 7060-5.

Chong ZZ, Li F, Maiese K (2007) Cellular demise and inflammatory microglial activation during beta-amyloid toxicity are governed by Wnt1 and canonical signaling pathways. *Cell Signal*, 19: 1150-62.

Chong SA, Benilova I, Shaban H, De Strooper B, Devijver H, Moechars D, Eberle W, Bartic C, Van Leuven F, Callewaert G (2011). Synaptic dysfunction in

hippocampus of transgenic mouse models of Alzheimer's disease: a multi-electrode array study. *Neurobiol Dis*, 44: 284-91.

Cimarosti H, Ashikaga E, Jaafari N, Dearden L, Rubin P, Wilkinson KA, Henley JM (2012). Enhanced SUMOylation and SENP-1 protein levels following oxygen and glucose deprivation in neurons. *J Cereb Blood Flow Metab*, 32: 17-22.

Citron M (2010). Alzheimer's disease: strategies for disease modification. *Nat Rev Drug Discov*, 9: 387-98.

Clarke JR, Cammarota M, Gruart A, Izquierdo I, Delgado-García JM (2010). Plastic modifications induced by object recognition memory processing. *Proc Natl Acad Sci U S A*, 107: 2652-7.

Corona C, Pensalfini A, Frazzini V, Sensi SL (2011) New theratpeutic targets in Alzheimer's disease: brain deregulation of calcium and zinc. *Cell Death Dis*, 2: e176.

Couvreur P, Barratt G, Fattal E, Legrand P, Vauthier C (2002). Nanocapsule technology: a review. *Crit Rev Ther Drug Carrier Sys*t, 19: 99-134.

Craggs L, Kalaria RN (2011). Revisiting dietary antioxidants, neurodegeneration and dementia. *Neuroreport*, 22: 1-3.

Crespo-Biel N, Canudas A, Pallàs M (2007). Kainate induces AKT, ERK and cdk5/GSK3β pathway deregulation, phosphorylates tau protein in mouse hippocampus. *Neurochem Int,* 50: 435-442.

Crews L, Masliah E (2010). Molecular mechanisms of neurodegeneration in Alzheimer's disease. *Hum Mol Genet*, 19: 12-20.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (2001). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, 378: 785-9.

Cunha GM, Canas PM, Melo CS, Hockemeyer J, Müller CE, Oliveira CR, Cunha RA (2008). Adenosine A2A receptor blockade prevents memory dysfunction caused by β-amyloid peptides but not by scopolamine or MK-801. *Exp Neurol,* 210: 776-781.

Dagvadorj J, Naiki Y, Tumurkhuu G, Noman AS, Iftekar-E-Khuda I, Koide N, Komatsu T, Yoshida T, Yokochi T (2009). Interleukin (IL)-10 attenuates lipopolysaccharide-induced IL-6 production via inhibition of IkappaB-zeta activity by Bcl-3. *Innate Immun*, 15: 217-224.

Decker H, Jürgensen S, Adrover MF, Brito-Moreira J, Bomfim TR, Klein WL, Epstein AL, De Felice FG, Jerusalinsky D, Ferreira ST (2010). N-methyl-D-aspartate receptors are required for synaptic targeting of Alzheimer's toxic amyloid-β peptide oligomers. *J Neurochem*, 115: 1520-9.

De Ferrari GV, Chacón MA, Barría MI, Garrido JL, Godoy JA, Olivares G, Reyes AE,Alvarez A, Bronfman M, Inestrosa NC (2003). Activation of Wnt signaling rescues neurodegeneration and behavioral impairments induced by β-amyloid fibrils. *Mol Psychiatry*, 8: 195-208.

de Oliveira AC, Candelario-Jalil E, Langbein J, Wendeburg L, Bhatia HS, Schlachetzki JC, Biber K, Fiebich BL (2012). Pharmacological inhibition of Akt and downstream pathways modulates the expression of COX-2 and mPGES-1 in activated microglia. *J Neuroinflammation*, 9: 2.

Dewachter I, Ris L, Jaworski T, Seymour CM, Kremer A, Borghgraef P, De Vijver H, Godaux E, Van Leuven F (2009). GSK3beta, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at serine-9. *Neurobiol Dis*, 35: 193-200.

Dinarello CA (2000). Proinflammatory cytokines. *Chest*, 118: 503-8.

Doble BW, Woodgett JR (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci*, 116: 1175-86.

Dodart JC, Mathis C, Bales KR, Paul SM (2002). Does my mouse have Alzheimer's disease? *Genes Brain Behav*, 1: 142-155.

Dorval V, Fraser PE (2007). SUMO on the Road to neurodegeneration. *Biochim Biophys Acta*, 1773: 694-706.

Dorval V, Mazzella MJ, Mathews PM, Hay RT, Fraser PE (2007). Modulation of Abeta generation by small ubiquitin-like modifiers does not require conjugation to target proteins. *Biochem J*, 404: 309–316.

Duman RS, Voleti B (2012). Signaling pathways underlying the pathophysiology and treatment of depression: novel mechanisms for rapid-acting agents. *Trends Neurosci*, 35: 47-56.

Eckert A, Hauptmann S, Scherping I, Meinhardt J, Rhein V, Dröse S, Brandt U, Fändrich M, Müller WE, Götz J (2008). Oligomeric and fibrillar species of β-

amyloid (Aβ42) both impair mitochondrial function in P301L tau transgenic mice. *J Mol Med,* 86: 1255-1267.

Faraji AH, Wipf P (2009). Nanoparticles in cellular drug delivery. *Bioorg Med Chem*, 17: 2950-62.

Feligioni M, Brambilla E, Camassa A, Sclip A, Arnaboldi A, Morelli F, Antoniou X, Borsello T (2011). Crosstalk between JNK and SUMO Signaling Pathways:deSUMOylation Is Protective against H2O2-Induced Cell Injury. *Plos One*, 6: 1-9.

Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M (2005). Global prevalence of dementia: a Delphi consensus study. *Lancet*, 366: 2112-17.

Ferrís-Tortajada J, Berbel-Tornero O, García-Castell J, Ortega-García JA, López-Andreu JA (2012). Dietetic factors associated with prostate cancer: protective effects of Mediterranean diet. *Actas Urol Esp*, 36: 239-45.

Forman MS, Trojanowski JQ, Lee VM (2004). Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. *Nat Med,* 10: 1055-1061.

Francis BM, Kim J, Barakat ME, Fraenkl S, Yücel YH, Peng S, Michalski B, Fahnestock M, McLaurin J, Mount HT (2012). Object recognition memory and BDNF expression are reduced in young TgCRND8 mice. *Neurobiol Aging*, 33: 555-63.

Fromherz P (2002). Electrical interfacing of nerve cells and semiconductor chips. *Chemphyschem*, 3: 276-84.

Frotscher M, Zafirov S, Heimrich B (1995). Development of identified neuronal types and of specific synaptic connection in slice cultures of rat hippocampus. *Prog Neurobiol*, 45: 143-164.

Frozza RL, Horn AP, Hoppe JB, Simão F, Gerhardt D, Comiran RA, Salbego CG (2009). A comparative study of beta-amyloid peptides Abeta1-42 and Abeta25-35 toxicity in organotypic hippocampal slice cultures. *Neurochem Res*, 34: 295-303.

Frozza RL, Bernardi A, Hoppe JB, Meneghetti AB, Matté A, Battastini AM, Pohlmann AR, Guterres SS, Salbego C. Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-Core Nanocapsules. *Mol Neurobiol, in press.*

Fuentealba RA, Farias G, Scheu J, Bronfman M, Marzolo MP, Inestrosa NC (2004). Signal transduction during amyloid-β-peptide neurotoxicity: role in Alzheimer disease. *Brain Res Rev,* 47: 275-289.

Furini CR, Rossato JI, Bitencourt LL, Medina JH, Izquierdo I, Cammarota M (2010). Beta-adrenergic receptors link NO/sGC/PKG signaling to BDNF expression during the consolidation of object recognition long-term memory. *Hippocampus*, 20: 672-83.

Ganguli M, Chandra V, Kamboh MI, Johnston JM, Dodge HH, Thelma BK, Juyal RC, Pandav R, Belle SH, DeKosky ST (2000). Apolipoprotein E polymorphism and Alzheimer's disease: the Indo-US Cross-National Dementia Study. *Arch Neurol*, 57: 824-830.

Gareau JR, Lima CD (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol*, 11: 861-871.

Garrido JL, Godoy J, Alvarez A, Bronfman M, Inestrosa NC (2002). Protein kinase C inhibits amyloid β-peptide neurotoxicity by acting on members of the Wnt pathway. *FASEB J*, 14: 1982-1984.

Garwood CJ, Pooler AM, Atherton J, Hanger DP, Noble W (2011). Astrocytes are important mediators of Aβ-induced neurotoxicity and tau phosphorylation in primary culture. *Cell Death Dis*, 2: e167.

Geiss-Friedlander R, Melchior F (2007). Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol,* 8: 947-956.

Gibertini M, Newton C, Friedman H, Klein TW (1995). Spatial learning impairment in mice infected with Legionella pneumophila or administered exogenous interleukin-1-beta. *Brain Behav Immun*, 9: 113-28.

Goedert M, Klug A, Crowther RA (2006). Tau protein, the paired helical filament and Alzheimer's disease. *J Alzheimers Dis*, 9: 195-207.

Goedert M, Spillantini MG (2006). A Century of Alzheimer's disease. *Science,* 314: 777-780.

Golde TE (2007). The Pathogenesis of Alzheimer's disease and the role of Aβ42. *CNS Spectr,* 12: 4-6.

Gómez-Pinilla F, Huie JR, Ying Z, Ferguson AR, Crown ED, Baumbauer KM, Edgerton VR, Grau JW (2007). BDNF and learning: Evidence that

instrumental training promotes learning within the spinal cord by up-regulating BDNF expression. *Neuroscience*, 148: 893-906.

Gong CX, Liu F, Grundke-Iqbal I, Iqbal K (2006). Dysregulation of protein phosphorylation/dephosphorylation in Alzheimer's disease: a therapeutic target. *Journal of Biomed Biotechnol,* 2006: 1-11.

Granger AJ, Shi Y, Lu W, Cerpas M, Nicoll RA (2013). LTP requires a reserve pool of glutamate receptors independent of subunit type. *Nature*, 493: 495-500.

Gavillet M, Allaman I, Magistretti P (2008). Modulation of astrocytic metabolic phenotype by proinflammatory cytokines. *Glia,* 56: 975-989.

Green HF, Nolan YM (2012). GSK-3 mediates the release of IL-1b, TNF-a and IL-10 from cortical glia. *Neurochem Int,* 61: 666-71.

Gureviciene I, Ikonen S, Gurevicius K, Sarkaki A, van Groen T, Pussinen R, Ylinen A, Tanila H (2004). Normal induction but accelerated decay of LTP in APP+PS1 transgenic mice. *Neurobiol Dis*, 15: 188–195.

Guterres SS, Alves MP, Pohlmann AR (2007). Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. *Drug Target Insights*, 2: 147-57.

Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB, Selkoe DJ (1992). Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature*, 359: 322-325.

Haass C, Selkoe DJ (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide. *Nature***,** 8: 101-112.

Haddad JJ (2002). Cytokines and related receptor-mediated signaling pathways. *Biochem Biophys Res Commun,* 297: 700-13.

Hardy J, Selkoe DJ (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the Road to therapeutics. *Science***,** 297: 353-356.

Harvey AL (2008). Natural products in drug discovery. *Drug Discov Today*, 13: 894-901.

Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. *Cell Mol Life Sci*, 65:1631-52.

Hayashi T, Seki M, Maeda D, Wang W, Kawabe Y, Seki T, Saitoh H, Fukagawa T, Yagi H, Enomoto T (2002). Ubc9 is essential for viability of higher eukaryotic cells. *Exp Cell Res*, 280: 212–221.

Heneka MT, O'Banion MK (2007). Inflammatory processes in Alzheimer's disease. J Neuroimmunol, 184: 69-91.

Hernández F, Gómez de Barreda E, Fuster-Matanzo A, Lucas JJ, Avila J (2010). GSK3: a possible link between beta amyloid peptide and tau protein. *Exp Neurol*, 223: 322-5.

Herrera F, Maher P, Schubert D (2012). c-Jun N-terminal kinase controls a negative loop in the regulation of glial fibrillary acidic protein expression by retinoic acid. *Neuroscience*, 208: 143-9.

Herz J, Beffert U (2000). Apolipoprotein E receptors: linking brain development and Alzheimer's disease. *Nat Neurosci,* 1: 51-58.

Hofmann F, Bading H (2006). Long term recordings with microelectrode arrays: Studies of transcription-dependent neuronal plasticity and axonal regeneration. *J Physiol*, 99: 125–132.

Holopainen IE (2005). Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal death, neuroprotection, and synaptic plasticity. *Neurochem Res,* 30: 1521-1528.

Hooper C, Killick R, Lovestone S (2008). The GSK3 hypothesis of Alzheimer's disease. J Neurochem, 104: 1433-9.

Hopkins SJ (2003). The pathophysiological role of cytokines. *Leg Med (Tokyo),* 5: S45-57.

Hoppe JB, Frozza RL, Horn AP, Comiran RA, Bernardi A, Campos MM, Battastini AM, Salbego C (2010). Amyloid-beta neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3beta, tau and neuroinflammation. *J Pineal Res*, 48: 230-8.

Horn AP, Gerhardt D, Geyer AB, Valentim L, Cimarosti H, Tavares A, Horn F, Lenz G, Salbego C (2005). Cellular death in hippocampus in response to PI3K pathway inhibition and oxygen and glucose deprivation. *Neurochem Res*, 30: 355-61.

Horwood JM, Dufour F, Laroche S, Davis S (2006). Signalling mechanisms mediated by the phosphoinositide 3-kinase/Akt cascade in synaptic plasticity and memory in the rat. *Eur J Neurosci*, 23: 3375-84.

Hou L, Liu Y, Wang X, Ma H, He J, Zhang Y, Yu C, Guan W, Ma Y (2011). The effects of amyloid-β42 oligomer on the proliferation and activation of astrocytes in vitro. *In Vitro Cell Dev Biol Anim*, 47: 573-80.

Huang HC, Chang P, Dai XL, Jiang ZF (2012). Protective effects of curcumin on amyloid-β-induced neuronal oxidative damage. *Neurochem Res*, 37: 1584-97.

Huang Z, Zhong XM, Li ZY, Feng CR, Pan AJ, Mao QQ (2011). Curcumin reverses corticosterone-induced depressive-like behavior and decrease in brain BDNF levels in rats. *Neurosci Lett*, 493: 145-8.

Huges RN (2004). The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neurosci Biobehav*, 28: 497–505.

Hurley LL, Akinfiresoye L, Nwulia E, Kamiya A, Kulkarni AA, Tizabi Y (2013). Antidepressant-like effects of curcumin in WKY rat model of depression is associated with an increase in hippocampal BDNF. *Behav Brain Res,* 15: 27-30.

Inestrosa NC, Varela-Nallar L, Grabowski CP, Colombres M (2007). Synaptotoxicity in Alzheimer's disease: the Wnt signaling pathway as a molecular target. *IUBMB Life,* 59: 316-21.

Ishige K, Takagi N, Imai T, Rausch WD, Kosuge Y, Kihara T, Kusama-Eguchi K, Ikeda H, Cools AR, Waddington JL, Koshikawa N, Ito Y (2007). Role of Caspase-12 in Amyloid β-peptide-induced toxicity in organotypic hippocampal slices cultured for long periods. *J Pharmacol Sci,* 104: 46-55.

Ittner LM, Götz J (2011). Amyloid-β and tau--a toxic pas de deux in Alzheimer's disease. *Nat Rev Neurosci,* 12: 65-72.

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.

Jantas D, Szymanska M, Budziszewska B, Lason W (2009). An involvement of BDNF and PI3-K/Akt in the anti-apoptotic effect of memantine on staurosporine-evoked celldeath in primary cortical neurons. *Apoptosis*, 14: 900-12.

Jaques JA, Doleski PH, Castilhos LG, da Rosa MM, Souza Vdo C, Carvalho FB, Marisco P, Thorstenberg ML, Rezer JF, Ruchel JB, Coradini K, Beck RC, Rubin MA, Schetinger MR, Leal DB (2013). Free and nanoencapsulated curcumin prevents cigarrete smoke-induced cognitive impairment and redox imbalance. *Neurobiol Learn Mem*, 100: 98-107.

Jarrard LE (1993). On the role of the hippocampus in learning and memory in the rat. *Behav Neural Biol*, 60: 9-26.

Jhamandas JH, Cho C, Jassar B, Harris K, MacTavish D, Easaw J (2001) Cellular mechanisms for amyloid beta-protein activation of rat cholinergic basal forebrain neurons. *J Neurophysiol*, 86: 1312–20.

Jimenez S, Torres M, Vizuete M, Sanchez-Varo R, Sanchez-Mejias E, Trujillo-Estrada L, Carmona-Cuenca I, Caballero C, Ruano D, Gutierrez A, Vitorica J (2011). Age-dependent accumulation of solubre beta (Abeta) oligomers reverses neuroprotectvive effect of soleble amyloid precursor protein-alpha (sAPP(alpha)) by modulating phosphatidylinositol 3-kinase (PI3K)/Akt-GSK-3beta pathway in Alzheimer mouse model. *J Biol Chem*, 286: 18414-25.

Jung DR, Cuttino DS, Pancrazio JJ, Manos P, Cluster T (1998) Cell-based sensor microelectrode array characterized by imaging x-ray photoelectron spectroscopy, scanning electron microscopy, impedance measurements, and extracellular recordings. *J Vac Sci Technol A*, 16: 1183–1188.

Jurgens HA, Johnson RW (2012). Dysregulated neuronal-microglial cross-talk during aging, stress and inflammation. *Exp Neurol*, 233: 40-8.

Kelsey NA, Wilkins HM, Linseman DA (2010). Nutraceutical antioxidants as novel neuroprotective agents. *Molecules,* 15: 7792-814.

Kim DS, Park SY, Kim JK (2001). Curcuminoids from Curcuma longa L. (Zingiberaceae) that protect PC12 rat pheochromocytoma and normal human umbilical vein endothelial cells from betaA(1-42) insult. *Neurosci Lett*, 303: 57-61.

Kim D, Chung J (2002). Akt: versatile mediator of cell survival and beyond. *J Biochem Mol Biol*, 35: 106-15.

Knowles RB, Wyart C, Buldyrev SV, Cruz L, Urbanc B, Hasselmo ME, Stanley HE, Hyman BT (1999). Plaque-induced neurite abnormalities: implications for disruption of neural networks in Alzheimer's disease. *Proc Natl Acad Sci USA*, 96: 5274-5279.

Koffie RM, Meyer-Luehmann M, Hashimoto T, Adams KW, Mielke ML, Garcia-Alloza M, Micheva KD, Smith SJ, Kim ML, Lee VM, Hyman BT, Spires-Jones TL (2009). Oligomeric amyloid b associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. *Proc Natl Acad Sci USA*, 106: 4012-4017.

Koffie RM, Hyman BT, Spires-Jones TL (2011). Alzheimer's disease: synapses gone cold. *Mol Neurodegener*, 6: 63.

Ksiezak-Reding H, Pyo HK, Feinstein B, Pasinetti GM (2003). Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. *Biochim Biophys Acta*, 1639: 159-68.

LaFerla FM, Green KN, Oddo S (2007). Intracellular amyloid-β in Alzheimer's disease. *Nat Neurosci,* 8: 499-509.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA,Klein WL (1998). Diffusible, nonfibrillar ligands derived from A beta(1–42) are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A*, 95: 6448–53.

Lavoie S, Chen Y, Dalton TP, Gysin R, Cuenod M, Steullet P, Do KQ (2009). Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit. *J Neurochem*, 108: 1410-22.

Lawlor MA, Alessi DR (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci*, 114: 2903-10.

Lee KY, Koh SH, Noh MY, Kim SH, Lee YJ (2008). Phosphatidylinositol-3-kinase activation blocks amyloid beta-induced neurotoxicity. *Toxicology*, 243: 43-50.

Lee SM, Tole S, Grove E, McMahon AP (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. *Development*, 127: 457-67.

Lee YS, Jang MS, Lee JS, Choi EJ, Kim E (2005). SUMO-1 represses apoptosis signal-regulating kinase 1 activation through physical interaction and not through covalent modification. *EMBO reports*, 6: 949-955.

Lewis S (2012). Neuronal circuits: Mapping the local field potential. *Nat Rev Neurosci*, 13: 75.

Li B, Zhong L, Yang X, Andersson T, Huang M, Tang S-J (2011). WNT5A signaling contributes to Aβ-induced neuroinflammation and neurotoxicity. PlosOne, 6: 1-10.

Li X, Bijur GN, Jope RS (2002). Glycogen synthase kinase-3β, mood stabilizers, and neuroprotection. *Bipolar Disord*, 4: 137-44.

Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM (2001). The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J Neurosci*, 21: 8370-7.

Lindén H, Tetzlaff T, Potjans TC, Pettersen K, Grün S, Diesmann M, Einevoll GT (2011). Modeling the Spatial Reach of the LFP. *Neuron*, 72: 859-72.

Lisman J, Yasuda R, Raghavachari S (2012). Mechanisms of CaMKII action in long-term potentiation. *Nat Rev Neurosci*, 13: 169-82.

Liu MG, Chen XF, He T, Li Z, Chen J (2012). Use of multi-electrode array recordings in studies of network synaptic plasticity in both time and space. *Neurosci Bull,* 28: 409-422.

Lou SJ, Liu JY, Chang H, Chen PJ (2008). Hippocampal neurogenesis and gene expression depend on exercise intensity in juvenile rats. *Brain Res*, 1210: 48-55.

Lu XH, Bradley RJ, Dwyer DS (2004). Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38. *Brain Res*, 1011: 58-68.

Luo XG, Chen SD (2012). The changing phenotype of microglia from homeostasis to disease. *Transl Neurodegener*, 1: 9.

Ly PT, Song W (2011). Loss of activated CaMKII at the synapse underlies Alzheimer's disease memory loss. *J Neurochem*, 119: 673-5.

Ma QL, Yang F, Rosario ER, Ubeda OJ, Beech W, Gant DJ, Chen PP, Hudspeth B, Chen C, Zhao Y, Vinters HV, Frautschy SA, Cole GM (2009). Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin recep tor substrate via c-Jun N-terminal kinase signaling: suppression by omega 3 fatty acids and curcumin. *J Neurosci*, 29: 9078-89.

Ma Z, Haddadi A, Molavi O, Lavasanifar A, Lai R, Samuel J (2008). Micelles of poly(ethylene oxide)-b-poly(epsilon-caprolactone) as vehicles for the solubilization, stabilization, and controlled delivery of curcumin. *J Biomed Mater Res A*, 86: 300-10.

Maheshwari RK, Singh AK, Gaddipati J, Srimal RC (2006). Multiple biological activities of curcumin: a short review. *Life Sci*, 78: 2081-7.

Mandelkow EM, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, Mandelkow E (1992). Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett*, 314: 315-21.

Mattson MP, Chan SL (2003). Neuronal and glial calcium signalin in Alzheimer's disease. *Cell Calcium,* 34: 385-397.

Mattson MP (2004). Pathways towards and away from Alzheimer's disease. *Nature***,** 430: 631-639.

Mattson MP, Magnus T (2006). Ageing and neuronal vulnerability. *Nat Rev Neurosci*, 7: 278-94.

Mattson MP (2008). Glutamate and Neurotrophic Factors in Neuronal Plasticity and Disease. *Annals of the New York Academy of Sciences,* 1144: 97–90.

Mazanetz MP, Fischer PM (2007). Untangling tau hyperphosphorylation in drug design for neurodegenerative disease. *Nat Rev Drug Discov,* 6: 464-479.

McAllister AK, Katz LC, Lo DC (1999). Neurotrophins and synaptic plasticity. *Annu Rev Neurosci*, 22: 295-318.

McGaugh JL (2000). Memory--a century of consolidation. *Science*, 287: 248-51.

McGeer EG, McGeer PL (2010). Neuroinflammation in Alzheimer's disease and mild cognitive impairment: a field in its infancy. *J Alzheimers Dis,* 19: 355-61.

McMillan LE, Brown JT, Henley JM, Cimarosti H (2011). Profiles of SUMO and ubiquitin conjugation in an Alzheimer's disease model. *Neurosci Lett*, 502: 201-208.

Medeiros R, Prediger RDS, Passos GF, Pandolfo P, Duarte FS, Franco JL, Dafre AL, Di Giunta G, Figueiredo CP, Takahashi RN, Campos MM, Calixto JB (2007). Connecting TNF-α signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: relevance for the behavioral and synaptic deficits induced by amyloid β protein. *J Neurosci*, 27: 5394–5404.

Mehan S, Meena H, Sharma D, Sankhla R (2001). JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. *J Mol Neurosci,* 43: 376-390.

Meier W (2000). Polymer nanocapsules. *Chem Society Rev*, 29: 295-303.

Mishra S, Palanivelu K (2008). The effect of curcumin (turmeric) on Alzheimer's disease: An overview. *Ann Indian Acad Neurol*, 11: 13-9.

Miyamoto E (2006). Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *J Pharmacol Sci*, 100: 433-42.

Mizuno S, Iijima R, Ogishima S, Kikuchi M, Matsuoka Y, Ghosh S, Miyamoto T, Miyashita A, Kuwano R, Tanaka H (2012). AlzPathway: a comprehensive map of signaling pathways of Alzheimer's disease. *BMC Syst Biol*, 6: 52.

Möller HJ, Graeber MB (1998). The case described by Alois Alzheimer in 1911. Historical and conceptual perspectives based on the clinical record and neurohistological sections. *Eur Arch Psychiatry Clin Neurosci*, 248: 111-122.

Moon DO, Kim MO, Choi YH, Park YM, Kim GY (2010). Curcumin attenuates inflammatory response in IL-1beta-induced human synovial fibroblasts and collagen-induced arthritis in mouse model. *Int Immunopharmacol*, 10: 605-10.

Motterlini R, Foresti R, Bassi R, Green CJ (2000). Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. *Free Radic Biol Med*, 28: 1303-12.

Mucke L, Selkoe DJ (2012). Neurotoxicity of amyloid beta protein: synaptic and networw dysfunction. *Cold Spring Harb Perspect Med*, 2: a006338.

Muller S, Berger M, Lehembre F, Seeler JS, Haupt Y, Dejean A (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. *J Biol Chem,* 275: 13321–13329.

Murer MG, Yan Q, Raisman-Vozari R (2001). Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol*, 63: 71-124.

Nacerddine K, Lehembre F, Bhaumik M, Artus J, Cohen-Tannoudji M, Babinet C, Pandolfi PP, Dejean A (2005). The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev Cell,* 9: 769–779.

Nassif M, Hoppe J, Santin K, Frozza R, Zamin LL, Simão F, Horn AP, Salbego C (2007). Beta-amyloid peptide toxicity in organotypic hippocampal slice culture involves Akt/PKB, GSK-3beta, and PTEN. *Neurochem Int*, 50: 229-35.

Ng TP, Chiam PC, Lee T, Chua HC, Lim L, Kua EH (2006). Curry consumption and cognitive function in the elderly. *Am J Epidemiol*, 164: 898-906.

Noraberg J, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J (2005). Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Curr Drug Targets CNS Neurol Disor* 4: 435-452.

Opal SM, DePalo VA (2000). Anti-inflammatory cytokines. *Chest*, 117: 1162-72.

Palop JJ, Mucke L (2010) Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat Neurosci*, 13: 812-818.

Pan R, Qiu S, Lu DX, Dong J (2008). Curcumin improves learning and memory ability and its neuroprotective mechanism in mice. *Chin Med J*, 121: 832-9.

Pangalos M, Donoso JR, Winterer J, Zivkovic AR, Kempter R, Maier N, Schmitz D (2013).Recruitment of orients-lacunosum-moleculare interneurons during hippocampal ripples. *Proc Natl Acad Sci USA*, 110: 4398-403.

Pap M, Cooper GM (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem*, 273:19929-32.

Parachikova A, Agadjanyan MG, Cribbs DH, Blurton-Jones M, Perreau V, Rogers J, Beach TG, Cotman CW (2007). Inflammatory changes parallel the early stages of Alzheimer disease. *Neurobiol Aging*, 28: 1821-33.

Park KM, Bowers WJ (2010). Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. *Cell Signal*, 22: 977-83.

Park SY, Kim HS, Cho EK, Kwon BY, Phark S, Hwang KW, Sul D (2008). Curcumin protected PC12 cells against beta-amyloid-induced toxicity through the inhibition of oxidative damage and tau hyperphosphorylation. *Food Chem Toxicol,* 46: 2881-7.

Patel D, Good T (2007). A rapid methods to measure beta-amyloid induced toxicity in vitro. *J Neurosci Methods*, 161: 1-10.

Philipson O, Lord A, Gumucio A, O'Callaghan P, Lannfelt L, Nilsson LN (2010). Animal models of amyloid-beta-related pathologies in Alzheimer's disease. *FEBS J*, 277: 1389-409.

Pi HJ, Otmakhovm N, El Gaamouch F, Lemelin D, De Koninck P, Lisman J (2010) CaMKII control of spine size and synaptic strength: role of phosphorylation states and nonenzymatic action. *Proc Natl Acad Sci USA*, 107: 14437-42.

Pimplikar SW (2009). Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int J Biochem Cell Biol*, 41: 1261-8.

Potter SM, De-Marse TB (2001). A new approach to neural cell culture for long-term studies. *J Neurosci Met*, 110: 17-24.

Prediger RD, Franco JL, Pandolfo P, Medeiros R, Duarte FS, Di Giunta G, Figueiredo CP, Farina M, Calixto JB, Takahashi RN, Dafre AL (2007). Differential

susceptibility following beta-amyloid peptide-(1-40) administration in C57BL/6 and Swiss albino mice: Evidence for a dissociation between cognitive deficits and the glutathione system response. *Behav Brain Res*, 177: 205-13.

Qian L, Zhao J, Shi Y, Zhao X, Feng G, Xu F, Zhu S, He L (2007). Brain-derived neurotrophic factor and risk of schizophrenia: an association study and meta-analysis. *Biochem Biophys Res Commun*, 353: 738-743.

Qin XY, Cheng Y, Yu LC (2010). Potential protection of curcumin against intracellular amyloid beta-induced toxicity in cultured rat prefrontalcortical neurons. *Neurosci Lett*, 480: 21-4.

Querfurth HW, LaFerla FM (2010). Alzheimer's disease. *N Engl J Med*, 362: 329-44.

Quintanilla RA, Orellana DI, González-Billault C, Maccioni RB (2004). Interleukin-6 induces Alzheimer-type phosphorylation of tau protein by deregulating the cdk5/p35 pathway. *Exp Cell Res*, 295: 245-57.

Ray B, Bisht S, Maitra A, Lahiri DK. Neuroprotective and neurorescue effects of a novel polymeric nanoparticle formulation of curcumin (NanoCurc) in the neuronal cell culture and animal model: Implications for Alzheimer's disease (2011). *JAD*, 23:61–77.

Refolo LM, Fillit HM (2004). New directions in neuroprotection: basic mechanisms, molecular targets and treatment strategies. *J Alzheimer Dis*, 6: 1-2.

Reichardt LF (2006). Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci*, 361: 1545-64.

Rennaker RL, Street S, Ruyle AM, Sloan AM (2005). A comparison of chronic multi-channel cortical implantation techniques: manual versus mechanical insertion. *J Neurosci Methods*, 142: 169-76.

Ribizzi G, Fiordoro S, Barocci S, Ferrari E, Megna M (2010). Cytokine polymorphisms and Alzheimer disease: possible associations. *Neurol Sci*, 31: 321-5.

Ringman JM, Frautschy SA, Teng E, Begum AN, Bardens J, Beigi M, Gylys KH, Badmaev V, Heath DD, Apostolova LG, Porter V, Vanek Z, Marshall GA,Hellemann G, Sugar C, Masterman DL, Montine TJ, Cummings JL, Cole GM (2012). Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. *Alzheimers Res Ther*, 4: 43.

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, Peterson PK (2004). Role of microglia in central nervous system infections. *Clin Microbiol Rev,* 17: 942-964.

Roney C, Kulkarni P, Arora V, Antich P, Bonte F, Wu A, Mallikarjuana NN, Manohar S, Liang HF, Kulkarni AR, Sung HW, Sairam M, Aminabhavi TM (2005). Targeted nanoparticles for drug delivery through the blood-brain barrier for Alzheimer's disease. *J Control Release*, 108: 193-214.

Rosales-Corral S, Tan DX, Reiter RJ, Valdivia-Velázquez M, Acosta-Martínez JP, Ortiz GG (2004). Kinetics of the neuroinflammation-oxidative stress correlation in rat brain following the injection of fibrillar amyloid-beta onto the hippocampus in vivo. *J Neuroimmunol*, 150: 20-8.

Ryder J, Su Y, Ni B (2004). Akt/GSK3b serine/threonine kinases: evidence for a signaling pathway mediated by familial Alzheimer's disease mutations. *Cell Signal*, 16: 187–200.

Ryu JK, McLarnon JG (2008). Thalidomide inhibition of perturbed vasculature and glial-derived tumor necrosis factor-alpha in an animal model of inflamed Alzheimer's disease brain. *Neurobiol Dis*, 29: 254-66.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*, 307: 1098-101.

Saresella M, Calabrese E, Marventano I, Piancone F, Gatti A, Alberoni M, Nemni R, Clerici M (2011). Increased activity of Th-17 and Th-9 lymphocytes and a skewing of the post-thymic differentiation pathway are seen in Alzheimer's disease. *Brain Behav Immun*, 25: 539-47.

Salinas PC, Zou Y (2008) Wnt signaling in neural circuit assembly. *Annu Rev Neurosci,* 31:339-58.

Schaffazick SR, Guterres SS, Freitas LL, Pohlmann AR (2003). Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. *Química Nova,* 26: 726-737.

Schaffazick SR, Pohlmann AR, Dalla-Costa T, Guterres SS (2003). Freeze-drying polymeric colloidal suspensions: nanocapsules, nanospheres and nanodispersion. A comparative study. *Eur J Pharm Biopharm*, 56: 501-5.

Selkoe DJ (2002). Alzheimer's disease is a synaptic failure. *Science*, 298: 789-91.

Selkoe DJ, Schenk D (2003). Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Annu Rev Pharmacol Toxicol,* 43: 545-584.

Selkoe DJ (2011). Resolving controversies on the path to Alzheimer's therapeutics. *Nat Med*, 17: 1060-5.

Shaikh J, Ankola DD, Beniwal V, Singh D, Kumar MN (2009). Nanoparticle encapsulation improves oral biovailability of curcumin by at least 9-fold when compared to curcumin administered with piperine as absorption enhancer. *Eur J Pharm Sci*, 37: 223-230.

Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL (2007). Natural oligomers of the Alzheimer amyloid beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci*, 27: 2866-75.

Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med*, 14: 837-42.

Shankar GM, Walsh DM (2009) Alzheimer's disease: synaptic dysfunction and Abeta. *Mol Neurodegener*, 4: 48.

Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ,Steward WP (2004). Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res*, 10: 6847-54.

Siegel M, Warden MR, Miller EK (2009). Phase-dependent neuronal coding of objects in short-term memory. *Proc Natl Acad Sci USA*, 106: 21341-6.

Simão F, Zamin LL, Frozza R, Nassif M, Horn AP, Salbego CG (2009). Protective profile of oxcarbazepine against oxygen-glucose deprivation in organotypic hippocampal slice culture could involve PI3K cell signaling pathway. *Neurol Res*, 31: 1044-8.

Sipos E, Kurunczi A, Kasza A, Horvath J, Felszeghy K, Laroche S, Toldi J, Parducz, A, Penke B, Penke Z (2007). β-amyloid pathology in the entorhinal cortex of rats induces memory deficits: Implications for Alzheimer's disease. *Neuroscience*, 147: 28-36.

Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat Res,* 579: 200-13.

Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE (2001). Biodegradable polymeric nanoparticles as drug delivery devices. *J Control Release*, 70: 1-20.

Sperling RA, Dickerson BC, Pihlajamaki M, Vannini P, LaViolette PS, Vitolo OV, Hedden T, Becker JA, Rentz DM, Selkoe DJ, Johnson KA (2010). Functional alterations in memory networks in early Alzheimer's disease. *Neuromolecular Med*, 12: 27-43.

Spira ME, Hai A (2013). Multi-electrode array technologies for neuroscience and cardiology. *Nat Nanotechnol*, 8: 83-94.

Stine WB Jr, Dahlgren KN, Krafft GA, LaDu MJ (2003). In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J Biol Chem*, 278: 11612-22.

Stoppini L, Buchs PA, Muller D (1991). A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37: 173-182.

Su CC, Wang MJ, Chiu TL (2010). The anti-cancer efficacy of curcumin scrutinized through core signaling pathways in glioblastoma. *Int J Mol Med*, 26: 217-24.

Sun Y, Dai M, Wang Y, Wang W, Sun Q, Yang GY, Bian L (2011). Neuroprotection and sensorimotor functional improvement by curcumin after intracerebral hemorrhage in mice. *J Neurotrauma*, 28: 2513-21.

Srinivasan R, Bibi FA, Nunez PL (2006). Steady-state visual evoked potentials: distributed local sources and wave-like dynamics are sensitive to flicker frequency. *Brain Topogr*, 18: 167-87.

Tan S, Ma G, Li Y, Li J, Yao W, Ren X, Liu X, Gao J (2012). Effects of Abeta1-42 on the current of KATP channels in cultured cholinergic neurons. *Neurol Res*, 34: 707-13.

Thakur A, Wang X, Siedlak SL, Perry G, Smith MA, Zhu X (2007). c-Jun phosphorylation in Alzheimer disease. *J Neurosci Res,* 85: 1668-1673.

Thangapazham RL, Puri A, Tele S, Blumenthal R, Maheshwari RK (2008). Evaluation of a nanotechnology-based carrier for delivery of curcumin in prostate cancer cells. *International Journal of Oncology,* 32: 1119-23.

Tong L, Balazs R, Soiampornkul R, Thangnipon W, Cotman CW (2008). Interleukin-1 beta impairs brain derived neurtotrophic factor-induced signal transduction. *Neurobiol Aging*, 29: 1380-93.

Tyler WJ, Perrett SP, Pozzo-Miller LD (2002). The role of neurotrophins in neurotransmitter release. *Neuroscientist*, 8: 524-31.

Valentim LM, Rodnight R, Geyer AB, Horn AP, Tavares A, Cimarosti H, Netto CA, Salbego CG (2003). Changes in heat shock protein 27 phosphorylation and immunocontent in response to preconditioning to oxygen and glucose deprivation in organotypic hippocampal cultures. *Neuroscience*, 118: 379-86.

Van Eldik LJ, Thompson WL, Ralay Ranaivo H, Behanna HA, Martin Watterson D (2007). Glia proinflammatory cytokine upregulation as a therapeutic target for neurodegenerative diseases: function-based and target-based discovery approaches. *Int Rev Neurobiol*, 82: 277-96.

Varghese K, Molnar P, Das M, Bhargava N, Lambert S, Kindy MS, Hickman J J (2010). A new target for amyloid beta toxicity validated by standard and high-throughput electrophysiology. *PLoS One,* 5: e8643.

Vivanco I, Sawyers CL (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, 2: 489-501.

Waetzig V, Herdegen T (2004). Neurodegenerative and physiological actions of c-Jun N terminal kinases in the mammalian brain. *Neurosci Lett*, 361: 64-67.

Wang DM, Yang YJ, Zhang L, Zhang X, Guan FF, Zhang LF (2013). Naringin Enhances CaMKII Activity and Improves Long-Term Memory in a Mouse Model of Alzheimer's Disease. *Int J Mol Sci*, 14: 5576-86.

Wang HM, Zhao YX, Zhang S, Liu GD, Kang WY, Tang HD, Ding JQ, Chen SD (2010). PPARgamma agonist curcumin reduces the amyloid-betastimulated inflammatory responses in primary astrocytes. *J Alzheimers Dis*, 20: 1189-99.

Wang HY, Crupi D, Liu J, Stucky A, Cruciata G, Di Rocco A, Friedman E, Quartarone A, Ghilardi MF (2011). Repetitive transcranial magnetic stimulation enhances BDNF-TrkB signaling in both brain and lymphocyte. *J Neurosci*, 31: 11044-54.

Wang J, Du XX, Jiang H, Xie JX (2009). Curcumin attenuates 6-hydroxydopamine-induced cytotoxicity by anti-oxidation and nuclear factor-kappa B modulation in MES23.5 cells. *Biochem Pharmacol*, 78: 178-83.

Weber WM, Hunsaker LA, Gonzales AM, Heynekamp JJ, Orlando RA, Deck LM, Vander Jagt DL(2006). TPA-induced up-regulation of activator protein-1 can be inhibited or enhanced by analogs of the natural product curcumin. *Biochem Pharmacol,* 72: 928-940.

Wilson CJ, Finch CE, Cohen HJ (2002). Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. *J Am Geriatr Soc*, 50: 2041-56.

Wilkinson KA, Henley JM (2010). Mechanisms, regulation and consequences of protein SUMOylation. *Biochem J*, 428: 133–145.

Wilkinson KA, Nakamura Y, Henley JM (2010). Targets and consequences of protein SUMOylation in neurons. *Brain Res Rev*, 64: 195–212.

Winters BD, Forwood SE, Cowell RA, Saksida LM, Bussey TJ (2004). Double dissociation between the effects of peri-postrhinal cortex and hippocampal lesions on tests of object recognition and spatial memory: Heterogeneity of function within the temporal lobe. *J Neurosci*, 24: 5901-08.

Winters BD, Bussey TJ (2005). Transient inactivation of perirhinal cortex disrupts encoding, retrieval, and consolidation of object recognition memory. *J Neurosci*, 25: 52-61.

Wiss-Coray T (2006). Inflammation in Alzheimer's disease: driving force, bystander or beneficial response? *Nat Med*, 12: 1005-1015.

Yan YD, Kim JA, Kwak MK, Yoo BK, Yong CS, Choi HG (2011). Enhanced oral bioavailability of curcumin via a solid lipid-based self-emulsifying drug delivery system using a spray-drying technique. *Biol Pharm Bull*, 34: 1179-86.

Yang W, Sheng H, Homi HM, Warner DS, Paschen W (2008). Cerebral ischemia/stroke and small ubiquitin-like modifier (SUMO) conjugation - a new target for therapeutic intervention? *J Neurochem*, 106: 989-99.

Yamauchi T (2005). Neuronal Ca2+/calmodulin-dependent protein kinase II--discovery, progress in a quarter of a century, and perspective: implication for learning and memory. *Biol Pharm Bull*, 28: 1342-54.

Yoshii A, Constantine-Paton M (2007). BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nature Neuroscience*, 10: 702-11.

Yuan J, Yankner BA (2000). Apoptosis in the nervous system. *Nature*, 407: 802-9.

Zaheer A, Zaheer S, Thangavel R, Wu Y, Sahu SK, Yang B (2008). Glia maturation factor modulates β-amyloid-induced glial activation, inflammatory cytokine/chemokine production and neuronal damage. *Brain Res,* 1208: 192-203.

Zamin LL, Dillenburg-Pilla P, Argenta-Comiran R, Horn AP, Simão F, Nassif M, Gerhardt D, Frozza RL, Salbego C (2006). Protective effect of resveratrol against oxygen-glucose deprivation in organotypic hippocampal slice cultures: Involvement of PI3-K pathway. *Neurobiol Dis,* 24: 170-82.

Zanotto-Filho A, Braganhol E, Edelweiss MI, Behr GA, Zanin R, Schröder R, Simões-Pires A, Battastini AM, Moreira JC (2012). The curry spice curcumin selectively inhibits cancer cells growth in vitro and in preclinical model of glioblastoma. *J Nutr Biochem*, 23: 591-601.

Zanotto-Filho A, Coradini K, Braganhol E, Schröder R, de Oliveira CM, et al. Curcumin-loaded lipid-core nanocapsules as strategy to improve pharmacological efficacy of curcumin in glioma treatment (2013). *Eur J Pharm Biopharm*, 83: 156-67.

Zeng Y, Zhao D, Xie CW (2010). Neurotrophins enhance CaMKII activity and rescue amyloid-β-induced deficits in hippocampal synaptic plasticity. *J Alzheimers Dis*, 21: 823-31.

Zhang L, Xu T, Wang S, Yu L, Liu D, Zhan R, Yu SY (2012). Curcumin produces antidepressant effects via activating MAPK/ERK-dependent brain-derived neurotrophic factorexpression in the amygdala of mice. *Behav Brain Res*, 235: 67-72.

Zhang X, Yin WK, Shi XD, Li Y (2011). Curcumin activates Wnt/β-catenin signaling pathway through inhibiting the activity of GSK-3β in APPswe transfected SY5Y cells. *Eur J Pharm Sci*, 42: 540-6.

Zhang YQ, Sarge KD (2008). Sumoylation of amyloid precursor protein negatively regulates Aβ aggregate levels. *Biochem Biophys Res Commun*, 374: 673–678.

Zhang Z, Feng SS (2006). In vitro investigation on poly(lactide)-Tween 80 copolymer nanoparticles fabricated by dialysis method for chemotherapy. *Biomacromolecules*, 7: 1139-46.

Zhao BL, Li XJ, He RG, Cheng SJ, Xin WJ (1989). Scavenging Effect of Extracts of Green Tea and Natural Antioxidants on Active Oxygen Radicals. *Cell Biophysics,* 14: 175–185.

Zhao D, Watson JB, Xie CW (2004). Amyloid beta prevents activation of calcium/calmodulin-dependent protein kinase II and AMPA receptor phosphorylation during hippocampal long-term potentiation. *J Neurophysiol*, 92: 2853-2858.

Zhou H, Beevers CS, Huang S. the targets of curcumin. *Curr Drug Targets*, 12: 332-47.

Zhuang H, Kim YS, Koehler RC, Doré S (2003). Potential mechanism by which resveratrol, a red wine constituent, protects neurons. *Ann N Y Acad Sci,* 993: 276-86.